Brief Communication

Determination of Circulating Endothelial Cells and Endothelial Progenitor Cells Using Multicolor Flow Cytometry in Patients with Thrombophilia

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Abstract

Background/Aims: Endothelial progenitor cells (EPCs) and circulating endothelial cells (CECs) have been described as markers of endothelial damage and dysfunction in several diseases, including deep venous thrombosis. Their role in patients with known thrombophilia has not yet been evaluated. Both EPCs and CECs represent extremely rare cell populations. Therefore, it is essential to use standardized methods for their identification and quantification. ***Methods:*** In this study, we used multicolor flow cytometry to analyze the number of EPCs and CECs in patients with thrombophilia with or without a history of thrombosis. Patients with hematological malignancies after high-dose chemotherapy and patients with acute myocardial infarction were used as positive controls. ***Results:*** EPC and CEC immunophenotypes were determined as CD45dim/−CD34+CD146+CD133+ and CD45dim/−CD34+CD146+CD133−, respectively. Increased levels of endothelial cells were observed in positive control groups. No significant changes in the number of EPCs or CECs were detected in patients with thrombophilia compared to healthy controls. ***Conclusion:*** Our optimized multicolor flow cytometry method allows unambiguous identification and quantification of endothelial cells in the peripheral blood. Our results support previous studies showing that elevated levels of CECs could serve as an indicator of endothelial injury or dysfunction. Normal levels of CECs or EPCs were found in patients with thrombophilia.

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Introduction

Endothelial progenitor cells (EPCs) and circulating endothelial cells (CECs) are extremely rare cell populations (<50 cells/mL in the peripheral blood of healthy controls) [1]. They were originally described by Asahara et al. [2] in 1997, and they occur in bone marrow, umbilical cord, and the peripheral blood [3].

The physiologic function of EPCs is to maintain vascular integrity, tissue regeneration, and tissue remodeling [3, 4]. EPCs are considered as endothelial cell renewal markers [5]. Their potential role in the pathogenesis of certain diseases is still the subject of studies. Changes in

EPC levels have been described in several clinical conditions, e.g., heart failure, diabetes mellitus, cardiovascular diseases, and malignant tumors (tumor growth or metastatic processes) [3–6]. An elevated number of EPCs has been associated with chronic exercise of professional runners [7]. Altered EPCs have been demonstrated in patients with pH-negative myeloproliferative neoplasms with thrombotic complications [8]. However, the significance of EPCs in the pathogenesis of thrombophilia, atherothrombotic diseases, and thromboembolic diseases is still uncertain.

CECs are mature cells which have been released from the vascular endothelium as a result of vascular damage [9]. CECs are therefore considered as markers of endothelial damage or dysfunction [5, 9]. Furthermore, associations with plasma markers of endothelial damage (von Willebrand factor and soluble E-selectin) have been described [10]. CECs do not have the progenitor potential [9, 11], and, in contrast to EPCs, they are difficult to cultivate [12]. Elevated CEC levels have been demonstrated in many diseases, such as infection [13], immune disorders [14], pulmonary artery hypertension [15], posttransplantation conditions [16], cancer [17], cardiovascular disease [18], and deep venous thrombosis [19]. Many diseases are associated with vascular damage, and hence the determination of the CEC number has been considered a promising tool to monitor disease activity with the potential to evaluate prognosis and response to treatment. However, the lack of standardized assays/methods and use of different CEC immunophenotype definitions resulted in a wide variability in the observed cell count [9].

Flow cytometry is the most useful method for the identification of CECs and EPCs allowing the evaluation of high numbers of cells in the suspension. Determination of CECs and EPCs should be based on the specific markers expressed on the surface of these cells. However, the immunophenotypes used for their identification and interpretation of acquired data highly vary among published studies. Some authors identified EPCs as CD45dim/−, CD34+, and CD133+ [20–22] and CECs as CD45dim/−, CD34+, and CD133− cells [20, 22, 23]. However, CD133 and CD34 antigens are expressed by numerous blood cell populations and are not specific for endothelial cells [24]. Therefore, other studies used more extensive immunophenotyping including CD146 and/or CD144 and/or KDR markers for EPC and CEC definition [1, 5, 9, 11, 19, 25, 26].

