

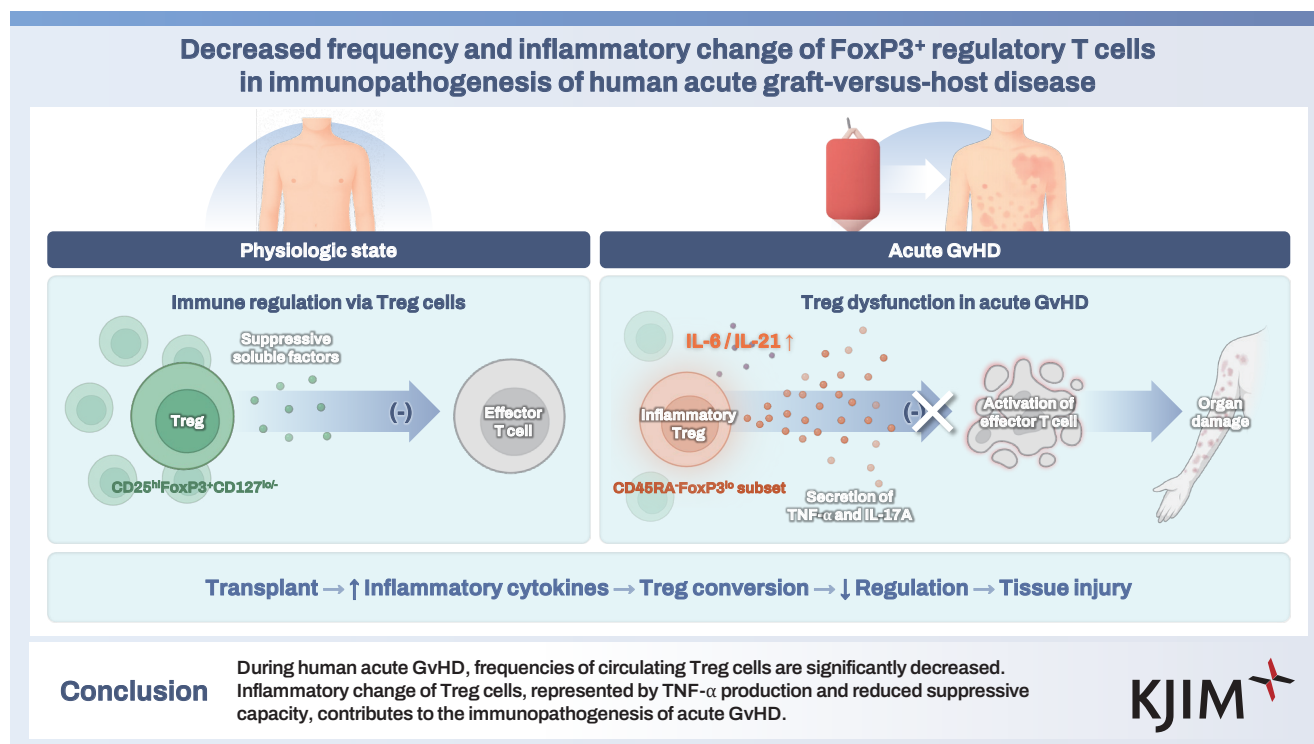


Decreased frequency and inflammatory change of FoxP3⁺ regulatory T cells in immunopathogenesis of human acute graft-versus-host disease

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Background/Aims: Acute graft-versus-host disease (GvHD) is a severe complication of allogeneic stem cell transplantation, characterized by immune-mediated tissue damage primarily affecting the skin, liver, and gastrointestinal tract. Regulatory T (Treg) cells play a critical role in maintaining immune homeostasis. However, the pathogenic roles of changes in Treg cell number and function on acute GvHD remain poorly understood. This study aimed to investigate the quantitative and qualitative changes in Treg cells and their clinical and pathogenic implication of acute GvHD.

Methods: A total of 62 patients who underwent allogeneic stem cell transplantation at a tertiary institution from 2019 to 2024 were enrolled. Peripheral blood mononuclear cells were isolated and analyzed by multicolor flow cytometry. Treg cell

subsets and cytokine production were assessed after T-cell receptor stimulation. Serum levels of inflammatory cytokines were measured using cytometric bead array, and Treg cell suppressive function was evaluated through co-culture experiments.

