Increased myeloid precursors in regenerating bone marrow; implications for detection of minimal residual disease in acute myeloid leukemia

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Presented study is focused on exact immunophenotypic definition of myeloid precursors and their following stages in regenerating bone marrow during treatment of ALL/AML for correct interpretation of the immunophenotype results and proper distinction from minimal residual disease (MRD) by multiparameter flow cytometry.

This study includes bone marrow samples from 36 controls, 27 patients with AML, 39 patients with B-ALL undergoing therapy who remained in complete remission after treatment and also 30 B-ALL patients one year after the end of therapy. We observed substantial expansion of immature bone marrow populations in the regenerating bone marrows, which were identified by expression of CD34 and/or CD117 markers by 4-color flow cytometry. Myeloid precursors were significantly increased after cessation of induction therapy cycle of B-ALL (1.27±2.04%, p=0.0064) and also AML patients (0.87±0.77%, p=0.001), but also during follow-up of B-ALL patients (1.42±2.36%, p=0.0001) when compared with non-treated controls (0.38±0.29%). Some cases where their frequencies achieved up to 12% reflect the massive regeneration of myeloid lineage in bone marrow after chemotherapy cycles. Especially in these cases accurate interpretation of such a high frequency of immature myeloid cells as myeloid precursors was very important to exclude incoming relapse or secondary leukemia. The myeloid precursors represented by $CD34^+$ in regenerating bone marrow expressed CD45 (94.8±5.5%), CD117 (38.3±26.2%), CD38 (91.4±5.7%), HLA-DR (90.6±7.6%), CD13 (73.0±20.8%) and CD33 (85.2±15.6%), while CD90 (2.7±2.5%), CD133 (10.0±8.2%) and T or B lymphocyte markers were negative. Comparing immunophenotypes with control bone marrows, only difference in expression of CD33 marker was found (85.2±15.6% versus 63.0±17.4% p=0.024). In addition, according to expression of these markers three different subsets of myeloid precursor cells were identified in regenerating bone marrow samples: $CD34^+$ $CD117^-$ $HLA\text{-}DR^+$, $CD34^+$ $CD117^+$ $HLA\text{-}DR^+$ and $CD34^-$ $CD117^+$ $HLA\text{-}DR^{-/+}$ without aberrant marker expression.

In conclusion for the correct discrimination of MRD in acute leukemia it is indispensable to define the range of normality in myeloid differentiation by extensive studies of bone marrows not only from healthy donors but also from regenerating bone marrow of patients undergoing therapy.

Key words: AML; regenerating bone marrow; minimal residual disease; precursors

In bone marrow $CD34^+$ populations contain progenitor cells committed to the myeloid, lymphoid and erythroid lineages, which are capable of long-term reconstitution [1, 2, 3]. CD34 cells that are committed to differentiate to myeloid cells are distinguished from those committed to lymphoid by surface expression of myeloid markers CD13 and CD33 [4, 5, 6] and progenitor antigen CD117 [7, 8, 9]. Although expression of myeloid markers (CD13, CD33) in CD34 and CD117 precursors indicates myeloid lineage association, the exact sequence of these markers appearing during differentiation is still matter of discussion [5, 10].

Distinction between normal and malignant cells is particularly important for regenerating bone marrow. Some reports on regenerating bone marrow after chemotherapy are done in acute lymphoblastic leukemia (ALL) to define precursors committed to B-cell lineage [11, 12]. We directed our study to the exact immunophenotypic definition of myeloid precursors and their successive stages during granulocytic and monocytic development in regenerating bone marrow, which often causes problems to distinguish from the pathological phenotypes (i.e. in detecting minimal residual disease in

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AML). We assessed their frequencies during treatment of acute leukemia (AL) and potential interference with minimal residual disease (MRD).

The results of 39 patients with ALL (precursors B-ALL) and 27 patients with acute myeloid leukemia (AML) in complete remission (CR) determined by multicolor flow cytometry are reported as well. For exact definition of non-leukemic myeloid precursors we chose bone marrow of non-AML patients to exclude the presence of malignant myeloid precursor populations at the time of bone marrow regeneration (MRD).