Thrombophilia is defined as an inherited or acquired predisposition to venous thrombosis. Congenital abnormalities associated with a high risk of venous thrombosis include deficiencies in antithrombin, protein C, or protein S, factor (F) V Leiden mutation (activated protein C resistance), and G20210A prothrombin mutations. The antiphospholipid syndrome is an example of acquired thrombophilia. Nevertheless, many other acquired conditions can increase the risk of thrombosis, such as acquired abnormalities in coagulation proteins (e.g., elevated levels of FVIII coagulation or deficiencies in the natural anticoagulants), and certain diseases (e.g., myeloproliferative neoplasms) [27, 28]. Data from a healthy population in the Czech Republic, i.e., the Central European region, showed a prevalence of the most frequent thrombophilia mutations in FV Leiden and G20210A prothrombin of 4.5 and 1.3%, respectively [29]. In general, more than 10% of the total population is affected by at least one identifiable thrombophilia [28].

The aim of our study was to further optimize and standardize CEC and EPC measurements by multicolor flow cytometry and to evaluate the significance of these cells in patients with thrombophilia.

Materials and Methods

Peripheral blood was collected using EDTA as an anticoagulant from patients with known thrombophilia, healthy controls, and patients with presumably increased numbers of EPCs and CECs, i.e., patients with hematologic malignancies after high-dose chemotherapy (autologous stem cell transplantation), patients with severe infection (sepsis), or patients with acute myocardial infarction.

All samples were first analyzed using the XN 3000 (Sysmex) blood analyzer and subsequently stained with 7-color assay (CD45 – Krome orange/CD31 – FITC/CD146 – PE/CD34 – PerCP-Cy5.5/CD117 – PE-Cy7/CD133 – APC/CD3 – APC-Alexa Fluor 750), using a modified EuroFlow standard operating protocol (SOP) for bulk lysis for minimal residual disease panels [30]. Multiparameter flow cytometry immunophenotyping was performed using a FACSVerse (Becton Dickinson) flow cytometer and analyzed with the Kaluza software (Beckman Coulter). The flow cytometer was set according to the EuroFlow instrument setup SOP [30]. Peripheral blood specimens were pre-incubated with FcR blocking reagent and then incubated with antibodies purchased from Beckman Coulter (CD3, CD45, CD117, CD144), BD Biosciences (CD31, CD34, CD146), and MACS Miltenyi Biotec (CD133, FcR, KDR). All samples were immediately measured and at least 2,000,000 leukocytes were acquired per tube. The absolute numbers of CECs and EPCs were calculated based on the results of the WBC parameter from the blood count.

