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## CD56<sup>bright</sup> natural killer regulatory cells in filgrastim primed donor blood or marrow products regulate chronic graft-versus-host disease: the Canadian Blood and Marrow Transplant Group randomized 0601 study results

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### ABSTRACT

Randomized trials have conclusively shown higher rates of chronic graft-versus-host disease with filgrastim-stimulated apheresis peripheral blood as a donor source than unstimulated bone marrow. The Canadian Blood and Marrow Transplant Group conducted a phase 3 study of adults who received either filgrastim-stimulated apheresis peripheral blood or filgrastim-stimulated bone marrow from human leukocyte antigen-identical sibling donors. Because all donors received the identical filgrastim dosing schedule, this study allowed for a controlled evaluation of the impact of stem cell source on development of chronic graft-versus-host disease. One hundred and twenty-one evaluable filgrastim-stimulated apheresis peripheral blood and filgrastim-stimulated bone marrow patient donor products were immunologically characterized by flow cytometry and tested for their association with acute and chronic graft-versus-host disease within 2 years of transplantation. The immune populations evaluated included, regulatory T cells, central memory and effector T cells, interferon  $\gamma$  positive producing T cells, invariate natural killer T cells, regulatory natural killer cells, dendritic cell populations, macrophages, and activated B cells and memory B cells. When both filgrastim-stimulated apheresis peripheral blood and filgrastim-stimulated bone marrow were grouped together, a higher chronic graft-versus-host disease frequency was associated with lower proportions of CD56<sup>bright</sup> natural killer regulatory cells and interferon  $\gamma$ -producing T helper cells in the donor product. Lower CD56<sup>bright</sup> natural killer regulatory cells displayed differential impacts on the development of extensive chronic graft-versus-host disease between filgrastim-stimulated apheresis

peripheral blood and filgrastim-stimulated bone marrow. In summary, while controlling for the potential impact of filgrastim on marrow, our studies demonstrated that CD56<sup>bright</sup> natural killer regulatory cells had a much stronger impact on filgrastim-stimulated apheresis peripheral blood than on filgrastim-stimulated bone marrow. This supports the conclusion that a lower proportion of CD56<sup>bright</sup> natural killer regulatory cells results in the high rate of chronic graft-versus-host disease seen in filgrastim-stimulated apheresis peripheral blood. *clinicaltrials.gov Identifier: 00438958*.

## Introduction

Filgrastim granulocyte-colony stimulating factor (G-CSF)-stimulated apheresis peripheral blood (G-PB) as a donor source is clinically well established due to rapid engraftment, ease of collection, and similar survival to marrow as a donor source. G-PB is limited by a significantly higher rate of chronic graft-versus-host disease (cGvHD),<sup>1-8</sup> purported to be due to the infusion of increased donor product T cell numbers.<sup>9</sup> Other studies have suggested that the CD34<sup>+</sup> cell donor load,<sup>10,11</sup> activated HLA-DR<sup>+</sup> T cells,<sup>12</sup> and possibly the total nucleated cell dose<sup>13</sup> impact on the development of cGvHD after G-PB transplantation, however, all such studies are limited by the lack of comparison to a control marrow transplanted population. Cell populations are associated with acute GvHD (aGvHD) after G-PB which includes dendritic cells<sup>14</sup> and Treg cells<sup>15</sup> but do not have any association with cGvHD. Other donor cell populations have found no association for either aGvHD or cGvHD.<sup>16</sup> To date no study has definitely established which immune cell populations are most responsible for the higher rate of cGvHD associated with the G-PB donor source compared to marrow. Until the specific, unique components in G-PB versus marrow as a source are identified, it remains difficult to develop graft manipulation strategies to modulate cGvHD.

The Canadian Blood and Marrow Transplant Group (CBMTG) undertook a definitive phase 3 trial comparing G-bone marrow (BM) with G-PB in sibling allografts for adults with hematologic malignancies. In that study, the CBMTG showed that cGvHD was lower with G-BM (HR=0.66; 95% CI 0.46 – 0.95;  $P=0.007$ ).<sup>17</sup> This study presented an unprecedented opportunity to evaluate the impact of graft source on the development of cGvHD with both donor sources receiving G-CSF treatment using an identical regimen. The population was relatively homogenous as only human leukocyte antigen (HLA)-identical sibling donor (8/8 or 7/8 HLA-match) was used for predominantly myeloid malignancies with the only variable being the method of collection (i.e., marrow harvest versus apheresis). We hypothesized that one of the immune cell populations previously identified by correlative cGvHD biology and biomarkers studies would correlate with the induction of cGvHD by G-PB donor product. To test this hypothesis, we evaluated both G-BM and G-PB donor grafts combined for donor product immune cells for any specific cell types correlation with the development of cGvHD. Once identified, we evaluated the relative impact of each immune cell population on cGvHD for the relative impact of the two donor sources, G-PB versus G-BM, on the development of cGvHD. The immune populations evaluated included: regulatory T cells, central memory and effector T cells, interferon (IFN) $\gamma$ <sup>+</sup> producing T cells, regulatory natural killer (NK) cells, invariant natural killer T (iNKT) cells, plasmacytoid

and myeloid dendritic cells, macrophages, activated B cells, and memory B cells.

## Methods

### Clinical Study Design

Samples for the current study were obtained as part of a larger clinical study (CBMTG 0601), a randomized phase 3, parallel group trial conducted by the CBMTG at 13 centers in Canada, Saudi Arabia, Australia, New Zealand, and the USA. The institutional research ethics board at each center approved the trial and recipients and donors both gave informed consent before randomization. Recipients were between 16 and 65 years of age and with a hematologic malignancy. Donors were 7/8 or 8/8 HLA-matched siblings medically fit to receive G-CSF and undergo a marrow harvest or apheresis. This study has been described previously.<sup>17</sup>

### Patient and Donor Characteristics

CBMTG 0601 comprised 223 donor-recipient pairs randomized between April 2007 and January 2012 with 223 evaluable pairs. Of the entire 223 evaluable patients from the clinical trial, 121 had evaluable samples for the current correlative studies. The primary analysis was performed on patients who had survived up to 2 years after BM transplantation (BMT) (> 95% of patients developed overall cGvHD by 2 years), with the omission of patients due to death and leukemia relapses that occurred before the onset of cGvHD (Table 1;  $n = 89$ ). We found no significant difference between the 121 evaluated and the 102 not included in the analysis for cGvHD (65% vs. 59%), death (34% vs. 44%), relapses (29% vs. 24%), or time to cGvHD (day 180 $\pm$ 112 vs. day 185 $\pm$ 124), respectively. We defined overall cGvHD as including both limited and extensive cGvHD, and will from now on refer to overall cGvHD as cGvHD, unless specified as extensive cGvHD. Confirmatory analysis was performed on all evaluable patients including those with a death or relapse before 2 years.

