

ORIGINAL ARTICLE

Circulating endothelial cell count: a reliable marker of endothelial damage in patients undergoing hematopoietic stem cell transplantation

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The physio-pathologic interrelationships between endothelium and GvHD have been better elucidated and have led to definition of the entity 'endothelial GvHD' as an essential early phase prior to the clinical presentation of acute GvHD. Using the CellSearch system, we analyzed circulating endothelial cells (CEC) in 90 allogeneic hematopoietic stem cell transplantation (allo-HSCT) patients at the following time-points: T1 (pre-conditioning), T2 (pre-transplant), T3 (engraftment), T4 (onset of GvHD) and T5 (1 week after steroid treatment). Although CEC changes in allo-HSCT represent a dynamic phenomenon influenced by many variables (that is, conditioning, immunosuppressive treatments, engraftment syndrome and infections), we showed that CEC peaks were constantly seen at onset of acute GvHD and invariably returned to pre-transplant values after treatment response. Since we showed that CEC changes during allo-HSCT has rapid kinetics that may be easily missed if blood samples are drawn at pre-fixed time-points, we rather suggest an 'on demand' evaluation of CEC counts right at onset of GvHD clinical symptoms to possibly help differentiate GvHD from other non-endothelial complications. We confirm that CEC changes are a suitable biomarker to monitor endothelial damage in patients undergoing allo-transplantation and hold the potential to become a useful tool to support GvHD diagnosis (*ClinicalTrials.gov* NCT02064972).

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INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) can be curative in several onco-hematologic diseases, but it is burdened by life-threatening complications.^{1–3} To date, the overall incidence of GvHD still remains between 30 and 60% and is approximately associated with a 50% mortality rate, especially in its severe form.^{1–3} Its current pathogenesis model represents a multiphase process in which the result of the conditioning regimen, the release of cytokines and the antigenic differences between recipient and donor lead donor lymphocytes to attack epithelial cells and mucous membranes in target organs.³ The release of cytokines and the immune attack result in ongoing tissue damage with further cytokine production and a continuous inflammatory positive feedback loop.⁴ However, T-lymphocytes firstly require a tight contact with cells of the vascular endothelium to reach the epithelial target organs.⁵ Therefore, the vascular endothelium is currently considered an early phase target of donor T-lymphocytes.^{6–8} During allo-HSCT, endothelial cells are affected by a series of events, resulting in endothelial damage. In fact, vascular endothelium is highly exposed to toxic drugs and inflammatory molecules released in the extracellular matrix by damaged cells and leukocytes.^{9,10} These insults can be the initial event of endothelial injury and may contribute to the development of immune reactions related to life-threatening complications, including GvHD.^{7,8} Nowadays, validated non-

invasive biomarkers used in routine clinical practice for GvHD diagnosis are lacking. Moreover, laboratory tests can hardly predict the risk of developing GvHD or its response to treatment.^{11–14} Thus, the identification of specific biomarkers from peripheral blood (PB) samples would represent a valuable tool to avoid invasive diagnostic procedures and help personalize treatments after allo-HSCT.¹⁵

In recent years, the direct count of circulating endothelial cells (CEC) has emerged as a valuable biomarker of endothelial damage in a variety of disorders.^{8,12,16–20} However, due to their very low numbers and complex phenotype, several published techniques have shown different degrees of variability, reporting a wide range of CEC values (0–7900 CEC/mL) in healthy subjects.^{12,21–24} We prospectively correlated CEC count changes to GvHD onset and response to treatment in 90 allo-HSCT patients. Moreover, in this study, we used the commercially available rare cell isolation platform CellSearch system to identify and count CEC and potentially translate results into clinical practice.