Statistical analysis was performed using Statistica 12 (StatSoft CR s.r.o., Czech Republic). The total statistical significance was calculated by ANOVA, individual groups were compared by the Mann-Whitney U test, and $p < 0.05$ was considered statistically significant.
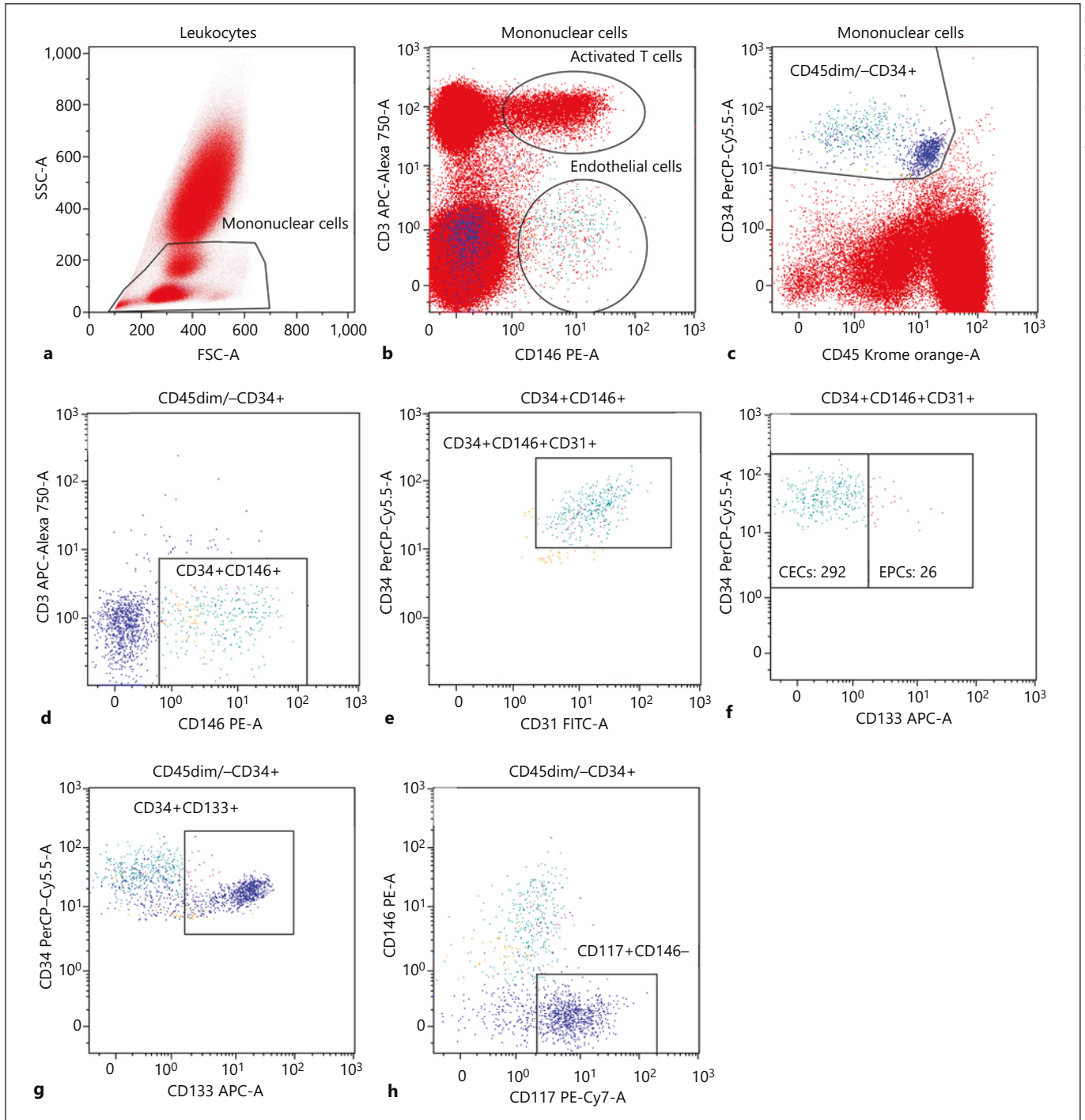
Fig. 1. Gating strategy for the determination of EPC and CEC immunophenotypes. **a** Gating of mononuclear cells. **b** Expression of CD146 on activated T cells and endothelial cells. **c** Gating of CD34+ progenitor and endothelial cells. **d**, **e** Identification of endothelial cells using CD31 and CD146. **f** Differentiation of EPCs and CECs using CD133 with the indicated number of cells. **g**, **h** Identification of hematopoietic progenitor cells using CD34, CD133, and CD117.

Table 1. Clinical and demographic data from two patient groups and a group of healthy controls and quantification of CECs and EPCs

	Thrombophilia patients ($n = 61$)	Healthy controls ($n = 28$)	Hematologic + AMI patients ($n = 31$)
Median of age (range), years	46 (25–82)	33 (18–64)	61 (19–81)
Gender, females:males	28:33	19:9	7:24
Positive VTE anamneses, n (%)	31 (50.8)	0 (0)	3 (9.7)
Positive thrombophilia	61 (100)	0 (0)	1 (3.2)
FV Leiden[1]	37 (60.7)		1 (3.2)
FII prothrombin heterozygote	7 (11.5)		0 (0)
Elevated FVIII	17 (27.9)		0 (0)
Antithrombin deficiency	1 (1.6)		0 (0)
Protein C deficiency	1 (1.6)		0 (0)
Median number of cells/mL (range)			
CECs	17.7 (3.1–40.0)	14.2 (3.1–51.9)	82.9 (32.8–1,241.4)
EPCs	3.5 (0–20.5)	2.8 (0–30.2)	17.8 (0–151.7)

The range was determined as 90% confidence interval of the population. AMI, acute myocardial infarction; VTE, venous thromboembolism. [1] Homozygote mutation – 1, heterozygote mutation – 36.
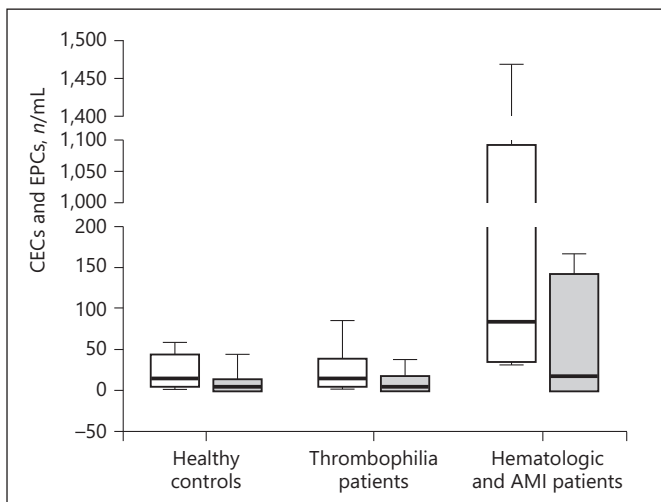
Fig. 2. Comparison of CEC (white box) and EPC (gray box) numbers per milliliter for each patient group with healthy controls. The box ranges are determined at 90% confidence intervals and median, minimum, and maximum are indicated.