Results: Patients with acute GvHD showed a decreased frequency of circulating Treg cells, with a notable increase in the CD45RA⁺FoxP3^{lo} pro-inflammatory subset. Treg cells produced inflammatory cytokines including TNF- α upon stimulation and exhibited reduced suppressive activity. The frequency of TNF- α ⁺ Treg cells correlated with the clinical severity of acute GvHD. Elevated serum levels of IL-6 and IL-21 were associated with the inflammatory conversion of Treg cells.

Conclusions: During human acute GvHD, frequencies of circulating Treg cells are significantly decreased. Inflammatory change of Treg cells, represented by TNF- α production and reduced suppressive capacity, contributes to the immunopathogenesis of acute GvHD.

Keywords: Hematopoietic stem cell transplantation; Graft-versus-host disease; Regulatory T cell; TNF-alpha; Inflammation

INTRODUCTION

Acute graft-versus-host disease (GvHD) remains a significant and potentially life-threatening complication following allogeneic hematopoietic stem cell transplantation (SCT) [1]. Acute GvHD is triggered by allo-reactive donor-derived T cells recognizing the recipient's tissues, initiating an immune response that results in widespread tissue damage. The primary target organs affected by acute GvHD include the skin, liver, and gastrointestinal tract, leading to a range of clinical manifestations such as skin rash, jaundice, and severe diarrhea. Despite advancements in transplant techniques and immunosuppressive therapeutic strategies, acute GvHD continues to be a major challenge to the successful allogeneic SCT, contributing to considerable morbidity and mortality in transplant recipients [2].

The pathophysiology of acute GvHD involves a complex interplay between donor T cells, host antigen-presenting cells (APCs), and pro-inflammatory cytokines [3,4]. Upon transplantation, donor T cells become activated by recognizing host allo-antigens presented by APCs, resulting in a cascade of various inflammatory cytokine production [5]. This inflammatory response further amplifies the immune response, causing immune-mediated tissue damage.

Regulatory T (Treg) cells, a specialized subset of CD4⁺ T cells characterized by the expression of CD25 and the transcription factor FoxP3, play a crucial role in controlling excessive and inappropriate immune responses [6,7]. Although numerous studies have reported the potential role of Treg cells in the development of acute GvHD, the precise mechanisms underlying the pathologic changes of Treg cell

population in this context remain unclear. Treg cells typically suppress the activation and proliferation of conventional T cells, thereby preventing excessive immune responses and maintaining immune homeostasis [8,9]. However, under inflammatory conditions, Treg cells can lose their suppressive function and acquire pro-inflammatory properties, a phenomenon known as "inflammatory conversion." The inflammatory conversion of Treg cells has been observed in various autoimmune and inflammatory diseases, including multiple sclerosis, rheumatoid arthritis and psoriasis [10-12]. Also, previous study from our group has shown that Treg cells exhibit lineage plasticity, acquiring Th17-like characteristics during acute viral infections, with these inflammatory changes correlating with disease severity [13]. However, the impact of inflammatory conversion of Treg cells on the pathogenesis of acute GvHD has not yet been fully elucidated.

In the present study, we aimed to investigate the quantitative and qualitative changes in Treg cells in patients with acute GvHD, especially focusing on their inflammatory conversion. We also analyze the association between the inflammatory change of Treg cells and clinically documented tissue damage.

METHODS

Study subjects and insolation of lymphocytes

This study was conducted from 2019 to 2024 and included adult patients (19 years or older) who underwent allogeneic SCT at Ajou University Medical Center for hematologic malignancies. Among the 154 patients who underwent alloge-

neic hematopoietic SCT during the study period, 40 patients diagnosed with acute GvHD were included in this study. The 22 patients in the control group who did not develop acute GvHD were selected based on matched clinical characteristics with the acute GvHD group to ensure comparability between the two groups. All Patients received peripheral blood stem cell transplants from matched sibling, matched unrelated, or haploidentical donors. For matched donor transplantation, the GvHD prophylaxis regimen consisted of a calcineurin inhibitor plus methotrexate and anti-thymocyte globulin [14]. For haploidentical donor transplantation, a calcineurin inhibitor and mycophenolate mofetil were administered in combination with post-transplant cyclophosphamide [15]. The severity of acute GvHD was assessed according to the Mount Sinai Acute GvHD International

Consortium (MAGIC) criteria [16]. Detailed characteristics of 62 enrolled patients are described in Table 1. The study was approved by the Institutional Review Board of Ajou University Hospital (IRB number. AJOUIRB-OBS-2021-484), and all participants provided written informed consent. Additionally, all experimental procedures involving patient-derived specimens were conducted in accordance with the approved IRB protocol.

