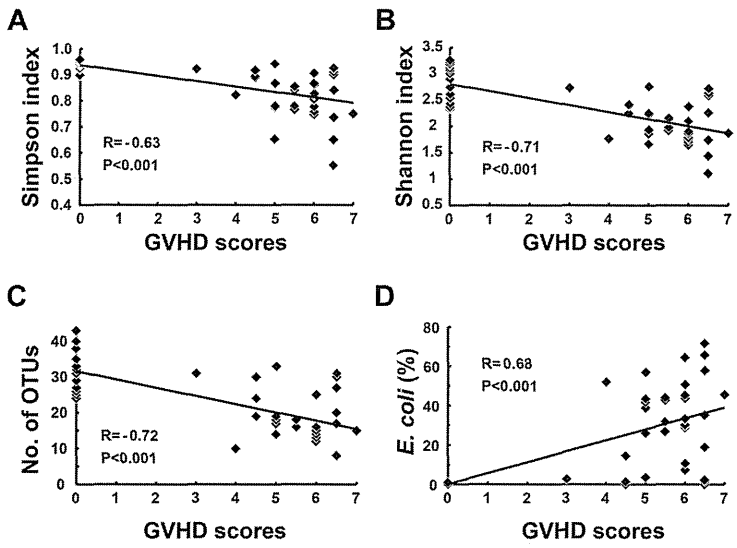

Figure 4. Correlation between the degree of flora changes and GVHD severity. Lethally irradiated B6D2F1 mice were transplanted with TCD BM with T cells from B6 donors (n = 6 / group). Fecal pellets were collected at day 0 and weekly thereafter and intestinal microbiota was characterized by RFLP analysis. Clinical GVHD scores and various parameters of the flora diversity and the proportion of *E coli* in the intestinal flora at various time points from each mice were plotted. Correlations of GVHD clinical scores and Simpson index (A), Shannon index (B), numbers of OTUs (C), and proportion of *E coli* (D). The regression line was plotted with all data. Data from 2 independent experiments were combined. R: correlation coefficient.

slower development of GVHD. It should be noted that normal flora diversity was recovered and *E coli* returned to a normally small population among the intestinal microbiota late after BMT, as GVHD severity reduced. No mortality was observed in allogeneic animals after regaining normal intestinal flora.

Loss of Paneth cells and the dysbiosis by a mechanism independent on conditioning

We addressed whether GVHD mediates Paneth cell injury and the alteration of the composition of the intestinal flora by a mechanism dependent on radiation-induced intestinal tract damage in the B6 → B6D2F1 BMT model without conditioning, as previously described.[7] Unirradiated B6D2F1 mice were intravenously injected with 12×10^7 splenocytes from syngeneic or allogeneic B6 donors on day 0. In this model, GVHD occurred early after BMT at a peak around day 20 but was spontaneously improved (Figure 6A-C). Numbers of Paneth cells were markedly reduced 2 weeks after BMT but gradually returned to normal levels thereafter in allogeneic animals (Figure 6D). The changes in the intestinal microbiota occurred in parallel with the degree of GVHD severity and Paneth cell injury; normal microbial diversity was lost with the outgrowth of *E coli*, but was gradually restored later after BMT in allogeneic animals (Figure 6E-H).

Oral administration of antibiotics inhibited the outgrowth of *E coli* and ameliorated GVHD

Finally, we evaluated whether modifying the enteric flora using oral antibiotics could ameliorate GVHD. Lethally irradiated B6D2F1 mice were transplanted with 5×10^6 TCD BM cells with or without 2×10^6 T cells from B6-Ly5.1 (CD45.1$^+$) donors. Polymyxin B (PMB), an antibiotic primarily effective against gram-negative bacteria, was administered by daily oral gavage at a dose of 100 mg/kg from day −4 until day 28 after BMT. Analysis of fecal pellets 7 days after BMT showed that the outgrowth of *E coli* was inhibited in mice treated with PMB compared with those treated with diluent (Figure 7A). PMB suppressed the outgrowth of *E coli* during PMB treatment; however, *E coli* levels increased after cessation of PMB treatment (Figure 7B). Notably, administration of PMB significantly reduced mortality and morbidity of GVHD (Figure 7C-D). Donor (CD45.1$^+$) T-cell expansion (Figure 7E) and pathology scores of the small intestine (Figure 7F) were significantly reduced in PMB-treated mice compared with those in controls.

Discussion

Intestinal GVHD is critical for determining the outcome of allogeneic BMT. Paneth cells are essential regulators of the

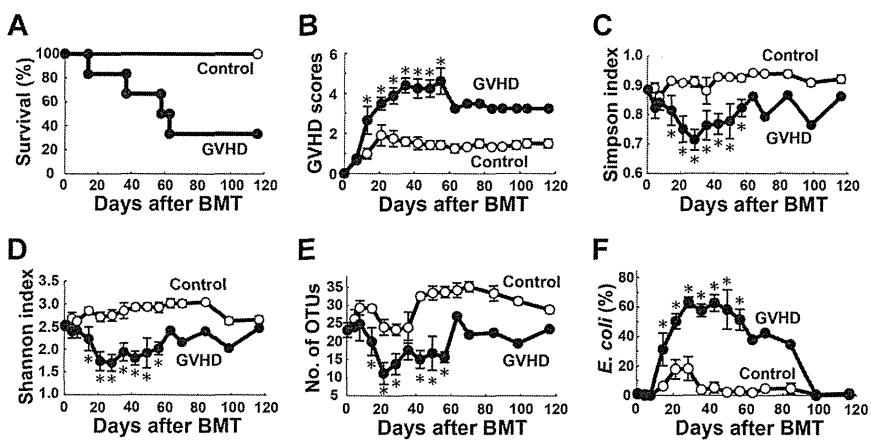

Figure 5. Delayed impairment of the intestinal ecology after MHC-matched BMT. B6 mice were transplanted with 5×10^6 TCD BM without (control group) or with (GVHD group) 2×10^6 T cells from C3H.Sw donors after 12 Gy TBI (n = 6/group). (A-B) Survival (A) and clinical GVHD scores (B, mean ± SE) in control group and GVHD group. Data are representative of 3 similar experiments. (C-F) Fecal pellets were collected once per week after BMT and intestinal microflora was characterized by RFLP analysis of 16S rRNA genes constructed from each sample of fecal pellets and digested with *Hha*I. Time course changes in flora diversity determined by Simpson index (C), Shannon index (D), and numbers of OTUs (E). (F) Time course changes in the proportion of *E coli*. Data are representative of 3 similar experiments and are shown as mean ± SE (*$P < .05$).

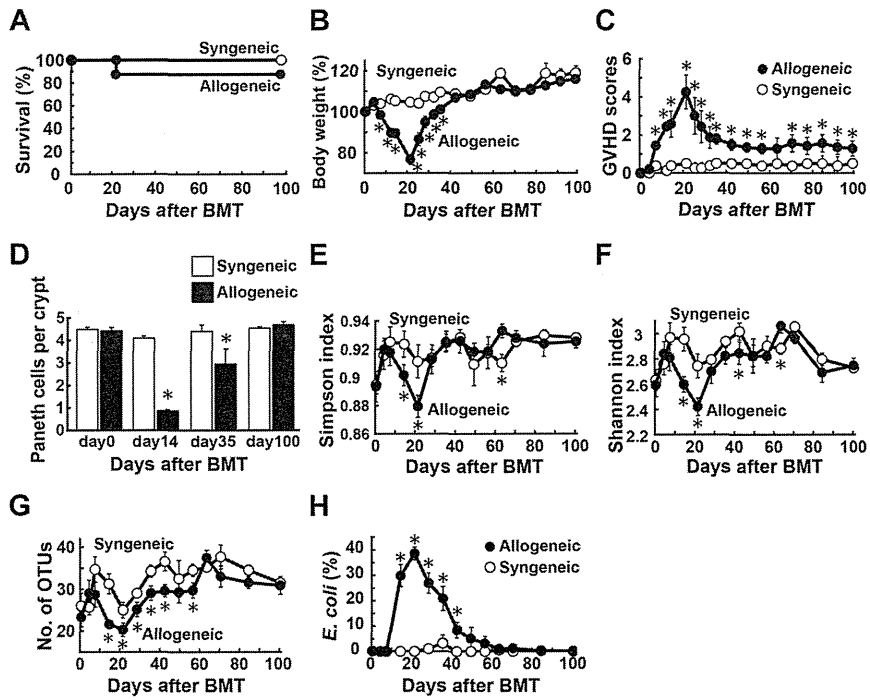

Figure 6. Paneth cell injury and the dysbiosis developed by a mechanism independent on conditioning. Unirradiated B6D2F1 mice were transplanted with 12×10^7 splenocytes from syngeneic or allogeneic MHC-mismatched B6 donors on day 0 (n = 6/group). Survival (A), changes in body weight (B, mean ± SE), clinical GVHD scores (C, mean ± SE), and (D) quantification of Paneth cells per crypt in the small intestine were shown. (E-H) Fecal pellets were collected before and after BMT weekly and intestinal microbiota was characterized by RFLP analysis of 16S rRNA gene libraries constructed from each sample of fecal pellets and digested with *Hha*I. Time course changes in flora diversity determined by using Simpson index (E), Shannon index (F), numbers of OTUs (G), and the proportion of *E coli* (H). Data from 2 independent experiments were combined and are shown as mean ± SE.

composition of commensal microbiota in the intestine, and they maintain the intestinal microbial environment by secreting various microbial peptides. In this study, we found that damage to Paneth cells by GVHD results in dramatically reduced expression of α-defensins in the small intestines and perturbed normal intestinal environment. These changes occurred in the absence of conditioning irradiation, thus indicating a mechanism dependent on allogeneic T-cell responses. However, Paneth cell loss occurred earlier and more prolonged in mice receiving irradiation than in unirradiated mice, suggesting that conditioning enhanced Paneth cell damage directly and indirectly by accelerating GVHD. The diversity of the intestinal microflora was lost with overwhelming expansion of specific bacteria, such as *E coli*, which are normally a very small proportion of the intestinal microbial communities. Paneth cells secret α-defensins into the intestinal lumen within minutes after sensing gram-negative and gram-positive bacteria and their components, such as LPS, through activation of pattern recognition receptors.[23,34] α-defensins are the most potent antimicrobial peptides and account for 70% of the bactericidal peptide activity released from Paneth cells.[11,23] α-Defensins are released in the small bowel lumen and persist as intact and functional forms throughout the intestinal tract.[35] Thus, they shape the composition of the microbiota in the entire intestine. Importantly, α-defensins have selective bactericidal activity against noncommensals, such as *Salmonella enterica*, *E coli*, *Klebsiella pneumoniae*, and *Staphylococcus aureus*, although exhibiting minimal bactericidal activity against commensals.[28,29] Such bacteria-dependent bactericidal activities of α-defensins are in tune with intestinal environment and may explain why the absence of α-defensins causes the alterations in the intestinal microbiota in GVHD. Because commensals have a profound influence on nutritional, physiologic, and metabolic function of the host,[14,36,37] reduction of commensals may have ill effects on the host with GVHD. In this study, *E coli* was the dominant enteric microbe in mice with GVHD among multiple strains of mice. However, the dominant species may differ between studies because of the differences in several factors, including differences in the maintenance protocols used to feed and care for the experimental animals.[32] Nonetheless, our study confirms and further extends a recent study showing the intestinal flora change, with an increase in gram-negative *Enterobacteriaceae* family members including *E coli*, after allogeneic BMT in mice.[18]

