Flow cytometric analysis of bone marrow (BM) samples is performed routinely for identification, frequency assessment, and further characterization of the various leukocyte subpopulations when abnormal hematopoiesis is suspected. The BM compartment is a complex tissue containing cells of multiple hematopoietic lineages with various maturational stages per lineage. In healthy individuals, the BM precursor cells guarantee continuous production of the various hematopoietic differentiation lineages. The differentiation and maturation can be monitored by changes in cytomorphology and immunophenotype. Especially in the B-cell lineage, multiple stages have been defined based on their immunophenotype (1–5). Also in the monocytic, granulocytic, erythroid and thrombocytic lineages several differentiation stages can be identified by immunophenotyping (6–8). Knowledge of the expression (levels) of various lineage-specific, immature, and mature markers in normal hematopoietic development provides a frame of reference for recognition of abnormal differentiation patterns.

Abnormal BM hematopoiesis may result from (1) an arrest during precursor B-cell differentiation; (2) BM regeneration after immunosuppressive therapy or as a response to infection; (3) dysplasia in one or more myeloid lineages; (4) increased turnover of hematopoietic cells.

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caused by peripheral cell depletion; or (5) unregulated expansion of neoplastic hematopoietic cells. Most of these abnormalities will present as major shifts in BM composition or as specific shifts in the relative distribution of subsets in one or more lineages. In the case of a B-cell differentiation arrest, the absence of the more mature B cells and the relative increase of precursor B cells will be the most striking observation (5). In regenerating BM after cytotoxic treatment, the immature precursor B cells will also be overrepresented, but mature B cells will still be detectable (9,10). In most BM-derived hematopoietic malignancies, such as precursor B-acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML), the relatively homogeneous leukemic cell population dominates the BM compartment with profound suppression of other hematopoietic lineages. These leukemic cells frequently have an aberrant immunophenotype not found in normal BM (11–16). Knowledge of the normal immunophenotypic differentiation patterns in BM facilitates recognition of ALL and AML cells, even if they occur at low frequencies (17–20).

Studies of normal BM hematopoiesis are improved by technological innovation of flow cytometry to the routine use of four-color analyses. To generate significant data from these multi-parameter analyses, it is important to attune the composition of the monoclonal antibody (mAb) combinations to the part of hematopoiesis under study: the relative distribution of the BM leukocytes over the major lineages; immature precursor cells; more mature differentiation stages; a specific lineage (B- or myeloid cells); or, a specific differentiation stage within a lineage.

Numerous leukocyte antigens have been studied extensively for their expression (levels) on various hematopoietic lineages and differentiation (11,21,22). It is not our aim to review the complete expression profiles of all leukocyte markers or to describe all minor BM subpopulations. We focus on a limited set of six four-color immunostainings, which were selected for rapid screening of all major leukocyte (sub)populations in BM in order to easily identify shifts or aberrancies in normal BM hematopoiesis (i.e., B-cell, myeloid, and erythroid lineages). We did not include immunostainings for identification of the natural killer (NK)/T-cell lineage, since NK/T-cell differentiation does not take place in BM.

**METHODOLOGY**

**Technical Considerations for the Design of the Four-Color Immunostainings**

The selection of the most informative mAb combinations should preferably be based on the properties of antibodies, antigens, and fluorochromes. First, several clones in the same cluster of differentiation (CD) recognize different epitopes or the same epitopes with different affinity (22). For example, CD15 mAb can recognize sia-lylated or nonsialylated epitopes, and CD34 antibodies (type I, II, and III mAb) recognize different epitopes with variable sensitivity for enzymes (23). Second, aspecific Fc-receptor (FcR) binding, causing an increased background signal, can be minimized by selecting mAbs of specific immunoglobulin subclasses or by using F(ab)_2_ fragments, thereby avoiding interaction with FcR’s (24). Third, most mAb are available with different fluorochrome-conjugates; the choice of fluorochrome-conjugate should depend on the expression level of the antigen. Because of their strong fluorescence signal, PE- and APC-conjugated mAb are the best choice for the detection of weakly expressed antigens, as well as for antigens with variable expression from weak to strong.

