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# Can Cytokine Detection Predict the Occurrence and Outcome of aGVHD after Allo-HCT? A Retrospective Study

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**Background: Many cytokines play essential roles in the occurrence and development of acute graft-versus-host-disease (aGVHD). This study aims to validate whether 11 proinflammatory and anti-inflammatory cytokines can be a candidate for aGVHD biomarkers to predict its occurrence and outcome.**

**Methods: Out of 178 patients who underwent allogeneic hematopoietic stem cell transplantation, we retrospectively enrolled 32 cases into the pre-transplant cohort and 45 cases into the post-transplant cohort. The serum cytokine concentrations were determined by flow cytometry. The control and experimental groups were non-aGVHD, I–II aGVHD and III–IV aGVHD groups, respectively. Risk factors and overall survival (OS) were also evaluated.**

**Results: In the pre-transplant cohort, interleukin (IL)-2 decreased in patients with aGVHD, and IL-4 only reduced in patients with III–IV aGVHD. In the post-transplant cohort, only IL-4 increased 1.79 times more in patients with III–IV aGVHD than in the other two groups. Patients with gastrointestinal (GI) aGVHD had lower IL-2, IL-4 and IL-17F levels pre-transplant and lower IL-2 post-transplant. None of the other cytokines was significantly different. Logistic regression analysis showed that no cytokine could predict the occurrence and outcome of aGVHD. Diarrhea within 15 days post-transplant is an independent risk factor for the occurrence of aGVHD and a risk factor for a fatal outcome. Patients without diarrhea had longer survival time of 672 (586–757) days vs 444 (229–548) days and better 2-year OS (85.7% vs 46.4%) than those with diarrhea. Compared to patients with aGVHD, patients without aGVHD had a longer survival time of 618 (530–706) days vs 449 (353–545) days and better 2-year OS (76.2% vs 47.1%).**

**Conclusions: Proinflammatory and anti-inflammatory cytokines can provide specific indications for the occurrence and progression of aGVHD. However, to truly guide the diagnosis and prognosis, cytokines with larger sample sizes, more detection time points and more accurate diagnostic efficacy need to be further studied.**

**Keywords:** hematopoietic stem cell transplantation; cytokine; acute graft-versus-host-disease

## Introduction

Acute graft-versus-host-disease (aGVHD) remains a major complication of hematopoietic stem cell transplantation (HSCT), causing early morbidity and mortality. The incidents of aGVHD were around 40–60% [1]. Allogeneic Hematopoietic Cell Transplantation (allo-HCT), especially haploidentical HSCT (haplo-HSCT), has been an effective alternative in the absence of human leukocyte antigen (HLA)-compatible donors, which has been widely applied in China in the last 10 years. Compared to HLA-matched HSCT, haplo-HSCT usually has a more powerful graft-versus-leukemia (GVL) response and an increased incidence of GVHD [2,3]. The diagnosis of aGVHD onset depends mainly on clinical manifestations and tissue biopsy. Noninvasive peripheral blood biomarkers that can accurately predict the occurrence and severity of aGVHD before clinical manifestations are needed to avoid invasive tissue biopsies.

Classically, aGVHD arises within the first 100 days after allo-HCT, mediated by donor T cells, leads to a cytotoxic effect against target organs, in which the skin, gastrointestinal (GI) tract, liver and lung are most commonly involved [4]. Other than direct T-cell-mediated cytotoxicity, researchers assumed that aGVHD is an immunologic cascade of events in which a network of cytokines may act as primary mediators of aGVHD and further induce tissue damage [5]. In the first phase of aGVHD, conditioning chemoradiotherapy and infections lead to inflammatory cytokines, such as interleukin (IL)-1$\beta$, IL-2, tumor necrosis factor (TNF)-$\alpha$ and IL-6, release and the expression of HLA and leukocyte adhesion molecules on target tissues increase