Material and methods

Patients and samples. The bone marrow (BM) aspirates considered as healthy control were obtained from 36 aged 1-72 (0-16 years n=17; ≥ 17 years n=18) individuals with non-leukemic disorders (suspected but not confirmed diagnosis of hematological malignancies). Cord blood sample was taken from one healthy full-term neonate. The regenerating bone marrows were taken from 39 precursor B-ALL patients (cALLA+ B-ALL) aged 0-78 years (0-16 years n=29; ≥ 17 years n=10) and 27 AML patients aged 0.5-68 years (0-16 years n=6; ≥ 17 years n=21) undergoing therapy. At least one year after the end of the therapy 30 B-ALL patients aged 5-62 years (0-16 years n=20; ≥ 17 years n=9) were also examined. Children were treated according to the BFM (Berline-Frankfurt-Münster) protocols (ALL IC BFM 2002, AML-BFM 98) while adults according to GMALL (German multicenter ALL) protocol for ALL. AML adult patients were treated according to standard protocols (combination of anthracycline and cytarabine -3+7) and those who had acute promyelocytic leukaemia received all-trans retinoic acid (ATRA).

All samples were collected in heparin anticoagulant at the initial diagnosis and during the follow-up.

Flow cytometric analysis and gating strategy. Multiparameter flow cytometric analysis was done on erythrocyte-lysed whole BM samples using a commercially available lysing solution. The staining procedure has been described in detail previously [13]. Cell samples were examined by staining with relevant combinations of antibodies. In each case the immunophenotype was done at the time of diagnosis, after induction therapy and during following treatment in 1-3 months intervals. To detect myeloid populations and their precursors in bone marrow compartment a quadruple marker staining with direct fluorochromes-labeled MoAbs, fluorescein isothiocyanate [FITC], phycoerythrin [PE], R-phycoerythrin-texas red [ECD] and phycoerythrincyanin 5 [PC5] were used in the following combinations: HLA-DR/CD13/CD45/CD34; CD11b/CD117/CD45/CD34; CD33/CD7/CD45/CD34; CD4(CD2)/CD56/CD45/CD34; CD14/CD15/CD45/CD13(CD34); CD10/CD20(CD19)/CD45/CD34. For the more detailed analysis of precursors populations in regenerating bone marrows, also some other combinations of monoclonal antibodies listed previously [14] were used. All antibodies directly conjugated by fluorochromes were purchased from Immunotech (Marseille, France) except CD133 (purchased from RD system). Precursor populations were detected in bone marrow by gating on CD34 and/or CD117 antigens. Antigen expressions were systematically analyzed by multiparameter flow cytometry (EPICS ALTRA flow cytometer equipped by Expo 32 program for analysis). In addition, gating on CD45 intensity versus side scatter (SSC) was used to detect BM populations and separate them from the cell-debris.

Statistical analysis. A case was considered as positive for a specific antigen if the antigen was expressed at least in 20% cells of the individual subpopulation in sample (20% cut off level) and when the fluorescence intensity was clearly separated from negative controls (isotypic controls or negative cell population for the examined antigen). The student T-test was used to estimate the statistical significance of differences observed between groups. $P < 0.05$ was considered statistically significance. All statistic analyses were done with the NSCC 2004 software program.

Results

Patients. In the current investigation 36 controls, 39 patients with non-AML, 27 with AML undergoing therapy and 30 B-ALL patients one year after the end of therapy were analyzed between January 2004 and April 2007 by multiparameter flow cytometry applying the panel of monoclonal antibodies described previously.

Immunophenotypic analysis of control and regenerating bone marrow populations. More extensive analysis of myeloid precursors was done on non-leukemic controls, patients with B-ALL one year after the end of treatment and regenerating bone marrows in order to find out possible immunophenotypic differences due to treatment.

Myeloid precursors during treatment. Populations of $CD34^+$ precursors homogenously express maturation-associated antigens CD45, CD38 and HLA-DR, while expression and intensity of CD117, CD33 and CD13 expression was heterogeneous. The expression of CD90 and CD133 was not proved. In some cases during the immunophenotypic characterization of myeloid cell differentiation a subset of cells that express CD117 and lacked CD34 was identified.

