



# Granulocyte concentrates: prolonged functional capacity during storage in the presence of phenotypic changes

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## ABSTRACT

### Background

Granulocyte transfusion has been proposed as a bridging therapy for patients with prolonged periods of chemotherapy-induced neutropenia, who suffer from severe fungal and bacterial infections. To recover, adequate numbers of granulocytes are required when the patients are refractory to standard treatment. The aim of this study was to assess the functional characteristics and efficacy of granulocyte colony-stimulating factor/dexamethasone-mobilized granulocytes used for transfusions.

### Design and Methods

Granulocytes from the leukapheresis products were tested for the expression of cell-surface antigens, interactions with endothelial cells, motility, killing of microbes and survival. The granulocytes were used *in vivo* for transfusion in 16 severely ill children, who were – apart from a patient with a granulocyte dysfunction – all suffering from prolonged neutropenia.

### Results

Mobilization of granulocytes with granulocyte colony-stimulating factor and dexamethasone caused phenotypic changes (decreased CD62L expression and increased levels of CD66b and CD177). The ability of the granulocytes to interact with endothelial cells (rolling, adhesion, transmigration) and to kill various types of pathogens was not affected by the mobilization, leukapheresis and irradiation procedures. The granulocytes were functionally indistinguishable from those isolated from untreated donors, even after 24 hours of storage. Granulocyte transfusion seemed to benefit 70% of patients, as 11 out of the 16 children showed clinical recovery within 1-2 weeks after beginning the transfusions.

### Conclusions

Although CD62L expression is downregulated on granulocytes used for granulocyte transfusions, concomitant CD177 upregulation may explain the intact interactions with endothelial cells. All other granulocyte functions tested were intact, including the ability to kill fungi. Granulocyte concentrates can be stored without loss of *in vitro* viability and functionality for at least 24 hours. As demonstrated *in vivo*, granulocyte transfusions may be an effective therapy for neutropenic pediatric patients suffering from life-threatening infections.

Key words: granulocytes, granulocyte colony-stimulating factor, phenotype, function, transfusion.

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## Introduction

Patients with prolonged neutropenia after chemotherapy are prone to serious bacterial and fungal infections.<sup>1</sup> Despite novel and appropriate antimicrobial therapies and the use of growth factors such as granulocyte colony-stimulating factor (G-CSF), patients may remain refractory to these therapies. To improve the outcome of these patients, granulocyte transfusions have been suggested as a promising therapy in addition to antimicrobial therapy. Recent studies on the use of recombinant G-CSF to mobilize bone-marrow granulocytes to the blood pool in granulocyte donors have provided encouraging results.<sup>2-10</sup> So far the most efficient way to increase the level of neutrophils is to administer a single dose of G-CSF plus corticosteroids<sup>11</sup> to increase the granulocyte yield 5- to 10-fold higher than a decade ago; this ensures a good therapeutic dose of granulocytes for transfusion. To provide effective therapy, not only must the numbers of granulocytes be sufficient, but the cells must also retain full functional capacity. Mobilized granulocytes given to a patient must be capable of reaching the sites of ongoing infection and effectively fighting the invading pathogens. Administration of G-CSF/dexamethasone to the donor has been described to change the phenotype of mobilized granulocytes, although their basic functions such as oxidative burst and phagocytosis, remain unaffected.<sup>12-14</sup> However, very little is known about the interaction between granulocytes obtained from granulocyte concentrates, used for transfusion, and endothelial cells or about their capacity to kill bacterial and fungal pathogens.

In this study, we first performed an extensive phenotypic and functional characterization of the granulocytes from the leukapheresis product, obtained after *in vivo* G-CSF/dexamethasone mobilization, focusing on aspects relevant for fighting infections, such as rolling over and adhesion to endothelial cells under flow conditions, transmigration, chemotaxis, respiratory burst and microbial killing. We also evaluated the effect of storing the granulocyte concentrates on the functional capacity of the cells. In addition, we followed the kinetics of the granulocytes transfused, as well as the efficacy of the transfused product *in vivo*, in a group of pediatric patients given pre-emptive or curative treatment with granulocyte transfusions.

## Design and Methods

### Patients

#### Selection of the patients and treatment indications

Seventeen hemato-oncology and stem-cell transfusion (SCT) patients and one patient with a primary immunodeficiency disorder were selected either for curative or pre-emptive treatment with granulocyte transfusions. The patients were followed at the Academic Medical Center (AMC) in Amsterdam, Erasmus Medical Center (Erasmus MC) in Rotterdam, Leiden University Medical Center (LUMC) in Leiden,

and the University Medical Center Utrecht (UMCU). Patients with proven fungal or bacterial infection, unresponsive to appropriate antimicrobial therapy or with progressing infection at 72 hours after the initiation of such therapy and with no neutrophil regeneration with G-CSF, were selected for curative treatment with granulocyte transfusions in addition to anti-microbial therapy. Those with poorly controlled fungal or bacterial infections prior to allogeneic SCT (for any indication and for whom a delay of the transplant was unacceptable) were selected for pre-emptive granulocyte transfusions in addition to anti-microbial treatment. The study was approved by the Ethics Committees of the AMC Hospital, UMCU Hospital, LUMC Hospital and Erasmus University Hospital and was conducted in accordance with the Declaration of Helsinki.

#### Granulocyte transfusion and follow-up

Granulocytes were transfused three times a week. The absolute neutrophil count (ANC) was measured 1 hour, 1 day and 2 days after each infusion. Patients with neutropenia received G-CSF (10 µg/kg/day), as did SCT recipients starting at day +7 after infusion of the stem cell graft.

