Hematopoiesis in patients with mature B-cell malignancies is deregulated even in patients with undetectable bone marrow involvement

Mature B-cell malignancies represent the most common types of hematologic tumors. Despite this, little information is available on the composition and function of hematopoiesis in patients with these malignancies. Hematopoietic stem and progenitor cells (HSPCs) might be influenced by at least three key factors: 1. HSPC-intrinsic mutations (that might predispose subjects to the development of these malignancies), 2. Direct or indirect impact of malignant lymphocytes present in the bone marrow (BM) or extramedullary tissue, and 3. Age-related changes.[1-5] The aim of the study herein was to analyze HSPC content in patients with thus far untreated mature B-cell malignancies.

We first confirmed the age-related changes observed by Kuranda *et al*. in our cohort of 22 control samples.[6] While the absolute numbers of hematopoietic stem cells (HSCs) did not change, we observed a negative correlation of their relative numbers with increasing age (Figure 1A,C). The absolute, but not relative numbers of multipotent progenitors (MPPs) positively correlated with age (Figure 1A,C). The most statistically significant change was observed in the compartment of multilymphoid progenitors (MLPs), where both the absolute and relative frequencies positively correlated with age (Figure 1A,C). Both the absolute and relative numbers of pro-B cells were significantly lower in the control samples of the elderly (Figure 1A,C). In addition to the age-related changes, we have recently demonstrated that healthy Caucasians have significantly increased proportions of BM-derived pro-B cells compared to Asians.[7] To avoid any potential age- or race-related biases in HSPC frequencies, the control cohort used in this study comprised BM samples obtained from age-matched healthy Caucasians (all patients were Caucasians as well). The flow cytometry gating strategy is explained in detail in the *Online Supplementary Materials and Methods*.

All patient samples were obtained after written informed consent according to the Helsinki Declaration of 1975 (revised in 1985). The study was approved by the Ethics Committee of the Charles University General Hospital in Prague. The patient cohort (n=125, median age 65 years) included samples of chronic lymphocytic leukemia (CLL, n=21), diffuse large B-cell lymphoma (DLBCL, n=35), follicular lymphoma (FL, n=24), mantle cell lymphoma (MCL, n=27), and multiple myeloma (MM, n=18). Surprisingly, most of the age-related changes observed among control samples were not discernible in patient samples (Figure 1B,D). The exceptions to the rule were absolute and relative frequencies of pro-