Both subgroups had similar patient characteristics to the entire population in the study (Table 1). A comprehensive immune evaluation of T cells, B cells, NK cells, iNKT cells, macrophages, and dendritic cell populations was performed on the donor product for a number of immune populations (*Online Supplementary Table S1*) and tested for association with aGvHD and cGvHD within 2 years of transplantation. Additional evaluations examined the association of identified immune cell populations and the development of aGvHD and cGvHD for the graft source (G-PB or G-BM) and other clinical factors, including transplant related mortality (TRM), relapse, previous aGvHD before the onset of cGvHD, donor-recipient sex differences, acute myeloid leukemia (AML) versus no AML, total body irradiation (TBI) versus no TBI, recipient age and donor age.

### Sample processing for biological studies using immunophenotyping and functional assays

Samples from allografts were couriered overnight at room temperature to a central laboratory located at BC Children's Hospital

Research Institute in Vancouver, Canada; peripheral blood mononuclear cells (PBMCs) were isolated and frozen on arrival. Batched samples were thawed using  $1 \times 10^6$  viable cells per assay. Immunophenotyping and functional assays evaluated T cell, B cell, dendritic cell, monocyte, and NK cell populations (*Online Supplementary Table S1*). Data were acquired using LSR II flow cytometer (BD Biosciences) and analyzed by FlowJo v10 (TreeStar, Ashland, OR, USA). Details of the cell immunophenotyping strategy can be found in Table 1. Graft composition was evaluated as the percentage of cell population per donor lymphocytes. On smaller cell count samples, there was a prioritization for assays with immunophenotyping to be carried out first, followed by functional stimulations assays for cytokine production if sufficient samples were available.

### Study endpoints

The primary biologic endpoint of the analysis was an association of cGvHD with a number of subpopulations of T cell, B cell, NK cell, iNKT cell, macrophages, and dendritic cell populations. aGvHD and cGvHD were characterized according to Przepiorka *et al.*<sup>18</sup> and Sullivan *et al.*,<sup>19</sup> respectively. Any GvHD was defined as chronic GvHD and/or acute GvHD (grade 1 – IV aGvHD). Chronic GvHD was defined as an initial diagnosis of cGvHD within 2 years of transplantation.

### Statistical analysis

The effect of candidate immune cell populations on the development of cGvHD (within a 2 year period from transplantation) was tested using a univariate logistic regression model. Patients

**Table 1. Baseline characteristics: overall vs. studied populations.**

Variable	Overall Population in clinical trial (N = 223)		Confirmatory population including early deaths, relapse <sup>1</sup> (N = 121)		Primary studied population excluding early deaths, relapses <sup>1</sup> (N = 89)	
	G-BM (%) N=113	G-PB (%) N=110	G-BM (%) N=52	G-PB (%) N=69	G-BM (%) N=39	G-PB (%) N=50
<b>Disease Stage</b>						
Early	71 (63)	77 (70)	34 (65)	49 (71)	26 (67)	33 (66)
Late	42 (37)	33 (30)	18 (35)	20 (29)	13 (33)	17 (34)
<b>Disease</b>						
CML	7 (6)	6 (5)	5 (10)	5 (7)	5 (13)	4 (8)
AML	53 (47)	54 (49)	26 (50)	35 (58)	18 (46)	27 (54)
MYELO	15 (13)	9 (8)	5 (10)	7 (10)	4 (11)	6 (12)
Other	38 (34)	41 (37)	16 (30)	22 (32)	12 (31)	13 (26)
<b>Conditioning Regimen</b>						
BU+CY	65 (58)	57 (51)	35 (67)	36 (52)	29 (74)	29 (58)
CY+TBI	2 (2)	2 (2)	0	0	0	0
Other:			0	0	0	0
Fludarabine + Melphalan	40 (35)	41 (37)	16 (30)	24 (35)	9 (23)	13 (26)
VP-16 + TBI	5 (4)	7 (6)	1 (3)	6 (9)	1 (3)	5 (10)
Fludarabine+Busulfan	1 (1)	3 (3)	0	2 (3)	0	2 (4)
<b>Donor Sex</b>						
Male	66 (58)	66 (60)	30 (58)	44 (64)	22 (57)	32 (64)
Female	47 (42)	44 (44)	22 (42)	25 (36)	17 (43)	18 (36)
<b>Donor median age (range)</b>						
	43 (18 – 66)	41 (18 – 64)	45.5 (18-62)	44 (18-66)	49 (20-62)	46 (19-64)
<b>Donor CMV Status</b>						
Positive	69 (61)	57 (52)	26 (50)	26 (38)	19 (49)	20 (40)
Negative	44 (39)	53 (48)	26 (50)	43 (62)	20 (51)	30 (60)
<b>Recipient Sex</b>						
Male	69 (61)	57 (52)	34 (65)	39 (57)	25 (64)	29 (58)
Female	44 (39)	53 (48)	18 (35)	30 (43)	14 (36)	21 (42)
<b>Recipient median age (range)</b>						
	43 (16 – 63)	46 (16 – 64)	48 (20-63)	46 (16-64)	49 (22-63)	47 (17-59)
<b>Recipient CMV Status</b>						
Positive	62 (55)	59 (54)	23 (44)	32 (46)	17 (44)	22 (44)
Negative	51 (45)	51 (46)	29 (56)	37 (54)	22 (66)	28 (56)

<sup>1</sup>Early deaths and relapses were defined as occurring before 2 years post BMT or before the onset of cGvHD. BU: busulfan; CY: cyclophosphamide; VP=16 = etoposide; CMV: cytomegalovirus; MYELO: myelodysplastic disease; CML: Chronic Myeloid Leukemia; AML: Acute Myeloid Leukemia; G-BM: G bone marrow; G-PB: G peripheral blood; TBI: total body irradiation.

who relapsed or those who died before occurrence of cGvHD were excluded from the logistic regression analysis, since it could not be established whether or not they would have developed cGvHD. Of the initial 121 patients, 89 met the inclusion criteria. As these were exploratory analyses no statistical adjustments were made for multiple comparisons, thus a *P*-value threshold of 0.01 was used in the primary analysis and 0.05 in all other secondary analyses. All analyses were performed using MATLAB. For the two cell populations that were found to be significant, due to smaller cell numbers in some donor graft samples, 7 of the 89 patients did not have immunophenotyping for CD56<sup>bright</sup> NK<sub>reg</sub> cells and 11 of the 89 patients did not have functional stimulation and immunophenotyping for the CD4<sup>+</sup> T cell IFN $\gamma$  (Figure 1).

To confirm that excluding patients who displayed relapse or died before cGvHD did not introduce biases, we also employed a univariate Cox proportional hazards model<sup>20</sup> with those patients included, and used the time to cGvHD onset as the response. The time to cGvHD for said patients was considered as censored under the Cox model.