Alteration of the intestinal microbiota has been shown in experimental and clinical inflammatory bowel diseases, allergies, diabetes, and obesity.[13,38-42] This study provides several lines of evidence that suggest a close association between dysbiosis and GVHD. Mice without GVHD maintained normal microbiota after BMT, and dysbiosis only occurred in mice with GVHD, independent of the murine models used. The normal intestinal environment was never restored as long as severe GVHD persisted, but was restored when tolerance was induced after transplant. In mice with GVHD, the degree of changes to the microflora was significantly correlated to GVHD severity and MHC disparity between the donor and recipient. Furthermore, modifying enteric flora by oral administration of antibiotics inhibited the outgrowth if *E coli* and ameliorated GVHD. The flora shift toward the widespread prevalence of gram-negative bacteria increases the translocation of LPS, the major component of the outer membrane of gram-negative bacteria, into systemic circulation and further accelerates GVHD by stimulates production of inflammatory cytokines, such as TNF-α and IL-1, which are critical effector molecules that mediate GVHD.[22,43-45] Thus, GVHD and the dysbiosis can lead to a positive feedback loop that increases the translocation of LPS, thereby resulting in further cytokine production, progressive intestinal injury, and systemic GVHD acceleration. Earlier seminal studies in the 1960s-1970s suggested that GVHD is reduced in germfree mice or by treatment with poorly absorbable antibiotics.[3-6] A recent study also demonstrated that modifying the enteric flora using a probiotic microorganism reduced GVHD in mice.[46] Thus, our study again highlights an important role of oral antibiotics administration on GVHD. Our study demonstrated that dominant bacteria in the intestinal microbiota cause systemic infection. There was a microbiologic evidence of infection in mice with severe GVHD and a correlation between severity of infection and GVHD, thus suggesting that severe bacteremia, probably caused by the translocation of enteric bacteria, can also contribute to GVHD mortality, as previously suggested.[6,46] Indeed, septicemia by gram-negative rods

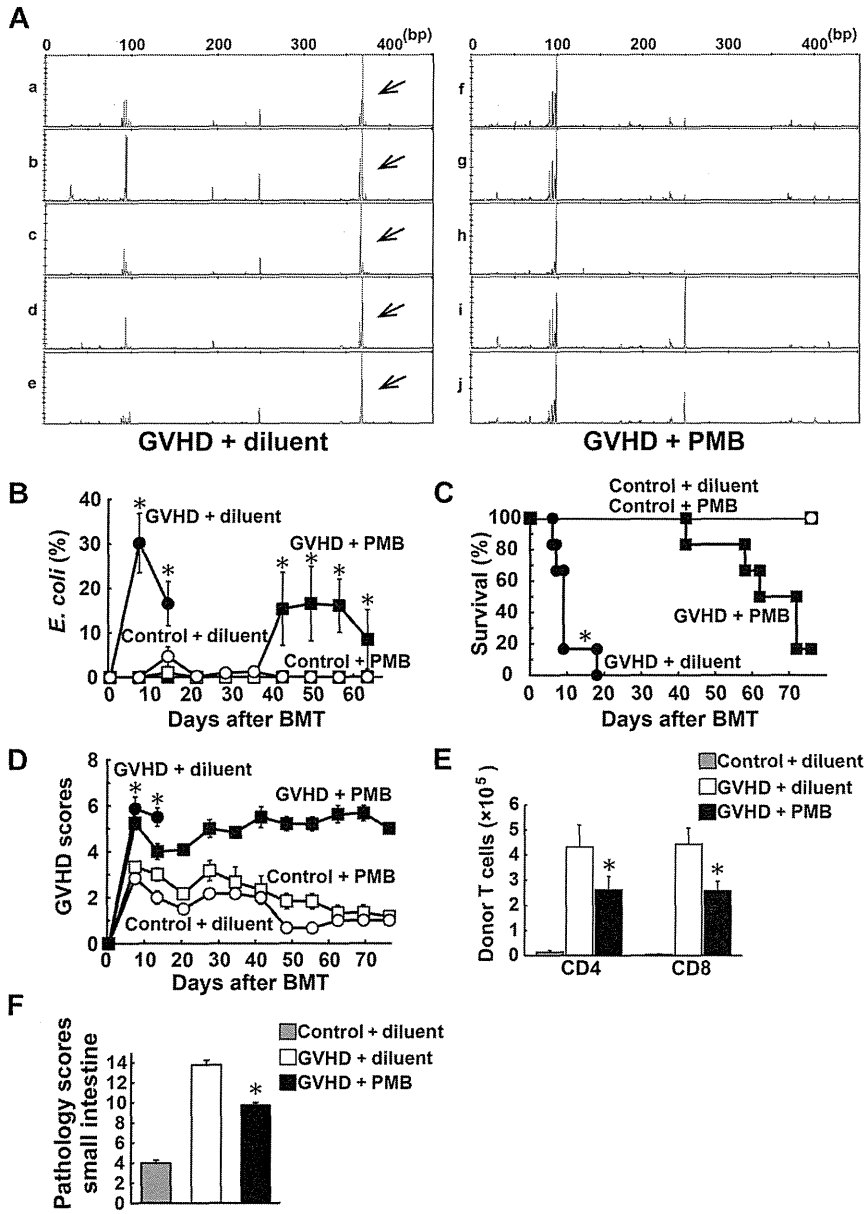

Figure 7. Oral administration of polymyxin B ameliorated GVHD. Lethally irradiated B6D2F1 mice were transplanted with 5×10^6 TCD BM without or with 2×10^6 T cells from B6 or B6-Ly5.1 (CD45.1$^+$) donors. Polymyxin B (PMB; 100 mg/kg) or diluent was administered by daily oral gavage from day −4 until day 28 after BMT. (A) Fecal pellets were collected once per week after BMT and intestinal microflora was characterized by RFLP analysis of 16S rRNA genes constructed from each sample of fecal pellets and digested with *HhaI*. Representative RFLP patterns are shown in mice with GVHD receiving diluent (a-e) and those with PMB (f-j) 7 days after BMT. Arrows indicate an OTU for *E coli*. (B) Time course changes in the proportion of *E coli* (n = 6-12 / group). Survival (C) and clinical GVHD scores (D, mean ± SE) after BMT are shown (n = 6-12/group). Data from 2 independent experiments were combined. (E) Numbers of donor (CD45.1$^+$) T cells in mLNs on day 5 (n = 20/group). (F) Pathology scores of the small intestine on day 7 (n = 20/group). Data from 3 independent experiments were combined and are shown as mean ± SE (*$P < .05$).

is one of the most frequent causes of death in patients with severe intestinal GVHD.

There are other interfaces that exist between the environment and the host, such as the skin and airways. Epithelial cells in these tissues can also release antimicrobial peptides such as β-defensins in response to bacteria and LPS.[47] GVHD-mediated epithelial cell damage of these tissues may also impair the local secretion of antimicrobial peptides, leading to aberrant overgrowth of pathogens and development of dermal infections or pneumonia, which are frequently observed in patients with GVHD. Furthermore, the development of these pathologic conditions may be associated with the unique tissue specificity of GVHD for tissues that are in contact with high microbial loads, such as the skin, liver, intestine, and lung.

Intestinal epithelial cells are continuously regenerated from ISCs, which are required to regenerate damaged sections of the intestinal epithelium.[48] Paneth cells are derived from ISCs and serve as a niche for ISCs.[8] Our previous[7] and current studies addressed intestinal GVHD at the cellular level, and demonstrated that ISCs and their niche Paneth cells could survive pretransplant conditioning and regenerate injured epithelium by conditioning in the absence of GVHD. However, both ISCs and Paneth cells are targeted by GVHD, resulting in an impairment of the physiologic repair mechanisms of injured epithelium, although it remains to be elucidated whether Paneth cell loss is induced by direct cytotoxicity to Paneth cell itself or secondary to the loss of ISCs. This phenomenon may explain the prolonged and refractory nature of clinical intestinal GVHD. These new insights will help to establish new therapeutic strategies that can be used to prevent and treat GVHD and related infections and improve the clinical outcome of allogeneic BMT.

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Authorship

Contribution: Y.E. and T.T. developed the conceptual framework of the study, designed the experiments, conducted studies, analyzed data, and wrote the paper; S.T., H.O., S. Shimoji, K.N., H.U., S. Shimoda, and H.I. conducted experiments; and N.S., T.A., and K.A. supervised experiments.

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Cytopenias after day 28 in allogeneic hematopoietic cell transplantation: impact of recipient/donor factors, transplant conditions and myelotoxic drugs

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ABSTRACT

Background
Secondary cytopenias are serious complications following hematopoietic cell transplantation. Etiologies include myelotoxic agents, viral infections, and possibly transplant-related factors such as the intensity of the conditioning regimen and the source of stem cells.

Design and Methods
We retrospectively analyzed data from 2162 hematopoietic cell transplant recipients to examine the effect of these factors on overall cytopenias occurring after 28 days in hematopoietic cell transplantation.

Results
Advanced age of the patient, recipient cytomegalovirus seropositivity, unrelated donor status, human leukocyte antigen mismatch and lower doses of transplanted $CD34^+$ cells ($\leq 6.4\times10^6$/kg) significantly increased the risk of cytopenias after day 28. Non-myeloablative hematopoietic cell transplantation had protective effects on anemia and thrombocytopenia after day 28 (adjusted odds ratio 0.76, probability value of 0.05 and adjusted odds ratio 0.31, probability value of <0.0001, respectively) but not on overall or ganciclovir-related neutropenia. This lack of protection appeared to be due to the use of mycophenolate mofetil in the majority of recipients of non-myeloablative hematopoietic cell transplants. Peripheral blood stem cells did not confer protection from cytopenias when compared to bone marrow.

Conclusions
Elderly patients appear to be more prone to cumulative toxicities of post-transplant drug regimens, but non-myeloablative conditioning, optimized human leukocyte antigen matching, and higher doses of $CD34^+$ cell infusions may reduce the risk of cytopenia after day 28.

Key words: non-myeloablative allogeneic hematopoietic stem cell transplantation, ganciclovir-related neutropenia, cytopenias after day 28.

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Introduction

Secondary cytopenia is a common complication after hematopoietic cell transplantation (HCT). Causes include viral infection, septicemia, graft-*versus*-host disease (GVHD), and myelotoxic drugs.[1-5] Of the commonly used drugs with myelotoxic potential, ganciclovir is particularly prone to cause neutropenia, which occurs in up to 40% of allograft recipients and may increase the risk of invasive bacterial and fungal infections.[1,6] The underlying mechanism of ganciclovir-related neutropenia is a dose-dependent inhibition of DNA-polymerase in hematopoietic progenitor cells.[7] We previously reported that ganciclovir-related neutropenia is associated with low marrow cellularity, hyperbilirubinemia, and elevated serum creatinine levels after myeloablative conditioning (M-HCT).[1] However, it is not known how non-myeloablative conditioning (NM-HCT) influences the incidences of secondary cytopenias in general and ganciclovir-related neutropenia in particular.