According to expression of several markers three subpopulations of myeloid precursors were defined in regenerating bone marrow: $CD34^+CD117^-HLA\text{-}DR^+$, $CD34^+CD117^+HLA\text{-}DR^+$ and $CD34^-CD117^+HLA\text{-}DR^{-/+}$ (Fig. 1.). Subpopulations of $CD34^+$ in controls and also in regenerating bone marrows expressed either low levels or no surface antigens associated with T- or B- cell lineage (Tab. 1). Mature myeloid associated antigens such as CD14, CD15 and CD11b were also negative (Tab. 1). No significant differences were observed in the expression of studied markers, except CD33 which was expressed in significantly higher rate in regenerating CD34 positive populations (85.2±15.6% versus 63.0±17.4% p=0.024). Aberrant expression was also not detected on $CD117^+$

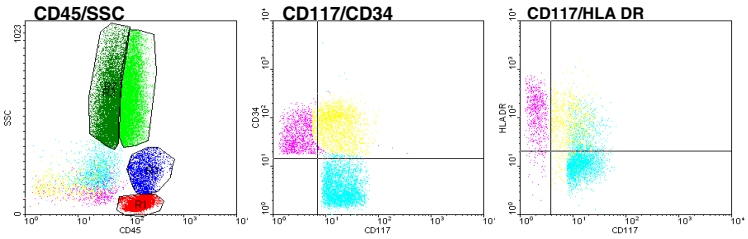

Fig. 1. Subpopulations of myeloid precursors present in regenerating bone marrow in a patient with precursor B-ALL. In the dot plots the immunophenotype profiles of 3 subpopulations are presented: CD34^{+}CD117^{-}HLA-DR^{+} (violet dots), CD34^{+}CD117^{+}HLA-DR^{+} (yellow dots) a CD34^{-} CD117^{+}HLA-DR$^{-/+}$ (turquoise dots).

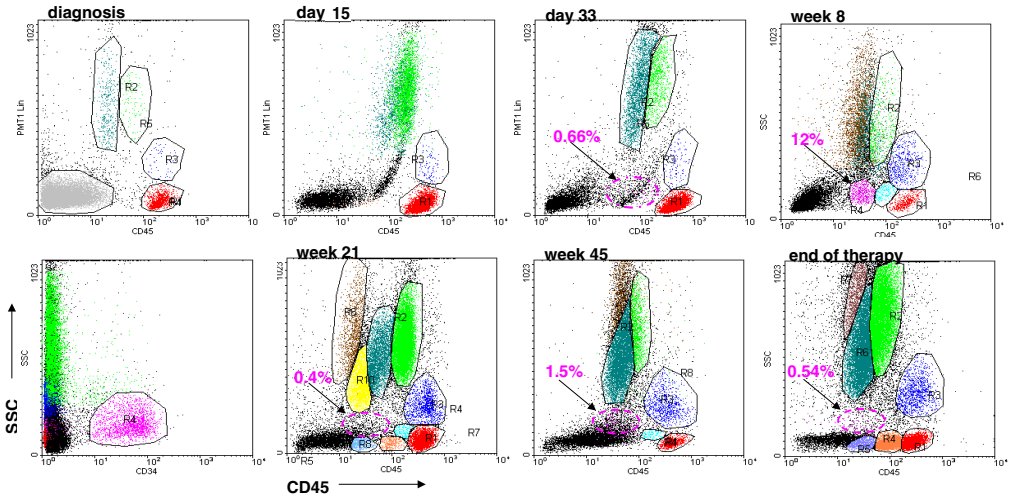

Fig. 2. Example of regenerating bone marrow in patient treated for precursor B-ALL. Myeloid precursors (violet dots, gated on CD34 and or CD117) are present already after induction therapy cycle (day 33), B-cell precursors-hematogones 1 a 2 (orange and light-blue dots) present during later stages of treatment (21st week) and also after the end of therapy. Presence of several granulocytes subpopulations with increasing maturations stages (brown to light-green dots) expressed by increasing intensity of CD45 antigens. Grey color represents pathologic gate, red lymphocyte and dark-blue monocyte populations.