As to visualization, the identified cell populations were plotted with values split by GvHD status. Multivariate analysis on all significant cell populations identified in the univariate analysis was also performed to test for the unique effect of each cell population. This analysis was applied only on patients that had all these cell populations, resulting in 75 and 94 patients being evaluated for the logistic regression model and Cox model, respectively (Figure 1). Furthermore, logistic regression was applied to examine the effect of aGvHD, sex, TBI, recipient age, donor age, AML, death, relapse as well as donor source on the identified cell populations. Moreover, with patients split by cGvHD status, optimal cut points for the identified cell populations were determined by plotting their receiver operating characteristic (ROC) curve and by finding the point on the ROC curve that is closest to the point of perfect sensitivity and specificity. Lastly, we examined the interaction effect between donor source and the identified significant markers on cGvHD status using logistic regression and the Cox model.

## Role of the funding source

The National Cancer Institute of the National Institutes of Health (NIH) funded the study herein following peer review, but had no direct influence on the study design, the collection, analysis, and interpretation of data, in the writing of the report or in the decision to submit the paper for publication. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

## Results

### Evaluation of donor graft composition for immune populations associated with the development of cGvHD

Immunophenotypic and functional evaluations were performed for a large number of CD4<sup>+</sup> and CD8<sup>+</sup> T cell, NK cell, B cell, macrophages, plasmacytoid and myeloid dendritic cell, iNKT cell, and regulatory T cell populations as outlined in *Online Supplementary Table S1* and correlated with the presence of cGvHD. We also evaluated the activation status of CD4<sup>+</sup> and CD8<sup>+</sup> T cells by CD25 and human leukocyte antigen – antigen D related (HLA-DR) expression and found no difference. Initial analyses evaluated candidate immune cell populations for correlation with cGvHD followed by analysis for extensive cGvHD. The two donor sources, G-PB and G-BM, were grouped together for these analyses. We found no significant associations (at *P*<0.01) with cGvHD except for two populations, CD56<sup>bright</sup> NK<sub>reg</sub> cells (Table 2; *P*=0.003) and IFN $\gamma$  CD4<sup>+</sup> T cells (*P*=0.002).

A confirmatory analysis was further performed that included patients who either died or developed a leukemia relapse before the onset of cGvHD and before 2 years following transplantation. These included 107 patients for CD56<sup>bright</sup> NK<sub>reg</sub> cells and 100 patients for IFN $\gamma$  CD4<sup>+</sup> T cells as a marker (Figure 1). We confirmed the results of

**Table 2.** Association between donor cell population numbers with development of cGvHD (excludes all patients with a death or relapse before 2 years or overall cGvHD).

Variable	Percentage cells per total lymphocytes – Independent variable <sup>1</sup>	
	<i>P</i> (Wald test)	Odds Ratio (95% CI)
CD56 <sup>bright</sup> NK <sub>reg</sub> cells		
Overall cGvHD (N = 821)	0.003	0.13 (0.03- 0.49)
Extensive cGvHD (N = 78)	0.005	0.19 (0.06-0.61)
CD4 <sup>+</sup> T cells IFN $\gamma$		
Overall cGvHD (N = 78)	0.002	0.77 (0.66- 0.91)
Extensive cGvHD (N = 75)	0.002	0.83 (0.74-0.94)
	Percentage cells per total lymphocytes – Multivariate analysis <sup>2</sup>	
CD56 <sup>bright</sup> NK <sub>reg</sub> cells		
Overall cGvHD	0.02	0.16 (0.03- 0.74)
Extensive cGvHD	0.01	0.19 (0.05-0.69)
CD4 <sup>+</sup> T cells IFN $\gamma$		
Overall cGvHD	0.007	0.77 (0.64- 0.93)
Extensive cGvHD	0.01	0.84 (0.73-0.96)

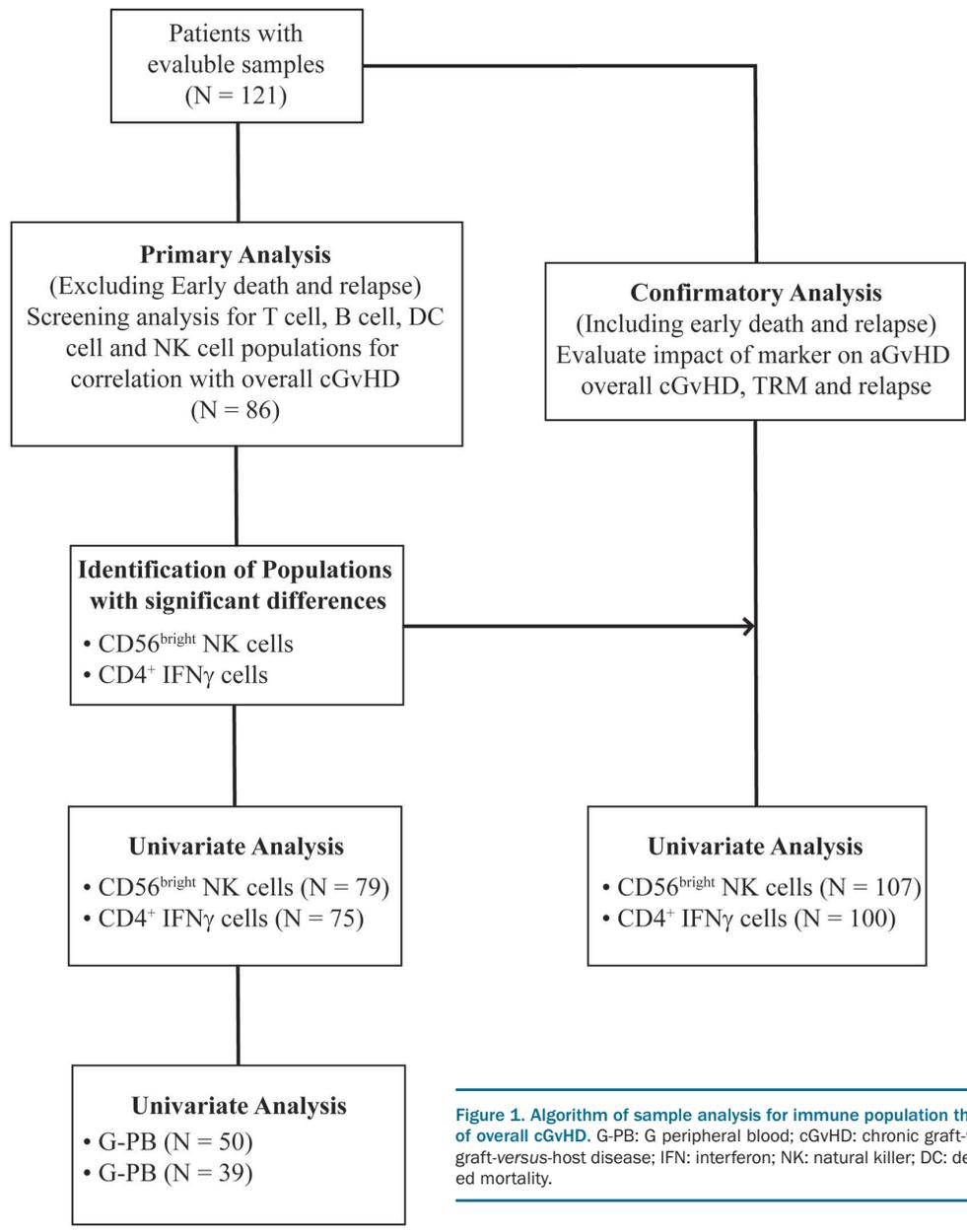
<sup>1</sup>The multivariate analysis based on logistic regression model was performed only on patients who had both values resulting in a decrease to N=75. <sup>2</sup>The multivariate analysis based on the Cox proportional hazards model was performed only on patients who had both values resulting in a decrease to N=94. cGvHD: chronic graft-versus-host disease; IFN: interferon; NK: natural killer.

