

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

Agata Drewniak,^{1,2} Jaap-Jan Boelens,³ Hans Vrielink,⁴ Anton T.J. Tool,¹ Marrie C.A. Bruin,³ Marry van den Heuvel-Eibrink,⁵ Lynne Ball,⁶ Marianne D. van de Wetering,² Dirk Roos,¹ and Taco W. Kuijpers^{1,2}

¹Sanquin Research, and Landsteiner Laboratory, Academic Medical Centre (AMC), University of Amsterdam, Amsterdam; ²Emma Children's Hospital, AMC, Amsterdam; ³Wilhelmina Children's Hospital, Department of Pediatric Immunology/Hematology and BMT, University Medical Center Utrecht (UMCU), Utrecht; ⁴Sanquin Blood Bank, Northwest Region, Amsterdam; ⁵Sophia Children's Hospital, Department of Pediatric Hemato-Oncology, Erasmus University Medical Center, Rotterdam; ⁶Willem Alexander Children and Adolescent Unit, Department of Pediatric Immunology, Hemato-oncology, BMT and Autoimmune Disease, Leiden University Medical Center (LUMC), Leiden, The Netherlands

Citation: Drewniak A, Boelens J-J, Vrielink H, Tool ATJ, Bruin MCA, van den Heuvel-Eibrink M, Ball L, van de Wetering MD, Roos D, and Kuijpers TW. Granulocyte concentrates: prolonged functional capacity during storage in the presence of phenotypic changes. *Haematologica* 2008 doi: 10.3324/haematol.12489

Supplementary Methods

Killing of Gram-positive and -negative micro-organisms

The bactericidal activity of granulocytes was determined as previously described (*Dedeval at al. 2006*). In brief, *Escherichia coli* (strain ML-35) and *Staphylococcus aureus* (strain 502A) were grown aerobically at 37°C overnight in LB broth. The next day, bacteria were collected by centrifugation, washed twice in phosphate-buffered saline (PBS) and resuspended in HEPES medium to an OD600 of 1 (i.e. 10⁹ bacteria/mL). After opsonisation (10% (v/v) pooled serum, 15 min, at 37°C), bacteria were added at a ratio of 5 : 1 neutrophil (4x10⁶ cells/mL). At the desired time points, 50-μL samples were diluted in 2.5 mL of water/NaOH at pH 11.0. At the end of the incubation period, the viable bacteria in each sample were determined by the pour-plate method in LB agar. The colony-forming units (CFU) were counted after overnight incubation at 37°C, and the percentage killing was calculated.

Killing of yeast and fungi

Killing of *C. albicans* was determined by means of FACS analysis of green fluorescent protein (GFP)-expressing cells, as described previously (*Webb JS. et al. 2001*). *Candida albicans* stably transfected with GFP was a generous gift from Dr. Alexander Johnson (Department of Microbiology and Immunology, University of California, San Francisco, CA, USA). Yeast was cultured in LB medium overnight at 30°C. Cells were collected by centrifugation at 2000 g for 5 min, washed twice in PBS, and suspended to the required absorbance at 630 nm.

The yeast to neutrophil ratio for the killing experiments was 4:1. The reaction was stopped by adding saponin at a final concentration of 0.2%. The t=0 time point was defined by adding saponin to the cells prior to the yeast. The assay was performed at 37°C while shaking gently. Samples were analyzed by FACS; GFP-positive cells were considered to be alive.

A strain of *Aspergillus fumigatus*, originally isolated from a patient with invasive aspergillosis, was used to assess the fungicidal activity of the granulocytes. After growth on yeast nitrogen base agar (Difco Laboratories, Detroit, MI, USA) with 2% (w/v) glucose for 4-5 days at 30°C, conidia were harvested in PBS, filtered through a sterile gauze and washed twice in PBS. A suspension of 10x10⁴ conidia/mL was prepared by making appropriate dilutions in yeast-nitrogen base supplemented with 2% (w/v) glucose. Aliquots of 1 mL were dispensed into the 24-mm-diameter wells of a Costar plate (Costar, Cambridge, MA, USA) and incubated for 14-16 hours until the conidia had germinated to produce 80-120-μm hyphae. Inhibition of hyphal growth was measured by the colorimetric MTT [3-(4,5dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide] assay (Sigma, St. Louis, MO, USA).

