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Proliferative and anti-apoptotic fractions in maturing hematopoietic cell lineages and their role in homeostasis of normal bone marrow

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Abstract

Recent developments in clinical flow cytometry allow the simultaneous assessment of proliferative and anti-apoptotic activity in the different hematopoietic cell lineages and during their maturation process. This can further advance the flow cytometric diagnosis of myeloid malignancies. In this study we established indicative reference values for the Ki-67 proliferation index and Bcl-2 anti-apoptotic index in blast cells, as well as maturing erythroid, myeloid, and monocytic cells from normal bone marrow (BM). Furthermore, the cell fractions co-expressing both proliferation and anti-apoptotic markers were quantified. Fifty BM aspirates from femoral heads of patients undergoing hip replacement were included in this study. Ten-color/twelve-parameter flow cytometry in combination with a software-based maturation tool was used for immunophenotypic analysis of Ki-67 and Bcl-2 positive fractions during the erythro-, myelo-, and monopoiesis. Indicative reference values for the Ki-67 and Bcl-2 positive fractions were established for different relevant hematopoietic cell populations in healthy BM. Ki-67 and Bcl-2 were equally expressed in the total CD34 positive blast cell compartment and 30% of Ki-67 positive blast cells also showed Bcl-2 positivity. The Ki-67 and Bcl-2 positive fractions were highest in the more immature erythroid, myeloid and monocytic cells. Both fractions then gradually declined during the subsequent maturation phases of these cell lineages. We present a novel application of an earlier developed assay that allows the simultaneous determination of the Ki-67 proliferative and Bcl-2 anti-apoptotic indices in maturing hematopoietic cell populations of the BM. Their differential expression levels during the maturation process were in accordance with the demand and lifespan of these cell populations. The indicative reference values established in this study can act as a baseline for further cell biological and biomedical studies involving hematological malignancies.

KEYWORDS

anti-apoptosis, Bcl-2, hematopoietic cell lineages, Ki-67, maturation, multiparameter flow cytometry, normal bone marrow, proliferation

1 | INTRODUCTION

Proliferative and apoptotic signaling are important factors in maintaining tissue homeostasis, and are directly related to the lifespan of cells [1]. Disturbances in these cellular mechanisms often lead to tumorigenesis, and the magnitude of their imbalance contributes to the aggressiveness of malignancies [2]. Therefore, markers for proliferative and apoptotic behavior have claimed a major role as objective parameters and proven their value in diagnostic histopathological analyses of solid tumors and lymphoma [3, 4].

However, histological examination of bone marrow (BM) tissue for the diagnosis of myeloid malignancies is challenging. Discriminating between the different hematopoietic cell lineages with the simultaneous objective assessment of their proliferating or apoptotic fraction is so far not possible in histological (Figure 1) or cytological patient samples. The diagnostic workflow of these malignancies comprises morphological,

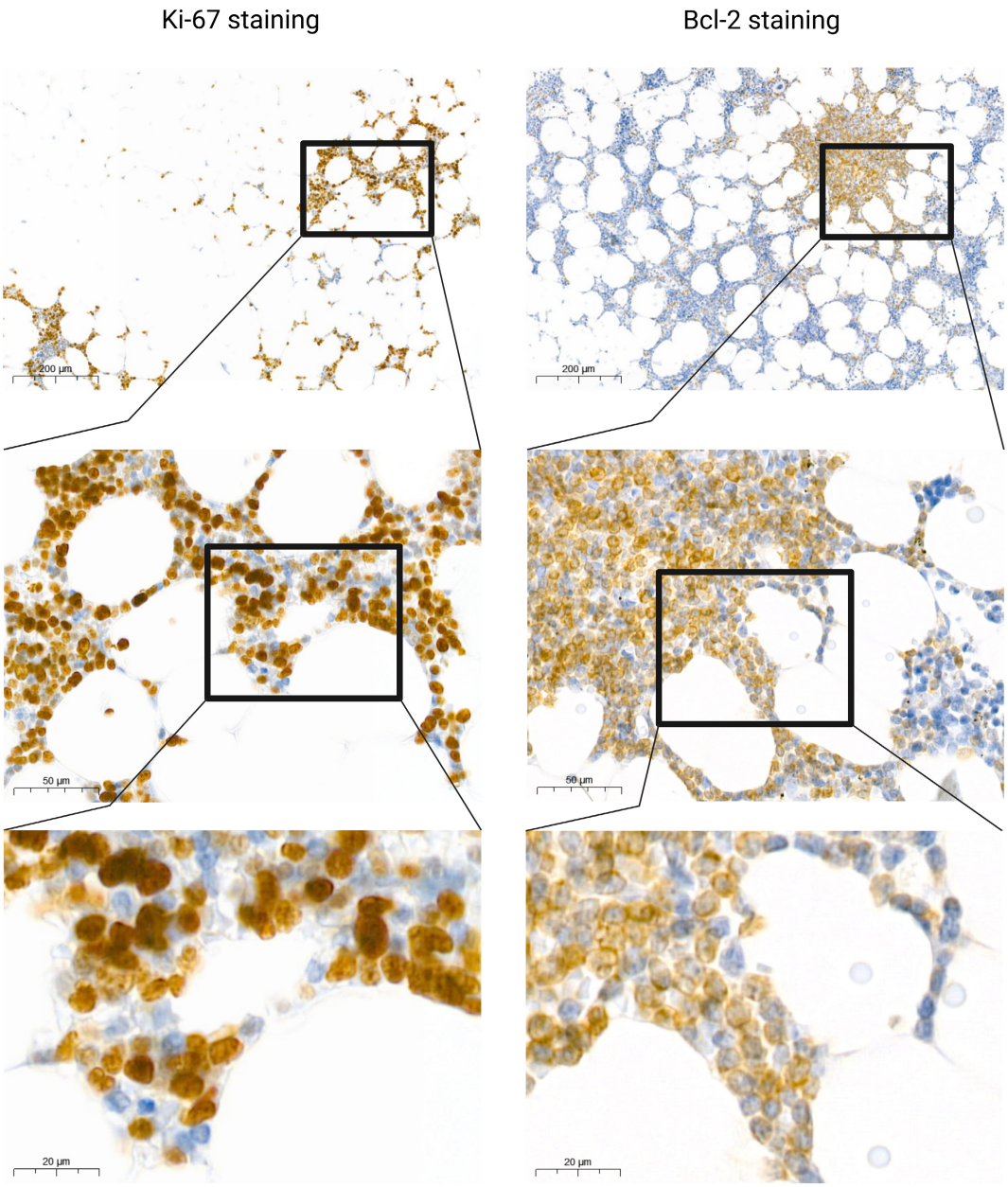


FIGURE 1 Immunohistochemical staining pattern of normal bone marrow (BM) tissue from a femoral head with the Ki-67 antibody MIB-1 and Bcl-2 antibody Bcl-2/100 (10×, 40×, and 100× magnification). Cells positive for Ki-67 or Bcl-2 can be recognized by their brown staining. In normal BM, the majority of cells are positive for Ki-67 and show a variable expression. Little cells are positive for Bcl-2 in normal BM tissue and Bcl-2 positivity is focused in specific regions. Ki-67 positive cells were more abundant in the normal BM than Bcl-2 positive cells.

molecular, cytogenetic and immunophenotypic (flow cytometric) analyses, all assessing different aspects of the malignancy [5–7]. These techniques combined are rather time-consuming and morphological and flow cytometric analyses are prone to a subjective interpretation, leading to discrepancies in diagnosis when comparing the results of different clinical centers [5, 6]. Additional objective biomarkers with diagnostic value may, therefore, lead to more straightforward diagnosis of myeloid malignancies.

