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Expansion of circulating extrafollicular helper T-like cells in patients with chronic graft-versus-host disease

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ABSTRACT

Chronic graft-versus-host disease (cGVHD) is a major cause of morbidity and mortality after allogeneic hematopoietic stem cell transplantation (allo-HSCT). Previous studies have shown that T follicular helper cells (Tfh) contribute to immune pathology in cGVHD, but the function of extrafollicular helper T cells during cGVHD pathogenesis remains largely unknown. In the current study, we identified circulating extrafollicular helper T-like cells ($CD44^{hi}CD62L^{lo}PSGL\text{-}1^{lo}CD4^{+}$, c-extrafollicular Th-like) in human peripheral blood. We performed phenotypic and functional analyses of c-extrafollicular Th-like cells from 80 patients after allo-HSCT to explore the role of these cells in the development of human cGVHD. Patients with active cGVHD had significantly higher frequencies and counts of c-extrafollicular Th-like cells than those of patients without cGVHD. The expansion of c-extrafollicular Th-like cells was more significant in patients with moderate/severe cGVHD than that of patients with mild cGVHD. C-extrafollicular Th-like cells from patients with active cGVHD exhibited increased functional abilities to induce plasmablast differentiation and IgG1 secretion compared to those of patients without cGVHD. Moreover, c-extrafollicular Th-like cell levels were highly correlated with the generation of autoreactive B cells, plasmablasts and IgG1 antibodies. Our studies provide new insights into human cGVHD pathogenesis and identify c-extrafollicular Th-like cells as a key element in the development of human cGVHD.

1. Introduction

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is a curative therapy for hematological malignancies [1]. Chronic graft-versus-host disease (cGVHD) is a major cause of morbidity and mortality after allo-HSCT [2]. The clinical manifestations of cGVHD are similar to those of systemic autoimmune diseases [3], but the exact mechanism of cGVHD is still unclear.

Interactions of $CD4^{+}$T and B cells play important roles in cGVHD pathogenesis [4–8]. Donor mature $CD4^{+}$T cells targeting alloantigens in the recipient and autoreactive $CD4^{+}$T cells generated de novo in a damaged thymic environment are responsible for the development of cGVHD [9]. Donor B cells augment clonal expansion of pathogenic $CD4^{+}$T cells via their antigen-presenting cell function and augment cGVHD development [10]. In addition, cGVHD is also associated with abnormalities of B cell reconstitution, aberrant B cell activation and deposition of antibodies [11,12]. $CD4^{+}$T and B cell interact at many levels to coordinate effective and specific immune responses. These interactions occur at the extrafollicular T-B border and follicular germinal centers (GCs) [13]. In brief, naive $CD4^{+}$T cells differentiate into Th1, Th2, Th17, and pre-T follicular helper cells (Tfh) under different cytokine and microenvironment regulation. First, $CD4^{+}$T cells downregulate the expression of PSGL-1 (P-selectin glycoprotein ligand-1), differentiate into pre-Tfh cells, and migrate to the T-B border [13]. Pre-Tfh and B cell interactions lead to the generation of short-lived plasma cells and low-affinity IgG1 and result in immunoglobulin isotype switching without somatic hypermutation [14–16]. Pre-Tfh cells subsequently upregulate the expression of CXCR5 and migrate further into the center of the B cell zone to form GCs. This T cell subset in the GC is termed Tfh. Tfh-mediated activation of B cells in the GC promotes differentiation of naive B cells into memory B cells and production of high affinity IgG, resulting in somatic hypermutation [13,17,18].

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The roles of Tfh and extrafollicular helper T cells in cGVHD pathogenesis have gained increasing attention in recent years. It has been reported that Tfh cells were reduced in the spleen of cGVHD mice [12,19] and in the blood of patients with cGVHD [20]. In murine cGVHD models, extrafollicular helper T cells ($CD44^{hi}CD62L^{lo}PSGL\text{-}1^{lo}CD4^{+}T$) expanded in the spleen, lung and liver [12,19]. Since extrafollicular helper T cells expanded in cGVHD mice, we sought evidence of this phenotype in human cGVHD. We identified a subset of $CD4^{+}T$ cells ($CD44^{hi}CD62L^{lo}PSGL\text{-}1^{lo}CD4^{+}T$) in human peripheral blood. These cells share similar phenotypic characteristics with extrafollicular helper T cells in secondary lymphoid organs, and we refer to them as circulating extrafollicular helper T-like cells (c-extrafollicular Th-like cells). The purpose of this study was to explore the role of c-extrafollicular Th-like cells in the development of human cGVHD.

2. Material and methods

2.1. Study design and patient eligibility

Patients with acute leukemia undergoing allo-HSCT were enrolled in this prospective, experimental study. Eligibility criteria were as follows: (1) > 3 months from time of allo-HSCT; (2) not receiving high-dose prednisone (≥0.3 mg/kg per day or ≥30 mg/day); and (3) never treated with rituximab (anti-CD20 mAb) or ibrutinib (inhibitor of Bruton's tyrosine kinase). Patients who did not achieve engraftment or who developed life-threatening infections and patients whose primary disease relapsed within 1 year of transplantation were excluded. The diagnosis and grade of cGVHD were established at the time of sample collection according to the National Institutes of Health (NIH) criteria [21]. Patients with active cGVHD were defined as requiring addition of high-dose prednisone or continued multiagent immunosuppression after sample collection [22]. Patients without cGVHD were defined as patients who had not developed cGVHD by the time of sample collection. Patients with previous cGVHD that had resolved or who became asymptomatic by the time of sample collection were not included [22]. Forty active cGVHD patients were closely matched to 40 patients without cGVHD according to time after transplantation, age, gender, stem cell source, conditioning regimen, GVHD prophylaxis and grade of acute GVHD. An additional 20 healthy stem cell transplant donors were recruited for this study. This study was conducted in accordance with the Declaration of Helsinki and approved by the institutional review board of Nanfang Hospital. All patients and healthy donors gave written informed consent to participate in the study.

2.2. Patient samples

Blood samples were collected from participants, stored in EDTA-containing tubes and processed within 5 h of collection. Samples were centrifuged for 15 min at 1500 rpm to collect plasma, which was stored at −80 °C. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation.

2.3. Flow cytometry analysis

The antibodies and reagents used for flow cytometry analysis were as follows: eFluor450-CD4 (OKT4), Streptavidin-PE-Cyanine 7, Streptavidin-APC-eFluor 780, PE-Cyanine7-CD44 (IM7), APC-PD-1 (eBioJ105), and APC-PSGL-1 (FLEG) were purchased from eBiosciences. Biotin-CXCR5 (MU5UBEE), PE-IgD (IA6-2), PE-Cyanine7-CD19 (HIB19), FITC-CD62L (DREG56), APC-CD27 (O323), eFluor450-CD38 (HB7), and Aqua fluorescent reactive dye for viability analysis were purchased from Invitrogen. eFluor450-CD21 (B-Ly4) was purchased from BD Biosciences. Streptavidin-PerCP was purchased from biolegend. All staining was performed according to the manufacturer's instructions. Flow cytometric analysis was performed on a FACS Canto™ II Flow Cytometer (BD Biosciences), and the resulting data were analyzed with FlowJo software (Tree Star, Ashland, OR).

