Profile of Toll-Like Receptors on Peripheral Blood Cells in Relation to Acute Graft-versus-Host Disease after Allogeneic Stem Cell Transplantation

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ABSTRACT

Toll-like receptors (TLRs) play a key role in the cross-talk between the innate and adaptive immune systems. Previous studies investigating associations between certain TLRs and acute graft-versus-host disease (aGVHD) have reported contrasting results, and no studies relating aGVHD to the expression and function of all human TLRs together have been published to date. We prospectively evaluated the expression of 9 TLRs on T lymphocytes and monocytes by flow cytometry in relation to aGVHD in 34 patients. Induction of TNF-α, IL-4, IFN-γ, and monocyte chemotactic protein 1 on TLR activation was assessed by ELISA on cell supernatants. Nineteen patients developed aGVHD, at a median time of 28 days (range, 20-50 days) after transplantation. A 2-step multivariate analysis was performed using principal component analysis and multifactor analysis of variance. The levels of TLR-5 expression on monocytes and T lymphocytes were positively correlated to aGVHD ($P = .01$), whereas levels of TLR-1 and -9 were negative predictors ($P = .03$ and .01, respectively). This profile of TLR-1, -5, and -9 can promote an overall immunostimulatory/proinflammatory response. If our findings are confirmed by further studies, this TLR profile could be a useful biomarker of aGVHD.

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INTRODUCTION

Acute graft-versus-host disease (aGVHD) is a primary T cell—mediated complication of allogeneic stem cell transplantation (SCT), occurring when donor-derived T cells are stimulated by host antigen-presenting cells (APCs), which are enhanced by proinflammatory cytokines, such as IL-1, IL-6, and TNF-α [1,2]. Recent studies have brought a renewed focus on the previously unrecognized role of adaptive T cell subsets and B cells in the induction and regulation of aGVHD [1]. Several novel innate immune cell subtypes (monocytes, dendritic cells [DCs], and NK cells) are known to affect the intensity of immunologic reactions in GVHD [1,3,4]. However, the complex interactions involving the innate and adaptive immune systems remain to be defined to provide a clearer understanding of the pathogenesis of aGVHD.

Pattern recognition receptors, such as Toll-like receptors (TLRs), play a key role in the cross-talk between the innate and adaptive immune systems [5,6]. TLRs belong to the type I transmembrane glycoprotein receptor family and are expressed by several cell types, including airway and epithelial cells, NK cells, DCs, B and T lymphocytes, monocytes, mast cells, macrophages, neutrophils, basophils, and endothelial cells [5-7]. TLRs recognize pathogen-associated molecular patterns (PAMPs), such as common protein, carbohydrate, and DNA/RNA pattern motifs [5,6]. They also serve as receptors for endogenous ligands and damaged tissues, suggesting that both pathogen-derived molecules and products of damaged tissues can trigger signals responsible for the regulation of innate and adaptive immune responses [5,6]. Extracellular ligands are recognized by surface TLRs (TLR-1, -2, -4, -5, and -6). Intracellular TLRs (TLR-3, -7, -8, and -9) bind mainly to foreign nucleic acids and sometimes detect self-DNA/RNA [5,6,8].

Very little is known about the in vivo expression and function of TLRs in patients who undergo allogeneic SCT and eventually develop aGVHD. The analysis of TLRs on cells of the immune system could be useful for identifying biomarkers of aGVHD and potential therapeutic targets. Specific TLR agonists and antagonists have shown promising results as immune-modulating agents in the treatment of other immune disorders, including noninfectious inflammatory and autoimmune diseases [9]. Thus, we prospectively evaluated the expression of TLR-1 to -9 on peripheral blood T lymphocytes and monocytes in relation to the onset of aGVHD. We also performed functional analysis after TLR stimulation to assess activity. We focused the analysis on T lymphocytes and monocytes, the former because they are primarily involved and the latter because of their emerging key role in the pathogenesis of aGVHD.

PATIENTS AND METHODS
Patients and Transplantation Procedures

Prospective evaluations of TLR expression and function were performed in 34 patients who underwent allogeneic SCT. All patients provided written consent in accordance with the Declaration of Helsinki and the Ethics Committee of the Hospital "Spedali Civili." Characteristics of the patients and transplants are reported in Table 1.

Antibiotic prophylaxis with levofloxacin was provided until complete neutrophil recovery was observed. Fluconazole or itraconazole was administered until all immunosuppressive drugs were withdrawn. Trimethoprim-sulphamethoxazole was given to prevent *Pneumocystis carinii* pneumonia. Cytomegalovirus (CMV) and human herpesvirus 6 (HHV-6) infections were

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Table 1
Patient and Transplant Characteristics

Characteristic	Value
Age at SCT, years, median (range)	46.5 (18-64)
Sex, n (%)	
Male	24 (71)
Female	10 (29)
Diagnosis, n (%)	
Acute leukemia	15 (44)
NHL/CLL	8 (24)
Other	11 (32)
Donor, n (%)	
Matched related donor	22 (65)
Matched unrelated donor	12 (35)
HLA match, n (%)	28 (82)
Conditioning, n (%)*	
MAC	19 (56)
RIC	15 (44)
Antithymocyte globulin, n (%)	
Yes	14 (41)
No	20 (59)
Stem cell source, n (%)	
Peripheral blood	30 (88)
Bone marrow	4 (12)
CD34$^+$ cell dose, $\times 10^6$/kg, median (range)	5 (1.1-7.9)
CD3$^+$ cell dose, $\times 10^7$/kg, median (range)	20.7 (2.3-48.7)
GVHD prophylaxis with CyA + MTX, n (%)	34 (100)
Bacterial infection before 30 days post-SCT, n (%)	18 (53)
IFI before 30 days post-SCT, n (%)	3 (9)
CMV infection before 30 days post-SCT, n (%)	5 (16)
HHV-6 infection before 30 days post-SCT, n (%)	2 (6)
aGVHD, n (%)	
Yes	19 (56)
No	15 (44)
Grade $\geq$II	11 (32)
Time, days, median (range)	28 (20-50)

CLL indicates chronic lymphoblastic leukemia; IFI, invasive fungal infection; MAC, myeloablative conditioning; NHL, non-Hodgkin lymphoma; RIC, reduced-intensity conditioning.