According to the European Hematology Association research roadmap for myeloid malignancies, a better understanding of mechanisms of leukemic stem/progenitor resistance to anti-cancer therapies is required to advance the field [8]. This could (partly) be achieved by characterization of the cellular and molecular mechanisms involved in myeloid malignancy development, progression and therapy resistance. Ultimately, the better understanding and characterization of leukemic stem/progenitor cells, together with the impact of the clonal architecture of leukemia and clonal evolution during the disease course will allow for precision medicine that targets these cells specifically.

The recent discoveries regarding the genetic complexity of myeloid malignancies allowed the development of precision medicine strategies. This led to the proposition of novel prognostic scoring systems, further tailoring the clinical decision of myeloid malignancies to the individual patient level. Biological behavioral parameters (such as proliferation, apoptosis and differentiation) are often assessed by means of these genetic markers/profiles [9, 10]. As myeloid malignancies are rather complex and heterogeneous disorders, these genetic markers/profiles often result in contradicting signals e.g. growth arrest versus cell cycling, for cell death versus cell survival. Therefore, the relationship between individual somatic mutations and clonal expansion of malignant cells remains unclear. Analysis of the biological behavior by detection of proteins that are involved in these processes may be complementary to the recently proposed genetic markers/profiles for prediction of prognosis and therapeutic decision making.

The potential of proliferative and apoptotic markers in establishing a better prognosis and prediction of treatment response in myeloid malignancies was studied in the past decades [11], but this did not lead to the incorporation of the intracellular dynamic markers in the diagnostic work-up of these malignancies. The number of parameters that could be analyzed by histopathological and flow cytometric analyses was limited at the time that these markers were initially studied. This made simultaneous differentiation between the different myeloid cell lineages based on their extracellular antigen expression together with the detection of intracellular dynamic (proliferative and apoptotic) markers challenging.

The major advances in the field of flow cytometry have led to a rapid increase in simultaneously measurable parameters at the level of the individual cell, which is crucial for the subsequent implementation of the above mentioned intracellular dynamic markers [12]. Implementing these markers in flow cytometric panels contributes to our understanding of the biological background of myeloid malignancies and to more accurate and straightforward diagnosis, prognostic stratification and contributes to development of precision medicine strategies in these malignancies.

One of the most frequently applied proliferation markers in clinical histopathology is Ki-67, which is a well-known marker for prognosis and treatment response in histopathological analyses of solid tumors and non-Hodgkin lymphoma [3, 4]. In a previous study, we developed a protocol for intranuclear staining of the proliferation marker Ki-67 in differentiating hematopoietic cells [12, 13].

However, tissue homeostasis is maintained by a combination of proliferation and (anti-) apoptotic signaling. In order to gain a better understanding of the cell biological aspects of the normal myeloid maturation process and myeloid malignancies, it is pivotal to quantify proliferation in conjunction with (anti-)apoptotic signaling. Furthermore, anti-apoptotic signaling is an important driver for carcinogenesis, progression of cancers and affects response to various anti-cancer therapies (e.g. chemotherapy, immunotherapy and Venetoclax therapy) [14].

Anti- and proapoptotic proteins of the B-cell lymphoma-2 (Bcl-2) family [15] have been described to contribute to the development and functioning of the fetal and adult hematopoietic systems and to influence the generation and maintenance of different hematopoietic lineages [16]. This family of proteins comprises various pro- and anti-apoptotic proteins that are critical for apoptotic signaling. These proteins regulate the mitochondrial pathway of apoptosis by controlling the permeabilization of the outer mitochondrial membrane. One of the anti-apoptotic protein of the Bcl-2 family is the Bcl-2 protein [17]. Expanding our assay with intracellular dynamic markers, such as Bcl-2, allows broader applicability of the assay and more elaborate analyses of cell behavior in healthy and malignant BM. Proper cell fixation and permeabilization is, however, crucial for the flow cytometric detection of both dynamic markers.

According to the state-of-the-art review of Kollek et al., there is some evidence that myeloid progenitor cells express the Bcl-2 protein to promote survival of these cells [16]. Furthermore, another study by Porwit-MacDonald and colleagues concluded that Bcl-2 expression tends to decrease during the myeloid and monocytic maturation in normal fetal BM [17]. Nevertheless, the Bcl-2 expression during the erythropoiesis is yet to be determined as strategies for flow cytometric analysis of this cell lineage were developed recently. Cell biological aspect of BM cells, such as proliferation and (anti-)apoptotic signaling, are prone to age-related alterations [18]. Therefore, the proliferative and (anti-)apoptotic fractions of fetal BM cells, as determined in previous studies, are no suitable reference for myeloid malignancies that are predominantly diagnosed in elderly individuals such as myelodysplastic syndromes and acute myeloid leukemia [8].

To our knowledge no studies exist that assess the Bcl-2 anti-apoptotic index during the process of erythro-, myelo- and monopoiesis the BM of elderly patients. Unraveling the Bcl-2 anti-apoptotic index during the maturation from blast cells to mature hematopoietic cells in relation to the Ki-67 proliferative index can explain the lifespan of these different cell progenitors/lineages and expands the knowledge about BM tissue composition and homeostasis. Ultimately, the results of the present study could act as a reference for future studies of these processes in myeloid malignancies. Therefore, we aimed to elucidate the Bcl-2 anti-apoptotic index in differentiating

hematopoietic cells during the myelo-, mono- and erythropoiesis in relation to the Ki-67 proliferation index during these maturation processes.

2 | MATERIALS AND METHODS

2.1 | Sample collection

This study included 50 BM aspirates from femoral heads of healthy patients undergoing hip replacement, which were consecutively collected and supplied by the department of Orthopedic Surgery of Zuyderland Medical Center. Femoral heads were extracted from 25 males with a median age of 70 years (range 45–84 years) and 25 females with a median age of 71 (range 51–89 years). Additionally, five separate BM aspirates from healthy patients were subjected to simultaneous staining of Ki-67 and Bcl-2. Data was acquired after informed consent in accordance with the Declaration of Helsinki, while also approval for this study was obtained from the Medical Ethical Committee of the Zuyderland Medical Center and Hogeschool Zuyd (METC Z registration nr. 15-N-201).