MATERIAL AND METHODS

Patients

During two study periods, June 2011–December 2012 and June 2014–October 2015, we prospectively evaluated 90 patients with malignant hematologic disorders who underwent allo-HSCT. Moreover, 10 healthy

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subjects served as controls. In patients and controls, CEC counts were performed with the CellSearch system (Janssen Diagnostics LLC, Raritan, NJ, USA). CEC were counted, starting from enrollment up to day +100, at the following time-points: T1 (pre-conditioning), T2 (pre-transplant), T3 (engraftment), T4 (day +28 or at onset of GvHD) and T5 (1 week after steroid treatment). CEC counts of patients without GvHD at day +28 were compared with those reported in patients at the onset of GvHD. In the first 20 patients, CEC counts were monitored at weekly intervals until day +100. Blood samples were drawn from a central catheter to reduce risks of endothelial cell contamination due to traumatic damage from venipuncture. Moreover, the first tube was invariably discarded to avoid accidental contamination. Samples were collected by using dedicated tubes (CellSave Preservative Tubes, Janssen Diagnostics LLC, Raritan, NJ, USA), stored at room temperature and analyzed within 48 h after blood draw.²⁰ The local research and ethics committee approved the study protocol and all patients provided written informed consent, in accordance with the Declaration of Helsinki (*ClinicalTrials.gov* NCT02064972).

Infections management and GvHD diagnosis

All patients received levofloxacin until complete neutrophil recovery, fluconazole or itraconazole until withdrawal of all immunosuppressive drugs, trimethoprim-sulphamethoxazole for *Pneumocystis jirovecii* pneumonia prevention. CMV infections were monitored weekly by quantitative real-time PCR in plasma, and patients tested positive were treated with ganciclovir or foscarnet. Fungal infections were evaluated and defined according to published revised criteria.²⁵ Diagnosis and grading of GvHD were based primarily on clinical findings according to commonly accepted diagnostic criteria.²⁶ Whenever possible or clinically indicated, clinical data were supported by histopathology studies on tissue biopsies from skin, liver and gastrointestinal tract.

CEC identification and count

CEC counts were performed by means of the Circulating Endothelial Cell isolation kit (research use only) in combination with the CellSearch system (Janssen Diagnostics LLC, Raritan, NJ, USA), that allows standardization of the whole process of sample collection, cellular selection, monoclonal antibodies labeling, analysis and enumeration of CEC, as previously described.²⁰ To be scored as a CEC, a CD146⁺ cell must have a nucleus (DAPI), express CD105, show the morphology of an intact cell, and be negative for CD45. Overall, CEC were defined as CD146⁺/CD105⁺/DAPI⁺/CD45⁻ cells. Counts were expressed as number of CEC per mL of PB.

Statistical analysis

Mann–Whitney *U*-test was used in univariate analysis for comparison of continuous variables, and χ^2 test was used for comparison of categorical variables. Multivariate logistic regression analysis was performed at each time-point, to assess the independent predictive value of CEC counts in the presence of possible interfering factors such as patient and/or transplant-related variables and/or infectious events. Analysis of the receiver operating characteristics curve was performed to establish a cut-off for predictive values of CEC at T3 and T4. CEC counts and their relative possible increase/decrease at each time-point (T) were included in all analyses. The relative increase of CEC value (%) was expressed using the following formula: (CEC at Tx – CEC at T-previous)/CEC at T-previous \times 100. A mixed model analysis for repeated measures was performed to further test differences in CEC counts over time between patients with and without GvHD. All *P*-values were two-sided and *P* < 0.05 was considered statistically significant.

RESULTS

Patients

Ninety patients, median age 48 years (range 18–69; 52 males/38 females), who underwent allo-HSCT from either HLA-matched familial (*n* = 20; 22%), unrelated (*n* = 54; 60%) or haploidentical donors (*n* = 16; 18%) were enrolled. Hematological diseases included 37 AML, 15 ALL, 11 HL, four NHL, four CLL, five myelodiplastic syndrome, four chronic myeloproliferative syndromes, eight multiple myeloma and two severe aplastic anemia. Forty-eight patients (53%) were in complete response (CR) or

upfront, 31 patients (35%) in partial response/CR > 1 and 11 patients (12%) in progression at the time of transplant.