* Types of conditioning: MAC subtypes included total body irradiation (12 Gy in 6 fractions) + cyclophosphamide (CY) in 6 patients and busulfan + CY in 13 patients. RIC subtypes included thiotepa + fludarabine + CY in 7 patients, thiotepa + CY in 6 patients, and thiotepa + fludarabine + melphalan in 2 patients.

monitored weekly by quantitative real-time PCR in plasma, and patients who tested were treated with ganciclovir or foscarnet. Fungal infections were evaluated and defined according to the revised criteria of the European Organization for Research and Treatment of Cancer Invasive Fungal Infections Cooperative Group/National Institute of Allergy and Infectious Diseases Mycoses Study Group Consensus Group [10]. Bloodstream infection was defined according to the modified criteria of Poutsiaka et al. [11]. Bacterial infections included all bloodstream infections with or without organ localization. Chimerism was assessed by the variable number tandem repeat PCR test on peripheral blood mononuclear cells (PBMCs), polymorphonuclear leukocytes, and lymphocytes. Diagnosis and grading of aGVHD were based primarily on clinical findings according to commonly accepted diagnostic criteria [12,13]. Whenever possible or necessary, the clinical data were supported by histopathologic findings of the skin, liver, and gastrointestinal tract. aGVHD prophylaxis included 3 mg/kg/day cyclosporine A (CyA) i.v. via continuous infusion starting on day -1 along with methotrexate (MTX) 15 mg/m^2 on day 1 and 10 mg/m^2 on days 3, 6, and 11 in myeloablative conditioning regimens, and 10 mg/m^2 on day 1 and 8 mg/m^2 on days 3 and 6 in reduced-intensity conditioning regimens. CyA was given orally in 2 doses as soon as the patient was able to tolerate oral intake. CyA blood levels were monitored daily by chemiluminescence microparticle immunoassay (Abbott Hematology–Diagnostics Division, Santa Clara, CA). CyA dose was adjusted to maintain blood levels between 150 and 250 ng/mL.

Assessment of TLRs

To investigate a possible correlation between aGVHD and the expression and function of TLRs in SCT recipients, we performed flow cytometry analysis of TLR expression on monocytes and T lymphocytes, along with a functional analysis by induction of cytokines from PBMCs on TLR stimulation. The assessment of TLRs was planned at days +30, +60, and +90 post-SCT and at the time of aGVHD onset; however, aGVHD developed before day +60 (median time, 28 days post-SCT) in all patients. Thus, the

assessment was performed at the onset of aGVHD and at day +30 for patients without aGVHD. We assessed TLR-1, -2, -4, and -6 (expressed on plasma membrane), receptors for lipid-based PAMPs; TLR-3, -7, -8, and -9 (expressed in cytoplasmic compartments), receptors for nucleic acid–based PAMPs; and TLR 5, surface receptor for flagellin of Gram-negative bacteria. We did not assess TLR-10, because its agonist for functional analysis is unknown and preliminary data on cell expression show no significant differences between patients with aGVHD and those without aGVHD. We assessed these TLRs in 17 healthy donors as well.

Analysis of TLR Expression by Flow Cytometry

PBMCs from healthy donors and patients were isolated by Ficoll-Paque density gradient centrifugation using standard procedures. Lymphocyte and monocyte gates were identified on forward-scatter and side-scatter plots. FITC-conjugated CD3 antibody (BD Biosciences, Mountain View, CA) was used to identify T lymphocytes. PBMCs were incubated with anti-TLR Abs as described below. Surface and intracellular staining was performed using PE-conjugated Abs. TLR-2, -3, -4, -5, -6, -7, -8, and -9 antibodies were purchased from Imgenex (San Diego, CA), and anti-TLR1 was obtained from e-Bioscience (San Diego, CA). Human immunoglobulin vein solution (Kedrion, Lucca, Italy) was used for FcR monocyte saturation to avoid binding of unspecific antibodies. PE-conjugated IgG1 isotype antibody was used as a negative control (BD Pharmingen, San Diego, CA).

For TLR-1,- 2, -4, -5 and -6, analysis was performed using the flow cytometric cell surface method. Cells were incubated for 30 minutes at 4°C in the dark, washed once with 2% FBS, and finally suspended with staining buffer solution (Imgenex). Intracellular staining for TLR-3, -7, -8, and -9 was performed using an Imgenex Intracellular Staining Flow Assay Kit according to the manufacturer's instructions. A minimum of 10,000 events were acquired and analyzed in both monocyte and T lymphocyte gates using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences). TLR expression was evaluated as mean fluorescence intensity (MFI), with negative control MFI subtracted.