Granulocytes were infused over 30 to 60 minutes, within 6 hours of collection. In the case of ABO incompatibility, the granulocytes were infused in 3 hours. To prevent allergic transfusion-related reactions in the patients, clemastine (0.05 mg/kg) was administered prior to the transfusion.

Granulocyte transfusions were stopped when the infection was under control or when the ANC was above 500/µL in the case of SCT patients receiving pre-emptive treatment. In addition to the ANC, C-reactive protein (CRP) and galactomannan (in cases of *Aspergillus* infection) were used as parameters to measure infection control. Patients were closely monitored for all transplantation-related morbidities, such as graft-versus-host disease (GvHD).

### Donor procedures

#### Donor selection, mobilization and apheresis of granulocytes

Granulocyte donors were selected from volunteers among family members of the patients, based on the general criteria for the selection of blood donors and absence of contraindications to the administration of G-CSF and/or dexamethasone. After informed consent, the potential donor was medically assessed by a trained, independent physician, and blood samples were taken for virology tests (e.g. CMV, HBV, HCV, HIV) and typing of ABO and Rhesus D (RhD) blood groups. Preferably, ABO RhD-compatible (and in the case of CMV-negative patients, CMV-negative) donors were selected from the available donor pool. In the case of ABO incompatibility, anti-A and/or B antibody titers of ≤1:64 (in indirect antiglobulin tests) were accepted.

Approximately 12 hours prior to the leukapheresis procedure, G-CSF (5 µg/kg, subcutaneously) and dexamethasone (8 mg, orally) were administered to the donor.

Leukapheresis procedures were performed with the COBE Spectra® apheresis system (Gambro BCT,

Lakewood, CO, USA) via a peripheral venous access (dual needle), with application of the PMN program. The product was collected into the PCV bags with the anticoagulant consisting of 30 mL of 46.7% (w/v) sodium citrate in 500 mL of 6% (w/v) hydroxyethyl starch (HES) solution. The anticoagulant-starch to whole-donor-blood volume ratio was set at 1:13. For pediatric patients, the target number of granulocytes to be harvested was about  $2.5 \times 10^9$ /kg bodyweight of the patient. In patients weighing more than 40 kg, the aim was to obtain a product with as many neutrophils as possible and more than  $50 \times 10^9$  granulocytes per unit to be transfused. Immediately after harvesting, the granulocyte concentrate was irradiated (25 Gy).

### Granulocyte isolation

For the *in vitro* functional studies, granulocytes were purified from the leukapheresis product or from heparinized venous blood collected from healthy volunteers or the granulocyte donors prior to the leukapheresis. The granulocytes were isolated as described previously.<sup>15,16</sup>

### Immunophenotyping of granulocytes

Cell surface expression of various receptors on granulocytes was assayed in total leukocyte samples by direct or two-step immunofluorescence flow cytometry (FACS), with saturating concentrations of commercially available monoclonal antibodies, labeled with either fluorescein isothiocyanate (FITC), phycoerythrin (PE) or unconjugated, according to the manufacturer's procedure. CD11b-FITC, CD18-FITC, CD16-FITC, CD64-FITC, CD66b-FITC and CD31 were from Sanquin Reagents, Amsterdam, The Netherlands; CD62L-PE and HLA-DR-PE were from BD PharMingen, San Diego, CA, USA; CD177 was from AbD Serotec, Oxford, UK; ICAM-2 (CD102) was from Santa Cruz Biotechnology, Santa Cruz, CA, USA; PSGL-1 (CD162) and CD63-FITC were from Immunotech, Marseille, France; CD99 was from Abcam, Cambridge, UK; goat F(ab')<sub>2</sub> anti-mouse-APC was from Southern Biotech (Birmingham, AL, USA). Cells were gated based on their forward and side scatter properties, and 10 000 gated events were collected per sample: 100% positive staining for CD16 and negative for CD36 (monocytes) or CD56 (NK cells) confirmed the purity of the analyzed population.

### Standard granulocyte chemotaxis and NADPH oxidase activity

Chemotaxis was assessed by means of Fluoroblock inserts (Falcon; Becton Dickinson, USA), as has been described in detail elsewhere.<sup>17</sup> NADPH-oxidase activity was assessed as the release of hydrogen peroxide, determined by an Amplex Red kit (Molecular Probes, Eugene, OR, USA) as described previously.<sup>18</sup>

### Rolling over and adhesion to endothelial cells under flow conditions

Human umbilical vein endothelial cells (HUVEC) were isolated from human umbilical cord veins. Cells were cultured in RPMI-1640 medium containing 20%

(v/v) human serum, 100 U/mL penicillin and 100 µg/mL streptomycin (Life Technologies, Paisley, UK) and grown to confluence in 5-7 days. Primary endothelial cells of the first or second passage were used in the experiments.<sup>19,20</sup>

HUVEC were first coated on glass cover slips, grown until confluence (i.e. for approximately 2 days), and then stimulated with tumor necrosis factor- $\alpha$  (100 U/mL; Boehringer Mannheim, Germany) for 6 hours before starting perfusion. Granulocytes ( $2 \times 10^6$ /mL) were perfused over the HUVEC for 10 minutes at a flow rate of 100 µL/min. During perfusion the flow chamber was mounted on a microscope stage (Axiovert 25; Zeiss, Oberkochen, Germany) equipped with a black-and-white charge-coupled device video camera (Sanyo, Osaka, Japan) coupled to a VHS video recorder. The number of adherent cells was evaluated using image analysis software OPTIMAS 6. Cells that were in contact with the surface appeared as bright white-centered cells after adjustment of the microscope during the recording.<sup>19,21</sup>

### Transendothelial migration assay

Transmigration assays were performed with 6.5-mm Transwells with 8-µm pore size (Costar, Cambridge, MA, USA). Inserts were coated with 20 µg/mL fibronectin (Sigma). HUVEC were seeded on the Transwells 2 days prior to the assay and cultured in a humidified atmosphere (37°C and 5% CO<sub>2</sub>). The integrity of the HUVEC monolayer was assessed microscopically. Before the experiment, cells were washed twice with HEPES medium.