**Table 1. Patient characteristics.**

| | Non-aGVHD (N = 21) | aGVHD (N = 36) | $\chi^2$/Z | *p* |
|---|---|---|---|---|
| Sex | | | 0.856 | 0.355 |
| Male | 9 | 20 | | |
| Female | 12 | 16 | | |
| Age | 39 (21–50) | 31 (19–46) | –0.571 | 0.568 |
| Diagnosis | | | 8.381 | 0.097 |
| AML | 8 | 21 | | |
| ALL | 3 | 7 | | |
| Lymphoma | 1 | 1 | | |
| CML | 2 | 2 | | |
| Acute mixed lineage leukemia | 3 | 5 | | |
| Others | 4 | 0 | | |
| Disease risk | | | 3.959 | 0.047 |
| Standard | 10 | 8 | | |
| High | 11 | 28 | | |
| Disease state | | | 4.852 | 0.088 |
| Remission | 12 | 14 | | |
| Partial remission | 4 | 3 | | |
| Unremission | 5 | 19 | | |
| HCT-CI | | | 0.000 | 1.000 |
| 1–3 | 19 | 32 | | |
| 4–6 | 2 | 4 | | |
| Donor | | | 0.902 | 0.342 |
| Non-related | 5 | 5 | | |
| Related | 16 | 31 | | |
| HLA-matching | | | 0.128 | 0.938 |
| 10/10 matched | 6 | 9 | | |
| 6–9/10 matched | 4 | 8 | | |
| 5/10 matched | 11 | 19 | | |
| White blood cell recovery within a median time of × days | 13 (11–16) | 13 (11–16) | 0.033 | 0.973 |
| Platelet recovery within a median time of × days | 18 (12–31) | 15 (12–28) | –0.635 | 0.532 |

AML, Acute Myeloid Leukemia; ALL, Acute Lymphocytic Leukemia; CML, Chronic Myeloid Leukemia; HCT-CI, Hematopoietic Cell Transplantation Specific Comorbidity Index. *p* value was evaluated between groups using chi-square test and the Mann-Whitney U test.

[6,7]. In the second phase, transplanted donor T cells recognize alloantigen, activate, proliferate and differentiate into pathogenic or regulatory T cells (Tregs), regulated by IL-12, IL-23, IL-6, IL-27, IL-10 and transforming growth factor (TGF)-$\beta$ [8]. In the last phase, additional inflammatory cytokines, such as interferon (IFN)-$\gamma$, TNF-$\alpha$, IL-2, and IL-17, contribute to target tissue injury [5,9]. Therefore, cytokines may be biomarkers for early diagnosis of aGVHD and assess its severity and outcome. A profile of proinflammatory and anti-inflammatory cytokines, such as IL-1$\beta$, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IL-17F, TNF-$\alpha$ and IFN-$\gamma$ are core cytokines in the pathological process of aGVHD [2,10–12] and some of them are correlated with the risk and severity of aGVHD [13]. However, clinical indicator validation studies showed that most single-cytokine measurements lack requisite sensitivity and specificity for clinical diagnosis, mainly due to the network and pleiotropy of their actions [14]. Thus, a profile of cytokines should be evaluated on a more specific clinical condition. In clinical practice, the most common conditions that affect the concentrations of the abovementioned cytokines are infections, such as bloodstream infection [15], pneumonia [16], and the application of immunosuppressants [17]. After excluding the stimulating effect of pathogenic microorganisms and the inhibitory effect of excessive immunosuppressants, the obtained cytokine levels may truly reflect patients' actual immune states of aGVHD.

This study aimed to determine 11 plasma proinflammatory and anti-inflammatory levels in patients undergoing allo-HSCT and to associate the cytokine concentrations with the presence of aGVHD and overall survival in these patients.

## Materials and Methods

### *Study Population*

Out of 178 patients who underwent allogeneic hematopoietic stem cell transplantation between June 2019