The mAb clones and fluorochrome-conjugates that were used for the six four-color immunostainings to assess the normal BM differentiation patterns are summarized in Table 1. Our standard immunophenotyping procedure uses ammonium chloride-lysed whole BM samples to prevent selective loss of cells and to conserve light scatter characteristics (25). Cells were permeabilized for intracellular immunostaining of TdT by using FACSBranding lysing solution (BD Biosciences; San Diego, CA) (26). Data were acquired on a FACS Calibur (BD Biosciences) using Cell Quest Pro Software and analysed using both Cell Quest Pro and Paint a Gate Pro software.

**Strategy for the Design of Four-Color Immunostainings**

In this report we present a limited set of six immunostainings that unravel the normal differentiation patterns within the four major hematopoietic lineages in BM. Because T-cell development normally does not occur in BM, no T- or NK-cell-specific immunostainings were included. In five of the six selected immunostainings, a lineage-specific marker is used to select the lineage of interest. The combination of CD19 or CD13, with side-scatter (SSC)
characteristics in a dot plot is useful to select and study the immunophenotypic differentiation patterns within the B cell and granulocytic/monocytic lineage, respectively. Apart from lineage-specific markers and immature or mature markers that are restricted to a particular cell lineage, markers with a much broader expression pattern can be useful in the study of normal differentiation patterns in BM. The pan-leukocyte marker CD45 gives additional information about the major subpopulations, as the CD45 antigen is differently expressed in various lineages and differentiation stages (6,27,28). CD45 expression is high on lymphocytes and monocytes, whereas granulocytes, precursor B cells, precursor granulocytic cells, and proerythroblasts are also CD45 positive, but at lower levels (29,30). In contrast, (more) mature erythroid cells are generally CD45 negative (6,31). Therefore, when combined with SSC characteristics, CD45 can help distinguish the major leukocyte (sub)populations in BM. CD34 is another non-lineage-restricted marker, expressed by the precursor stages of the various cell lineages in BM (32,33). The normal differentiation patterns within a specific lineage can be displayed by the application of “dynamic” markers, i.e., markers that are gradually up- or downregulated during differentiation. The immunophenotypic changes are best illustrated when mAb directed against markers with overlapping expression profiles are combined in one staining. For example, CD10 and CD20 (in B-cell development) and CD13 and CD16 (in myeloid development) are informative combinations (4,7). Ideally, the selected antibody combinations cover multiple consecutive differentiation stages, resulting in dot plots that provide direct insight in the completeness and relative distribution of the major subpopulations in the differentiation lineage under study.

The composition of the selected immunostainings was based on our extensive experience obtained during the last decade with 900–1,200 BM samples analysed each year. All flow cytometric analyses of BM samples were performed routinely with three-color immunostainings during 1996–1999 and with four-color immunostainings from 1999 until the present.

We selected a set of six four-color immunostainings for the analysis of the B-cell, monocytic, granulocytic and erythroblast cell lineages. Per lineage, the major subpopulations are described and illustrated in the respective plots and differentiation schemes. Normal age-related variations in the expression profiles are discussed followed by examples of disease-induced shifts. We also discuss some alternative markers or four-color immunostainings that can be used for the same purpose.

**BONE MARROW DIFFERENTIATION PATTERNS**

**B-Cell Differentiation**

Normal B-cell differentiation. Early B-cell differentiation has been extensively studied in BM using flow cytometric immunophenotyping. Initially, most studies focused on precursor B-acute lymphoblastic leukemia (precursor B-ALL) to unravel early B-cell development. Later, studies on the B-cell compartment in normal BM further unraveled the consecutive expression of antigens (2–5,20). Refinements in multiparameter flow cytometry allowed the analysis of large numbers of B cells by which minor B-cell populations could be identified (4,5,34). The two selected quadruple immunostainings have proved extremely useful for fast screening of the BM B-cell compartment, enabling the identification of (pathological) abnormalities in B-cell differentiation.

**Immunostaining 1: TdT/CD20/CD19/CD10.** The combination of CD10 and CD20 with a pan-B-cell marker (CD19) is a well-established combination for analysis of B-cell differentiation (2,4). Within the CD19+ B cells, at least four sequential maturation stages can be discriminated based on CD10 and CD20 expression (Fig. 1A: stages I-IV). These four differentiation stages form a continuous staining pattern in a CD10/CD20 plot (Fig. 1D) as a result of the stepwise loss of CD10 and the gradual gain of CD20 during maturation. Plasma cells, when present, reveal a minor discrepant population in this dot plot, being CD10-CD20+. As both CD10 and CD20 show a wide range in expression levels during B-cell differentiation, APC- or PE-conjugates of these mAbs are preferred for discrimination of the various subpopulations. Adding TdT to the CD10/CD20 combination of mAbs enables a better discrimination between the immature CD10bright cells that are TdT- and the more mature CD10- cells that are TdT+ (Fig. 1C). As detection of TdT concerns an intracellular staining, the addition of TdT delays the otherwise very quick screening. In our opinion, however, the advantage of a better discrimination between the CD10- precursor B-cell populations compensates the more complex intracellular staining. First, a low expression of TdT combined with a high CD10 expression is characteristic for precursor B-ALL (20). Second, the ratio between TdT-CD10+ and TdT+CD10+ precursor B cells can be indicative for (drug-induced) regeneration of BM (9,10).