Table 1
Patient characteristics.

Characteristic	Chronic GVHD		
	No (n = 40)	Active (n = 40)	*P*
Age, median (range), y	31.0 (15–61)	29 (16–53)	0.37
Gender, n (%)			0.48
Male	28 (70)	24 (60)	
Female	12 (30)	16 (40)	
Time from HSCT to sample collection (range), m	6.9 (3.13–12.00)	7.3 (3.17–11.97)	0.91
GVHD prophylaxis, n (%)			0.67
ATG + MMF + CSA + MTX	21 (52.5)	21 (52.5)	
CSA + MTX + MMF	15 (37.5)	17 (42.5)	
CSA + MTX	4 (10)	2 (5.0)	
Primary Disease, no (%)			0.37
AML	25 (62.5)	20 (50)	
ALL	15 (37.5)	20 (50)	
Conditioning regimen, no (%) [a]			0.65
Myeloablative	15 (37.5)	18 (45.0)	
Intensified	25 (62.5)	22 (55.0)	
Transplant type, no (%)			1.00
MSD	19 (47.5)	19 (47.5)	
HID	21 (52.5)	21 (52.5)	
Source of stem cell, no (%)			0.82
PBSC	19 (47.5)	18 (45.0)	
BM + PBSC	21 (42.5)	22 (55.0)	
Acute GVHD grade, no (%)			0.18
0-1	25 (62.5)	18 (45)	
2-4	15 (37.5)	22 (55)	
No. of immunosuppressive therapies before study inclusion, no (%) [b]			0.001
None	27 (67.5)	8 (20)	
1	13 (32.5)	4 (10)	
2	0 (0)	15 (37.5)	
≥3	0 (0)	13 (32.5)	

GVHD, graft-versus-host disease; HSCT, hematopoietic stem cell transplantation; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; MSD, matched sibling donor; HID, Haplo-identical donor; PBSC, peripheral blood stem cell; BM, bone marrow; ATG, antithymocyte globulin; CsA, ciclosporin A; MMF, mycophenolate mofetil. MTX, methotrexate.

[a] Myeloablative conditioning regimens include TBI (total body irradiation) + Cy (cyclophosphamide), Bu (busulfan) + Cy, and Bu + Flu (fludarabine). Intensified conditioning regiments include TBI + Cy + etoposide, and Flu + cytarabine + TBI + Cy.

[b] Immunosupressive drugs include cyclosporin A (CsA), tacrolimus (Tac), mycophenolate mofetil (MMF), and steroids. None of the patients without cGVHD received steroids therapy.

Table 2
Clinical characteristics of cGVHD.

	Mild	Moderate	Severe
	N = 11	N = 20	N = 9
Skin (%)	6 (15)	14 (35)	4 (10)
Eyes (%)	1 (2.5)	7 (17.5)	1 (2.5)
Oral mucosa (%)	6 (15)	7 (17.5)	0
Liver (%)	1 (2.5)	3 (7.5)	2 (5)
GI (%)	1 (2.5)	4 (10)	5 (12.5)
Lungs (%)	0	0	1 (2.5)
Joints (%)	0	1 (2.5)	0
Genital Tract (%)	0	0	1 ((2.5)
Steroid treatment (%)	3 (27.3)	9 (45.0)	8 (88.9)

2.4. Serum immunoglobulin analysis

Total IgG, IgM, and IgA were measured in plasma by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's

