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Evolution of $CD4^+CD25^{hi}$ T cell subsets in *Aspergillus*-infected liver transplantation recipients reduces the incidence of transplantation rejection via upregulating the production of anti-inflammatory cytokines

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ABSTRACT. Infection is the leading risk factor of liver transplantation-related death. Aspergillosis is a life-threatening complication in immune-compromised patients, and is the cause of approximately 2/3 of deaths in liver transplant recipients. In our previous studies, we found a regulatory T cell (Treg) population that showed significantly increased immune tolerance in *Aspergillus*-infected liver transplant recipients. Furthermore, interleukin (IL)-17 production was also increased, and an IL-17-producing Treg cell subset was identified in these patients. Functional studies of the role of these IL-17-producing Treg cells in the induction of immune tolerance are needed to help reduce the death rate of liver transplantation recipients. This study included 75 liver transplant recipients with and

without histologically confirmed aspergillosis after liver transplantation. The percentage of T cell population subsets producing cytokines was detected by fluorescence-activated cell sorting and enzyme-linked immunosorbent assay in peripheral blood. Complements in blood serum were also examined. The risk of acute rejection was lower in *Aspergillus*-infected liver transplant recipients compared to the non-*Aspergillus*-infected group; the $CD4^+CD25^{hi}$ T cell population in peripheral blood was higher and the $CD4^+CD45RA\text{-}CD45RO^+$ T cell population was lower. There was no significant difference between the $CD4^+CD25^{lo}CD45RA^+$ and $CD4^+CD25^{lo}CD45RA\text{-}$ T cell populations. Moreover, IL-6 decreased and IL-4 increased in the blood serum of *Aspergillus*-infected liver transplant recipients. Together, these results indicate that the incidence of graft rejection in liver transplantation recipients with *Aspergillus* infections was lower than that of the non-infected group, and suggests a mechanism for this effect.

Key words: Liver transplantation; Aspergillosis; Treg cells; Cytokines

INTRODUCTION

Aspergillosis is caused by the spores of *Aspergillus*, which is ubiquitously distributed in the environment under certain conditions, such as in patients with immune suppressive therapy or in those who have undergone solid organ and stem cell transplantation, and reaches approximately 80% mortality (Oren and Goldstein, 2002). Of the nearly 185 *Aspergillus* species, *A. fumigatus* is the most prevalent pathogen, and accounts for more than 90% of all infections reported (Chen et al., 2011). The incidence of invasive aspergillosis occurs in 1 to 8% of liver transplant recipients and the mortality rate ranges from 60 to 80% (Fortún et al., 2002; Singh and Paterson, 2005).

Aspergillosis can occur as a local or a systemic infection, depending primarily on the functions of helper T (Th) cell subsets and regulatory T (Treg) cells. Both Th1, Th2, and Th17 responses in mice were shown to evolve during repeated pulmonary exposure to *Aspergillus* (Murdock et al., 2011). Treg cells involved in the development of aspergillosis provide the host with adequate defense without necessarily eliminating the fungus or causing unacceptable levels of host damage (Montagnoli et al., 2006).

After solid-organ transplant, it is critical to find the balance between immuno-suppression to block rejection and prevention of infection. Recent studies have focused on the co-evolution of innate and adoptive immune cells, such as dendritic cells and Th cell subsets, in the process of the recognition and elimination of *Aspergillus*. In our previous studies, we found that CD4+CD25hi Treg cells were increased in *Aspergillus*-infected liver transplantation recipients; however, the mechanism of immune responses by Treg cells in the elimination of *Aspergillus* remains unclear.

MATERIAL AND METHODS

Patients

We examined data from 75 liver transplant recipients (67 male, 8 female; average age,

42.3 years) with or without histologically and polymerase chain reaction (PCR) confirmed aspergillosis after liver transplantation from March 1, 2010 to March 31, 2011 in our hospital. Clinical samples (sputum, blood, and liver tissue) were examined for fungal infections by microscopic examination and culture on Sabouraud dextrose agar (Merck; Germany). Blood samples were collected at 1-week intervals for molecular examinations. Sera from the patients were extracted for *Aspergillus* DNA detection using the QIAamp DNA Minikit (Qiagen; Germany) in accordance with manufacturer recommendations. Nested PCR was performed with 2 sets of primers according to Yamakami et al. (1996). Based on comparison of the 18S rRNA sequence genes of *Aspergillus*, the *Aspergillus* species (panfungal aspergillosis) was identified.

