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Granulocyte maturation determines ability to release chromatin NETs and loss of DNA damage response; these properties are absent in immature AML granulocytes

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ABSTRACT

Terminally-differentiated cells cease to proliferate and acquire specific sets of expressed genes and functions distinguishing them from less differentiated and cancer cells. Mature granulocytes show lobular structure of cell nuclei with highly condensed chromatin in which HP1 proteins are replaced by MNEI. These structural features of chromatin correspond to low level of gene expression and the loss of some important functions as DNA damage repair, shown in this work and, on the other hand, acquisition of a new specific function consisting in the release of chromatin extracellular traps in response to infection by pathogenic microbes. Granulocytic differentiation is incomplete in myeloid leukemia and is manifested by persistence of lower levels of HP1γ and HP1β isoforms. This immaturity is accompanied by acquisition of DDR capacity allowing to these incompletely differentiated multi-lobed neutrophils of AML patients to respond to induction of DSB by γ-irradiation. Immature granulocytes persist frequently in blood of treated AML patients in remission. These granulocytes contrary to mature ones do not release chromatin for NETs after activation with phorbol myristate-12 acetate-13 and do not exert the neutrophil function in immune defence. We suggest therefore the detection of HP1 expression in granulocytes of AML patients as a very sensitive indicator of their maturation and functionality after the treatment. Our results show that the changes in chromatin structure underlie a major transition in functioning of the genome in immature granulocytes. They show further that leukemia stem cells can differentiate *ex vivo* to mature granulocytes despite carrying the translocation BCR/ABL.

1. Introduction

To understand development of leukemia, it is important to know how changes of chromatin structure are orchestrated during the $CD34^+$ stem cells differentiation and how they correlate with chromatin functions in normal differentiated cells and leukemia conditions. New blood cells of different cell lineages are formed from bone marrow hematopoietic stem cells (HSCs), which provide a constant supply of blood cells throughout life. HSCs show considerable plasticity and can reprogram their gene expression in response to signals produced by a combination of cytokines giving rise to different clones of blood cells with specific functions. For example, granulocytic colony stimulating factor (G-CSF) is used to mobilize HSCs to peripheral blood for using peripheral blood stem cells (PBSC) in transplantation regimes [1] and also to stimulate granulocytic differentiation of HSCs in vitro. HSCs express several specific antigens on the surface (particularly $CD34^+$) and do not express lineage antigens of differentiated cells (Lin^- cells) [2].

Terminal cell differentiation involves a large-scale chromatin remodeling that turns most of the open euchromatin into condensed heterochromatin [3]. During this process, most genes that are required for cell "housekeeping" functions acquire heterochromatin organization and become repressed [4,5]. Heterochromatin is associated with certain types of histone lysine methylation (e.g. at histone H3 lysines 9 and 27 and histone H4 lysine 20), which have a role in preserving heterochromatin structure and rendering histones less susceptible to activatory modifications [6]. The preservation and spreading of heterochromatin is mediated through the direct interaction of dimethylated histone H3 at lysine 9 (H3K9me2) with heterochromatin protein 1 (HP1) [6–8]. However, recent data show that the HP1 is dramatically reduced in mature erythrocytes, lymphocytes and especially granulocytes [9–13]. On

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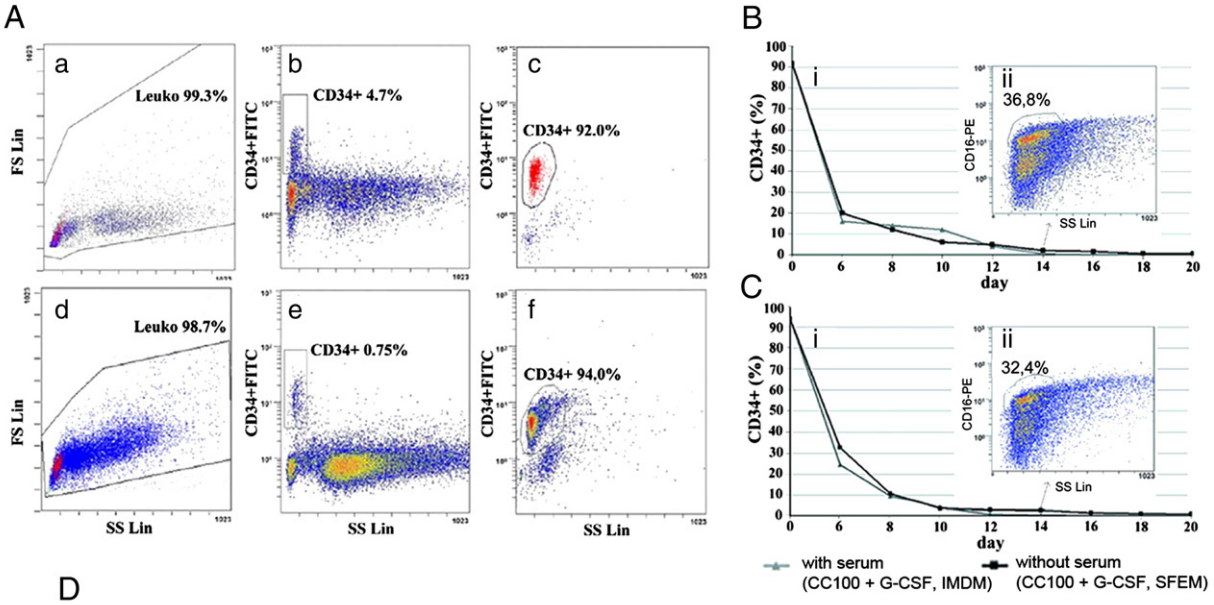
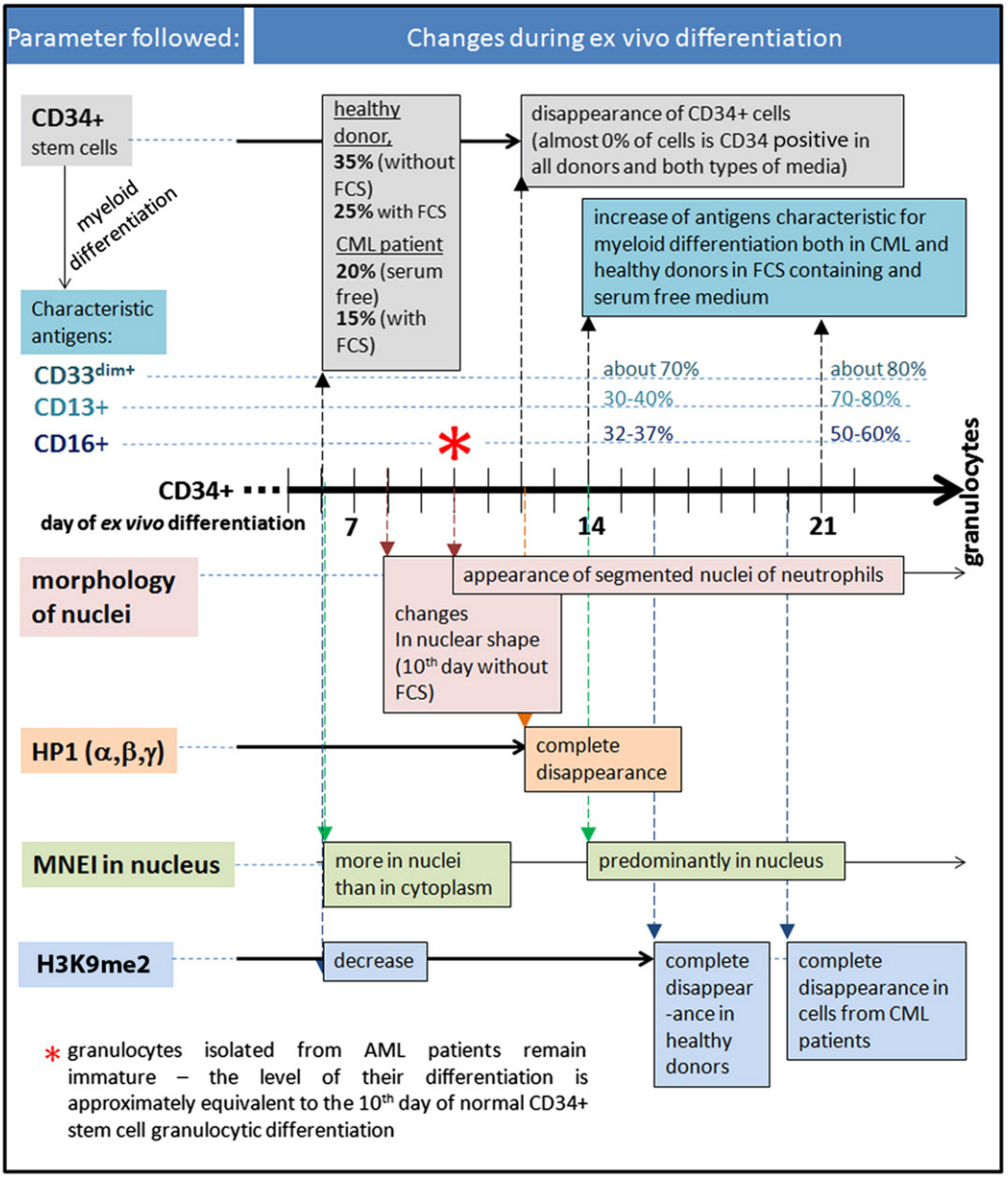