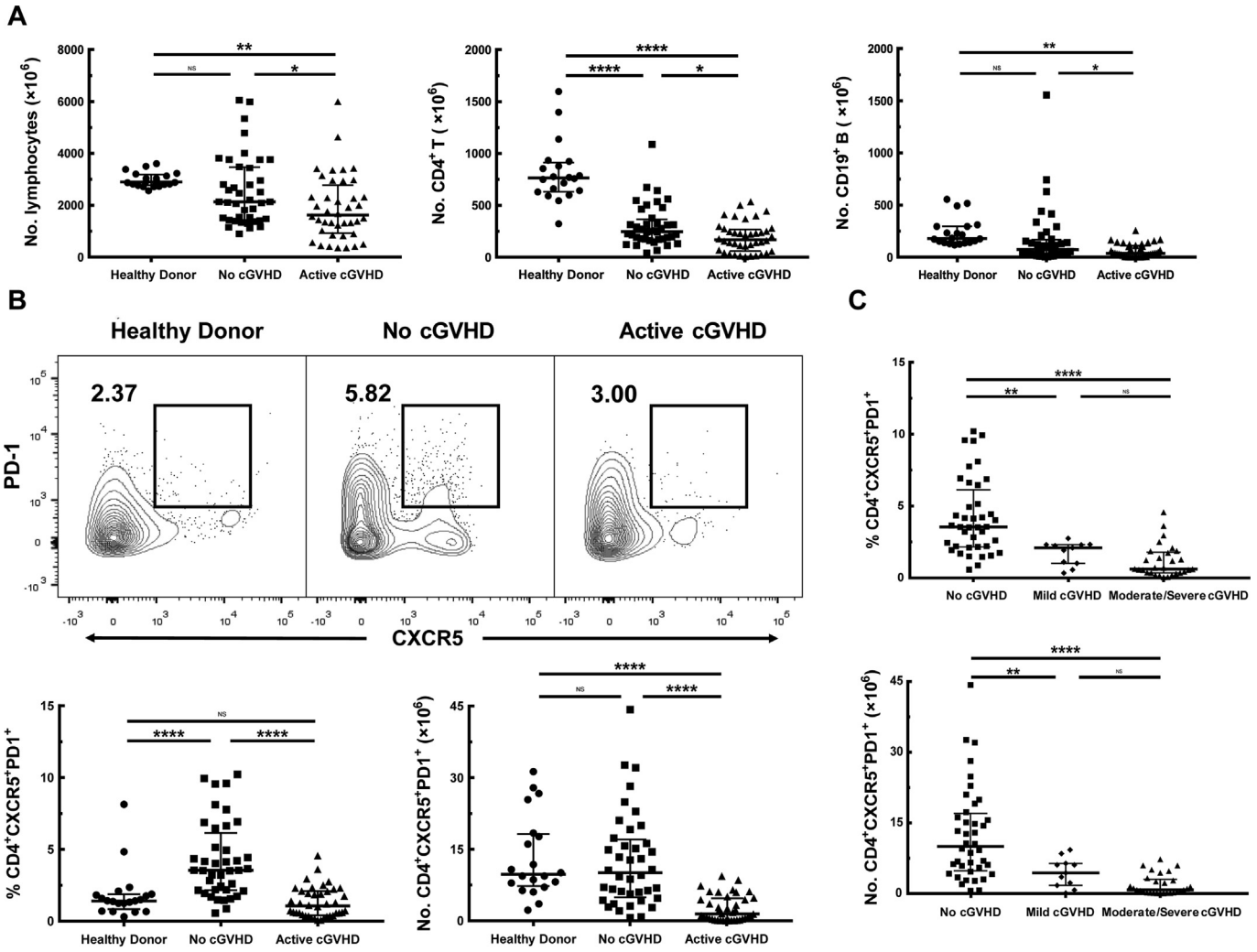

Fig. 1. Patients with active cGVHD had lymphopenia and decreased cTfh cells. (A) Total counts of lymphocytes, $CD4^+$T cells and $CD19^+$B cells in healthy donors, patients without cGVHD and patients with active cGVHD. (B) Frequencies and counts of cTfh cells in healthy donors, patients without cGVHD, and patients with active cGVHD. (C) Frequencies and counts of cTfh cells in patients with different severity of cGVHD. Black bars in each figure represent 75th percentile, median and 25th percentile values. NS, not significant; *P < 0.05, **P < 0.01, and ****P < 0.0001.

protocols (R&D Systems). IgG1, IgG2, IgG3, and IgG4 were measured in plasma by a MILLIPLEX® MAP Human Immunoglobulins Isotype Multiplex Assay kit (Merck).

2.5. T cell and B cell coculture

For purification of c-extrafollicular Th-like cells and naive B cells, whole EDTA-anticoagulated blood (8–10 mL) was obtained from post-transplantation patients or healthy individuals. C-extrafollicular Th-like cells ($CD44^{hi}CD62L^{lo}PSGL\text{-}1^{lo}CD4^+$) and naive B cells ($CD19^+IgD^+CD38^{lo}CD27^-$) were sorted by flow cytometry at more than 98% purity (MoFlo XDP, Beckman Coulter). Purified cells were plated at 20000 cells per well at a 1:1 ratio in RPMI 1640 complete medium supplemented with 10% fetal bovine serum, L-glutamine, penicillin, streptomycin, 10 μg/mL phytohemagglutinin (PHA, Sigma-Aldrich), and 10 ng/mL lipopolysaccharide (LPS, Sigma-Aldrich) for 7 days. Plasmablast differentiation ($CD19^+IgD^{lo/-}CD38^{hi}CD27^+$) and IgG1 were measured at day 7.

2.6. Statistical analysis

A descriptive analysis of all variables was performed, including median, range, minimum and maximum values for continuous variables and numbers and frequencies for categorical variables. For categorical variables, the chi-square statistic or Fisher exact test was used to establish differences in their distribution; the Wilcoxon rank-sum test was used to compare continuous variables. Univariable linear regression analysis was performed for the factors listed in Tables 1 and 2 to identify variables that were associated with the frequency of c-extrafollicular Th-like cells. Factors that were significant at the 0.1 level from the univariable analysis were included in the multivariable analysis. Prior to linear regression analysis, the percentage of the c-extrafollicular Th-like cell values were natural log-transformed to meet the normality assumption. Correlation studies were performed using the nonparametric Spearman rank test. Tests for significance were 2-sided, with a significance *P* level of 0.05 or less [11]. All statistical analyses were performed using GraphPad Software (Prism Version 6.0; GraphPad Software, San Diego, CA) and SPSS version 22.0 (SPSS, Chicago, IL).

3. Results

3.1. Patient characteristics

Eighty patients were enrolled in this study, and there were 52 males and 28 females, with a median age of 30-years-old (range: 15–61