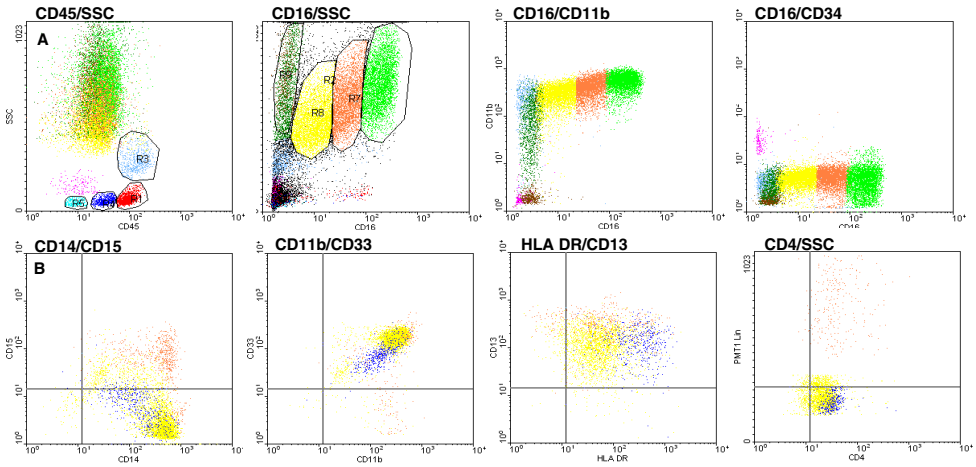

Fig. 3. Immunophenotype of granulocytic and monocytic subpopulations in regenerating bone marrow. A-Increasing expression and intensity of CD16 a CD11b in combination with SSC allows us to identify 5 granulocytes subpopulations with increasing maturations stages (brown to light-green dots) and their precursors (violet dots). B-cell precursors were also present (dark blue and turquoise dots). B-Three subpopulations of monocytes differed in intensity of expression HLA-DR, CD33, CD11b, CD15 and CD14 are presented. Orange dots represent the double positive CD14^{+}CD15^{+} subpopulation with high value of SSC parameter.

Tab. 1. Expression of antigens on myeloid precursors (CD34+)

antigen	% expression on CD34+ (average ± SD)		p (T-test)
	control BM	regenerating BM	
CD45	95.6 ± 3.5	94.8 ± 5.5	**n.s.**
CD117	43.5 ± 26.3	38.3 ± 26.2	**n.s.**
CD90	2.0 ± 1.0	2.7 ± 2.5	**n.s.**
CD133	5.3 ± 4.9	10.0 ± 8.2	**n.s.**
CD38	80.7 ± 12.2	91.4 ±5.7	**n.s.**
HLA-DR	91.4 ± 7.8	90.6 ± 7.6	**n.s.**
CD13	55.6 ± 26.6	73.0 ± 20.8	**n.s.**
CD33	63.0 ± 17.4	**85.2** ± 15.6	**0.05**
CD11b	5.8 ± 8.4	6.7 ± 4.8	**n.s.**
CD14	6.7 ± 2.5	14.0 ± 8.5	**n.s.**
CD15	10.3 ± 11.1	10.3 ± 11.6	**n.s.**
T-antigens	3.0 ± 2.3	2.7 ± 2.8	**n.s.**
B-antigens	2.8 ± 3.8	2.8 ± 2.7	**n.s.**

n.s. – not significant

subpopulations. In cord blood (n=1) cell population with immunophenotype CD34+CD117+HLA-DR+ formed 0.2% and hematogones 0.7% of total events of all BM cells. The expression of other markers was similar to subpopulation CD34+ presents in bone marrow (Tab. 1).

In the regenerating bone marrow the higher amount of immature bone marrow populations was detected. During the first stage of treatment (immediately after induction therapy cycle, day 33) the presence of myeloid precursors was found, while the presence of B-lymphoid precursors was not yet present. They were characteristic for the later stages of treatment (Fig. 2).