At the start of the assay, 10<sup>6</sup> neutrophils were placed in the upper compartment of the Transwell and allowed to migrate for 1 hour at 37°C to a medium containing chemokines (C5a or interleukin-8, both at 10 nM) added to the lower compartment. Migrated cells were collected from the lower compartment and quantified by flow cytometry in the presence of fixed amounts of fluorescent beads. The percentage of migrated cells was calculated as a fraction of the total cell input, as follows:

$$\% \text{ of migrated cells} = \left[ \frac{\text{number of transmigrated cells}}{\text{number of beads}} \right] / \left[ \frac{\text{number of input cells}}{\text{number of beads}} \right] \times 100\%.$$

### Statistics

Results are presented as means  $\pm$  SD unless otherwise specified. Groups were compared using two-tailed t test analysis. Differences were considered statistically significant if  $p < 0.05$ .

## Results

### Characteristics of the granulocytes used for infusion: phenotype and function

G-CSF/dexamethasone exposure changes the granulocyte phenotype

Granulocytes obtained from granulocyte donors (treated with G-CSF and dexamethasone) had lower

surface expression of the early activation markers CD62L and CD16 (FcγRIIIb) compared to control cells obtained from untreated donors, whereas the level of CD64 (FcγRI) for prolonged activation was increased (Figure 1A). More impressive upregulation was seen for the neutrophil-specific NB1 antigen, CD177.<sup>22,23</sup>

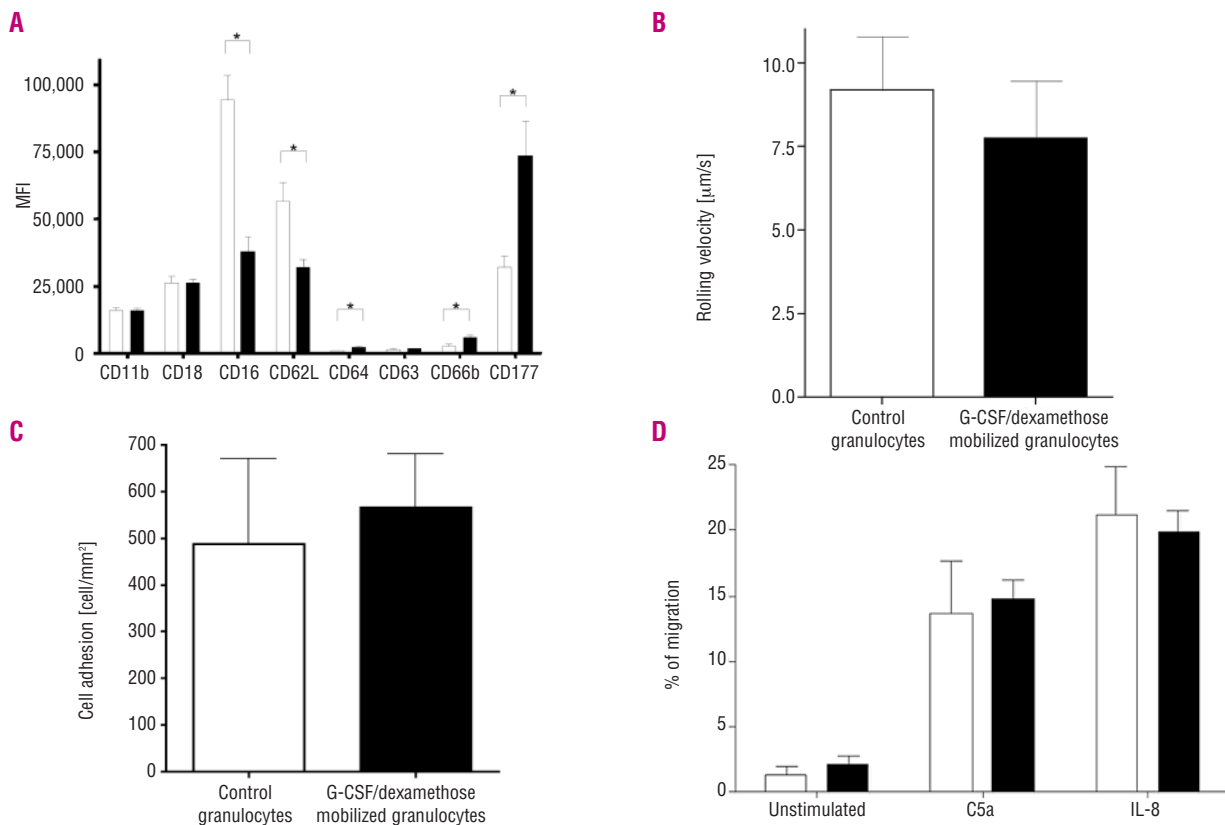
CD66b expression on neutrophils from the granulocyte donors was upregulated compared to that on control cells. On the other hand, surface expression of CD11b and CD18, which are partially expressed in the same compartments as CD66b, was similar. Surface expression of additional adhesion molecules involved in the process of transmigration (CD31, CD99, ICAM-2, and PSGL-1) was unchanged (*data not shown*). The tetraspanin molecule CD63, a marker of degranulation of azurophilic granules,<sup>15</sup> was unchanged by prior *in vivo* exposure of the donor to G-CSF/dexamethasone.

Upon *in vitro* activation the upregulation of CD11b/CD18, CD66b and CD63 was normal (*data not shown*), which demonstrates the granulocytes' normal functional capacity to degranulate. No change in expression was noted for the HLA class-II molecule.