Allografting

Seventy-two patients (80%) received hematopoietic stem cells from mobilized PB and 18 (20%) from bone marrow; conditioning regimens were myeloablative in 47 patients (52%) and reduced intensity in 43 patients (48%). Neutrophil engraftment was obtained in 87 patients with a median time to engraftment of 22 days (range 14–40) whereas two patients died from infection and one from transplant-related toxicity during neutropenia. No differences in clinical and transplant characteristics were recorded between patients with GvHD and those without, with the exception of the use of FBU2 as conditioning (*P* = 0.04) and incidence of fungal infections (*P* = 0.01) (Table 1). Overall, 39/90 patients (43%) presented with acute GvHD at a median of 24 days (range 12–113) post transplant. GvHD was grade I in 10/39 (26%), II in 26/39 (67%) and III in 3/39 patients (7%). In 29/39 patients (74%) GvHD presented with skin involvement, in 16/39 patients (41%) with gut involvement and in 4/39 (10%) with liver involvement.

CEC counts pre-transplant

The median CEC/mL at T1 (pre-conditioning) was 24 (range 2–786) as compared with 2 (range 1–14) in healthy controls (*P* < 0.0001) (Figure 1). No differences were detected in CEC counts between patients with GvHD and those without before (T1, pre-conditioning) and at the end of the conditioning regimen (T2, pre-transplant). CEC counts did not change at any time-point regardless of age or gender (data not shown). At T1 (pre-conditioning) multiple myeloma patients had lower CEC values as compared with AL patients (*P* = 0.004) (Figure 2a), while no differences in CEC counts were recorded in relation to disease status at transplant (Figure 2b). At T2 (pre-transplant) no differences in CEC counts were seen by type of conditioning (myeloablative conditioning vs reduced intensity conditioning) (Figure 2c), type of HPC donor (Figure 2d) or GvHD prophylaxis (Figure 2e).

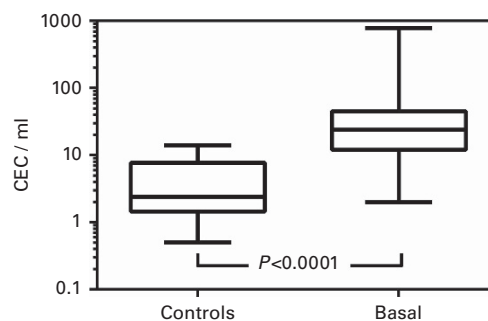
CEC counts post transplant

At T3 (engraftment) no differences in CEC counts were seen by type of conditioning, donor type, stem cell source or GvHD prophylaxis, while multiple myeloma patients had lower CEC values as compared with AL (*P* = 0.01) and lymphoma/CLL patients (*P* = 0.05). Patients with bacterial infections showed lower CEC values at T3 time-point (47; range 10–162 vs 84; range 17–436; *P* = 0.001). Median CEC/mL was 57 (range 16–295) in patients with GvHD and 91 (range 10–436) in those without GvHD (*P* = 0.003) (Table 2). This difference remained significant by multivariate analysis (OR 0.992, 95% CI 0.985–0.99; *P* = 0.029) (Table 3). At T4 (day +28 or at onset of GvHD) no differences in CEC counts were recorded by donor type, stem cell source or GvHD prophylaxis. Myeloablative conditioning was associated with higher CEC values as compared with reduced intensity conditioning (*P* = 0.02). Multiple myeloma patients had lower CEC values as compared with AL patients (*P* = 0.003). Patients with documented bacterial infections showed lower CEC values at T4 time-point (62; range 16–135 vs 94; range 13–658; *P* = 0.001). Median CEC/mL were 71 (range 13–658) in patients with GvHD and 87 (range 26–436) in those without GvHD (*P* = NS) (Table 2). At GvHD onset (T4), the relative increase of CEC counts (T4 vs T3) was 13% (range –79 to 723%) in patients with GvHD vs 0% (range –70 to 332%) in patients without GvHD (*P* = 0.026) (Table 2). This finding was also confirmed by multivariate analysis (T4 vs T3 > 0%: OR 4.2, 95% CI 1.6–10.8; *P* = 0.002) (Table 3). CEC counts inversely correlated with acute GvHD grade as follows: at T4 (at onset of GvHD) median

