Biology

The Expression of Th17-Associated Cytokines in Human Acute Graft-versus-Host Disease

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A B S T R A C T
The role of Th17 cells and Th17-associated cytokines in the development of acute graft-versus-host disease (aGVHD) in clinical allogeneic hematopoietic stem cell transplantation (allo-HSCT) recipients is not well established. In the current study, a cohort of 69 allo-HSCT patients was examined for the percentages of Th17 and FoxP3+ Treg cells and the expressions of RORyt and FoxP3 in peripheral blood mononuclear cells (PBMCs). The Th17 percentage and RORyt expression were significantly higher, whereas Treg percentage and FoxP3 expression were significantly lower in severe aGVHD (grade 3 to 4) and mild aGVHD (grade 1 to 2) patients than in patients without aGVHD (grade 0) and healthy donors. We then investigated the expressions of Th17-associated cytokines, including TGF-β, IL-6, IL-1β, IL-17, IL-21, IL-22, IL-23, as well as IL-23R in the PBMCs of patients after allo-HSCT. The expressions of IL-17 and IL-22 in CD4+ T cells were also examined. The results showed that the expressions of IL-6, IL-1β, IL-17, IL-21, IL-23, and IL-23R were all increased, whereas IL-22 expression was decreased in aGVHD patients. The changes were also correlated with the severity of aGVHD. We also investigated the dynamic changes of Th17/Treg cells and Th17-associated cytokines in patients during the onset and resolution of aGVHD. The results demonstrated a reciprocal relationship between Treg and Th17 cells. Th17-associated cytokine expressions, namely IL-17 and IL-23, were closely related to the occurrence and resolution of aGVHD. We conclude that the dynamic balance between the Th17 and FoxP3+ Treg cells and the changes of Th17-associated cytokines could be the indicators of the disease progression and promising candidates of prognostic biomarkers of aGVHD.

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INTRODUCTION
Acute graft-versus-host disease (aGVHD) is a leading cause of nonrelapse mortality following allogeneic hematopoietic stem cell transplantation (allo-HSCT) [1]. aGVHD is a systemic disease mainly targeting the skin, liver, and gastrointestinal tract [2,3]. It is characterized by exaggerated inflammatory responses, during which the production of proinflammatory cytokines enhances the ability of donor T lymphocytes to attack the host tissues and to produce more proinflammatory cytokines, perpetuating the disease process by contributing to the cytokine storm that initiates aGVHD.

Th17 cells were characterized by production of interleukin-17 (IL-17) A, IL-17F, IL-21, and IL-22 [4-7]. Th17 differentiation requires TGF-β and IL-6 [8]. Stabilization of Th17 phenotype requires IL-23, together with TNF and IL-1β, whereas their expression is mainly dependent on IL-21 [9,10]. Studies with murine models showed conflicting results, with some data suggesting that Th17 is protective [11] and others indicating a pathogenic role in aGVHD [12,13]. In humans, there are also divergent results with retrospective reports that circulating and/or tissue localized Th17 cells can be increased [14,15] or decreased [16] in aGVHD. Although the role of Th17 in aGVHD needs to be further determined, some Th17-related cytokines, particularly IL-6 and IL-23, have been shown to play critical roles in aGVHD [17-21]. Furthermore, both IL-21 blockade and abrogation of donor T cell IL-21 signaling reduced GVHD mortality [22,23]. On the other hand, a recent study showed that IL-22 regulated tissue sensitivity to aGVHD and protected mice from aGVHD tissue damage and mortality [24]. Despite the extensive studies in the murine models, the expression of Th17-related cytokines in aGVHD patients is not well studied.

CD4+CD25+FoxP3+ regulatory T cells (Tregs) play a pivotal role in regulation and maintenance of immune tolerance to self-antigens. Many studies have shown that the increasing frequencies of Tregs are associated with a lower incidence of aGVHD in patients who have undergone allo-HSCT [25-29]. It has been suggested that the Th17/Treg ratio could be a sensitive and specific biomarker of aGVHD. Therefore, the balance between Treg and Th17 may be essential for maintaining immune homeostasis in aGVHD patients. However, the decrease of Tregs was correlated with increases in the frequency of Th17 cells only in a very small subset of patients in 1 study [14].

The aim of this study was to determine the percentages of peripheral Treg and Th17 cells and the expression of their related cytokines in aGVHD patients. We also monitored the dynamic changes of Treg and Th17 cells, as well as IL-17,
To inaugurate aGVHD grade 2 was treated by increasing the dose of post-transplantation immunomodulatory suppressive drugs and/or substituting FK506 for CSA.