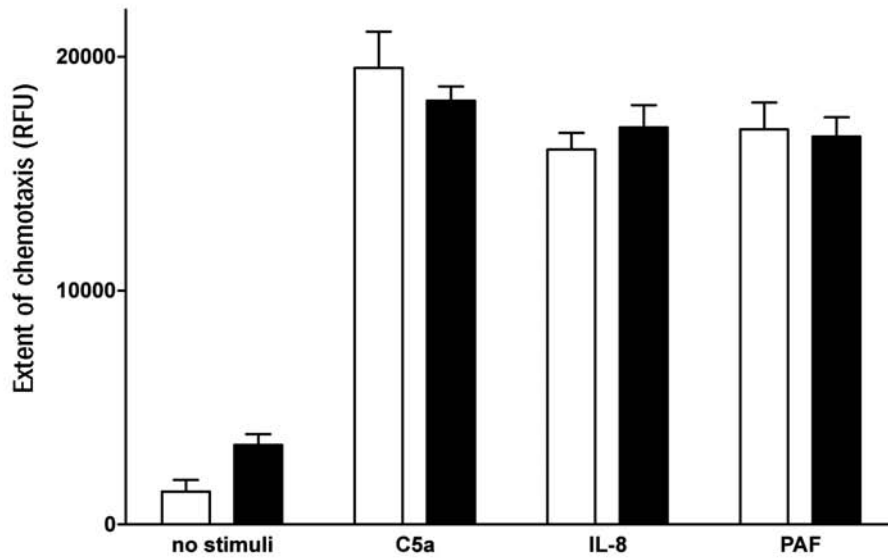
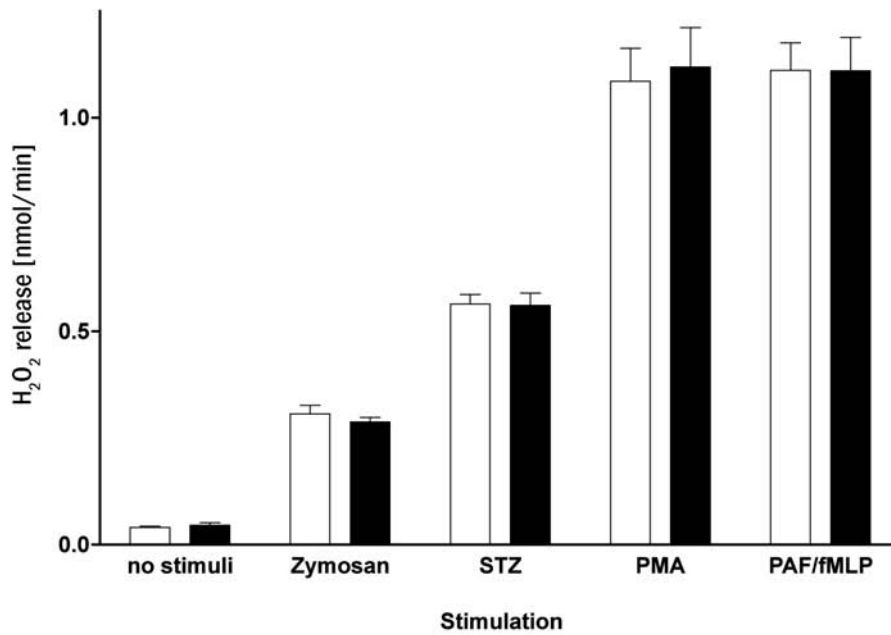
Cell count and viability

Total white blood cell counts were measured with an automated counter (Advia, Bayer, Medcompare, San Fransico, CA, USA). Viability was assessed with flow cytometry, by annexinV-FITC/PI staining (*Maianski NA, et al. 2003*) and by cell morphology based on cytopins and standard electron microscopy (EM) pictures.