Less toxic non-myeloablative conditioning regimens that can be successfully used in elderly patients and/or patients with comorbidities have been developed.[8-13] Non-myeloablative conditioning does not eradicate host hematopoiesis and allows relatively prompt hematopoietic recovery within 28 days after transplantation.[14,15] NM-HCT may, therefore, be associated with a lower incidence of cytopenias, including ganciclovir-related neutropenia. In addition, the increased use of hematopoietic growth factors for secondary neutropenia at moderate levels in recent years may also be associated with a lower risk of profound levels of neutropenia.

The purpose of this study was to examine risk factors for the occurrence of cytopenias 28 days after HCT as a surrogate for secondary neutropenia overall, and ganciclovir-related neutropenia in particular.

Design and Methods

Study population

This retrospective study population consisted of 2162 consecutive patients who underwent HCT between 1998 and 2006 at the Fred Hutchinson Cancer Research Center (FHCRC) (Seattle, WA, USA). The retrospective analysis was approved by the Institutional Review Board of the FHCRC. Informed consent was obtained from all the patients before HCT. We compared events between 534 patients undergoing NM-HCT and 1628 contemporaneous patients undergoing M-HCT who served as a comparison group (Table 1). Clinical and laboratory data were extracted from the computerized database and from patients' charts.

The most common regimens for NM-HCT were fludarabine (30 mg/m^2/day for 3 consecutive days) together with low-dose total body irradiation (2 Gy, day 0), or low-dose total body irradiation (2 Gy, day 0) alone. In contrast, many different types of conditioning regimens were used for M-HCT. The most common regimen for M-HCT consisted of cyclophosphamide (60 mg/kg/day for 2 consecutive days) followed by total body irradiation (12 Gy or 13.2 Gy) or busulfan (4 mg/kg/day for 4 consecutive days) followed by cyclophosphamide (60 mg/kg/day for 2 consecutive days). The NM-HCT group included more elderly patients, almost exclusive use of peripheral blood stem cells as the source of stem cells, and higher doses of transplanted CD34$^+$ cells than those in the M-HCT group (Table 1).

In terms of GVHD prophylaxis, M-HCT patients most commonly received a combination of a calcineurin inhibitor (either cyclosporine or tacrolimus) and short-term methotrexate (15 mg/m^2 intravenously on day 1, and 10 mg/m^2 on days 3, 6, and 11). All NM-HCT patients received post-grafting immunosuppressants including mycophenolate mofetil (MMF) and a calcineurin inhibitor, cyclosporine or tacrolimus (Table 1)

MMF was administered at a dose of 15 mg/kg orally twice a

Table 1. Characteristics of the patients for the neutropenia/transfusion analysis (allogeneic transplant from 1998 to 2006, hematologic malignancy n=2162).

	Myeloablative (n=1628)	Non-myeloablative (n=534)
Patient's age		
≤ 40 years	770 (47%)	95 (18%)
> 40 years	858 (53%)	439 (82%)
Donor's age[a]		
≤ 40 years	788 (56%)	196 (42%)
> 40 years	618 (44%)	273 (58%)
Patient self-reported race[b]		
Caucasian	1297 (80%)	475 (90%)
Non-Caucasian	315 (20%)	52 (10%)
Donor self-reported race[c]		
Caucasian	826 (80%)	274 (88%)
Non-Caucasian	211 (20%)	39 (12%)
Patients' CMV status[d]		
Negative	799 (49%)	212 (40%)
Positive	827 (51%)	322 (60%)
Donors' CMV status[e]		
Negative	972 (60%)	305 (57%)
Positive	654 (40%)	229 (43%)
Patients' gender		
Male	918 (56%)	333 (62%)
Female	710 (44%)	201 (38%)
Donors' gender		
Male	865 (53%)	285 (53%)
Female	763 (47%)	249 (47%)
Sex mismatch		
Other	1224 (75%)	383 (72%)
Female into male	404 (25%)	151 (28%)
Donor		
Related	777 (48%)	283 (53%)
Unrelated	851 (52%)	251 (47%)
HLA mismatch		
No	1331 (82%)	485 (91%)
Yes	297 (18%)	49 (9%)
Stem cell source		
Peripheral blood stem cells	929 (57%)	491 (92%)
Bone marrow	699 (43%)	43 (8%)
Use of mycophenolate mofetil		
No	1490 (92%)	0
Yes	138 (8%)	534 (100%)
CD34 cell dose[f]		
PBSC (median, range×10^6)	7.47 (0.02-57.9)	7.99 (0.76-42.6)
Marrow (median, range×10^6)	3.20 (0.02-35.6)	4.83 (0.71-22.3)
> 6.4×10^6	569 (42%)	333 (64%)
≤ 6.4×10^6	774 (58%)	186 (36%)
ABO mismatch[g]		
No	810 (50%)	307 (57%)
Yes	816 (50%)	227 (43%)
Major	337 (21%)	93 (17%)
Minor	365 (22%)	110 (21%)
Bi-directional	114 (7%)	24 (5%)

[a]unknown for 287; [b]unknown for 23; [c]unknown for 812; [d]unknown for 2; [e]unknown for 2; [f]unknown for 300; [g]unknown for 2; PBSC: peripheral blood stem cells.

day from day 0 to day 27 and discontinued for the human leukocyte antigen (HLA) matched-related NM-HCT patients; while for the unrelated NM-HCT patients MMF was given at a dose of 15 mg/kg orally two or three times a day from day 0 to day 40, with tapering to day 96. For the single HLA-antigen and combined HLA-antigen and allele-mismatched NM-HCT patients, 15 mg/kg MMF was given three times a day and then tapered at day 100 over 2 months.[8,9,12,16,17]

Transfusion requirements

Red blood cell transfusions

Red blood cells were routinely transfused when the hematocrit fell below 26%. In patients with severe uremia, other causes of platelet dysfunction, active bleeding, or thrombocytopenia refractory to platelet transfusion, the hematocrit was maintained at 30% or above. A hematocrit of at least 30% was also maintained in patients with a history of cardiac or peripheral vascular disease, and in patients over 65 years old.

Platelet transfusions

A platelet threshold for transfusion of 1.0×10^{10}/L was used for clinically stable, afebrile patients without evidence of hemorrhage, infection, or uncontrolled GVHD. Transfusions at higher platelet levels were given to patients receiving anti-coagulant medications and patients with abnormal coagulation times, platelet dysfunction such as uremia, or other bleeding diatheses. Invasive procedures, anticoagulation management, prevention of blood clots and management of central venous catheter-associated thrombosis required maintenance of higher platelet levels.

Infection surveillance, prophylaxis and pre-emptive therapy against cytomegalovirus

Cytomegalovirus (CMV) surveillance with polymerase chain reaction (PCR) analysis or the pp65 antigenemia assay was performed on a weekly basis until day 100 as previously described.[18,19] After day 100, surveillance and pre-emptive therapy were recommended for CMV intermediate- and high-risk patients on a weekly or biweekly basis until day 365.

Pre-emptive ganciclovir treatment was started when CMV pp65 antigenemia/PCR became positive during the first 100 days after HCT. Ganciclovir (5 mg/kg IV twice daily) for 7 to 14 days was administered as induction therapy followed by a half-dose of ganciclovir (5 mg/kg IV daily) or valganciclovir 900 mg once a day orally as maintenance therapy until negative surveillance or day 100.[20] All doses were adjusted based on the patients' renal function according to the manufacturers' recommendation. After day 100, pre-emptive therapy was recommended for patients with CMV pp65 antigenemia or more than 1000 copies/mL (assessed by PCR) as previously described.[18,19]

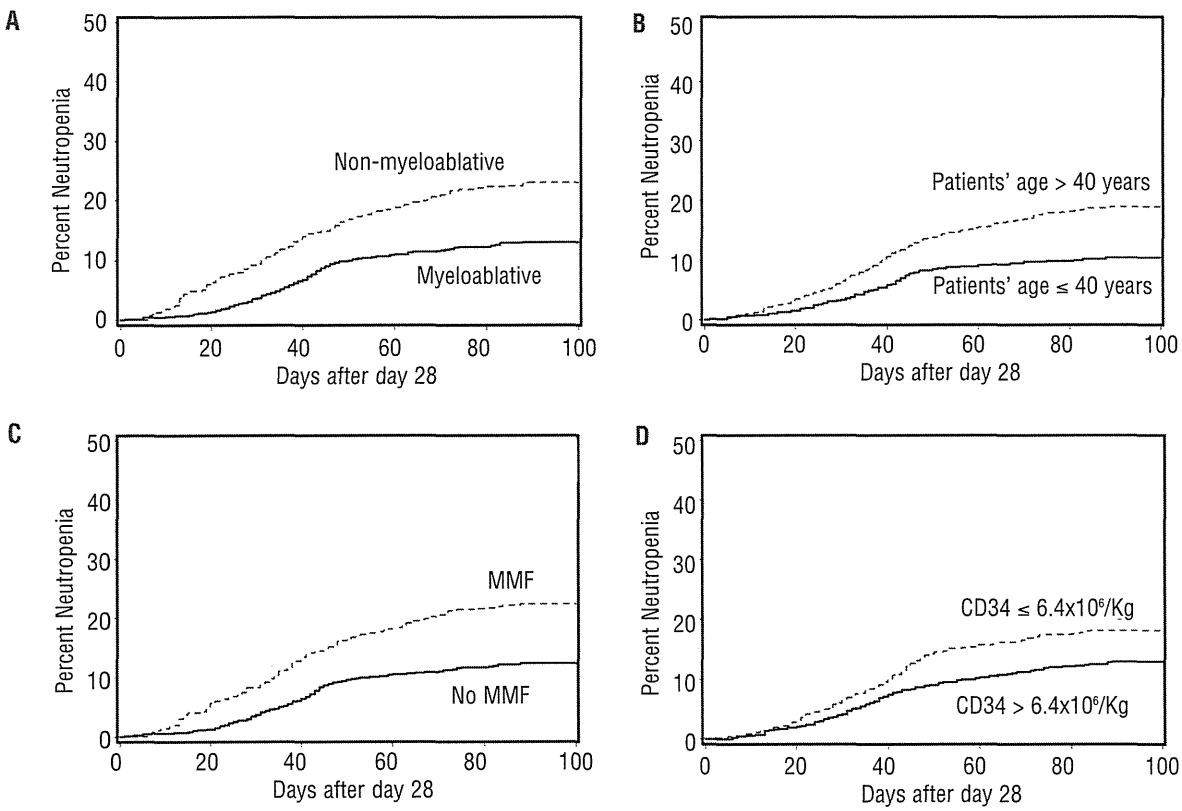

Figure 1. Cumulative incidence of neutropenia after day 28. The probabilities of neutropenia after day 28 (absolute neutrophil count < 500/L) according to (A) non-myeloablative (NM-HCT) vs. myeloablative hematopoietic stem cell transplantation (M-HCT), (B) patients' age (≤ 40 vs. > 40 years), (C) MMF use (yes vs. no) and (D) CD34⁺ cell dose (≤ 6.4×10^6/kg vs. > 6.4×10^6/kg) are illustrated. The cumulative incidence of development of neutropenia was higher for NM-HCT than it was for M-HCT. However, in a multivariate analysis NM-HCT was not a significant risk factor for development of neutropenia.