the logistic regression analysis for both CD56<sup>bright</sup> NK<sub>reg</sub> cells with cGvHD (*Online Supplementary Table S2*; OR=0.54, *P*=0.02) and IFN $\gamma$ <sup>+</sup> CD4<sup>+</sup> T cells with cGvHD (*Online Supplementary Table S2*, OR=0.93, *P*=0.001). Further analysis focused on these two populations (CD56<sup>bright</sup> NK<sub>reg</sub> cells and IFN $\gamma$ <sup>+</sup> CD4<sup>+</sup> T cells) as outlined below.

**Association of donor CD56<sup>bright</sup> NK<sub>reg</sub> cell composition with the development of cGvHD**

Significant associations between a lower percentage of donor CD56<sup>bright</sup> NK<sub>reg</sub> cells per total lymphocytes and development of any GvHD (aGvHD and/or cGvHD; *P*=0.003) as well as cGvHD only (Figure 2A and Table 2; OR=0.13; *P*=0.003) were found. Further analysis, limited to extensive cGvHD alone, was similar with logistic regression (Figure 2B, OR=0.19; *P*=0.005). A significant

association was also found with aGvHD status (*P*=0.02). We confirmed that the CD56<sup>bright</sup> NK<sub>reg</sub> cell population was the classic regulatory NK (NK<sub>reg</sub>) population by further evaluation for expression of CD335 (NKp46), CD336 (NKp44), and CD337 (NKp30) on all samples from the study population of G-BM and G-PB NK<sub>reg</sub> cells (Figure 2C). The expression of CD335 (NKp46) was found to be significantly higher in CD56<sup>bright</sup> NK<sub>reg</sub> cells with comparable expression of CD337 in both subpopulations consistent with the NK<sub>reg</sub> phenotype.<sup>21,22</sup> From now on, we will refer to this population (CD3<sup>-</sup>, CD56<sup>bright</sup>/CD335<sup>bright</sup>/perforin<sup>-</sup>/granzyme B<sup>-</sup>/CD16<sup>+/+</sup>) as CD56<sup>bright</sup> NK<sub>reg</sub> cells. We evaluated whether cytomegalovirus (CMV) seropositivity of the donor impacted on the presence of CD56<sup>bright</sup> NK<sub>reg</sub> cells present, and found no significant difference in CD56<sup>bright</sup> NK<sub>reg</sub> cells for CMV seropositive (0.59±0.50% CD56<sup>bright</sup> NK<sub>reg</sub> cells per total lymphocytes) versus CMV



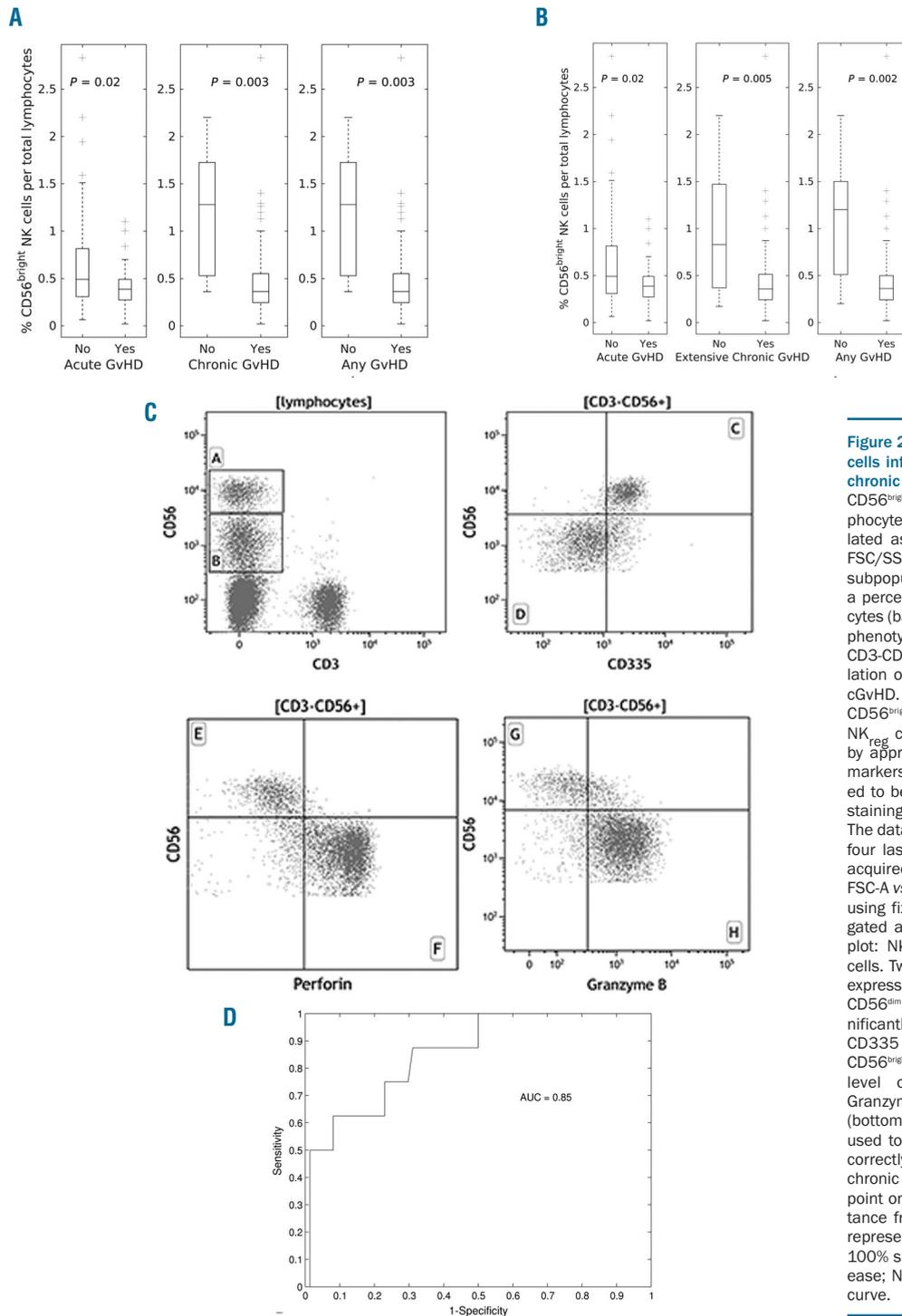
**Figure 1.** Algorithm of sample analysis for immune population that correlate with the development of overall cGvHD. G-PB: G peripheral blood; cGvHD: chronic graft-versus-host disease; aGvHD: acute graft-versus-host disease; IFN: interferon; NK: natural killer; DC: dendritic cells; TRM: transplant related mortality.

seronegative ( $0.55 \pm 0.42\%$ ,  $P=0.65$ ) donors. A ROC curve to predict the development of cGvHD by 2 years was calculated for the CD56<sup>bright</sup> NK<sub>reg</sub> population, and we found an area under the curve (AUC) of 0.85 (Figure 2D).