**Immunostaining 2: CD45/CD34/CD19/CD22.** The second quadruple staining concerns the combination of CD45 with CD22, and CD45 and CD19. Again, four major B-cell subpopulations (Fig. 1A: stages I-IV) can be discriminated within the CD19+ B cells. The most immature B cells from the first immunostaining (TdT-CD10- cells) globally correspond to the CD34+CD22-CD45dim cells in this immunostaining (Fig. 1E,F). The CD45 expression increases during maturation followed by an increased CD22 expression (Fig. 1F). The mature CD10-CD20+ B cells from the first immunostaining globally correspond to the CD22brightCD45bright cells in the CD22/CD45 dot plot. When present, CD22+CD19-CD34+ pro-B cells can be identified as a minor population in addition to the earlier described four stages of B-cell differentiation (5,25).

In a standardized setting, with a calibrated flow cytometer and using the same mAb clones, the staining patterns from both immunostainings as displayed in the respective plots are quite stable. Shifts in the relative sizes of the subpopulations or a different location in the plot may therefore indicate (ab)normal variations or aberrancies in the B-cell development.
Normal variations and abnormalities in B-cell differentiation. The expression profiles from the two selected immunostainings are quite stable, but shifts in the relative sizes of the subpopulations can be found dependent on the age of the individual (4). In children the CD10⁺ subpopulations predominate, while in adults the CD10⁻CD20⁺ B cells are more frequent. In the elderly (generally >75 years), the mature B-cell population can even occupy most of the BM B-cell compartment (Fig. 2A–C). Moreover, the CD10⁻CD20⁻ plasma cells represent a substantial subpopulation of the CD19⁺ B cells in the BM of an older person (cyan colored). These age-related shifts should be regarded as normal.

Irrespective of age, shifts in the relative distribution of the (precursor) B-cell subpopulations can also be found in regenerating BM (Fig. 3A–C). Precursor B-cell regeneration can be observed after chemotherapy for acute leukemia or during temporary therapy stops. Differences in the precursor B-cell regeneration patterns are known to be related to the intensity of the preceding treatment (9,10). Following a high-intensity therapy block such as induction treatment of ALL patients, the more immature TdT⁺CD10⁺ precursor B cells dominate over the TdT⁻CD10⁺ precursor B cells during BM regeneration, whereas the ratio between the TdT⁺CD10⁺ and TdT⁻CD10⁺ precursor B cells is inversed when the preceding therapy block has been less intense (9).
FIG. 2. Age-related shift in B-cell compartment of elderly individuals. In bone marrow (BM) of elderly individuals, the relative size of the (CD10⁻) precursor B-cell population is decreased when compared to BM of young individuals (see Fig. 1). The CD19⁺ B-cell population in the adult BM (A) is predominated by the more mature TdT⁺ CD10⁻ CD20⁺ B cells (B,C). The cyan dots represent the plasma cell population (CD19⁺ CD10⁻ CD20⁻ TdT⁻), which can be a substantial subpopulation in the BM of elder individuals. Note the change in using CD19-PE instead of CD19-PerCP, and CD20-PerCP instead of CD20-PE.

FIG. 3. Regenerating precursor B cells in bone marrow (BM) after cytotoxic treatment. In BM from a patient treated for precursor B-acute lymphoblastic leukemia (BALL), massive regeneration of the precursor B-cell compartment is seen as soon as the therapy is (temporarily) stopped after induction treatment. Analysis of CD19⁺ B cells (A) shows high numbers of (nonleukemic) CD10⁺ precursor B cells in the BM sample, with relatively low numbers of more mature CD10⁻ CD20⁺ B cells (B,C).