A
a
Leuko 99.3%
FS Lin
b
CD34+ 4.7%
CD34+FITC
c
CD34+ 92.0%
CD34+FITC
d
Leuko 98.7%
FS Lin
SS Lin
e
CD34+ 0.75%
CD34+FITC
SS Lin
f
CD34+ 94,0%
CD34+FITC
SS Lin
B
i
CD34+ (%)
day
ii
36,8%
CD16-PE
SS Lin
C
i
CD34+ (%)
day
ii
32,4%
CD16-PE
SS Lin
with serum
(CC100 + G-CSF, IMDM)
without serum
(CC100 + G-CSF, SFEM)

D


Parameter followed:
Changes during ex vivo differentiation
CD34+
stem cells
myeloid differentiation
Characteristic antigens:
CD33dim+
CD13+
CD16+
CD34+
day of ex vivo differentiation
healthy donor,
35% (without FCS)
25% with FCS
CML patient
20% (serum free)
15% (with FCS)
disappearance of CD34+ cells
(almost 0% of cells is CD34 positive in all donors and both types of media)
increase of antigens characteristic for myeloid differentiation both in CML and healthy donors in FCS containing and serum free medium
about 70%
30-40%
32-37%
about 80%
70-80%
50-60%
7
14
21
granulocytes
morphology of nuclei
appearance of segmented nuclei of neutrophils
changes
In nuclear shape
(10th day without FCS)
HP1 (α,β,γ)
complete disappearance
MNEI in nucleus
more in nuclei than in cytoplasm
predominantly in nucleus
H3K9me2
decrease
complete disappear-ance in healthy donors
complete disappearance in cells from CML patients
* granulocytes isolated from AML patients remain immature – the level of their differentiation is approximately equivalent to the 10th day of normal CD34+ stem cell granulocytic differentiation

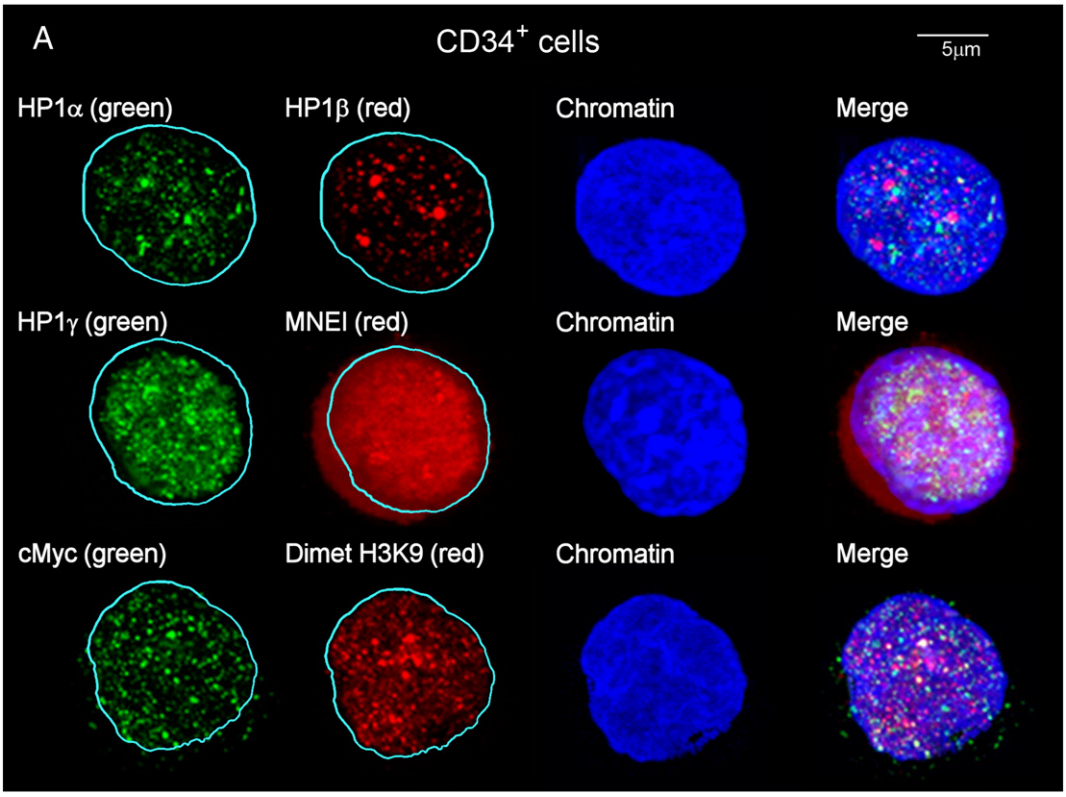


Fig. 2. CD34$^+$ cells isolated from the bone marrow of a CML patient. Cells were fixed by paraformaldehyde and submitted to co-immunodetection with antibodies derived from a rabbit and a mouse to HPlα and HP1β; HP1γ, and MNEI; cMYC and H3K9me2. Total images of nuclei (composed from 40 slices of 0.2 μm thin) were obtained by the confocal microscope Leica DM RXA.

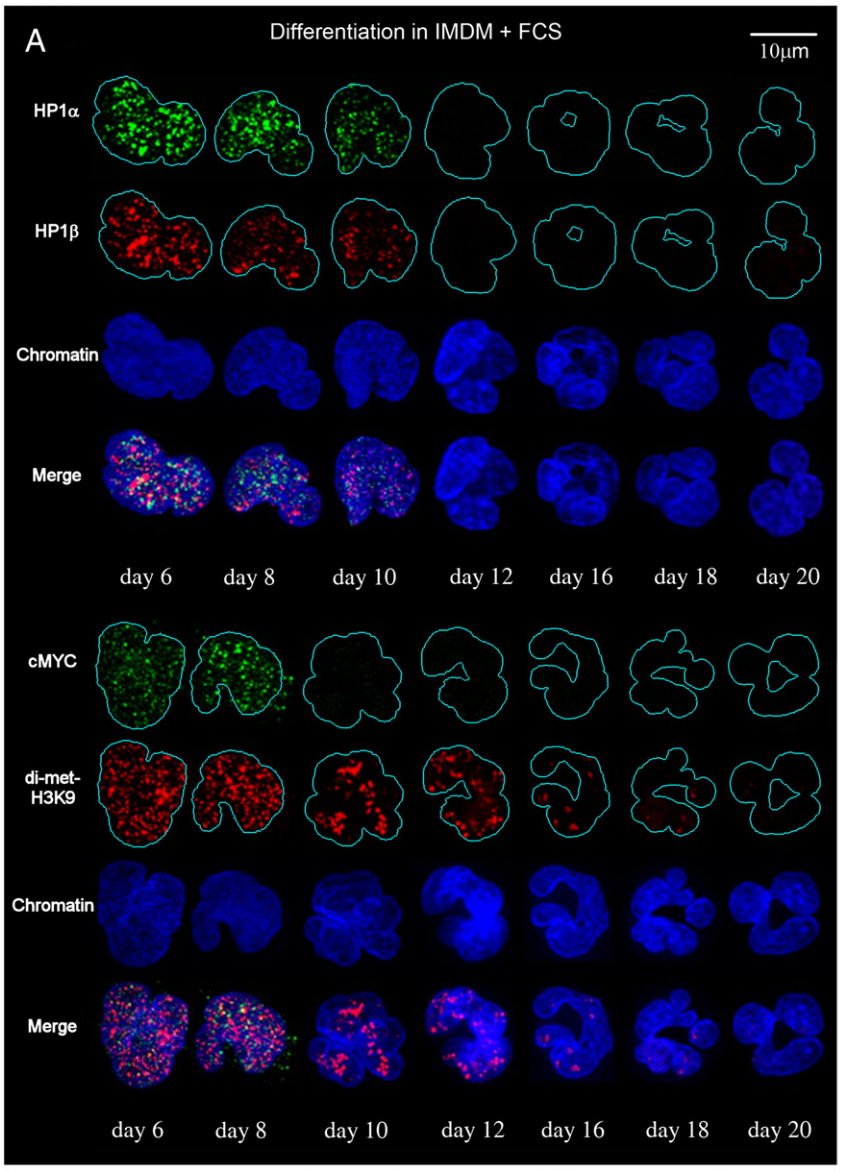
the other hand, it was shown that the level of H3K9me2 in mature granulocytes is high [14] despite this epitope being immunochemically undetectable [13] due to its weak exposure in the tightly condensed chromatin. Nevertheless, a distinct level of H3K9me2 was detected by immunofluorescence in neutrophils of patients in the chronic phase of chronic myeloid leukemia (CML) without detectable HP1 proteins. Dimethylated histone became more easily detected in granulocytes of CML patients in blast crisis and in acute myeloid leukemia (AML) [13,14] where it was also accompanied by high levels of HP1 proteins. The accessibility of the histone H3K9 for immunodetection indicates an incomplete chromatin condensation of AML neutrophils, also frequent in patients in remission.