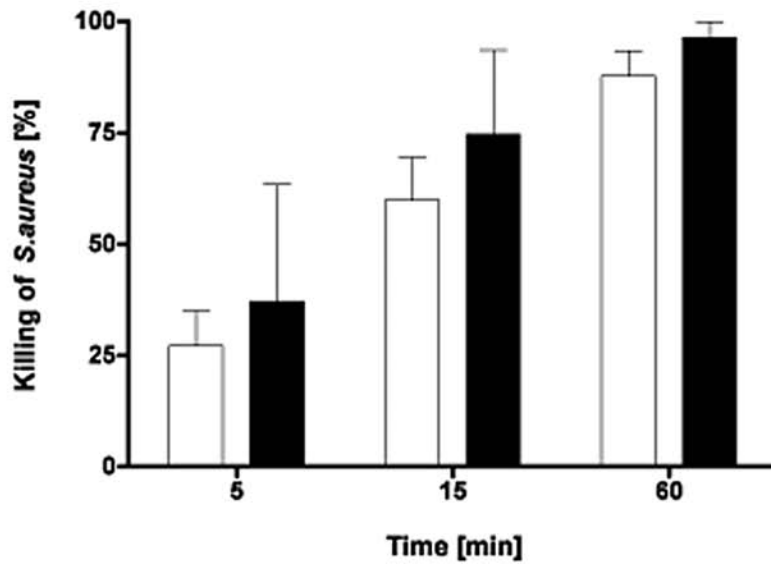
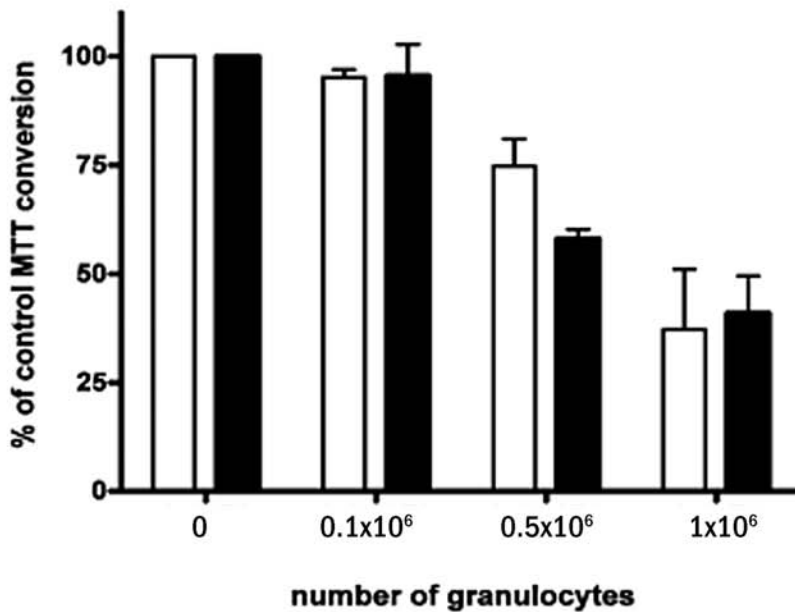
Supplementary Table S1. Composition of granulocyte products obtained by centrifugation of leukapheresis products from G-CSF/dexamethasone stimulated donors.

Volume (mL)	319 ± 53
White blood cells (x10 ⁹ /L)	187 ± 39
Granulocytes (x10 ⁹ /L)	168 ± 41
Hemoglobin (mmol/L)	3.4 ± 0.6
Red blood cells (x10 ¹² /L)	2.1 ± 0.7
Platelets (x10 ⁹ /L)	348 ± 84

