Cytometry PART A
Journal of Quantitative Cell Science
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Determination of the Proliferative Fractions in Differentiating Hematopoietic Cell Lineages of Normal Bone Marrow

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Received 16 April 2018; Revised 20 June 2018; Accepted 21 June 2018

Additional Supporting Information may be found in the online version of this article.

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Published online 00 Month 2018 in Wiley Online Library (wileyonlinelibrary.com)

DOI: 10.1002/cyto.a.23564

Abstract

Because of the proven prognostic value of Ki-67 as a proliferation marker in several types of solid cancers, our goal is to develop and validate a multiparameter flow cytometric assay for the determination of the Ki-67 expression in hemato-oncological diseases. The aim of the present study was to establish the reference values for the fraction of Ki-67 positive cells in and during maturation of individual hematopoietic cell lineages present in normal bone marrow.

Aspirates derived from femoral heads of 50 patients undergoing a hip replacement were used for the flow cytometric quantification of Ki-67 expression in the different hematopoietic cell populations of healthy bone marrow. Furthermore, the proliferative index was investigated in detail for the maturation steps during erythro-, myelo-, and monopoiesis using recently described immunophenotypic profiles in combination with a software-based maturation tool.

Reference values for the proliferative index were established for different relevant hematopoietic cell populations in healthy bone marrow. During maturation, the size of the Ki-67 positive fraction was the highest in the most immature compartment of the myeloid, monocytic, and erythroid cell lineages, followed by a steady decline upon cell maturation. While proerythroblasts showed a proliferative activity of almost 100%, the myelo- and monoblast showed a lower proliferative index of on average of 50%, indicating that a relatively large proportion of these cells exist in a quiescent state.

In conclusion, we can state that when using a novel combination of immunophenotypic markers, the proliferation marker (Ki-67) and a software-based maturation tool, it was possible to determine the proliferative fractions in the diverse hematopoietic cell lineages in bone marrow, in particular during maturation. Using this approach, the proliferative indices for the normal myelo-, mono-, and erythropoiesis were determined, which can be used as a reference in future studies of hematologic malignancies originating from bone marrow.

Key terms

proliferation; multiparameter flow cytometry; Ki-67 antigen; maturation pathway

Introduction

Cell proliferation is of interest since abnormal proliferation appears to be a precursor of tumorigenesis, and also because the quantitative description of cell proliferation in tumors can be used to predict the biological behavior of a particular neoplasia. There are several reliable methods of studying cell proliferation in tissues. One of the most important and most used methods is the immunohistochemical detection of the Ki-67 antigen. Ki-67 is a nuclear and nucleolar protein, which is centrally involved in cell proliferation (1). Expression of Ki-67 is seen during the late G1 phase and throughout S, G2, and M phases (2). This antigen is localized in the

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nucleus and associated with the chromosomes of proliferating cells, but absent in non-proliferating differentiated and quiescent cells. The fraction of Ki-67 expressing cells correlates with histological grades of tumors and can also be predictive of clinical outcome.

Despite the widespread use of Ki-67 in histopathology, its use in flow cytometric analysis of hematologic malignancies is sparse (see for example (3–5)). A possible explanation is that since 1983, at the time of the first description of this monoclonal antibody by Gerdes et al. (6), only one-, and 10 years later two-fluorescence parameter flow cytometry was available. Bone marrow is a very heterogeneous tissue population with, next to stromal tissue, different hematopoietic cell lineages including their complete maturation pathways. With one- or two-fluorescence flow cytometry, the Ki-67 expression could only be determined for the total bone marrow cell population without being able to differentiate between the different cell lineages. Normal bone marrow is composed of a number of cell types, each with a regulated phenotype, arranged in a specific spatial organization. This is controlled by the proliferative activity of progenitor cells, as well as by the number of grow-arrested cells and cells undergoing apoptosis (7,8). Bone marrow tissue is not kinetically homogeneous, but is composed of a range of related hematopoietic cell lineages, each with different properties. This concept of cell heterogeneity is also valid in hematopathologic disorders. Recent technological advances in flow cytometry allow for more parameters to be analyzed at an individual cell level, making simultaneous identification of the different cell lineages and even maturation phases of cells possible (9–11). Flow cytometric quantification of the cycling cells offers greater time-efficiency and reproducibility as compared to the microscopic examination of proliferative activity (12). These relatively recent developments, therefore, allow the detailed analysis of different cell populations in hemato-oncological diseases.

However, one of the necessities in this field, in order to achieve correct information about proliferation in hemato-oncological patients, is the standardization and knowledge about the Ki-67 expression in and during the maturation of different hematopoietic cell lineages in normal bone marrow. Therefore, the aim of the present study is to develop and validate a multiparameter flow cytometric assay for the determination of reference Ki-67 values in the different hematopoietic cell lineages, as well as its expression during maturation of the myelo-, mono-, and erythropoiesis.