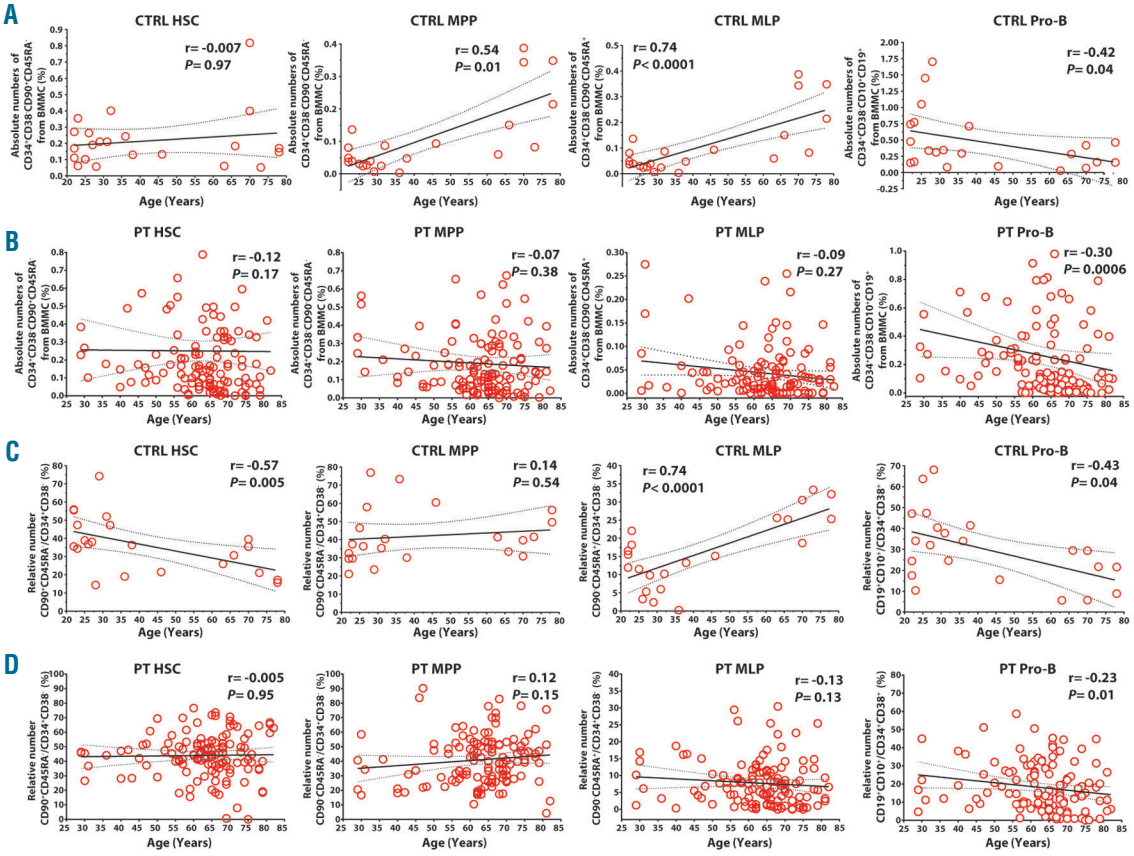

Figure 1. Correlation of hematopoietic stem and progenitor cells with age. Age-related changes of absolute (A,B) and relative (C,D) numbers of hematopoietic stem and progenitor cell (HSPC) populations in the BM-derived samples obtained from healthy controls (CTRL) (n=22) (A,C), and patients (PT) with mature B-cell malignancies (n=125) (B,D). Linear regression analysis demonstrates the correlation of HSPC frequencies with age. Pearson´s correlation coefficients (r), and *P*-values are shown. HSC: hematopoietic stem cell; MPP: multipotent progenitors; MLP: multilymphoid progenitors; BMCC: bone marrow mononuclear cell.

B cells, which negatively correlated with increasing age (Figure 1B,D). In contrast, the most statistically significant age-related change observed in controls, the increased absolute and relative numbers of MLP, was lost in patient samples (Figure 1B,D). The precise molecular mechanism underlying the observed loss of difference in the absolute and relative numbers of HSC, MPP, and especially MLP populations between the young and elderly patients remains elusive. The data, however, strongly suggests that hematopoiesis of the elderly, but not of the young, is impacted by the presence of malignant B cells, either in the BM, or in extramedullary locations (Figures 2A,B and 3D,E). As a consequence the disease-related alterations of the hematopoiesis in the elderly might override the age-related changes normally observed in healthy individuals.

After the age-related analysis we analyzed HSPC changes in the whole patient cohort compared to age-matched healthy controls. While absolute numbers of HSCs (from unselected bone marrow mononuclear cells (BMMCs)) were not changed, their relative numbers (from $Lin^{-}CD34^{+}CD38^{-}$ BMMC) were significantly increased (44.4 ± 16.8% *versus* 33.5 ± 14.9%, P=0.02) (Figure 2A,B). While absolute numbers of MPP were significantly decreased (0.1896 ± 0.2061% *versus* 0.3495 ± 0.0808%, P=0.01, Figure 2A), their relative numbers were not changed (Figure 2B). Both the absolute and relative numbers of MLP were significantly decreased in patients compared to controls: 0.04317 ± 0.05524% *versus* 0.1421 ± 0.1370% (P<0.0001), and 7.789 ± 6.584% *versus* 18.32 ± 11.38% (P<0.0001), respectively (Figure 2A,B). The downregulation of MLP was also observed in the analyses of individual diagnoses, where all subtypes of mature B-cell malignances showed reduced absolute and relative MLP numbers (Figure 2C,D). Similarly, both absolute (from unselected BMMC) and relative (from $CD34^{+}CD38^{+}$ BMMC) numbers of pro-B cells were not statistically different in patients compared to controls (Figure 2A,B). The fact that HSPC deregulations were also observed in patient samples with undetectable BM infiltration strongly suggests that even extramedullary located lymphoma might impact normal hematopoiesis remotely. Precise molecular mediators that could remotely deregulate hematopoietic processes might include cytokines, growth factors, or other types of messengers (e.g., microRNA) produced by malignant lymphocytes and secreted into the plasmatic pool.