Pneumocystis jirovecii prophylaxis consisted of trimethoprim sulfamethoxazole as the primary agent and dapsone as the secondary agent. Identical doses of both drugs were used for all patients, regardless of conditioning regimen.[21] However, recipients of non-myeloablative conditioning regimens started prophylaxis at day 28 after HCT while recipients of myeloablative transplantation received pretransplant dosing which was then resumed after neutrophil engraftment.

Definitions of cytopenias after day 28

We evaluated neutropenia, anemia and thrombocytopenia after day 28, and ganciclovir-related neutropenia. Ganciclovir-related neutropenia was defined as non-relapse–related neutropenia (absolute neutrophil count < 500/μL and < 200/μL) after the start of pre-emptive therapy for pp65 antigenemia /PCR positivity in patients with an absolute neutrophil count greater than 1000/μL at the time of CMV infection. Neutropenia after day 28 was defined as absolute neutrophil counts less than 500/μL and less than 200/μL occurring any time between day 28 post-HCT and day 120 among relapse-free patients. We used transfusion support after day 28 as a surrogate marker of anemia and thrombocytopenia. Significant anemia and thrombocytopenia beyond day 28 were both defined as the upper 25th percentile of transfusion support after day 28, up to the first of day 80, death or relapse. Specifically, we defined patients who received more than 0.8 units of red blood cell transfusions per week as cases with anemia after day 28; similarly, we defined patients who were given more than 1.6 units of platelets per week up to day 80 as cases with thrombocytopenia.

Statistical analysis

The characteristics of NM-HCT and M-HCT patients were summarized using frequency counts and percentages for categorical variables and medians and ranges for continuous variables. The cumulative incidence of neutropenia after day 28 was estimated by previously described methods, with death or relapse treated as a competing risk. Univariate and multivariate Cox regression models were used to estimate hazard ratios and 95% confidence intervals (95% CI) for risk factors associated with neutropenia after day 28 and ganciclovir-related neutropenia as defined above. Univariate and multivariate logistic regression models were used to estimate odds ratios for risk factors associated with anemia and thrombocytopenia. Cox regression was used to perform a landmark analysis among patients alive and disease-free at day 100, to evaluate the impact of prior cytopenias and other risk factors on subsequent non-relapse mortality, defined as any death without prior relapse. Covariates included recipient/donor age and sex, recipient/donor race, donor CMV serostatus, sex mismatch, HLA disparity, donor relationship, intensity of conditioning, stem cell source, T-cell-depleted conditioning, year of transplantation, disease risk, GVHD prophylaxis, acute GVHD and chronic GVHD. Acute and chronic GVHD and other post-transplant factors were analyzed as time-dependent variables. Variables with a significance level of less than 0.05 in the univariate models were candidates for the multivariate models. All *P* values are two-sided and unadjusted for multiple comparisons.

Results

Risk factors for cytopenias after day 28

Among the 1818 patients with neutrophil engraftment at day 28, 711 (39%) had at least one form of cytopenia after day 28: 103 (6%) had neutropenia only, 128 (7%) had anemia only, 102 (6%) had thrombocytopenia only and 123 (7%) had all three cytopenias. Neutropenia after day 28 was significantly more frequent in NM-HCT than in M-HCT (23% and 13%, respectively) (Figure 1A).

In univariate analysis, the risk factors for neutropenia (< 500/μL) after day 28 included the patients' age (> 40 years), recipient CMV seropositivity, patients at higher risk of CMV, unrelated donor status, receipt of NM-HCT, use of MMF, lower CD34$^+$ cell dose (≤ 6.4×10^6/kg) in the graft, chronic GVHD, high bilirubin level (> 6 mg/dL), and elevated creatinine (> 2 mg/dL) (Table 2). In a multivariate model, we identified patients' age, CMV seropositivity, unrelated donor status, HLA mismatched donor, MMF use and lower CD34$^+$ cell dose (≤ 6.4×10^6/kg) as significant risk factors for neutropenia after day 28 in both HCT with bone marrow and peripheral blood stem cells (Table 3) (Figure 1B-D). Analysis of a lower threshold for defining neutropenia (< 200/μL) did not reveal additional risk factors (*data not shown*).

Results of univariate analyses for anemia and thrombocytopenia after day 28 are presented in Table 4. ABO-mismatched donors, patients' age (> 40 years), female donor, patients at higher risk of CMV, unrelated donor, HLA-mismatched donor, bone marrow as the stem cell source and lower CD34$^+$ cell dose (≤ 6.4×10^6/kg) were risk factors for anemia after day 28. Risk factors for thrombocytopenia after day 28 included ABO-mismatched donor, unrelated donor, HLA-mismatched donor, bone marrow as the stem cell source and CD34$^+$ cell dose (≤ 6.4×10^6/kg) (Table 4). In a multivariate model, ABO-mismatched donor, patients' age (> 40 years), CMV infection, unrelated donor, HLA-mismatched donor and lower CD34$^+$ cell dose (≤ 6.4×10^6/kg) were identified as common risk factors for anemia and thrombocytopenia after day 28 in both HCT with bone marrow and peripheral blood stem cells (Table 5). CMV serostatus was no longer significant when active post-transplant CMV infection was included in the model.

NM-HCT was significantly associated with a lower incidence of anemia and thrombocytopenia after day 28 in both univariate and multivariate models (Tables 4 and 5).

Risk factor for ganciclovir-related neutropenia

The cumulative incidence of ganciclovir-related neutropenia was similar for NM-HCT and M-HCT recipients (26% and 22%, respectively). A univariate model for ganciclovir-related neutropenia is shown in Table 2. In the univariate model, we found patients' age (> 40 years), unrelated donor status, MMF use, lower CD34$^+$ cell dose (≤ 6.4×10^6/kg) and high bilirubin level (> 6 mg/dL) to be significant risk factors for ganciclovir-related neutropenia (Table 2). All factors except MMF use and high bilirubin levels remained statistically significant risk factors for ganciclovir-related neutropenia (Table 3). Analysis of a lower threshold for neutropenia (< 200/μL) did not reveal additional risk factors (*data not shown*).

The impact of cytopenias after day 28 on non-relapse mortality

In a multivariate analysis of non-relapse mortality, we included acute GVHD, age of the patients and donors, sex of the patients and donors, patients' CMV status, donor relation, HLA disparity, stem cell source, MMF use and the intensity of the conditioning regimen as covariates. After adjustment for these factors, neutropenia, anemia and thrombocytopenia after day 28 were all significant inde-

Table 2. Univariate risk factors for neutropenia (absolute neutrophil count <500/μL).

	Ganciclovir-induced[1]			Neutropenia after day 28[2]		
	N	HR (95% CI)	P value	N	HR (95% CI)	P value
Patients' age						
≤ 40 years	225	1.0		718	1.0	
> 40 years	468	1.90 (1.3-2.8)	0.0004	1100	1.96 (1.5-2.6)	<0.0001
Donors' age						
≤ 40 years	320	1.0		819	1.0	
> 40 years	284	1.35 (1.0-1.9)	0.08	771	1.28 (1.0-1.6)	0.06
Patients' sex						
Male	350	1.0		1046	1.0	
Female	343	0.78 (0.6-1.1)	0.12	772	0.85 (0.7-1.1)	0.17
Donors' sex						
Male	376	1.0		976	1.0	
Female	317	0.91 (0.7-1.2)	0.56	842	1.04 (0.8-1.3)	0.73
Sex mismatch						
Others	540	1.0		1356	1.0	
F into M	153	1.13 (0.8-1.6)	0.53	462	1.28 (1.0-1.6)	0.06
Patients' race						
Caucasian	521	1.0		1474	1.0	
Others	160	0.75 (0.5-1.1)	0.15	323	0.94 (0.7-1.3)	0.69
Donors' race						
Caucasian	325	1.0		917	1.0	
Others	109	0.75 (0.5-1.2)	0.25	219	0.81 (0.5-1.2)	0.31
Patients' CMV status						
Negative	64	1.0		848	1.0	
Positive	628	1.22 (0.7-2.2)	0.48	968	1.98 (1.5-2.5)	<0.0001
Donors' CMV status						
Negative	313	1.0		1053	1.0	
Positive	379	0.74 (0.5-1.0)	0.06	764	1.09 (0.9-1.4)	0.46
CMV risk group						
D-/R-	13	1.0		602	1.0	
D+/R-	51	0.80 (0.2-2.9)		246	1.39 (0.9-2.1)	
R+	628	1.02 (0.3-3.2)	0.73	968	2.21 (1.6-3.0)	<0.0001
Donor relation						
Related	324	1.0		907	1.0	
Unrelated	369	1.56 (1.1-2.1)	0.007	911	1.42 (1.1-1.8)	0.004
HLA mismatch						
No	572	1.0		1523	1.0	
Yes	121	1.22 (0.8-1.8)	0.33	295	1.31 (1.0-1.8)	0.08
Stem cell source						
Peripheral blood	463	1.0		1266	1.0	
Bone marrow	230	1.14 (0.8-1.6)	0.44	552	0.95 (0.7-1.2)	0.68
Conditioning						
Myeloablative	522	1.0		1361	1.0	
Non-myeloablative	171	1.25 (0.9-1.8)	0.22	457	1.85 (1.5-2.4)	<0.0001
MMF use						
No	468	1.0		1243	1.0	
Yes	225	1.41 (1.0-1.9)	0.04	575	1.93 (1.5-2.4)	<0.0001
CD34+cell dose (10^6)						
> 6.4/kg	290	1.0		807	1.0	
≤ 6.4/kg (PB)	161	1.72 (1.2-2.5)	0.004	407	1.59 (1.2-2.1)	0.0008
≤ 6.4/kg (BM)	153	1.36 (0.9-2.0)	0.13	370	1.20 (0.9-1.6)	0.24
Time-dependent associations						
Acute GVHD						
0–I	125	1.0		408	1.0	
II–IV	568	1.38 (0.9-2.1)	0.12	1410	0.90 (0.7-1.2)	0.42
Chronic GVHD						
No	287	1.0		790	1.0	
Yes	406	0.41 (0.1-1.2)	0.06	1028	0.07 (0.0-0.1)	<0.0001
Bilirubin						
≤ 6 mg/dL	571	1.0		1522	1.0	
> 6 mg/dL	122	1.86 (1.3-2.7)	0.003	296	2.01 (1.5-2.7)	<0.0001
Creatinine						
≤ 2 mg/dL	479	1.0		1311	1.0	
> 2 mg/dL	214	1.45 (1.0-2.1)	0.05	507	1.40 (1.1-1.8)	0.01
Antigenemia/PCR						
≤ 5 and ≤ 1000	408	1.0				
6–10 or 1001–10^4	107	0.90 (0.5-1.5)				
> 10 or > 10^4	178	1.53 (1.1-2.2)	0.05			

continued in next column

[1]Based on 693 patients positive for CMV by antigenemia or PCR, without prior relapse, and absolute neutrophil count (ANC) > 1000/μL; [2]based on 1818 patients relapse-free at day 28, and with ANC > 1000/μL.