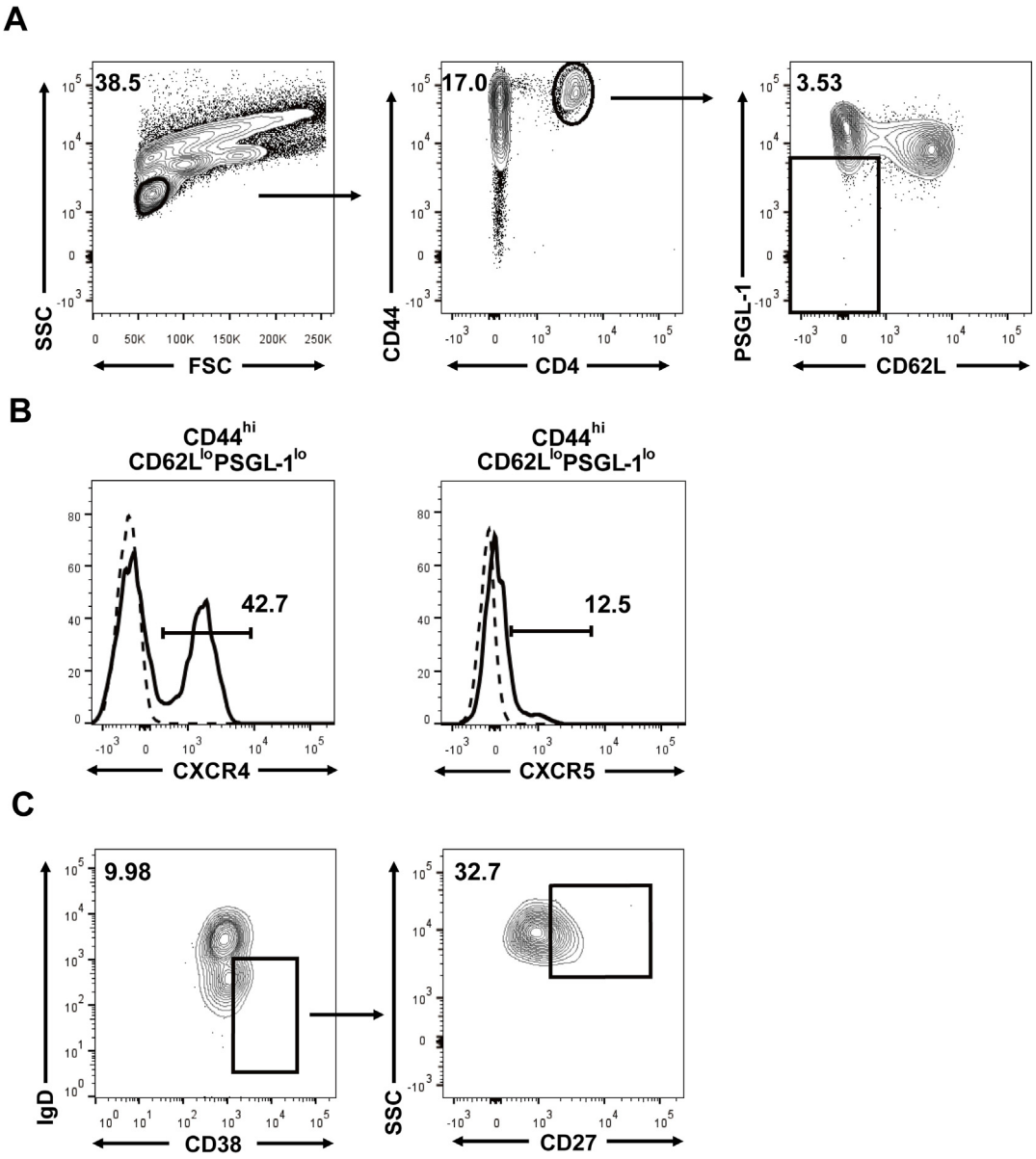

Fig. 2. Extrafollicular Th-like cells could be identified in human peripheral blood. (A) Representative flow patterns of extrafollicular Th-like cells in the peripheral blood of healthy donors. (B) Expression levels of the chemokine receptors CXCR4 and CXCR5 were showed in extrafollicular Th-like cells from healthy donors. Specific staining is indicated by the solid line, and FMO staining is indicated by the dotted line. Values indicate the percent positive minus FMO. (C) Representative flow patterns of plasmablasts. Plasmablasts ($CD19^+IgD^{lo/-}CD38^{hi}CD27^+$) were measured after naive B cells were cultured with extrafollicular Th-like cells for 7 days. Extrafollicular Th-like cells and naive B cells were purified by cell sorting (purity > 98%) from healthy donor samples. Abbreviations: forward side scatter (FSC); side scatter (SSC); fluorescence minus one (FMO).

years). There were no significant differences in age, gender, time after transplant, GVHD prophylaxis, primary disease, conditioning regimen, source or type of graft, or grade of acute GVHD between patients with and without cGVHD in our study (Table 1). As expected, there was a significant difference in immunosuppressive treatment on the date of sample collection between patients with active cGVHD compared with that of patients without cGVHD. Among patients with active cGVHD, the most frequent organ manifestations of cGVHD were skin (60%) and oral mucosa (32.5%). Twenty-two patients (55%) had more than 2 organs involved. Clinical manifestations of cGVHD are summarized in Table 2.

3.2. Lymphopenia and decreased circulating Tfh cells in patients with active cGVHD

We evaluated total lymphocytes, $CD4^+$T cells, and $CD19^+$B cells in patients with or without cGVHD and in healthy donors. In line with Dulude et al. [23], patients with active cGVHD had lymphopenia ($P < 0.05$) (Fig. 1A). Patients with active cGVHD had significantly lower $CD4^+$T and $CD19^+$B cell counts than those of patients without cGVHD (median, $167.1*10^6$ vs $249.6*10^6$; $P < 0.05$; and median, $36.64*10^6$ vs $74.43*10^6$; $P < 0.05$) and those of healthy donors (median, $167.1*10^6$ vs $768.5*10^6$; $P < 0.0001$; and median, $36.64*10^6$ vs $179.3*10^6$; $P < 0.01$) (Fig. 1A). In our previous study, cGVHD mice showed destruction of lymphoid follicles, absence of GCs, and a decrease of Tfh cells [12]. In humans, Tfh cells can be identified in the periphery, herein referred to as circulating Tfh (cTfh) cells

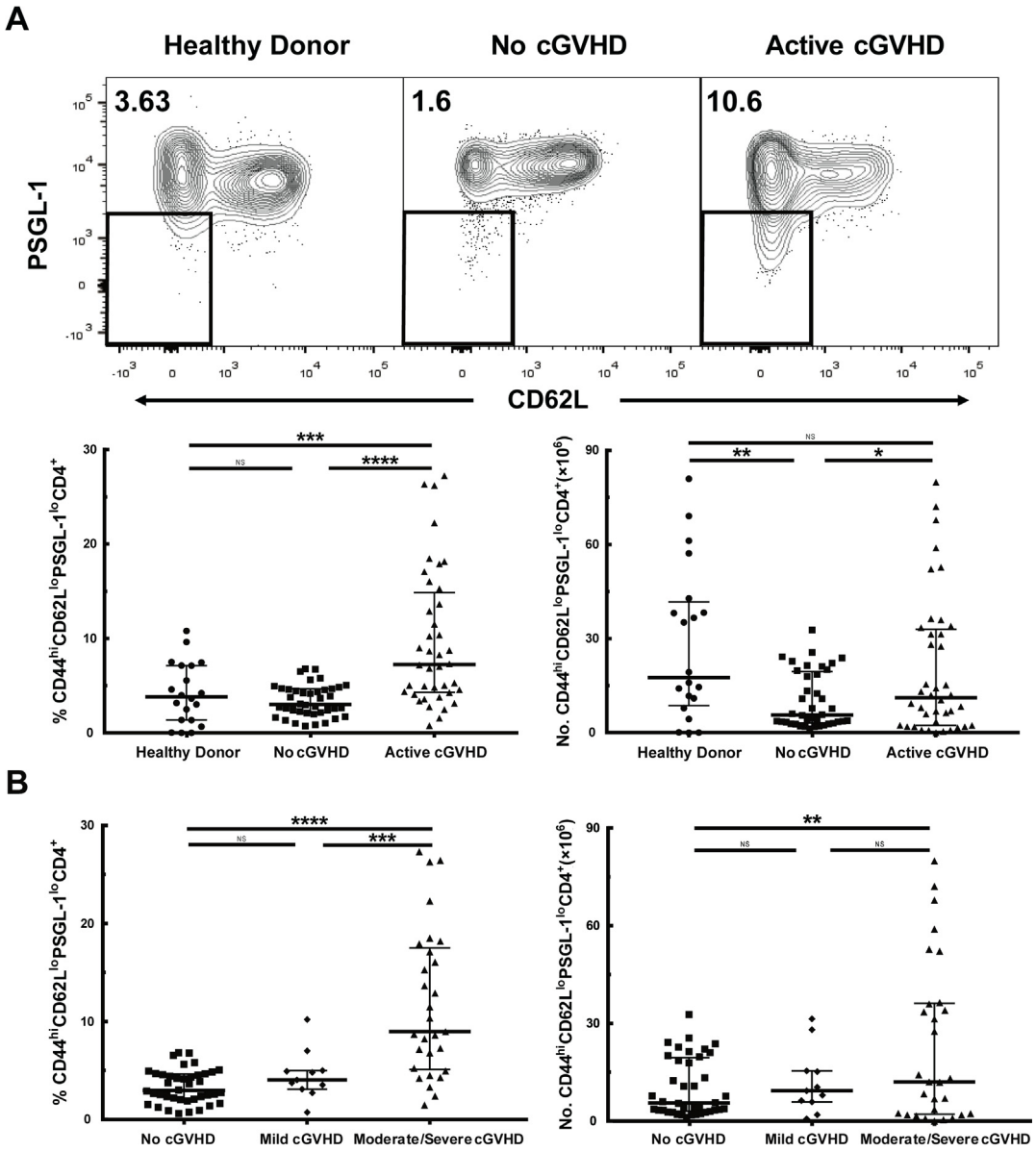

Fig. 3. C-extrafollicular Th-like cells expanded in patients with active cGVHD. (A) Frequencies and counts of c-extrafollicular Th-like cells in healthy donors, patients without cGVHD, and patients with active cGVHD. (B) Frequencies and counts of c-extrafollicular Th-like cells in patients with different severity of cGVHD. Black bars in each figure represent 75th percentile, median and 25th percentile values. NS, not significant; *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