While the presence of myeloid precursors in non-leukemic BM was in minority (0.38±0.29%) and similarly 1 year after the end of treatment (0.34±0.35%), in regenerating bone marrow of both adults and children treated for ALL or adults for AML their proportion was significantly higher. In several cases (B-ALL) their amount achieved almost 8-12% of total events of all BM cells (Fig. 2. Tab. 2). A significant increase of myeloid precursors was observed shortly after the induction therapy of B-ALL (1.27±2.04%, p=0.0064) and also of AML patients (0.87±0.77%, p=0.001), but even during follow-up of B-ALL patients (1.42±2.36%, p=0.0001) in comparison with non-treated controls (Tab. 2). Up to the present time the detection of myeloid precursors was analyzed in our AML patients only after the induction therapy due to the low number of patients investigated during follow-up for their worse survival.

Immunophenotype of myelo-monocyte populations. In regenerating bone marrows similarly to controls, several maturation stages of granulocytes were observed. During the regeneration the less mature stages of granulocytic differentiation dominated. To investigate the degree of their maturation, several antigens were examined. Except precursor markers (CD34, CD117) other myeloid markers (CD11b, CD13, CD33, CD15, CD16, CD65), monocyte markers (CD14, CD36), and marker CD10 characteristic mainly for different stages of B-cell precursors were investigated. All granulocyte subpopulations present in regenerating bone marrow homogenously expressed only markers CD33+, CD15+ a CD65+. According to different expression and intensity of expression of other markers several subpopulations were defined (Tab. 3). Besides antigen expression the light scattering properties were also useful. The high values of SSC parameter were characteristic for the mature granulocytes (CD11b+ CD13+ CD10+), but were rarely observed even on immature subpopulation (CD11b- CD13- CD33+).

Within granulocytic differentiation in regenerating bone marrow, the population of basophilic granulocytes was identified in minority (0.75±0.6%), and expressed mainly CD13+,

Tab. 2. Prevalence of myeloid precursors in controls and regenerating bone marrows

	control BM			regenerating BM					
				B ALL				AML	
	non leukemic	1 year after the end of the treatment	T-test	after induction	T-test	following treatment	T-test	after induction	T-test
adults average±SD % (range)	**0.36±0.27 (0-0.87)**	0.41±0.59 (0-1.9)	**n.s.**	**1.71***±1.23 (0.2-5.5)	**0.04**	**0.75***±1.05 (0 – 5.2)	**0.04**	**0.96***±0.82 (0-3.7)	**0.003**
children average±SD % (range)	**0.37±0.31 (0-1.1)**	0.31±0.16 (0-0.48)	**n.s.**	**1.17***±2.14 (0–8)	**0.03**	**1.97***±2.92 (0–12)	**0.03**	0.57±0.52 (0.15-1.6)	**n.s.**
total average±SD % (range)	**0.38±0.29 (0–1.1)**	0.34±0.35 (0–0.48)	**n.s.**	**1.27***±2.04 (0–8)	**0.006**	**1.42***±2.36 (0-3.7)	**0.0001**	**0.87***±0.77 (0-3.7)	**0.002**

n.s. – not significant

** – statistically significant in comparison with control/non-leukemic bone marrows*

$CD33^+$, $CD38^+$, $CD11b^+$ antigens. They were localized on CD45/SSC dot plots in regions typical for NK cells and merged into myeloblast region.

Similar to granulocytic differentiation, three monocytic subpopulations representing different stage of monocyte maturation in regenerating bone marrow were identified. These subpopulations in control and regenerating bone marrow differed in expression especially of CD45, CD4, CD13, HLA-DR a CD33 (Fig. 3). Within monocytic differentiation the double positive $CD14^+CD15^+$ cell subpopulations with high value of SSC parameter were identified (Fig. 3).