For patients diagnosed with acute GvHD, peripheral blood samples were collected at the time of diagnosis corresponding to the respective grades of acute GvHD. The median time of sampling was day 56 post-transplantation (range, 17 to 89 days). For patients in the control group who did not develop acute GvHD, blood samples collected on day 35 post-transplantation were used. Peripheral blood mononu-

Table 1. Clinical characteristics of patients enrolled in the current study

Characteristic	Acute GvHD (+) (n = 40)	Acute GvHD (-) (n = 22)	Total (n = 62)
Age (yr)	49 (21–67)	52 (22–65)	51 (21–67)
Sex			
Male	23 (57.5)	11 (50.0)	34 (54.8)
Female	17 (42.5)	11 (50.0)	28 (45.2)
Donor type			
Matched sibling	11 (27.5)	8 (36.4)	19 (30.6)
Matched unrelated	12 (30.0)	10 (45.6)	22 (35.5)
Haploidentical	10 (25.0)	11 (50.0)	21 (33.9)
Conditioning regimen			
Myeloablative conditioning	22 (55.0)	11 (50.0)	33 (53.2)
Reduced-intensity conditioning	18 (45.0)	11 (50.0)	29 (46.8)
Primary hematologic disease			
Acute leukemia	30 (75.0)	17 (77.3)	47 (75.8)
Myelodysplastic syndrome	10 (25.0)	3 (13.6)	13 (21.0)
Lymphoma	0 (0.0)	2 (9.1)	2 (3.2)
Disease status at transplantation			
CR1	27 (67.5)	14 (63.6)	41 (66.2)
CR2	13 (32.5)	6 (27.3)	19 (30.6)
Not applicable	0 (0.0)	2 (9.1)	2 (3.2)
Severity of acute GvHD			
Grade I	11 (27.5)	0 (0.0)	-
Grade II	24 (60.0)	0 (0.0)	-
Grade III	5 (12.5)	0 (0.0)	-

Values are presented as median (range) or number (%).

Severity of acute GvHD was graded according to the MAGIC criteria.

GvHD, graft-versus-host disease; CR, complete response; MAGIC, Mount Sinai Acute GvHD International Consortium.

clear cells (PBMCs) were isolated from whole blood by standard Ficoll-Paque (GE Healthcare, Uppsala, Sweden) density gradient centrifugation. Serum samples were also obtained for cytokine analysis.

Flow cytometry and immunophenotyping

The following fluorochrome-conjugated monoclonal antibodies used for multicolor flow cytometry: anti-CD3 (UCHT-1), anti-CD4 (RPA-T4), anti-CD14 (M5E2), anti-CD19 (HIB19), anti-CD25 (M-A251), anti-CD45RA (HI100) (all from BD Biosciences, San Jose, CA, USA); anti-CD39 (eBioA1) (eBioscience, San Diego, CA, USA); anti-CD127 (40131) (R&D Systems, Minneapolis, MN, USA). Cryopreserved or freshly isolated lymphocytes were stained with fluorochrome-conjugated antibodies against surface markers for 30 minutes on ice and then washed. In all samples for flow cytometric analyses, dead cells were excluded by using Live/Dead fixable cell stain kit (Invitrogen, Carlsbad, CA, USA).

For intracellular staining, PE-conjugated anti-Foxp3 monoclonal antibody (PCH101) and anti-Helios (22F6) (all from eBioscience). Surface-stained cells were permeabilized using FoxP3 staining buffer kit (eBioscience) according to the manufacturer's instruction and further stained for intracellular molecules.

Flow cytometry was performed on an LSR II instrument using FACSDiva software (BD Biosciences), and the data were analyzed using FlowJo software (Treestar, San Carlos, CA, USA).

In vitro stimulation of T cells and intracellular cytokine staining

Cryopreserved PBMCs were thawed, resuspended in RPMI 1640 containing 5% fetal bovine serum (FBS) and 2 mM L-glutamine, and rested overnight at 37°C. PBMCs were stimulated in the presence of anti-CD3 antibody (0.1 µg/mL) and anti-CD28 antibody (1 µg/mL), and brefeldin A (GolgiPlug; BD Biosciences) and monensin (GolgiStop; BD Biosciences) were added 1 hour later. After another 5 hours of incubation, PBMCs were first stained with Live/Dead fixable cell stain kit to exclude dead cells and then stained with fluorochrome-conjugated antibodies against surface markers. Stained cells were permeabilized using FoxP3 staining buffer kit and further stained for intracellular molecules with anti-FoxP3, either anti-IFN-γ (BD Biosciences) or anti-IL-17A (eBioscience) and anti-TNF-α (BD Biosciences).