[24,25]. We tested whether abnormalities of cTfh cells are present in cGVHD patients. Compared with that of healthy donors, a trend of decreased frequency of cTfh cells was detected in patients with active cGVHD (median, 1.40% vs 1.06% of $CD4^+$T cells; P = 0.59). The frequency of cTfh cells significantly decreased in patients with active cGVHD compared with that of patients without cGVHD (median, 1.06% vs 3.54% of $CD4^+$T cells; P < 0.0001) (Fig. 1B). Comparisons of absolute numbers of cTfh cells showed a similar trend in which patients with active cGVHD had significantly lower numbers of cTfh cells than those of patients without cGVHD (median, $1.45*10^6$ vs $10.03*10^6$; P < 0.0001) and those of healthy donors (median, $1.45*10^6$ vs $9.74*10^6$; P < 0.0001) (Fig. 1B). Furthermore, these decreases were more significant in patients with moderate/severe cGVHD (median, 0.62% vs 3.54% of $CD4^+$T cells; P < 0.0001 and median, $0.75*10^6$ vs $10.03*10^6$; P < 0.0001) compared to those of patients without cGVHD (Fig. 1C).

3.3. Extrafollicular helper T-like cells could be identified in human peripheral blood

In mice, extrafollicular helper T cells are found in secondary lymphoid organs and downregulate PSGL-1. Extrafollicular helper T cells are identified as $CD44^{hi}CD62L^{lo}PSGL\text{-}1^{lo}CD4^+$ T cells with decreased expression of CXCR5 [19,26]. In our study, we identified a subset of $CD4^+$T cells ($CD44^{hi}CD62L^{lo}PSGL\text{-}1^{lo}CD4^+$T) in human peripheral blood (Fig. 2A). Since these cells share similar phenotypic characteristics with extrafollicular helper T cells in secondary lymphoid organs, we referred to them as circulating extrafollicular helper T-like cells (c-extrafollicular Th-like). These c-extrafollicular Th-like cells expressed high levels of CXCR4 and low levels of CXCR5 (Fig. 2B). We sorted $CD44^{hi}CD62L^{lo}PSGL\text{-}1^{lo}CD4^+$ T cells from human peripheral blood to examine their functional capacity. C-extrafollicular Th-like cells could induce naive B cell differentiation to mature plasmablasts (Fig. 2C).

Table 3
Linear regression analysis for factors associated with percentage of c-extrafollicular Th-like cells.

Clinical factor	Contrast	Univariable		Multivariable		
		Mean difference	*P*	LS Mean difference	SE	*P*
Age	< 30 vs ≥ 30	0.50	0.72			
Gender	Male vs female	−1.38	0.34			
Transplant type	MSD vs HID	−0.51	0.73			
Source of stem cell	PBSC vs PBSC + BM	−0.59	0.86			
GVHD prophylaxis	ATG + MMF + CSA + MTX vs CSA + MTX + MMF vs CSA + MTX	−0.17, 4.11	0.29			
Conditioning regimen[a]	Myeloablative vs intensified	1.84	0.42			
aGVHD grade	0-1 vs 2-4	−3.11	0.07			0.56
cGVHD grade	No vs Mild, Moderate/Severe	−1.18, −8.34	< 0.001	−0.02, −0.4	0.04, 0.03	< 0.01
GVHD location	Skin vs GI, Liver, Mucosal, Eyes	3.95, 0.44, 3.40, −5.55	0.43			

GVHD, graft-versus-host disease; MSD, matched sibling donor; HID, Haplo-identical donor; PBSC, peripheral blood stem cell; BM, bone marrow; ATG, antithymocyte globulin; CsA, ciclosporin A; MMF, mycophenolate mofetil. MTX, methotrexate.
In multivariable analysis, factors with P < 0.1 from univariable analysis were included. LS, least squares; LS mean difference, least squares mean difference among groups; SE, standard error of the LS mean difference.
[a] Myeloablative conditioning regimens include TBI (total body irradiation) + Cy (cyclophosphamide), Bu (busulfan) + Cy, and Bu + Flu (fludarabine). Intensified conditioning regiments include TBI + Cy + etoposide, and Flu + cytarabine + TBI + Cy.

3.4. C-extrafollicular Th-like cells expanded in patients with active cGVHD

The frequency of c-extrafollicular Th-like cells increased in patients with active cGVHD compared with that of patients without cGVHD (median, 7.28% vs 3.02% of $CD4^+$T cells; P < 0.0001) and that of healthy donors (median, 7.28% vs 3.82% of $CD4^+$T cells; P < 0.001). The absolute numbers of c-extrafollicular Th-like cells in patients with active cGVHD also increased compared with those of patients without cGVHD (median, 11.22 $*10^6$ vs 5.64 $*10^6$; P < 0.05) (Fig. 3A). We further explored whether the expansion of c-extrafollicular Th-like cells was associated with disease severity. Patients with moderate/severe cGVHD had greater expansion of c-extrafollicular Th-like cells than that of patients with mild cGVHD (median, 9.02% vs 4.10% of $CD4^+$T cells; P < 0.001) and that of patients without cGVHD (median, 9.02% vs 3.02% of $CD4^+$T cells; P < 0.0001) (Fig. 3B). Although the counts of c-extrafollicular Th-like cells in patients with moderate/severe cGVHD were slightly higher than those of patients with mild cGVHD, the counts were extremely elevated compared with those of patients without cGVHD (median, 12.06$*10^6$ vs 9.39$*10^6$; P = 0.15; median, 12.06 $*10^6$ vs 5.64$*10^6$; P < 0.01) (Fig. 3B). Further, multivariable linear regression analysis confirmed that moderate/severe cGVHD was a significant risk factor for a high percentage of c-extrafollicular Th-like cells after adjusting for other transplant characteristics (P < 0.01) (Table 3).

3.5. Enhanced function of c-extrafollicular Th-like cells in patients with active cGVHD

Next, we compared the functional capacity of c-extrafollicular Th-like cells from patients and healthy donors. C-extrafollicular Th-like cells from patients with active cGVHD induced greater plasmablast differentiation than that of patients without cGVHD and that of healthy donors when the c-extrafollicular Th-like cells were cultured with autologous naive B cells (median, 18.1% vs 9.04% of $CD19^+$B cells; P < 0.01 and median, 18.1% vs 5.86% of $CD19^+$B cells; P < 0.01) (Fig. 4A). Previous studies demonstrated that B cells were constitutively activated in cGVHD, and this finding might simply reflect the increased sensitivity of B cells to stimulation in cGVHD patients [11,27]. We also examined the functional capacity of c-extrafollicular Th-like cells from patients to induce plasmablast differentiation of naive B cells from healthy donors. C-extrafollicular Th-like cells from patients with active cGVHD induced greater plasmablast differentiation than that of patients without cGVHD and that of healthy donors when cultured with normal naive B cells (median, 10.02% vs 4.65% of $CD19^+$B cells; P < 0.05; and median, 10.02% vs 5.08% of $CD19^+$B cells; P < 0.05) (Fig. 4B). As expected, c-extrafollicular Th-like cells from patients with active cGVHD drove more IgG1 secretion than that of patients without cGVHD and that of healthy donors when cultured with autologous (median, 525.9 ng/mL vs 282.6 ng/mL; P = 0.22; and median, 525.9 ng/mL vs 190.6 ng/mL; P < 0.05) or normal naive B cells (median, 657.9 ng/mL vs 246.3 ng/mL; P < 0.05; and median, 657.9 ng/mL vs 107.9 ng/mL; P < 0.001) (Fig. 4C).