Fluorescence-activated cell sorting (FACS) analysis

Cells were collected from patients' peripheral blood with the human $CD4^+$ lymphocytes enrichment kit (Miltenyi Biotec) for flow cytometry analysis. The cells were stained with a cocktail containing phycoerythrin (PE)-CD4, allophycocyanin (APC)-CD25, fluorescein isothiocyanate (FITC)-CD45RA, and peridinin chlorophyll protein (PerCP)-Cy5.5-CD45RO (Ebioscience). All cells were analyzed on a FACSAria system.

Cytokine enzyme-linked immunosorbent assay (ELISA)

To determine the cytokine levels in blood serum, 100 µL 100 µg/mL coating antibodies in Hank's balanced salt solution (HBSS; Invitrogen Corp.) was used to coat 96-well microtiter plates (Corning Inc.) overnight at 4°C. After washing in 0.05% Tween-20 in phosphate-buffered saline (PBS), the plates were blocked with 200 µL 10% fetal bovine serum in PBS for 2 h at room temperature, and then washed again as before. One hundred microliters serum was added to each well, and the plates were incubated overnight at 4°C. After additional washes, the plates were incubated for 5 h at 37°C with 200 µL 1:1000 diluted detective antibodies (Ebioscience). The plates were washed and incubated at room temperature for 30 minutes in the dark with 100-µL substrate (Sigma-Aldrich), and absorbance was read at 405 nm using a microplate reader. Values in the linear range were multiplied by the dilution factor and expressed as optical density $(OD)_{405} \pm SD$.

Real-time PCR

Total RNA from peripheral blood cells was purified (Naonoprep Kit; Stratagene) and M-MLV reverse transcriptase (Invitrogen) was used for reverse transcription into cDNA. RedTaq PCR reagents (Sigma) and the following primers were used for PCR: β-actin, F: 5'gagctacgagctgcctgacg3', R: 5'gtagtttcgtggatgccacag3'; RORγt, F: 5'gtgctggttaggatgtgccg3', R: 5'gtgggagaagtcaaagatgga3'; FOXP3, F: 5'gtggcatcatccgacaagg3', R: 5'tgtggaggaactctgggaat3'; TLR4, F: 5'ggccagcaaattacctgtgtg3', R: 5'aggttcaggatgtccgcct3'.

Western blot analysis

Transcription factors were analyzed by Western blot as previously described (Ostroukhova et al., 2004). For MyD88, RORγt, FOXP3, and TLR4 detection, successive over-

night incubations at 4°C with primary and secondary antibody were carried out.

Statistical analysis

All data entry and statistical analysis were performed with SPSS for Windows version 13. The Student *t*-test was used to compare continuous variables, and the χ^2 test was used to compare qualitative variables. Continuous and normally distributed data are reported as means ± standard deviation (SD) with values of $P < 0.05$ considered to be statistically significant.

RESULTS

Confirmation of *Aspergillus* infection in patients

In order to account for *Aspergillus* infection and its clinical influence on T cell subsets, we first confirmed *Aspergillus* infection in patients' peripheral blood and liver tissues before and after liver transplantation (Figure 1). Seventy-five liver transplant patients (67 male, 8 female; average age, 42.3 years) were tested histologically and for *Aspergillus*-specific PCR proliferation. Among these patients, 43 (36 male and 7 female) were found to be infected with *Aspergillus* after and/or before liver transplantation via PCR, and 24 patients (23 male and 1 female) had no *Aspergillus*-specific gene fragments in either their blood or liver tissues. Notably, no *Aspergillus* DNA could be found in either blood or liver tissues of all of rejection patients. These findings were consistent with the histological detection results. Furthermore, the blood samples of 30 healthy volunteers and 26 aspergillosis patients without liver transplantation were collected as control groups.