Functional Analysis of TLRs

The functional evaluation of TLRs was performed on total PBMCs to avoid possible aspecific activation by cell selection procedures [14]. A total of 100,000 cells/well were plated in duplicate in 96-well round-bottomed plates and stimulated by the following TLR agonists: Pam3CSK4 for TLR-1/2, heat-killed preparation of *Listeria monocytogenes* for TLR-2, Poly(I:C) Low Molecular Weight for TLR-3, Lipopolysaccharide for TLR-4, flagellin for TLR-5, FSL1 for TLR-6/2, imiquimod for TLR-7, ssRNA40 for TLR-8, and ODN2006 for TLR-9 (InvivoGen, San Diego, CA). Stimuli were used after dilution in endotoxin-free RPMI complete medium at final concentrations according to the manufacturer's instructions. After 24 hours of cell stimulation at 37°C in a 5% CO$_2$ atmosphere, the plate was centrifuged, and conditioned media were harvested and stored at -20°C before ELISA assays. Supernatants were thawed at room temperature and assessed using ELISA kits (Bender MedSystem, Vienna, Austria) according to the manufacturer's instructions. The cytokines analyzed were IFN-γ (Th1 cytokine), IL-4 (Th2 cytokine), TNF-α (proinflammatory cytokine), and monocyte chemotactic protein 1 (MCP-1; C-C chemokine). Detection limits were as follows: 1.65 pg/mL for TNF-α, 2.31 pg/mL for MCP-1, 0.66 pg/mL for IL-4, and 0.99 pg/mL for IFN-γ.

Results are reported as relative induction and expressed using the following formula: (induction of cytokine with TLR ligand activation - induction of cytokine of medium control)/induction of cytokine of medium control.

Lymphocyte Count and Routine Immunophenotyping

Lymphocyte count and routine immunophenotyping were performed by flow cytometry in the laboratory of the University of Brescia's Clinical Biochemistry Department. Peripheral blood samples were collected and incubated with pretitrated saturating dilutions of the following FITC- or PE-conjugated mAbs: CD14, CD45, CD3, CD4, CD8, CD19, CD16, and CD56 (BD Biosciences). Samples were analyzed with a FACScan flow cytometer and CellQuest software (BD Biosciences).

Statistical Analysis

Univariate analysis of the variables in relation to the onset of aGVHD was performed using the Mann-Whitney U test and χ^2 test. The Mann-Whitney U test was used to compare continuous values, and the χ^2 test was used to compare differences in percentage. Monocyte count, lymphocyte subset counts, TLR expression on monocytes and T lymphocytes, and cytokine induction on TLR activation were analyzed. The following patient- and transplant-related variables were also analyzed: age at SCT, sex, diagnosis, type of donor, HLA mismatch, type of conditioning (reduced-intensity or conventional myeloablative; chemotherapy-based or TBI-based), use of

Table 2
Comparison of Clinical Characteristics and Monocyte and Lymphocyte Values in Patients with and without aGVHD

	With aGVHD	Without aGVHD	P Value
	(n = 19)	(n = 15)	
Age at SCT, years, median (range)	48 (24-64)	44 (18-62)	.57
Sex, n (%)			
Male	13 (68)	11 (73)	.95
Female	6 (32)	4 (27)	
Diagnosis, n (%)			
Acute leukemia	9 (47)	6 (40)	.93
NHL/CLL	4 (21)	4 (27)	.98
Other	6 (32)	5 (33)	.80
Donor, n (%)			
Matched related donor	13 (68)	9 (60)	.88
Matched unrelated donor	6 (32)	6 (40)	
HLA mismatch, n (%)	4 (21)	2 (13)	.89
Conditioning, n (%)*			
MAC	12 (63)	7 (47)	.49
RIC	7 (37)	8 (53)	
Conditioning, n (%)†			
Chemotherapy-based	16 (84)	12 (80)	.89
Total body irradiation–based	3 (16)	3 (20)	
Antithymocyte globulin, n (%)			
Yes	8 (42)	6 (40)	.82
No	11 (58)	9 (60)	
Stem cell source, n (%)			
Peripheral blood	18 (95)	12 (80)	.43
Bone marrow	1 (5)	3 (20)	
CD34+ cell dose, × 10^6/kg, median (range)	5.1 (1.1-6.6)	4.8 (1.5-7.9)	.58
CD3+ cell dose, × 10^7/kg, median (range)	18.2 (2.3-48.7)	21.9 (2.8-36.4)	.81
Bacterial infection before 30 days post-SCT, n (%)	8 (42)	10 (67)	.28
IFI before 30 days post-SCT, (%)	1 (5)	2 (13)	.83
CMV infection before 30 days post-SCT, n (%)	4 (21)	1 (7)	.49
HHV-6 infection before 30 days post-SCT, n (%)	2 (11)	0 (0)	.57
Lymphocytes, cells/μL, mean ± SD			
CD3+	380 ± 261	552 ± 465	.24
CD3+/CD4+	143 ± 108	183 ± 128	.40
CD3+/CD8+	213 ± 146	344 ± 403	.27
CD19+	37 ± 47	59 ± 49	.21
CD16+/CD56+	115 ± 132	144 ± 52	.47
Monocytes, cells/μL, mean ± SD	480 ± 363	539 ± 277	.73

CLL indicates chronic lymphoblastic leukemia; IFI, invasive fungal infection; MAC, myeloablative conditioning; NHL, non-Hodgkin lymphoma; RIC, reduced-intensity conditioning.

* Types of RIC and MAC did not differ in patients with aGVHD and those without aGVHD.

† Total body irradiation was used only in MAC.