Tab. 3. Several subpopulations of neutrophilic granulocytes in regenerating bone marrow

	$CD11b^-CD13^-$	$CD11b^+CD13^-$	$CD11b^+CD13^+CD10^+$
CD11b	-	++	+++
CD13	-	-	+++
CD14	-	-	+
CD36	-	-	+
CD10	-	-	+
CD16	-	++	+++
CD33	+++	++	+
CD38	+++	++	-
CD45	+	++	+++

-antigen not present, +low intensity of expression, ++ mediate intensity of expression, +++ high intensity of expression

Discussion

Results of immunophenotypic analysis of populations in regenerating bone marrow of non-leukemic and leukemic (AML and B-ALL during and after treatment) specimens (individuals) by multiparameter flow cytometry are presented.

The purpose of the study was to characterize phenotypes mainly of cell precursors for their exact discrimination from pathological cells in the detecting minimal residual disease. Monitoring of MRD becomes increasingly important for a more accurate stratification of the therapy, considering the risk of relapse in acute leukemia [15, 16].

Knowledge of the expression of various lineage-specific, maturation-associated and mature markers in normal hematopoietic differentiation provides a frame of reference for identification of abnormal differentiation patterns [6] even if they occur at low frequencies. The normal differentiation patterns within a specific lineage can be displayed by the application of markers that are gradually up- or down-regulated during differentiation (´dynamic´ markers). There is a wide scale of normal variations and abnormalities in cell differentiation, mainly in B-cell and myeloid lineages, depending on the age of the individual or BM regeneration. This should be considered with great watchfulness. Many reports are focused on investigation of immunophenotype of healthy bone marrow populations [6, 8, 19, 10]. Recently it have been shown, that for correct discrimination of MRD also examination of regenerating bone marrow populations and physiological or therapy related shifts are needed [11, 20, 21].

Some reports confirmed the value of B-cell precursors investigation which were found overrepresented in regenerating bone marrow during and after chemotherapy [11, 12]. In the present study we focused on myeloid differentiation during treatment of ALL/AML for correct interpretation of the immunophenotype results and proper distinction from MRD. More extensive analysis of myeloid precursors was done in control bone marrows (non-leukemic) and regenerating bone marrows in order to find out possible immunophenotypic differences due to treatment.

We observed changes in regenerating bone marrow concerning relative distribution of several subpopulations according to the treatment. In control bone marrows myeloid precursors represented minor proportion of total events of all BM cells (0.38%) and these findings are in accordance with results of Macedo et al. [23].

In the regenerating bone marrow the higher frequency of immature bone marrow populations was detected. During the first stage of treatment (after induction therapy) we found presence of myeloid precursors, while absence of B-lymphoid precursors was observed (Fig.2). The immunophenotypic profiles of several subpopulations of B-cell precursors were described previously [17]. Later occurrence of B-cell precursors after treatment is in accordance with results of others [11], while Van Lochem et al. [12] found B-cell precursors also 2 weeks after induction treatment.

The substantial expansion of normal B-cell precursors in regenerating bone marrow related to the preceding treatment block was already proved [11, 12, 22]. Van Lochem et al. stressed the correct interpretation of the regeneration of normal B-cells, which should not to be mistaken for a relapse [12]. Our results concerning relative distribution of myeloid precursors in regenerating bone marrows extended the results on their existence in control BM aspirates [5, 12]. According to our knowledge results on myeloid precursors in regenerating bone marrows were not yet described.

Myeloid precursors were overrepresented immediately after cessation of induction therapy cycle (1.27%, p=0.012) and also during subsequent stages of treatment (1.42%, p=0.0005) in comparison with control BM. Their values observed during the follow-up were seldom as high as 12% of total events of all BM cells. This apparently reflects the massive regeneration of myeloid lineage in bone marrow. Especially in these cases accurate interpretation of such a high frequency of immature myeloid cells as myeloid precursors it is very important to exclude incoming relapse or secondary leukemia. These observations suggest that the range of normality in myeloid differentiation need to be established by extensive studies of bone marrows not only from healthy donors but also from patients with non-myeloid leukemia undergoing therapy.

In contrast to B-cell differentiation with complete maturation spectrum of defined antigens [6, 22, 21, 17], the precise sequence of myeloid antigens expression occurring very early during normal myeloid differentiation is still unclear [5].