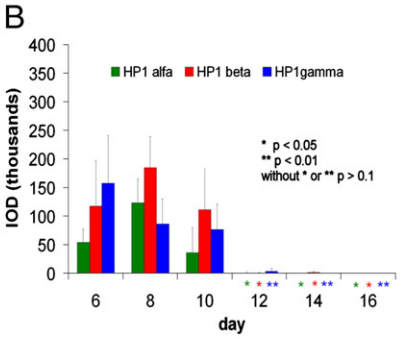
The leukocytes of healthy donors, chronic CML and AML patients express a nucleo-cytoplasmic serpin MNEI (monocyte neutrophil elastase inhibitor) that was proposed to replace HP1 during the terminal myeloid differentiation [14] similar to the closely related serpin MENT (myeloid and erythroid nuclear termination stage-specific protein) expressed in terminally differentiated avian granulocytes [15,16]. MNEI accumulates in the nuclei of neutrophils at one of the late stage of their maturation [17]. The presence of HP1 proteins in multi-lobed nuclei of neutrophils of leukemic individuals and decreased MNEI thus indicate that chromatin of these cells is not completely condensed and that these cells are immature.

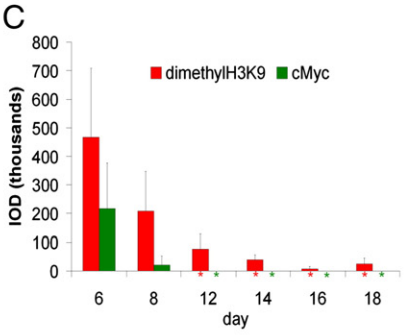
To determine whether elimination of HP1 proteins, increase of nuclear MNEI and chromatin condensation occur coordinately during granulopoiesis of normal and leukemic stem cells or possibly reflect the presence of genetic translocation between BCR-ABL genes in these last cells, we followed *ex vivo* differentiation of CD34$^+$ cells isolated from the bone marrow of patients with chronic-phase CML with complete hematological response to imatinib mesylate (Glivec), the PBSCs of healthy donors and from patients with non-Hodgkin's lymphoma (NHL). In patients with NHL, the differentiation into granulocytic lineage should not be affected, thus these samples served as an additional control for comparison with the CML sample.

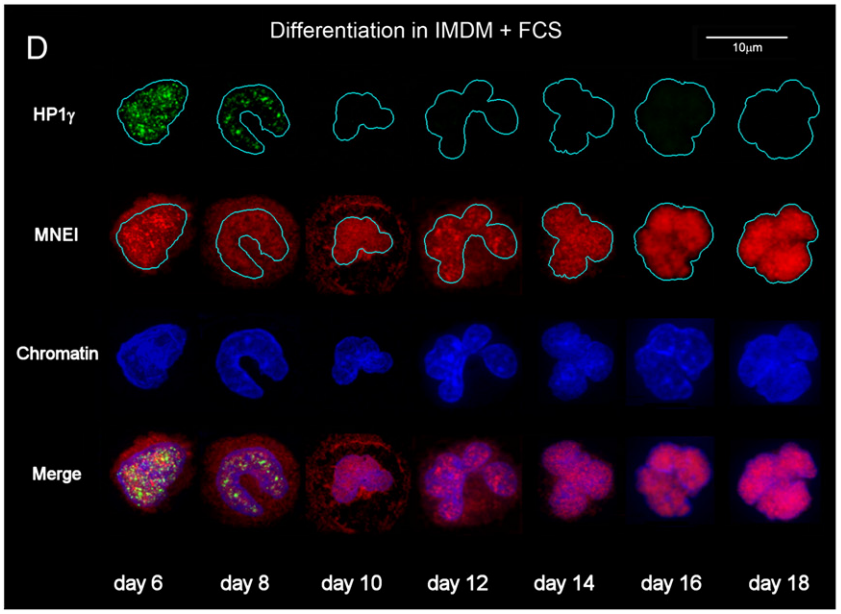
Next to changes in chromatin structure, we focused on functional differences between terminally differentiated granulocytes from healthy donors and incompletely differentiated blood cells taken from AML patients. Due to the stable repression of many genes accompanied by profound changes in chromatin structure, differentiated cells dramatically change their metabolic activity and responsiveness to many signals; however, they mostly preserve the ability to maintain the genome integrity including the detection, signaling and repair of DNA damage. Here we studied differentiation-dependent changes in the DNA damage response (DDR) induced by one of the most profound damages to the genome the interruption of both DNA strands called double-strand break

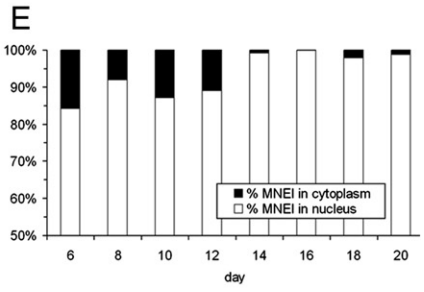
Fig. 1. CD34$^+$ cells isolation and *ex vivo* differentiation. (A) Flow cytometric data of CD34$^+$ cell fractions from bone marrow of a CML patient and PBSC of a healthy donor before and after immunomagnetic separation: (A-a, A-b) flow cytometric analysis of bone marrow of a CML patient before immunomagnetic selection of CD34$^+$ cells. (A-a) Forward scatter vs. side scatter, (A-b) CD34$^+$ labeled with FITC vs. side scatter. (A-c) Flow cytometric analysis of CD34$^+$ purity (92%) after immunomagnetic selection. (A-d, A-e) flow cytometric analysis of PBSC from a healthy donor before immunomagnetic selection. (A-d) Forward scatter vs. side scatter, (A-e) CD34$^+$ labeled with FITC vs. side scatter. (A-f) Flow cytometric analysis of CD34$^+$ purity (94%) after immunomagnetic selection. (B, C) Progress of HSC differentiation (measured as decrease in CD34 expression) during culture of the enriched fraction of CD34$^+$ mononuclear cells in serum-free (black line, squares) and serum-supplemented (blue line, triangles) medium: (B) Cells from the bone marrow of a CML patient; (C) cells from PBSC of a healthy donor. Insets (Bii, Cii): CD16 expression after 14-day culture in serum-free medium. (D) A scheme showing the timing of the changes in the antigens expression, nuclear morphology and epigenetic markers of chromatin structure during *ex vivo* differentiation of CD34$^+$ cells to granulocytes. The arrows emanating from the time-axis indicate the appearance of the particular changes (described in the boxes).


A
Differentiation in IMDM + FCS
10μm
HP1α
HP1β
Chromatin
Merge
day 6
day 8
day 10
day 12
day 16
day 18
day 20
cMYC
di-met-H3K9
Chromatin
Merge
day 6
day 8
day 10
day 12
day 16
day 18
day 20


B
HP1 alfa
HP1 beta
HP1gamma
* p < 0.05
** p < 0.01
without * or ** p > 0.1
IOD (thousands)
day


C
dimethylH3K9
cMyc
IOD (thousands)
day


D
Differentiation in IMDM + FCS
10μm
HP1γ
MNEI
Chromatin
Merge
day 6
day 8
day 10
day 12
day 14
day 16
day 18


E
% MNEI in cytoplasm
% MNEI in nucleus
day

(DSB). Within several minutes after irradiation, the nucleosomal histone H2AX is phosphorylated on serine 139 (γH2AX) by ATM kinase in the proximity of DSBs. This phosphorylation spreads up to the distance of about 2Mbp of DSB flanking chromatin forming thus foci known as IRIFs [18–20]. These foci are generally used for the detection of DSBs. Many repair proteins including sensors, transducers and effectors are rapidly attached to γH2AX to repair this damage. MDC1, NBS1, MRE11, RAD50, ATM kinase, 53BP1 colocalize with γH2AX as the first and their presence in IRIF indicates the ongoing process of DSB repair [20]. Global chromatin condensation was shown to inhibit DDR [21] while HP1 proteins may play a positive role in DDR [22].

We examined whether the DDR induced by ionizing radiation is preserved in terminally differentiated mature granulocytes that have condensed chromatin, a short life span, are capable to unravel neutrophil extracellular traps (NETs) to capture and kill microbes [23], but lack HP1 [13]. This ability was examined also in the immature granulocytes and incompletely differentiated precursors emerging in peripheral blood of leukemic individuals before the disease treatment as well as in remission. In addition, we investigated whether the ability of mature neutrophils to release chromatin NETs after activation [23–28] is also preserved by immature neutrophils of AML patients expressing HP1γ.

We show that CML stem cells can differentiate *ex vivo* to mature granulocytes despite the presence of the BCR/ABL translocation and that mature granulocytes from healthy donors neither express DNA repair proteins nor undergo proper DDR contrary to immature leukemia granulocytes. Immature neutrophils of AML patients are not able to release chromatin into neutrophil extracellular traps (NETs) after activation with phorbol 12-myristate 13-acetate (PMA) and thus execute the principal function of neutrophils. Taken together, a tight correlation between chromatin structure and cell function has been observed giving an interesting picture of mechanisms governing granulocytic differentiation.