Table 1: Clinical Characteristics of Patients (n = 69)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>n (%)</th>
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<tbody>
<tr>
<td>No. of patients</td>
<td>69</td>
</tr>
<tr>
<td>Age, yr, median (range)</td>
<td>34 (11 to 57)</td>
</tr>
<tr>
<td>Sex (female/male)</td>
<td>28/41</td>
</tr>
<tr>
<td>Diagnosis</td>
<td></td>
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<tr>
<td>Acute lymphoblastic leukemia</td>
<td>20 (29.0)</td>
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<tr>
<td>Acute myeloid leukemia</td>
<td>29 (42.1)</td>
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<tr>
<td>Chronic myeloid leukemia</td>
<td>7 (10.1)</td>
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<tr>
<td>Lymphoma</td>
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<tr>
<td>Myelodysplastic syndrome</td>
<td>6 (8.7)</td>
</tr>
<tr>
<td>Severe aplastic anemia</td>
<td>4 (5.8)</td>
</tr>
<tr>
<td>Paroxysmal nocturnal hemoglobinuria</td>
<td>1 (1.4)</td>
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<tr>
<td>Conditioning regimen</td>
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</tr>
<tr>
<td>Bu-based</td>
<td>52 (75.4)</td>
</tr>
<tr>
<td>TBI-based</td>
<td>14 (20.3)</td>
</tr>
<tr>
<td>Flu + CTX + ATG</td>
<td>3 (4.3)</td>
</tr>
<tr>
<td>Acute GVHD grade</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>12 (17.4)</td>
</tr>
<tr>
<td>2</td>
<td>16 (23.2)</td>
</tr>
<tr>
<td>3</td>
<td>5 (7.2)</td>
</tr>
<tr>
<td>4</td>
<td>12 (17.4)</td>
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<tr>
<td>Site of aGVHD</td>
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<tr>
<td>Skin</td>
<td>14 (20.3)</td>
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<tr>
<td>Liver</td>
<td>2 (2.9)</td>
</tr>
<tr>
<td>Gut</td>
<td>20 (29.0)</td>
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<tr>
<td>Multiorgans</td>
<td>9 (13.0)</td>
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<tr>
<td>Donor type</td>
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<tr>
<td>Sibling</td>
<td>39 (56.5)</td>
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<tr>
<td>MUD</td>
<td>20 (29.0)</td>
</tr>
<tr>
<td>Haplo-HSCT</td>
<td>7 (10.2)</td>
</tr>
<tr>
<td>UCBT</td>
<td>3 (4.3)</td>
</tr>
<tr>
<td>Days to aGVHD onset, median (range)</td>
<td>32 (15 to 105)</td>
</tr>
</tbody>
</table>

Bu indicates busulam; TBI, total body irradiation; GVHD, graft-versus-host disease; MUD, matched unrelated donor; Haplo-HSCT, haploidentical hematopoietic stem cell transplantation; UCBT, umbilical cord blood transplantation; Flu, fludarabine; ATG, antithymocyte globulin; CTX, cyclophosphamide.

IL-23, and IL-22 serum levels during the onset of aGVHD. These studies provided important evidence in human aGVHD that Treg and Th17 cells played important roles during aGVHD, and some of their related cytokines could be targeted for therapeutic purposes.

Isolation of Human CD4+ T Cells

CD4+ T cells were purified from patients’ peripheral blood using Human CD4+ T cell Enrichment Cocktail (Stemcell, Vancouver, BC). Briefly, Human CD4+ T cell Enrichment Cocktail was added at 50 µL of whole blood and incubated for 20 minutes at room temperature. Samples were diluted with an equal volume of PBS + 2% fetal bovine serum (FBS) (Gibco, Grand Island, NY) and mixed gently. Dissociated cells were then layered on top of the density medium (Pharmacia, Piscataway, NJ) and centrifuged for 20 minutes at 1200 g at room temperature. The enriched cells were collected from the density medium for RNA extraction.

RNA Extraction and Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction (PCR)