### Granulocyte functions and interaction with endothelial cells

Standard granulocyte functions, such as chemotaxis and respiratory burst, were then investigated. No significant differences were noted in the directed motility in response to C5a, IL-8 or PAF, as compared to that of the control cells (*Online Supplementary Figure S1A*). The only difference was observed in the case of random mobility of unstimulated cells (chemokinesis), which was significantly enhanced in granulocytes from the leukapheresis product (*Online Supplementary Figure S1A*). The production of reactive oxygen species upon neutrophil activation by various stimuli was normal (*Online Supplementary Figure S1B*).

The interaction of neutrophils with endothelial cells was investigated under various flow conditions. Rolling of neutrophils over HUVEC monolayers and subsequent firm adhesion to these endothelial cells were unaffected (Figures 1B and C), despite the 50% reduction in surface expression of CD62L (which is a major molecule involved in cell-cell interactions between granulocytes and endothelial cells) after G-CSF/dexamethasone mobilization. The transmigration of granulo-



**Figure 1.** Effect of G-CSF/dexamethasone mobilization on the phenotype of granulocytes, and their interaction with endothelial cells. (A) Expression of various surface antigens on granulocytes. CD11b, CD18, CD16, CD62L, CD64, CD63, CD66b and CD177 binding (all antibodies were used at a concentration of 20 μg/mL) was compared on granulocytes isolated from healthy controls (open bars) and on cells obtained from the leukapheresis products (black bars). Results are presented as mean fluorescent intensity (MFI) (mean ± SEM); statistically significant differences ( $p < 0.05$ ) are indicated by an asterisk (\*). The data represent results of experiments performed on granulocytes obtained from five different granulocyte donors and ten healthy untreated controls. (B) Rolling capacity of neutrophils over stimulated HUVEC under flow conditions. (C) Granulocyte adhesion to HUVEC stimulated by tumor necrosis factor- $\alpha$ . (D) Transmigration of granulocytes isolated from healthy controls (open bars) and leukapheresis products (black bars) through unstimulated HUVEC in response to C5a or IL-8 (both at 10 nM). All data of B, C and D represent the mean ± SEM of the results of three independent experiments.

cytes through the endothelial cell layer induced by IL-8 or C5a was also comparable to that of control cells (Figure 1D).

### **Killing of micro-organisms**

Because G-CSF and dexamethasone have been described to have pronounced and contradictory effects on the killing capacity of micro-organisms by neutrophils *in vitro*, especially on their fungicidal activity,<sup>24</sup> we determined the capacity of granulocytes mobilized for transfusion to kill a variety of different micro-organisms, including *E. coli*, *S. aureus*, *C. albicans* and *A. fumigatus*, which are all clinically relevant pathogens. All micro-organisms were efficiently killed, and statistically significant differences were not observed between control granulocytes and the mobilized granulocytes (n=3-7 experiments in triplicate) (*Online Supplementary Figures S2A and S2B and data not shown*).

### **Storage of granulocytes and concomitant changes in phenotype and function: survival and decay in functional capacity**

Granulocyte concentrates collected by leukapheresis are usually transfused as quickly as possible, preferably within 6 hours. Longer storage periods are avoided because of assumed poor granulocyte survival and function.<sup>25,26</sup> G-CSF/dexamethasone-mobilized granulocytes did, however, have a greatly prolonged lifespan during *in vitro* cultures, as determined by annexinV-FITC/PI-staining and cell morphology, when compared with control granulocytes (*data not shown*).

After 24 hours of storage, the total white blood cell counts of mobilized granulocyte concentrates had decreased by approximately 5%. The viability of the neutrophils was excellent, as determined by annexinV binding (<5% apoptotic neutrophils, i.e. annexinV-FITC<sup>+</sup>; *data not shown*) and cell morphology (*Online Supplementary Figures S3A and S3B*).

Neutrophil functions (such as chemotaxis, adhesion, respiratory burst and bactericidal and antifungal activity) all remained unchanged after storage of the granulocyte transfusion product for 24 hours (Figure 2 and *data not shown*). Small changes were observed in surface antigen expression after storage of the leukapheresis product: CD62L expression decreased further during storage. In addition, there was a slight increase in CD11b and CD18 expression (which may be upregulated from secretory vesicles or specific granules), whereas the expression of CD63, a marker of azurophil granule release, remained unchanged (*data not shown*).

### **Patients and granulocyte transfusions**

Between December 2002 and October 2006, 16 patients fulfilled criteria for treatment with granulocyte transfusions (curative group), 15 of whom had persistent neutropenia due to treatment for pediatric cancer and one of whom had a granulocyte dysfunction.<sup>18,27</sup> Four children received pre-emptive granulocyte transfusions during allogeneic or autologous SCT (Table 1), of whom two had also received transfusions earlier in a curative setting (#1 and #8).

In the curative group, the first infusion of granulocytes

was administered after a prolonged period of neutropenia (median, 21 days; range, 12-140), whereas in the pre-emptive group granulocyte infusions were started on the first day of expected neutropenia. The median number of granulocyte infusions in the curative group was three (range, 1-10) and in the pre-emptive group six (range 4-9). Each infusion contained a median of  $2 \times 10^9$  granulocytes/kg (range,  $0.3-5.0 \times 10^9$ /kg, Table 2; additional information about other cells present in the granulocyte concentrates are included in the *Online Supplementary Table S1*). The increment in ANC in the peripheral blood of the patients, measured 1 hour after the granulocyte transfusion, was more than  $1 \times 10^9$ /L, and stayed above that level until 24 hours after the infusion. Thereafter, the number decreased again in most cases (Figure 3A).