In regenerating bone marrow following chemotherapy, we have defined three physiological maturation phases of myeloid precursors with immunophenotype: $CD34^+$ $CD117^-$ $HLA\text{-}DR^+$, $CD34^+CD117^+HLA\text{-}DR^+$ and $CD34^-CD117^+HLA\text{-}DR^{-/+}$. Myeloid precursors in regenerating BM constantly express differentiation-associated markers CD45, HLA-DR and CD38, while expression and its intensity of CD117, CD33 and CD13 was irregular. There were no immunophenotypic differences between regeneration of myeloid precursors in BM of ALL or AML patients undergoing therapy. These results are consistent with those reported by Gaipa et al. [5] and Zamir et al. [10] in healthy bone marrow.

In order to determine the impact of treatment to bone marrow populations, i. e. if differences between untreated individuals (controls) and bone marrow populations of patient undergoing therapy exist, their immunophenotypes were compared. Only minor differences were found (Tab. 2), which concerned CD33 marker expressed in significantly higher rate in regenerating CD34 positive populations (85.2±15.6% versus 63.0±17.4% p=0.024). Very important finding was that all subpopulations lacked aberrant antigen expression (Tab. 2). These observations allow the discrimination of MRD in AML patients also in cases where coexistence with the regenerating myeloid precursors was present.

Besides myeloid precursors the shift in relative distribution and phenotypic profile of granulocytic and monocytic subpopulations with prevalence of immature types was observed in regenerating bone marrow. Three distinct subpopulations of monocytes were identified, having significant differences of intensity of HLA-DR, CD33, CD11b, CD14 and expression of CD15. These results are partially consistent with those reported by Zamir et al. [10] in healthy bone marrow, but we complemented these observations also for another markers as CD14, CD15 and CD11b. These markers allowed us to identified monocyte subpopulation with double positive expression of CD14/CD15 cells with higher SSC parameter. During granulocytic regeneration increasing expression and intensity of CD16 a CD11b in combination with SSC allowed us to identified 5 granulocyte subpopulations with increasing maturations stages, similar to observation of Van Lochem [6] in healthy bone marrow.

In conclusion, it follows from our results that the multiparameter flow cytometry means an unambiguous contribution to the study of exact phenotypic characteristics of subpopulations during myeloid differentiation and their proportion in regenerating bone marrow. Knowledge concerning shifts in regenerating bone marrow is necessarily for correct interpretations of immunophenotypic data for exact discrimination of MRD during follow-up of acute leukemia by comparing leukemic cells with those present in both healthy and regenerating bone marrow.

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References

[1] BENDER JG, UNVERZAGT KL, WALKER DE et al. Identification and comparison of CD34-positive cells and their subpopulations from normal peripheral blood and bone marrow using multicolor flow cytometry. Blood.1991 Jun 15; 77: 2591–6.

[2] TERSTAPPEN LW, SAFFORD M, UNTERHALT M et al. Flow cytometric characterization of acute myeloid leukemia: IV. Comparison to the differentiation pathway of normal hematopoietic progenitor cells. Leukemia. 1992 Oct; 6: 993–1000.

[3] MACEDO A, ORFAO A, CIUDAD J et al. Phenotypic analysis of CD34 subpopulations in normal human bone marrow and its application for the detection of minimal residual disease. Leukemia 1995 Nov; 9: 1896–901.

[4] PIERELLI L, TEOFILI L, MENICHELLA G et al. Further investigations on the expression of HLA-DR, CD33 and CD13 surface antigens in purified bone marrow and peripheral blood CD34+ haematopoietic progenitor cells. Br J Haematol. 1993 May; 84: 24–30.

[5] GAIPA G, COUSTAN-SMITH E, TODISCO E et al. Characterization of CD34+, CD13+, CD33- cells, a rare subset of immature human hematopoietic cells. Haematologica. 2002 Apr; 87: 347–56.

[6] VAN LOCHEM EG, VAN DER VELDEN VH, WIND HK et al. Immunophenotypic differentiation patterns of normal hematopoiesis in human bone marrow: reference patterns for age-related changes and disease-induced shifts. Cytometry. 2004; 60B: 1–13.