Materials and Methods

Sample Collection

Bone marrow (BM) aspirates from femoral heads (n = 50) of healthy patients receiving a hip replacement were collected and supplied by the department of Orthopaedic Surgery of Zuyderland Medical Center (MC). These femoral heads were derived from 23 male and 27 female patients. The mean age of the male and female patients was 66 ±11 years and 66 ±9 years, respectively. The study was performed according to the Helsinki Declaration and was approved by the Medical Ethical Committee of the Zuyderland MC. Inclusion for the use of the femoral head was approved after the written informed consent, which was obtained before surgery.

Bone Marrow Extraction

BM aspirate was extracted from the femoral heads by the use of a 16ga x 2.688in MAX bone marrow aspiration needle (Argon Medical Devices Inc., Athens, USA). The aspiration needle was used for multiple punctures of the spongy bone. A pipette was inserted into the puncture holes and approximately 10 ml of 3.8% sodium citrate (Merck KGaA, Darmstadt, Germany) was used to flush the BM cells out of the femoral head. The BM sample was washed twice by centrifugation with phosphate-buffered saline (PBS) pH 7.4 for 5 min at 300×*g*.

Immunocytochemical Staining Protocols (Table 1)

Different combinations of antibodies were used to separate different hematopoietic cell lineages. This resulted in four different antibody combinations, as shown in Table 1.

Tube 1 was used as a negative control and contained the fluorochrome-labeled antibodies directed against CD45, CD13, HLA-DR, and CD34, and no FITC-labeled Ki-67. All antibodies used in this study were carefully titrated. To correct for background staining by the conjugated primary antibodies, a pilot-study was conducted in which a fluorescence-minus-one (FMO) approach was compared to a negative control using FITC-directly conjugated to non-relevant, isotype-specific mouse immunoglobulins. This pilot-study showed the best results with a negative control using the latter approach.

The antibody combination in tube 2 was designed for the analysis of the Ki-67 proliferative activity in different cellular components present in normal bone marrow, as well as for the expression of Ki-67 during the maturation of the myelo- and monopoiesis.

The antibody combination in tubes 3 and 4 was designed for the analysis of the Ki-67 proliferative activity during the maturation of the erythropoiesis. Due to the fact that CD36-APC and CD71-APC-A700 showed bleed through, which could not be compensated for (data not shown), these two antibodies were separated over tubes 3 and 4. Since these two tubes also contain the backbone markers CD45, HLA-DR, CD117, and CD13, the data obtained with these antibody combinations can be merged using the "Merge and calculate"-function of the software program Infinicyt™ v.1.8 (Cytognos SL, Salamanca, Spain), so that the expression of CD36 and CD71 can be investigated simultaneously during erythropoiesis.

For the establishment and validation of the negative control (tube 1) and the myelo-/monopoietic lineage (tube 2) bone marrow samples derived from 25 femoral heads were used. Likewise, for the analysis of the erythropoietic lineage 25 additional bone marrow samples were used. There were no differences in the preparation and staining between these four tubes.

Table 1. Composition of the four different test tubes with combinations of antibodies that were used to separate different hematopoietic cell lineages.

TUBES	ANTIGEN	CLONE	FLUOROCHROME	MANUFACTURERS	AB VOLUME (μℓ)
1	Non-relevant IgG	VI-AP	FITC	N-M	5
	CD13	Immu103.44	PE-Cy 5.5	BC	5
	CD34	581	Pe-Cy7	BC	5
	HLA-Dr	Immu-357	PB	BC	5
	CD45	J.33	KO	BC	2
2	Ki-67	MIB-1	FITC	DAKO	5
	CD14	RMO52	r-PE	BC	5
	CD64	22	ECD	BC	2
	CD13	Immu103.44	PE-Cy 5.5	BC	5
	CD34	581	Pe-Cy7	BC	5
	CD16	3G8	APC	BC	1
	CD10	ALB1	APC-A700	BC	3
	CD11b	Bear1	APC-A750	BC	1
	HLA-Dr	Immu-357	PB	BC	5
	CD45	J.33	KO	BC	2
3	Ki-67	MIB-1	FITC	DAKO	5
	CD105	1G2	r-PE	BC	10
	CD123	SSDCLy107D2	ECD	BC	5
	CD13	Immu103.44	PE-Cy 5.5	BC	5
	CD117	104D2D1	Pe-Cy7	BC	5
	CD71	YDJ1.2.2	APC-A700	BC	1
	CD235a	11EUB-7-6	APC-A750	BC	5
	HLA-Dr	Immu-357	PB	BC	5
	CD45	J.33	KO	BC	2
4	Ki-67	MIB-1	FITC	DAKO	5
	CD13	Immu103.44	PE-Cy 5.5	BC	5
	CD117	104D2D1	Pe-Cy7	BC	5
	CD36	CB38	APC	BD	5
	HLA-Dr	Immu-357	PB	BC	5
	CD45	J.33	KO	BC	2

FITC: fluorescein isothiocyanate; r-PE: r-phycoerythrin; ECD: electron-coupled dye (also known as phycoerythrin-Texas Red-X; PE-CY5.5: phycoerythrin-cyanin 5.5; PE-Cy7 = phycoerytrein-cyan7; APC: allophycocyanin; APC-A700: allophycocyanin-A7; APC-A750: allophycocyanin A750; PB = Pacific Blue; KO: Krome Orange; BC: Beckman Coulter, Marseille, France; BD = BD Biosciences, San Jose, CA, USA; DAKO: DAKO A/S, Glostrup, Denmark; N-M: Nordic-Mubio, Susteren, the Netherlands.