2.2 | Bone marrow extraction

BM aspirates were extracted from the femoral heads using a 16ga x 2.688in MAX bone marrow aspiration needle (Argon Medical Devices Inc.) and subsequent flushing of the BM tissue with 3.8% sodium citrate (Merck KGaA). This aspiration needle was used to puncture the spongy tissue of each femoral head multiple times. A pipette was then inserted into the puncture holes and approximately 10 ml of 3.8% sodium citrate was used to flush as much BM cells as possible out of the tissue. The acquired BM aspirate was washed twice by centrifugation for 5 min at 300×*g* and resuspending with 4 ml phosphate-buffered saline (PBS) pH 7.4. After washing, the white blood cell count of the BM aspirates was quantified on a XN-9000 hematology analyzer (Sysmex Europe GmbH).

2.3 | Flow cytometry

Ten-color/twelve-parameter flow cytometry was utilized for immunophenotypic analysis as described previously [12]. The Ki-67 and Bcl-2 positive fractions during erythro-, myelo- and monopoiesis were determined by a panel of seven different tubes with a combination of antibodies as described in Table S1. The antibody panels used for simultaneous staining of Ki-67 and Bcl-2 are displayed in Table S2. Characteristics of the different monoclonal antibodies used in this study are depicted in Table S3. Analysis of these tubes was performed using the "merge and calculate" function of Infinicyt v2.0 (Cytognos SL) as described previously [12].

Briefly, 50 µl of BM aspirate (properly diluted to a white blood cell count <20 × 10^9/L) was incubated with the antibodies staining the extracellular proteins (Table S1). After incubation for 15 min at room temperature (RT) in the dark, the cells were washed with 4 ml of phosphate buffered saline pH 7.4 (PBS) and centrifuged for 5 min at 300×*g*.

Non-nucleated cell lysis and fixation and permeabilization of the remaining nucleated BM cells for intracellular staining of Ki-67, Bcl-2 and their respective IgG1 isotype controls was performed with the Fix&Perm buffer set (GAS-002; Nordic-MUbio) according to the manufacturer's protocol. After intracellular staining with fluorescein-isothiocyanate (FITC)-labeled Ki-67 (Agilent, diluted 1:30) and Phycoerythrein-CF594 (PE-CF594)-labeled Bcl-2 (BD Biosciences; diluted 1:60) for 15 min at RT, the cells were washed in 4 ml PBS, centrifuged for 5 min at 300×*g* and resuspended in 0.5 ml of PBS. The samples were analyzed within 2 h after immunolabeling.

Data collection was performed with the Navios™ Flow Cytometer in combination with the Navios Tetra software (Beckman Coulter). The instrument setup was performed according to standard procedures. Verification of the optical alignment and fluidics system of the Navios™ Flow Cytometer was performed using Flow-Check™ Pro Fluorospheres (Beckman Coulter). The verification of the compensation for each fluorochrome was established using Flow-Set™ Pro Fluorospheres (Beckman Coulter) and was performed weekly. A minimum number of 500,000 relevant events were aimed to be collected per tube. Relevant events were determined by excluding debris with the FSC-Area vs. SSC-Area plot without excluding erythroblasts.

For analyses of the erythropoiesis and in case of an insufficient number of events, multiple tubes of the same patient were merged by the Infinicyt 2.0 software package (Cytognos). A minimum of 1000 events of the cell population of interest were included in the "Merge and Calculate" strategy. Backbone markers were included according to the guidelines of the EuroFlow consortium [19]. Tubes 1, 2, and 5 contained the backbone markers FSC, SSC, CD13, CD34, CD45, CD117, and HLA-DR that allowed proper selection of homogeneous populations (blast cells, myeloid cells and monocytic cells). The antibody combination in tubes 3, 4, 6, and 7 were designed for the analysis of the Ki-67 proliferation index and Bcl-2 anti-apoptotic index during the maturation of the erythropoiesis, respectively. CD36-APC and CD71-APC-A700 showed significant spill over due to tandem degradation of the CD71-APC-A700 antibody, which could not be sufficiently compensated for. Tubes 3, 4, 5, and 6 contained the backbone markers FSC, SSC, CD33, CD45, CD117, and HLA-DR, which allowed proper selection of the erythroid cells before merging the different tubes for further analyses. This allowed the use of the "Merge and calculate"-function of the software program Infinicy 2.0 software package (Cytognos SL, Salamanca, Spain), for further definition of the erythroid cells based on their CD36 and CD71 expression and subsequent determination of the Ki-67 proliferative and Bcl-2 anti-apoptotic indices during the erythropoiesis.

Validation of the "Merge and Calculate" strategy for the different BM cell populations and comparison to the results of the double staining protocol is shown in Figure S1. For the validation, the Ki-67 positive and/or Bcl-2 positive fractions of five patient samples were used. These comprised 1 non-clonal cytopenic patient, 1 MDS patient

and two AML patients, and guarantee a broad range of values for subsequent linear regression. Furthermore, coefficients of variation (% CV) between the two methods were within acceptable ranges (10%–25%; 30%–35% for rare populations as described by Selliah et al.) in 77 out of 80 measurements (data not shown) [20]. Only the %CVs of the Ki-67 negative Bcl-2 negative blast population of the MDS and 2 AML patients exceeded these acceptable ranges, but were not considered clinically relevant deviations as this population was present in a low frequency in these patients. The high correlation between the results of the two methods and high similarity between the produced Ki-67 and Bcl-2 positive fractions validated the use of the "Merge and Calculate" strategy for determination of these fractions.

2.4 | Data analysis and gating strategy

An overview of the different hematopoietic cell lineages, their morphological sub-staging and the corresponding CD marker expression profiles are shown in Figure S2. Data analysis was performed with the Infinicyt 2.0 software package (Cytognos). The gating strategy utilized for the analysis of Bcl-2 expression in the various hematopoietic cell lineages is presented in Figure S3.

Briefly, single cells were gated by excluding debris and doublets (Figure S3A). Subsequently, blast cells and lineage-committed blast cells were gated. The blast cells were selected based on CD34-positivity and by selecting the blast region in the SSC vs. CD45 plot (Figure S3B). Lineage-committed blast cells included early erythroid progenitors, myeloblasts and monoblasts (Figure S3C). Early erythroid progenitors were defined by selecting the CD34-positive CD105-positive cells. Myeloblasts were gated based on their CD34-positivity and CD13-positivity. Monoblasts were selected by gating the CD34-positive and CD64-positive cells.