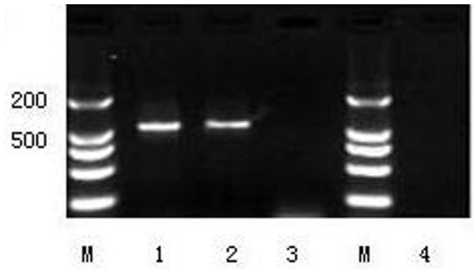

Figure 1. *Aspergillus* specific gene fragment proliferation in patients. *Lane M* = DL2,000 DNA marker; *lane 1* = postive control; *lane 2* = patients with *aspergillus* infection; *lane 3* = negative control; *lane 4* = patients without *aspergillus* infection.

Evolution of the $CD4^+CD25^{hi}$ regulatory T cell subset in liver transplantation recipients with *Aspergillus* infections

We stained Treg subsets and activated (effector) T cell subsets in liver transplantation recipients with or without *Aspergillus* infections. The results suggested that aspergillosis, in both liver transplantation recipients and other patients, led to an increase in $CD4^+CD25^{hi}$ regulatory T cell subsets (13.2 ± 2.33 in aspergillosis patients and 12.84 ± 2.18 in liver recipients; P = 0.3574). Although the precise mechanism for this difference is unclear, local inflammatory and tissue damages are well

known to cause changes in the proportion of Treg cells in local tissues via transforming growth factor (TGF)-β and interleukin (IL)-10 secretions. The $CD4^+CD45RA\text{-}CD45RO^+$ cell subset was also found to be increased in *Aspergillus*-infected liver recipients (8.20 ± 1.21 in infected recipients and 3.96 ± 0.87 in non-infected recipients; $P < 0.0001$), which suggested that T cell responses were efficiently activated after *Aspergillus* infection. No difference was found in the $CD4^+CD25^{lo}CD45RA^+$ naive cell subset (15.29 ± 4.92 in *Aspergillus*-infected recipients and 13.21 ± 2.93 in non-infected recipients; $P = 0.0818$) or in the total amount of T lymphocytes (Table 1).

Table 1. T cell subsets in peripheral blood.

Group (%)	$CD4^+CD25^{hi}$	$CD4^+CD25^{lo}CD45RA^+$	$CD4^+CD45RA\text{-}CD45RO^+$
Health control	11.14 ± 1.48	13.22	5.62
Aspergillosis without liver transplantation	13.2 ± 2.33	13.36 ± 2.39	5.29 ± 1.29
Non-aspergillosis after liver tansplantation	11.38 ± 14.49	13.21 ± 2.93	3.96 ± 0.87
Aspergillosis liver transplantation recipients after transplantation	12.84 ± 2.18	15.29 ± 4.92	8.20 ± 1.21

We also evaluated the protein levels of the transcriptional factors RORγt and FOXP3 in blood samples by Western blotting. Considering that TLR4 is the specific sensor for fungal infections and is activated in *Aspergillosis* patients, we also tested for TLR4 as a positive control. The results suggested that *Aspergillus* infections in non-liver transplantation patients caused an increase in the Th17 cell subset (3.42 ± 0.28 in infected patients and 2.07 ± 0.67 in the healthy group; $P < 0.0001$), and a decrease in the Treg population (1.63 ± 0.28 in infected patients and 2.09 ± 0.34 in the healthy group; $P < 0.0001$). However, in the liver transplantation groups, immuno-suppressive therapy did not lead to downregulation of Treg cells (2.28 ± 0.40 in liver recipients and 2.09 ± 0.34 in the healthy group; $P = 0.0828$). The percentage of Treg subsets in aspergillosis was higher than in recipients without *Aspergillus* infection (2.92 ± 0.23 in *Aspergillus*-infected recipients and 0.96 ± 0.39 in non-infected recipients; $P < 0.0001$), as shown in Table 2. These results were consistent with those obtained from the PCR arrays (Table 3).

Table 2. Transcriptional factors in peripheral blood.