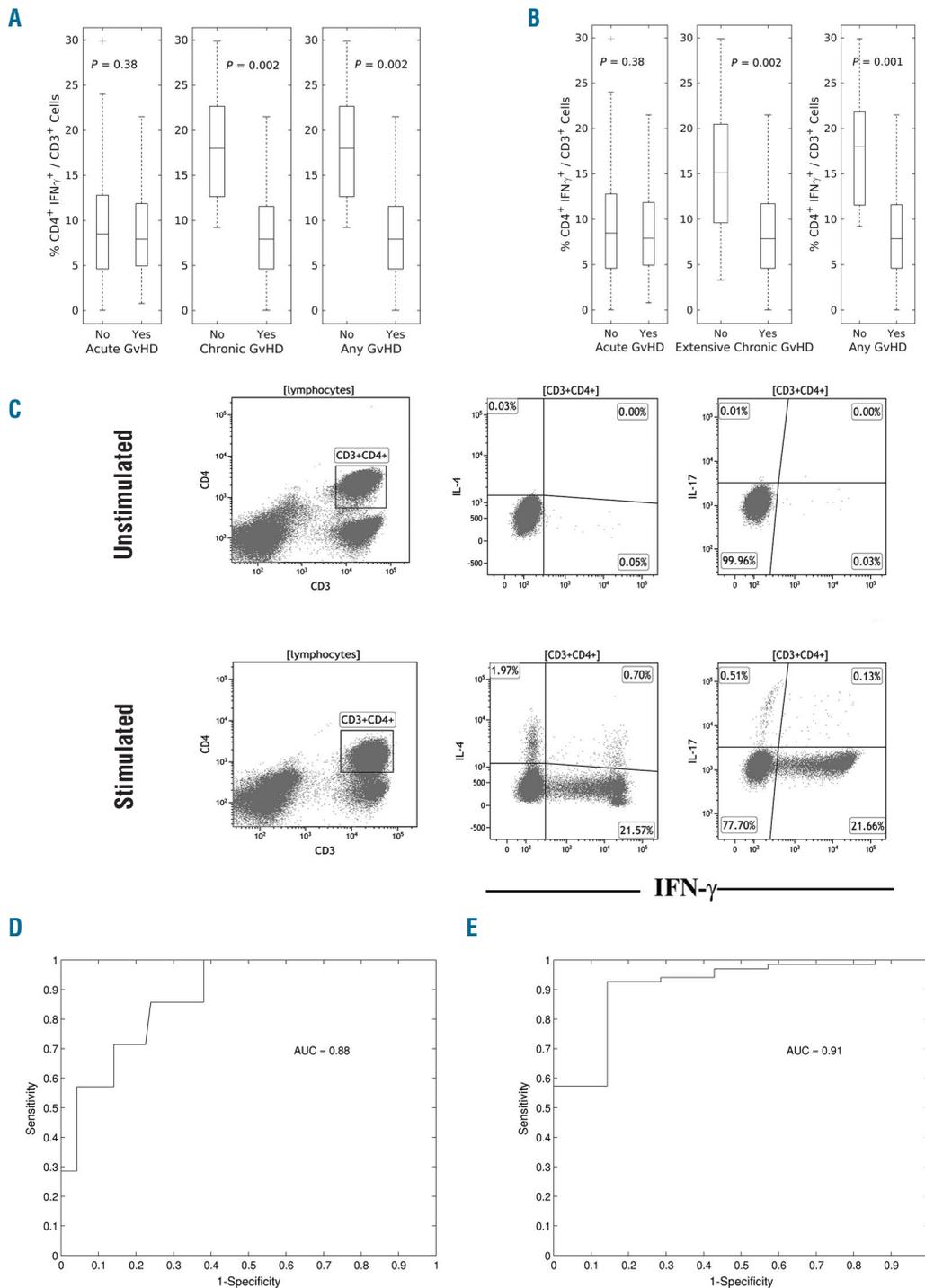
### Association of donor IFN $\gamma$ producing CD4<sup>+</sup> T cells with the development of cGvHD

Our group, and others, have previously shown that lower numbers of IFN $\gamma$  producing cells were associated with cGvHD.<sup>23</sup> Cytokine production was measured after

mitogen stimulation *in vitro* (PMA/Ionomycin). We identified a significant association between lower numbers of IFN $\gamma$ -producing CD4<sup>+</sup> T cell population (CD4<sup>+</sup>/CD3<sup>+</sup>/IFN $\gamma$ <sup>+</sup>/IL-4<sup>-</sup>/IL-17<sup>-</sup>) and development of any GvHD (either aGvHD and/or cGvHD;  $P=0.002$ ) and cGvHD alone (Figure 3A and Table 2, OR=0.77,  $P=0.002$ ). Further analysis, limited to extensive cGvHD alone, was similar for logistic regression (Figure 3B and Table 2, OR=0.83,  $P=0.001$ ). A ROC analysis revealed an AUC of 0.88 (Figure 3C).



**Figure 2. Correlation of donor CD56<sup>bright</sup> NK<sub>reg</sub> cells infusion characteristics with acute and chronic GvHD.** (A) Box and whisker plot of CD56<sup>bright</sup> NK<sub>reg</sub> cells percentage per total lymphocytes for overall cGvHD, which was calculated as the total nucleated cells gated on FSC/SSC plots, then the percentage of each subpopulation of interest was determined as a percentage of the total number of lymphocytes (based on forward and side scatter). The phenotype of CD56<sup>bright</sup> NK cells was CD56<sup>bright</sup> CD3-CD16-perforin-granzyme B. (B) The correlation of CD56<sup>bright</sup> NK<sub>reg</sub> cells with extensive cGvHD. (C) Representative dot plot showing CD56<sup>bright</sup> and CD56<sup>dim</sup> subpopulations of NK<sub>reg</sub> cells. Mononuclear cells were stained by appropriate conjugated mAbs for surface markers. The cells were then fixed and treated to be permeable, followed by intracellular staining (Online Supplementary Table S1). The data was acquired by LSRII equipped with four lasers. A minimum of  $1 \times 10^6$  cells were acquired. Single cells were gated based on FSC-A vs. FSC-H and dead cells were excluded using fixable viability dye. Lymphocytes were gated as FSC lo and SSC lo. Upper left dot plot: NK cells were defined as CD3<sup>+</sup>CD56<sup>+</sup> cells. Two populations are revealed based on expression of CD56, CD56<sup>bright</sup> (gate A) and CD56<sup>dim</sup> (gate B). CD56<sup>bright</sup> cells express a significantly higher level of activating receptor CD335 (NKp46) compared to CD56<sup>dim</sup>. CD56<sup>bright</sup> cells express a significantly lower level of molecules involved in killing, Granzyme B (bottom left dot plot) and Perforin (bottom right dot plot). (D) A ROC analysis was used to determine an 'optimal' cut point for correctly predicting the occurrence of overall chronic GvHD. 'Optimal' was defined as the point on the ROC curve with the shortest distance from the point (0, 1). The point (0, 1) represents the ideal, 100% sensitivity and 100% specificity. GvHD: graft-versus-host disease; NK: natural killer; AUC: area under the curve.