Subsequently, the cell populations of the erythropoiesis, myelopoiesis (neutrophilic granulopoiesis) and monopoiesis were gated (Figure S3C–E, respectively). Erythroblasts were selected by gating the CD45-negative and CD45 dim cells. CD33 negative and dim-cells were then selected for robust exclusion of myeloid cells. The erythroblasts were then separated from other contaminating myeloid

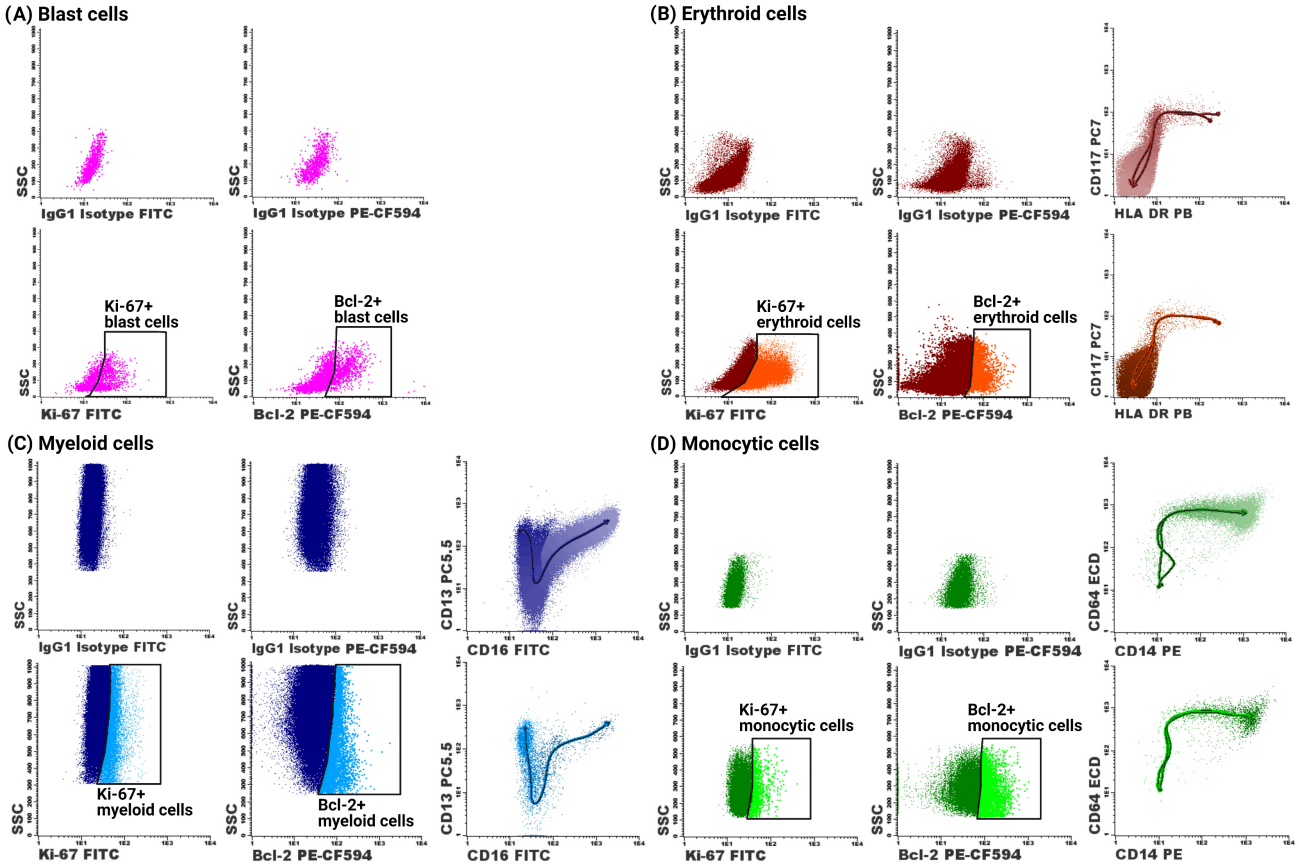


FIGURE 2 Gating strategy of Ki-67 and Bcl-2 in the blast cells (A) and during the erythro- (B), myelo- (C) and monopoiesis (D) according to the threshold that was based on their respective IgG1 isotype controls. After gating the Ki-67 or Bcl-2 positive fraction, the maturation pathway for the total cell population and for the Ki-67/Bcl-2 positive fraction was drawn in the Infinicyt software. For erythroid cells, the CD117 versus HLADR plot was used to draw the maturation pathway. The maturation of the myeloid cells was drawn by the CD13 versus CD11b plot or the CD13 versus CD16 plot. The maturation of the monocytic cells was plotted by assessment of the CD64 versus CD14 expression. After determining the total number of cells and the Ki-67/Bcl2 positive cells per maturation step of the erythro-, myelo- and monopoiesis, the Ki-67/Bcl-2 positive fractions of cells per maturation step was calculated.

cells, lymphoid cells and platelets. The myelopoiesis was gated by selecting the granulocytes in a SSC vs. CD45 plot, excluding basophils and other cell populations. Then the myeloid cells were selected, while monocytes and eosinophils were excluded. The maturation stages of the myelopoiesis were determined based on expression of CD13 and CD11b/CD16. Monocytes were selected in the SSC versus CD45 plot by backgating CD14-positive cells, while non-myeloid cells were excluded.

After defining the various cell lineages, the threshold set for the FITC- and PE-CF594-signal was based on the IgG1 isotype control and was used to determine the Ki-67 and Bcl-2 positive fraction (Figure 2). Maturation pathways were studied for the total cell population, and for the Ki-67 or Bcl-2 positive cells in each cell lineage [12]. The maturation pathways were all drawn by the use of the "Draw Maturation" function in the Infinicyt v2.0 software package.

Gating of cells that were simultaneously labeled with the Ki-67-FITC and Bcl-2-PE-CF594 antibodies was performed by plotting the IgG1 isotype control-FITC against the IgG1 isotype control-PE-CF594 for determining the thresholds for Ki-67 and Bcl-2 positivity (Figure 3). These thresholds were then used to gate the simultaneously labeled cells in a Ki-67 versus Bcl-2 plot.

2.5 | Statistical analysis

Statistical analysis was performed by use of the GraphPad Prism 8.0 software package (GraphPad Software, San Diego, USA). Continuous variables were expressed as mean ± standard deviation.

3 | RESULTS

3.1 | Ki-67 and Bcl-2 positive fraction of hematopoietic cell populations in normal bone marrow

The Ki-67 and Bcl-2 positive fractions of the blast cells (defined by CD34 positivity), erythroid, myeloid, and monocytic cells, determined as shown in Figure S1, are graphically depicted in Figure 4. The blast

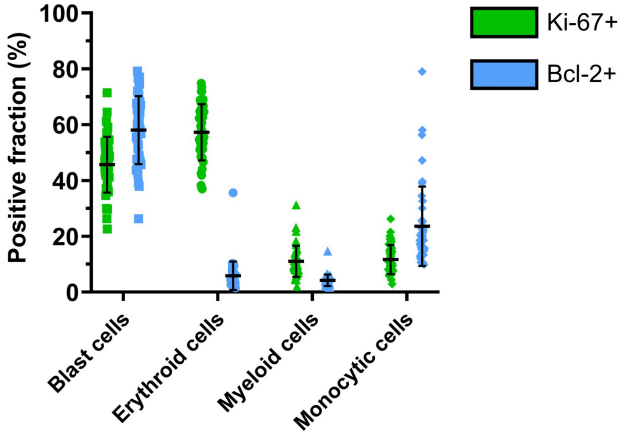


FIGURE 4 Graphic representation of the Ki-67 positive and the Bcl-2 positive fraction of blast, erythroid, myeloid, and monocytic cells. While the Ki-67 positive fraction was highest in erythroid cells, a low Bcl-2 positive fraction was observed. in myeloid cells, a low Ki-67 and low Bcl-2 positive fraction was seen. Monocytic cells displayed intermediate numbers of cells with expression of Bcl-2 and low numbers with expression of Ki-67. Blast cells showed an intermediate frequency of cells with expression of Ki-67 and Bcl2.