Total RNA was isolated from PBMCs of CD4+ T cells using TRIzol Reagent according to the manufacturer’s directions (Invitrogen, Carlsbad, CA). cDNA was synthesized using M-MuLV reverse transcription, random hexamer primers, and 10 mM dNTP (Promega, Madison, WI). Real-time PCR was performed in triplicates using SYBR Green PCR. The primer sequence genes were as follows: Foxp3 forward primer: GTGGCATCATCCATCCAGAAC; Foxp3 reverse primer: TCTTGAGACTCTTCTGGAAT; RORγt forward primer: CCGTGTAGATTGRTCAGG; RORγt reverse primer: GATGTTGGCTGGCTGATGA; IL-17a forward primer: CAATCCCAAGATCCAGT; IL-17a reverse primer: GCAGTACATTCGCA; IL-10 forward primer: GGCAGGAGCA; IL-10 reverse primer: TTCTGCTACTTGCTGCT; IFNγ forward primer: TTTGGTGGGTGTGTCAGAT; IFNγ reverse primer: GGCAGGAGCA; IL-23 forward primer: TCTCTGATTGTAGCTTTCCT; IL-23 reverse primer: AAGACGTCACAGCCTGGT; IL-23 reverse primer: ATGCCTGCTTACCT; IL-22 forward primer: GATGTTGGCTGGCTGATGA; IL-22 reverse primer: TTCTGCTACTTGCTGCT; IL-23 forward primer: GGAGATGAG. The mRNA expression was determined by real-time PCR using SYBR Green Master Mix (Applied Biosystems, Foster City, CA). Thermocycler conditions comprised an initial holding at 50°C for 2 minutes and a subsequent holding at 95°C for 10 minutes, which was followed by a 2-step PCR program at 95°C for 15 seconds and 60°C for 60 seconds for 40 cycles. Data were collected and quantitatively analyzed on an ABI Prism 7500 HT Sequence Detection System (ABI, Foster City, CA). Human GAPDH gene was used as an endogenous control for sample normalization. Results were presented as folds relative to the expression of GAPDH. Relative transcripts were determined by the formula: 2^((-ΔΔCt)) and permitted to set values below detection levels.

Measurement of Cytokines

Serum samples from consecutive patients were collected as soon as aGVHD was diagnosed and before therapy was begun. We also collected blood samples from 18 age-matched healthy donors as controls.

Flow Cytometry and Intracellular Cytokine Staining

PBMCs were separated by density gradient centrifugation with lymphocyte separation medium (Invitrogen Life Technologies, Carlsbad, CA) in the presence of 5 g/mL brefeldin A (eBioscience, San Diego, CA) in a tissue culture incubator at 37°C and 5% CO2. The cells were labeled with anti-CD4 mAbs. After fixation and permeabilization, cells were stained with anti-Foxp3 PE and analyzed on a BD FACS (Becton Dickinson [BD], San Jose, CA). For IL-17 and IFN-γ staining, PBMCs were incubated for 4 hours with 50 ng/mL phorbol myristate acetate and 750 ng/mL ionomycin (both from Becton Dickinson, San Jose, CA or Lightspeed, Carlsbad, CA) in the presence of 5 g/mL brefeldin A (eBioscience, San Diego, CA) in a tissue culture incubator at 37°C and 5% CO2. The cells were labeled with anti-CD4 mAbs. Afterward, cells were fixed and permeabilized with FACs Permeabilizing Solution (Becton Dickinson) and stained with anti-IFN-γ-APC (eBioscience, San Diego, CA) and anti-IL-17-PE mAbs (BD Pharmingen, San Diego, CA). As a control, cells were also stained with isotype mAbs. Cells were washed in ice-cold PBS that contained BSA (1%) and azide (0.1%) and analyzed on a BD FACS using Cell Quest software (BD Biosciences, San Jose, CA).

PATIENTS, MATERIALS, AND METHODS

Patients and Data

Sixty-nine patients who underwent allo-HSCT between August 2010 and September 2011 at the Center for Hematopoietic Stem Cell Transplantation at the First Affiliated Hospital of Soochow University were included in this study. The characteristics of these patients are shown in Table 1. Informed consent was obtained from all patients, with approval of the institutional review board. The conditioning regimen consisted of either busulfan (8 mg/kg intravenously [i.v.] every 6 hours for 12 doses) followed by cyclophosphamide (CTX) (1.8 g/m2 i.v. every 6 hours for 12 doses) or total body irradiation (TBI) (850 cGy daily for 1 day) followed by Cy. All patients with matched unrelated donor transplantsations and haplo-identical related donor transplantsations were given ATG (Thymoglobulin; Sangstat, Fremont, CA) 4 mg/kg/day to 8 mg/kg/day for 3 to 5 days, given from day -5 or day -3 until day -1. Twenty-three patients were given peripheral blood stem cells (PBSCs) from donors treated with granulocyte colony-stimulating factor. The other 46 patients received mobilized bone marrow (BM) cells plus PBSCs. GVHD prophylaxis consisted of a combination of either cyclosporine A (CSA) and short-term methotrexate (MTX) or CSA, short-term MTX, and mycophenolate mofetil. The dosage of CSA was 2.5 mg/kg/day, i.v. from day -9. MMF was administered orally, 5 g every 12 hours from day -9 to engraftment. The dosage of MTX was 15 mg/m2 administered i.v. on day +1 and 10 mg/m2 on days +3, +6, and +11.