Table 1. Comparison of clinical and transplant characteristics in patients with and without acute GvHD (n = 90)

Characteristics	GvHD (%)	No GvHD (%)	P-value
Age (years), median (range)	47 (18–67)	49 (18–69)	0.41
Sex			
Male	22 (56)	30 (59)	0.81
Female	17 (44)	21 (41)	
Diagnosis			
Acute leukemias	24 (62)	28 (55)	0.53
Lymphomas/CLL	8 (20)	11 (21)	0.90
MDS/CMS	2 (5)	7 (14)	0.18
MM	5 (13)	3 (6)	0.25
SAA	0 (0)	2 (4)	0.21
Disease status			
CR/upfront ^a	21 (54)	27 (53)	0.93
PR/CR > 1	14 (36)	17 (33)	0.80
Progression	4 (10)	7 (14)	0.62
Donor			
MUD	23 (59)	31 (61)	0.86
MRD	6 (15)	14 (27)	0.17
Haploid	10 (26)	6 (12)	0.09
HPC source			
MPB	32 (82)	40 (78)	0.67
BM	7 (18)	11 (22)	
Conditioning regimen			
MAC	23 (59)	24 (47)	0.26
RIC	16 (41)	27 (53)	0.25
TBI (yes)	9 (23)	7 (14)	
MA conditioning			
BU/CY	7 (18)	13 (25)	0.39
FBu4	3 (8)	2 (4)	0.44
TBI/CY	7 (18)	4 (8)	0.15
TBI/F/Th	2 (5)	1 (2)	0.41
TBF	4 (10)	4 (8)	0.69
RIC conditioning			
Th/CY/F	5 (12.5)	7 (14)	0.90
TBI/F/Th(r)	0 (0)	2 (4)	0.21
FBu2	0 (0)	5 (10)	0.04
Th/CY	5 (12.5)	7 (14)	0.90
TBF(r)	3 (8)	1 (2)	0.19
Th+F/Cy+F	0 (0)	3 (5)	0.12
F/Mel/Th	3 (8)	2 (4)	0.44
GvHD prophylaxis ^b			
CyA/MTX	29 (74)	44 (86)	0.15
CyA/MMF	10 (26)	7 (14)	
ATG	20 (51)	28 (55)	0.73
Bacterial infections ^c	14 (36)	13 (25)	0.29
Fungal infections ^c	8 (21)	2 (4)	0.01
CMV infections ^c	8 (21)	8 (16)	0.53

Abbreviations: ATG = anti-lymphocyte globulin; BM = bone marrow; BU = busulfan; CMS = chronic myeloproliferative syndromes; CY = cyclophosphamide; CyA = Cyclosporin A; F = fludarabine; Haploid = haploidentical related donor; HPC = hematopoietic progenitor cells; MAC = myeloablative conditioning; MA = myeloablative; MDS = myelodysplastic syndrome; MM = multiple myeloma; MMF = mofetil micofenolate; MPB = mobilized peripheral blood; MRD = matched related donor; MTX = methotrexate; MUD = matched unrelated donor; RIC = reduced intensity conditioning; r = RIC; SAA = severe aplastic anemia; TBF = Th/BU/F; Th = thiotepa. ^aUpfront: MDS only. ^bIn haploidentical transplantation GvHD prophylaxis included CyA/MMF and cyclophosphamide post stem cell reinfusion (100 mg/kg total dose: day+3 and +5). ^cBefore GvHD onset. Bold values indicate that they are statistically significant.