Table 3. Multivariate risk factors for neutropenia (absolute neutrophil count < 500/μL).

	Ganciclovir-induced[1]			Neutropenia after day 28[2]		
	N	HR (95% CI)	P value	N	HR (95% CI)	P value
Patients' age						
≤ 40 years	225	1.0		718	1.0	
> 40 years	468	1.72 (1.2-2.6)	0.008	1098	1.66 (1.3-2.2)	0.0004
Donors' age						
≤ 40 years	320	1.0				
> 40 years	284	1.53 (1.0-2.3)	0.03			
(missing)	89	0.96 (0.6-1.6)	0.87			
Patients' CMV status						
Negative				848	1.0	
Positive				968	1.84 (1.4-2.4)	<0.0001
Donor relation						
Related	324	1.0		906	1.0	
Unrelated	369	2.03 (1.4-3.0)	0.0004	910	1.45 (1.1-1.9)	0.003
HLA mismatch						
No				1522	1.0	
Yes				294	1.44 (1.1-2.0)	0.02
MMF use						
No	468	1.0		1241	1.0	
Yes	225	1.39 (1.0-2.0)	0.07	575	2.02 (1.5-2.6)	<0.0001
CD34+cell dose (10^6)						
> 6.4/kg	290	1.0		807	1.0	
≤ 6.4/kg (PB)	161	1.79 (1.2-2.7)	0.004	407	1.57 (1.2-2.1)	0.003
≤ 6.4/kg (BM)	153	1,61 (1.0-2.6)	0.04	370	1.74 (1.2-2.5)	0.002
(missing)	89	1.51 (0.9-2.6)	0.13	234	1.74 (1.2-2.6)	0.006

[1]Based on 693 patients positive for CMV by antigenemia or PCR, without prior relapse, and with an absolute neuthophil count (ANC) > 1000/μL; [2]based on 1816 patients relapse-free at day 28, and ANC > 1000/μL; PB: peripheral blood; BM: bone marrow.

pendent risk factors for non-relapse mortality after HCT (neutropenia: HR=1.81, 95% CI 1.4–2.4, P<0.0001; anemia: HR=1.56, 95% CI 1.2–2.1, P=0.002; thrombocytopenia: HR=2.35, 95% CI 1.7–3.2, P<0.0001) (Table 6).

Discussion

This study provides novel and somewhat unexpected results on the risk of cytopenias after HCT. Older recipient age, low CD34+ cell dose, an unrelated donor, and HLA mismatch were risk factors for cytopenias after transplantation. Non-myeloablative conditioning was associated with significantly reduced incidences of anemia and thrombocytopenia after day 28, but not of neutropenia.

Table 4. Univariate risk factors for significant anemia and thrombocytopenia after day 28.

	Anemia[1]			Thrombocytopenia[2]		
	N	OR (95% CI)	*P* value	N	OR (95% CI)	*P* value
ABO mismatch						
No	1068	1.0		1068	1.0	
Yes	973	1.78 (1.5-2.2)	<0.0001	973	1.51 (1.2-1.8)	<0.0001
Patients' age						
≤ 40 years	827	1.0		827	1.0	
> 40 years	1216	1.25 (1.0-1.5)	0.03	1216	1.01 (0.8-1.2)	0.92
Donors' age						
≤ 40 years	931	1.0		931	1.0	
> 40 years	841	0.88 (0.7-1.1)	0.24	841	0.99 (0.8-1.2)	0.94
Patients' sex						
Male	1179	1.0		1179	1.0	
Female	864	1.16 (1.0-1.4)	0.13	864	0.91 (0.7-1.1)	0.34
Donors' sex						
Male	1090	1.0		1090	1.0	
Female	953	1.23 (1.0-1.5)	0.03	953	0.96 (0.8-1.2)	0.71
Sex mismatch						
Others	1524	1.0		1524	1.0	
F into M	519	1.11 (0.9-1.4)	0.34	519	1.08 (0.9-1.4)	0.49
Patients' race						
Caucasian	1666	1.0		1666	1.0	
Others	355	0.92 (0.7-1.2)	0.54	355	0.98 (0.8-1.3)	0.90
Donors' race						
Caucasian	1038	1.0		1038	1.0	
Others	241	0.96 (0.7-1.3)	0.82	241	1.04 (0.7-1.4)	0.83
Recipient CMV serostatus						
Negative	960	1.0		960	1.0	
Positive	1081	1.28 (1.1-1.6)	0.01	1081	1.14 (0.9-1.4)	0.19
Donor CMV serostatus						
Negative	1205	1.0		1205	1.0	
Positive	837	0.97 (0.8-1.2)	0.79	837	0.87 (0.7-1.1)	0.19
CMV serostatus risk group						
D-/R-	692	1.0		692	1.0	
D+/R-	268	1.18 (0.9-1.6)		268	1.03 (0.7-1.4)	
R+	1081	1.35 (1.1-1.7)	0.03	1081	1.15 (0.9-1.4)	0.42
Donor relation						
Related	1010	1.0		1010	1.0	
Unrelated	1033	1.58 (1.3-1.9)	<0.0001	1033	2.15 (1.7-2.6)	<0.0001
HLA Mismatch						
No	1716	1.0		1716	1.0	
Yes	327	1.60 (1.2-2.1)	0.0003	327	2.03 (1.6-2.6)	<0.0001
Stem cell source						
Peripheral blood	1348	1.0		1348	1.0	
Bone marrow	695	1.45 (1.2-1.8)	0.0003	695	1.82 (1.5-2.2)	<0.0001
Conditioning						
MA	1544	1.0		1544	1.0	
NMA	499	0.71 (0.6-0.9)	0.004	499	0.30 (0.2-0.4)	<0.0001
MMF use						
No	1420	1.0		1420	1.0	
Yes	623	0.77 (0.6-1.0)	0.02	623	0.44 (0.3-0.6)	<0.0001
CD34⁺cell dose (10^6)						
> 6.4/kg	855	1.0		855	1.0	
≤ 6.4/kg (PB)	447	1.43 (1.1-1.8)	0.004	447	1.31 (1.0-1.7)	0.04
≤ 6.4/kg (BM)	462	1.81 (1.4-2.3)	<0.0001	462	1.93 (1.5-2.4)	<0.0001

[1]Based on 2043 patients relapse-free at day 28; significant anemia post-day 28 is defined as red blood cell transfusions > 0.8 units per week from day 28 to the first of day 80, death or relapse. [2]Based on 2043 patients relapse-free at day 28; significant thrombocytopenia post-day 28 is defined as platelet transfusions > 1.6 units per week from day 28 to the first of day 80, death or relapse. PB: peripheral blood; BM: bone marrow; MA: myeloablative; NMA: non-myeloablative.

Table 5. Multivariate risk factors for significant anemia and thrombocytopenia after day 28.

	Anemia[1]			Thrombocytopenia[2]		
	N	OR (95% CI)	*P* value	N	OR (95% CI)	*P* value
ABO mismatch						
No	1066	1.0		1066	1.0	
Yes	973	1.67 (1.4-2.0)	<0.0001	973	1.27 (1.0-1.6)	0.03
Patients' age						
≤ 40 years	825	1.0		825	1.0	
> 40 years	1214	1.43 (1.1-1.8)	0.001	1214	1.36 (1.1-1.7)	0.007
CMV infection[3]						
Negative	1301	1.0		1301	1.0	
Positive	738	1.39 (1.1-1.7)	0.002	738	1.36 (1.1-1.7)	0.005
Donor relation						
Related	1008	1.0		1008	1.0	
Unrelated	1031	1.30 (1.1-1.6)	0.02	1031	1.86 (1.5-2.3)	<0.0001
HLA Mismatch						
No	1715	1.0		1715	1.0	
Yes	324	1.33 (1.0-1.7)	0.04	324	1.46 (1.1-1.9)	0.007
Conditioning						
MA	1540	1.0		1540	1.0	
NMA	499	0.78 (0.6-1.0)	0.06	499	0.32 (0.2-0.4)	<0.0001
CD34⁺cell dose (10^6)						
> 6.4/kg	855	1.0		855	1.0	
≤ 6.4/kg (PB)	446	1.54 (1.2-2.0)	0.002	446	1.52 (1.1-2.0)	0.004
≤ 6.4/kg (BM)	462	1.79 (1.4-2.3)	<0.0001	462	1.57 (1.2-2.1)	0.002
(missing)	276	1.45 (1.1-2.0)	0.02	276	1.43 (1.0-2.0)	0.03

[1]Based on 2039 patients relapse-free at day 28; significant anemia post-day 28 is defined as red blood cell transfusions > 0.8 units per week from day 28 to the first of day 80, death or relapse. [2]Based on 2039 patients relapse-free at day 28; significant thrombocytopenia post-day 28 is defined as platelet transfusions > 1.6 units per week from day 28 to the first of day 80, death, or relapse. [3]CMV infection defined as any active CMV infection before day 100; CMV serostatus; a separate multivariate model that included CMV recipient serostatus instead showed a significant association of CMV seropositivity with anemia (adjusted OR 1.33, 95% CI 1.1-1.6, P=0.005) and thrombocytopenia (adjusted OR 1.25, 95% CI 1.0-1.5, P=0.04).PB: peripheral blood; BM: bone marrow; MA: myeloablative; NMA: non-myeloablative.

We hypothesized that non-myeloablative conditioning is associated with less neutropenia after day 28. Surprisingly, in this study we did not find a significant reduction of neutropenia either overall or in the context of ganciclovir use. Overall, neutropenia after day 28 occurred in 13% of patients. The exact contribution of MMF to the relatively high rates of neutropenia in NM-HCT recipients cannot be determined since MMF was given to all patients receiving non-myeloblative conditioning. MMF was significantly associated with neutropenia even after controlling for donor relatedness (which determined the duration of drug use). However, neutropenia is an important adverse effect of MMF and cumulative toxicity with ganciclovir is plausible and has been described.[22] Our study also identified other factors that might explain the high rate of neutropenia in NM-HCT. We found older recipient age to be a risk factor for both neutropenia after day 28 and ganciclovir-related neutropenia. NM-HCT is more commonly done in older patients. The effect of older recipient age may be mediated by subclinical renal dysfunction (especially tubular function[23]), which may lead to inadvertent overdosing of myelotoxic drugs that are eliminated through the kidneys and whose doses are adjusted only by creatinine clearance (which does not measure tubular function). Such an effect would be consistent with the pharmacokinetic properties and the toxicity profile of ganciclovir, which

Table 6. Multivariate risk factors for non-relapse mortality. Landmark analysis at day 100, n=1489, 331 events.