**Figure 3. Correlation of donor IFN $\gamma$  producing CD4 $^+$  T cells infusion characteristics with acute and chronic GvHD.** (A) Box and whisker plot of the correlation of IFN $\gamma$  producing CD4 $^+$  T cells percentage with overall cGvHD. The population was calculated as a subpopulation of CD3 $^+$  lymphocytes. PBMCs were stimulated with PMA (100ng/ml)/ionomycin (1  $\mu$ g/ml) in the presence of monensin for 6 hours. Unstimulated cells treated with monensin were used as control. At the end of incubation, the cells were harvested and surface markers were stained for CD3, CD4, and CD8, in addition to fixable viability to distinguish dead cells. Intracellular staining was performed to detect production of IL-17, IL-4 and IFN $\gamma$ . The data was acquired using BD LSR II and analyzed by FlowJo v9. Hierarchical gating; 1: lymphocytes were selected based on FSC/SSC, 2: exclusion of dead cells, 3: selection of CD3 $^+$  T cells, and 4: determination of IFN $\gamma$  producing CD4 $^+$  T-cells. The data is presented as % of CD4 $^+$ IFN $\gamma^+$  T cells per CD3 $^+$  lymphocytes. (B) Correlation of IFN $\gamma$  producing CD4 $^+$  T cells with extensive cGvHD; (C) A representative dot plot of IFN $\gamma^+$  T helper cells. Mononuclear cells were seeded at a density of 1x10 $^6$  per milliliter of culture medium (RPMI-1640 supplemented with 10% heat-inactivated FBS and 2mM l-glutamine), and the cells incubated in CO2 incubator providing 95% oxygen and 5% CO2 at 37 degrees centigrade. The cells were stimulated for 6 hours with 100ng/ml PMA and 1  $\mu$ g/ml Ionomycin in presence of golgi inhibitor monensin (BD Biosciences; following manufacturers' instructions). As control, cells were cultured without stimulator, but received monensin. The cells were then harvested and surface staining was performed to detect CD3 $^+$ CD4 $^+$  cells. Then the cells were fixed and treated to be permeable. Intracellular staining was performed to detect IFN $\gamma$ , IL-4 and IL-17. At least 1x10 $^6$  cells were acquired. Gating hierarchy; 1: single cells (FSC-A vs. FSC-H), 2: viable cells (fixable viability dye negative), and 3: lymphocytes 4-CD3 $^+$ CD4 $^+$  cells. The percentage of Th1 (IFN $\gamma^+$ ), Th17 (IL-17 $^+$ ) and Th2 (IL-4 $^+$ ) were determined after setting quadrant based on unstimulated cells (upper row). Dot plots in lower row show cytokine expression after stimulation. D) A ROC analysis was used to determine an 'optimal' cut point for correctly predicting the occurrence of chronic GvHD. The optimal cut point for IFN $\gamma$  producing CD4 $^+$  T cells/CD3 $^+$  T cells was 13.9%. (E) Logistic regression was performed with the two identified markers as predictors and overall cGvHD status as response. Each sample was given an estimated probability of overall cGvHD based on the fitted model. The ROC was generated by applying different probability thresholds. GvHD: graft-versus-host disease; IFN: interferon; AUC: area under the curve.

A multivariable logistic regression analysis was performed on samples with both CD56<sup>bright</sup>NK<sub>reg</sub> cells and IFN $\gamma$ <sup>+</sup> CD4<sup>+</sup> T cells (N=94). The decrease from the original 121 to 94 patients was due to small cell numbers in donor samples and patients with relapse or death before cGvHD who were further removed (Table 2). We found that both IFN $\gamma$ <sup>+</sup> CD4<sup>+</sup> T cells (OR=0.84;  $P=0.01$ ) and CD56<sup>bright</sup>NK<sub>reg</sub> cells (OR=0.19;  $P=0.01$ ; Table 2) maintained their significance, suggesting that each of these cell populations has some unique attributes that significantly relate to cGvHD status. We also found that the combination of CD56<sup>bright</sup>NK<sub>reg</sub> cells and IFN $\gamma$ <sup>+</sup> CD4<sup>+</sup> T cells resulted in a higher ROC AUC of 0.91 (Figure 3D) than that of using each cell population alone.

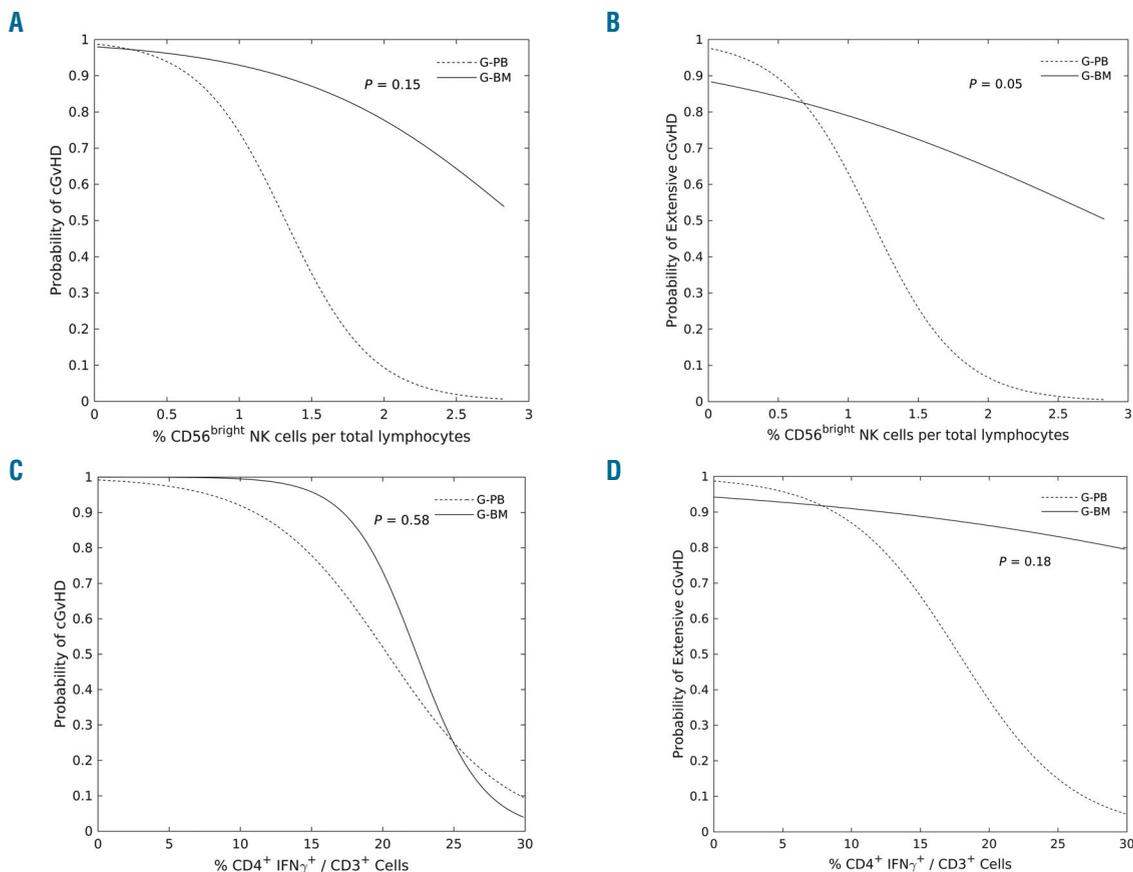
### Correlation of clinical factors and donor immune populations