During the first incubation step, 50 μl samples of properly pre-diluted BM aspirate (white blood cell count < 30×10^9/L) were incubated with the antibodies for the cell surface markers.

The cells were incubated in the dark at room temperature (RT) for 15 min, after which they were washed with 4 ml of PBS, followed by centrifugation for 5 min at 300×*g*. For intranuclear staining of Ki-67, all cell samples were thereafter fixed and permeabilized with the Fix&Perm buffer set (Nordic-MUbio, Susteren, The Netherlands) according to the manufacturer's protocol. During the fixation step, the cells were incubated for 15 min at RT with fixation reagent A. After a washing step, the FITC-labeled Ki-67 (DAKO A/S Glostrup, Denmark) and FITC-labeled isotype-specific Ig negative control (Nordic-MUbio) were added simultaneously with the permeabilization buffer and incubated for 15 min. After washing in 4 ml PBS and resuspension in 0.5 ml of PBS, the cell samples were analyzed within 2 hours.

Flow Cytometry

Data collection was performed using the Navios™ Flow Cytometer in combination with Navios tetra software (Beckman Coulter, Marseille, France). Instrument setup was performed according to the standard procedures. Daily verification of the Navios Flow Cytometer optical alignment and fluidics system was performed using Flow-Check™ Pro Fluorospheres (Beckman Coulter). Compensation was performed weekly for each fluorochrome using the Flow-Set Pro Fluorospheres (Beckman Coulter). At least 500,000 relevant events were collected for each sample.

Data Analysis and Gating Strategy

Establishment of reference values of Ki-67 positivity in different hematopoietic cell lineages (Figures 1 and 2). Data analysis was performed using Kaluza Flow Analysis Software (Beckman Coulter). Hematopoietic cell lineage classification

was based on side scatter (SSC) and fluorescence positivity using the gating strategy, as shown and described in Figures 1 and 2. Within the above-mentioned gate settings, the Ki-67 positive population is determined by using the FITC-conjugated isotype-negative control for threshold setting.

Determination of Ki-67 positivity during myelo-, mono-, and erythropoiesis (Supplemental Figure S1.A-C). For the determination of Ki-67 positivity during different maturation stages of myelo-, mono-, and erythropoiesis, the software package Infinicyt v.1.8 (Cytognos SL, Salamanca, Spain) was used. Single cells were selected from the Forward Scatter (FSC) Peak versus FSC-INT plot. All cells that were out of range of the set SSC values were considered debris and excluded from further analysis. For a proper gating of the cells that take part in the process of myelopoiesis, all non-relevant cell populations (erythroblasts, lymphoblasts, lymphocytes, and eosinophils) were identified based on selection of combinations of the well-described classical immunophenotypic features (see above, Fig. 1 and 2) (10,11,13–18) and excluded from further analysis. A similar strategy was used for the definition of the mono- and erythropoiesis.

The myeloid maturation pathway was determined using the "Maturation Drawn" application within the Infinicyt software package and was based on CD13 versus CD11b positivity (Figure S1A; upper row)(10,16). This resulted in a multiparametric differentiation pathway consisting of an arbitrary number of 20 immunophenotypic stages of maturation. The Ki-67 positive cell fraction was selected using a Ki-67 versus Time plot using the FITC-conjugated isotype negative control for threshold setting. For the Ki-67 positive cells, the same maturation pathway was drawn in a CD13 versus CD11b dot plot (Figure S1A; lower row). The number of Ki-67 positive events per fraction was divided by the total amount of myeloid cells in the same fraction, thus resulting in the proliferation rate of 20 consecutive stages of maturation. The monocytic maturation pathway was drawn using the CD14 versus CD64 plot (Figure S1B) (15), while for the erythropoiesis the CD71 versus CD235a plot was used (Figure S1C) (13,14,17,18). Again, total events within each maturation stage were compared to the number of Ki-67 positive events.

Statistical Analysis

Continuous variables are expressed as mean ± standard deviation for variables with a normal distribution and median (interquartile range) for skewed variables. Normality was tested by the Kolmogorov–Smirnov test. Statistical analysis was performed using Microsoft Excel (Microsoft, Redmond, WA) and GraphPad Prism 5.0 (GraphPad Software, San Diego, CA) software. Box-and-whiskers plots show the data graphically.

Results

In Table 2, the mean values as well as the 95% ranges of the different bone marrow cell constituents are shown.