	HR (95% CI)	P value
Neutropenia[1]		
No	1.0	
Yes	1.81 (1.4-2.4)	<0.0001
Anemia[2]		
No	1.0	
Yes	1.56 (1.2-2.1)	0.002
Thrombocytopenia[3]		
No	1.0	
Yes	2.35 (1.7-3.2)	<0.0001
GVHD		
0-I	1.0	
II-IV	1.37 (1.0-1.8)	0.03
Patients' age		
≤40 years	1.0	
>40 years	1.51 (1.2-2.0)	0.002
Donors' age		
≤40 years	1.0	
>40 years	1.12 (0.9-1.5)	0.42
(missing)	1.03 (0.7-1.5)	0.87
Donor/patient sex		
Other	1.0	
Female/male	1.25 (1.0-1.6)	0.07
Patient CMV status		
Negative	1.0	
Positive	1.05 (0.8-1.4)	0.72
Donor relation		
Related	1.0	
Unrelated	1.08 (0.8-1.4)	0.59
HLA mismatch		
No	1.0	
Yes	1.62 (1.2-2.2)	0.001
Stem cell source		
Peripheral blood	1.0	
Bone marrow	0.87 (0.7-1.1)	0.31
MMF use		
No	1.0	
Yes	2.12 (1.4-3.3)	0.0006
Conditioning		
Myeloablative	1.0	
Non-myeloablative	0.84 (0.5-1.3)	0.44

[1]Neutropenia was defined as an absolute neutrophil count (ANC) < 500/μL occurring any time between day 28 post-HCT and day 120 among patients, relapse-free at day 28, and ANC > 1000/μL. [2]Significant anemia post-day 28 is defined as red blood cell transfusions > 0.8 units per week from day 28 [3]Significant thrombocytopenia post-day 28 is defined as platelet transfusions > 1.6 units per week from day 28 to the first of day 80, death, or relapse.

includes predominantly neutropenia but not thrombocytopenia and anemia.

There are limited data on cytopenia after day 28 relative to the intensity of the conditioning regimen. Severe GVHD, myelotoxicity associated with drugs such as ganciclovir, trimethoprim sulfamethoxazole or MMF, as well as viral and severe fungal or bacterial infections have all been associated with an increased risk of neutropenia after day 28.[1,2,4,22] Furthermore, Bruno *et al.* previously reported unrelated donor, grade II-IV acute GVHD, impaired renal function, the combination of busulfan and cyclophosphamide, total body irradiation, stem cell dose and infections as risk factors for secondary failure of platelet recovery among M-HCT.[5] The present study extended our previous findings that NM-HCT may also have protective effects against thrombocytopenia and anemia.[24] We identified HLA mismatch, CMV serostatus and the CD34+ cell count as additional risk factors for both outcomes in multivariable models. We speculate that the higher doses of CD34+ cells and the reduced intensity of the conditioning regimen used in NM-HCT contributed to the lower rates of anemia and thrombocytopenia.

The CD34+ cell dose rather than the stem cell source *per se* was an important risk factor for all cytopenias examined in this study. When the cell dose was included in the multivariable models the stem cell source was no longer significant, suggesting that the protective effect of peripheral blood stem cells for anemia and thrombocytopenia seen in the univariate analyses was mediated by the higher dose of CD34+ cells (Tables 3 and 5).

CMV serostatus of the recipient was a risk factor for both anemia and thrombocytopenia requiring blood products (Table 5), a finding not previously appreciated in HCT recipients.[25] When CMV serostatus and active CMV infection were included in a multivariable analysis, active CMV infection remained significant while CMV serostatus was no longer significant, suggesting that active CMV infection or pre-emptive therapy was responsible for the effect. The relative contribution of CMV infection compared to that of its treatment cannot be determined from this study. Ganciclovir has not been associated with thrombocytopenia or anemia or an increased use of blood products in several placebo-controlled randomized trials in HCT recipients.[6,26,27] A previous risk factor analysis in myeloablative HCT recipients between 1990 and 1997 did not identify CMV serostatus as a risk factor for thrombocytopenia, but the use of platelet products was not analyzed in that study.[5] Based on the lack of association with anemia and thrombocytopenia in randomized trials of ganciclovir, we speculate that CMV infection itself might be responsible for the effect.[4,28]

Our study has several limitations, including the retrospective nature of the analysis and that the analysis of concomitant medications was performed by protocol only. With regard to the non-myeloablative conditioning, the results can probably not be extrapolated to other types of reduced-intensity conditioning regimens. However, the strength of the analyses lies in the large sample size, the number of clinically important factors analyzed, a homogeneous transplant protocol, and highly standardized supportive care strategies.

In conclusion, the study provides a comprehensive analysis of factors associated with cytopenias after day 28 in HCT recipients. Unexpectedly, NM-HCT did not reduce the risk of neutropenia after day 28 overall or in the context of ganciclovir treatment. The high rates of neutropenia appear to be linked to the use of MMF and ganciclovir, emphasizing the need for less toxic immunosuppressive and anti-CMV drugs or strategies. In contrast, NM-HCT showed a protective effect against anemia and thrombocytopenia after day 28, probably through less toxic conditioning and higher doses of CD34+ stem cells or almost exclusive use of peripheral blood stem cells. Finally, the study identified potentially modifiable factors that could be used before transplantation to minimize the risk of post-transplant cytopenias, including non-myeloablative conditioning, optimized HLA matching, and higher doses of CD34+ cell infusions.

Authorship and Disclosures

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Impact of the mobilization regimen and the harvesting technique on the granulocyte yield in healthy donors for granulocyte transfusion therapy

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BACKGROUND: Granulocyte mobilization and harvesting, the two major phases of granulocyte collection, have not been standardized.

STUDY DESIGN AND METHODS: The data on 123 granulocyte collections were retrospectively investigated for the effect of the mobilization regimen and the harvesting technique. After a single subcutaneous dose (600 μg) of granulocyte–colony-stimulating factor (G-CSF) with (n = 68) or without (n = 40) 8 mg of orally administered dexamethasone, 108 granulocyte donors underwent granulocyte collections. Moreover, 15 peripheral blood stem cell (PBSC) donors who had received 400 μg/m^2 or 10 μg/kg G-CSF for 5 days underwent granulocyte collections on the day after the last PBSC collections (PBSC-GTX donors). Granulocyte harvesting was performed by leukapheresis with (n = 108) or without (n = 15) using high-molecular-weight hydroxyethyl starch (HES).

RESULTS: Granulocyte donors who received mobilization with G-CSF plus dexamethasone produced significantly higher granulocyte yields than those who received G-CSF alone ($7.2 \times 10^{10} \pm 2.0 \times 10^{10}$ vs. $5.7 \times 10^{10} \pm 1.7 \times 10^{10}$, p = 0.006). PBSC-GTX donors produced a remarkably high granulocyte yield ($9.7 \times 10^{10} \pm 2.3 \times 10^{10}$). The use of HES was associated with better granulocyte collection efficiency (42 ± 7.8% vs. 10 ± 9.1%, p < 0.0001).

CONCLUSION: G-CSF plus dexamethasone produces higher granulocyte yields than G-CSF alone. Granulocyte collection from PBSC donors appears to be a rational strategy, since it produces high granulocyte yields when the related patients are at a high risk for infection and reduces difficulties in finding granulocyte donors. HES should be used in apheresis procedures.

Bacterial and fungal infections during the neutropenic period remain one of the most important causes of mortality in patients with aplastic anemia or in those who undergo chemotherapy or stem cell transplantation. Several studies have shown that the transfusion of granulocyte concentrates, which are collected from healthy donors after mobilization with granulocyte–colony-stimulating factor (G-CSF), result in a substantial increase in the patient's absolute neutrophil count (ANC).[1-3] The efficacy of granulocyte transfusions in the improvement of clinical outcomes has been suggested in several studies, although large, randomized controlled trials should be conducted to corroborate this.[4,5]

Granulocyte collections from healthy donors consist of two major phases: granulocyte mobilization and harvesting. To date, neither the mobilization regimen nor the harvesting technique has been standardized. The reported mobilization regimens in the G-CSF era include administration of G-CSF at 300 to 600 μg as a standard dose or 5 μg/kg with or without dexamethasone.[6-10] The combined administration of G-CSF and dexamethasone

ABBREVIATIONS: ANC = absolute neutrophil count; G-CSF + Dex = granulocyte–colony-stimulating factor plus dexamethasone; PBSC(S) = peripheral blood stem cell(s); PBSC-GTX = granulocyte donation after PBSC harvest; pre-ANC = preapheresis absolute neutrophil count.

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may further increase the ANC relative to G-CSF alone but may also increase the risk of posterior subcapsular cataracts in donors who repeatedly donate granulocytes with this regimen.[11,12] Meanwhile, peripheral blood stem cell (PBSC) donors receive G-CSF for 4 to 5 days with the aim of PBSC collection. Because granulocytes are mobilized during this procedure, the collection of granulocytes from these donors appears reasonable once a sufficient number of PBSCs has been obtained.

While the usefulness of the "bag method" has been reported in granulocyte collection for pediatric patients,[13] granulocyte harvesting is usually performed by leukapheresis. High-molecular-weight hydroxyethyl starch (HES) is often used during the apheresis procedure to achieve an adequate separation of granulocytes from the red blood cells (RBCs) and may thereby result in higher granulocyte yields. However, the use of HES was avoided in a study by Ofran and colleagues[14] because of concerns about the adverse effects, including anaphylactic reactions.

Although the effect of the variations in each procedure is a critically important consideration when standardizing these procedures, a limited amount of information is available on the effect of this variation. Here, we retrospectively analyzed the data on granulocyte collections at our institution to investigate the effect of the mobilization regimen and the harvesting technique on the granulocyte yield.

MATERIALS AND METHODS

Donors

From February 2007 to July 2011, a total of 89 donors (57 men and 32 women) underwent 123 granulocyte collections. Among these, 108 granulocyte collections were obtained by a single dose of a mobilization regimen (GTX donors), while 15 were obtained on the next day after the last PBSC collection (PBSC-GTX donors).

The eligibility criteria for GTX donors included an age range from 15 to 65 years; compatibility or minor incompatibility for ABO blood types; and negativity for infections such as hepatitis B and C viruses, human immunodeficiency virus, human T-cell lymphotropic virus Type I, and syphilis. GTX donors were selected from family members or close friends of the patients. From January 2010, GTX donors were chosen from family members in accordance with Japanese guidelines,[15] except for the donors of one pediatric patient with aplastic anemia, who needed repeated granulocyte transfusions and could not find enough donor candidates from family members. Donors underwent a thorough history, blood tests, and physical examination by a hematologist before the first granulocyte mobilization.

PBSC-GTX donors were related donors, and the age-based eligibility criterion was 10 to 65 years. The eligibility of donors at 10 to 15 years of age was carefully determined after a deliberate examination. This study was approved by the institutional review board of the Hyogo College of Medicine. All donors signed an approved consent form before taking part in the study.

Granulocyte mobilization

GTX donors underwent granulocyte mobilization either with G-CSF alone or with G-CSF plus dexamethasone (G-CSF + Dex). Donors in both groups received 600 μg of filgrastim subcutaneously 12 to 18 hours before granulocyte collection. The G-CSF + Dex group also received 8 mg of dexamethasone orally at the same time as the filgrastim injection. Granulocyte collections were allowed to be performed three times per episode from a single donor. In repeated collections, the same mobilization regimen (i.e., doses of G-CSF and dexamethasone) as the previous collections was used and time intervals from the previous collections were equal to or more than 3 days.