**Table 2. Cytokine levels before and after allo-HCT in patients with/without aGVHD.**

| | Cohort I pre-transplant | | | | | Cohort II post-transplant | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Non-aGVHD (N = 11) | Grade I–II aGVHD (N = 18) | Grade III–IV aGVHD (N = 3) | H | *p* | Non-aGVHD (N = 13) | Grade I–II aGVHD (N = 28) | Grade III–IV aGVHD (N = 4) | H | *p* |
| IL-1$\beta$ | 0.57 (0.14–1.33) | 0.57 (0.22–1.74) | 1.14 (0.02–2.54) | 0.725 | 0.696 | 1.38 (0.06–2.9) | 1.23 (0.56–2.50) | 0.41 (0.23–0.44) | 3.108 | 0.211 |
| IL-2 | 7.41 (4.81–9.1) | 3.84[a] (2.13–4.63) | 3.36[a] (3.07–8.01) | 7.850 | 0.020 | 5.04 (4.04–6.12) | 3.99 (3.17–5.49) | 6.71 (3.94–8.06) | 3.486 | 0.175 |
| IL-4 | 2.51 (1.86–3.38) | 2.1 (1.72–2.35) | 1.51[b] (0.02–1.56) | 7.019 | 0.030 | 2.19 (1.8–2.56) | 2.13 (1.62–2.78) | 3.93[b] (3.13–5.0) | 6.944 | 0.031 |
| IL-6 | 38.16 (3.49–97.88) | 79.34 (12.25–238.34) | 43.65 (12.01–462.41) | 1.602 | 0.449 | 37 (13.91–48.67) | 24.43 (14.15–113.03) | 50.12 (8.87–101.52) | 0.130 | 0.937 |
| IL-8 | 72.67 (37.43–190.01) | 62.78 (39.86–128.06) | 42.58 (36.6–352.8) | 0.051 | 0.975 | 73.59 (26.54–246.35) | 35.93 (22.42–57.26) | 19.21 (16.18–74.86) | 3.592 | 0.166 |
| IL-10 | 4.42 (3.49–28.92) | 7.95 (2.29–20.28) | 6.76 (6.67–29.32) | 0.393 | 0.821 | 9.5 (2.71–39.44) | 5.40 (3.34–11.96) | 32.13 (3.96–130.33) | 1.870 | 0.393 |
| IL-12p70 | 4.14 (3.47–11.48) | 3.86 (3.43–4.34) | 4.07 (3.2–9.23) | 1.895 | 0.388 | 4.19 (3.70–7.18) | 4.05 (3.65–4.30) | 7.42 (3.65–10.31) | 2.361 | 0.307 |
| IL-17A | 2.4 (1.89–7.78) | 2.23 (1.85–2.85) | 2.24 (1.78–2.7) | 0.487 | 0.784 | 2.72 (1.99–3.52) | 2.55 (1.64–3.07) | 1.53 (0.98–2.1) | 4.668 | 0.097 |
| IL-17F | 2.65 (2.11–7.5) | 2.19 (1.92–2.72) | 2.03 (1.71–6.2) | 3.864 | 0.145 | 2.35 (1.74–4.96) | 2.34 (2.13–4.93) | 5.11 (3.13–7.11) | 4.266 | 0.119 |
| TNF-$\alpha$ | 3.14 (1.47–6.61) | 1.67 (1.39–2.28) | 1.68 (1.46–5.22) | 2.911 | 0.233 | 1.84 (1.89–2.85) | 1.71 (1.42–2.56) | 3.31 (1.39–4.66) | 1.689 | 0.430 |
| IFN-$\gamma$ | 2.17 (0.89–7.48) | 2.93 (1.87–3.8) | 2.43 (1.63–5.66) | 0.327 | 0.849 | 4.7 (2.66–10.39) | 3.74 (2.74–6.51) | 5.55 (2.61–11.79) | 0.749 | 0.687 |

Values are expressed as median (interquartile). *p* value was evaluated among groups using the Kruskal-Wallis H test. [a]Compared with non-aGVHD group; [b]Compared with non-aGVHD and grade I–II aGVHD group.

and August 2020 at Aerospace Center Hospital in China, we retrospectively enrolled 32 patients into cohort I and 45 patients into cohort II, where complete clinical information was available. Among them, 20 patients had pre- and post-transplant cytokine test results, bringing the total cohort to 57. Patient characteristics are described in Table 1. In cohort I, patients had serum cytokine levels detected at day 7 pre-transplant, while in cohort II, 45 patients tested serum cytokines at day 14 post-transplant. All cytokines were measured before aGVHD clinical onset. All patients with indications for HSCT, including Acute Myeloid Leukemia (AML), Acute Lymphocytic Leukemia (ALL), Chronic Myeloid Leukemia (CML), and Lymphoma were included in the study. Patients with bloodstream infection, pneumonia, or excessive blood immunosuppressant concentrations at the time of cytokine detection and patients with aGVHD or death before day 14 post-transplant were excluded. All patients received calcineurin inhibitor or/and Mycophenolate Mofetil (MMF) for GVHD prophylaxis.

### *aGVHD Grading*

Standard clinical criteria were used for aGVHD grading [18]. Among the 36 cases with aGVHD, 12 patients had multiple organ involvement, in which the GI tract, skin, liver and lung were involved in 29 (80.5%), 13 (36.1%), 5 (13.9%) and 3 (8.3%) cases, respectively. The control and experimental groups were non-aGVHD, I–II aGVHD and III–IV aGVHD groups. In addition, 11, 18 and 3 cases were included in cohort I in 3 groups, respectively, while 13, 28 and 4 cases were included in cohort II.

### *Risk Factors and Outcome*

Risk factors such as remission or not before allo-HCT, HCT-Specific Comorbidity Index (HCT-CI) [19], number of HLA mismatched loci, donor-recipient blood group compatibility, number of mononuclear cells and CD34+ cells in graft, diarrhea occurred within 15 days after transplantation and the cause of death were retrospectively collected from electronic medical records. The endpoint was death or 2-years after HSCT. Survival time was calculated from the day of HSCT to the event. The patients were followed for a median of 730 days (54–757 days).

### *Serum Cytokines Measurement*

#### Separation of Plasma from Peripheral Blood

A volume of 3 mL of peripheral blood was drawn from subjects. Plasma was extracted within 4 h of specimen collection and centrifuged at 1000 $\times$g for 15 min. The uppermost layer of plasma was analyzed using flow cytometry.