[7] REISBACH G, BARTKE I, KEMPKES B et al. Characterization of hemopoietic cell populations from human cord blood expressing c-kit. Exp Hematol. 1993 Jan; 21: 74–9.

[8] BASSO G, LANZA F, ORFAO A at al. Clinical and biological significance of CD34 expression in acute leukemia. J Biol Regul Homeost Agents. 2001 Jan-Mar; 15: 68–78.

[9] ESCRIBANO L, OCQUETEAU M, ALMEIDA J et al. Expression of the c-kit (CD117) molecule in normal and malignant hematopoiesis. Leuk Lymphoma. 1998 Aug; 30: 459–66.

[10] ZAMIR E, GEIGER B, COHEN N et al. Resolving and classifying haematopoietic bone-marrow cell populations by multi-dimensional analysis of flow-cytometry data. Br J Haematol. 2005; 129: 420–31.

[11] VAN WERING ER, VAN DER LINDEN-SCHREVER BE, SZCZEPANSKI T et al. Regenerating normal B-cell precursors during and after treatment of acute lymphoblastic leukaemia: implications for monitoring of minimal residual disease. Br J Haematol. 2000; 110: 139–46.

[12] VAN LOCHEM EG, WIEGERS YM, VAN DEN BEEMD R et al. Regeneration pattern of precursor-B-cells in bone marrow of acute lymphoblastic leukemia patients depends on the type of preceding chemotherapy. Leukemia. 2000 Apr; 14: 688–95.

[13] ZELEZNIKOVA T, BABUSIKOVA O. The value of dot plot patterns and leukemia-associated phenotypes in AML diagnosis by multiparameter flow cytometry. Neoplasma. 2005; 52: 517–22.

[14] ZELEZNIKOVA T, BABUSIKOVA O. The impact of cell heterogeneity and immunophenotypic changes on minimal residual disease monitoring in acute myeloid leukemia. Neoplasma. 2006; 53: 500–6.

[15] VENDITTI A, BUCCISANO F, DEL POETA G et al. Level of minimal residual disease after consolidation therapy predicts outcome in acute myeloid leukemia. Blood. 2000; 96: 3948–3952.

[16] FELLER N, VAN DER POL MA, VAN STIJN A et al. MRD parameters using immunophenotypic detection methods are highly reliable in predicting survival in acute myeloid leukaemia. Leukemia. 2004 Aug; 18: 1380–90.

[17] BABUSIKOVA O, ZELEZNIKOVA T, MLCAKOVA A et al. The knowledge on the 3rd type hematogones could contribute to more precise detection of small numbers of precursor B-acute lymphoblastic leukemia. Neoplasma. 2005; 52: 502–9.

[18] BASSO G, BULDINI B, DE ZEN L et al. New methodologic approaches for immunophenotyping acute leukemias. Haematologica. 2001; 86: 675–692.

[19] KERN W, HAFERLACH T. Quantification of minimal residual disease by multiparameter flow cytometry in acute myeloid leukemia. From diagnosis to prognosis. Med Klin. 2005; 100: 54–59.

[20] CAMPANA, D., COUSTAN-SMITH, E. Minimal residual disease studies by flow cytometry in acute leukemia. Acta Haematol. 2004; 112: 8–15.

[21] MCKENNA RW, ASPLUND SL, KROFT SH. Immunophenotypic analysis of hematogones (B-lymphocyte precursors) and neoplastic lymphoblasts by 4-color flow cytometry. Leuk Lymphoma. 2004; 45: 277–85.

[22] MCKENNA RW, WASHINGTON LT, AQUINO DB et al. Immunophenotypic analysis of hematogones (B-lymphocyte precursors) in 662 consecutive bone marrow specimens by 4-color flow cytometry. Blood. 2001; 98: 2498–507.

[23] MACEDO A, ORFAO A, CIUDAD J et al. Phenotypic analysis of CD34 subpopulations in normal human bone marrow and its application for the detection of minimal residual disease. Leukemia. 1995 Nov; 9: 1896–901.