Proliferative Activity of the Hematopoietic Cell Lineages Present in Normal Bone Marrow (Figure 1–3)

Flow cytometric determination of reference values for the Ki-67 positive fraction in the major hematopoietic cell lineages populating normal BM reveals a clear heterogeneity of proliferative activity in these different cell lineages. The total cell population present in the BM comprises a Ki-67 positive fraction of 10.7% (5.5–16.3%). The cell population with the highest proliferative activity can be found within the erythroid lineage (70.3% (53.7–77.4%)), while also a high Ki-67 positive fraction was found in the progenitor cells, i.e., the CD34 positive (blast) population (58.1% (45.7–67.9%)) and the CD10 positive/low side scatter lymphocyte progenitor cells (30.5% (13.5–43.7%)). A moderate proliferative activity of 21.0% (12.9–30.6%)) is found for the myeloid total cell population, including its precursors. Also, plasma cells (15.0% (5.1–24.7%)) and cells that can be found in the moderate SSC/CD45 positive blast region (15.0% (8.4–22.4%)) show a moderate proliferative activity. Finally, the monocytic and lymphocytic lineages show low Ki-67 positive fractions of 9.7% (4.0–12.6%) and 1.8% (1.0–3.9%), respectively, most likely representing the monoblast and lymphoblast population.

Proliferative Activity During Myelopoiesis (Figure 4A; See also Supplemental Figure S1.A)

Using the Maturation Pathway option in the Infinicyt software program, the proliferative activity during myelopoiesis was quantified based on the immunophenotypical expression of CD13, CD11b, and CD16. For this purpose, the myelopoietic process is divided into 20 equal maturation stages. Figure 2A depicts the mean values and standard deviations of the analyzed normal bone marrow samples. As starting point of the myelopoiesis (as well as the monopoiesis; see below), the CD34 strongly positive/CD10 negative/CD45 moderately positive blast cell population was used (maturation step 0). From this figure, it becomes clear that the myeloblast population starts with a Ki-67 positive fraction of 50.0 ± 13.0%, which remains stable up to the myelocyte stage. Upon further maturation, a steep decrease in proliferative activity takes place.

Proliferative Activity During Monocytopoiesis (Figure 4B; See also Supplemental Figure S1.B)

Starting with a 50.0 ± 13.0% Ki-67 positive blast population (see above), a gradual decrease in proliferative fraction was noticed during further maturation into the promonocyte and monocyte stage.

Proliferative Activity During Erythropoiesis (Figure 4C; See also Supplemental Figure S1.C)

In the first stages of the erythropoiesis up to the polychromatic erythroblast stage, the Ki-67 positive fraction remains above 90%. Then, the size of the Ki-67 positive fraction decreases upon further maturation. A possible explanation for the relatively high percentage of Ki-67 positive cells (25.9 ± 16.5%) at the end of the maturation curve is due to