Before initiation of the treatment, patients underwent a thorough evaluation to ascertain the severity and extent of their aGVHD, including a physical examination, laboratory evaluations, and a consultation without the tissue biopsy results. Each organ (skin, liver, and gut) was staged 1 through 4 for aGVHD according to modified criteria based on the schema of Glucksberg et al. [30]. Patients were also assigned a grade of aGVHD (1 through 4) based on overall severity [31]. aGVHD grade 2 was treated with high-dose methylprednisolone starting at 2 mg/kg/day, MTX, and a CD25 monoclonal antibody (Novartis, Basel, Switzerland) was given to subjects intolerant of or unresponsive to methylprednisolone. Acute GVHD grade 2 was treated by increasing the dose of post-transplantation immunomodulatory suppressive drugs and/or substituting FK506 for CSA.
of IL-17 (eBioscience, San Diego, CA), IL-22, and IL-23 (both from R&D systems, Minneapolis, MN). All assays were performed in triplicates according to the manufacturers' instructions.

**Statistical Analysis**

Data were analyzed by Pearson correlation and Mann-Whitney test with the use of Prism 5.00 for Windows software (Graph Pad Software, San Diego, CA). *P* values < .05 were considered statistically significant.

**RESULTS**

**GVHD**

All patients achieved a sustained and stable donor engraftment. Among the 69 HSCT patients, 45 (65.2%) patients developed aGVHD. Among the 45 patients who developed aGVHD, 12 (26.7%) were grade 1, 16 (35.5%) were grade 2, 5 (11.1%) were grade 3, and 12 (26.7%) were grade 4. The median day of onset of aGVHD was 32 (range, 15 to 94). At 100 days after transplantation, the cumulative incidence was 47.8% for grade 2 to 4 aGVHD, and 24.6% for grade 3 to 4 aGVHD. Thirty-three episodes of grade 2 to 4 aGVHD were treated with methylprednisolone, and 21 (63.6%) episodes were treated successfully, whereas the 12 episodes that lacked adequate response to the primary treatment were treated with intravenous MTX at a dose of 10 mg once every 7 days.

![Figure 1](image_url)

**Figure 1.** CD4<sup>+</sup>FoxP3<sup>+</sup>Treg cells decreased and Th17 cells increased in PBMCs of aGVHD patients. Peripheral blood of transplant patients was analyzed for CD4<sup>+</sup>FoxP3<sup>+</sup>Treg and Th17 cell percentages. (A) Representative plot of flow cytometric detection of CD4<sup>+</sup>FoxP3<sup>+</sup>Treg and Th17 cells. (B) (C) The percentages of Tregs and Th17 cells were evaluated in patients without aGVHD (grade 0), mild aGVHD (grade 1 to 2), severe aGVHD (grade 3 to 4), and healthy donors (HD). *P* < .05, *P* < .01, *P* < .001.

![Figure 2](image_url)

**Figure 2.** The expression of FoxP3 was down-regulated and RORγt expression was up-regulated in aGVHD patients. (A) Shows FoxP3 expression from PBMCs of patients without aGVHD (grade 0), patients with mild aGVHD (grade 1 to 2), patients with severe aGVHD (grade 3 to 4), and healthy donors (HD). (B) Shows RORγt expression from PBMCs of patients without aGVHD (grade 0), patients with mild aGVHD (grade 1 to 2), patients with severe aGVHD (grade 3 to 4), and healthy donors (HD). (C) RORγt expression negatively correlated with FoxP3 expression (*r^2 = .1237, P* < .01). *P* < .05, *P* < .01, *P* < .001.
anti-CD25 monoclonal antibody, or mesenchymal stem cell therapy. Patients were scheduled to receive at least 2 doses of MTX administrations for evaluation of the drug’s efficacy. Eight episodes showed improvement, but the remaining 4 patients had exacerbated GVHD and subsequently died.

**CD4⁺ FoxP3⁺ Treg Cells Decreased and Th17 Cells Increased in PBMCs of aGVHD Patients**

PBMCs from patients without aGVHD (grade 0), mild aGVHD (grade 1 to 2), severe aGVHD (grade 3 to 4), and healthy donors were collected and stained for CD4, IL-17, and FoxP3. Representative flow cytometry results are shown in Figure 1A. CD4⁺FoxP3⁺ T cells from patients with aGVHD (grade 1 to 2 and grade 3 to 4) were significantly decreased compared with patients without aGVHD (grade 0) (P < .05, P < .001) and healthy donors (P < .05, P < .01) (Figure 1B). Furthermore, the percent of CD4⁺FoxP3⁺ Tregs was even lower in severe aGVHD (grade 3 to 4) patients compared with that of patients with mild aGVHD (grade 1 to 2) (P < .05), suggesting the percent of CD4⁺FoxP3⁺ Tregs could be correlated with the severity of the aGVHD. Percent of Th17 cells in PBMCs from severe aGVHD patients (grade 3 to 4) was significantly increased compared with patients with mild aGVHD (grade 1 to 2) (P < .01), patients without aGVHD (grade 0) (P < .001), and healthy donors (P < .01) (Figure 1C).
Patients with mild aGVHD (grade 1 to 2) showed slight increases in the percent of Th17 cells compared with patients without aGVHD and healthy donors, but did not reach statistical significance.