3.6. Increased autoreactive B cells, plasmablasts and IgG1 levels in patients with active cGVHD

Previous studies have demonstrated that B cells and the production of alloantibodies might play critical roles in the immune pathology of cGVHD [5,7,12]. Consistent with previous reports [5,28], patients with active cGVHD had a higher frequency of autoreactive B cells and plasmablasts compared to those of patients without cGVHD (median, 23.08% vs 17.43% of $CD19^+$B cells; P < 0.05; and median, 2.26% vs 1.24% of $CD19^+$B cells; P < 0.05) (Fig. 5A). In our study, the levels of IgM and IgA were not significantly different between patients with active cGVHD and patients without cGVHD (Fig. 5B). In contrast, a trend of increased levels of IgG was detected in patients with active cGVHD compared to those of patients without cGVHD (median, 11.38 mg/mL vs 9.97 mg/mL; P = 0.10) and those of healthy donors (median, 11.38 mg/mL vs 9.78 mg/mL; P = 0.23) (Fig. 5C). We further identified the IgG subclass, and we observed that IgG1 subclasses were elevated in patients with active cGVHD compared to those of patients without cGVHD (median, 4.79 mg/mL vs 3.56 mg/mL; P < 0.05). Moreover, the level of IgG1 showed a relationship to the severity of cGVHD. Patients with moderate/severe cGVHD had higher levels of IgG1 compared with those of patients without cGVHD (median, 5.07 mg/mL vs 3.56 mg/mL; P < 0.05) (Fig. 5D).

3.7. C-extrafollicular Th-like cell levels correlated with the generation of autoreactive B cells, plasmablasts and IgG1 in patients with active cGVHD

We further examined whether c-extrafollicular Th-like cells that we identified in active cGVHD were correlated with the B-cell phenotype in these patients. In our study, the frequencies of c-extrafollicular Th-like cells were strongly correlated with the generation of autoreactive B cells and plasmablasts (r = 0.321, P < 0.01 and r = 0.280, P < 0.05) (Fig. 6A and B). The frequencies of c-extrafollicular Th-like cells were also strongly correlated with the level of IgG1 (r = 0.334, P < 0.01), but not with the level of IgG2, IgG3, and IgG4 (Fig. 6C).

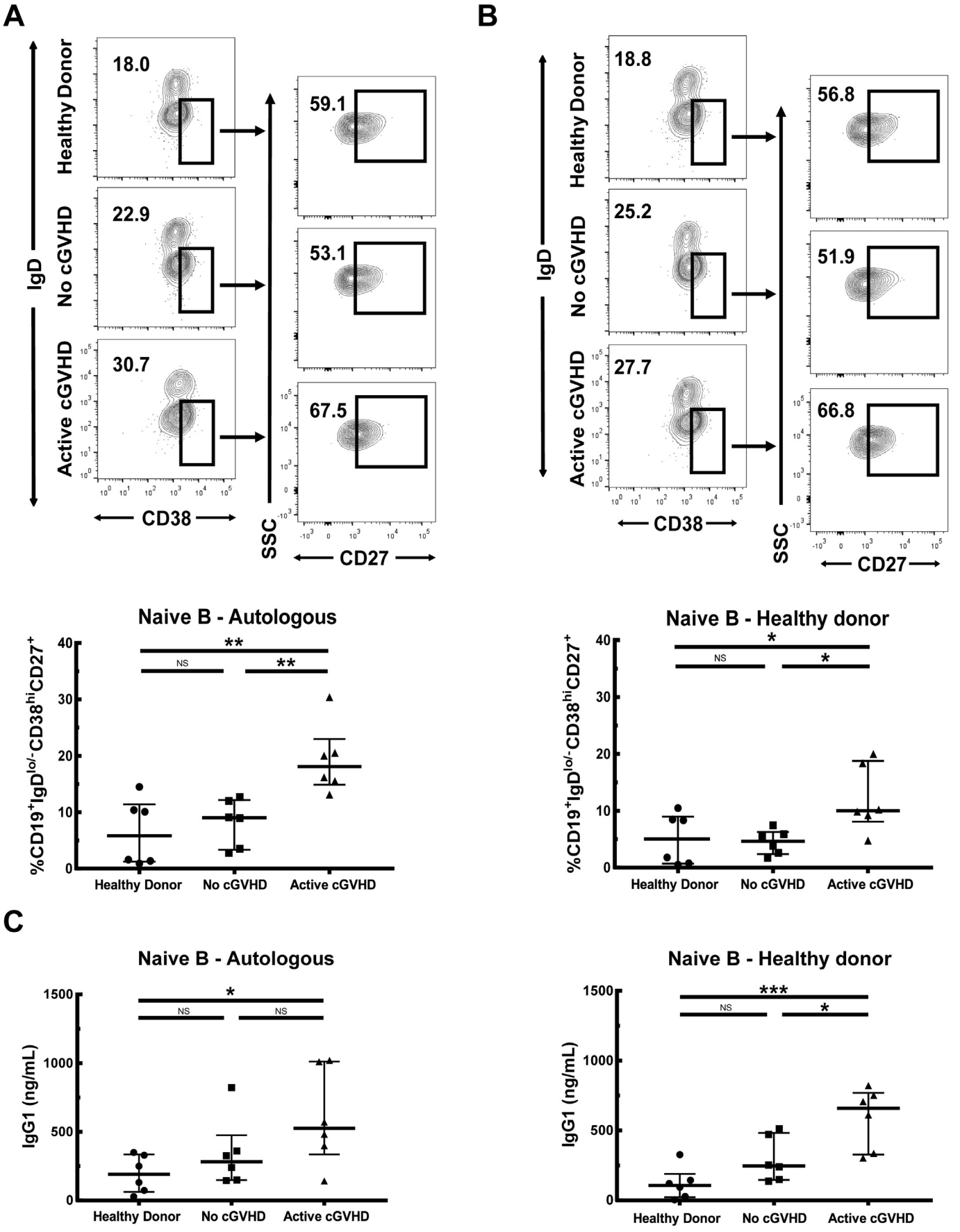

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Fig. 4. Enhanced function of c-extrafollicular Th-like cells in patients with active cGVHD. Frequencies of plasmablasts ($CD19^+IgD^{lo/-}CD38^{hi}CD27^+$) were measured after naive B cells were cultured with c-extrafollicular Th-like cells for 7 days. C-extrafollicular Th-like cells were purified by cell sorting (purity > 98%) from healthy donor and patient samples and cultured with autologous naive B cells ($CD19^+IgD^+CD38^{lo}CD27^-$) (Auto B) or naive B cells from allogeneic healthy donors (Allo B). Results are shown in (A–B). (C) IgG1 production by naive B cells (autologous or allogeneic healthy donors) in vitro coculture with c-extrafollicular Th-like cells. Black bars in each figure represent 75th percentile, median and 25th percentile values. NS, not significant; *P < 0.05, **P < 0.01, and ***P < 0.001.