**Table 3. Cytokine levels before and after allo-HCT in patients with/without GI aGVHD.**

| | Cohort I pre-transplant | | | | Cohort II post-transplant | | | |
|---|---|---|---|---|---|---|---|---|
| | Non-GI aGVHD (N = 16) | GI aGVHD (N = 16) | Z | *p* | Non-GI aGVHD (N = 20) | GI aGVHD (N = 25) | Z | *p* |
| IL-1$\beta$ | 0.55 (0.16–1.16) | 1.1 (0.25–2.03) | 1.128 | 0.210 | 1.94 (0.14–3.03) | 0.79 (0.41–1.65) | –1.167 | 0.243 |
| IL-2 | 6.06 (4.39–8.02) | 3.66 (2.33–4.3) | –2.959 | 0.002 | 5.04 (4.04–8.09) | 3.63 (3.09–5.69) | –2.159 | 0.031 |
| IL-4 | 2.45 (1.93–3.16) | 1.89 (1.52–2.18) | –2.281 | 0.021 | 2.28 (1.89–2.82) | 2.12 (1.57–3.22) | –0.434 | 0.664 |
| IL-6 | 42.1 (4.97–89.64) | 100.93 (12.39–248.21) | 0.980 | 0.341 | 39 (13.71–135.11) | 22.74 (12.88–89.44) | –0.525 | 0.599 |
| IL-8 | 74.82 (39.89–225.21) | 59.54 (39.83–99) | –0.603 | 0.564 | 55.06 (27.08–150.34) | 33.38 (16.27–56.06) | –1.462 | 0.144 |
| IL-10 | 6.3 (3.55–27.56) | 7.95 (2.58–19.34) | –0.151 | 0.897 | 10.51 (2.7–21.01) | 5.2 (3.42–17.0) | –0.548 | 0.584 |
| IL-12p70 | 4.25 (3.54–10.69) | 3.86 (3.28–4.07) | –1.508 | 0.138 | 4.06 (3.79–4.46) | 4.05 (3.54–6.14) | –0.069 | 0.945 |
| IL-17A | 2.08 (1.81–4.13) | 2.39 (1.96–2.9) | 0.509 | 0.616 | 2.72 (2.27–3.41) | 2.06 (1.39–2.99) | –1.863 | 0.062 |
| IL-17F | 2.57 (2.11–7.18) | 2.09 (1.89–2.61) | –1.999 | 0.047 | 2.34 (2.21–2.63) | 2.63 (2.13–6.04) | 1.063 | 0.288 |
| TNF-$\alpha$ | 2.75 (1.49–5.96) | 1.65 (1.44–2.1) | –1.621 | 0.110 | 1.9 (1.59–3.47) | 1.66 (1.36–3.0) | –1.028 | 0.304 |
| IFN-$\gamma$ | 2.89 (1.94–5.28) | 2.48 (1.66–3.65) | –0.509 | 0.616 | 3.68 (3.12–8.65) | 4.11 (2.39–7.19) | –0.377 | 0.706 |

Values are expressed as median (interquartile). *p* value was evaluated between groups using the Mann-Whitney U test.

Flow Cytometry

The serum cytokine concentrations were determined by flow cytometry using the AimPlex assay kits (Quanto-Bio, Beijing, China, product number: C60011) on FACSCalibur flow cytometer (version number: LS2033wH, BD Biosciences, San Diego, CA, USA). The analytical procedure was performed following the manufacturer's instructions.

### *Statistical Methods*

SPSS 22.0 software (IBM SPSS statistics, Chicago, IL, USA) was used for statistical analysis. For discrete variables, numbers and proportions were calculated. For continuous factors, data were presented by the median and range. Differences in patient characteristics between non-aGVHD and aGVHD groups were assessed with the Mann-Whitney U test for continuous values and chi-square tests for categorical values. The Kruskal-Wallis H test was used to compare cytokine profiles according to the grades of aGVHD. Cytokine levels were compared using the Mann-Whitney U test between patients with different outcomes. Univariate and multivariate logistic regression analyses were used to study the effects of risk factors on the incidence of aGVHD and death outcomes. Univariate logistic regression analysis was used for the original data, and due to the small sample size of the population, multivariate logistic regression was used for factors with $p < 0.1$. The survival distributions for overall survival (OS) were calculated using the Kaplan–Meier methodology. The comparisons were made using the log-rank test. Differences were considered statistically significant when $p < 0.05$.