We further analyzed whether the detected changes in absolute and/or relative numbers of HSPCs might be associated with the extent of BM infiltration by neoplastic cells. 77 out of 125 patients (61.6%) had detectable BM infiltration by immunohistochemical (IHC) analysis

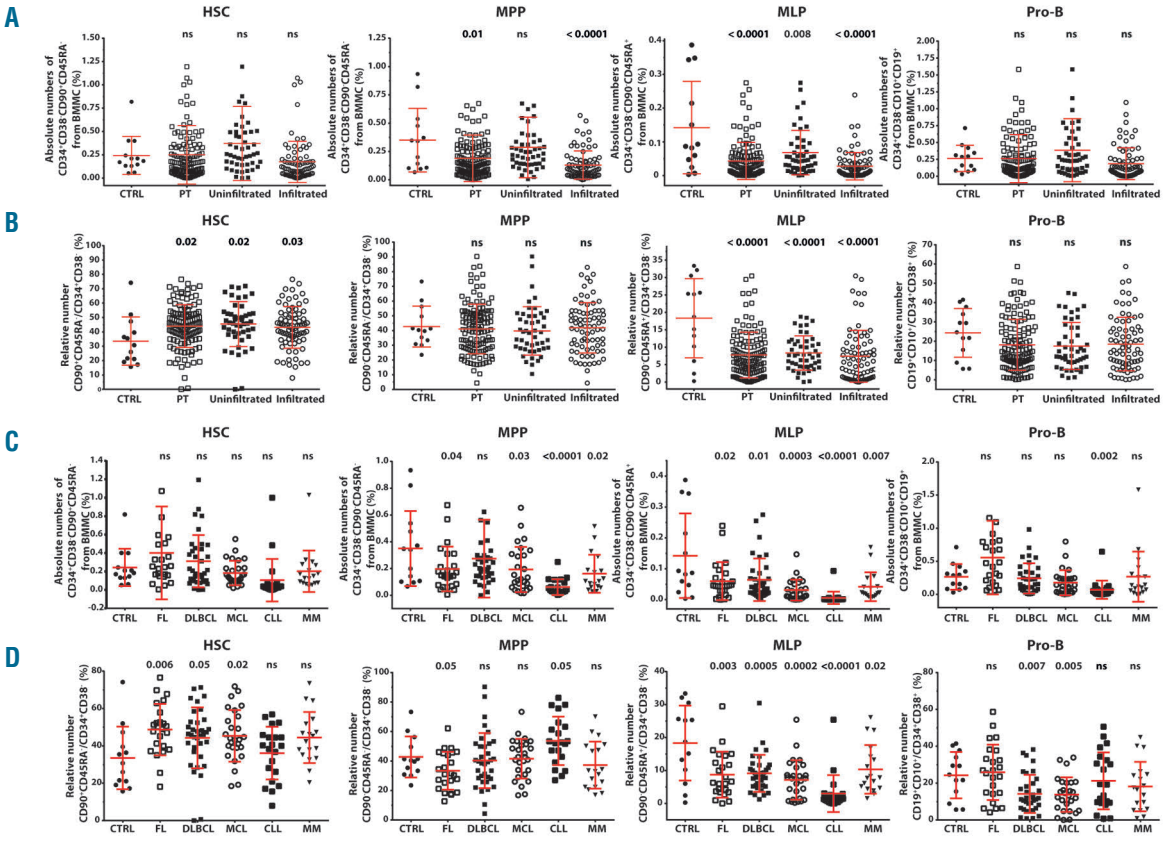

Figure 2. Analysis of hematopoietic stem and progenitor cell frequencies in various diagnosis and in infiltrated *versus* uninfiltrated patients. Absolute (A,C) and relative (B,D) hematopoietic stem and progenitor cell frequencies in all patient samples (A,B) and particular B-cell malignancies (C,D) compared to age-matched controls. Subanalyses of samples with detectable and undetectable BM infiltration are demonstrated. *P*-values of unpaired Student's *t*-tests are shown at the top of each column. Means and standard deviations are graphically expressed. ns: not significant; HSC: hematopoietic stem cell; MPP: multipotent progenitors; MLP: multilymphoid progenitors; BMCC: bone marrow mononuclear cell; PT: patient; CTRL: control; FL: follicular lymphoma; DLBCL: diffuse large B-cell lymphoma; MCL: mantle cell lymphoma; CLL: chronic lymphocytic leukemia; MM: multiple myeloma.