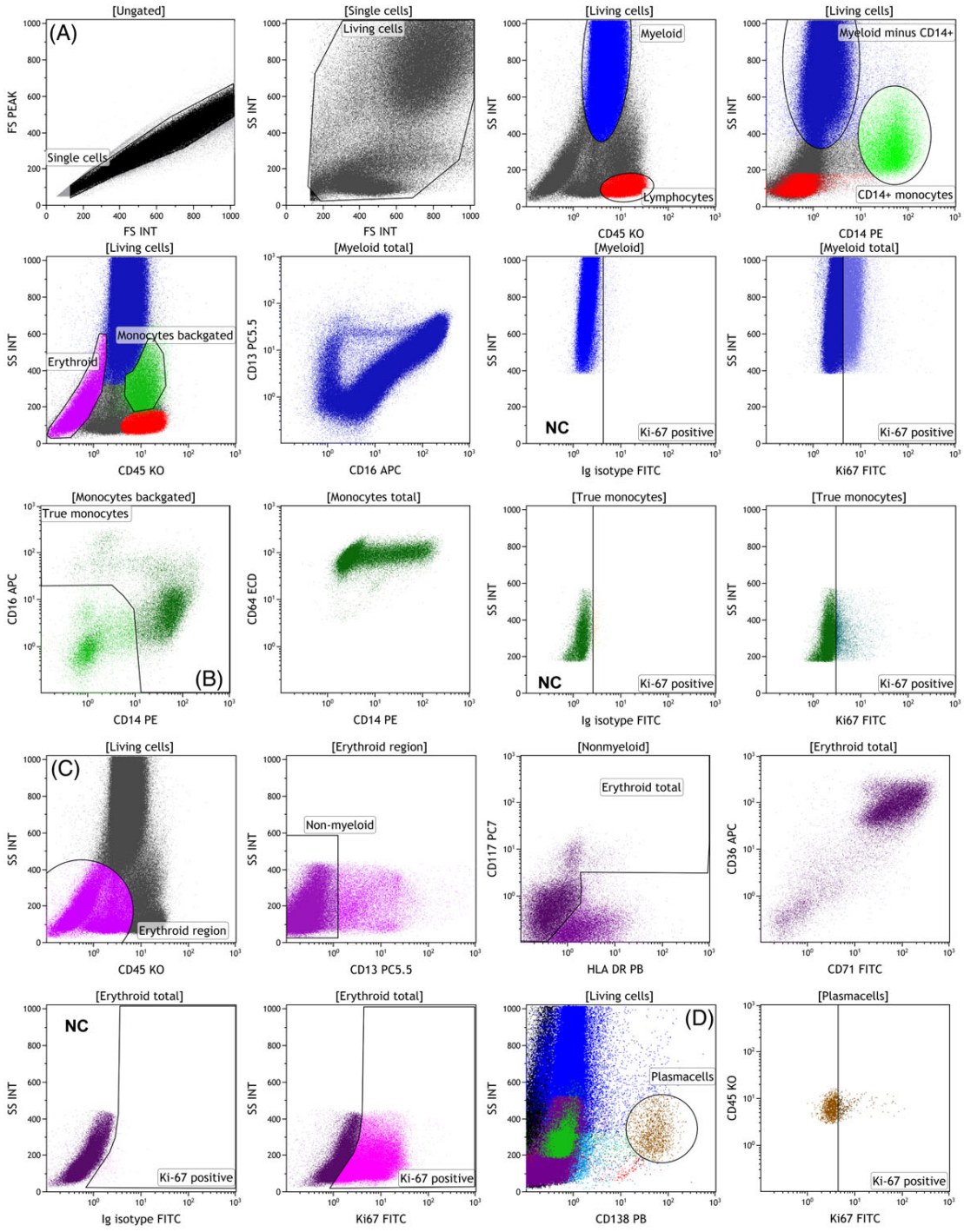

Figure 1. Gating strategy for the determination of the different cell populations present in bone marrow. For all four tubes the first two steps are identical. Single cells were selected in FSC-INT vs. FSC-Peak plot. Then debris was excluded in a FSC/SSC plot. Lymphocytes were gated in the $CD45^{high}$ vs. SSC^{low} region of the CD45/SSC plot. (A.) Selection of the myeloid population, starting with a rough selection of myeloid cells in a CD45/SSC plot, followed by excluding possible monocytes based on CD14-expression (Myeloid minus CD14+). (B.) Selection of the monocytic cell lineage is performed by backgating the CD14+ cells in the CD45/SSC plot (see A.). Exclusion of admixture with myeloid cells and lymphocytes is performed in a CD13/HLA-Dr plot. (C.) The selection of the erythroid cell lineage starts with the selection of $CD45^{negative\text{-}to\text{-}dim}$ with $SSC^{low\text{-}to\text{-}int}$ in a CD45/SSC plot. Remaining myeloid cells are excluded by the selection of CD13 negative cells. B-cell progenitors and other contaminating non-erythroid cells may be excluded by their $CD117^{-}HLA\text{-}Dr^{+}$ phenotype. (D.) Plasma cells were defined based on selecting $CD138^{positive}$ with SSC^{int} cells. Determination of the Ki-67 positive fraction in the above-described cell populations is based on gates that were set according to the negative control (NC). [Color figure can be viewed at wileyonlinelibrary.com]