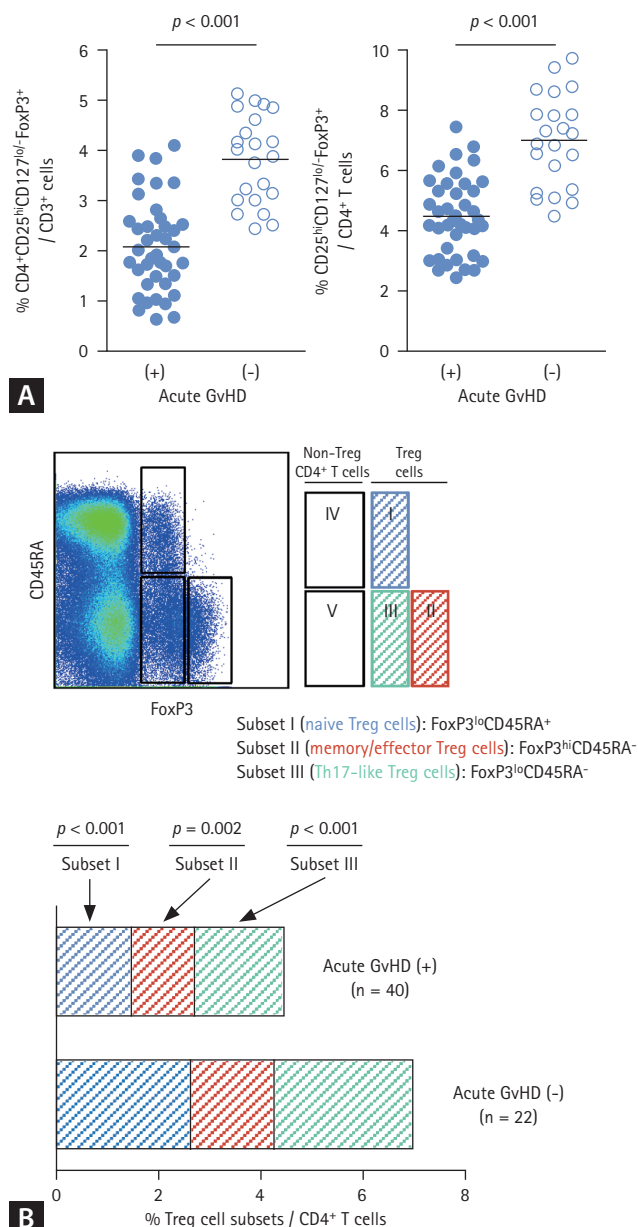


Figure 1. Frequency of circulating Treg cells in acute GvHD. (A, B) Frequencies of CD25^{hi}CD127^{lo/-}FoxP3⁺ CD4⁺ T cells in PBMCs from recipient of allogeneic SCT were determined by flow cytometry (n = 62). (A) Percentages of CD25^{hi}CD127^{lo/-}FoxP3⁺ cells in peripheral blood CD4⁺ T cells were measured and compared according to occurrence of acute GvHD (n = 40 for patients with acute GvHD and n = 22 for patients without acute GvHD). (B) Flow cytometry plot shows three distinct subsets of human CD4⁺FoxP3⁺ Treg cells defined based on the expression of CD45RA and FoxP3. Percentages of each Treg cell subset among total CD4⁺ T cells are determined and compared according to occurrence of acute GvHD (n = 40 for patients with acute GvHD and n = 22 for patients without acute GvHD). GvHD, graft-versus-host disease; PBMCs, peripheral blood mononuclear cells; SCT, stem cell transplantation; Treg, regulatory T.

Standard Treg cell suppression assay

The suppressive function of Treg cells was assessed using co-culture experiments with CFSE-labeled responder T cells as described elsewhere. Briefly, CFSE-labeled Treg-depleted responder CD4⁺ T cells (Tresp) were cocultured with magnetically isolated Treg cells in a range of ratios as indicated within RPMI 1640 containing 5% FBS and 2 mM L-glutamine. Treg cells are isolated from PBMCs using CD4⁺CD25⁺CD127^{lo/-} regulatory T cell isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's protocol. At the end of coculture in the presence of soluble anti-CD3 anti-body (0.1 µg/mL; Beckman Coulter Immunotech, Marseille, France) and anti-CD28 antibody (1 µg/mL; BD Biosciences) stimulation for 96 hours, cells were analyzed for proliferation assessed by percentage of CFSE^{lo} dividing cells. Percentage of suppression was calculated as $[1 - (\% \text{ T cell proliferation with Treg cells} / \% \text{ T cell proliferation without Treg cells})] \times 100$.