## Results

### *aGVHD*

In the pre-transplant cohort, neither of the cytokine levels was observably different in the patients with grade I–II, grade III–IV aGVHD and the patients without aGVHD pre-transplant except for IL-2 and IL-4 (Table 2). The former decreased in all patients with aGVHD while the latter only reduced in patients with III–IV aGVHD. In the post-transplant cohort, only IL-4 remarkably increased 1.79 times in patients with III–IV aGVHD than in the control group.

IL-6, IL-8, IL-10 and IFN-$\gamma$ showed no statistically significant difference among the three groups before and after transplantation. The median of IL-10 increased dramatically in patients with III–IV aGVHD, but the distribution showed no statistically significant with the other two groups ($p = 0.393$).

Compared with patients without GI aGVHD, patients with GI aGVHD had lower IL-2, IL-4 and IL-17F levels before transplantation ($p < 0.05$) and lower IL-2 post-transplant ($p = 0.031$) (Table 3).

### *Risk Factors*

Univariate and multivariate analysis showed that diarrhea within 15 days post-transplant is an independent risk factor for aGVHD and a risk factor for fatal outcomes. Older recipient age is an independent risk factor for death. Neither of the cytokine levels can predict the onset of aGVHD and the death outcome (Table 4).

**Table 4. Univariate and multivariate analysis of risk factors for the occurrence of aGVHD and death outcome.**

| Factors | aGVHD | | | | | | | | | | Death outcome | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Univariate | | | | | Multivariate | | | | | Univariate | | | | | Multivariate | | | | |
| | B | S.E. | OR (95% CI) | Wald $\chi^2$ | $p$ | B | S.E. | OR (95% CI) | Wald $\chi^2$ | $p$ | B | S.E. | OR (95% CI) | Wald $\chi^2$ | $p$ | B | S.E. | OR (95% CI) | Wald $\chi^2$ | $p$ |
| Donor gender | –2.249 | 1.117 | 0.105 (0.012–0.942) | 4.055 | 0.044 | –1.276 | 0.781 | 0.279 (0.060–1.290) | 2.669 | 0.102 | –1.36 | 0.856 | 0.873 (0.163–4.675) | 0.025 | 0.874 | | | | | |
| Recipient age | –0.098 | 0.044 | 0.906 (0.832–0.988) | 5.074 | 0.025 | 0.028 | 0.023 | 1.028 (0.982–1.076) | 1.392 | 0.238 | 0.043 | 0.026 | 1.044 (0.992–1.098) | 2.723 | 0.099 | –0.049 | 0.021 | 0.953 (0.915–0.552) | 5.437 | 0.020 |
| Remission or not before allo-HCT | –1.365 | 1.008 | 0.255 (0.035–1.841) | 1.835 | 0.176 | | | | | | 0.175 | 0.737 | 1.191 (0.281–5.050) | 0.056 | 0.813 | | | | | |
| HCT-CI | 1.004 | 0.585 | 2.730 (0.867–8.599) | 2.944 | 0.086 | –0.177 | 0.312 | 0.838 (0.455–1.543) | 0.323 | 0.570 | –0.019 | 0.334 | 0.982 (0.510–1.887) | 0.003 | 0.956 | | | | | |
| Number of HLA mismatched loci | 1.004 | 0.585 | 2.113 (0.171–26.165) | 0.350 | 0.554 | | | | | | 0.861 | 1.115 | 2.365 (0.266–21.033) | 0.596 | 0.440 | | | | | |
| Donor-recipient blood group compatibility | –2.83 | 1.268 | 0.059 (0.005–0.709) | 4.978 | 0.026 | –1.379 | 0.724 | 0.252 (0.061–1.041) | 3.625 | 0.057 | –0.351 | 0.719 | 0.704 (0.172–2.879) | 0.238 | 0.625 | | | | | |
| Graft composition mononuclear cells | 0.203 | 0.177 | 1.225 (0.865–1.734) | 1.310 | 0.252 | | | | | | –0.029 | 0.147 | 0.972 (0.729–1.296) | 0.038 | 0.844 | | | | | |
| Graft composition CD34+ cells | 0.146 | 0.205 | 1.157 (0.74–1.728) | 0.505 | 0.477 | | | | | | 0.002 | 0.135 | 1.002 (0.769–1.305) | 0.000 | 0.987 | | | | | |
| Diarrhea within 15 d post-transplant | 3.807 | 1.431 | 45.024 (2.727–743.369) | 7.082 | 0.008 | 1.998 | 0.814 | 7.375 (1.496–36.372) | 6.024 | 0.014 | 1.462 | 0.840 | 4.313 (0.832–22.355) | 3.031 | 0.082 | 1.280 | 0.767 | 3.597 (0.800–16.176) | 2.786 | 0.095 |
| IL-4 | –0.019 | 0.183 | 0.982 (0.686–1.405) | 0.010 | 0.919 | | | | | | 0.061 | 0.215 | 1.063 (0.698–1.620) | 0.081 | 0.775 | | | | | |
| IL-2 | –0.106 | 0.068 | 0.899 (0.787–1.027) | 2.454 | 0.117 | | | | | | –0.052 | 0.081 | 0.950 (0.810–1.113) | 0.405 | 0.524 | | | | | |
| IL-6 | 0.000 | 0.001 | 1.000 (0.998–1.001) | 0.022 | 0.882 | | | | | | 0.000 | 0.001 | 1.000 (0.999–1.002) | 0.208 | 0.648 | | | | | |