**Figure 1.** CEC counts at baseline (T1, pre-conditioning) in patients undergoing allo-HSCT in comparison to healthy subjects (controls). Boxes represents values from the first to the third quartile, horizontal line shows the median value and the whiskers indicate the min and max value.

CEC/mL was 93 (range 46–299) in grade I, 64 (range 13–658) in grade II and 36 (range 34–89) in grade III (grade I vs III $P=0.05$). No significant differences were reported in CEC counts when considering skin alone (22/39 patients; 56%) vs gut/liver (17/39 patients; 44%) involvement (Table 4). Mixed models analysis confirmed the correlation between CEC counts and GvHD at T3 ($P=0.004$) and the increase of CEC values in patients with GvHD from T3 to T4 ($P=0.001$). At T5 (1 week after steroid treatment) median CEC counts (48 CEC/mL, range 6–184) returned to pre-transplant values (T2, pre-transplant) (44 CEC/mL, range 8–718) (Table 2).

In receiver operating characteristics analysis, a value of 66 CEC/mL at T3 was 78% specific and 61% sensitive for the development of GvHD (area under the curve, AUC = 0.71; $P=0.005$). At T4, a relative increase >0% of CEC count (T4 vs T3) was 79% specific and 53% sensitive (AUC = 0.70). The AUC values demonstrated a moderate discriminatory power of the test. Finally, when plotting CEC counts over time (weekly determinations) in the first 20 patients, CEC changes represented a dynamic phenomenon, influenced by many variables, but invariably showed an increase in CEC counts, starting 1–2 weeks prior to the onset of GvHD, with a peak at GvHD diagnosis; the same CEC counts decreased rapidly, returning to pre-transplant values, in patients in which steroid treatment was able to control GvHD manifestations (Supplementary Figure 1).

DISCUSSION

In recent years, the physio-pathological interrelationships between the endothelium and GvHD have been better elucidated and have led to define the entity 'endothelial GvHD' as an essential early phase prior to the clinical presentation of acute GvHD.^{5–8} However, GvHD diagnosis still almost completely relies on clinical symptoms, clinicians expertise and should be confirmed by histology studies.²⁷ Therefore, the identification of specific biomarkers detectable in PB samples could clearly become a valuable diagnostic and clinical tool to avoid invasive diagnostic procedures such as tissue biopsies. Moreover, it may help personalize treatment strategies in GvHD patients. CEC are considered specific and sensitive markers of endothelial dysfunction and have been used in a variety of pathological conditions to monitor vascular damage.^{12,16–19,28} The commercial availability of the CellSearch system for CEC identification and count allows to easily monitor CEC changes as a function of endothelial damage in allo-HSCT patients.¹⁹ Since validated noninvasive biomarkers for GvHD diagnosis have not yet routinely been implemented in clinical practice,¹⁴ we propose the CellSearch system as a valid and reliable approach to help/support clinicians in their diagnosis of acute GvHD. Since vascular complications have a strong impact on the outcome of allo-HSCT, CEC changes have been correlated with