Serum cytokine analysis

Levels of soluble factors including IL-6 and IL-21 in sera of acute GvHD patients were quantified using CBA (BD Biosciences). Briefly, capture beads and PE-conjugated detection reagent for each soluble factor analyzed were incubated with serum of patients. Concentration in sera was determined by measuring fluorescence intensity of beads captur-

ing soluble factors with LSRII flow cytometer and interpolating the value into standard equation.

Statistical analyses

Statistical analyses were performed using the Prism software version 5.0 (GraphPad, La Jolla, CA, USA). The Mann–Whitney U test was used for comparisons between two groups, and the Wilcoxon matched-pairs signed-rank test was used for paired values. A *p* value ≤ 0.05 was considered statistically significant.

RESULTS

Decreased frequency of circulating Treg cells during acute GvHD

We first determined the frequency of Tregs in the peripheral blood of patients with acute GvHD, which was collected at the time of diagnosis of GvHD. Flow cytometric analysis revealed a significant reduction in the percentage of CD25^{hi}CD127^{lo/-}FoxP3⁺ CD4⁺ Treg cells in patients with acute GvHD compared to non-acute GvHD control (Fig. 1A). We also investigated the association between quantitative and qualitative changes in Treg cells and clinical factors that could influence acute GvHD. However, no significant associations were identified. The human CD4⁺CD25^{hi}Foxp3⁺ T

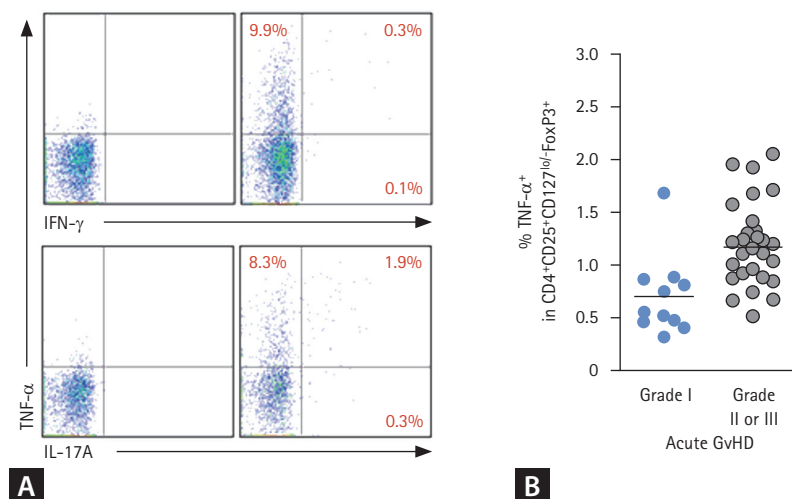


Figure 2. Production of inflammatory cytokines by Treg cells from acute GvHD patients. (A, B) PBMCs from acute GvHD patients were stimulated with ancti-CD3/CD28 antibodies and cytokine production (TNF-α, IFN-γ, and IL-17A) were analyzed with multicolor flow cytometry following intracellular cytokine staining. (A) Representative flow cytometry dot plots of a patient are shown. Plots are gated on CD4⁺CD25⁺CD127^{lo/-}FoxP3⁺ T cells. (B) Percentage of cells producing TNF-α among CD4⁺CD25⁺CD127^{lo/-}FoxP3⁺ T cells from the patients were determined and analyzed according to grade of acute GvHD (n = 11 for grade I and n = 29 for grade II or III). GvHD, graft-versus-host disease; PBMCs, peripheral blood mononuclear cells; Treg, regulatory T.

cell population is known to be functionally heterogeneous and composed of three distinct subpopulations such as CD45RA⁺Foxp3^{lo} resting Tregs, CD45RA⁺Foxp3^{hi} activated Tregs and cytokine-secreting CD45RA⁺Foxp3^{lo} non-suppressive cells. When we analyzed for the composition of Treg cell subsets in more detail, we observed that the propor-

tion of CD45RA⁺Foxp3^{hi} (actively suppressive) Treg cells was decreased and the proportion of CD45RA⁺Foxp3^{lo} (cytokine-producing non-suppressive) Treg cells was increased in patients with acute GvHD (Fig. 1B). Proportion of CD45RA⁺Foxp3⁺ (naïve) Treg cells was also decreased in patients with acute GvHD (Fig. 1B).