Table 3
Comparison of TLR Expression on T Lymphocytes and Monocytes in Patients with and without aGVHD

	Without aGVHD (n = 15)	With aGVHD (n = 19)	P Value
T lymphocytes, MFI			
TLR-1	4.3 ± 2.9 (0.9-10.8)	1.6 ± 2.2 (0-8.0)	**.02**
TLR-2	1.2 ± 0.8 (0-2.6)	1.4 ± 0.9 (0-3.1)	.53
TLR-3	1.6 ± 1.1 (0-4.3)	1.3 ± 1.2 (0-4.3)	.50
TLR-4	1.8 ± 1.3 (0-4.7)	1.7 ± 1.3 (0-6.0)	.74
TLR-5	1.9 ± 1.6 (0-6.2)	3.5 ± 2.3 (0.8-8.2)	**.03**
TLR-6	1.9 ± 1.8 (0-6.2)	2.6 ± 2.1 (0.2-8.0)	.38
TLR-7	2.9 ± 1.7 (0.7-6.5)	2.0 ± 2.1 (0-6.4)	.16
TLR-8	0.8 ± 1.7 (0-6.8)	0.5 ± 0.6 (0-1.8)	.61
TLR-9	108.8 ± 51.5 (16.9-207.4)	63.8 ± 50.4 (0-165)	**.01**
Monocytes, MFI			
TLR-1	54.9 ± 37.4 (12.7-130.7)	21.4 ± 21.9 (0.6-66.3)	**.005**
TLR-2	4.9 ± 3.6 (0-12.9)	7.9 ± 10.5 (0.7-43)	.50
TLR-3	4.1 ± 1.9 (1.4-8.3)	3.1 ± 1.9 (0-7.1)	.15
TLR-4	11.1 ± 8.3 (0-28.9)	10.8 ± 6.9 (2.3-22.9)	.91
TLR-5	9.0 ± 4.7 (3.2-16.6)	25.8 ± 24.3 (0.6-80)	**.01**
TLR-6	9.2 ± 6.5 (2.4-26.8)	10.9 ± 12.4 (0-34.3)	.40
TLR-7	7.0 ± 4.4 (3.2-18.3)	4.6 ± 3.9 (0-12.9)	.14
TLR-8	1.9 ± 1.7 (0-5.3)	1.3 ± 1.4 (0-9.1)	.22
TLR-9	168.5 ± 77.9 (39.6-283.5)	85.3 ± 73.9 (0-310)	**.002**

MFI values are expressed as mean ± standard deviation (range). Significant P values are in bold type.

predictor variables for aGVHD. PCA transforms a number of possibly correlated variables into a smaller number of uncorrelated variables; these principal components (PCs) are linear combinations of the original variables. Each PC is a cluster of correlated variables. PCs are chosen in sequence as the best descriptor of the data. The first extracted PC accounts for the largest part of the total variance in the dataset, the second PC has the second largest amount of the variance, and so on for each subsequent component. The last few PCs do not account for much of the variance and thus can be ignored. We used the eigenvalue-1 criterion (ie, Kaiser criterion) to select an adequate number of PCs, with only PCs with eigenvalue >1 extracted. Only the variables with component loading >0.5 (absolute value) were included in each PC extracted. Loadings vary in value from −1 to 1 and represent the degree to which each of the variables correlates with each PC. Variables with negative loadings have a meaning opposite to that of variables with positive loadings.

Multivariate analysis was completed by multifactor analysis of variance (ANOVA) using the PC scores, which were values of the PCs extracted by PCA for each patient. All P values were 2-sided, and a P value <.05 was considered statistically significant.
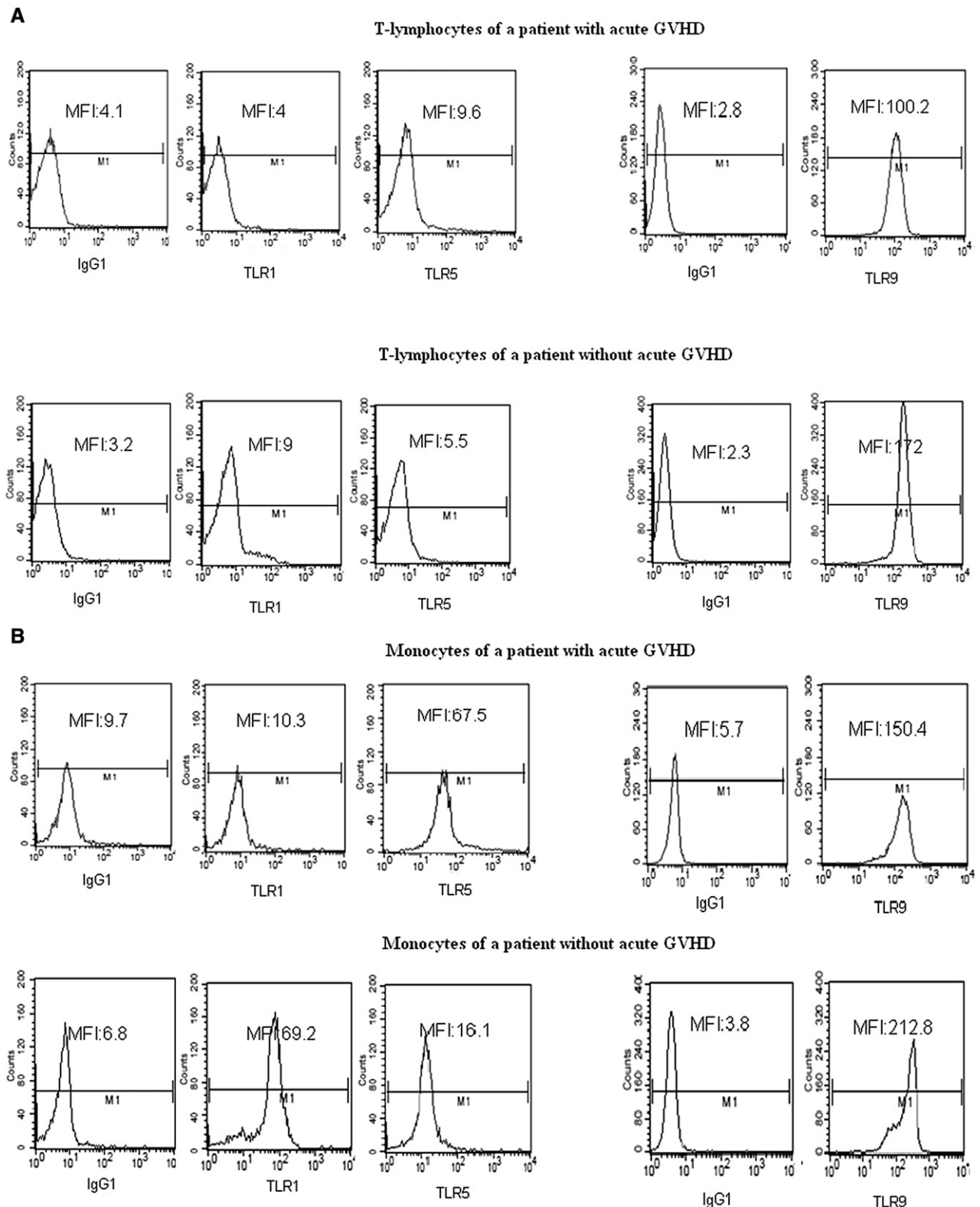
RESULTS