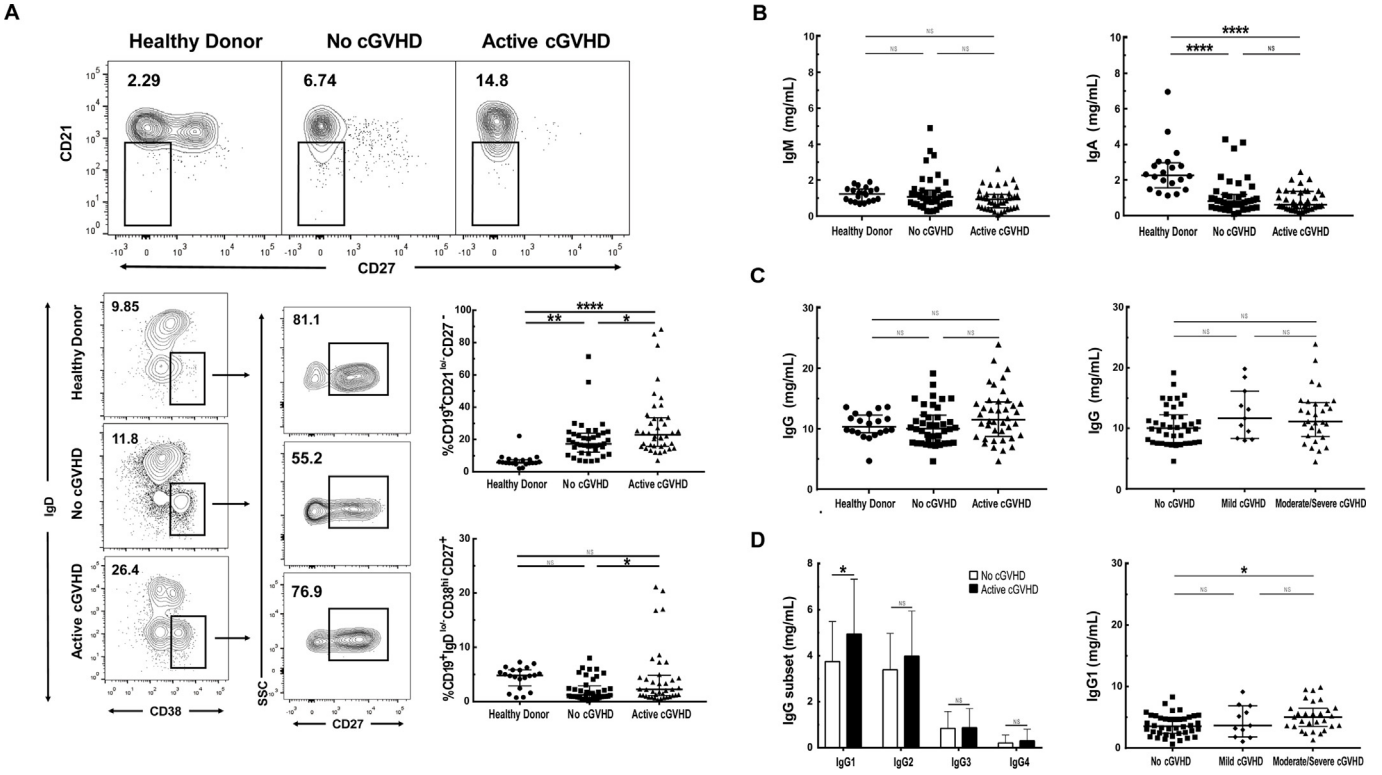

Fig. 5. Increased autoreactive B cells, plasmablasts and IgG1 levels in patients with active cGVHD. (A) Frequencies of autoreactive B cells and plasmablasts in healthy donors, patients without cGVHD, and patients with active cGVHD. (B) Serum IgM and IgA levels in healthy donors, patients without cGVHD and patients with active cGVHD. (C) Serum IgG levels in healthy donors, patients without cGVHD, patients with active cGVHD and patients with different severity of cGVHD. (D) Serum IgG subset levels in patients without cGVHD and patients with active cGVHD. Serum IgG1 levels of patients with different severity of cGVHD. Black bars in each figure represent 75th percentile, median and 25th percentile values. NS, not significant; *P < 0.05, **P < 0.01, and ****P < 0.0001.

4. Discussion

The role of GC $CD4^+$T and B cell interactions in cGVHD pathogenesis remain controversial. Blazar et al. demonstrated that similar to certain autoimmune diseases, the aberrant expansion of Tfh cells and GC formation were required for cGVHD and bronchiolitis obliterans (BO) in mouse models [7,29]. Ibrutinib, an inhibitor of Bruton's tyrosine kinase, ameliorated cGVHD by reducing GC reactions and tissue immunoglobulin deposition in mouse models [30]. However, our previous study and other studies suggested that GC formation is dispensable for the induction of cGVHD in mouse models [12,19]. Human studies have shown that patients who underwent allo-HSCT have reduced immunoglobulin somatic hypermutation at 1year post-transplantation, which is a process that requires GC formation. These finding suggested that GC formation is lacking in cGVHD patients. In this study, the frequency and count of cTfh cells were markedly decreased in patients with active cGVHD. This observation was also consistent with the results of Knorr's study, which showed that patients with active cGVHD had low numbers of cTfh cells in the blood [20]. Moreover, we found that these decreases were more significant in patients with moderate/severe cGVHD compared to those of patients without cGVHD.