Graft composition mononuclear cells $\times 10^8$/kg recipient body weight; Graft composition CD34+ cells $\times 10^6$/kg recipient body weight.

**Table 5. Cytokine levels before and after allo-HCT in patients with different outcomes.**

| | Cohort I pre-transplant | | | | Cohort II post-transplant | | | |
|---|---|---|---|---|---|---|---|---|
| | Survivor (N = 16) | Non-survivor (N = 16) | Z | *p* | Survivor (N = 25) | Non-survivor (N = 20) | Z | *p* |
| IL-1$\beta$ | 0.58 (0.39–1.52) | 0.60 (0.19–2.02) | 0.170 | 0.867 | 1.23 (0.06–2.57) | 0.98 (0.47–2.46) | –0.389 | 0.697 |
| IL-2 | 4.73 (3.64–7.47) | 4.07 (2.35–6.85) | 1.131 | 0.270 | 4.25 (3.50–7.52) | 4.63 (3.36–5.72) | 0.297 | 0.767 |
| IL-4 | 2.25 (1.74–2.83) | 2.05 (1.52–2.35) | 0.886 | 0.381 | 2.14 (1.80–3.06) | 2.33 (1.76–2.96) | –0.548 | 0.583 |
| IL-6 | 35.40 (4.97–105.53) | 79.34 (20.29–405.16) | –1.432 | 0.160 | 24.54 (12.33–66.64) | 45.86 (15.70–147.01) | –0.868 | 0.385 |
| IL-8 | 56.71 (27.34–126.51) | 79.50 (43.88–236.66) | –1.281 | 0.210 | 46.12 (21.86–79.32) | 38.54 (17.83–84.08) | 0.297 | 0.767 |
| IL-10 | 6.22 (2.55–20.21) | 9.30 (3.49–23.02) | –0.773 | 0.445 | 5.33 (2.71–20.53) | 6.30 (3.42–37.48) | –0.777 | 0.437 |
| IL-12p70 | 3.88 (3.48–5.77) | 4.03 (3.30–4.29) | 0.283 | 0.780 | 4.03 (3.56–4.27) | 4.21 (3.93–8.08) | –1.555 | 0.120 |
| IL-17A | 2.21 (1.92–2.84) | 2.32 (1.81–3.01) | –0.170 | 0.867 | 2.72 (1.73–3.25) | 2.08 (1.42–3.07) | 0.949 | 0.343 |
| IL-17F | 2.31 (2.02–4.81) | 2.13 (1.89–3.78) | 1.056 | 0.305 | 2.35 (2.04–2.89) | 2.45 (2.16–6.52) | –0.663 | 0.508 |
| TNF-$\alpha$ | 2.24 (1.46–5.96) | 1.70 (1.46–2.32) | 0.980 | 0.341 | 1.75 (1.36–2.70) | 2.03 (1.51–3.79) | –1.028 | 0.304 |
| IFN-$\gamma$ | 2.62 (1.27–4.08) | 2.61 (1.89–4.66) | –0.188 | 0.867 | 3.44 (2.47–5.95) | 4.94 (3.13–10.67) | –1.314 | 0.189 |

Values are expressed as median (interquartile). *p* value was evaluated between groups using the Mann-Whitney U test.

### *Survival*

In total, 24 cases died within 2 years post-transplant, of which seven patients had two main causes of death. Infection is the leading cause of death (14/24 58.3%), followed by relapse (7/24 29.2%), GVHD (7/24 29.2%) and hemorrhage or thrombosis events (3/24 12.5%). Neither of the cytokine levels was observably different in the non-survivor patients within 730 days post-transplant and in survivors (Table 5). Patients without diarrhea within 15 days post-transplant had a longer survival time of 672 (586–757) days vs 444 (229–548) days and better 2-year OS (85.7% vs 46.4%) than those with diarrhea (Fig. 1A). Compared to patients with aGVHD, patients without aGVHD had a longer survival time of 618 (530–706) days vs 449 (353–545) days and better 2-year OS (76.2% vs 47.1%, Fig. 1B).