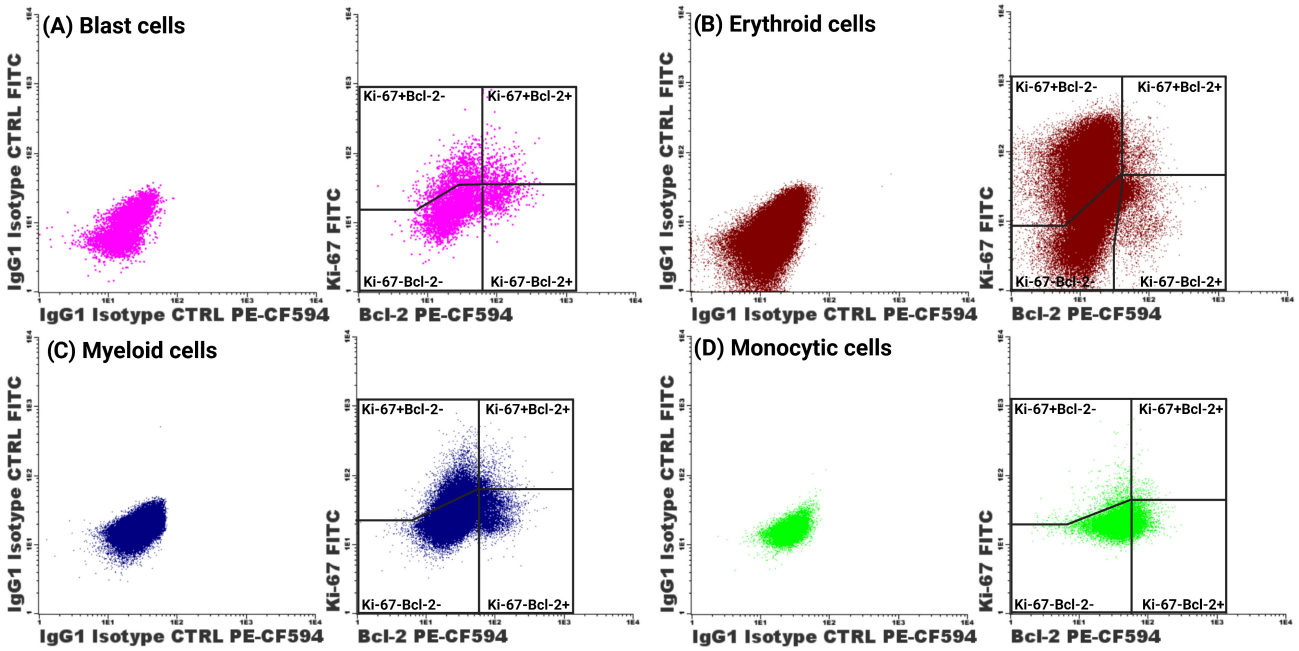


FIGURE 3 Gating procedure for the flow cytometric double staining of Ki-67 and Bcl-2 for (A) blast cells, (B) erythroid cells, (C) myeloid cells, and (D) monocytic cells. Gating thresholds were based on the IgG1 isotype control-FITC versus IgG1 isotype control-PE-CF594 plot for each of the cell populations. After determining these thresholds, the Ki-67 negative/Bcl-2 negative, Ki-67 positive/Bcl-2 negative, Ki-67 negative/Bcl-2 positive, and Ki-67 positive/Bcl-2 positive fractions were determined for the different cell populations.

cells showed a high Ki-67 positive fraction (46 ± 10%) and Bcl-2 positive fraction (58 ± 12%). The erythroid cells displayed a high Ki-67 positive fraction (57 ± 10%), in combination with a low Bcl-2 positive fraction (6 ± 5%). Myeloid cells showed a low positive fraction for both Ki-67 (11 ± 6%) and Bcl-2 (4 ± 2%). Finally, monocytic cells presented with a relatively low Ki-67 positive fraction (12 ± 5%), in combination with a moderate Bcl-2 positive fraction (24 ± 14%).

3.2 | Decreasing Ki-67 and Bcl-2 positive cell fractions during the erythro-, myelo- and monopoiesis

The mean proliferative and anti-apoptotic activity, as respectively determined by the Ki-67 and Bcl-2 positive fractions, during the erythro-, myelo- and monopoiesis (determined as shown in Figure 2) is shown in Figure 5. These maturation pathways were divided into 20 equal maturation stages by the Infinicyt software package. For stage 0, the Ki-67 or Bcl-2 positive fraction of the lineage-committed blast cells were used.

The early erythroid progenitors (Figure 5, left row; A) showed a Ki-67 positive fraction as well as a Bcl-2 positive fraction of almost 100%. When the proerythroblasts (B) mature to basophilic erythroblasts (C), the Ki-67 and Bcl-2 positive fraction remained around 90%. However, a low fraction of cells reside in these early maturation stages. When basophilic erythroblasts matured to polychromatophilic erythroblasts (D) and subsequently to orthochromatophilic erythroblasts (E), the Ki-67 and Bcl-2 positive fractions decreased further to 30% and 0%, respectively. The cell fraction increased