### **Clinical outcome**

In the group treated curatively, the granulocyte transfusions helped to control the infection in 11 of the 16 patients in whom antimicrobial therapy for >72 hours had not resulted in any clinical improvement. The increase in the number of circulating granulocytes was followed by a decrease in CRP levels (Figure 3B). Eight of the 11 patients with a proven *Aspergillus* species infection showed clinical recovery, with galactomannan levels becoming negative within 10 days after starting the transfusions (Table 2). In three patients with Gram-negative infections (#6, #10 and #11) the infection resolved.

Five patients died; two patients died 18 and 19 days after initiation of transfusions from cerebral hemorrhage and multiorgan failure, respectively (#5 and #7), and three from rapidly progressive fungal infection less than 1 week after starting treatment with granulocyte transfusions (#12, #14, #15); in two of these no increment in neutrophils above  $1 \times 10^9$ /L at 1 hour after granulocyte infusion was observed (Tables 1 and 2).

All children in the group treated pre-emptively survived their transplant procedure and showed no evidence of disseminated infection. Three are alive and well more than 1 year after SCT. One died 4.5 months after allogeneic SCT because of EBV-lymphoproliferative disease.

## **Discussion**

We studied the effects of G-CSF and dexamethasone prestimulation of the donor, as well as the leukapheresis and irradiation procedures on the viability, function, and morphology of granulocytes used for transfusion of neutropenic patients. We also assessed the clinical yield and efficacy of the transfused cells in pediatric oncology patients.

The morphologic appearance of granulocytes from granulocyte transfusion products and their basic functions, such as respiratory burst, interaction with endothelial cells, migration, and killing capacity, were all normal. Although various immunological defects have been reported to be caused by glucocorticoids *in vivo* and *in vitro*,<sup>28</sup> including decreased fungicidal activity of granulocytes,<sup>24</sup> we did not observe these effects, perhaps because of the concomitant administration of G-CSF.

**Table 1. Patients' characteristics.**

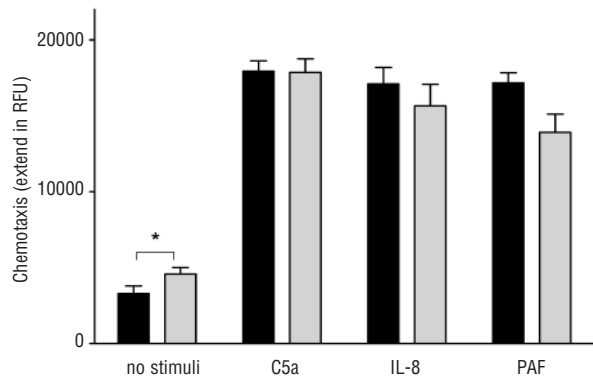
Pt.n.	Diagnosis	Age (years)	(Chronic) infection	Refractory to	Therapy	CMV	Weight (Kg)
<b>Curative group</b>							
1	HLH	5	intestinal aspergillosis ( <i>Aspergillus fumigatus</i> )	voriconazole	HLH-2004 protocol induction phase	neg	25
2	ALL	8	cutaneous aspergillosis ( <i>Aspergillus flavus</i> )	voriconazole	ALL-10 protocol: induction phase	neg	29
3	HLH	21	pulmonary aspergillosis ( <i>Aspergillus fumigatus</i> )	voriconazole	post-SCT (day +28: double-unrelated cord blood [4/6 match either direction], Bu/Cy/VP16/ATG conditioning)	pos	75
4	BD	5	pulmonary aspergillosis ( <i>Aspergillus fumigatus</i> )	amphotericin B → voriconazole	pre-SCT (day -20 [10/10 match], Flud/Mabcampath/TBI conditioning)	neg	18
5	MDS	2	pulmonary aspergillosis (HRCT changes & pos galactomannan)	voriconazole	post-SCT (day +35, rejection 1st unrelated cord blood: Bu/Mel/Cy/ATG, day +14 2nd unrelated cord blood [5/6 match], without conditioning)	pos	13.5
6*	LAD	11	bilateral pneumonia ( <i>Pseudomonas aeruginosa</i> )	ceftazidime	immunodeficiency	neg	31
7	Hepatoblastoma	7	bloodstream and ascites ( <i>Staphylococcus aureus</i> and <i>Candida albicans</i> )	gentamycin, flucloxacillin; amphotericin B & flucytosine	SIOPEL IV protocol first course	neg	25
8	Neuroblastoma stage IV	3	sinusitis ( <i>Aspergillus fumigatus</i> )	voriconazole	after last course chemotherapy, 8 additional COJEC courses of chemotherapy before SCT	neg	17
9	X-ALD, metabolic disease	6	pulmonary aspergillosis ( <i>Aspergillus fumigatus</i> )	liposomal amphotericin B (Ambisome®)	post-SCT (day +104, rejection BMT [10/10 match], Bu/Cy/ATG; day +39 rejection 2nd PBSCT same donor, OKT3 conditioning; day +8 autologous BM rescue)	neg	26
10	AML, relapse	13	perianal cyclospori and necrosis ( <i>Escherichia coli</i> )	ceftazadime, meronem, tobramycin & surgical drainage	post-reinduction FLAG-daunosome	pos	42.5
11	AML	1	portacath infection/necrosis & sepsis ( <i>Pseudomonas aeruginosa</i> )	ceftazadime, tobramycin; vancomycin, meronem; surgical debridement	post-2 <sup>nd</sup> FLAG-IDA induction therapy	neg	9.0
12	SAA	5	pulmonary aspergillosis ( <i>Aspergillus fumigatus</i> )	liposomal amphotericin B (Ambisome®) → voriconazole	immunosuppression (ATG, prednisolone, cyclosporine A)	neg	27
13	ALL, relapse	9	pulmonary aspergillosis ( <i>Aspergillus fumigatus</i> )	meronem, voriconazole & surgery	post-reinduction chemotherapy for relapsed ALL	neg	52
14	Wilms' tumor, relapse	1	influenza A, CoNS and possible <i>Aspergillus</i> , HRCT	liposomal amphotericin B (Ambisome®) → voriconazole vancomycine	post-intensive reinduction chemotherapy for relapsed Wilms' tumor	neg	8.6
15	HLH	7	sepsis ( <i>Candida albicans</i> )	voriconazole plus flucytosine; meronem	steroids, VP16, cyclosporine A; reactivation HLH due to EBV infection	neg	20
16	ALL, relapse	5	pulmonary aspergillosis ( <i>Aspergillus fumigatus</i> )	voriconazole & surgery; vancomycin, meronem	post-CLASP	neg	20
<b>Pre-emptive granulocyte transfusions</b>							
17	X-CGD	10	liver abscesses ( <i>Staphylococcus aureus</i> )	linezolid	BMT sibling [10/10 match], Bu/Cy/ATG	neg	22
18	ALL	13	mucormycosis	amphotericin B & surgery	BMT MUD [9/10 match: HLA-C mismatch], VP16/ATG/TBI	pos	40
19a (1)	HLH	6	intestinal aspergillosis ( <i>Aspergillus fumigatus</i> )	voriconazole	unrelated cord blood [5/6 match: HLA-DR mismatch], BU/Cy/VP16/ATG	neg	25
19b (1)				voriconazole	BMT MUD [9/10 match: HLA-DR mismatch], VP16/Mabcampath/TBI	neg	25
20 (8)	Neuroblastoma Stage IV	3	sinusitis ( <i>Aspergillus fumigatus</i> )	voriconazole	autologous SCT	neg	17.5