In addition to Tfh cells, recent studies have shown that extrafollicular helper T and B cell interactions play key roles in the pathogenesis of autoimmune diseases [26,31]. In MRL^{lpr} lupus mice, extrafollicular helper T cells ($PSGL\text{-}1^{lo}CXCR4^{hi}CD4^+T$) expanded in the spleen [26]. Ectopic infiltration of Tfh-like cells have been observed in the lung tissues of a murine airway inflammation model [32] and in inflamed kidney tissues of patients with systemic lupus erythematosus [33]. In murine cGVHD models, it was observed that extrafollicular helper T cells expanded in the spleen, lung and liver [19]. In the present study, we identified c-extrafollicular Th-like cells in the human peripheral blood. We explored the role of these cells in the development of human cGVHD. We found that the frequency and count of c-extrafollicular Th-like cells markedly increased in patients with active cGVHD. The expansion of c-extrafollicular Th-like cells correlated with the clinical grade of cGVHD. Patients with moderate/severe cGVHD had a higher frequency of c-extrafollicular Th-like cells than patients with mild cGVHD ($P < 0.001$). Further, multivariable linear regression analysis also confirmed that moderate/severe cGVHD was a significant risk factor for a high percentage of c-extrafollicular Th-like cells after adjusting for other transplant characteristics ($P < 0.01$). C-extrafollicular Th-like cells from patients with active cGVHD also induced greater plasmablast differentiation than those of patients without cGVHD and those of healthy donors. The surface phenotype and B-cell helper function of c-extrafollicular Th-like cells in patients need to be further explored.

Extrafollicular T and B cell interactions lead to the generation of short-lived plasma cells and production of low-affinity IgG1 without somatic hypermutation [14–16]. Our previous study showed that IgG antibodies from donor B cells contributed to the destruction of B-cell

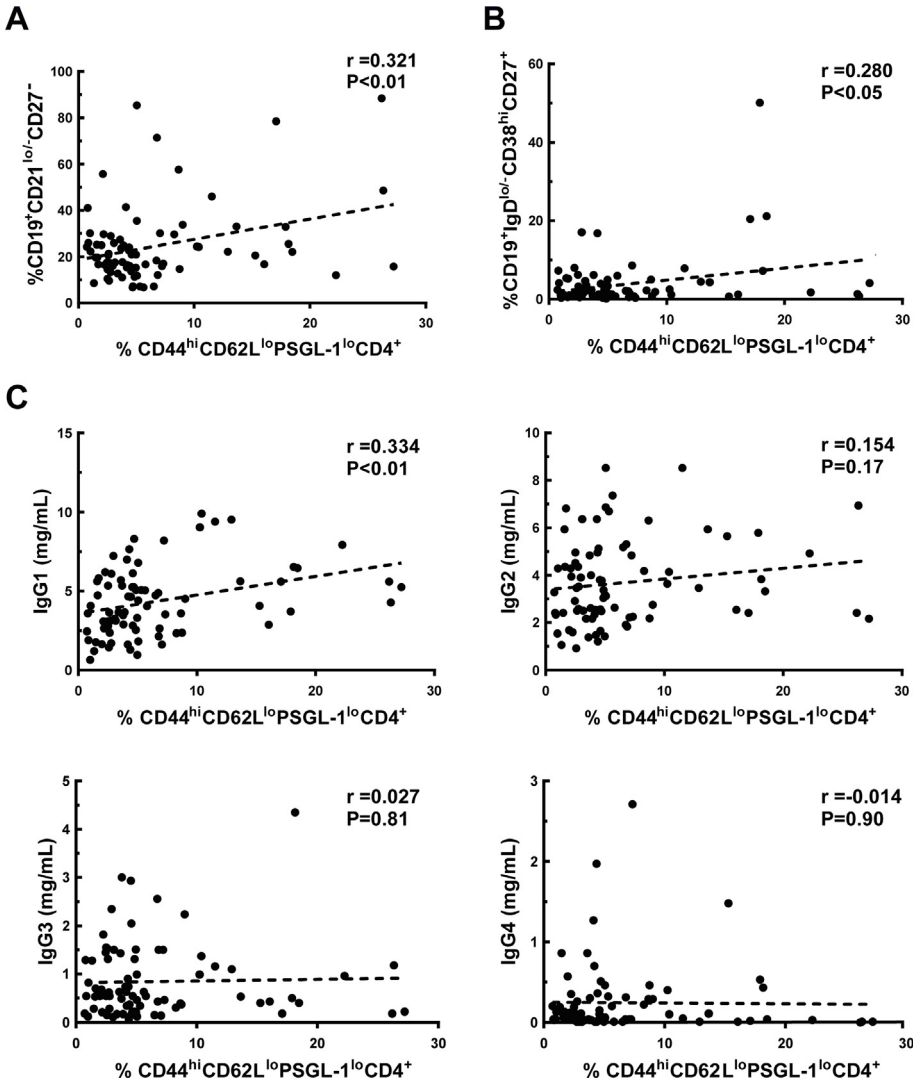

Fig. 6. C-extrafollicular Th-like cell levels correlated with generation of autoreactive B cells, plasmablasts and IgG1 in patients with active cGVHD. (A) Correlation between percentage of c-extrafollicular Th-like cells and percentage of autoreactive B cells in patient samples. (B) Correlation between percentage of c-extrafollicular Th-like cells and percentage of plasmablasts in patient samples. (C) Correlation between percentage of c-extrafollicular Th-like cells and IgG subset level in patient samples.

follicles and GCs in a murine cGVHD model [12], suggesting that IgG antibodies might result from extrafollicular helper T and B cell interactions. Odegard et al. showed that extrafollicular helper T cells elicited IgG1 production via IL-21 in systemic autoimmunity [26,34,35]. In systemic lupus mice, extrafollicular development of IgG^+ plasma cells was impaired when extrafollicular helper T cells were absent [26]. In our analysis, c-extrafollicular Th-like cells from patients with active cGVHD induced more IgG1 secretion. The frequency of c-extrafollicular Th-like cells was strongly correlated with the generation of autoreactive B cells, plasmablasts, and the levels of IgG1. This observation was also consistent with previous studies that showed that allo-HSCT recipients had a gradual recovery of serum IgM, IgG1, and IgG3, but not IgG2 or IgA and had reduced-immunoglobulin somatic hypermutation at 1year post-transplantation. Patients with active cGVHD had lymphopenia, and loss of GCs might have resulted from damage to lymphoid niches due to the deposition of IgG1 antibodies. In mouse cGVHD models, extrafollicular helper T cells expressed low levels of CXCR5, indicating that they interacted with B cells to produce low-affinity IgG1 at the T-B border. Further studies are needed to explore whether IgG1 are somatic hypermutation during the development of human cGVHD.

5. Conclusions

Our study demonstrates that the development of human cGVHD is associated with expansion of c-extrafollicular Th-like cells and reduction of the percentages of cTfh cells. The levels of c-extrafollicular Th-like cells correlated with the severity of cGVHD and the generation of autoreactive B cells, plasmablasts and IgG1 antibodies. Therefore, our findings support future monitoring of c-extrafollicular Th-like cell development and investigation of c-extrafollicular Th-like cell-based therapy to prevent or attenuate cGVHD.

Author contributions

H.J. designed study, performed research, analyzed the data, and wrote the manuscript; H.Z., and K.Y. performed experiments, collected and analyzed the data; Y.C. performed experiments; H.Q., Z.F., F.H., L.X., R.L., and K.Z. assisted in the research; Q.L. supervised the research and critically revised the manuscript; all authors approved the final version of publication.

Conflict of interest disclosure

The authors declare that they have no competing interests.

Acknowledgments

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