Group (fold-changes with beta-actin)	RORγt	FOXP3	TLR4
Healthy control	2.07 ± 0.67	2.09 ± 0.34	3.29 ± 0.98
Aspergillosis without liver transplantation	3.42 ± 0.28	1.63 ± 0.28	4.22 ± 0.85
Non-aspergillosis before liver tansplantation	1.99 ± 0.53	2.28 ± 0.40	3.30 ± 0.38
Non-aspergillosis after liver tansplantation	1.09 ± 0.28	1.28 ± 0.32	1.92 ± 0.27
Non-aspergillosis liver transplantion recipients before rejection	1.94 ± 0.33	1.97 ± 0.29	2.89 ± 0.69
Non-aspergillosis after liver tansplantation	3.98 ± 0.84	0.96 ± 0.39	4.96 ± 0.72
Aspergillosis liver transplantation recipients before transplantation	2.19 ± 0.39	2.03 ± 0.31	3.39 ± 0.37
Aspergillosis liver transplantation recipients after transplantation	3.21 ± 0.39	2.92 ± 0.23	3.89 ± 0.39

Table 3. Transcriptional factors in peripheral blood (real-time PCR).

Group (fold-changes with beta-actin)	RORγt (x100)	TLR4 (lx1000)	FOXP3 (x100)
Healthy control	0.79 ± 0.21	0.53 ± 0.22	0.49 ± 0.19
Aspergillosis without liver transplantation	1.12 ± 0.32	1.02 ± 0.31	0.27 ± 0.20
Non-aspergillosis before liver tansplantation	0.77 ± 0.25	0.49 ± 0.30	0.47 ± 0.17
Non-aspergillosis after liver tansplantation	0.42 ± 0.22	0.37 ± 0.27	0.25 ± 0.15
Non-aspergillosis liver transplantion recipients before rejection	0.82 ± 0.19	0.55 ± 0.31	0.52 ± 0.21
Non-aspergillosis after liver tansplantation	1.42 ± 0.33	0.98 ± 0.19	0.22 ± 0.18
Aspergillosis liver transplantation recipients before transplantation	0.83 ± 0.28	0.54 ± 0.26	0.45 ± 0.16
Aspergillosis liver transplantation recipients after transplantation	1.29 ± 0.27	1.02 ± 0.36	0.32 ± 0.18

Aspergillus infection downregulated the production of anti-inflammatory cytokines in liver recipients

The Treg/Th17 cell ratio is critical for host anti-fungal immune responses. Treg cells play their roles either by directly inhibiting effector T or innate immune cells or by regulating the production of pro/anti-inflammatory cytokines. We tested the cytokine production levels in blood serum from patients, and found that the production of IL-4, which is generally produced in response to the T cell defense against fungal infections (Kreindler et al., 2010), was increased in *Aspergillus*-infected liver recipients (29.28 ± 3.98 in infected recipients and 23.92 ± 8.37 in non-infected recipients; $P < 0.0001$). However, the anti-inflammatory cytokine IL-6 was found to be decreased in *Aspergillus*-infected liver recipients (165.29 ± 49.92 in infected recipients and 187.29 ± 34.98 in non-infected recipients; $P = 0.0664$) (Table 4). The sources of these cytokines and the mechanism underlying the regulation of cytokines by Treg cells remain unclear based on this data.

Table 4. Cytokines levels in peripheral blood.