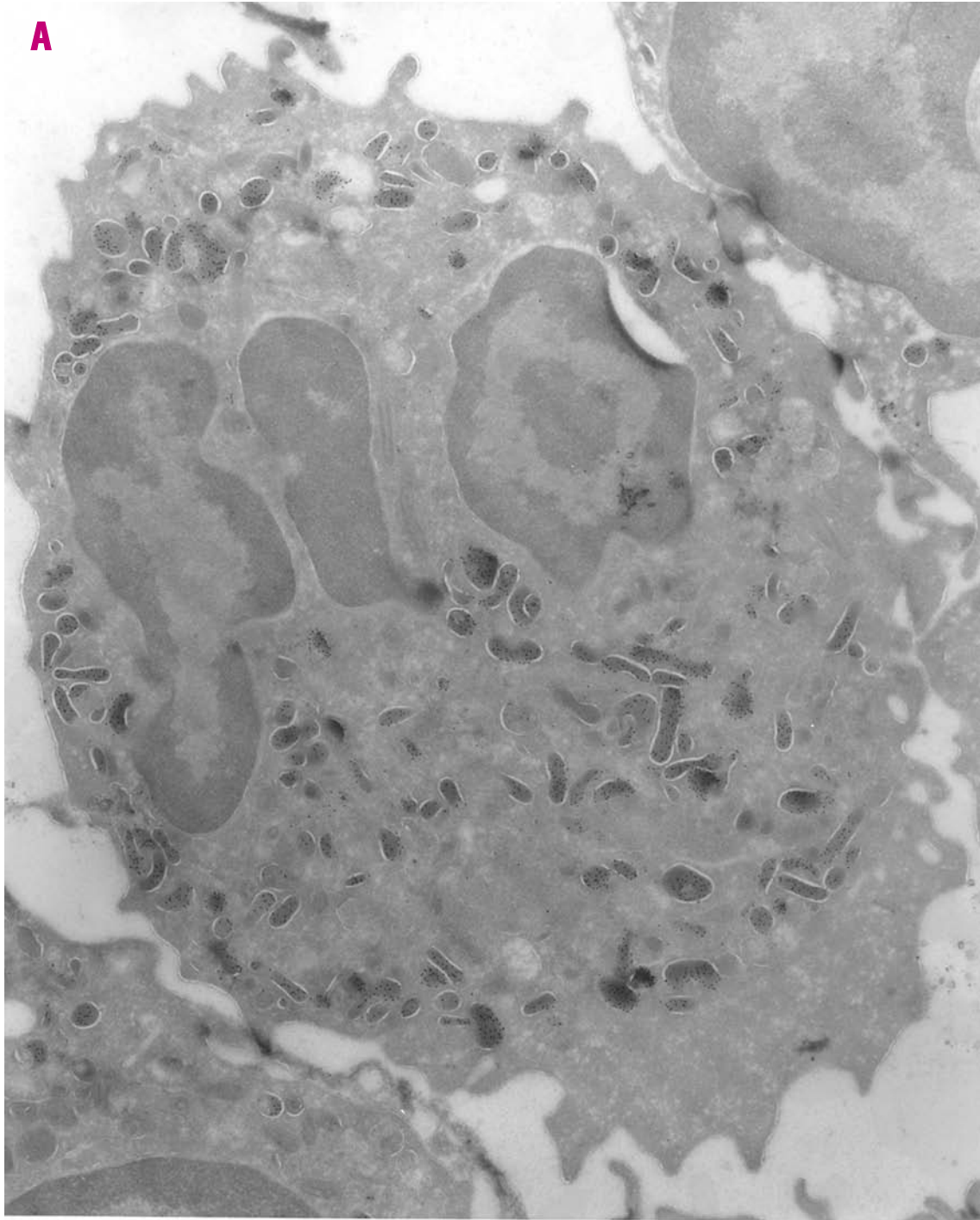
Data are presented as mean ± SD

A**B**

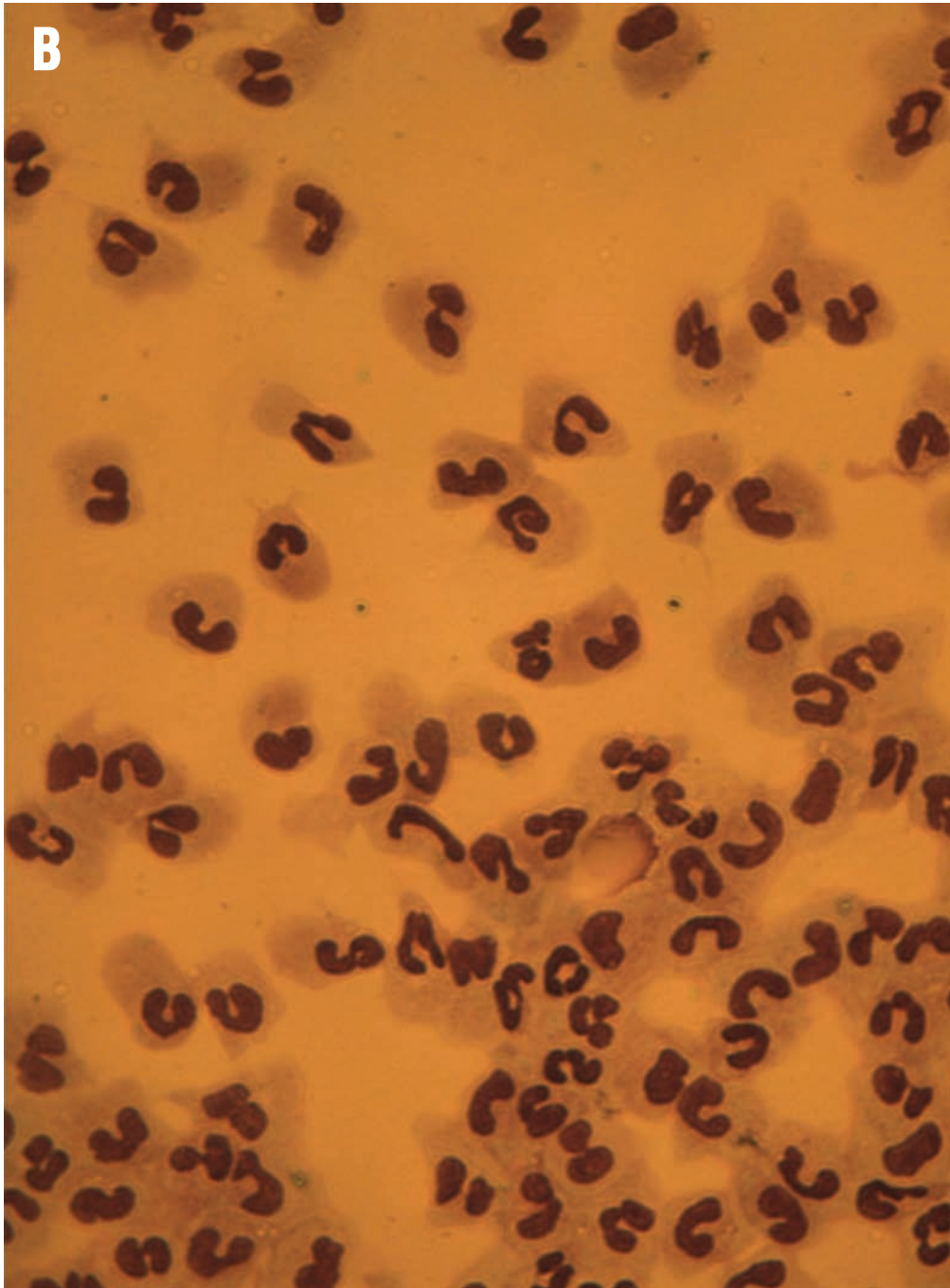
Supplementary Figure S1. Comparison between neutrophil functions of cells obtained from control blood samples and the granulocyte transfusion concentrates. **(A)** Chemotaxis measured in response to C5a (10 nM), IL-8 (10 nM) and PAF (100 nM)²⁷ **(B)** Release of H₂O₂ by granulocytes in response to different stimuli (1 μM PAF/fMLP, unopsonized zymosan, STZ (both 1 mg/mL) or PMA (100 ng/mL), based on H₂O₂-mediated oxidation of Amplex Red²⁸. Granulocytes were isolated from healthy controls (open bars) and from leukapheresis products (black bars). Results from eight separate experiments (mean ± SEM) are presented. Statistically significant differences ($p < 0.05$) are indicated by an asterisk (*).

A**B**

Supplementary Figure S2. Killing capacity of G-CSF/dexamethasone mobilized granulocytes: (A) Killing of *S. aureus* was assessed by a colony-forming unit (CFU) assay. Control (open bars) and mobilized (black bars) granulocytes were incubated with bacteria; at the indicated times samples were taken, cells were lysed, and bacteria were seeded on LB agar plates. Colonies were counted after overnight incubation. Results are presented as a reverse percentage of CFU at time = 0. (B) Damage to *Aspergillus* hyphae by granulocytes. The indicated numbers of control (open bars) or mobilized (black bars) granulocytes were added to wells in which 10×10^4 conidia had been germinated, and were incubated together for 2 hours at 37°C. Data are means \pm SEM of three to five independent experiments.



Supplementary Figure S3. Effect of storage on granulocyte viability. (A) Morphology of donor-derived neutrophils from clinical grade granulocyte concentrates stored for 24 hours at room temperature, as shown by electron microscopy, and (B) on a cytopsin preparation.



Supplementary Figure S3. Effect of storage on granulocyte viability. (B) Morphology of donor-derived neutrophils from clinical grade granulocyte concentrates stored for 24 hours at room temperature, as shown on a cytopsin preparation.