2. Material and methods

2.1. Cells

Mature granulocytes and mononuclear cells (lymphocytes and monocytes) were isolated from peripheral blood as described earlier [13]. The resulting cell population contained more than 95% of mature neutrophils as assessed by microscopy analysis. Immature neutrophils were isolated by the same procedure from the peripheral blood of patients with acute myeloid leukemia (AML). The informed consent of all blood donors was obtained.

2.2. Patients

The peripheral blood was obtained from ten AML patients from the Hemato-Oncology Clinic of Faculty Hospital in Brno–Bohunice and three patients from the Hemato Oncology Clinic of Faculty Hospital in Olomouc. The causal mutations of the disease were different at these patients. Patients were divided into 2 groups according to presence or absence of HP1 proteins in their neutrophils isolated from the peripheral blood without respect to genetic changes.

2.3. Cell irradiation

Cells were irradiated with 3 Gy of γ-rays (^{60}Co, 1 Gy/min) in RPMI medium with 10% fetal calf serum (FCS) immediately after isolation and maintained in this medium for next 30 min before fixation or RNA and protein isolation.

2.4. Hemopoietic stem cells (HSCs) isolation, enrichment and separation of the CD34$^+$ cell population

CD34$^+$ cells for *ex vivo* differentiation were enriched from bone marrow (BM) of a patient with CML and, for comparison, also from PBSCs of two patients with lymphoma undergoing autologous transplantation and from two healthy donors. HSCs of lymphoma patients and of healthy donors were taken from the leukapheresis product of peripheral blood (PBSC graft) after recombinant granulocyte-colony stimulating factor (rhG-CSF) (Filgrastim; Inc., Amgen, USA) administration. Approximately 50 ml of bone marrow were obtained from a patient with CML in complete hematological remission during the collection of the back-up graft. Leukapheresis products were enriched for CD34$^+$ cells as described in the Supplement.

2.5. Stem cells expansion and differentiation

Two different systems were used for the expansion of CD34$^+$ cells. The first expansion system consisted of Iscove's modified Dulbecco's medium (IMDM; Sigma-Aldrich, USA) completed with 10% FCS (Sigma-Aldrich, USA). The second system contained serum-free expansion medium (SFEM) (StemCell Technologies, Inc., Vancouver, Canada) without FCS. Both types of media were completed with 100 ng/ml of rhG-CSF (Filgrastim, Inc., USA) and a mixture of recombinant human cytokines (CC100; StemCell Technologies, Inc., Canada) containing 100 ng/ml of stem cell factor (SCF), 20 ng/ml of interleukin-3 (IL-3), 20 ng/ml of interleukin 6 (IL-6) and 100 ng/ml of stem cell tyrosine kinase-1 ligand (Flt-3-L). The initial concentration of CD34$^+$ cells was 2×10^4/ml in both types of systems. Cells were cultured for 21 days and counted in two-day intervals using an automatic cell counter (Abbott, USA) to determine the absolute leukocyte count in the samples. The culture medium was half exchanged every two days, and its volume was adjusted to keep the cell concentration below 1×10^6/ml in order to prevent cell overgrowth. A constant concentration of cytokines was maintained. Samples of 2×10^5 cells were taken in two-day intervals from the 6th day for the immunodetection of chromatin structure markers and DSB repair proteins. The vitality of growing cells was assessed regularly by a LIVE/DEAD® Viability/Cytotoxicity Kit (Molecular Probes, Eugene,USA).

2.6. Flow cytometry

The phenotype of growing cells was analyzed after 14 and 21 days of culture, and the expression of CD34 antigen was followed every 2 days on a Cytomics FC500 cytometer (Beckman-Coulter, USA). Number of cells taken for these measurements was 1×10^6 in all samples. The myeloid lineage specific antigens CD33, CD14 and CD16 were detected

Fig. 3. Changes in the presence of HP1α, β, cMYC, dimethylated H3K9 (A, B, C), HP1γ and MNEI (D, E) detected by immunofluorescence during *ex vivo* differentiation of CD34$^+$ cells of a CML patient in IMDM completed with FCS. (A, D) Images of DNA inside the cell nuclei (blue, TOPRO3) represent the most progressive fraction of cells at the given time of the process. (A) The levels of HP1α and β decreased on day 10 and disappeared from the nuclei by day 12. cMYC expression was stopped from day 10. Dimethylated histone was detectable until day 18 of the process in neutrophils originated from CD34$^+$ cells of the CML donor; but its level decreased considerably from day 12. (B, C) Histograms of the integrated optical density (IOD) express levels of specific proteins during differentiation of CD34$^+$ cells through granulocytes. IOD was measured as described in Section 2.8 for 80–130 nuclei per specific time. Attached cells preserved their natural shape and were not deformed. All values are expressed as mean standard deviation (s.d.) of (n) number nuclei in specific time of observation and given in IOD. High s.d. show differences in IOD of individual nuclei present on slides for specific times of differentiation. These differences are likely connected with different stages of cells differentiation on the slide. A statistically significant difference of results relative to the values obtained for day 6 was calculated using the Student's *t*-test: $^*P<0.05$, $^{**}P<0.01$. (D) The level of MNEI in cells at given times of the differentiation process. The histograms (E) present relation between the content of MNEI in the nucleus and cytoplasm expressed as % of the mean value of IOD measured in the central slice (0.2 μm thin) through the cells; the mean was calculated from about 100 cells in each time. The proportion of the IOD in the nucleus and the cytoplasm was calculated from the total cellular value for each cell separately. The level of MNEI in the cytoplasm was significantly lower ($P<0.05$) than in the nucleus from day 6 and decreased to a negligible value from day 14. RGB channels were nonlinearly increased by the Photoshop curves function in order to allow the figure printing.

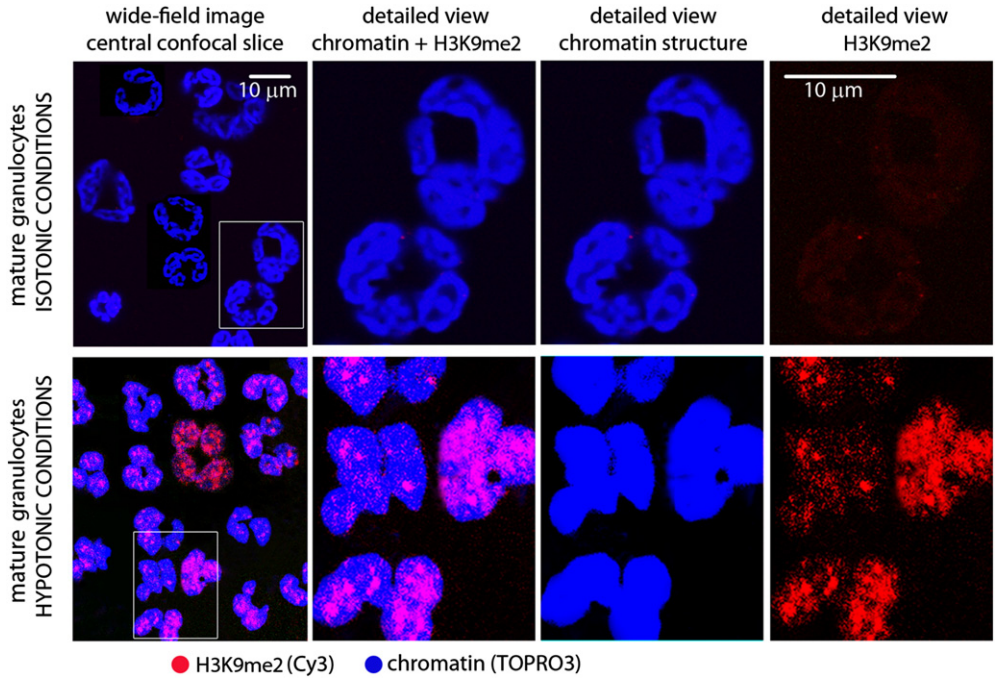


Fig. 4. Changes in chromatin structure of mature neutrophils after short exposure to hypotonic treatment. Freshly isolated mature neutrophils of a healthy donor were incubated in 0.5× PBS for 10 min and then immediately submerged to 4% paraformaldehyde for fixation. Chromatin relaxation in neutrophils obtained by this treatment enabled positive immunodetection of histone H3K9me2 (lower range of images). In neutrophils that were immediately fixed after isolation, the immunodetection of this antigen was negative (upper range of images).

with the monoclonal antibodies directly labeled with FITC, R-PE or PE-Cy5 (Becton-Dickinson, USA; Caltag Lab, USA).

2.7. Cell fixation and immunostaining

Cells harvested at different time intervals from the growing culture and freshly isolated granulocytes from the peripheral blood were processed as described earlier [13] and in the Supplement.