CMV: cytomegalovirus; HLH: hemophagocytic lympho-histocytosis; ALL: acute lymphoblastic leukemia; BD: Blackfan-Diamond; X-CGD: X-linked chronic granulomatous disease; SAA: severe aplastic anemia; HRCT: high resolution; CT-scanning; SCT: stem cell transplantation; MUD: matched unrelated donor; Bu: busulfan; Cy: cyclophosphamide; VP16: etoposide; ATG: anti-thymocyte globulin; Flud: fludarabine; TBI: total body irradiation; LAD: leukocyte adhesion deficiency; COJEC: chemotherapy (vincristine, etoposide, carboplatin); FLAG: fludarabine and high-dose cytosine arabinoside; CLASP: high dose cytosine and L-asparaginase.

**Table 2.** Product infused, granulocyte counts after granulocyte transfusion and outcome.

Pt. n.	N. of GTX	Day of starting GTX (before SCT)	G/kg infused median (range)	G/L +1h median (range)	G/L +1d median (range)	G/L +2d median (range)	GTX stopped (day after initiation)	CRP (mg/L: start → 2GTX)	Galactomannan (start → +10 days)	Current status
<b>Curative group</b>										
1	2	No SCT	1.1	2.2 (1.7-3.3)	0.4 (0.3-0.6)	(0.1-2.6*)	5	307→54	pos (5)→neg	See pre-emptive group: patient #19
2	4	No SCT	1.3 (1.2-1.5)	1.4 (0.24-1.8)	1.3 (0.2-1.8)	NT	12	285→120	pos (2)→neg	Chemotherapy for B-ALL
3	5	+28	1.0 (0.5-1.2)	1.4 (0.3-3.2)	1.5 (0.2-2.6)	0.6 (<0.1-1.1)	13	634→184	pos (2)→neg	Died of pulmonary insufficiency/enterococci sepsis (+ 70d)
4	10	-20	2.1 (0.8-2.7)	2.6 (0.7-3.9)	1.0 (0.1-2.4)	<0.1	7	61→12	pos (?)→neg	A&W, 100% donor
5	7	+35	3.3 (2.5-4.1)	1.3 (0.8-4.2)	0.8 (0.1-2.1)	0.1 (0.1-0.3)	18 (died)	287→181	pos (5)→pos (4)	Multiple problems, died of MOF (+ 21d after 2nd graft)
6 <sup>a</sup>	4	No SCT	1.0 (0.8-1.2)	20.7 (17-28)	15.6 (12.6-18.5)	12.6	8	280→165	NA	Ongoing infections but stable BMT successful at +320d
7	3	No SCT	2.1 (1.7-2.6)	2.5 (0.9-2.1)	1.3 (0.9-2.8)	0.6	5	342→273	NA	Died of cerebral bleeding (+18d)
8	3	No SCT	2.7 (1.8-2.8)	6.8 (5.8-7.8)	4.3 (2.3-6.1)	1.0 (0.4-1.7)	8	248→95	pos (2.5)→?	See pre-emptive group: patient #20
9	2	+104	1.8 (1.7-1.9)	3.6 (2.8-4.4)	3.0 (2.2-3.8)	1.2 (0.4-2.0)	5	329→34	pos (?)→neg	Survived infection but died one year later due to underlying disease
10	2	No SCT	2.0 (1.8-2.2)	1.5 (1.4-1.6)	2.0 (1.9-3.1)	0.6 (0.5-0.6)	5	257→61	NA	Resolved underwent further chemotherapy and BMT, died due to acute GvHD
11	7	No SCT	0.22 (0.1-0.35)	3.8 (2.0-12.2)	2.0 (1.0-6.6)	0.9 (0-1.8)	10	286→36	NA	Stable, further chemotherapy; pre-emptive antibiotics and GTX planned for future aplastic episodes
12	2	No SCT	1.0 (0.9-1.1)	0.1	<0.1	Not evaluated	4 (died)	325→196	Not evaluated	Progressive infection, died in aplasia
13	6	No SCT	1.5 (0.3-3.0)	Not evaluated	Not evaluated	Not evaluated	13	157→86	Not evaluated	Alive complete resolution of infection continued chemotherapy
14	1	No SCT	0.8	0.01	<0.1	Not evaluated	2 (died)	120→80	Not evaluated	Died of progressive infection
15	2	No SCT	1.7 (1.3-2.0)	2.1 (1.1-3.1)	1.4 (0.9-2.7)	Not evaluated	5 (died)	410→327	NA	Died due to respiratory insufficiency and progressive underlying disease
16	3	No SCT	2.6 (0.7-3.7)	0.4 (0.1-0.7)	2.6 (0.4-9.4)	Not evaluated	8	264→47	Pos (7.5)→neg	<i>Aspergillus</i> resolution post-surgery. BMT; died of cardiac failure
<b>Pre-emptive group</b>										
17	5	-3	2.3 (1.2-3.5)	1.2 (1.1-3.1)	0.5 (0.2-1.8)	0.1 (<0.1-0.1)	12	70→<7	NA	A&W
18	9	1	1.0 (0.7-1.7)	0.6 (0.4-0.6)	0.2 (<0.1-0.3)	<0.1	26	<7 <sup>a</sup>	NA	A&W, 100% donor, cured from GvHD III (skin, liver)
19a (1)	9	-1	2.7 (2.3-3.4)	9.3 (1.6-16.9)	5 (1.7-9.9)	2 (<0.1-4.7)	34	<7 <sup>a</sup>	NA	Engrafted; HLH flare associated with adenovirus infection
19b (1)	6	-7	2.0 (1.3-2.3)	6.3 (2.9-9.9)	5.2 (0.23-6.4)	0.3 (0.1-2.5)	26	<7 <sup>a</sup>	NA	100% donor, GvHD gr.IV (liver). Died of EBV-LPD (+150d)
20 (8)	4	+5	3.0 (1.9-5.0)	3.2 (2.7-3.7)	0.2 (<0.1-0.3)	0.2 (<0.1-0.3)	14	11→43	NA	A&W