To confirm the changes of Treg and Th17 subsets, PBMCs from the patients were measured for FoxP3 and ROR
\[ \text{transcript levels by real-time PCR. All transplantation} \]
\[ \text{patients had lower levels of FoxP3 expression compared} \]
\[ \text{with healthy donors (Figure 2A). FoxP3 expression in} \]
\[ \text{PBMCs from patients at the onset of aGVHD (grade 1 to 2} \]
\[ \text{and grade 3 to 4) (within 24 to 48 hours of onset and before} \]
\[ \text{active treatment) were significantly decreased compared} \]
\[ \text{with patients without aGVHD (grade 0) (P < .01, P < .001) and healthy donors (both P < .001). In addition, patients} \]
\[ \text{with severe aGVHD (grade 3 to 4) showed significant lower} \]
\[ \text{FoxP3 expression than the patients with mild aGVHD} \]
\[ \text{(grade 1 to 2) (P < .05), correlating with the flow cytometry} \]
\[ \text{results.} \]

On the contrary, patients with aGVHD (grade 1 to 2 and grade 3 to 4) displayed significantly higher expression of the Th17 master gene regulator ROR\[ \text{t than patients without aGVHD (grade 0) (P < .01, P < .001) and healthy donors (P < .05, P < .01) (Figure 2B). Similar to the flow cytometry} \]
\[ \text{results, the ROR\[ \text{t expression was also higher in severe} \]
\[ \text{aGVHD patients (grade 3 to 4) than that in mild aGVHD} \]
\[ \text{patients (grade 1 to 2) (P < .001). Moreover, the ROR\[ \text{t} \]
\[ \text{expression was negatively correlated with the expression of} \]
\[ \text{FoxP3 in all transplantation patients (r^2 = .237, P < .01) (Figure 2C). These data demonstrated the decrease of Treg} \]
\[ \text{and increase of Th17 cells in aGVHD patients, suggesting the} \]
\[ \text{imbalance of the 2 CD4^+ T cell subsets may play important} \]
\[ \text{role during the onset of aGVHD.} \]

Figure 4. Correlation between Th17-related cytokine and FoxP3 and IL-17 expressions. Correlations among TGF-β (A), IL-6 (B) expressions, and FoxP3 expression were analyzed. Correlations among IL-17 expression and TGF-β (C), IL-6 (D), IL-1β (E), IL-21 (F), IL-22 (G), IL-23 (H), serum levels of IL-23 (I), and IL-23R (J) expressions are shown.
Expression of Th17-Associated Cytokines in aGVHD Patients

The expressions of the Th-17-associated cytokines TGF-β, IL-6, IL-1β, IL-21, IL-22, IL-23, and IL-23R in the PBMCs of patients who underwent allo-HSCT were investigated. Both IL-17 and IL-23 mRNA levels in PBMCs from patients at the onset of severe aGVHD (grade 3 to 4) were significantly increased compared with patients of mild aGVHD (grade 1 to 2) (both $P < .001$, $P < .05$), patients without aGVHD (grade 0) (both $P < .01$, $P < .001$), and healthy donors ($P < .05$, $P < .01$) (Figure 3A,B). The IL-17 expressions in CD4$^+$ T cells showed similar differences, except that the increase of expressions in mild aGVHD reached statistical significance ($P < .05$). Furthermore, the serum levels of IL-17 and IL-23 were significantly higher in patients with severe aGVHD (grade 3 to 4) than those in patients with mild aGVHD (grade 1 to 2) ($P < .001$, $P < .05$) and patients without aGVHD (both $P < .01$, $P < .001$). Patients with mild aGVHD (grade 1 to 2) also showed higher IL-17 serum levels than the patients without aGVHD ($P < .01$). Interestingly, IL-23R expression was also higher in aGVHD patients (grade 1 to 2 and grade 3 to 4) than in patients without aGVHD (grade 0) ($P < .01$, $P < .001$) and healthy donors ($P < .05$, $P < .01$) (Figure 3H). IL-6 and IL-21 were up-regulated in aGVHD (grade 1 to 2 and grade 3 to 4) patients compared with patients without aGVHD (grade 0) ($P < .01$, $P < .05$) (Figure 3E,F). All transplantation patients had...
increased levels of IL-1β compared with healthy donors (Figure 3G). It was even higher in aGVHD (grade 1 to 2 and grade 3 to 4) patients than in patients without aGVHD (grade 0) (both $P < .001$). Severe aGVHD patients (grade 3 to 4) also showed higher IL-1β expression than mild aGVHD patients (grade 1 to 2) ($P < .001$). Unlike the above cytokines, IL-22 expression in PBMCs significantly decreased in severe aGVHD (grade 3 to 4) patients compared with patients without aGVHD and healthy donors (both $P < .05$). IL-22 expression in CD4$^+$ T cells showed the same results. Moreover, it also significantly decreased in mild aGVHD patients (grade 1 to 2) compared with that in the patients without aGVHD ($P < .05$). Furthermore, the serum levels of IL-22 were significantly reduced in patients with aGVHD and correlated with the severity of aGVHD. TGF-β expression was not significantly changed among all patients and healthy donors, except the expression in severe aGVHD patients (grade 3 to 4) was significantly reduced compared with the patients without aGVHD ($P < .05$).