of trephine biopsy specimens with a range of between 2.5% and nearly 100% and a median of 40% (Figure 3A). Absolute numbers of all analyzed populations of HSPCs, with the exception of HSCs (i.e., MPP, MLP and pro-B), negatively correlated with the extent of BM involvement (Figure 3B). Plausibly, the data reflects a situation whereby malignant lymphocytes present in the BM oppress normal hematopoiesis. Not surprisingly, the most significant suppression of the absolute HSPC numbers was observed in CLL, which has the highest extent of BM involvement out of all of the analyzed B-cell malignancies (Figure 2C,D). While relative numbers of HSC and MLP demonstrated negative correlation with BM infiltration, relative numbers of MPP showed positive correlation with the extent of infiltration (Figure 3C). These data point to a more complex deregulation of hematopoiesis than mere spatial oppression of hematopoiesis by the presence of malignant B cells. There was no significant correlation between relative numbers of pro-B and BM infiltration (Figure 3C).

Hematopoiesis is a complex process orchestrated by dozens of hematopoietic transcription factors and other important genes. We asked whether the observed differences in the composition of HSPCs between patients and healthy controls would be associated with transcriptional deregulation of these important molecules. Gene expression analyses of 27 selected genes, including hematopoietic stem cell / B-cell / T-cell transcription factors, apoptosis-related genes, cell cycle regulators, stem cell markers and adhesion molecules, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control were carried out on sorted HSC samples obtained from