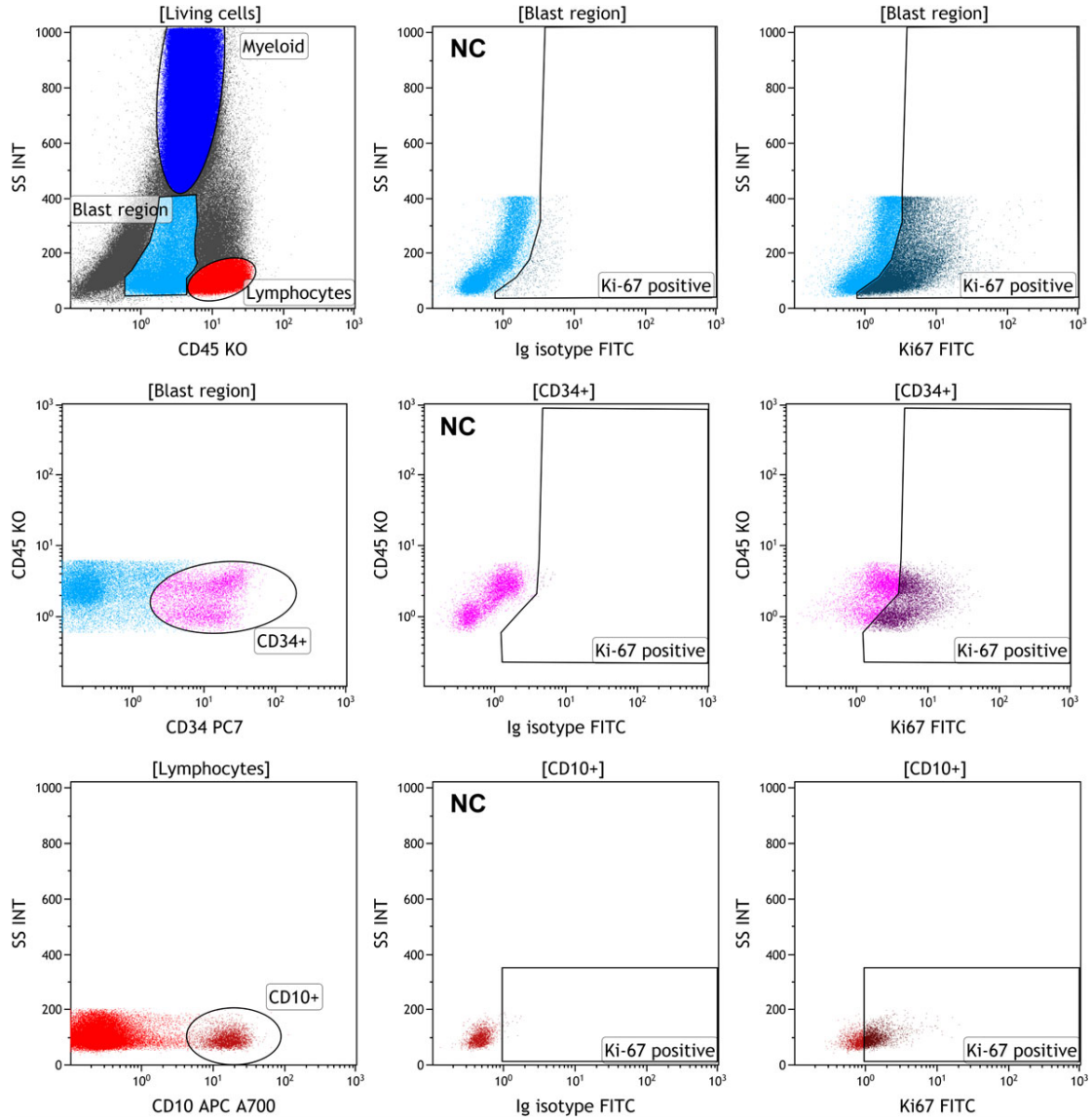

Figure 2. The gating strategy for the progenitor cells starts also by exclusion of doublets and debris, as explained in Figure 1A. The blast region was based on $CD45^{dim}$ with $SSC^{low\text{-}to\text{-}int}$ in a CD45/SSC plot. The CD34-positive blasts were selected in the blast region population in a CD34/CD45 plot. Finally, within the lymphocyte gate, the B-cell progenitor cells were selected based on their CD10-positivity.Also in these different progenitor populations, the determination of the Ki-67 positive fraction is based on gates that were set according to the negative control (NC). [Color figure can be viewed at wileyonlinelibrary.com]

the lysis of the reticulocytes and enucleated immature erythrocytes, a part of the final stages of the nucleated immature erythrocytes are lost in our assay.

Discussion

Hematopoiesis is a complex and tightly regulated process of blood cell formation that is hierarchically coordinated. During normal hematopoiesis, different blood cell types are produced in the BM depending on physiological requirements. In the last few years, increasing knowledge is gathered about specific cell surface protein expression patterns during maturation of different hematopoietic cell types, in particular for the process of erythropoiesis (9,10,13,15,16,18,19). Parallel developments in flow cytometry allow for multiple parameters to be analyzed at a single cell level, making the simultaneous identification of different cell lineages and maturation stages during blood cell formation possible (9–11). As a result, these individual cell populations can now be better discriminated, which allows a more precise determination of their proliferative activity.

For this study, bone marrow aspirates were derived from femoral heads of patients undergoing a hip replacement. The average age of these patients was 66 $\pm$ 11 years; and as a

Table 2. Normal values for the bone marrow differential cell count of the femoral heads used in this study. These values were determined by multiparameter flow cytometric analysis. Furthermore, a comparison is made between our data and those published by Bain et al. (20) obtained after morphologic examination of iliac crest aspirates.

BONE MARROW	% FOUND IN THIS STUDY		% AFTER BAIN ET AL. (20)	
CELL TYPES	MEAN VALUES	RANGE	MEAN VALUES	RANGE
Erythroblasts	21.6	14.0–27.2	23.6	14.7–32.6
Neutrophils	60.4	39.6–71.5	49.2	30.2–68.1
Monocytes	4.1	3.7–4.8	2.5	0.5–4.6
Lymphocytes	8.8	5.4–11.4	16.1	6.0–26.2
Plasma cells	0.1	0.0–0.3	1.9	0–3.8
Blasts	1.0	0.3–1.9	0.4	0–1.3

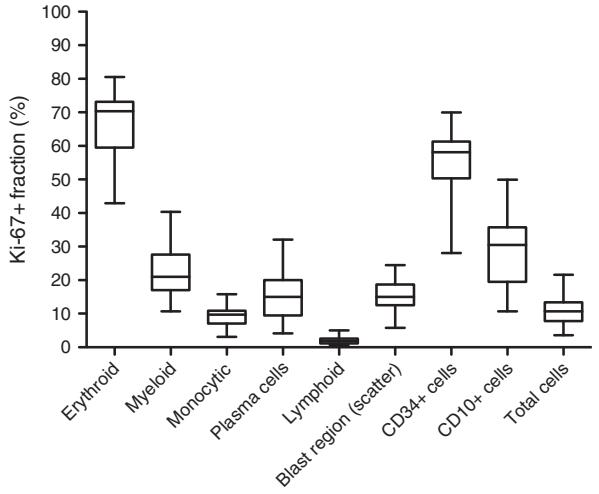

Figure 3. Normal values of Ki-67-positive fractions of the diverse hematopoietic cell lineages residing in normal bone marrow (n = 50). Results are expressed as median using interquartile ranges.

result, the data presented in this study are restricted to the older patient. Therefore, this dataset represents properly matched reference values since most hematopathologic disorders, such as myelodysplasia, myeloproliferative disorders, and acute myeloid leukemia, present themselves at a higher age.