GTX: granulocyte transfusion; SCT: stem cell transplantation; G: granulocyte ( $1 \times 10^9$ ); \*includes some autologous reconstitution; NT: not tested; NA: not applicable; <sup>a</sup>CRP at start, #LAD with pre-existing leukocytosis, mainly of dysfunctional neutrophils. A&W = alive and well. EBV-LPD = EBV-associated lymphoproliferative disease.



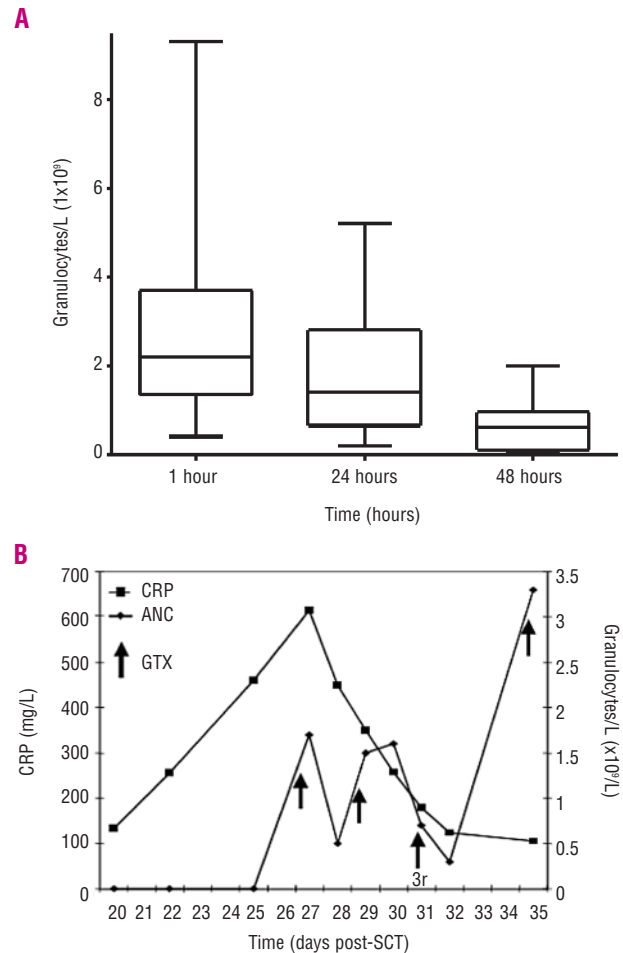
**Figure 2.** Effect of storage on granulocyte chemotaxis. Chemotaxis of granulocytes purified from healthy controls and from freshly prepared and stored leukapheresis products. Chemotaxis in response to C5a (10 nM), IL-8 (10 nM) and PAF (100 nM) was measured in granulocytes isolated from the granulocyte concentrates at time 0 (black bars) and after storage for 24 hours (gray bars). Results are presented as the extent of chemotaxis (in RFU). The results are from eight separate experiments (mean  $\pm$  SEM). Statistically significant differences ( $p < 0.05$ ) are indicated by an asterisk (\*).

Thus, granulocytes given to the patients were fully capable of fighting fungal infections as well as bacterial ones.