2.8. Fluorescence microscopy

Images were obtained with a high-resolution Leica DM RXA confocal cytometer (Leica, Wetzlar, Germany) described in [29,30], equipped with a CSU-10a confocal unit (Yokogawa, Japan), a CoolSnap HQ charged-coupled device camera (Photometrix, Melbourne, Australia) and an Ar-Kr laser Inova 70C Spectrum (Coherent, Hilton SA, Australia). The oil immersion Plan Fluotar objective (100×/NA 1.3) was used. Forty optical sections at 0.2 μm steps were acquired for each nucleus using the software FISH 2.0 [29,30] at the constant temperature of 26 °C. The exposition time and dynamic range of the camera in the red, green and blue channels were adjusted to the same values for all slides to obtain quantitatively comparable images. Integrated optical density (IOD) in red or green color channels was measured using the image analysis software Image-Pro Plus, version 5.1.2.59 (Media Cybernetics, Inc., MA, USA). IOD registers average intensity per measured object. It was measured in the central, i.e., 20th slice (0.2 μm thick), through the cell nucleus. At least 50 nuclei were scored for the measurements of specific antibodies IOD. The statistical significance (P) of the values obtained during the differentiation relative to the values on day 6 was determined according to the normal distribution by the Student t-test.

2.9. RT-PCR

The total RNA was isolated from granulocytes and mononuclear cells by High Pure RNA Isolation Kit (Roche). 10^6 cells of each type were used. cDNA synthesis was performed using Transcriptor High Fidelity cDNA Synthesis Kit with oligo $(dT)_{18}$ as a primer (Roche). PCR amplification was carried out using HotStarTaq Master Mix Kit (Qiagen) with specific primers listed in the Supplement.

2.10. Protein extraction, gel electrophoresis and western blotting

Protein extraction, gel electrophoresis and western blotting were done according to the standard procedures, as described in the Supplement.

2.11. Activation of neutrophils by phorbol 12-myristate 13-acetate to generate neutrophil extracellular traps

Brinkmann et al. [23] disclosed the capacity of mature neutrophils to release chromatin and proteins from cytoplasmic granules to form extracellular fibers (NETs) that capture and kill microorganisms. This capacity of neutrophils can be stimulated not only by pathogenic microorganisms but also by alternative stimuli [26,28], the most potent of which is phorbol 12-myristate 13-acetate (PMA). We used PMA to detect the capability of NETs formation by AML neutrophils that are not completely mature, express HP1γ protein and have immunodetectable H3K9me2. Granulocytes were isolated from the peripheral blood of 2 healthy donors and 5 AML patients after elimination of erythrocytes using Ficoll-Hypacque centrifugation gradient. Freshly isolated neutrophils were resuspended in RPM1 medium containing 2% of FCS to 1×10^6 cells/ml. 200 μl of this cell suspension was seeded to a positively charged microscopic slide and incubated for 1 h in CO_2 incubator at 37°C. Then, 4 ml of 21 nM PMA or 10,5 nM PMA in RPMI with 2% FCS were added to cells attached to the slides to obtain the final 20 nM or 10 nM concentration of PMA. Cells were incubated in these media for 150 min and 240 min in a CO_2 incubator at 37^0C before fixation in 4% paraformaldehyde for 10 min. Cells were then washed 3 times in PBS, permeabilized in PBS containing 0.2% Triton X-100 and immunostained with anti-histone H2B antibody from Millipore as described in Supplement.

2.12. Hypotonic treatment of granulocytes

Granulocytes isolated from peripheral blood of 2 healthy donors were resuspended in PBS to 1×10^6 cell/ml. 200 μl of this cell suspension were seeded to a microscopic slide and incubated for 30 min in a CO_2 incubator at 37 °C. Next, 4 ml of 0.525× PBS were added and cells were incubated in this low-salt (150 mOsm) medium (0.5× PBS) for 10 min, fixed in 4% paraformaldehyde for 10 min, permeabilized and immunostained with anti-histone H3K9me2 antibody (Upstate). (Normal culture medium or 1× PBS have the osmolarity of 300 mOsm).

3. Results

3.1. Purity of the CD34$^+$ cells

To analyze changes in chromatin structure during the CD34$^+$ cells differentiation into granulocytes and the ability of CD34$^+$ cells isolated from CML patients to differentiate (in the presence of the BCR/ABL translocation), CD34$^+$ cells were isolated, differentiated *ex vivo*, and immunologically analyzed for the expression of specific surface antigens and chromatin structure determining proteins. The purity of CD34$^+$ cells from bone marrow is usually lower than the purity of cells obtained from a PBSC graft after single-step separation. Therefore, the bone marrow cells were purified in a two-step procedure. The average purity of the enriched CD34$^+$ cell fraction from PBSC grafts was 94% and that of BM of a CML patient was 92% (Fig. 1A). The PCR analysis in combination with FISH method confirmed the presence of BCR/ABL translocation in 70% of enriched CD34$^+$ cells of a CML patient.

3.2. Ex vivo cell expansion and differentiation of healthy and CML blood stem cells

Cells from all samples expanded much more efficiently in the SFEM medium without than with FCS in IMDM (Figure S1). Cells of a CML patient expanded approximately 876× in serum free medium and only 96× in a medium supplemented with FCS. Cells from PBSC graft of a healthy donor expanded 39× in serum free medium and 7× in the medium supplemented with FCS. Massive expansion of leukemia cells from a CML patient in SFEM medium was about 20 times higher than the expansion of cells from a healthy PBSC in this medium and about 13 times higher in the medium completed with FCS.

The lower expansion of both cell types in IMDM medium containing 10% FCS could be, probably due to a more rapid cell differentiation, indicating that there are some, not yet known cytokines in FCS that are favorable for terminal granulocyte differentiation and maturation and were not added into the system. Very similar results were obtained with stem cells of different donors in repeated experiments. More rapid differentiation of stem cells in IMDM medium completed with FCS was manifested by more rapid loss of CD34 antigen (Fig. 1 Bi, Ci) at both types of donors and also by earlier disappearance of HP1γ protein and later accumulation of MNEI in cell nuclei of stem cells of a CML patient during ex vivo differentiation (Fig. 3D, Figure S2).

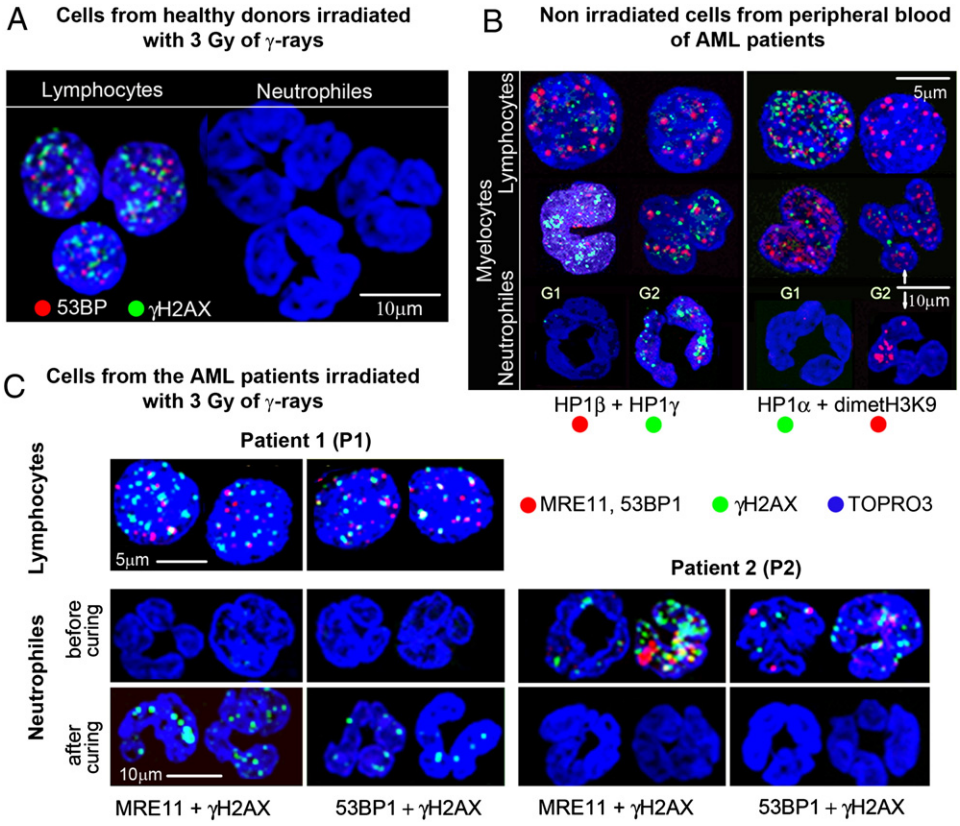


Fig. 5. Presence of chromatin structure markers and DSB repair proteins in nuclei of blood cells isolated from healthy donors and AML patients. (A) The comparison of the expression of γH2AX and repair proteins in human lymphocytes and neutrophil granulocytes isolated from the blood of healthy donors and irradiated with the dose of 3 Gy of γ-rays. There are neither repair proteins nor γH2AX in irradiated mature neutrophils contrary to irradiated lymphocytes. (B) HP1α, β, γ and H3K9me2 immunodetected in lymphocytes, myelocytes and neutrophils of peripheral blood of AML patients at the time of the disease diagnosis. HP1 proteins and H3K9me2 are present in lymphocytes however they are not in neutrophils of the G1 group of AML patients contrary to those of the G2 group. (C) Immunodetection of γH2AX and repair proteins in γ-irradiated lymphocytes and neutrophils isolated from the blood of AML patients P1 (group1) and P2 (group2) before and after the leukemia treatment. The cells were irradiated with 3 Gy of γ-rays and fixed 30 min PI. While all detected proteins were found in lymphocytes, they were not found in neutrophils of the group 1 patients, represented by P1, before the treatment and in neutrophils of the group 2 represented by P2 after the treatment. Chromatin was counterstained with TOPRO-3. RGB channels were nonlinearly increased by the Photoshop curves function in order to allow the figure printing.