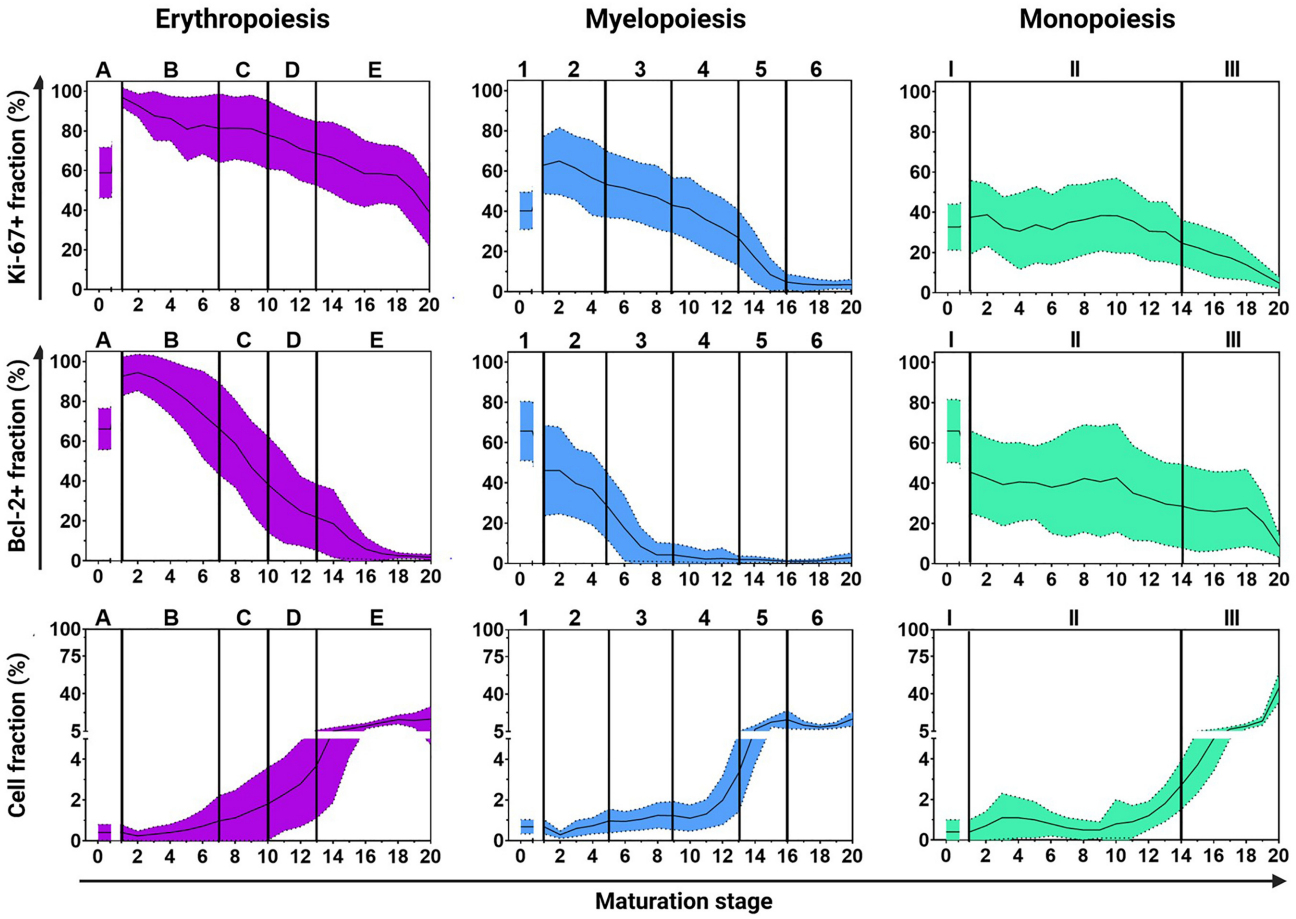


FIGURE 5 Ki-67 positive fractions and Bcl-2 positive fractions in the different maturation stages of erythro-, myelo- and monopoiesis. Black solid lines in each graph indicate the mean percentages of Ki-67 positive or Bcl-2 positive events for each maturation stage. The areas surrounding the solid line illustrate the standard deviation. For stage 0, the lineage-committed blasts of the erythro-, myelo- and monopoiesis were displayed. The Ki-67 and Bcl-2 positive fractions are highest in the early erythropoiesis (near 100%) and declined to 90%, 30% and 0%, respectively. During the early stages of the myelopoiesis, approximately 50% of the cells were positive for Ki-67, while 50% of the cells were positive for Bcl-2. Subsequently, the Ki-67 positive fraction rose slightly to 60%, followed by a gradual decline to 0%. The Bcl-2 positive fraction gradually declined to approximately 0% as the myeloid cells become mature. At stage 1 of the monopoiesis, the Ki-67 and Bcl-2 positive fractions were both 40%, and gradually declined to 0% as these cells undergo the maturation process. Morphological staging is indicated by capital letters, Arabic numbers and Roman numbers as follows. (A) early erythroid progenitors; (B) proerythroblasts; (C) basophilic erythroblasts; (D) polychromatophilic erythroblasts; (E) orthochromatophilic erythroblasts; 1: myeloblasts; 2: promyelocytes; 3: myelocytes; 4: metamyelocytes; 5: band cells; 6: neutrophils; I: monoblasts; II: promonocyte; III: monocytes.

simultaneously during these maturation stages from approximately 0.1%–25%.

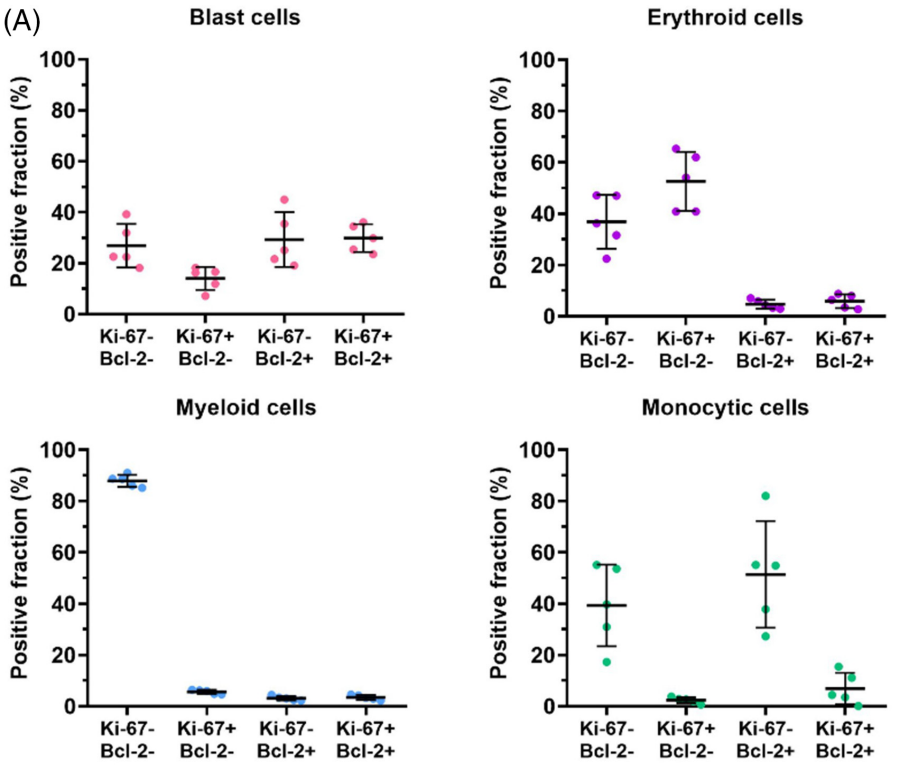
Myeloblasts (Figure 5, middle row, 1) started with a Ki-67 and Bcl-2 positive fraction that were both around 50%, while the fraction of myeloblasts from the total of myeloid cells is small (0.5%). The Ki-67 positive fraction in promyelocytes (2) slightly increased to 60%, while the Bcl-2 positive fraction slightly decreased to 40% and the cell fraction remained almost the same (approximately 0.5% on average). Upon maturation into myelocytes (3), metamyelocytes (4) and band cells (5), the Ki-67 fraction gradually decreased, reaching 0% when cells matured to neutrophils (6). The Bcl-2 positive fraction steeply decreased to virtually 0% when promyelocytes matured to myelocytes and remained very low or absent in the later maturation stages. Nevertheless, the cell fraction increased steeply during the maturation process of the myelopoiesis to 25%.