FIG. 4. Alternative immunostainings for normal B-cell development in bone marrow (BM). Alternative immunostainings that show the different stages in B-cell development are CyIg/H9262/SmIgM/CD20/CD10 and SmIg/SmIg/H9260/SmIg/H9261/CD20/CD10. When gated on CD10⁻ and CD20⁻ small cells (lymphocyte scatter) (A,B), the stepwise gain of CyIg and gradual increase in SmIgM expression is observed (C) using alternative staining CyIg/SmIg/CD20/CD10: CyIg⁺ CD10⁻ CD20⁺ precursor B cells differentiate to mature SmIgM⁺ CD10⁻ CD20⁺ B lymphocytes. Using a comparable gating strategy, the differentiation into either Igκ⁺ or Igλ⁺ positive B cells can be shown (D) using alternative staining SmIgκ/SmIgλ/CD20/CD10.
Besides the shifts in the relative sizes of the subpopulations, shifts in expression levels of markers can be observed. This should be considered with great suspicion. Precursor B-ALL frequently display aberrant marker expression by which the malignant population will occupy a so-called empty space of the dot plot template: the cells are located in a space where normally no cells are found (20). Examples include very high expression of CD10 combined with low expression or negativity for TdT or low expression or negativity for CD45 combined with a relatively high CD22 expression (20,35,36).

**Other possible quadruple immunostainings for studying B-cell differentiation.** The two selected quadruple immunostainings (i.e., TdT/CD20/CD19/CD10 and CD45/CD34/CD19/CD22) are efficient for first screening of the B-cell compartment in BM. They quickly reveal shifts or abnormal patterns in the B-cell differentiation profile, which then need to be further analyzed in detail. It is obvious that these two immunostainings are not the only possibilities for screening of the B-cell compartment in BM.

In combination with CD10, CD20, and TdT, one can argue the use of CD22 instead of CD19, as a (minor) subpopulation of precursor B cells (pro-B cells) is known to be CD19 negative (5). On the other hand, normal plasma cells are generally CD22 negative (37,38). Since both CD19− and CD22− B cells concern only minor subpopulations of B cells, both markers are useful as pan-B-cell markers for the major B-cell subpopulations. In the two B-cell immunostainings we prefer to use CD19 as the pan-B-cell marker for gating the B-cell lineage, because of its homogeneous expression. The expression of CD22 is much more heterogeneous, varying from dim to bright positive, as is illustrated in Figure 1F. This heterogeneous expression and the expression of CD22 on basophils (39) makes CD22-based B-cell gating more complicated. In some cases, relatively high background staining of CD19 can be seen on monocytes: this is caused by binding of the mAb to FcR. This background staining generally does not affect B-cell gating if a combination of CD19 positivity with low SSC characteristics is used as a selection gate.

As indicated, the intracellular TdT staining may delay the screening process. For reasons of efficiency, the immunostainings CD45/CD20/CD19/CD10 or CD34/CD20/CD19/CD10 will be reasonable alternatives to discriminate the various immature B-cell subsets, taking into account that the expression patterns of TdT, CD45 and CD34 are different (Fig. 1).

CD10 and CD20 can also be combined with many other differentiation-stage specific markers like cytoplasmic immunoglobulin μ chain (CyIgμ), surface membrane (Sm)IgM or SmIg light chain. These immunostainings will give extended information about the maturational stage of the various subpopulations (Fig. 4), but will not identify additional major B-cell subpopulations in BM.

**Monocytic Cell Differentiation**

**Normal monocytic differentiation.** Immunostaining of cells of the monocytic lineage can be hampered by the aspecific binding of mAb via FcR on monocytes or by autofluorescence of the monocytes. For specific staining of more mature monocytes, IgG2a mAb should be avoided whenever possible because monocytes express high levels of the IgG2a binding FcR’s (CD64) (24).

The most immature monocytic precursor cell (monoblast) can immunophenotypically not be discriminated from the most immature granulocytic precursor cell (myeloblast) or from their common progenitor. Immunostaining 3 was selected to identify the myeloid/promonocytes. Two additional differentiation stages can immunophenotypically be identified with an immunostaining specific for monocytic differentiation (Fig. 5A; immunostaining 4).

**Immunostaining 3. CD34/CD117/CD45/CD13.33.** Both CD33 and CD13 are specific markers for the granulocytic and monocytic lineage. As their expression level is heterogeneous during differentiation, a mixture of CD13 and CD33 mAb was used in this third immunostaining to optimize the staining of all cells that belong to the granulocytic and monocytic lineage.