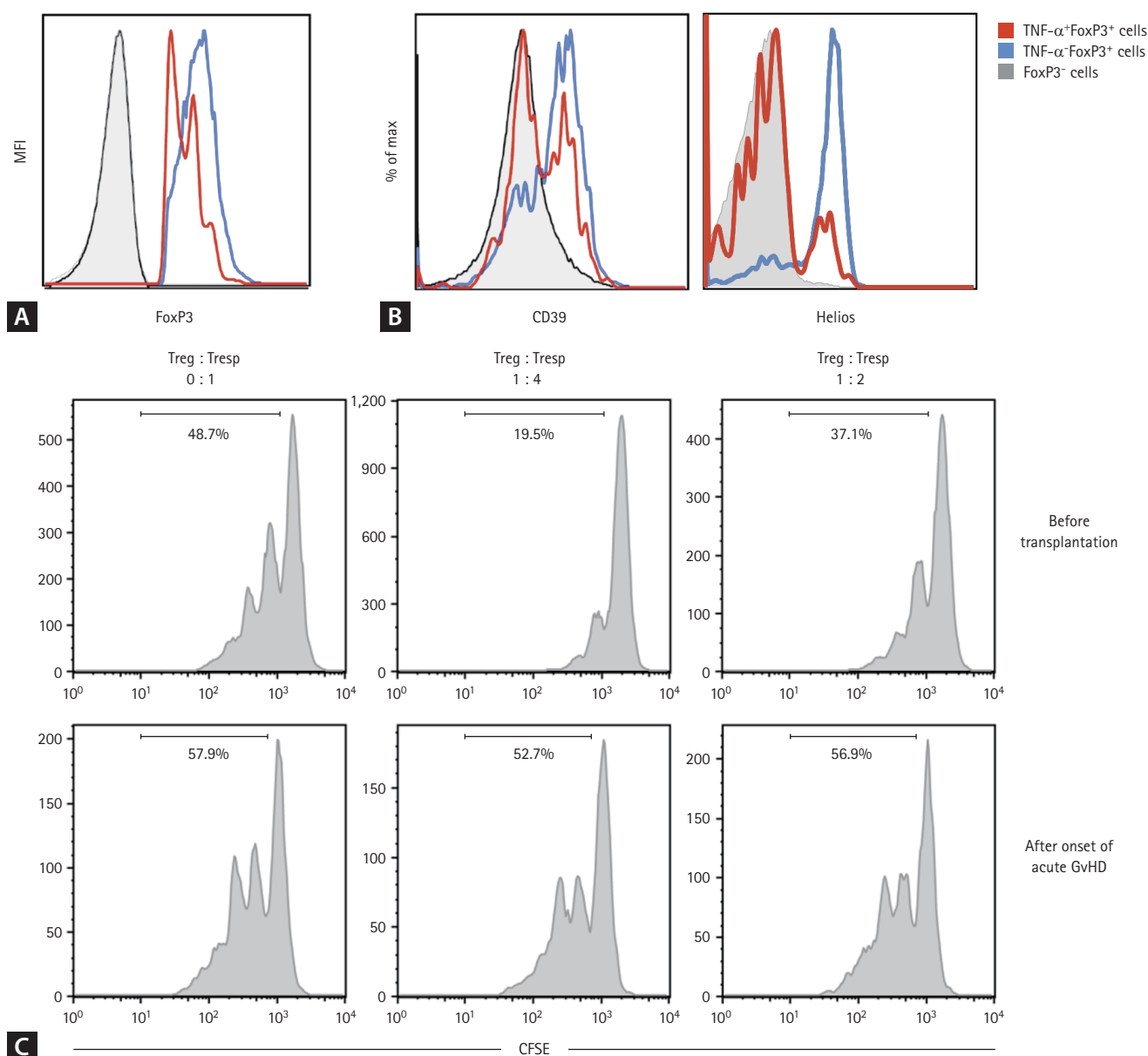


Figure 3. Functional characteristics of TNF- α producing Treg cells. (A, B) Several immunophenotypes known to be related to Treg cell suppressive function were analyzed by flow cytometry. (A) Expression levels of FoxP3 based on MFIs in TNF- α ⁺ and TNF- α ⁻ Treg cells are measured and compared. (B) Expression levels of CD39 and Helios based on percentage of CD39⁺ cells and Helios⁺ cells in TNF- α ⁺ and TNF- α ⁻ Treg cells are measured and compared. (C) Histograms showing Tresp cell proliferation in the absence or presence of different ratio of Treg cells were analyzed with CFSE assay. A representative data of a subject both at baseline and after development of acute GvHD are shown. MFI, mean fluorescence intensity; Treg, regulatory T; Tresp, responder T; CFSE, carboxyfluorescein succinimidyl ester; GvHD, graft-versus-host disease.