PBSC-GTX donors received either 400 $\mu g/m^2$ filgrastim or 10 μg/kg lenograstim daily in divided doses (morning and evening). Two sessions of PBSC collections were performed on Days 4 and 5 of the G-CSF administration. PBSC-GTX donors received an evening dose of G-CSF (200 $\mu g/m^2$ filgrastim or 5 μg/kg lenograstim) on Day 5 and underwent granulocyte harvesting in the morning on Day 6.

Granulocyte harvesting

Granulocytes were harvested with a blood cell separator (COBE Spectra, CaridianBCT, Lakewood, CO) by using a granulocyte collection program; 7 L of whole blood was processed with trisodium citrate anticoagulant in approximately 3 hours by using peripheral venous access. High-molecular-weight HES (Hespan, Braun Medical, Irvine, CA) was used as a RBC-sedimenting agent (500 mL of HES to 30 mL of trisodium citrate) until the completion of collections; total volume of HES per procedure was approximately 500 mL when 7 L of whole blood was processed. HES was not used in the granulocyte collections performed between April 2009 and January 2010.

Follow-up investigations

Immediately before and after apheresis, donors completed questionnaires regarding adverse effects of the mobilization and apheresis, respectively. At 1 month after collection, donors received a physical examination and blood tests, including complete blood counts, differentials, and biochemistries. Subsequently, at 3 months and annually up to 5 years thereafter, the donors received another questionnaire regarding their overall condition and particular symptoms possibly related to mobilization and apheresis.

Definitions and statistical analysis

Preapheresis ANC (pre-ANC) was defined as ANC examined in the morning of the day of granulocyte collection. All data are provided as mean ± standard deviation unless otherwise noted. Granulocyte collection efficiency was calculated by dividing the granulocyte yield by the total number of granulocytes processed, in accordance with previous studies.[16-18] The number of granulocytes processed was obtained from the mean ANC of the pre-ANC and the postapheresis ANC multiplied by the net volume of processed blood.

Statistical comparisons were performed among GTX donors who received G-CSF alone or G-CSF + Dex. Ages and characteristics were compared between GTX donors who received G-CSF alone or G-CSF + Dex by using Mann-Whitney's U test and Fisher's exact test, respectively. Pre-ANC and granulocyte yields were compared between these two different groups of donors by using the t test. Granulocyte collection efficiency was compared between donors who underwent leukapheresis using HES and those who underwent it without using HES. The effects of the mobilization regimen on the pre-ANC and granulocyte yields were analyzed only in the first granulocyte donation to exclude the effect of repeated mobilization. Collections where the processing volume was less than 6 L due to donor discomfort or venous access problems (n = 4) were excluded from the analysis of the granulocyte yield. In donors who underwent granulocyte collections repeatedly, the pre-ANC and granulocyte yield at the time of collection were compared with those of the previous collection by using the paired t test.

RESULTS

Donor characteristics

The donor characteristics for 123 collections are shown in Table 1 with collection-based numbers. A total of 108 collections were from GTX donors, that is, 68 who received mobilization with G-CSF alone and 40 who received G-CSF + Dex; 15 collections were from PBSC-GTX donors. Only one PBSC-GTX donor was 10 to 15 years of age. The donor was a 13-year-old female, with a body height and weight comparable to those of the adult female donors. The donor was chosen as an HLA one-antigen-mismatched PBSC donor for her father, who had chemoresistant leukemia and lacked related or unrelated HLA-identical donors. Informed consent was obtained from both the donor and her mother after a thorough explanation from a coordinator and an attending physician, who were not involved in the treatment of the patient. The apheresis procedure in the donor was performed with peripheral venous access, and the 7 L of processing was completed without problems, except for mild numbness. Age, body weight, baseline white blood cell (WBC) counts, baseline ANC, and baseline platelet (PLT) counts were not statistically different among GTX donors. The ratio of male donors was higher in the GTX donors who received G-CSF alone than in those who received G-CSF + Dex ($p = 0.009$).

Effect of the mobilization regimen on pre-ANC

The pre-ANC of the GTX donors who received mobilization for the first time either with G-CSF alone or with G-CSF + Dex and of the PBSC-GTX donors are shown in Fig. 1. The mobilization with G-CSF + Dex resulted in a significantly higher pre-ANC than the one with G-CSF alone ($34 \times 10^9 \pm 5.5 \times 10^9$/L vs. $28 \times 10^9 \pm 7.6 \times 10^9$/L; $p = 0.0045$). A subgroup analysis of the male donors also showed a significantly higher pre-ANC in donors who received G-CSF + Dex than those who received G-CSF alone ($35 \pm 5.4 \times 10^9$/L vs. $30 \pm 8.3 \times 10^9$/L, $p = 0.016$). The pre-ANC of PBSC-GTX donors was similar to that of the GTX donors mobilized by G-CSF and Dex ($38 \times 10^9 \pm 9.6 \times 10^9$/L).

In donors who underwent granulocyte collections repeatedly, the pre-ANC at the time of collection relative to that of the previous collection was related to the length of the time interval between them (Fig. 2). A significantly higher pre-ANC was observed for a collection relative to a previous one when the interval was within 2 weeks, while a significantly lower pre-ANC was observed when the interval was more than 8 weeks.

TABLE 1. Granulocyte donor characteristics (per collection)

Mobilization methods	G-CSF alone (n = 68)	G-CSF + Dex (n = 40)	PBSC-GTX (n = 15)
Age (years), median (range)	34 (18-53)	39 (18-61)	27 (13-50)
Sex (male/female)	41/27	34/6	6/9
Body weight (kg)	63 ± 14	64 ± 12	58 ± 9.6
Baseline WBC count (×10⁹/L)	6.1 ± 1.6	6.0 ± 2.4	5.3 ± 1.2
Baseline ANC (×10⁹/L)	3.7 ± 1.3	3.7 ± 1.8	3.2 ± 0.7
Baseline PLT count (×10⁹/L)	222 ± 43	223 ± 45	221 ± 42
Number of previous granulocyte donations			
0	46	28	15
1	18	9	0
2	4	2	0
3	0	1	0

Effect of the use of HES on granulocyte collection efficiency

HES was used in 105 granulocyte collections and not in 18 collections. The effect of the use of HES is shown in Fig. 3. Leukapheresis with the use of HES resulted in significantly better granulocyte collection efficiency than that without the use of HES ($42 \pm 7.8\%$ vs. $10 \pm 9.1\%$, $p < 0.0001$; Fig. 3A). Moreover, the use of HES was associated with significantly decreased contamination from RBCs and PLTs in the granulocyte concentrates (Figs. 3B and 3C).

Effect of mobilization regimens on granulocyte yield

Based on the above observations, the effect of mobilization regimens on granulocyte yield was evaluated only in collections using HES. A significantly higher granulocyte yield was achieved in donors who received G-CSF + Dex than in those who received G-CSF alone ($7.2 \times 10^{10} \pm 2.0 \times 10^{10}$ vs. $5.7 \times 10^{10} \pm 1.7 \times 10^{10}$, $p = 0.006$; Fig. 4), reflecting the difference in the pre-ANC. However, a subgroup analysis of the male donors showed only a marginal effect from the use of Dex on the granulocyte yield ($7.5 \times 10^{10} \pm 2.0 \times 10^{10}$ vs. $6.3 \times 10^{10} \pm 1.7 \times 10^{10}$, $p = 0.059$). PBSC-GTX donors produced a remarkably high granulocyte yield ($9.7 \times 10^{10} \pm 2.3 \times 10^{10}$). The interval from the previous collection affected the granulocyte yield in a manner similar to that for the pre-ANC: A significantly higher granulocyte yield was obtained for a collection relative to a previous one when the interval was 7 to 13 days (Fig. 5).

Adverse events in donors

Adverse events in the donors are shown in Table 2. Mobilization-related adverse events were observed in

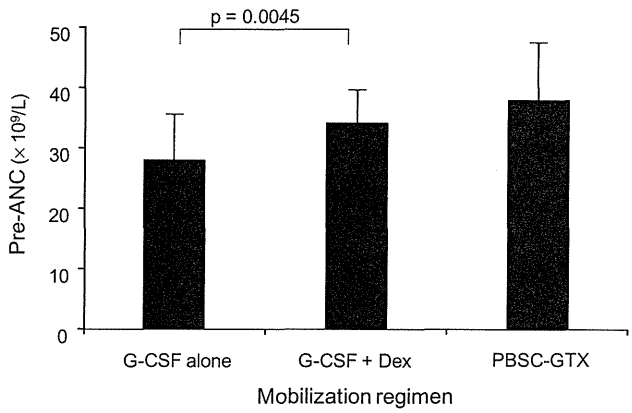

Fig. 1. Pre-ANC in donors who underwent granulocyte mobilization for the first time with G-CSF alone (n = 41) or with G-CSF + Dex (n = 25) and in those who underwent granulocyte collection after PBSC harvest (n = 15). G-CSF + Dex resulted in significantly higher pre-ANC than G-CSF alone.

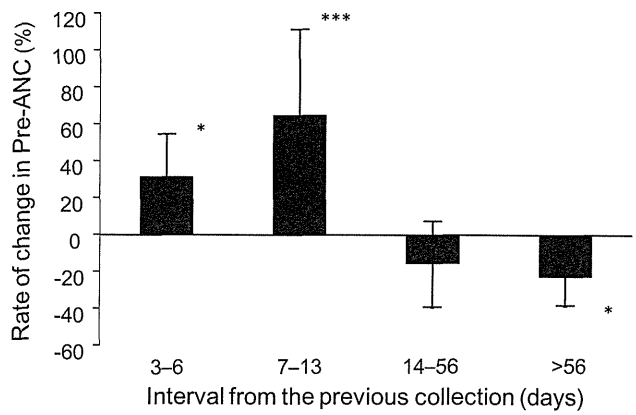

Fig. 2. Rate of change in pre-ANC between the current and previous granulocyte collections according to the length of the time interval between them. *p < 0.05, **p < 0.01, and ***p < 0.001, respectively.