For the identification of myeloid/promonoblasts, a combination of CD45 expression and SSC pattern is useful in the gating procedure (Fig. 5B). A dim expression of CD45 is seen on generally all precursor cells in BM: myeloblasts, monoblast, precursor B cells, and erythroblasts. CD34 is expressed on precursors-cells of granulocytic, monocytic and B-cell lineage, while CD13.33 and CD117 are expressed predominantly on precursor cells of the granulocytic/monocytic lineage. CD117 is also expressed on the (CD34dimCD13.33−) promyelocytes (Fig. 5C), on a minor subpopulation of precursor B cells and at very high levels on mast cells (40–42). The latter populations is however hardly detectable in normal BM. The CD34+CD117−CD45dimCD13.33+ cells in this immunostaining represent the myeloid/promonoblasts, while the majority of CD34+CD117−CD45dimCD13.33− cells are precursor B cells (Fig. 5B.D).

**Immunostaining 4. CD14/CD33/CD45/CD34.** The progression from monoblast to promonocyte is marked by an increase in CD33 expression and the disappearing of CD34 expression, while the cells maintain intermediate levels of CD45 (Fig. 5E,G). The CD34−CD33high/CD45intermediate promonocytes express low levels of CD15 on their surface. Subsequently, the CD45 expression is increased and the cells gain CD14 (Fig. 5F). This CD14 expression marks the monocytic stage. In contrast to the neutrophils, the monocytic cells retain HLA-DR during their maturation.

**Normal variations and abnormalities in monocytic development.** Shifts in the relative distribution of the different immunophenotypically identified monocytic subpopulations are rarely seen as a result of normal (age-related) variations. Monoblasts or promonocytes are overrepresented in cases of monoblast-predominant AML (AML-M5a) or promonocyte-predominant AML (AML-M5b), respectively (Fig. 6). Consequently, the shifts in the immunophenotypic profiles will be as prominent as the
shifts in the relative frequency of the leukemic cell population. Maturation asynchronisms such as coexpression of CD34/CD11b or CD34/CD15; cross-lineage marker expression such as CD19, CD2, or CD7; and aberrant intensities of expression may be correlated with the prognosis of the disease in AML (16,17,43).

In cases of myeloid dysplastic syndrome (MDS) shifts in the frequency of monocytes (in normal BM, 1–8%) can be seen. Either monocytosis or a lack of monocytes can distinguish different types of MDS (44,45). For those cases, immunostaining 4 (CD14/CD33/CD45/CD34) is useful to quantify the CD14+/CD45dim monocytes.

Other possible quadruple immunostainings for studying monocyctic differentiation. For evaluation of monocyctic development, CD36, CD64, or CD4 (dimly expressed on monocytes) can be used in addition to CD14. CD36 expression and dim CD4 expression often precede CD14 expression and may indicate the monocytic differentiation of an otherwise CD34+ AML. In addition, CD68 is useful to identify the final stage of monocyctic differentiation, i.e., macrophages. With their increased FSC and SSC, macrophages can be distinguished from their precursors. However, in BM generally no macrophages are detectable.

As mentioned before, transient dim CD15 expression is seen on cells of the monocyctic lineage preceding the CD14 expression and is only detectable with high affinity CD15 mAb (22,24). However, low expression of CD15 on CD34+ precursor cells identifies not only commitment to monocyctic development, but also granulocytic precursor cells in transition from CD34+/CD15− blasts to CD34+/CD15+ promonocytes (46).
Granulocytic Cell Differentiation

**Normal granulocytic (neutrophil) differentiation.**
The granulocytic differentiation in BM generally concerns neutrophil differentiation, as eosinophils and basophils comprise only minor subpopulations in normal BM. As mentioned before, the most immature granulocytic precursor cell (myeloblast) cannot be discriminated immunophenotypically from the most immature monocytic pre-
...cursor cell (monoblast). One of the two immunostainings we selected for the monocytic differentiation (immunostaining 3) identifies both the monocytic and granulocytic precursor cells, while the other (immunostaining 5) unravels the further granulocytic differentiation.