CD4⁺CD25^{hi}CD127^{lo/-}FoxP3⁺ Treg cells from acute GvHD patients produce inflammatory cytokine TNF- α

We assessed the production of inflammatory cytokines from Treg cells following TCR stimulation of peripheral blood lymphocytes with anti-CD3/CD28 antibody and intracellular cytokine staining. As a result, we found that a remarkable proportion of CD4⁺CD25^{hi}FoxP3⁺ Treg cells of acute GvHD patients produced TNF- α upon TCR stimulation (Fig. 2A). Notably, a significant proportion of TNF- α -producing Treg cells also secreted IL-17A simultaneously (Fig. 2A). These findings align with the increased CD45RA-FoxP3^{lo} fraction observed among Treg cell subsets in aGvHD patients (Fig. 1B).

To further explore the clinical implications of this inflammatory Treg cell population, we analyzed their correlation with the progression and severity of acute GvHD. The frequency of TNF- α ⁺ cells among circulating Treg cells significantly correlated with the severity of acute GvHD, with higher proportion of TNF- α ⁺ Treg cells in patients with the higher grade of acute GvHD (Fig. 2B). This suggests that the pathological conversion of Treg cells to a pro-inflammatory state, characterized by TNF- α production, contributes to immune-mediated tissue injury during acute GvHD.

Treg cell suppressive function is reduced during acute GvHD

Next, we evaluated immunophenotypes known to be related to Treg cell suppressive function using multicolor flow cytometry. Our analysis revealed that TNF- α ⁺ Treg cells of acute GvHD patients exhibited lower expression of FoxP3, a master transcriptional factor of Treg cell function, compared to the TNF- α ⁻ counterpart (Fig. 3A). Similarly, the proportion of CD39⁺ cells, a marker associated with Treg cell suppressive activity, was reduced in TNF- α ⁺ Treg cells relative to TNF- α ⁻ cells (Fig. 3B). Additionally, we observed a decreased frequency of Helios-expressing Treg cells, a key determinant of Treg cell suppressive capacity, in TNF- α ⁺ Treg cells from acute GvHD patients (Fig. 3B).

We further performed Treg cell suppression assay to quantify their functional capacity on a per-cell basis, using peripheral blood T cells of recipient of allogeneic SCT both at baseline and post-acute GvHD onset. Notably, the suppressive activity of Treg cells during acute GvHD was significantly diminished compared to pre-SCT controls, as evidenced by reduced inhibition of non-Treg CD4⁺ T cell proliferation in co-culture experiments (Fig. 3C). These findings indicate

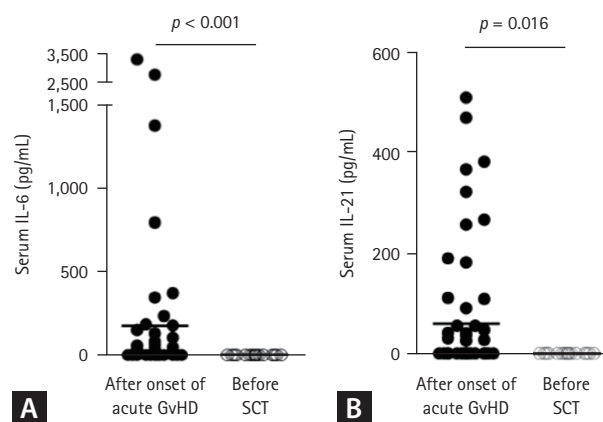


Figure 4. Polarizing cytokines in sera of acute hepatitis A patients. (A, B) In sera, concentrations of lineage-polarizing cytokines, including IL-6 (A) and IL-21 (B) were measured using cytometric bead array and ELISA respectively. The serum concentrations of cytokines were measured both at baseline and after development of acute GvHD and compared between the two time points. ELISA, enzyme-linked immunosorbent assay; GvHD, graft-versus-host disease; SCT, stem cell transplantation.

that the impaired suppressive function of Treg cells, coupled with their inflammatory conversion, may contribute to the immunopathogenesis of acute GvHD.