Monoblasts (Figure 5, right row, I) showed a Ki-67 and Bcl-2 positive fraction of about 50%, while this maturation stage comprised of a small fraction of cells (0.5%). When monoblasts matured to promonocytes (II), the Ki-67 and Bcl-2 positive fraction decreased to approximately 40%, while the cell fraction increased slightly to 1% on average. The Ki-67 and Bcl-2 positive fraction further decreased when cells became mature monocytes (III), ultimately reaching 0%–5%. During the maturation from promonocytes (II) to mature monocytes (III), the cell fraction increased from approximately 1%–40%.

3.3 | Simultaneous expression of Ki-67 and Bcl-2 in blast, erythroid, myeloid and monocytic cells

Flow cytometric double immunolabeling for Ki-67 and Bcl-2 expression in the blast cell compartment, erythroid, myeloid, and monocytic cells, determined as shown in Figure 3, reveals that a variable fraction of these cells can be simultaneously positive for both these cell biological markers (Figure 6).

Only the blast cells displayed a substantial fraction of cells that proliferate while simultaneously showing anti-apoptotic potential. All fractions of the blast cells were almost equally distributed in terms of Ki-67 and Bcl-2 positivity. Although erythroid cells are highly proliferative, only a limited number of cells contain the anti-apoptotic protein Bcl-2. Myeloid cells are mainly non-proliferative and do not express Bcl-2. However, monocytic cells highly express Bcl-2, with only a few cells being proliferative.



(B)

Population	Ki-67- Bcl-2-	Ki-67+ Bcl-2-	Ki-67- Bcl-2+	Ki-67+ Bcl-2+
Blast cells	27 ± 9%	14 ± 4%	29 ± 11%	30 ± 5%
Erythroid cells	37 ± 11%	53 ± 11%	5 ± 2%	6 ± 3%
Myeloid cells	88 ± 2%	6 ± 1%	3 ± 1%	3 ± 1%
Monocytic cells	39 ± 16%	2 ± 1%	51 ± 21%	7 ± 6%

FIGURE 6 Flow cytometric double immunostaining of blast cells, erythroid cells, myeloid cells and monocytic cells. (A) Graphical representation of double staining for Ki-67 and Bcl-2 in the different cell populations. Horizontal lines depict the mean fraction of cells, and the whiskers indicate the standard deviation. (B) Tabular visualization of double staining for Ki-67 and Bcl-2 in blast cells, erythroid cells, myeloid cells and monocytic cells. The blast cell population showed the largest fraction of Ki-67/Bcl-2 double positive cells of all myeloid cell compartments. In contrast, the committed cell populations (erythroid, myeloid, and monocytic cells) comprised almost no Ki-67/Bcl-2 double positive cells. Blast cells displayed an almost equal distribution of Ki-67 and Bcl-2 positivity. A large fraction of erythroid cells was Ki-67 positive, while little cells were Bcl-2 positive. The majority of myeloid cells were Ki-67/Bcl-2 double negative, while the other fractions were small. Monocytic cells displayed the largest Bcl-2 positive fraction of all cell populations, while only a small number of cells were Ki-67 positive.

4 | DISCUSSION

Using a fixation and permeabilization protocol for flow cytometry, the Ki-67 proliferative and Bcl-2 anti-apoptotic indices can be simultaneously determined in subsequent maturation stages of hematopoietic BM cell populations. This study thereby not only provides an indication of reference values for the Ki-67 and Bcl-2 positive fractions of the different hematopoietic cell lineages in BM of adults, but also yields separate data for the Ki-67 and Bcl-2 positive fractions during the erythro-, myelo- and monopoiesis. As such, a new dimension is added to the analysis of functional biological processes in a heterogeneous cell population such as the BM tissue, which in turn can lead to novel applications in clinical practice of BM derived benign and malignant diseases.

4.1 | Proliferative and anti-apoptotic activity in hematopoietic cell lineages of normal BM

In the CD34 positive blast cell compartment, the proliferative and anti-apoptotic fraction were approximately 50%. Simultaneous expression of Ki-67 and Bcl-2 was almost exclusively observed in the blast cell compartment, in contrast to the committed erythroid, myeloid and monocytic cell lineages. This indicates that blast cells are capable of proliferation, while simultaneously being protected by anti-apoptotic signaling. Existing literature hypothesized, based on *in-silico* modeling and the available knowledge about the BM endosteal and vascular niche, that blast cells are not exclusively in a quiescent state or proliferative state [21, 22]. Instead, a transitional state exists in which blast cells can be quiescent as well as proliferative simultaneously and these processes are delicately balanced to maintain tissue homeostasis. The relatively high percentage of proliferative cells in this cellular compartment that is also protected from apoptosis may be crucial to retain sufficient blast cell numbers. Our results confirm that these undifferentiated cells can be simultaneously proliferative, as well as being protected from apoptosis, which makes these cells more susceptible for tumorigenesis as compared to their more mature counterparts. Our data also confirms that proliferation and apoptosis are processes that are delicately balanced in order to maintain the normal tissue homeostasis, as an comparable number of cells showed (1) no Ki-67 and Bcl-2 expression, (2) either Ki-67 or Bcl-2 expression, and (3) simultaneous expression of Ki-67 and Bcl-2.

Similar Ki-67 positive fractions for all myeloid cell compartments were observed in an earlier study of our group [12]. However, prior studies by other investigators assessing the proliferative and anti-apoptotic fraction demonstrated significantly lower Ki-67 and Bcl-2 fractions in the CD34 positive progenitor cells (see for example Parker et al. [23]). This is most probably related to methodological differences and differences in determination of fluorescence thresholds. Defining gating thresholds for the Ki-67 and Bcl-2 positive cells is a crucial step for proper quantification of these fractions and is prone to subjectivity. Although Parker et al. [23] used the same fixation and permeabilization protocol as compared to the current study, incubation times and temperatures differed. In their study, BM cell suspensions were incubated in the permeabilization medium for 20 minutes at 4°C for intracellular staining with the monoclonal antibodies directed against Ki-67 and Bcl-2, while we incubated the cells for 15 min at RT. The lower incubation temperature in the study of Parker and colleagues [23] influences the binding capacity of the monoclonal antibodies negatively and explains their lower Ki-67 and Bcl-2 positive cell fractions. In addition, the study by Matarraz et al. [24] showed similar patterns for proliferation during the myelopoiesis, but approximately half the number of proliferating blast cells, myeloid and monocytic cells as compared to the present study. However, Matarraz et al. used normal as well as reactive BM samples for establishment of their reference values, while we exclusively used normal BM samples. Furthermore, a different BM cell harvesting and staining procedure was performed as compared to our study. This may explain the differences in the proliferating fractions between the study of Matarraz and colleagues and the present study.

4.2 | Proliferative and anti-apoptotic activity during the erythro-, myelo- and monopoiesis

With regard to the proliferative and apoptotic activity during maturation, the observed Ki-67 positive fractions in this study are in accordance with our previously published data [12]. The underlying study thus validates our earlier observation that the Ki-67 positive fraction is highest during erythropoiesis, followed by myelopoiesis and monopoiesis.