**Immunostaining 3:** CD34/CD117/CD45/CD13.33. For screening of abnormalities in the immature granulocytic/monocytic precursor cells, we selected a quadruple staining that covers the early precursors of both lineages: combination of the precursor markers CD34 and CD117 with CD45 and a mixture of CD13 and CD33 will reveal eventual shifts in the relative size or immunophenotype of the myelo/monoblasts (Fig. 5B–D). This immunostaining also identifies promyelocytes as CD117+/CD34dim/CD13.33high cells with an increased SSC.

**Immunostaining 5:** CD16/CD13/CD45/CD11b. The second quadruple immunostaining for the granulocytic differentiation concerns the combination of CD45 and CD13 with CD16 and CD11b. An intermediate CD45 expression discriminates the cells of the granulocytic lineage from the CD45high lymphocytes and monocytes. CD45 expression, in combination with the granulocytic/monocytic lineage marker CD13 and light scatter characteristics, is useful to select and study the granulocytic differentiation patterns (45,47).

CD13 is a unique marker in the granulocytic differentiation as it is dynamically expressed during granulocytic differentiation. It is expressed at high levels on myeloblasts and promyelocytes. CD13 is downregulated and dimly expressed on myelocytes and is gradually upregulated again as the granulocytic cells develop into segmented neutrophils (Fig. 7D). Combination of this dynamic marker with CD11b and CD16 in one immunostaining reveals four sequential maturation stages in the neutrophil development (Fig. 7A; stage II–V; Fig. 7E–G). CD11b and CD16 are initially expressed at low levels, but their expression increases during the development process, particularly in the last 2 stages (stages IV and V; Fig. 7E).

With the quadruple immunostaining 5 (CD16/CD13/CD45/CD11b), the myelo/monoblasts can be identified as CD16−CD13+/CD45intermediate−CD11b−. They have an intermediate forward scatter (FSC) but still a low SSC and globally correspond to the CD34+/CD117+/CD45intermediate−CD13.33− cells from immunostaining 3 (Fig. 7). When the myeloblasts progress to the stage of promyelocytes, a dramatic increase in SSC is seen (Fig. 7B,D). From this moment, the cells express CD15 and retain their high SSC. In the subsequent maturation stages CD11b is acquired, followed by CD16 (Fig. 7E–G). Note that the relatively high fluorescence signal for CD16-fluoroscein isothiocyanate (FITC) of promyelocytes and myelocytes in Figure 7E,G has nothing to do with early CD16 expression but with the high autofluorescence of these subpopulations in the FL1 (FITC) channel.

Like B-cell development, the staining patterns of immunostaining 5 for normal granulocytic differentiation in BM, are quite stable with regard to the immunophenotypic characteristics and relative distribution of the various sub-populations (provided that the same mAb clones are used).

**Normal variations and abnormalities in neutrophil differentiation.** The myeloid differentiation mainly concerns the maturation to neutrophilic granulocytes, as the other end-stage granulocytes (i.e., the basophils and eosinophils) comprise only minor cell populations in normal BM. However, as a result of immune responses to specific agents or in case of a chronic myeloid leukemia (CML), shifts can be observed in the relative distribution of the various end-stage granulocytes (48,49). In BM of a patient with a CML-accelerated phase, eosinophils and basophils can be identified as prominent subpopulations of the granulocytic lineage (Fig. 8). On the FSC/SSC dot plot, eosinophils show a high SSC and lower FSC compared to the neutrophils (Fig. 8A). Expression of CD16 on eosinophils is dim and also expression of CD11b and CD13 is somewhat lower compared to neutrophils (Fig. 8C). In contrast, CD45 expression on eosinophils is consistently higher then on neutrophils (Fig. 8B). Basophils have a much lower SSC compared to the other granulocytic subpopulations and are hard to distinguish from lymphocytes and, to a lesser extend, monocytes based on light scatter characteristics. An intermediate CD13 expression on basophils (high on monocytes and absent on lymphocytes) and a lower CD45 expression (Fig. 8B), help identify these cells. Furthermore, basophils have a unique immunophenotype, different from the other granulocytic cells. They express CD22, but are negative for other B-cell markers (39). An increase in the relative size of the basophilic cell population can be seen in the chronic and accelerated phase of CML (48).