We then explored the correlations between the examined cytokines and FoxP3, as well as IL-17 expressions, in aGVHD (grade 1 to 4) patients (Figure 4). TGF-β, but not IL-6, was positively associated with FoxP3 expression (Figure 4A B). IL-1β, IL-21, IL-23, and IL-23R expression were positively associated with IL-17 expression (Figure 4E–H). The serum levels of IL-23 were also positively correlated with IL-17 serum levels (Figure 4I). It seemed that IL-6 was positively associated with IL-17 expression and IL-22 was negatively associated with IL-17 expression, but the correlation did not reach the statistical significance (Figure 4D,G). TGF-β showed no correlation with IL-17 expression (Figure 4C). The results demonstrated that the expression of most Th-17-associated cytokines increased in aGVHD patients and their expressions were significantly associated with IL-17.

### Dynamic Changes of Th1, Th17, and FoxP3$^+$ Treg Cells and IL-17, IL-22 Expression during the Onset and Resolution of aGVHD

To investigate the dynamic changes of Th1, Th17, and FoxP3 Treg cells during the onset of aGVHD, 4 patients were followed for their Th1, Th17, and FoxP3 Treg cell percentages in PBMCs during the occurrence and resolution of aGVHD (Figure 5A–D). In the patient with unique patient number (UPN) 3 gut aGVHD (grade 3) occurred at days +84 and resolved after application of methylprednisolone (2 mg/kg) in the next few days. Accordingly, we demonstrated relatively higher levels of Th1 and Th17 and a lower level of FoxP3$^+$ Treg cell proportion in the PBMCs on day +84 (Figure 5A). During the progressive resolution of aGVHD, Th1 and Th17 cells decreased to almost undetectable levels, with an increase of Treg cells on day +98. In line with clinical manifestation and Th17 percentages, serum levels of IL-17 and IL-23, as well as IL-17 and RORγt transcript levels in CD4$^+$ T cells, dropped, but IL-22 serum levels and mRNA expression in CD4$^+$ T cells were lightly increased. In UPN 5, we detected a very low level of Th1 and Th17 cells on day +59 after HSCT, corresponding to stable donor engraftment and no aGVHD (Figure 5B). On day +74, patients with large area skin rashes (>75%) received methylprednisolone (2 mg/kg). Subsequently, abdominal pain and diarrhea occurred; FK506 and MTX 10 mg were given weekly. Corresponding to aGVHD activation, we observed an increase of Th1 and Th17 levels with a decrease of Treg cells. Serum levels of IL-17 and IL-23, as well as IL-17 and RORγt transcript levels in CD4$^+$ T cells, increased with a slight decrease of IL-22. In UPN 13, active GVHD (skin and liver involvement) occurred at day +36, which resolved after application of methylprednisolone and MTX in the next few days. Accordingly, we detected relatively higher levels of Th1 and Th17 cells and lower levels of Treg cells in PBMCs on day +36, which reversed when aGVHD was resolved on day +75 (Figure 5C). Serum levels of IL-17 and IL-23, as well as IL-17 and RORγt transcript levels in CD4$^+$ T cells, dropped after GVHD improvement with a slight increase of IL-22 serum levels. In UPN 16, who presented with a stable disease, we detected relatively lower Th1 and Th17 levels and progressively increased Treg levels from day +48 to +83 (Figure 5D). This trend was inverted after a severe aGVHD flare occurring on day +94. This patient suffered from multiple organ involvement (skin, liver, and gut) and was treated with FK506, MTX, and CD25 monoclonal antibody. At this time point, we detected an increase of Th1 and Th17 cells and a significant drop of Treg cells. Serum levels of IL-17 and IL-23, as well as IL-17 and RORγt transcript levels in CD4$^+$ T cells, increased simultaneously. Moreover, when aGVHD resolved to a stable disease on day +108, Th1 and Th17 cells decreased with an increase of Treg cells. Serum levels of IL-17 and IL-23, as well as IL-17 and RORγt transcript levels in CD4$^+$ T cells, also dropped. Both serum and transcription levels of IL-22 were very low in this patient. However, the serum level of IL-22 did slightly decrease when aGVHD occurred.