The rate of differentiation characterized by the loss of CD34 antigen was higher for stem cells from the CML donor (Fig. 1 Bi) compared to that for PBSC (Fig. 1 Ci) of healthy donors in both types of media. A "timetable" summarizing the changes in antigen expression during the cell differentiation is displayed and compared with changes in chromatin structure and cell morphology on Fig. 1D. CD34 antigen almost disappear from cells of PBSC cultured in serum-free and serum-supplemented media at day 12 and the day 14 from cells of a CML donor (Fig. 1 Bi, Ci, D) and the disappearance of CD34 antigen is accompanied by the increase of cells expressing antigens characteristic of myeloid differentiation ($CD33^{dim+}$, $CD13^{+}$ and $CD16^{+}$). The number of cells expressing these antigens increased, and, on day 21, some 80% of cells from the BM of a CML patients and the PBSC of a healthy donor were expressing $CD33^{dim+}$, 70%–80% expressed $CD13^{+}$ and 50%–60% expressed $CD16^{+}$ in both culture systems (Fig. 1 Bii, Cii, D). Vitality of cells in all systems was above 90% from the beginning to the end of the culture process.

3.3. Changes of HP1, MNEI and H3K9me2 during stem cell differentiation

Stem cells express CD34 antigen, all HP1 proteins, cMYC and H3K9me2 (Fig. 2). These proteins were successively eliminated during ex vivo differentiation that was accompanied also by changes in the shape of cell nuclei. The rate of differentiation was not the same for all cells in the culture, as shown by differences in the nuclear shape and different levels of detected proteins at the appropriate time of cell examination (Fig. 3A, B, C, D). The homogeneity of cells increased with the time of differentiation and was higher in the presence of FCS. Many banded cells were already present by the 8th day of the process (Fig. 1D). The changes in the shape of the nuclei were accompanied by a decrease in the level of HPl proteins and H3K9me2 signal on the one hand (Fig. 1D, 3A, B, C) and by an increased nuclear relocation of MNEI on the other (Fig. 1D, 3D, E). Cells expressing HP1α and HP1β proteins disappeared completely by the 12th day, cells with HP1γ by the 10th day of the differentiation and the level of MNEI in the nucleus was higher than in the cytoplasm from the 6th day (Fig. 1D, 3D, E); from the 14th day and later, this protein was observed predominantly in the nucleus (Fig. 1D, 3D, E). The disappearance of HP1 proteins was correlated with the appearance of higher number segmented nuclei of neutrophils (Fig. 1D). Importantly, the differentiation process was not significantly influenced by the origin of $CD34^{+}$ cells (healthy donor, BCR/ABL translocation or NHL patient), despite the H3K9me2 signal persisted in neutrophils coming from the $CD34^{+}$ cells of the CML patient some days longer (from 16th to 18th day) than in the healthy and NHL cells. To show that inaccessibility of H3K9me2 for immunodetection is caused by a tighter chromatin condensation during the differentiation of $CD34^{+}$ cells to granulocytes, we exposed the granulocytes isolated from peripheral blood of healthy donors to a short (10 min) incubation in a hypotonic solution of 0.5× PBS before fixation. Chromatin structure of granulocytes exposed to this lower-salt solution became more relaxed enabling H3K9me2 detection (Fig. 4).

It thus appears that there are coordinated consecutive changes of heterochromatin factors during the granulocyte differentiation process that include cessation of protein expression (HP1), cytoplasm-nuclear relocation (MNEI) and protection of antigenic epitopes due to chromatin condensation (H3K9me2).

3.4. Capacity of white blood cells for DDR induced by γ-rays

Next, we analyzed how changes in chromatin structure established in terminally differentiated granulocytes are reflected in their functions, and whether there are some differences between mature terminally differentiated granulocytes obtained from healthy donors and immature granulocytes of AML patients. Since DNA repair was reported to be active in majority of cell types, we followed the DDR induced by DSBs that are the most serious DNA lesions. Granulocytes and mononuclear cells (lymphocytes and monocytes) isolated from the peripheral blood of healthy donors were irradiated with 3 Gy of γ-rays and formation of IRIF foci at the sites of DSBs was followed by immunodetection 30 min post-irradiation (PI). While in lymphocytes and monocytes (Fig. 5A) as well as in stem cells (not shown), the IRIFs appeared very soon after DSB induction and their colocalization with different repair proteins was observed, neither the IRIFs nor the repair proteins were detected in mature neutrophils of healthy donors (Fig. 5A). These

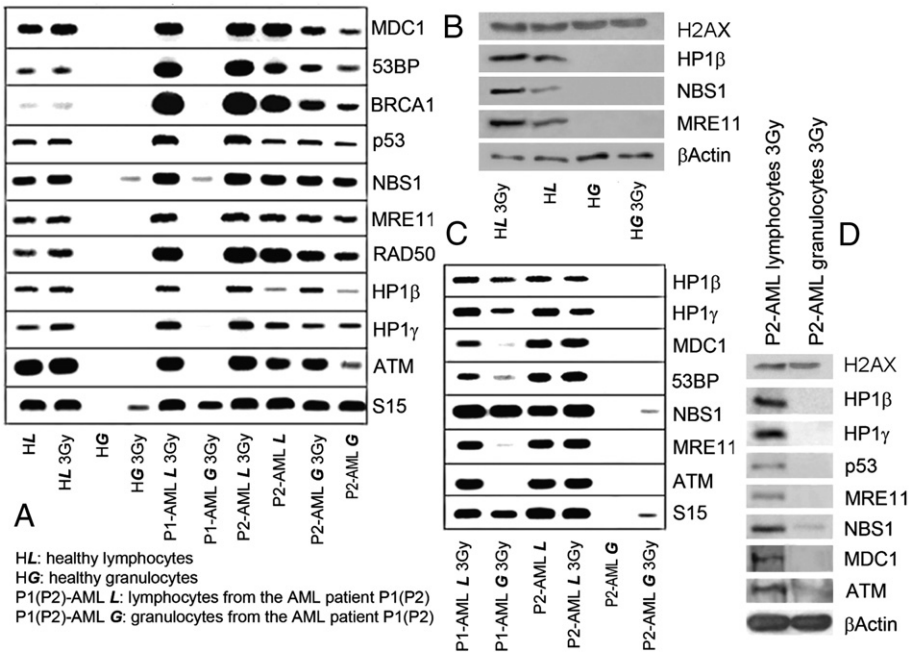


Fig. 6. Transcription activity (RT-PCR) of HP1β, HP1γ and selected DSB repair genes in lymphocytes and granulocytes of a healthy donor, AML patients P1 and P2 (representing the group 1 and 2). (A, C) Transcription activity before and after the treatment, respectively. (B, D) Levels (WB) of HP1β and HP1γ and some repair proteins before (B) and after (D) the treatment.

neutrophils did not express any of HP1 proteins (not shown) contrary to mononuclear cells and myelocytes isolated from the peripheral blood of AML patients before the treatment and incompletely differentiated AML neutrophils (Fig. 5B, C). The presence of HP1 proteins in neutrophils of some AML patients made us to divide them into groups for further analyses: While the neutrophils of the group 1 (G1) AML patients did not present any HP1 proteins similarly as the healthy mature neutrophils, those of the group 2 (G2) expressed small amount of HP1 proteins even if they had multi-lobed nuclei, typical for mature neutrophils (Fig. 5B). The cells of G2 patients expressed also repair proteins and phosphorylated H2AX after γ-irradiation; however the colocalization of γH2AX with repair proteins was rare 30 min post-irradiation (PI) (Fig. 5C). The expression of HP1γ, HP1β as well as repair proteins after irradiation thus represent marks of incompletely mature granulocytes. The absence of the repair proteins in the nuclei of terminally differentiated neutrophils (from healthy donors and G1-patients) was consistent with missing of the corresponding gene transcription detected by RT-PCR and western blotting (Fig. 6 A, B). The exception was the expression of small amount of Nibrin (NBS1) in irradiated terminally differentiated granulocytes of healthy donors and G1 patients.