## Discussion

With regard to proinflammatory and anti-inflammatory cytokines [20] as well as novel biomarkers, such as monocyte chemoattractant protein-1 (MCP-1) [21], the issue of false positives and false negatives should be considered for the evaluation of diagnostic efficacy and severity assessment of aGVHD. Infections and inflammation were significant sources of false positives, while immunosuppression caused low immunity and lessened production of cytokines might lead to false negatives.

After excluding the main interfering factors, we found that patients with above III grade aGVHD had lower IL-2 and IL-4 levels pre-transplant and a higher IL-4 level post-transplant. The median of IL-10 post-transplant increased dramatically in patients with III–IV aGVHD, but the data distribution showed no statistically significant with the other two groups. All the other proinflammatory and anti-inflammatory cytokines, including IL-6, IL-8 and IFN-$\gamma$, showed no statistically significant difference among the three groups before and after transplantation, implying that they may be associated with infections or other inflammatory states other than aGVHD. Many cytokine results were within the reference range, perhaps due to the exclusion of patients with apparent infections, and 86% of subjects were treatment-related leukopenia at the time of cytokine detection.

IL-2 is known to induce alloreactivity and aGVHD [14], which also show pleiotropy [22]. Similar to our results, Malwina *et al.* [20] divided 62 subjects into four groups depending on the presence of aGVHD and clinical manifestations of infection and reported that serum IL-2 levels in patients with AML were low between 0 and 20 days post-transplant due to intensive treatment and T lymphocytes disorders. The study also determined IFN-$\gamma$, whose function is also to induce alloreactivity, are more related to infection than the occurrence of aGVHD. Serum TNF-$\alpha$ is similar to IL-2 and also associated with aGVHD. However, it is not specific due to its production enhanced under plentiful inflammatory conditions, such as infection, pulmonary toxicity and veno-occlusive disease [23]. By excluding patients with such inflammatory stations, we found no changes in IFN-$\gamma$ and TNF-$\alpha$ specific to aGVHD.

IL-4 can induce the expansion of Tregs, modulate intestinal microbiota and suppress inflammatory cytokine output both before and after HSCT [24]. Patients who developed severe aGVHD and GI GVHD may have more severe T cell disorder and lower IL-4 level pre-transplant, and thus generate more inflammatory cytokines and fewer Tregs. IL-4 and IL-10, produced by host natural killer (NK) T cells, also play an essential role in mitigating GVHD after transplantation [25,26]. They are elevated in aGVHD with the theory that it is produced in response to downregulating the synthesis of proinflammatory cytokines [27].

Except for IL-2 and IL-4, we found that patients with GI aGVHD had decreased IL-17F levels pre-transplant. Pre-transplant cytokine levels mainly represent recipient cell origin. The IL-17 family included seven members (IL-17A–F and the viral homologue vIL-17). It has been demonstrated that recipient-origin IL-17 promotes GI tract

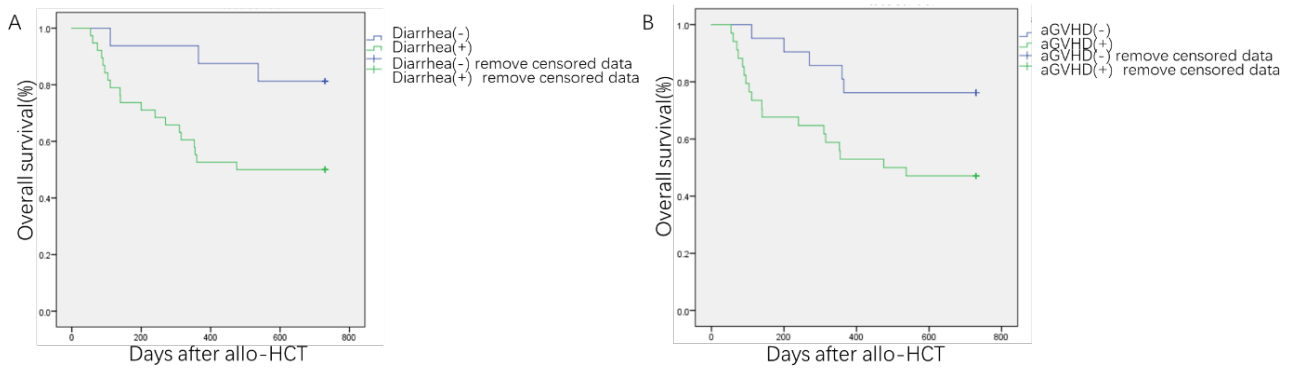


**Fig. 1. 2-year overall survival by diarrhea within 15 days post-transplant and aGVHD.** (A) Patients without diarrhea within 15 days post-transplant had better 2-year OS ($p = 0.015$). (B) Patients without aGVHD had better 1-year and 2-year OS ($p = 0.027$).

integrity, limits the donor alloantigen presentation [28] and prevents dysbiosis [29,30], whose effect was to avoid GI GVHD.