To study the dynamic changes of Th17-associated cytokine levels in the serum during the onset and resolution of aGVHD, 9 patients were measured for their serum IL-17, IL-23, and IL-22 levels before, during, and after aGVHD episodes (Figure 5E). All patients showed increased IL-17 and IL-23 serum levels during the onset of aGVHD and the cytokine levels dropped during the remission. On the contrary, patients showed decreased IL-22 serum levels during the onset of aGVHD and the cytokine levels increased during the remission.

Collectively, the results demonstrated a reciprocal relationship between Treg and Th17 cells. Th17-associated cytokine expression, especially IL-17 and IL-23, were closely related to the occurrence and resolution of aGVHD. The dynamic balance between the Th17 and FoxP3$^+$ Treg cells and changes of Th17-associated cytokines could be the indicators of the disease progression.

### DISCUSSION

In a total of 69 allo-HSCT patients, we first extensively studied the expression of Th17-associated cytokines, as well as Th17 and Treg percentages, and compared them among aGVHD patients, patients without aGVHD, and healthy donors. Th17 and most Th17-associated cytokines were up-regulated in aGVHD patients. The dynamic changes of Th17 and Treg cells, as well as Th17-associated cytokine levels, during the onset and resolution of aGVHD suggested they could be the indicators or therapeutic targets of aGVHD. Many reports have already confirmed the correlation between the frequency of Tregs and the severity of aGVHD. Miuia et al. [32] reported that FoxP3 mRNA expression was significantly decreased in PBMCs from patients with GVHD compared with patients without GVHD. Expression of FoxP3 was negatively correlated with the severity of GVHD. Rezvani et al. [25] found that patients who received HSCT with lower absolute numbers of CD4$^+$ FoxP3$^+$ T cells had a greater risk of developing GVHD. Our study confirmed the negative correlation between the frequency of Tregs and the severity of GVHD.

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aGVHD (Figure 1A). Patients with aGVHD (grade 3 to 4) had significantly decreased frequency of CD4⁺FoxP3⁺ Treg cells as compared with patients with aGVHD (grade 1 to 2). The expression of FoxP3 was also lower in aGVHD patients (Figure 2A).

Controversy exists in both mice and humans regarding the role of Th17 cells in the pathogenesis of aGVHD, although most of the studies indicated that Th17 promoted aGVHD. Carlson et al. [33] reported that a high dose of in vitro–differentiated Th17 cells mediated GVHD-related severe cutaneous and pulmonary lesions. IL-17 was also found to contribute to the early development of CD4⁺-mediated GVHD [12]. On the contrary, Yi et al. showed that IL-17⁻⁻⁻ donor T cells had elevated Th1 differentiation and induced severe aGVHD damage [11]. Iclozan et al. reported Th17 cells were sufficient but not necessary to induce aGVHD [13]. It was also suggested that Th17 might augment tissue-specific damage in skin and lung [34]. In humans, Dander et al. showed Th17-mediated aGVHD [14]. However, a study suggested that a Th17 to Treg ratio <1 was correlated with severe clinical and pathologic GVHD [16]. There was a recent human study showing that aGVHD was not associated with significant changes in the absolute numbers of the Th1 or Th17 cell subsets [35]. However, the proportions of the Th subsets could be more relevant to the overall function of the CD4⁺ T cells. Our results from aGVHD patients demonstrated that higher levels of Th17 and most Th17-associated cytokines were associated with clinical aGVHD, suggesting a possible role of Th17 and related cytokines in aGVHD pathogenesis. Moreover, we also demonstrated an inverse correlation between the proportion of Th17 and Treg cells during the onset and resolution of aGVHD. This result suggested a lower ratio of Th17 to Treg could indicate the progression of aGVHD remission compared with the ratio at the onset of the disease. Despite the possible involvement of the Th17 cells in aGVHD, Th1 cells may still play a dominant role in aGVHD, suggested by the similar and bigger changes of the Th1 cells in the representative patients (Figure 5).

Although antithymocyte globulin (ATG) is recommended for GVHD prophylaxis after allo-HSCT, evidence of efficacy of ATG is conflicting [36]. It has been suggested that ATG could induce the generation of Treg cells [37]. A recent study also showed that ATG during conditioning could reduce the frequencies of Th17 cells [35]. Therefore, ATG may influence the distributions of Th17 and Treg cells and the incidence of aGVHD. There were 30 patients who were treated with ATG during conditioning, and the overall frequencies of Th17 and Treg cells could be influenced by this treatment.