In Table 2, it is shown that the ranges of the different bone marrow cell populations are in concordance with those found in the literature (20,21). These data derived from iliac crest or sternum do not deviate significantly from those of the femoral heads used in this study.

From our reference values for the proliferative activities in normal BM, based on Ki-67 immunolabeling, it can be concluded that the proliferative fraction comprises approximately 10% of the total cell population residing in the BM. The results in the present study are in concordance with results found in earlier studies (3,4,22), in which BM samples

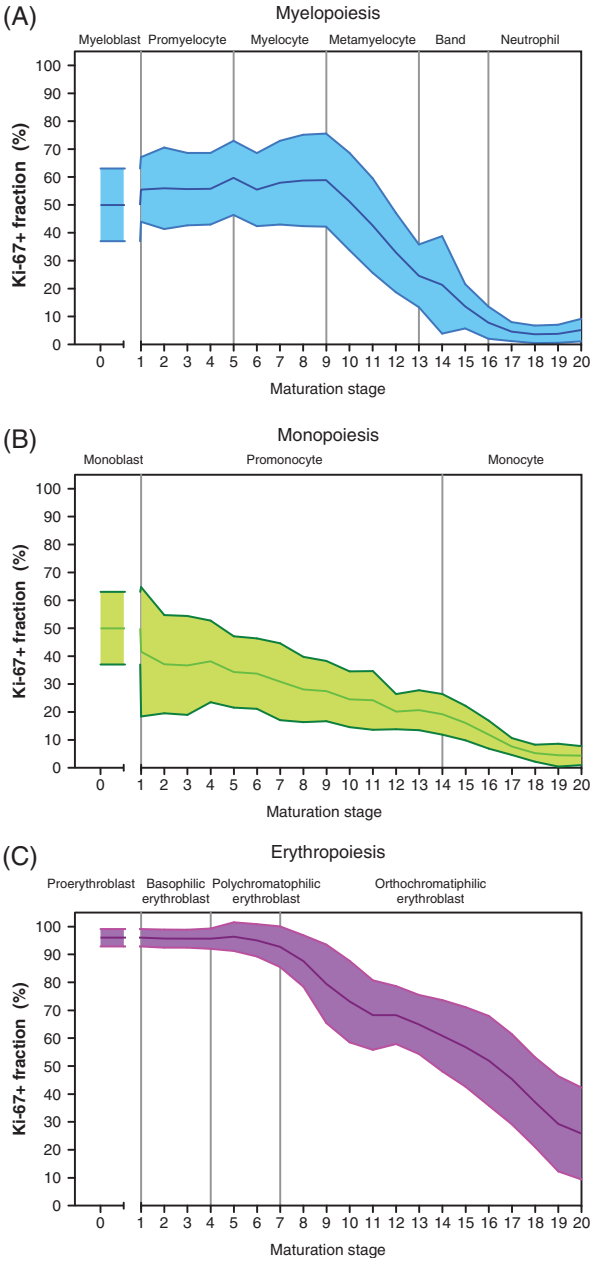

Figure 4. Size of the Ki-67-positive fraction of the different maturation stages of the myelopoiesis (A; n = 25), monopoiesis (B; n = 25), and erythropoiesis (C; n = 25). The solid line in each graph indicates the mean percentage of Ki-67-positive events for each maturation stage. The area surrounding the solid line illustrates the standard deviation of Ki-67-positive events. For stage 0 of the myelo- and monopoiesis, the Ki-67-positive fraction in the CD34-positive blast cells is used (see also Figure 2). [Color figure can be viewed at wileyonlinelibrary.com]

were analyzed for Ki-67 expression by one- or two-parameter flow cytometry or by immunohistochemistry. Noteworthy, Van Bockstaele et al. could not detect any convincing Ki-67 positivity on nuclei from normal BM samples (5). They stated that in normal bone marrow the cycling compartment consist

of a minor progenitor compartment (Ki-67 negative) and a major differentiating compartment (Ki-67 positive), resulting in a very low overall Ki-67 positivity. However, they used a two-color (Ki-67/DNA) flow cytometric approach, and were not able to analyze the Ki-67 expression in different individual hematopoietic cell populations. Next to this, it was also not possible for them to determine the Ki-67 expression during the maturation process.

Myelopoiesis

A moderate proliferation rate of approximately 22% is detected for the total myeloid cell population residing in the bone marrow. This relatively low proliferative activity in the total myelopoietic cell compartment was surprising, since neutrophils represent a large fraction of circulating cells present in peripheral blood. In addition, the circulating pool of neutrophils accounts for only 1–2% of the total mature neutrophil population (23). A major part of neutrophils reside in the bone marrow storage compartment. Also, once released into the blood stream, neutrophils have a short half-life of only 6–9 hours (24). The basal neutrophil production rate in the BM, therefore, is high and ranges between 5 and 10 $\times$ 10^{10} cells/day (25).