Shifts in the relative distribution of the granulocytic subpopulations and in the immunophenotypic characteristics are seen in myeloid malignancies like AML and myeloid dysplastic syndrome (MDS) (50,51). An increase of immature granulocytic precursor cells or a reduction in well-differentiated granulocytes can be seen in both diseases. The two immunostainings were not selected for the identification of all sorts of immunophenotypic aberrancies that can be expected in patients with AML or MDS, but they are useful for a quick screening of BM for such abnormalities. First, immunostaining 3 (CD34/CD117/CD45/CD13.33) will illustrate that most CD34+ precursor cells in MDS or AML BM belong to the myelo/monocytic lineage, and not to the B-cell lineage, as they are CD117+ and/or CD13.33+. In addition, the intermediate CD45 expression in combination with SSC identifies increased numbers of myelo/monoblasts in both immunostainings. Second, morphological abnormalities like hypo- or hypergranulation will be reflected in reduced or increased SSC values (Fig. 9). Third, in MDS an overexpression of CD13 and CD33 and a reduced expression of CD11b and CD16 is frequently seen and consequently the CD13/CD16 and CD13/CD11b dot plots of immunostaining 5 will show an aberrant expression profile of the granulocytic cells (Fig. 9).
Other possible quadruple immunostainings for studying granulocytic differentiation. As mentioned before, both CD33 and CD13 are specific markers for the granulocytic and monocytic lineage that can be expressed heterogeneously during differentiation. To optimize the staining of all cells that belong to the granulocytic and monocytic lineage, a mixture of CD13 and CD33 mAb can be used. An aberrant cross-lineage expression of CD13 and/or CD33 is seen in 40–60% of precursor B-ALL patients. CD65 can be used as an alternative pan-myeloid marker to cover a large part of the granulocytic differentiation from myeloblasts, until end-stage granulocytes.

Although described as a pan-granulocytic marker, CD15 expression is not confined to the granulocytic differentiation. In precursor cells committed to the monocytic differentiation (like promonocytes), CD15 can be expressed at low levels. Low-affinity mAb will only stain the granulocytic lineage, while high-affinity mAb (e.g., clone MM1; BD Biosciences) will also stain the immature monocytic cells. This mAb is useful in combination with CD34 and monocytic markers to identify the monocytic precursor cells.

CD66b expression precedes CD16 expression comparable to CD11b, being present on (pro-) myelocytes. Consequently, CD66b in combination with a pan-myeloid marker, like CD13 or CD33, will also reveal different stages in the granulocytic differentiation and is therefore an alternative mAb for CD11b. We selected the combination of CD11b with CD16 as CD11b distinguishes the early differentiation stages in granulocytic differentiation very well, whereas CD16 distinguishes the more mature differentiation stages very well. In addition to CD16 and CD11b, other markers that are not restricted to the granulocytic or monocytic lineage, like CD64 or CD35, will also show variations in their expression levels during granulocytic differentiation and are useful in combination with CD13 and/or CD33 (52).

Erythroid differentiation

Normal erythroid differentiation. To minimize noise when measuring leukocytes, erythrocytes in BM samples are generally lysed. The many commercially available permeabilizing or lysing reagents and ammonium chloride (NH₄Cl) preserve the lyse-resistant nucleated red cells of the erythroid lineage for immunophenotypic analyses.

Fig. 8. Eosinophilic and basophilic granulocytes in bone marrow (BM) of a chronic myeloid leukemia (CML) patient. In BM of a patient with CML-accelerated phase, relatively high numbers of eosinophilic (yellow dots) and basophilic (red dots) granulocytes can be identified. Eosinophilic granulocytes occupy a discrepant position in the forward scatter/side scatter (FSC/SSC) dot plot (A) and show a CD45 expression comparable to monocytes (B; blue dots). Basophilic granulocytes are hard to distinguish from lymphocytes in a FSC/SSC dot plot (A; green dots). An intermediate CD45 expression comparable to neutrophilic granulocytes however, discriminates the basophilic granulocytes (B). In the CD11b/CD13 dot plot, the basophilic and eosinophilic granulocytes show a slightly lower CD11b expression than the mature neutrophilic granulocytes (C).

Fig. 9. Hypogranular granulocytes in bone marrow (BM) of a MDS patient. BM of an MDS patient displays various aberrancies in the granulocytic lineage. Hypogranularity of the granulocytes results in a reduced SSC (A). Furthermore, the staining patterns in the CD11b/CD13 and CD13/CD16 dot plots are clearly changed with strongly reduced promyelocytic and myelocytic subsets (B, C).
**Immunostaining 6. CD71/CD235a/CD45/CD117.**

This selected quadruple immunostaining clearly identifies two sequential differentiation stages of nonlysed nucleated erythroid cells in BM samples after red cell lysis (Fig. 10A). Cells of the erythroid lineage are generally identified by the gradual loss of CD45 (6). Only the CD117⁺ CD71⁺ erythroid-precursors and pro-erythroblasts do express CD45 dimly. During maturation, the expression of the transferrin receptor CD71 slightly increases, whereafter CD235a (glycophorin A) is being expressed. The red cells retain CD235a but lose CD71 together with loss of the nucleus (6,31).