Each of the two cell markers was evaluated for any impact that the following clinical factors may have on their interpretation: clinical donor and recipient age, sex mismatch between donor and recipient, AML *versus* no AML, TBI *versus* no TBI, and presence or absence of aGvHD. Because all donors were related, 7/8 or 8/8 HLA

matches and received a myeloablative preparative regimen these variables were not evaluated. Only the IFN $\gamma$ <sup>+</sup> CD4<sup>+</sup> T cell donor population correlated with a modest decrease in transplant related mortality (*Online Supplementary Table S3*).

### Evaluation of the impact of donor immune populations on probability of G-BM and G-PB developing cGvHD

The CBMTG 0601 trial was a prospective randomized non-blinded study comparing donor G-CSF stimulated marrow *versus* G-CSF stimulated peripheral blood. Specific, well-defined clinical endpoints, including cGvHD, were documented up to 2 years post-transplant; this allowed us to directly compare the impact of each cell population in marrow (G-BM) *versus* peripheral blood (G-PB) allografts. Using an interaction test, we evaluated whether either of the two populations were different in terms of their impact on G-BM *versus* G-PB. While the CD56<sup>bright</sup> NK<sub>reg</sub> cell population showed no significant impact on overall cGvHD in either donor source (Figure 4A;  $P=0.15$ ), it did show a significant impact on the development of extensive cGvHD (Figure 4B;  $P=0.05$ ) after G-PB transplantation compared to G-BM. By contrast, IFN $\gamma$ <sup>+</sup> CD4<sup>+</sup> T cells appeared to have no significant impact



**Figure 4.** Impact of donor IFN $\gamma$  producing CD4<sup>+</sup> T cells and CD56<sup>bright</sup> NK<sub>reg</sub> cells infusion characteristics on cGvHD by donor source using G-PB or G-BM transplantation. (A) The estimated probability of overall cGvHD by treatment (donor source G-BM versus G-PB) as a function of the CD56<sup>bright</sup> cells per total lymphocytes. (B) Estimated probability of extensive cGvHD as a function of CD56<sup>bright</sup> cells per total lymphocytes by donor source (G-PB or G-BM). (C) Estimated probability of overall cGvHD as a function of donor IFN $\gamma$  producing CD4<sup>+</sup> T cells by donor source (G-PB or G-BM); (D) Estimated probability of extensive cGvHD as a function of donor IFN $\gamma$  producing CD4<sup>+</sup> T cells by donor source (G-PB or G-BM). GvHD: graft-versus-host disease; IFN: interferon.

on either G-BM or G-PB and later development of cGvHD (Figure 4C;  $P=0.58$ ) or extensive cGvHD (Figure 4D;  $P=0.18$ ).

## Discussion

G-CSF-mobilized peripheral blood apheresis donor product is used by a large number of BMT centers, despite the fact that it has a significantly higher rate of cGvHD compared to harvested bone marrow donor product. This major limitation could be minimized if the immune cellular component that influences the higher rate of cGvHD associated with G-PB were characterized. The CBMTG 0601 protocol comparing marrow *versus* apheresis peripheral blood donor product offered a unique opportunity to evaluate the impact of apheresis PB when both donor populations were treated with an identical dosing schedule of G-CSF and collected with identical timing. Moreover, the study population were all adults (> 16 years of age) with related donors (HLA 8/8 and 7/8 matching); using a minimization randomization ensured that the recipient populations were matched for important contributing factors such as preparative regimen and underlying disease.<sup>17</sup> A comprehensive evaluation of donor immune cell components that had previously been associated with the development of cGvHD allowed for an identification not only of populations associated with both donor sources, but, more importantly, of those associated with G-PB. We initially evaluated both G-BM and G-PB together and found two significant associations in two donor cell populations for both overall and extensive cGvHD. Both had inverse relationships with the development of overall cGvHD (IFN $\gamma$ <sup>+</sup> CD4<sup>+</sup> T cells and CD56<sup>bright</sup>NK<sub>reg</sub> cells) suggesting regulatory functions. We then looked at the populations in a broader context to ensure that the observations were consistent, and found: a) no correlation with other factors except aGvHD in NKreg cell and TRM in the T cell population, and b) the association was consistent when a secondary analysis included patients that died or relapsed before 2 years after BMT. We also found that the inclusion of both populations together increased the association with cGvHD. Having looked at the two populations in the overall population, we subsequently looked at the impact on cGvHD in the two donor sources of graft (G-BM and G-PB) separately. We found that the NK<sub>reg</sub> population had a proportionately greater impact on extensive cGvHD in G-PB compared to G-BM. This controlled evaluation supports the importance of CD56<sup>bright</sup>NK<sub>reg</sub> cells as a suppressive immune population on cGvHD in related donor G-PB transplantation.