The level of granulocytic maturation in the peripheral blood was followed in detail at 1 patient (P1) of the group 1 and one of the group 2 (P2) again 10 month after the beginning of leukemia treatment. At this time, the amount of WBC of P1 was almost normal, (3.41×10^9/L), however with only 1.33×10^9/L of neutrophils (presenting 39% of WBC). The level of P2 WBC was completely normal (8.8 x 10^9/L with 62.8% of neutrophils). The neutrophils of the latter patient were entirely mature; they expressed none of HP1 proteins, did not form γH2AX foci and expressed none of repair proteins after γ-radiation. (Fig. 5C, 6C). On the other hand, about 50% of neutrophils of the P1 were not mature. Many of them were in the stage of band cells. Both, the band and segmented cells expressed HP1 proteins, detected in the nuclei by specific antibodies (not shown). The activity of genes coding for HP1 isoforms and repair proteins detected by RT-PCR in extracts from granulocytes significantly contrasted for P1 and P2 after the treatment (Fig. 6C). RT-PCR showed active mRNA expression of HP1β and HP1γ and low expression of genes for DNA repair proteins in case of P1, however none of these genes were expressed in P2 granulocytes. In spite of low expression of several repair protein genes (MDC1, 53BP,

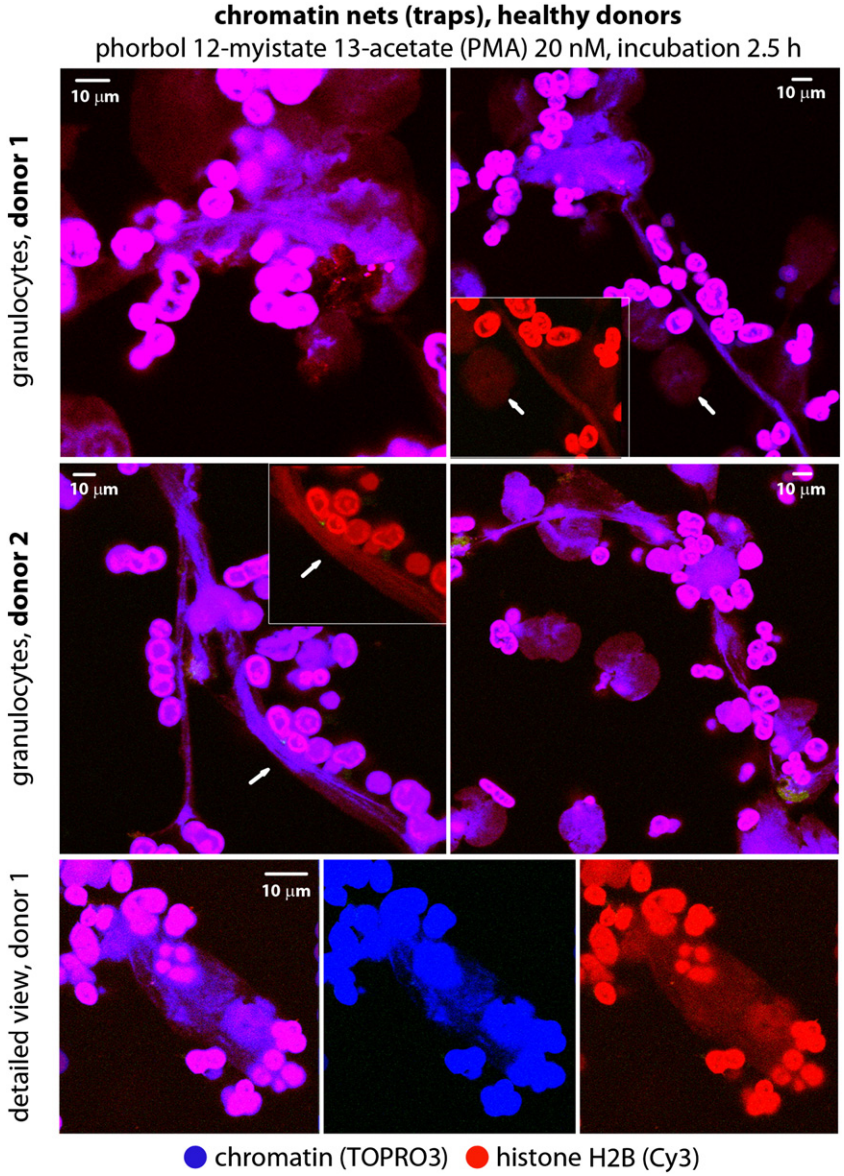


Fig. 7. Mature neutrophils isolated from healthy donors release extracellular traps (NETs) after activation with phorbol 12-myristate 13-acetate (PMA). Freshly isolated mature neutrophils were incubated in PMA (20 nM) for 2.5 h at 37 °C before fixation in paraformaldehyde and immunodetection of histone H2B.

MRE11) in P1 granulocytes, the proteins encoded by these genes were not found by immunodetection on cell nuclei (Fig. 5C, 6D); however, γH2AX foci marking regions of DSB were detected in the P1 nuclei after irradiation (Fig. 5C). Their number was smaller as compared with the lymphocytes irradiated with the same dose.

In some cases when the treatment of leukemia at patients of the group 2 lead to the attainment of normal mature granulocytes, this situation was not stable and the patients had to undergo further consolidation treatments. Therefore, we conclude that only the treatment of AML that removes the cells with causal mutations and simultaneously leads to persistent granulocyte maturation could effectively prevent AML relapse. The state of granulocyte maturation was followed for patients of the G1 and G2 groups for additional 10 to 19 months by immunological detection of HP1β and HP1γ expression. All five patients of the G2 had normal number of white blood cells and neutrophils did not show the marks of immature cells. Two patients of this group were observed after the stem cells allogenic transplantation. All three patients of the group 1 underwent at least one additional consolidation treatment during this time, however even though they have a normal number of white blood cells at present, their neutrophils contain HP1 proteins indicating an incomplete differentiation.

3.5. The incomplete maturation of AML neutrophils negatively affects the function of these cells to form neutrophil extracellular traps (NETs)

The ability to release chromatin NETs upon incubation with PMA was detected with granulocytes from 2 healthy donors (Fig. 7) and 5 AML patients (Fig. 8). Neutrophils of two of them expressed HP1γ in about 43% cells; one patient had HP1γ in about 70% of neutrophils, and two other expressed it in 90% and 94% neutrophils. The neutrophils of the last two patients did not respond to activation with PMA used at two different concentrations (10 nM and 20 nM) for 2.5 h and 4 h. The NETs were not found in any neutrophils among about 2×10^5 cells fixed on the microscopic slide and expressing HP1γ protein in about 90% of cells. The similar results were also obtained with neutrophils expressing HP1γ protein in about 70% cells. Nuclei of majority of these cells preserved the shape and structure of chromatin, (Fig. 8C), while at mature neutrophils exposed to 20 nM PMA for 4 h., the majority of nuclei lost their lobular shape, expanded and released chromatin NETs into the extracellular space (Fig. 8A).

The ability to release NETs and corresponding changes of chromatin structure were also observed in neutrophils, where the expression of HP1γ was found in about 40–43% of the cells (Fig. 8B). These results suggest that remodeling of chromatin structure during neutrophil differentiation enable these cells to rapidly react to pathogenic microbes in a body by releasing chromatin to extracellular space and forming NETs for capture and killing these microbes and thus exert the basic function of granulocytes in fighting bacterial infections. However, when the process of differentiation is not completed and neutrophils continue to express HP1 proteins, their capability to release chromatin NETs is precluded.

4. Discussion

In this work, the consequent changes of chromatin structure during differentiation of normal and leukemia blood progenitors to granulocytes were followed and the capability of differentiated cells to repair DNA DSB was analyzed. We show that heterochromatin epigenetic markers change substantially during the *ex vivo* differentiation of $CD34^+$ cells. These changes consist of the progressive disappearing of HP1 proteins from differentiating neutrophils, inaccessibility of H3K9me2 for immunodetection, and nuclear accumulation of a nucleocytoplasmic serpin MNEI (Figs. 1, 3D, E). The complete absence of all three heterochromatin proteins HP1 isoforms from differentiated HSCs $CD34^+$ is in excellent agreement with our previous study showing that the loss of these epigenetic markers distinguishes mature granulocytes from other human differentiated cells [13].

In order to determine the differences in the behavior of normal and leukemic cells during the differentiation process, dynamic changes of chromatin structure were followed for $CD34^+$ cells isolated from the peripheral blood of healthy and NHL donors and from bone marrow of a CML patient in chronic phase and after treatment with Glivec (while still preserving BCR/ABL translocation in the majority of cells). Changes of chromatin markers during cell differentiation were followed by *in situ* immunodetection as it is more favorable than "averaging" methods working with cell lysates for several reasons: It is applicable for a low number of cells (very important in case of stem cells) and as already mentioned, provides reliable information about the expression of antigens in individual cells during their differentiation and thus precludes "averaging" of results typical for western blotting and RT-PCR. It is really very important in this case due to the differences in the progress of differentiation between individual cells. By maintaining the same conditions in all experiments, we observed that the changes of studied markers during differentiation were not influenced by the origin of the $CD34^+$ cells and that $CD34^+$ cells can differentiate in mature granulocytes *ex vivo*, even in the presence of BCR/ABL translocation (Fig. 3). It means that the negative influence of the translocated BCR/ABL tyrosine kinase can be overcome by specific cytokines *ex vivo*.

In parallel with altering expression of surface antigens and changing morphology of the nuclei during differentiation, we observed several changes of higher-order chromatin structure. Soon after stopping proliferation, HP1 proteins disappear, followed by almost complete relocation of MNEI from the cytoplasm to the nucleus (Fig. 3D). Simultaneously, the accessibility of H3K9me2 for immunodetection decreases (Fig. 3A). Association of MNEI with condensed nuclear chromatin after the elimination of HP1 indicates that this protein could replace HP1 in chromatin condensation, similar to the homologous serpin MENT in chicken granulocytes [9,15,31].