**Normal variations or abnormalities in erythroid development.** The nucleated erythroid population can be much more predominant in cases of a severe aplastic anemia or MDS, or during BM regeneration after drug-induced BM suppression. In regenerating BM of children treated for ALL, the erythroid cell population can occupy up to 50% of the cells in the lysed BM sample (53). These CD45⁺ CD71⁺ CD235a⁺ cells include both the nucleated erythroid cells and the reticulocytes, which tend to be more resistant to lysis when present in high numbers in young children (Fig. 11A–C). Although shifts in the relative frequency of nucleated red cells can be seen in regenerating

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**Fig. 10. Normal erythroid development in bone marrow (BM).** A: In this scheme for normal erythroid development in BM, differentiation stages are depicted that can be identified by the selected four-color immunostaining 6. The colors in the differentiation scheme globally correspond to the colors in the underlying dot plots and represent different subpopulations. When gated on SSClow in combination with CD45neg-low, only the erythroblasts are seen in normal BM (B,C). Pro-erythroblasts will form a minor population in normal BM and erythrocytes are lysed in the sample procedure.

**Fig. 11. Erythroid development in bone marrow (BM) of a patient with regenerating BM.** In regenerating BM of a patient treated for acute lymphoblastic leukemia (ALL), three differentiation stages can be discriminated in the erythroid development: CD117⁺ CD45−/− CD71⁺ CD235a⁺ pro-erythroblasts (red dots), CD117⁺ CD45−/− CD71⁻ CD235a⁺ erythroblasts (green) and CD117⁺ CD45−/− CD71⁺ CD235a⁻ nonlysed erythrocytes (blue dots) can be discriminated.
BM, severe anemia, megaloblastic anemia or brisk hemolytic anemia, or BM of very young children, immunophenotypic shifts as a result of aberrant marker expression are rare. In contrast, in MDS patients aberrant expression of CD71 and/or CD235a is frequently observed (50).

CONCLUDING REMARKS

We present six quadruple immunostainings that detect all major leukocyte populations in BM. All six quadruple immunostainings reveal remarkably stable immunostaining patterns and are therefore useful as a frame of reference for quick identification of shifts in normal BM development. These shifts can either be age-related or disease- or drug-induced. Age-related shifts in normal BM development generally concern shifts in the relative distribution of the various subpopulations within the respective lineages. Although several subpopulations may become more predominant than others during aging, the shape of the immunophenotypic staining pattern remains unaltered. Alterations in the relative distribution of the subpopulations can also be seen in regenerating or reactive BM when erythroid, precursor B-cell or myelo/monoblast populations are clearly overrepresented. In most cases of malignant hematopoiesis, such as MDS or acute leukemia, the aberrant immunophenotype often positions the malignant cell population at a separate place in the dot plot. Such immunophenotypic shifts should therefore always be considered as an abnormality in the hematopoiesis. Further detailed flow cytometric analyses of the relevant cell lineage is then recommended.

In this report we do not include immunostainings for identification of the NK/T-cell lineage, since NK/T-cell differentiation does not take place in BM. The T-cell population found in normal BM comprises mature blood derived T lymphocytes. T-cell immunostainings of normal BM samples will therefore reveal nondynamic immunostaining patterns with a homogeneous T-cell population and no clear subpopulations other than CD4+ and CD8+ T lymphocytes. Adding a single T-cell marker to the six selected quadruple stainings will not give additional information, as changes in the relative number of T cells in the BM will mainly reflect the quality of the aspired BM. However, for screening BM involvement in immature or mature T-cell malignancies, we suggest the addition of two specific T-cell immunostainings: CD2/CD5/CD3/HLA-DR (19).

Reference values for the major leukocyte subpopulations in BM are only partly available, but the dot plot templates of the six selected immunostainings can serve as a reference. The normal immunophenotypic expression profiles help identify normal and abnormal variations in hematopoiesis and are therefore useful as first screening.

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LITERATURE CITED