Th17-polarizing cytokines are abundant in the serum of acute GvHD patients

To investigate a potential mechanism underlying inflammatory changes in Treg cells, we quantified various soluble factors in systemic circulation before and after onset of acute GvHD. Our analysis demonstrated that serum levels of IL-6 (Fig. 4A) and IL-21 (Fig. 4B) were significantly elevated following the onset of acute GvHD compared to baseline levels. These findings suggest that these Th17-polarizing cytokines may play a pivotal role in driving the inflammatory conversion of Treg cells during the course of acute GvHD, thereby contributing to immune dysregulation and tissue injury.

DISCUSSION

Our study underscores the critical role of the inflammatory conversion of Treg cells in the pathogenesis of human acute GvHD. Beyond a reduction in the frequency of circulating Treg cells, these cells undergo functional changes, acquiring the ability to produce pro-inflammatory cytokines such as TNF- α and IL-17A, which significantly contribute to im-

mune-mediated tissue damage. Furthermore, these inflammatory Treg cells demonstrated diminished suppressive activity and were strongly correlated with the clinical severity of acute GvHD, highlighting their pathogenic roles.

Many studies have demonstrated that the frequency of peripheral blood Treg cells is inversely associated with tissue inflammation in various autoimmune disorders [17]. Similarly, our findings showed a reduction in the number of circulating Treg cells at the onset of acute GvHD. However, given that human Treg cells comprise heterogeneous functional subpopulations, the absolute number of total Treg cells may not fully reflect their pathophysiological significance [18-20]. In this study, we conducted a detailed analysis of three distinct functional subsets of Treg cells. Specifically, we observed a decrease in the proportion of CD45RA⁺FoxP3^{hi} Treg cells, known for their strong suppressive activity, alongside an increase in CD45RA⁺FoxP3^{lo} Treg cells, which exhibit cytokine-producing, pro-inflammatory characteristics. These shifts in Treg cell subpopulations suggest that the balance between suppressive and pro-inflammatory Treg cells plays a pivotal role in the progression of acute GvHD.

The mechanisms underlying the functional alterations of Treg cells during acute GvHD remain poorly understood. It has been generally hypothesized that the inflammatory environment present in various immune disorders contributes to these unexpected functional changes [21,22]. In this study, we aimed to identify potential drivers of the inflammatory conversion of Treg cells and propose that elevated levels of pro-inflammatory cytokines, such as IL-6 and IL-21, found in the peripheral blood of acute GvHD patients, may play a crucial role. Both IL-6 and IL-21 are well-known for promoting the differentiation of naïve CD4⁺ T cells into the Th17 lineage in both in vitro and in vivo settings, resulting in IL-17A production and subsequent tissue inflammation [23]. Consistent with this, our study documented that TNF- α -producing Treg cells also produced IL-17A, indicating a simultaneous acquisition of multiple pro-inflammatory properties during acute GvHD.

In addition to uncovering the underlying mechanisms of Treg cell conversion, our findings suggest that elevated levels of Th17-polarizing cytokines may have significant implications for the development of novel therapeutic strategies against acute GvHD. Targeting these cytokines and preventing the inflammatory conversion of Treg cells could help mitigate the severity of acute GvHD. In clinical practice, monitoring the frequency and phenotype of inflammatory

Treg cells could serve as a valuable biomarker for predicting acute GvHD severity and treatment response [24]. Furthermore, therapeutic approaches aimed at stabilizing Treg cell function or preventing their conversion into pro-inflammatory cells have the potential to significantly improve clinical outcomes for patients with acute GvHD [25,26].

Inflammatory conversion represents a broader paradigm of immune regulation, with relevance to a variety of immune-mediated disorders. Future research should explore additional molecular mechanisms affecting Treg cell plasticity and stability. A deeper understanding of Treg cell plasticity and inflammatory conversion could pave the way for targeted interventions that enhance immune regulation while preventing pathological immune responses across multiple immune-mediated conditions.

KEY MESSAGE

1. During human acute GvHD, frequencies of circulating Treg cells are significantly decreased.
2. Treg cells undergo inflammatory conversion, producing pro-inflammatory cytokines TNF- α and exhibiting reduced suppressive activity.
3. This conversion is closely associated with clinical severity, suggesting a role in the immunopathogenesis of acute GvHD.

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Conflicts of interest

The authors disclose no conflicts.

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