Clinical Characteristics of Patients with aGVHD

Nineteen patients (56%) developed aGVHD at a median time of 28 days (range, 20-50 days) after SCT. The aGVHD was at least grade II in 11 patients (32%) and grade III in 3 patients (9%). The most common target of aGVHD was skin, in 84% of cases, with gastrointestinal tract and liver involved in 32% and 16% of cases, respectively.

Eight patients (42%) had a bacterial infection, and 1 patient (5%) had an invasive fungal infection. CMV and HHV-6 infections were detected in 4 (21%) and 2 (11%) patients, respectively (Table 2).

TLR Expression on Monocytes and T Lymphocytes

The analysis of TLR expression on monocytes and T lymphocytes was performed by flow cytometry in 17 healthy donors at the onset of aGVHD and at day +30 in patients without aGVHD. The median time of aGVHD onset was 28 days post-SCT. All patients achieved stable full chimerism at the time of TLR assessment. The analysis of TLR expression did not show significant differences between healthy donors and patients without aGVHD (data not shown). Data on TLR expression in SCT recipients are summarized in Table 3. In patients with aGVHD, monocytes and T lymphocytes

antithymocyte globulin in conditioning, stem cell source, CD34 and CD3 cell doses infused, and development of any bacterial, fungal, or viral infections before TLR assessment (ie, before the onset of aGVHD and before day +30 post-SCT in patients without aGVHD).

Principal component analysis (PCA) was performed as the first step of multivariate analysis to solve the problem of the high number of variables compared with the relatively limited and heterogeneous pool of patients, as described by Skert et al. [15]. PCA is typically used to reduce the dimensionality of a large number of interrelated variables while retaining as much information as possible [16]. Along with reducing a large dataset to a few components that can be easily overviewed, PCA has important noise-reducing properties in small populations of patients, analogous to the reduction in noise gained by using large populations of patients. Thus, we used PCA with varimax rotation to reduce the number of variables in relation to the number of patients and to perform a later multivariate analysis of

A

T-lymphocytes of a patient with acute GVHD

T-lymphocytes of a patient without acute GVHD

B

Monocytes of a patient with acute GVHD

Monocytes of a patient without acute GVHD

Figure 1. Expression of TLR-1, -5, and -9 on T lymphocytes and monocytes. Histogram plots of TLR-1, -5, and -9 expression in a patient with aGVHD and in a patient without aGVHD. The expression of surface (TLR-1 and -5) and intracellular (TLR-9) TLRs is reported with the corresponding negative control (mouse IgG1-PE). MFI is shown for each histogram. T lymphocytes of the patient with aGVHD have higher MFI values of TLR-5 and lower MFI values of TLR-1 and -9 compared with the patient without aGVHD (A). An analogous TLR profile is displayed by monocytes (B).

expressed lower levels of TLR-1, a surface receptor for lipid-based PAMPs ($P = .005$ and $.02$, respectively), and TLR-9, an intracellular receptor for nucleic acid–based PAMPs ($P = .002$ and $.01$, respectively), whereas levels of TLR-5, a surface receptor for flagellin of Gram-negative bacteria, were significantly increased ($P = .01$ and $.03$, respectively).