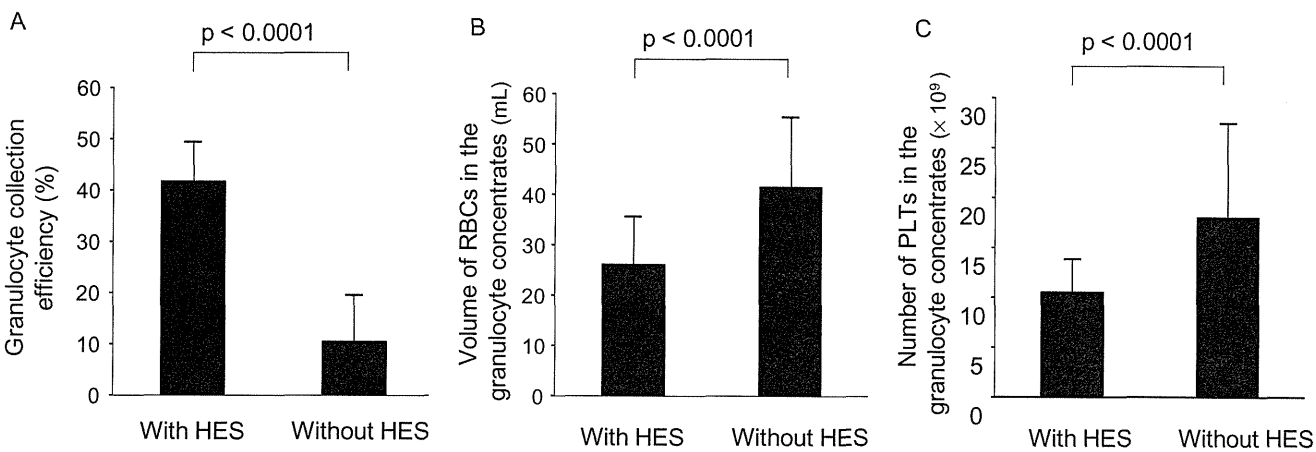

Fig. 3. Effect of the use of HES in leukapheresis. Granulocyte collection efficiency (A) and contamination from RBCs (B) and PLTs (C) in the granulocyte concentrates obtained by leukapheresis using HES or not using HES. The use of HES was associated with significantly better granulocyte collection efficiency and significantly less contamination from RBCs and PLTs.

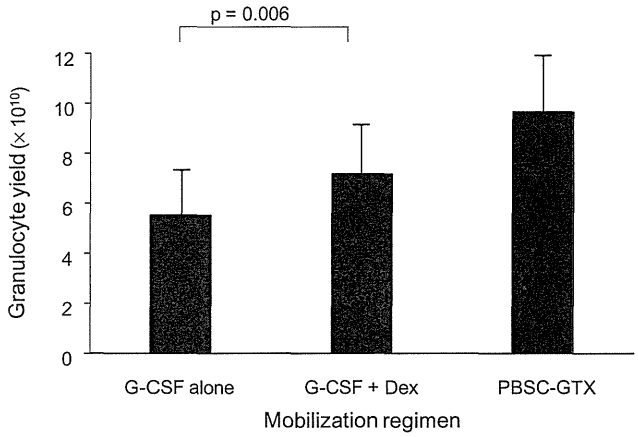

Fig. 4. Granulocyte yield by apheresis using HES from donors who underwent granulocyte mobilization for the first time with G-CSF alone or with G-CSF + Dex and in those who underwent granulocyte collection after PBSC harvest. G-CSF + Dex produced a significantly higher granulocyte yield than G-CSF alone.

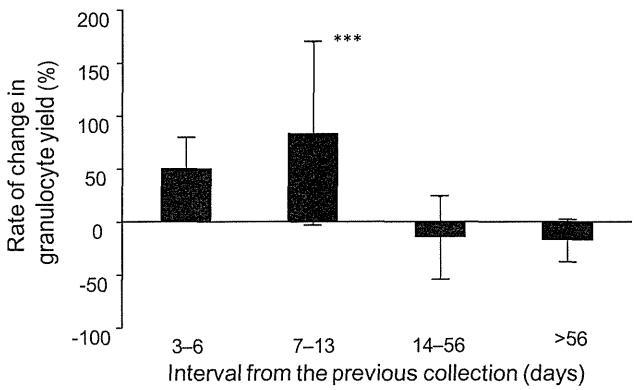

Fig. 5. Rate of change in granulocyte yield between current and previous granulocyte collections according to the length of the time interval between them. ***p < 0.001.

eight collections; no differences in the incidence rate were observed among mobilization regimens. The reason for the low incidence of mobilization-related adverse events compared to previous studies is unknown, but mild events might be underreported in the questionnaire. Meanwhile, apheresis-related events were observed in 20 collections, including mild thrombocytopenia (PLT count 50×10^9-100×10^9/L) in eight collections. Thrombocytopenia was attributable to the repeated apheresis in all but one collection. Adverse events that could potentially be related to the use of HES (e.g., allergic reactions and hemorrhagic tendencies) were not observed. Follow-up investigations with questionnaires have not revealed any serious long-term complications related to the granulocyte collections. The median follow-up time was 19 months (range, 4-59 months).

TABLE 2. Adverse events in granulocyte donors

Adverse effect	Number of events (percentage of collections)
Mobilization related	
Bone pain	4 (3.3)
Insomnia	2 (1.6)
Syncope*	1 (0.8)
Elevation of liver enzyme (NCI-CTC grade ≥2)	1 (0.8)
Apheresis related	
Thrombocytopenia (PLT count <100 × 10⁹/L)	8 (6.3)
Venous access problems†	5 (4.1)
Numbness	4 (3.3)
Nausea and/or headache	3 (2.4)

* Due to the vasovagal reflux after subcutaneous injection of G-CSF.
† Problems included multiple intravenous line placement attempts, poor blood flow, line coagulations, and hematomas.

DISCUSSION

This study had several significant findings. We showed that mobilization with G-CSF + Dex produces higher granulocyte yields than G-CSF alone, which is consistent with the findings of the previous studies.[6,9,10] However, the effect of the use of Dex was not significant when only male donors were analyzed. With some exceptions, most of the previous studies have shown that higher granulocyte doses lead to a greater increment of ANC in transfused patients.[6,19] In this respect, the combination of G-CSF with Dex may result in a clinical benefit in patients undergoing granulocyte transfusion. However, based on the previous reports suggesting an increased risk of posterior subcapsular cataracts in donors who had repeatedly received the Dex-containing mobilization regimen,[11,12] G-CSF alone may be preferable, particularly in donors undergoing repeated collections.

This study has particular significance in suggesting the utility of using PBSC donors for granulocyte collection. We showed that PBSC-GTX donors who had received G-CSF for 4 or 5 days can provide a remarkably high granulocyte yield. Previous studies have shown that G-CSF administration with a schedule similar to that for PBSC mobilization markedly decreased the apoptotic rate of the neutrophil, while it mildly impaired killing activity.[20,21] Patients undergoing SCT usually develop neutropenia within a few days after transplantation, when they develop a particularly high risk of developing de novo or recurrent infections. Indeed, the median time from SCT to the first GTX was reported to be 2 days in a randomized Phase III study of granulocyte transfusions.[22] Thus, the timing of the availability of granulocyte from PBSC donors (i.e., Day 2) matches the timing of the need for granulocyte transfusion. In addition, the use of PBSC donors reduces the difficulties in finding granulocyte

donors. This is particularly helpful in Japan, where the eligibility for granulocyte donors is limited to family members in most institutions, in accordance with Japanese guidelines.[15] Hence, we believe that this strategy of collecting granulocytes from PBSC donors on the day after the completion of the PBSC collections is a rational one.

We also showed that the interval between repeated granulocyte collections is associated with changes in the granulocyte yields. In donors who underwent granulocyte collections within 2 weeks of the previous collections, the pre-ANC and granulocyte yields were higher than in the previous collections. In contrast, repeated granulocyte collection after a greater than 2-week interval resulted in decreases in the pre-ANC and granulocyte yields. This finding may coincide with the previously reported finding that PBSC donors show a long-lasting decrease in ANC after PBSC collections.[23]

Finally, we clearly showed the advantage of using high-molecular-weight HES during an apheresis procedure. The use of HES resulted in a much more efficient harvesting: higher granulocyte yields and reduced contamination from RBCs and PLTs were achieved. None of the donors in this study developed an anaphylactic reaction. Several previous studies have shown that high-molecular-weight HES is superior to low-molecular-weight HES in achieving a significantly higher granulocyte yield.[16,24] When these results are taken together, the use of high-molecular-weight HES is highly recommended in combination with cautious observation during the procedure.

This study has several limitations. The number of granulocyte collections for each variation of the different methods was small. Moreover, this study is not a prospective controlled one: the choice of mobilization regimen and the collection techniques were dependent on the period when the granulocyte collections were performed. Nevertheless, only a small difference in the period is unlikely to affect the results.

In conclusion, our study showed the effect of the mobilization regimen and the harvesting technique on the granulocyte yield from healthy donors. Granulocyte collections from PBSC donors are an easy and useful option without providing new risk. The protocol for granulocyte collections should be standardized on the basis of the findings of this study and the previous ones. We recommend G-CSF alone for mobilization to avoid possible adverse events with the use of dexamethasone and strongly recommend the use of high-molecular-weight HES during an apheresis procedure.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest relevant to the manuscript submitted to **TRANSFUSION.**

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Effect of Early Posttransplantation Tacrolimus Concentration on the Development of Acute Graft-versus-Host Disease after Allogeneic Hematopoietic Stem Cell Transplantation from Unrelated Donors

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Only limited data are available regarding the relationship between blood concentration of tacrolimus and its efficacy in preventing acute graft-versus-host disease (aGVHD). We retrospectively evaluated the effects of the whole blood concentration of tacrolimus, which was measured by an automated microparticle enzyme immunoassay, early after allogeneic hematopoietic stem cell transplantation (HSCT) upon the development of aGVHD. Sixty patients, who underwent allogeneic HSCT from serologically human-leukocyte antigen-matched unrelated donors and received continuous infusion of tacrolimus with short-term methotrexate for GVHD prophylaxis, were included in this study. The target range of the blood concentration of tacrolimus was set at 10 to 20 ng/mL, and the level was maintained within this range in all patients. However, the mean blood concentration of tacrolimus during the third week after HSCT was significantly associated with the grades of aGVHD (17.3 ± 2.1 in patients with grades 0-I vs 15.9 ± 2.8 in II-IV and 14.8 ± 2.1 in III-IV; $P < .05$ and $<.01$, respectively). Multivariate analysis also demonstrated that higher age (≥35) of donor (odds ratio [OR] = 4.28) and lower mean blood concentrations of tacrolimus during the second (OR = 0.75; 95% confidence interval [CI]: 0.58-0.98) and third weeks (OR = 0.76; 95% CI: 0.58-0.98) after HSCT were significant risk factors for grades II-IV aGVHD ($P < .05$). We conclude that the early posttransplantation blood concentration of tacrolimus had a significant impact on the development of moderate-to-severe aGVHD after allogeneic HSCT from an unrelated donor.

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KEY WORDS: Tacrolimus, Graft-versus-host disease, Blood concentration, Allogeneic hematopoietic stem cell transplantation, Unrelated donor

INTRODUCTION

Graft-versus-host disease (GVHD) remains 1 of the major life-threatening complications of allogeneic hematopoietic stem cell transplantation (HSCT), despite the introduction of calcineurin inhibitors such as cyclosporine A (CsA) and tacrolimus. Tacrolimus possesses 100 times greater in vitro inhibitory activity against T cells than CsA, and has been widely used for the prophylaxis of GVHD alone or in combination with methotrexate (MTX) in patients undergoing allogeneic HSCT who are at high risk for developing GVHD [1,2]. There have been 3 randomized trials comparing the efficacy of CsA and tacrolimus in the prophylaxis of GVHD after allogeneic HSCT, all of which indicated that tacrolimus with short-term MTX could prevent the development of acute GVHD (aGVHD) more effectively than CsA with short-term MTX [3-5]. However, the target ranges of the blood concentration of tacrolimus early after transplantation varied significantly among these 3 studies [3-5]. In addition, the descriptions of the actual duration of intravenous administration of tacrolimus, as well as

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