It was shown recently that nuclear MNEI plays a role in maintaining heterochromatin compactness in neutrophils preventing premature formation of NETs [17]. Our results showing that the mature granulocytes accumulating nuclear MNEI have inhibited DDR further suggest that replacing HP1 with MNEI might be associated with DDR inhibition.

The mechanistic link between the HP1 disappearance from chromatin during terminal differentiation of human neutrophils, its possible replacement by MNEI and chromatin condensation remains to be examined. Nevertheless, our results indicate that the timing and extent of HP1 loss, nuclear accumulation of MNEI, and inaccessibility of H3Kme2 reflect the level of chromatin compaction and, likely, differentiation. It follows from these results that there is a coordinated temporal order of changes in chromatin proteins and structure and that these changes are intimately linked with one of the basic functions of neutrophils, the formation of extracellular NETs, in which chromatin serves as the principal tool.

It thus appears that the purpose of the above mentioned changes in chromatin structure during differentiation is to enable the unique function of neutrophils in immune defence. The tightly condensed chromatin and gene repression in terminally differentiated neutrophils must be rapidly reversed upon their activation by microbial infection to allow increased changes in gene expression [32–34] and chromatin relaxation into extracellular fibers NETs to kill bacteria [23]. The rapid and extensive changes in gene expression and chromatin relaxation in neutrophils are unique phenomena among the terminally differentiated cells. We show in this work, that the chromatin condensation in mature neutrophils in absence of HP1 is tighter than in its presence as indicted by the low accessibility of H3K9me2 for immunodetection. The fact that a short exposure of the neutrophils to hypotonic conditions makes the N-tails of histone H3 accessible to immunodetection (Fig. 4) shows that the level of H3K9me2 remains high in these cells and that this high chromatin

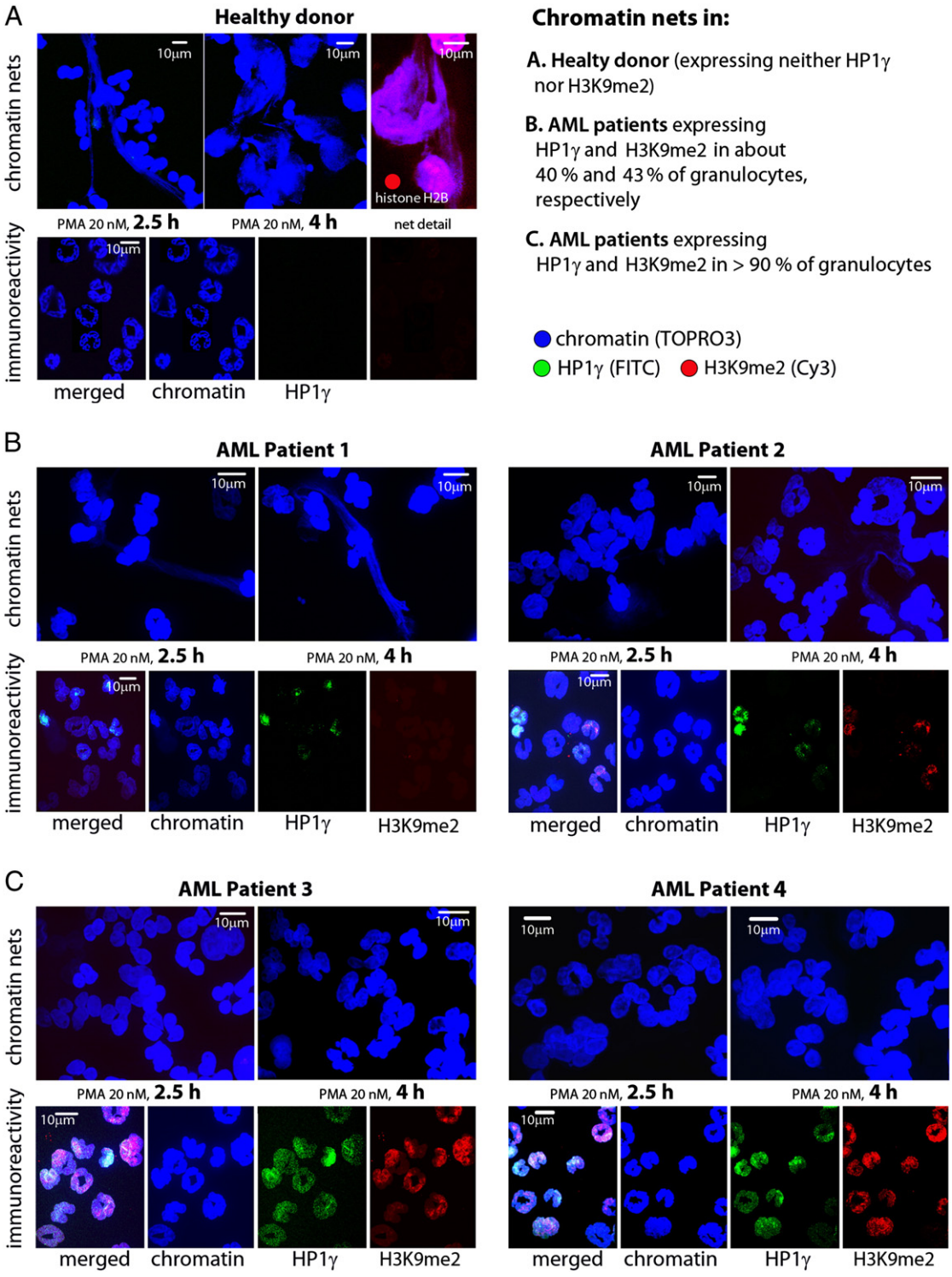


Fig. 8. Immature neutrophils expressing HP1γ do not form NETs after activation with PMA. Formation of nets was observed in peripheral neutrophils of the AML patients 1 and 2 expressing HP1γ in about 43% of these cells. No NETs were released by neutrophils of the AML patients 3 and 4, that expressed HP1γ in 90% and 93% of cells.

condensation rather than histone H3K9 demethylation is responsible for protection of H3K9me2 in mature granulocytes.

This tight chromatin condensation can be abruptly reversed by concerted action of peptidylarginine deaminase catalyzing histone citrullination and unfolding of chromatin higher-order structure [35] and by specific proteases (such as neutrophil elastase) translocating into the nuclei after their release from granules and causing large-scale digestion of histones [36]. MNEI which is a specific inhibitor of neutrophil elastase [37] may fulfill its function in protecting chromatin from decondensation by promoting chromatin higher-order folding and simultaneously inhibiting the proteases leaking into the nucleus prior to massive NET activation.

The short lifespan of neutrophils and specific role of their chromatin in forming NETs seem to be the reasons for silencing of genes participating in DNA double-strand break repair in mature neutrophils as we show in this work (Fig. 6). Obviously, the conformational transition of chromatin during NET formation does not require the integrity of genome and therefore DSBs need not to be repaired. Importantly,

granulocytic differentiation is incomplete in myeloid leukemia triggered by mutations in some genes and we show in this work that these immature cells do not have the ability to release chromatin into extracellular space and form the NETs. This may have a direct impact on the ability of AML patients in remission to fight bacterial infection. Bacterial and fungal infections are an important cause of mortality during AML therapy [38,39]. Different genetic changes inducing AML have apparently a crucial influence on progress and curability of the disease. At present, not all genetic mutations leading to AML (and their impact on granulocytic differentiation) are known. Some patients have mutation in one or more genes that occur frequently in AML (for ex. FLT3, c-KIT, RAS, NPM1, translocation AML1/ETO); nevertheless, the sequencing of AML genome showed that leukemia cells also contained acquired mutations in several genes that had not previously been associated with the disease [40]. Therefore the relationship between the clinical parameters of successful treatment (normal level of white blood cells, etc.), markers of complete maturation of granulocytes suggested in this work (including the ability to express HP1 and form chromatin NETs) and specific genetic changes should be studied in complex to disclose the cause of incomplete granulocytic maturation during the treatment. We believe that successful elucidation of the important issue why consolidation therapy does in some cases lead to attainment of mature granulocytes and in other cases does not, could make the leukemia treatment more efficient.

5. Conclusions

We show in this work that proteins expressed in human blood stem cells (e.g. CD34, all HP1, cMYC) are successively eliminated during ex vivo differentiation that is accompanied by changes in the shape of cell nuclei, chromatin condensation, and accumulation of the MNEI protein in the nuclei. Terminally differentiated granulocytes loss their ability to recognize and repair DNA damage but acquire another function — creation of chromatin extracellular traps (NETs) as a response to infection. The incompletely differentiated granulocytes of AML patients expressing HP1 proteins behave differently from the mature ones. When irradiated with ionizing radiation, they activate the DDR. The presence of the DDR markers in nuclei of immature neutrophils shows that these cells are not equivalent to mature cells. One of the most important consequences of the incomplete maturation of these cells is the loss of their natural function to release NETs to capture and kill infecting microbes. Importantly, AML treatment does not always lead to complete maturation of granulocytes, even if it restores the level of total blood cells including granulocytes to the normal values. We suggest detection of HP1 proteins in peripheral granulocytes of AML patients as a very sensitive indicator of their successful maturation as well as functionality after the treatment.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2012.12.012.

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