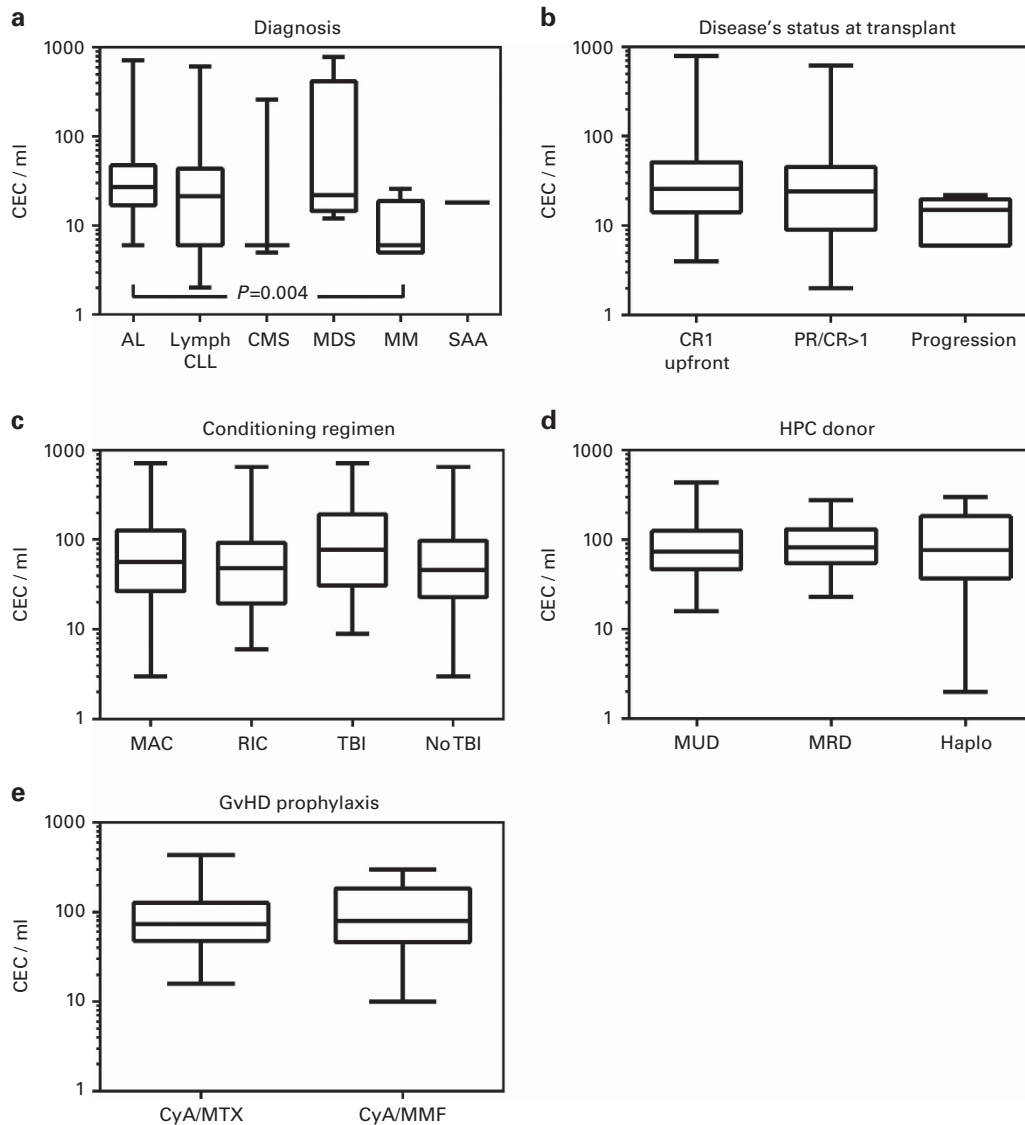


Figure 2. CEC counts in patients during allo-HSCT. CEC counts at T1 (pre-conditioning) are plotted in graph (a) in relation to diagnosis and in graph (b) in relation to disease status at transplant; while CEC counts at T2 (pre-transplant) are plotted in graph (c) in relation to conditioning, in graph (d) in relation to HPC donor and in graph (e) in relation to GvHD prophylaxis. Boxes represent values from the first to the third quartile, horizontal line shows the median value and the whiskers indicate the min and max value.

the occurrence of endothelial complications. Touzot *et al.* showed that high CEC values are associated with a wide spectrum of endothelial complications including sinusoidal obstruction syndrome, thrombotic microangiopathy, capillary leak syndrome and pulmonary arterial hypertension in 34 children undergoing allo-HSCT for primary immunodeficiency. However, their encouraging findings need to be confirmed in larger studies to establish the real role of CEC as a biomarker of endothelial damage.²⁹ Not only does our study confirm a preliminary report¹⁹ but it also represents the largest series of allo-HSCT patients where the clinical usefulness of CEC changes over time has prospectively been correlated to the diagnosis of GvHD and treatment response. Although CEC changes in allo-HSCT represent a dynamic phenomenon that can be influenced by many variables (among others, conditionings, immunosuppressive treatments, engraftment syndrome, infections), we clearly showed that CEC peaks were constantly seen prior to the onset of acute GvHD and invariably returned to pre-transplant values after treatment response. However, it is important to point out that our results conflict with a previous study conducted in a series of allo-