However, when analyzing the different maturation stages of myelopoiesis, a clear heterogeneity of proliferation rates becomes evident. In approximately 50% of the CD34-positive blasts Ki-67 expression is observed, which can be explained by the limited proliferative activity of the early hematopoietic stem cells, in order to prevent depletion of the progenitor pool (26). The remaining half of CD34-positive blasts represents most likely the quiescent hematopoietic progenitor cell compartment. Ki-67 expression remains stable during the promyelocytic and myelocytic maturation stages, which make up the mitotic pool of the myelopoiesis (27). The consecutive rapid decrease of Ki-67 positivity indicates a loss of proliferative capacity and entrance into the postmitotic pool, which consists of metamyelocytes, band neutrophils, and segmented neutrophils (25). During the final maturation stage, Ki-67 expression is almost completely lost since terminally differentiated neutrophils are no longer capable of cell division (25).

Monopoiesis

Also, the monocytic cell population shows a low proliferative profile with only approximately 10% of this cell lineage being Ki-67 positive (24). This can be explained by the fact that monocytes remain present in the peripheral circulation for 8–72 hours before infiltrating extravascular tissues and differentiating into mature resident macrophages (24). These macrophages have a long life span and are generally quiescent. While the overall monocytic population is characterized by a relatively small proliferative fraction, approximately 50% of the CD34-positive progenitor cells (for both the mono- and myeloblast lineage) exhibit Ki-67 positivity. The fraction of Ki-67-positive cells declines gradually during maturation, with the lowest proliferative capacity representing the pool of mature monocytes ready for release from the BM reserve (24).

Erythropoiesis

For the first time, this study describes the quantitative analysis of the proliferative activity in the different cell populations during the maturation process of erythropoiesis. Our analyses show that the highest proliferative fraction during blood cell formation occurs in this cell lineage, with the Ki-67 positive fraction in the very first stages of erythropoiesis, i.e., proerythroblasts, basophilic, and polychromatic erythroblasts, reaching almost 100%. Although unexpected in light of the foregoing results in myelo- and monopoiesis, this finding can be explained by the high physiological demand for erythropoiesis, with approximately a quarter of all cells residing in the bone marrow belonging to the erythroid lineage (28). A steady decline in the proliferative activity begins at the transition from polychromatic to orthochromatic erythroblasts (18). When interpreting the data for erythropoiesis, it has to be kept in mind that our flow cytometric approach detects only nucleated erythroblasts due to the usage of lysing reagents. As a result, the most differentiated, non-proliferating cell populations (reticulocytes and erythrocytes) can be lost and are for that reason not incorporated in these analyses.

Clinical Significance

Our findings may have clinical implications and may explain some pathophysiological aspects of hematological disorders.

For example, our study shows that almost 100% of the most immature erythroblasts in the BM show proliferative activity, in contrast to the progenitors of myelo- and monopoiesis, of which almost half shows Ki-67 expression. This is reflected in the effect of external influences that intervene with cell proliferation, such as irradiation (29), chemotherapy (29), or a Parvo B19 infection in patients with chronic hemolytic anemia (30). In these situations, the erythropoietic cell compartment is most affected and regresses transiently in the BM.

With respect to the myelo- and monopoiesis, the large compartment of quiescent, Ki-67-negative cells represents an interesting cell population in view of treatment strategies for myelo-/monocytic malignancies. These cells are not sensitive to therapies that interfere with cell proliferation, such as chemotherapy, radiotherapy, or other types of therapy from which these Ki-67-negative progenitor cells will escape. Detailed knowledge about the size of the Ki-67-negative fraction of these hemato-oncological processes can become leading in the choice of treatment options or in predicting clinical response to therapy.

In conclusion, this study shows that the proliferative fraction of the diverse hematopoietic cell lineages residing in BM can be reliably determined using a 10-color/12-parameter flow cytometric approach, by combining cell surface markers with Ki-67 using a Fix&Perm strategy. The three major hematopoietic cell lineages populating the normal BM show proliferation rates according to their life span and physiological demands. The immature cell populations show high percentages of Ki-67 expression, which diminishes upon consecutive maturation stages. While almost all proerythroblasts are Ki-67 positive, the

myelo- and monoblast showed a lower proliferative index of approximately 50%, which can indicate that these cells belongs to the non-proliferating compartment (G_0/early G_1 of the cell cycle) and thus exists in a quiescent state.

This knowledge about the proliferative activity in the different hematopoietic cell lineages during maturation in normal bone marrow can be used in future studies of hematologic malignancies originating from BM.

Acknowledgments

The authors would like to thank the staff of the Dept. of Orthopaedic Surgery of Zuyderland MC as well as their patients for delivering the femoral heads. Next to this, we appreciate the valuable assistance of the medical technicians of the subdivision Bone Marrow Diagnostics, Dept. of Clinical Chemistry & Hematology of Zuyderland MC.

Conflict of Interest Disclosures

R.M.J.R. is CEO and F.C.S.R. is CSO at Nordic-MUbio, The Netherlands.

Authorship Contributions

F.C.S.R., R.M.J.R. and M.P.G.L. designed and conceptualized the research. K.P.H.N., R.K., K.H.K.L., and R.J.M.R.D. performed the flow cytometric analyses. K.P.H.N., R.K. and M.P.G.L wrote the first concept of the article. F.C.S.R. and M.P.G.L. coordinated all aspects of this study.

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