Table 4
Comparison of Cytokine Relative Induction on TLR Activation in Patients with and without aGVHD

	IFN-γ (relative induction)			MCP-1 (relative induction)			TNF-α (relative induction)		
	Without aGVHD (n = 15)	With aGVHD (n = 19)	P	Without aGVHD (n = 15)	With aGVHD (n = 19)	P	Without aGVHD (n = 15)	With aGVHD (n = 19)	P
TLR-1	237 ± 553 (0-1484)	77 ± 157 (−1 to 472)	.52	2210 ± 6079 (−0.4 to 18,370)	1117 ± 3524 (−1 to 11,739)	.67	667 ± 1964 (−0.4 to 5902)	1.4 ± 3.1 (−1 to 10)	.32
TLR-2	983 ± 1770 (0.3-4923)	74 ± 219 (−1 to 698)	**.03**	14,176 ± 29,534 (−0.1 to 84,240)	2975 ± 8601 (−0.9 to 27,391)	.25	1664 ± 4644 (−0.4 to 14,040)	10 ± 18 (−1 to 56)	.12
TLR-3	241 ± 427 (−0.3 to 1124)	−0.4 ± 0.5 (−1 to 0)	**.008**	4287 ± 11,838 (−0.9 to 35,815)	916 ± 2095 (−1 to 6460)	.29	5.2 ± 9.1 (−0.3 to 26)	0.3 ± 1.1 (−1 to 3)	.31
TLR-4	868 ± 1771 (0-4823)	54 ± 92 (−1 to 217)	**.04**	3211 ± 5375 (−0.2 to 14,348)	3035 ± 5884 (−1 to 15,420)	.99	1617 ± 3950 (−0.1 to 11,955)	38 ± 81 (−1 to 241)	.08
TLR-5	597 ± 1561 (−1 to 4137)	184 ± 503 (−1 to 1604)	.80	12,196 ± 30,630 (−0.4 to 92,446)	1765 ± 3919 (−1 to 11,956)	.66	897 ± 2575 (−1 to 7763)	2.1 ± 5.6 (−1 to 19)	.09
TLR-6	−0.1 ± 0.4 (−1 to 0.3)	3.5 ± 8.3 (−1 to 23.9)	.83	3524 ± 7817 (0.2 to 23,206)	804 ± 2495 (−0.8 to 8315)	.88	39 ± 64 (−0.9 to 157)	2.2 ± 4.5 (−1 to 13.4)	.16
TLR-7	537 ± 1368 (−1 to 3637)	3.9 ± 13 (−1 to 42)	.29	4222 ± 11,871 (−0.4 to 35,820)	60 ± 161 (−1 to 543)	.29	112 ± 295 (−1 to 780)	0.7 ± 2.4 (−1 to 8)	.45
TLR-8	941 ± 1845 (0-4864)	195 ± 611 (−1 to 1934)	.15	1150 ± 3020 (−0.3 to 9141)	1926 ± 5633 (−1 to 17,920)	.40	2079 ± 5754 (−0.7 to 16,320)	18 ± 48 (−1 to 161)	.43
TLR-9	3.4 ± 8.3 (0-21)	0.3 ± 2.6 (−1 to 8)	**.04**	555 ± 1040 (0-2554)	1067 ± 3199 (−0.9 to 10,163)	.45	50 ± 118 (0-338)	18 ± 60 (−1 to 200)	.07

Relative induction = (induction of cytokine with TLR ligand activation - induction of cytokine of medium control)/induction of cytokine of medium control. Values of relative induction are expressed as mean ± standard deviation (range). Significant P values are in bold type.

Representative histogram plots of TLR-1, -5, and -9 expression are shown in Figure 1.

Functional Analysis of TLR

We analyzed cytokine induction from PBMCs on TLR stimulation concomitantly with flow cytometry analysis. All TLR agonists induced the production of IFN-γ, TNF-α, and MCP-1 in both healthy donors and SCT recipients, whereas IL-4 production was not induced. Results are reported in Table 4 as relative induction: (induction of cytokine with TLR ligand activation - induction of cytokine of medium control)/ induction of cytokine of medium control. Healthy donors and patients without aGVHD did not differ in terms of cytokine production (data not shown). Patients with aGVHD had lower IFN-γ relative induction on stimulation with HKLM (TLR-2 ligand; $P = .03$) and LPS (TLR-4 ligand; $P = .04$). IFN-γ relative induction was also decreased in patients with aGVHD in response to LMW (TLR-3 ligand; $P = .008$) and ODN2006 (TLR-9 ligand; $P = .04$).

### Univariate and Multivariate Analyses of Predictor Variables for aGVHD

In univariate analysis, clinical and transplant characteristics, monocyte count, and lymphocyte subset counts were not significantly different in patients with aGVHD and those without aGVHD (Table 2). Moreover, the 2 groups demonstrated no significant differences in terms of immunosuppressive therapy (MTX doses and CyA blood levels) and type of reduced-intensity or myeloablative conditioning regimens (data not shown). Monocytes and T lymphocytes of patients with aGVHD had an analogous profile, expressing higher levels of TLR-5 and lower levels of TLR-1 and -9. These patients also had lower IFN-γ relative induction.

A 2-step multivariate analysis with PCA and multifactor ANOVA was used to solve the problem of the high number of variables in comparison with the relatively limited and heterogeneous pool of patients. Four clusters of variables (Cs) were identified by PCA, with each cluster including only variables with loading >0.5 (Figure 2). On multifactor ANOVA, C2, C3, and C4 were significantly associated with the onset of aGVHD (Table 5). C2 included levels of TLR-5 expression on monocytes and T lymphocytes associated with the development of aGVHD (positive loading). Levels of TLR-9 and -1 on monocytes and T lymphocytes (C3 and C4, respectively) were inversely correlated with aGVHD (negative loading). In summary, multivariate analysis with PCA and multifactor ANOVA confirmed the results of univariate analysis, except for the negative correlation of IFN-γ relative induction.

DISCUSSION

TLRs are involved in maintaining immune tolerance and eliminating pathogenic microorganisms [5,6]. They also play a role in amplifying immune response in several inflammatory diseases, including systemic lupus erythematosus (SLE), arthritis, and inflammatory bowel disease, which have similarities to GVHD [5,6,17,18].