The progression of aGVHD depends heavily on the balance of proinflammatory and anti-inflammatory cytokines in the milieu, in which donor-directed T cell response occurs. In addition, the inflammatory milieu may destabilize the program of both natural and induced Tregs, converting them into inflammatory, effector-like phenotypes. Therefore, modulation of the Th17-Treg balance by cytokine blockade is a realistic and attractive strategy to prevent aGVHD. A large amount of data have shown that Th17 generation, expansion, stabilization, and function could be influenced by a set of cytokines, including (TGF)-β, IL-1β, IL-6, IL-21, IL-22, IL-17, and IL-23. We examined the expressions of the complete panel of the cytokines that may influence the frequencies of the Th17 cells with samples of the single cohort of patients. In addition, IL-17 and IL-22 expression were also examined with the purified CD4⁺ T cells to exclude the influence of other IL-17- and IL-22-producing cells. Furthermore, the serum levels of IL-17, IL-22, and IL-23 were also measured and tracked before, during, and at the remission stage of aGVHD to give a more complete picture of the expressions of Th17-associated cytokines. The results from the representative patients also supported a close correlation between Th17 cells and their associated cytokines, especially IL-17 and IL-23, at both transcriptional and serum levels.

IL-23 is not involved in the initial Th17 differentiation, but is necessary for the generation of a completely functional Th17 response [38]. Expression of IL-23 and IL-23R genes was up-regulated during GVHD in mouse colon [19]. Das et al. demonstrated that the selective protection of the colon that occurs as a consequence of inhibition of IL-23 signaling reduced GVHD without loss of the graft-versus-leukemia effect [21]. Another study indicated that deficiency of p19 in the allogeneic donor transplantation might reduce the inflammation caused by aGVHD [20]. In our study, in accordance with the already described IL-23/Th17 axis, IL-23 mRNA expressions in patients with aGVHD were significantly higher than those in healthy donors, and IL-23 and IL-23R expression were positively correlated with IL-17 expression. IL-23 serum levels were also elevated during the onset of aGVHD and decreased during disease remission. Our results strongly suggested a close correlation between IL-23 and aGVHD, as well as Th17 function. It could be a promising candidate for therapeutic targeting in aGVHD patients.

IL-6, IL-1β, and IL-21 were also elevated in severe aGVHD patients. Blockade of IL-1β has been shown to reduce aGVHD-related mortality [39]. However, the clinical trial using the same reagent did not show effectiveness in preventing aGVHD [40]. IL-6 is one of the central mediators of inflammation, which has a pivotal function in dictating whether T cells differentiate into Tregs or Th17 cells. Tawara et al. reported that anti-IL-6R mAb-mediated attenuation of GVHD was independent of the direct effects on effector T cell expansion or donor Tregs [18]. However, it may not be a good candidate for therapeutic blockade because of its regulatory role on both Th17 and Treg cells. IL-21 is produced by Th17 cells, and inhibition of IL-21 signaling on donor T cells attenuated GVHD, particularly in the intestine [22,23]. In humans, skin and colon samples obtained from patients with no GVHD or grade 2 to 4 GVHD were analyzed for IL-21 protein expression. IL-21 protein-producing cells were present in all gastrointestinal tract samples and 54% of skin samples obtained from GVHD patients, but not from GVHD-free controls. In a human xenogenic GVHD model, human IL-21-secreting cells were present in the colon of GVHD recipients and were associated with elevated serum IL-21 levels [41]. Our results showed elevated IL-21 expression in PBMCs of patients with aGVHD (grade 1 to 2 and grade 3 to 4) compared with patients without aGVHD (grade 0), suggesting the increased IL-21-producing cells in the circulation of aGVHD patients.

IL-22 is expressed predominantly in Th1 cells and Th17 cells. It was the only cytokine we examined that decreased and showed a negative correlation with IL-17 in aGVHD patients. A recent study showed that IL-22 could protect intestinal stem cells from inflammation-mediated tissue damage and reduce the sensitivity to aGVHD [24]. Our human studies support the protective role of IL-22 in aGVHD. The discrepancies between the increase of Th17 cells and the reduction of IL-22 expression in CD4⁺ T cells and serum levels suggest that Th17 may not be the main source of IL-22, which plays a role in aGVHD.
In conclusion, Th17 cells and most Th17-associated cytokines were up-regulated in aGVHD patients. The dynamic changes of Th17 and Treg proportions in PBMCs, as well as IL-17 and IL-23 serum levels, could be indicators of the onset and resolution of aGVHD. The imbalance of Th17 to Treg cells and their related cytokines could contribute to the immune dysregulation associated with aGVHD, which could be targeted for the development of therapeutic strategies.

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