Group (pg/mL)	TNF-β	IFN-γ	IL-1	IL-4	IL-6	IL-17	IL-23
Healthy control	54.18 ± 20.67	98.19 ± 17.28	69.17 ± 21.28	30.29 ± 8.24	96.28 ± 27.21	23.28 ± 5.25	77.98 ± 35.28
Aspergillosis without liver transplantation	100.27 ± 24.43	78.28 ± 27.28	124.38 ± 13.28	24.28 ± 7.17	178.36 ± 22.39	53.29 ± 4.29	132.28 ± 46.39
Non-aspergillosis before liver tansplantation	65.19 ± 22.17	90.28 ± 20.37	78.26 ± 17.29	29.28 ± 8.36	112.38 ± 23.18	27.28 ± 7.20	106.87 ± 23.29
Non-aspergillosis after liver tansplantation	23.28 ± 14.49	53.84 ± 14.28	32.29 ± 8.93	20.72 ± 5.98	43.21 ± 12.93	16.96 ± 3.87	35.87 ± 23.61
Non-aspergillosis liver transplantion recipients before rejection	63.46 ± 27.19	88.22 ± 19.26	77.29 ± 28.21	27.29 ± 7.39	110.28 ± 25.29	28.29 ± 20.20	103.28 ± 30.28
Non-aspergillosis after liver tansplantation after rejection	168.36 ± 34.89	65.08 ± 15.42	15.87 ± 56.93	23.92 ± 8.37	187.29 ± 34.98	69.36 ± 8.02	128.07 ± 39.96
Aspergillosis liver transplantation recipients before transplantation	70.27 ± 30.32	80.7 ± 18.25	83.20 ± 21.29	28.37 ± 6.68	98.18 ± 20.19	29.29 ± 8.93	90.29 ± 25.98
Aspergillosis liver transplantation recipients after transplantation	98.48 ± 34.48	50.32 ± 9.22	118.20 ± 11.98	29.28 ± 3.98	165.29 ± 49.92	48.20 ± 9.21	120.93 ± 34.02

DISCUSSION

Patients who have received liver or other solid-organ transplants have to face two challenges: transplantation rejection and pathogen infections. Immuno-suppressive therapy weakens the immune system to avoid unnecessary organ rejection; however, the dysfunctional immune cells increase the potential of the occurrence of a rare infection, such as aspergillosis, which is rarely found in healthy people.

Acute or clinical infection of the fungus *Aspergillus* causes a series of serious immune responses. One study showed that different components of *Aspergillus* could lead to the differentiation of different Th subsets. Specifically, secreted proteins mostly activated IL-4-producing T cell clones, whereas glycolipid components mostly led to Th17 responses (Crameri, 2002). In addition, polysaccharides were found to variably activate interferon (IFN)-γ-, IL-17-, or IL-10-producing T cell clones (Sarfati et al., 2006). Furthermore, most of the antigens from *Aspergillus* can evolve humoral immunity in both mice and humans. Research into the molecular mechanisms has also shown that Vitamin D3 was able to attenuate Th2 responses to *Aspergillus* (Kreindler et al., 2010), and that humoral immunity, promoted by Th2 cells, is

more critical than $CD8^+$ T cell responses in the elimination of fungi (Feldmesser, 2005). Moreover, although the tight regulation of the elimination of fungi was shown to be controlled by the Treg/Th17 ratio (Zelante et al., 2009), the variation of Treg and Th17 subsets in *Aspergillosis* remains unclear.

In this study, we first reported that the Treg cell subset increased in the peripheral blood of liver transplantation recipients with *Aspergillus* infections compared with uninfected recipients, and that *Aspergillus*-infected patients also had a lower risk of transplantation rejection. We also examined other Th subsets, such as IFN-γ-producing Th1 cells or IL-17-producing Th17 subsets, and found no significant differences. Considering humoral immunity and its critical roles in the process of *Aspergillus* infection, we hypothesized that Treg cells down-regulate immune responses via secreting anti-inflammatory cytokines. We examined cytokine levels in blood serum with ELISA and real-time PCR and found that IL-2, IL-6, IL-10, and granulocyte macrophage-colony-stimulating factor (GM-CSF) levels were all decreased in *Aspergillus*-infected patients. IL-2 and GM-CSF are critical for the proliferation and homeostasis maintenance of lymphocytes and $CD11b^+$ innate immune cells. IL-6 and IL-10 play roles in local inflammation. However, these data were not sufficient to identify the molecular mechanism of Treg in *Aspergillus* elimination.

In conclusion, the results of the present study demonstrated the phenotypes of T cell subsets and the levels of cytokines produced in liver transplantation recipients' peripheral blood. The results of the statistical analysis suggested that *Aspergillus*-infected liver transplantation recipients have a lower risk of rejection. However, this data was not sufficient to identify the specific role of Treg in *Aspergillus* elimination.

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