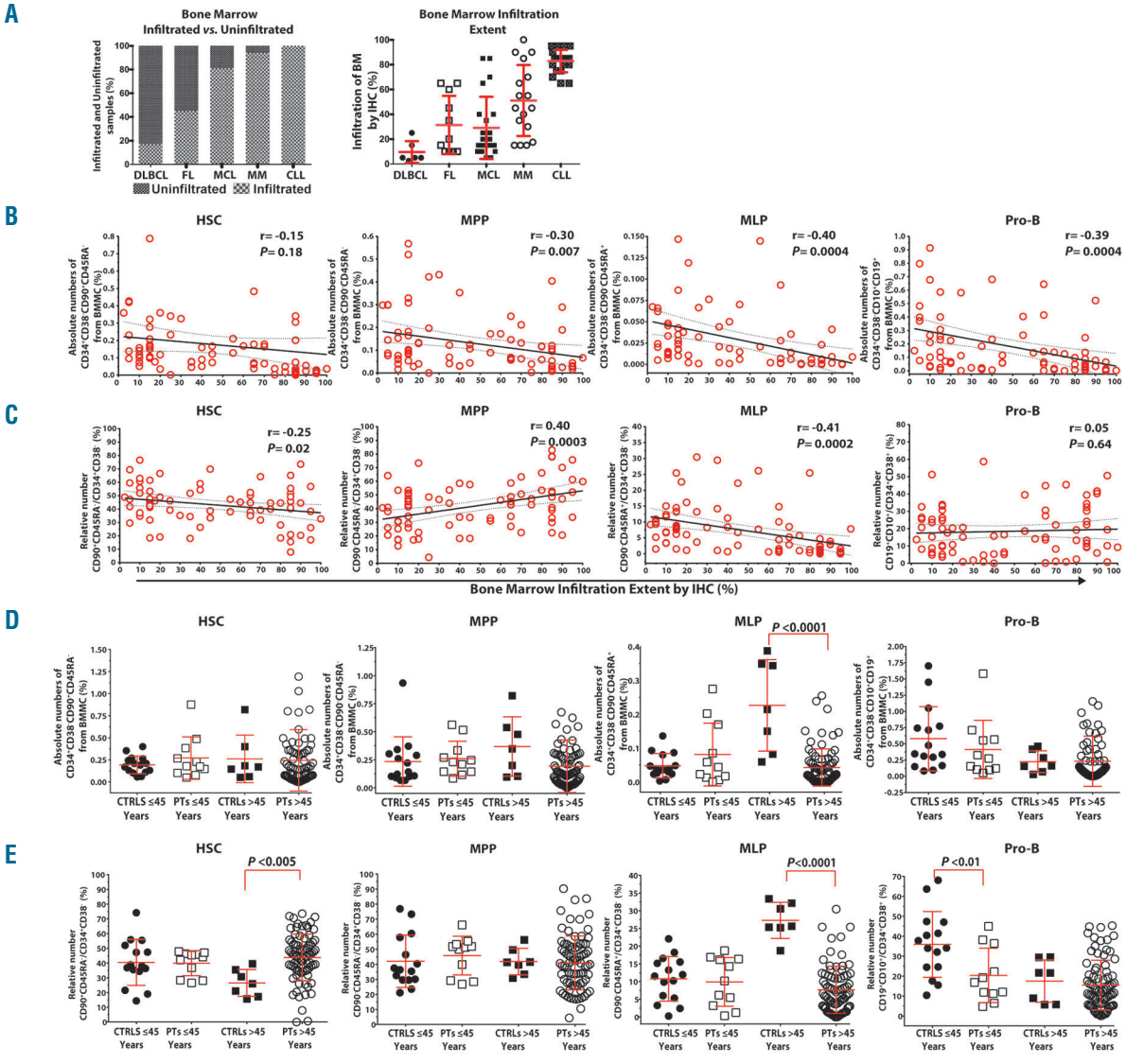

Figure 3. Correlation of both infiltration extent and younger *versus* older patients with hematopoietic stem and progenitor cell frequencies. Overview of the extent of BM involvement per diagnosis (A) and correlation of absolute (B) and relative (C) HSPC frequencies with the extent of BM infiltration in patient samples with detectable BM infiltration. Pearson's correlation coefficients (r), and *P*-values are shown. Panels D and E show absolute (D) and relative (E) hematopoietic stem and progenitor cell (HSPC) frequencies in BM-derived patient (PT) and control (CTRL) samples obtained from the young (≤45) and the elderly (>45 years). Means and standard deviations together with Student's *t*-test *P*-values are shown at the top of the paired columns. ns: not significant; HSC: hematopoietic stem cell; MPP: multipotent progenitors; MLP: multilymphoid progenitors; BMCC: bone marrow mononuclear cell; FL: follicular lymphoma; DLBCL: diffuse large B-cell lymphoma; MCL: mantle cell lymphoma; CLL: chronic lymphocytic leukemia; MM: multiple myeloma; IHC: immunohistochemistry.

63 patients (MCL= 16; FL= 13, DLBCL= 15, CLL= 7 and MM= 12) and 13 healthy volunteers. We considered a particular gene to be expressed only if its transcription was detectable before 40 cycles of real-time polymerase chain reaction (qPCR) in more than 25% of the particular samples. Using this criterion we observed 3 groups of gene expression patterns in HSC samples. Group 1 consisted of genes expressed in >25% of cases (both patients and controls; *Online Supplementary Table S1*). Group 1a comprised genes with significantly increased relative messenger RNA (mRNA) expression in the patients compared to controls, and included transcription factors *RUNX1, IKZF1 (IKAROS), BCL11A, GATA2, FOXP1, SPI1, MYC*, apoptosis related genes *MCL1, BCL2L1*, stem cell markers and adhesion molecules *CD44, PROM1 (CD133)*, and an efflux pump *ABCB1*. Group 1b included genes with similar expression frequency between the patient and control samples, and included transcription factors *IRF8, BMI1, FOXO1, SOX11,* and a stem cell marker CD34. Group 2 consisted of genes that were expressed in patients, but not in controls, and included cell proliferation, differentiation and apoptosis related genes *CCND1, NOTCH1,* and *ZAP70*. Group 3 comprised genes with undetectable expression in both patients and controls and included transcription factors *EBF1, IRF4, PRDM1, LEF1, BCL6,* an anti-apoptotic gene *BCL2*, and an NF-κB regulator *MALT1*. The gene expression data showed significantly higher expression of most of the tested transcription factors in patients compared to controls (*Online Supplementary Table S1*). This finding suggests that HSCs obtained from patients with mature B-cell malignancies are more transcriptionally active compared to HSCs obtained from age-matched healthy volunteers. Kikushige *et al*. described upregulation of *IKZF1*, but not *RUNX1* or *SPI1* in CLL-derived HSCs compared to normal HSCs.[1] Whether the upregulated transcription might correlate with the observed increased relative numbers of HSCs in patients compared to controls remains to be elucidated. The increased transcriptional activity and increased expression of transcription factors, including early lymphoid differentiation associated transcription factors (e.g., *IKZF1*, *SPI1*, *BCL11A*), in sorted HSCs does not correlate with the observed suppression of the earliest lymphoid progenitors. This might be explained by the bystander effect of the ongoing malignant process assuming the production of external factors that might modify the function and differentiation of hematopoietic stem cells through the initiation of various epigenetic changes.

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