The proliferative and anti-apoptotic fractions during the early stages of the erythropoiesis mount up to almost 100% and it can thus be concluded that these cells are highly proliferative and at the same time conserved in their immature stages. This observation can be related to the high demand of the differentiated erythrocytes, while the fraction of erythroblasts in the BM is limited [25, 26]. Approximately 200×10^9 erythrocytes are produced by normal BM on a daily basis [27], meaning that a high level of conservation and proliferation is necessary in order to provide the blood with sufficient numbers of erythrocytes. The data on the final stages of the erythropoiesis must be interpreted with caution, since these cells are vulnerable to non-nucleated cell lysis that occurs during the fixation and permeabilization procedure. Hence, these results may be less representative of the true proportions in the BM [28]. Nevertheless, the Fixation and Permeabilization reagents were essential for the intracellular and intranuclear staining of Ki-67, Bcl-2 and their respective IgG1 isotype controls.

During the myelopoiesis, the proliferative and anti-apoptotic fraction are approximately 50% in the early stages of myelopoiesis. Furthermore, granulocytes lose Bcl-2 expression rapidly during their maturation, which is in accordance with expression patterns described in the previous literature [29]. The total fraction of myeloid cells in the BM is 50–60%, thereby representing the largest fraction of leukocytes in BM [26, 30]. The BM produces roughly $50–100 \times 10^9$ granulocytes per day, with a lifespan of less than 1 day [31]. However,

large numbers of mature neutrophils are stored in the BM [32]. The intermediate proliferative and anti-apoptotic fraction in maturing myeloid progenitor cells in combination with the large number of mature neutrophils present in the BM explain how the peripheral blood can be supplemented with sufficient numbers of these cells.

The proliferative and anti-apoptotic fractions during the monopoiesis are the lowest, which are around 40% in the very early stages of monopoiesis and gradually decrease to 0%. This is in accordance with the fact that only 2% of the total BM cells consists of monocytic cells. The demand for this subpopulation of leukocytes is relatively low and hence these cells are normally not highly proliferative while being moderately conserved [26, 30].

As the relative fraction of immature cells (which express high levels of Bcl-2) is extremely low compared to the fraction of mature cells, Bcl-2 positive cells are rarely encountered in histopathological samples of normal BM. In contrast, high levels of Ki-67 are observed in histopathological samples of this tissue. This can be explained by the relatively frequent expression of Ki-67 in the mature erythroid compartment and the fact that this is the most cell rich compartment in normal BM [33].

When interpreting these data, one has to realize that only part of the (extracellular matrix-bound) BM cells is assessed in our study and that some cell populations may be underrepresented. Particular cell types, such as blast cells and subsequent lineage-committed blast cells in the BM are known to interact with the extracellular matrix of the so-called BM niches. This interaction induces various processes, including anti-apoptotic signaling and differentiation [34]. In our protocol cells were flushed out of the BM tissue with a sodium citrate solution, which may leave behind cells that were strongly bound to the matrix of BM niches. The cells that remained bound to the matrix were thus not considered in the flow cytometric work-up of these aspirates. These strongly bound cells may, however, represent an interesting population, since these cells are likely to show undifferentiated, progenitor characteristics such as quiescence and can play an important role in the origin and maintenance of myeloid malignancies [34]. Studies in progress that specifically focus on retrieval of these matrix-bound cells should elucidate their behavioral characteristics and the clinical significance of this cell population.

4.3 | Clinical significance

By examining the proliferative and anti-apoptotic potential of normal maturing hematopoietic cells, a new dimension is added to the analysis of functional biological processes in a heterogeneous cell population such as that of BM tissue. Based on the studies more insight can be obtained in the biological background of BM malignancies, which in turn could lead to novel applications for clinical practice, including diagnostic and monitoring strategies. However, the use of clinical control samples instead of BM from femoral heads is recommended in order to accurately reflect the patient population that are admitted for BM investigation for diagnosis of specific myeloid malignancies [35]. A high level of standardization of the flow cytometric and gating procedure for clinical application is also recommended, as this reduces intra- and inter-assay/operator variability.

Effects of treatment modalities for myeloid malignancies can be predicted based on the proliferative and anti-apoptotic capacity in the different hematopoietic cell lineages [11]. Erythroblasts are found to be highly proliferative and, hence, therapies that counter-act on proliferating cells (e.g. chemotherapy or radiation) are highly toxic for these cells. These regimens induce excessive apoptosis of erythroblasts, which results in the commonly observed chemotherapy-induced anemia [36].

Since proliferation and apoptosis are processes that are tightly linked with response to chemotherapy regimens, introducing markers that can give information on these processes can lead to new therapy management strategies [37, 38]. Additionally, other treatment modalities such as the FDA approved Bcl-2 inhibitor Venetoclax and CAR-T cell therapy are known to induce apoptosis in malignant cells [39, 40]. Since our assay detects Ki-67 expression as well as the anti-apoptotic protein Bcl-2, it can potentially be used to predict treatment response to different combinations of these therapies.

Concluding, a novel application of this previously developed assay is presented, which allows simultaneous determination of the Ki-67 proliferative and Bcl-2 anti-apoptotic indices in maturing hematopoietic cell populations of the BM. These cells show differential expression levels of Ki-67 and Bcl-2 during their maturation process. Immature cells showed the highest fractions of Ki-67 and Bcl-2 expression and diminish during the maturation process of these cells. This study validates the previously established method for assessment of the Ki-67 proliferation index in maturing hematopoietic cells residing in the BM using multi-parameter flow cytometry. Next to those for Ki-67, an indication of reference values for the anti-apoptotic potential, as assessed by the Bcl-2 positive fraction, were established and further explain how tissue homeostasis is maintained in the BM in order to provide sufficient blood cells according to their life span and physiological demand. Future studies on the applicability of these markers in hematological malignancies can elucidate their value in a clinical setting.

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AUTHOR CONTRIBUTIONS

Stefan G.C. Mestrum: Conceptualization (supporting); data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); supervision (supporting); writing – original draft (lead); writing – review and editing (equal). **Roanalis B.Y. Vanblarcum:** Data curation (supporting); formal analysis (equal); investigation (equal); writing – original draft (supporting); writing – review and editing (supporting).

Roosmarie J.M.R. Drent: Formal analysis (equal); investigation (supporting); methodology (supporting); supervision (supporting); writing – original draft (supporting); writing – review and editing (supporting). **Bert T. Boonen:** Methodology (supporting); resources (equal); writing – original draft (supporting); writing – review and editing (supporting). **Wouter L.W. van Hemert:** Methodology (supporting); resources (equal); writing – original draft (supporting); writing – review and editing (supporting). **Frans C.S. Ramaekers:** Conceptualization (lead); data curation (equal); investigation (lead); methodology (equal); supervision (equal); writing – original draft (supporting); writing – review and editing (equal). **Anton H.N. Hopman:** Conceptualization (lead); data curation (equal); investigation (equal); methodology (equal); supervision (lead); writing – original draft (supporting); writing – review and editing (equal). **Mathie Leers:** Conceptualization (lead); data curation (equal); investigation (lead); methodology (equal); supervision (equal); writing – original draft (supporting); writing – review and editing (equal).

CONFLICT OF INTEREST

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SUPPORTING INFORMATION

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