It is now well established that strategies that impact the graft cellular composition at the time of transplant can impact the development of cGvHD many months later. As an example, *in vivo* depletion of T cell and B cells with either anti-thymocyte globulin or alemtuzumab, *in vivo* depletion of activated T cell and B cell populations using post transplantation cyclophosphamide, and *ex vivo* depletion of T and B cell populations in haploidentical transplants can reduce the cumulative incidence of cGvHD.<sup>24-30</sup>

A number of donor cell populations have been associated with the onset of cGvHD. These include T cells, B cells and dendritic cells.<sup>31-33</sup> We have previously shown that activated B cells (CpG oligodeoxynucleotide (ODN) responsive, TLR9<sup>+</sup>) are associated with increased cGvHD,

whereas regulatory T cells and IFN $\gamma$  producing T cells are associated with decreased cGvHD.<sup>23,34</sup> G-CSF administration may influence allograft cellular composition in marrow and peripheral blood products.<sup>35</sup> One study found that the number of donor naive and memory T cell subsets correlated with infections and aGvHD, and were impacted by whether the graft source was unstimulated marrow or G-CSF-stimulated apheresis donor product.<sup>21</sup> The most comprehensive study was BMT CTN 0201,<sup>36</sup> which evaluated the impact of donor G-PB *versus* unstimulated marrow as the donor product. The BMT CTN 0201 study analysis differed from our study in that their primary analysis focused on: a) unrelated donor sources, b) unstimulated marrow as the control rather than G-BM as in our analysis, and c) overall survival rather than cGvHD. They found that plasmacytoid dendritic cells (pDCs) and naive T cells were associated with improved overall survival but not with cGvHD. Similar to our study, they found that the T cell content of the G-PB was higher than that of BM grafts. In spite of these differences, they found no increased incidence of cGvHD associated with donor graft CD8<sup>+</sup> or CD4<sup>+</sup> T cell populations, including those expressing CD45RA, CCR7, and CD62L, CD127, and Ki-67, for regulatory cells or for NKT cells. In the BMT CTN 0201 study, it appears that neither of the two populations identified in the current study, CD56<sup>bright</sup>NK<sub>reg</sub> cells or IFN $\gamma$  producing CD4<sup>+</sup> T cells, were included in their evaluations.

Our study reports a strong association of CD56<sup>bright</sup>NK<sub>reg</sub> cells with a lower rate of cGvHD in both G-BM and G-PB. CD56<sup>bright</sup>NK cells were first described in 1992 as IL-2 responsive group with the high affinity IL-2 receptor.<sup>37</sup> CD56<sup>bright</sup>NKp46 cells (NKregs) have been associated with lower GvHD in other small trials.<sup>38</sup> The CD56<sup>bright</sup>NK<sub>reg</sub> population has abundant immunoregulatory cytokines, is located primarily in secondary lymphoid tissues, and has low cytotoxicity. The cytokine-secreting CD56<sup>bright</sup>CD16<sup>dim</sup> cells express high levels of inhibitory CD94/NKG2A complex, CD25, and CD117, recognize HLA-E but lack inhibitory major histocompatibility complex (MHC) class 1a allele specific KIRs.<sup>39</sup> Unfortunately, KIR data was not collected as part of these studies and could not be further evaluated in these analyses. Expression of CD117 and NKp46 are typical for some populations of innate lymphocytes, associated with a lack of acute GVHD,<sup>40</sup> which we observed in this study. Our group has previously shown an inverse relationship of CXCR3<sup>+</sup> CD56<sup>bright</sup>NK cells with the onset of cGvHD in a large adult population,<sup>41</sup> further supporting the important role of this population in cGvHD. Moreover, the impact of G-CSF on the induction of CD34<sup>+</sup> progenitors for growth into an innate lymphoid effector population appears to be different in marrow *versus* PB.<sup>42</sup>

The other immune suppressive population that had an equal impact regardless of donor source, G-BM and G-PB, was the association of lower proportion IFN $\gamma$ <sup>+</sup> CD4<sup>+</sup> T cells with cGvHD. We have previously observed that an increase in IFN $\gamma$  was associated with a lower onset of late cGvHD in pediatric hematopoietic stem cell transplantation (HSCT) recipients, but had hypothesized that it would have been secreted by an NK<sub>reg</sub> population<sup>21</sup> as opposed to a CD4<sup>+</sup> T cell population. Murine models have shown that the role of IFN $\gamma$  in GvHD appears to be variable depending on specific times post BMT, as early administration of recombinant IFN $\gamma$  prevents CD4<sup>+</sup> T cell-mediated GVHD.<sup>43</sup> Support for this hypothesis

comes from the fact that donors who have microsatellite polymorphisms with decreased IFN $\gamma$  production have higher rates of cGvHD.<sup>44</sup> In mouse models, high IFN $\gamma$  production by NK T cells results in lower rates of cGvHD.<sup>45</sup> Interestingly, IFN $\gamma$  is not necessary for the development of GvHD in many murine GvHD models,<sup>46,2</sup> and disease can progress despite a lack of IFN $\gamma$ . The role of IFN $\gamma$ <sup>+</sup> CD4<sup>+</sup> T cells in the induction of immune tolerance is not well understood. One mechanism may be that classic Th1 IFN $\gamma$ <sup>+</sup> CD4<sup>+</sup> helper T cells induce immune tolerance *via* the activation of Th1 natural T<sub>reg</sub> (nT<sub>reg</sub>).<sup>47</sup> Another possibility is that IFN $\gamma$  inhibits donor T-cell expansion by promoting apoptosis and suppressing proliferation, thereby eliminating alloreactive T cells in GvHD tissues by interacting with recipient non-hematopoietic cells and upregulating programmed cell death (PD)-1L expression.<sup>48</sup> A third possibility is that the IFN $\gamma$ <sup>+</sup> CD4<sup>+</sup> T cell population represents a Th1 Treg population<sup>49</sup> that is primed to progress to an IL-10 producing Treg or Tr1 cell population. Lastly, IFN $\gamma$ -licensed mesenchymal stem cells inhibit proliferation of activated T cells through both an indoleamine 2,3-dioxygenase (IDO) and, possibly, PD-1 dependent manner.<sup>50</sup> Whatever their role, this population requires further study in its potential to predict a later onset of cGvHD.

One question is whether we could define a threshold of either CD56<sup>bright</sup>NK<sub>reg</sub> cells or IFN $\gamma$ <sup>+</sup> CD4<sup>+</sup> T cells which are required to be infused per Kg of the recipient. We found that the proportion in the donor product (cells per lym-

phocytes) and not the infused number of cells per Kg for both CD56<sup>bright</sup>NK cells ( $P=0.64$ ) and IFN $\gamma$ <sup>+</sup> CD4<sup>+</sup> T cells ( $P=0.94$ ) was of the greatest importance, suggesting that for regulatory cells there is a proportional relationship with other cell populations. Thus, focusing on the proportion of the regulatory cell populations such as CD56<sup>bright</sup>NK cells and IFN $\gamma$ <sup>+</sup> CD4<sup>+</sup> T cells in relation to the total cells infused is more relevant as a strategy, rather than that of achieving a certain threshold dose.

In summary, while controlling for the potential impact of G-CSF on marrow, our studies demonstrated that CD56<sup>bright</sup>NK<sub>reg</sub> cells had a much stronger impact on G-PB than on G-BM. This supports the conclusion that a lower proportion of CD56<sup>bright</sup>NK<sub>reg</sub> cells results in the high rate of cGvHD seen in G-PB, thus validating the development of strategies to increase the proportion of CD56<sup>bright</sup>NK<sub>reg</sub> cells after G-PB transplantation. Strategies could include alternative mobilization agents that selectively increase CD56<sup>bright</sup>NK<sub>reg</sub> cells, expansion *ex vivo* followed by adoptive transfer, and *in vivo* CD56<sup>bright</sup>NK<sub>reg</sub> cell expansion *via* the administration of low dose IL-2 after transplantation.

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