HSCT patients by Beije *et al.*³⁰ This may partly be explained by the different CEC count monitoring schedules that may have prevented to correlate CEC changes right at the onset of GvHD. Undoubtedly, onset of GvHD remains unpredictable over a rather long post-transplant period (at least up to day +100/+120 post transplant).³¹ We showed that CEC changes during allo-HSCT had a rapid kinetic that may have easily been missed if blood samples had been drawn only at given time-points. As a consequence, we suggest an 'on demand' evaluation of CEC counts right at the onset of GvHD clinical symptoms to possibly help differentiate GvHD from other non-endothelial complications. Moreover, the strength of our study was the use of the CellSearch system that guarantees a standardization of CEC counts with high-level reproducibility, specificity and sensitivity. Indeed, the CD146 immunomagnetic selection step, followed by an identification strategy based on positive/negative selection criteria, allows reduction of analytical variability also when rare events are acquired.²⁰ Furthermore, the use of standardized criteria for signal intensity and automated image analysis guarantees a better reproducibility by reducing inter-operator variability.²⁰

Table 2. Analysis of CEC values at the different time points in patients with and without acute GvHD and comparison of relative increases (%) in CEC values at the indicated timepoints

Time points	CEC/mL		P-value
	GvHD	No GvHD	
<i>T1 (pre-conditioning)</i>			
Median (range)	25.5 (2–718)	22 (3–786)	0.52
Mean ± s.d.	62.7 ± 131.2	62.7 ± 142.6	
<i>T2 (pre-transplant)</i>			
Median (range)	44 (8–718)	63.5 (3–648)	0.35
Mean ± s.d.	97.1 ± 150.5	99.7 ± 126.5	
<i>T3 (engraftment)</i>			
Median (range)	57 (16–295)	91 (10–436)	0.003
Mean ± s.d.	79.2 ± 64.8	119.8 ± 86.7	
<i>T4 (day +28 or GvHD onset)</i>			
Median (range)	71 (13–658)	87 (26–436)	0.11
Mean ± s.d.	108.2 ± 121.9	116.4 ± 84.7	
<i>T5 (1 week after steroid treatment)</i>			
Median (range)	48 (6–184)		
Mean ± s.d.	63.9 ± 51.9		
T2 vs T1 (relative increase %)	81.5 (–94 to 4417)	117 (–86 to 10 700)	0.44
T3 vs T1 (relative increase %)	147.5 (–98 to 2848)	314 (–95 to 3500)	0.05
T3 vs T2 (relative increase %)	33 (–89 to 679)	112.5 (–89 to 2933)	0.21
T4 vs T1 (relative increase %)	219.5 (–94 to 1729)	305.5 (–91 to 3043)	0.29
T4 vs T2 (relative increase %)	62 (–91 to 1050)	82 (–93 to 2200)	0.74
T4 vs T3 (relative increase %)	13 (–79 to 723)	0 (–70 to 332)	0.026

Abbreviation: CEC = circulating endothelial cells. Bold values indicate that they are statistically significant.