Minor changes were observed in surface antigen expression, possibly caused by the prior administration of G-CSF and dexamethasone to the donor<sup>25,29,30</sup> or by the leukapheresis procedure. CD62L expression was the surface antigen most affected by the mobilization and subsequent leukapheresis procedures. This member of the selectin family has been described to be required for the initial contact with the endothelium in post-capillary venules prior to extravasation.<sup>31,32</sup> However, we observed that rolling over and firm adhesion to HUVEC *in vitro*, under flow conditions, were unchanged despite the greatly reduced levels of CD62L expression. The expression of other adhesion molecules, such as CD31, CD11b and CD18, remained unchanged. The unaltered granulocyte-endothelial cell interactions may be due to the strongly increased expression of CD177 on the mobilized granulocytes. CD177, also known as human neutrophil antigen 2a (HNA2a or NB1), has recently been reported to function as a ligand for endothelial CD31 (PECAM-1), thus playing an important role in the adhesion and transmigration of neutrophils through endothelial cell monolayers.<sup>33</sup>

Another highly complex process, directed motility or chemotaxis, remained unaffected by the *in vivo* and *in vitro* manipulations required for the preparation of a granulocyte transfusion product. Only a minor enhancement of random migration (chemokinesis) was observed. This suggests a certain degree of pre-activation of the cells, which was probably due to the leukapheresis procedure, because the chemokinesis of granulocytes isolated directly from the blood of the same donors was comparable with that of control granulocytes (*data not shown*).

The life-span of granulocytes obtained from granulocyte transfusion donors is prolonged in *in vitro* cultures as well as *in vivo*.<sup>2,13,14,26,30,34</sup> Given the inherent association of apoptosis with functional decay during storage<sup>35</sup> and the



**Figure 3.** Granulocyte infusion and clinical response. (A) Number of granulocytes present in the blood of patients at 1 hour, 1 day and 2 days after granulocyte transfusion. Results are presented as a median with range (for more details see also Table 2). (B) Absolute neutrophil count (ANC) and c-reactive protein (CRP) levels during treatment with granulocyte transfusions in a representative patient (arrows indicate the moment of granulocyte transfusion, GTX). The third leukapheresis procedure resulted in a poor product ( $0.2 \times 10^9$  granulocytes/kg), which explains the lack of increment in the number of circulating granulocytes.

possible influence on survival of G-CSF *in vivo*, we tested the effects of 24-hour storage of the granulocyte concentrates. Total white blood cell counts within the granulocyte transfusion bags were maintained at levels of more than 95% of the starting counts, with an equally high viability. The cell morphology of mobilized granulocytes after 24 hours of storage showed no differences from that of fresh control cells on cytopins and electron microscopy pictures, and there were no signs of apoptosis or cell activation. Furthermore, we did not detect any release of elastase or IL-8 from the granulocytes into the fluid of the bags during storage (*data not shown*). The release of pro-inflammatory cytokines, including IL-8, may potentially cause adverse reactions after a granulocyte transfusion, but the levels of IL-8 in the bag of the granulocyte concentrates appeared not to increase before 36-48 hours of storage, and then rose up to 72 hours – as was also shown by others.<sup>36,37</sup>



One of the first functional abnormalities that occurs in stored granulocytes is a reduction of their motility. In our study, mobilized neutrophils from granulocyte concentrates retained the ability to undergo chemotaxis in response to a panel of chemoattractants (IL-8, C5a, PAF) after storage at ambient temperature, which is in agreement with the observations of Leavey *et al.* and those of Hubel *et al.* on the effects of storage at room temperature or 10°C, respectively, for 24h.<sup>38,39,45</sup>

Another important issue is whether stored granulocytes retain antifungal activity. Individuals with severely prolonged neutropenia or neutrophil dysfunction have an increased risk of developing fungal infections.<sup>1</sup> Granulocytes from leukapheresis products stored at ambient temperature retained the ability to kill opportunistic fungi efficiently (*data not shown*). Hubel *et al.* also described that granulocytes stored for 24h at 10°C had more than 80% efficacy in killing fungal pathogens, as compared to the baseline killing.<sup>38</sup> Thus, we may infer that granulocytes from G-CSF/dexamethasone-mobilized donors, despite some minor phenotypic changes, can still be used therapeutically for up to 24 hours after leukapheresis. This possibility of using stored granulocytes could be particularly relevant in small pediatric patients, since it would allow concentrates to be split for other patients. The *in vivo* yield and efficacy of stored granulocytes now need to be evaluated in a small pilot study.

The final part of this study was carried out to assess the yield and efficacy of granulocyte transfusions in severely neutropenic children suffering from life-threatening infections unresponsive to standard treatment. The clinical response (e.g. remarkable decrease in CRP, and galactomannan becoming negative) after the start of granulocyte transfusions suggests their *in vivo* efficacy. Once infused, granulocytes remained detectable in

the blood for more than 24 hours, apart from in three patients, two of whom died of rapidly progressive aspergillosis.

In this study, granulocyte transfusions seemed to benefit at least 70% of the patients in the group treated curatively, as infections were eradicated or controlled in 11 out of 16 patients in this group. This result is superior to that in the past in similar patients who did not receive granulocyte transfusion treatment, and is in agreement with more recently published reports.<sup>3,5,40-42</sup> Prophylactic granulocyte transfusion has already been reported to be beneficial in such patients.<sup>8,9,40,43,44</sup> None of our patients treated pre-emptively showed progression of prior low-grade (fungal) infection during the period of neutropenia after SCT conditioning. Our experience with a small number of patients precludes firm conclusions, although all the indications are that these patients do indeed benefit from granulocyte transfusions.

Taken together, our data show that G-CSF/dexamethasone-mobilized granulocytes have a completely intact ability to respond to signs of infection, migrate towards an ongoing infection and kill invading pathogens, as shown by various *in vitro* assays and a small clinical *in vivo* study.

## Authorship and Disclosures

AD performed part of the *in vitro* research, analyzed the data and wrote the paper. JJB analyzed many of the patients' clinical data. ATJT performed part of the *in vitro* research. JJB, MCAB, MvdH, LB, MDvdW and TWK treated the patients. HV, DR and TWK designed the study and TWK supervised and coordinated the study. The authors declare no potential conflict of interests to declare.

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