In addition to the role of the disease, the drug effect should also be considered. For example, tacrolimus and cyclosporin are regularly used for the prevention and treatment of GVHD, and the effect of calcineurin inhibitors mainly reduces the transcription of IL-2, IL-4, IL-10 and IL-17 [31].

IL-6 is a cytokine that previously has been associated with proinflammatory effects. However, it also mediates strong anti-inflammatory effects, and higher levels of IL-6 is important mediators and therapeutic target in aGVHD [32]. Mariela *et al.* [33] and Mahdiyar *et al.* [34] found no significant association between IL-6 and the presence of aGVHD in both haplo-HSCT patients and non-haplo transplant patients, which were consistent with the results of this study. However, dysregulation of IL-6 exists both before and after allo-HCT, probably because both recipient dentritic cell (DC) and donor DC play a role in the cellular source of IL-6 dysregulation during aGVHD [9].

IL-8 plays an important role in immune cell homeostasis and is protective against GVHD. In previous studies with relatively small population of 20 to 84 patients, IL-8 concentration can be elevated or decreased in aGVHD [35–37]. In a study with 424 patients, IL-8 increased in aGVHD patients. The area under the receiver operating characteristic curve of a diagnostic panel including IL-8 was around 0.9 [38]. These controversial results, taken in our study, may be due to different cell origins and detection time.

Mohamad Mohty *et al.* [39] conducted research on 113 French people who underwent reduced-intensity HCT, tested inflammatory cytokine levels at day –7, 0, 30, 60 and 90, showed that among 10 inflammatory cytokines, only IL-12p70 plasma levels correlate with aGVHD incidence and severity and the time point with the most significant difference is 30 days. However, our study cannot reproduce the results, probably due to the earlier detection timepoint.

Li *et al.* [40] conducted research in patients who had already onset aGVHD, that compared with patients without aGVHD, the expression of IL-2, IL-4, IL-10 and IL-17A were significantly increased. Furthermore, IL-6 level was extremely high in patients with III–IV aGVHD compared with I–II aGVHD. Therefore, there might have been more positive findings if our study had postponed the post-transplant cytokine testing time to 30 days or when aGVHD had been clinically presumed.

Recent studies showed that high IL-6 level is a principal cause of early death in HSCT patients [33], whose early elevation at +7 days post-transplant was significantly associated with worse OS. Another complication that implies an increase in IL-6 production is cytokine release syndrome (CRS). Higher grade CRS was the only factor associated with aGVHD and poorer GVHD- and relapse-free survival [41]. Olle Ringdén *et al.* [42] reported a trend ($p = 0.07$) for better survival in patients with lower than median IFN-$\gamma$ levels at +7 days post-transplant compared to those with higher IFN-$\gamma$ levels. However, the cytokine level is not a risk factor for aGVHD and the fatal outcome in this study. In future clinical practice, choosing a proper detection time or dynamic monitoring of cytokines may have certain implications for the occurrence and development of aGVHD.

Diarrhea within 15 days post-transplant is the only independent risk factor for the occurrence of aGVHD and the risk factor for death. Clinical studies supported the association of alterations in the gut microbiome diversity and composition during allo-HSCT, with aGVHD and OS [43,44]. Homeostasis of the immune response in the gut mucosa is maintained by the balance between proinflammatory cells and intestinal microbiota, which also regulate the expression of proinflammatory cytokines and increase T cell proliferation. With the maturity of preconditioning and GVHD prophylaxis protocols, the role of many traditional risk factors is gradually diluted, and we need to pay more attention to new prophylaxis targets, such as the regulation of gut microbiota in the future.

## Conclusions

Proinflammatory and anti-inflammatory cytokines can provide specific indications for the occurrence and progression of aGVHD in patients who underwent allo-HSCT. However, to truly guide the diagnosis and prognosis of clinical practice, multicenter studies of cytokines with larger sample sizes, more detection time points and more accurate diagnostic efficacy need to be further conducted.

## Availability of Data and Materials

The authors confirm that the data supporting the findings of this study are available within the article.

## Author Contributions

QG and WJZ—designed the research and interpretation of data; DZ, QH and YA—performed the research; QG—performed the statistical analysis and drafted the manuscript. GWL—guided the design of the study and provided help on the methodology of data analysis and revised the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

## Ethics Approval and Consent to Participate

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

The study was retrospectively approved by the Ethics Committee of Aerospace Center Hospital (approval number: 2022065).

This is a retrospective study, thus, consent to participate is not applicable.

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## Conflict of Interest

The authors declare no conflict of interest.

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