Table 3. Univariate and multivariate analysis to evaluate CEC values as independent predictor variable for GvHD at time of hematopoietic engraftment and at time of GvHD onset

Variables	OR (95% CI)	P-value
<i>T3-engraftment</i>		
<i>Univariate analysis</i>		
Diagnosis of AL vs other	1.3 (0.6–3.1)	0.53
Diagnosis of MM vs other	2.3 (0.5–10.5)	0.26
Conditioning RIC (FBu2) vs other	nv	0.95
Bacterial infections	1.3 (0.5–5.3)	0.58
Fungal infections	6.3 (1.2–31.7)	0.03
CEC (cells/mL)	0.992 (0.985–0.99)	0.029
CEC relative increase (T3 vs T1)	0.999 (0.998–1.00)	0.17
<i>Multivariate analysis</i>		
CEC (cells/mL)	0.992 (0.985–0.99)	0.029
<i>T4-GvHD onset</i>		
<i>Univariate analysis</i>		
Diagnosis of AL vs other	1.3 (0.6–3.1)	0.53
Diagnosis of MM vs other	2.3 (0.5–10.5)	0.26
Conditioning RIC (FBu2) vs other	nv	0.95
MAC vs RIC	1.7 (0.7–4.1)	0.19
Bacterial infections	1.6 (0.7–4.1)	0.29
Fungal infections	6.3 (1.2–31.7)	0.03
CEC relative increase (T4 vs T3) > 0%	4.2 (1.6–10.8)	0.005
<i>Multivariate analysis</i>		
CEC relative increase (T4 vs T3) > 0%	4.2 (1.6–10.8)	0.002

Abbreviations: CEC = circulating endothelial cells; MAC = myeloablative conditioning; MM = multiple myeloma; NV = not measurable; RIC = reduced intensity conditioning. Bold values indicate that they are statistically significant.

Table 4. Acute GvHD presentation (grade and involvement) and related CEC counts

Characteristics	Patients	Median CEC/mL (range)	P-value
<i>GvHD grade</i>			
Grade I	10/39 (26%)	93 (46–299)	I vs II 0.66
Grade II	26/39 (67%)	64 (13–658)	I vs III 0.05
Grade III	3/39 (7%)	36 (34–89)	II vs III 0.11
<i>Target organs</i>			
Skin (only)	22/39 (56%)	68 (16–658)	0.36
Gut/liver	17/39 (44%)	77 (13–304)	
Median GvHD onset (range): d +24 (12–113)			

Abbreviation: CEC = circulating endothelial cells. Bold values indicate that they are statistically significant.

become possible to guide the timely introduction, in non-responders, of additional immunosuppressive treatments whereas actions might be shifted to less aggressive approaches in responders.

Data availability

The data sets generated during the current study are available from the corresponding author on reasonable request.

CONFLICT OF INTEREST

KB is employee of Janssen Diagnostics. The study was conducted with the support of the Investigator-Initiated Study Program of Janssen Diagnostics, LLC to CA. The remaining authors declare no conflict of interest.

As we previously reported,¹⁹ we further confirmed, on a larger patient series, that statistically significant higher CEC counts ($P < 0.003$) are seen at engraftment in patients with no GvHD vs those who develop GvHD. This puzzling finding may also be correlated with the decreased CEC counts associated with higher GvHD grades we documented, although with the limitation of the low number of GvHD grade III patients and the lack of GvHD grade IV patients in our series. In addition to the exhaustion of endothelial cell shedding from vascular walls, as a consequence of end-stage endothelial damage observed with GvHD worsening, higher CEC counts in low grade GvHD may be the result of a putative protective effect of CEC.³² However, this hypothesis requires in depth investigations and potential clinical implications can only be speculative for the time being.

We believe that CEC monitoring in allo-HSCT may facilitate diagnosis of acute GvHD and might help identify non-responders before the overt development of refractory disease. However, before clinical translation, our results need to be confirmed in a larger series of GvHD patients. Consequently, it could

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AUTHOR CONTRIBUTIONS

CA conceived and designed the study, interpreted the results and wrote the manuscript; CS enrolled patients, performed statistical analysis and helped to write the manuscript; BB enrolled patients and helped to write the manuscript; RV revised CellSearch analysis; ADP, AB, AN, S Braga and G Piccinelli performed CellSearch analysis; M Malagola, S Bernardi, LG, LB and MF enrolled patients; G. Piovani, KB, DR and M Marini critically revised the final manuscript. All authors read and approved the final manuscript.

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