Previous studies dealing with TLRs and aGVHD in SCT recipients and mouse models have focused mainly on TLR-4 and -9 and have reported contrasting results [3,19-28]. To our knowledge, no studies to date have examined the expression and function of all human TLRs together in relation to aGVHD, even in a single-cell subset of the immune system. Thus, we performed a prospective study correlating aGVHD to expression of TLR-1 to -9 and cytokine production on
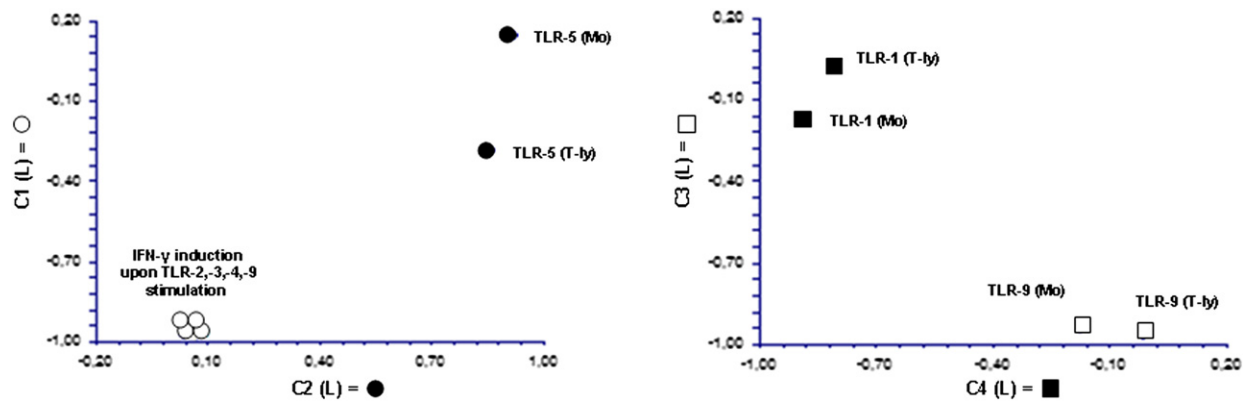
Figure 2. Clusters of variables identified by PCA after SCT. In the first step of the multivariate analysis, 4 clusters of variables were extracted by PCA. The position of each variable in the loading plot indicates its relationship with each cluster. Only variables with component loading >0.5 (absolute value) are included in each cluster. Loadings vary in value from −1 to 1 and represent the degree to which each of the variables correlates with each cluster. Variables with negative loadings have a meaning opposite to that of variables with positive loadings. C, cluster of variables; L, loading; ○, variables included in C1; ●, variables included in C2; □, variables included in C3; ■, variables included in C4. TLR-x (x = 1, 5, or 9) (Mo) represents TLR-x expression on monocytes; TLR-x (x = 1, or 5, or 9) (T-ly) represents TLR-x expression on T lymphocytes.

activation of specific ligands in monocytes and T lymphocytes of peripheral blood, with T lymphocytes primarily involved and the role of monocytes in the pathogenesis of aGVHD becoming increasingly evident [1,4,29]. Although monocytes function as weaker APCs compared with DCs, they may differentiate to DCs when exposed to inflammatory stimuli [30,31]. Monocytes may contribute to tissue damage through their effector functions, such as production of TNF-α and O_2 radicals, or by stimulating effector T lymphocytes [32,33].

Our 2-step multivariate analysis revealed a significant correlation between aGVHD and a similar TLR expression profile on monocytes and T lymphocytes, which were characterized by higher levels of TLR-5 and lower levels of TLR-1 and -9.

TLR-5 detects flagellin, a protein that autopolymerizes to form the flagella of a wide variety of pathogenic and nonpathogenic bacteria, including those of intestinal microbiota [5,6]. The interaction between TLR-5 and flagellin in monocytes and lymphocytes may be a critical point in the pathogenesis of aGVHD, with intestinal microflora playing a known role in its development [1-3]. Furthermore, immune cells also may encounter flagellin following the bacterial translocation from the bowel lumen to the systemic circulation [3]. Increased expression of TLR-5 on monocytes has been associated with increased inflammatory responses in elderly individuals [34] and in patients with SLE [35], similar to our results in patients with aGVHD. Increased expression of TLR-5 on monocytes could induce their differentiation in M1 macrophages and in immunogenic APCs/DCs, with

a consequent Th1 cell response [33,36]. Overexpression of TLR-5 on monocytes could alter its intracellular trafficking and localization, leading to a shift from an overall anti-inflammatory to an overall proinflammatory immune response. In fact, there is evidence suggesting that changes in intracellular trafficking of extracellular TLRs may activate different signaling pathways and consequently regulate the type and intensity of immune responses [37].

The expression and function of TLR-5 on lymphocytes have been analyzed in several previous studies that focused mainly on T cells of healthy donors or of patients with autoimmune/inflammatory diseases [7,35,38-42]. We found higher TLR-5 expression on T lymphocytes of patients with aGVHD. Both effector and regulatory T cells (Tregs) express TLR-5 at physiologically relevant levels, explaining in part its ambivalent immunoregulatory/immunostimulatory function [39-42]. TLR-5 stimulation may enhance the suppressive capacity of Tregs and the proliferation/activation of effector T cells, especially of memory T cells, balancing immune responses in physiological conditions [39,42]. In aGVHD, this homeostatic mechanism may break down, because an amplified inflammatory environment may persist and effector T cells may prevail over Tregs. Inflammatory cytokines and costimulatory molecules may lead to strong activation of effector T cells, which would maintain their resistance to Treg suppression [39].

Immune reactions in aGVHD could be promoted by decreased expression of TLR-1, which works as a heterodimer with TLR-2 to recognize lipopeptides of different pathogens, including bacteria, fungi, and viruses [5,6]. As a heterodimer with TLR-2, TLR-1 may influence the response to endogenous danger signals from injured tissue, such as heat shock proteins and high-mobility group box 1 proteins [5,6]. In monocytes, decreased surface expression of TLR-1 could preferentially activate the canonical nuclear factor-κB pathway, resulting in the induction of inflammatory cytokines and immunogenic APC maturation [43,44]. Previous studies have shown that TLR-2–mediated stimulation of Tregs influences their expansion and functions [40,45]; thus, a defective TLR-2/1 heterodimer stimulation could dysregulate the proliferative and suppressive efficiency of Tregs.

TLR-9 signaling may function as dampener or promoter of immune reactions, likely with a prevalence of protective

Table 5
Clusters of Variables Correlated with aGVHD by Multifactor ANOVA

Cluster	P Value
C1 (IFN-γ relative induction on TLR-2, -3, -4, and -9 stimulation)	.16
C2 (TLR-5/Mo, TLR-5/T-ly)	**.01**
C3 (TLR-9/Mo, TLR-9/T-ly)	**.01**
C4 (TLR-1/Mo, TLR-1/T-ly)	**.03**

TLR-x (x = 1, 5, or 9)/Mo: TLR-x expression on monocytes; TLR-x (x = 1, 5, or 9)/T-ly: TLR-x expression on T lymphocytes. Four clusters of variables were identified by PCA. IFN-γ relative induction on TLR stimulation and TLR expression on monocytes and on T lymphocytes included in each cluster are in parentheses. C2, C3, and C4 are correlated with aGVHD. Significant P values are in bold type.

effects in autoimmune diseases. Opposite effects of TLR-9 in the pathogenesis of GVHD have been demonstrated in mouse models and SCT recipients [3,21-23,26,27], whereas only 1 previous study correlated TLR-9 high-expressing B cells with the onset of extensive chronic GVHD [46].

TLR-9 recognizes double-stranded DNA viral, protozoan, and bacterial genoma [5,6]. Phosphorothioate linkages and unmethylated CpG motifs, which are lacking in mammalian DNA, are not the only foreign signature that triggers TLR-9 [8]. Localization of DNA in the endosomal compartment, rather than its sequence, species origin, covalent modification, or double versus single strandedness, triggers TLR-9 activation [8]. Endogenous DNA can indeed stimulate TLR-9 activation, especially in abnormal conditions, as occurs in SLE, whereas under normal conditions, endogenous nucleic acids hardly gain access to the intracellular compartment [5,8,17,18]. Self-derived nucleic acids can bypass this protective mechanism in conditions of abundant autoantigen release, such as tissue injury, increased apoptosis, defective clearance of cell debris, or inflammatory states [8,17,18,47]. Furthermore, in inflammatory milieu, oxidation of DNA/RNA by reactive oxygen species and hypomethylated DNA deriving from mitochondrial damage may lead to increased immunogenicity of self-nucleic acids [18,48]. All of these conditions may characterize aGVHD and thus may explain TLR-9 involvement, even in the absence of viral or bacterial infections. Patients with aGVHD expressed lower levels of TLR-9 on both monocytes and T lymphocytes, highlighting TLR-9's potential protective role. TLR-9 shares a common signaling pathway with TLR-7 and TLR-8, which induces proliferation and cytokine release in B cells, T cells, IFN-α, and proinflammatory cytokines in plasmacytoid DCs [8,18]. These cytokines may promote myeloid DC differentiation and T cell activation. Activation of this signaling pathway should produce an overall inflammatory/immunostimulatory effect, in contrast with the emerging protective role of TLR-9, as shown by our results and those of others [17,18,47]. Emerging evidence shows that the prevalence of TLR-9 stimulation may inhibit TLR-7 activation by RNA complexes [18,47] and its univocal proinflammatory effects on monocytes, which also promotes their differentiation in tolerogenic DCs. The antagonism with TLR-7 could positively modulate Treg efficiency [18,47]. Thus, decreased expression of TLR-9 could shift the balance toward a prevalence of TLR-7 activation and of the proinflammatory pathway.

Our multivariate analysis did not confirm IFN-γ induction on the activation of TLR-2, -3, -4, or -9 as a negative predictor of aGVHD. Furthermore, the different expression of TLRs in patients with aGVHD was not always related to changes in cytokine production. On the other hand, in vitro analysis of cytokine production can provide only a partial assessment of the effects of TLR activation, taking into account the different functions of TLRs in the cells of the immune system.

In conclusion, both monocytes and T lymphocytes were characterized by an analogous TLR profile (including TLR-1, -5 and -9 expression), which likely promotes an overall immunostimulatory/proinflammatory response. Changes in TLR expression and correlated signaling pathways could modulate the type and the intensity of immune reactions by interacting not only with microbial agents, but also with endogenous danger signals of damaged tissues, such as self-DNA, heat shock proteins, and high-mobility group box 1 protein.

In this preliminary study, PCA was used to overcome the problem deriving from the high number of variables compared with the relatively limited pool of patients. Studies involving a larger number of patients would be useful to confirm our findings, and to validate this TLR profile as a biomarker of aGVHD. Furthermore, studies on different lymphocyte subsets could help clarify the roles of TLR-1, -5, and -9 in adaptive immunity in the setting of aGVHD.

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