Results

We optimized a panel of monoclonal antibodies to determine CECs and EPCs according to their immunophenotype. We used anti-CD146 as endothelial lineage-specific marker, which is also expressed by activated T cells that serve in combination with anti-CD3 as an internal positive control. Based on our analysis, we determined the immunophenotype of CECs and EPCs such as CD45-dim/–CD34+CD146+CD31+CD133– and CD45dim/–CD34+CD146+CD31+CD133+, respectively. The immunophenotype of progenitor cells is CD45dim/CD34+CD146–CD133+CD117+ (Fig. 1).

Healthy donors without hereditary thrombophilia were used to set normal ranges of CECs and EPCs ($n = 28$). Blood samples from 19 women (age range, 19–64 years) and 9 men (age range, 18–50 years) were measured with no difference in CEC and EPC counts between gender ($p = 0.9216$ and $p = 0.4606$, respectively).

Cases with known thrombophilia ($n = 61$) included patients with FV Leiden or FII prothrombin mutations, antithrombin or protein C deficiencies, or elevated FVIII (Table 1). Thirty-one patients had a positive history of thrombosis, and 30 patients had no evidence of previous thrombosis. We did not observe any changes in CEC ($p = 0.7605$) or EPC ($p = 0.2811$) levels compared with healthy controls nor significant differences between the groups with or without a history of thrombosis ($p = 0.5786$, $p = 0.1917$, respectively) (Table 1). In addition, normal levels of EPCs and CECs were observed in 3 patients with acute thrombosis (data not shown).

Increased numbers of CECs, but not EPCs, were detected in 2 patients with acute myocardial infarction. Significantly elevated levels of CECs ($p < 0.0001$) were observed in patients with hematological malignancies after high-dose chemotherapy or in patients with severe infec-

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tion ($n = 29$; Fig. 2). These patients had also significantly higher counts of EPCs compared to healthy controls ($p < 0.0001$), however EPCs above the limit of detection of the method were detected in 4 cases only.

Discussion/Conclusion

Numbers of EPCs and CECs are usually <50 cells/mL peripheral blood [1], and therefore an optimal process of peripheral blood analysis is crucial. We have used a modified EuroFlow SOP for bulk lysis for minimal residual disease panels, which was developed to analyze rare cell populations in the peripheral blood [30]. Therefore, we analyzed whole blood samples without mononuclear cell isolation and thus avoided potential cell loss during the isolation process. The cells were first incubated with FcR blocking reagent to minimize nonspecific binding and to reduce the risk of falsely positive results.

Due to the low EPC and CEC numbers in the peripheral blood, it is essential to properly determine their immunophenotype with specific antibody combinations. Published data highly vary in the selection of antibodies used for EPC and CEC identification. Some works characterized EPCs and CECs with a set of CD45, CD34, CD133, and CD31 markers. Antigen CD45 was always negative or dimly positive, CD34 and CD31 were always positive, and CD133 expression was used to distinguish EPCs (CD133+) from CECs (CD133–) [20–23]. However, this panel of monoclonal antibodies does not include any specific endothelial marker and therefore cannot be used to properly identify EPCs and CECs [24]. This was confirmed by our analysis (Fig. 1g, h), where the CD34+CD133+ population, previously identified as EPCs, is CD146– and CD117+. Therefore, hematopoietic stem cells were falsely referred to as endothelial cells. These results underline the need for specific endothelial lineage markers. Published works inconsistently used mainly KDR and/or CD146 and/or CD144 and/or CD105 for CEC and EPC identification [1, 5, 9, 11, 19, 21].

In our study, we tested KDR, CD146, and CD144 markers to determine the endothelial lineage (data not shown). CD146 appeared to be the best marker for the identification of endothelial cells. Firstly, it exhibits the strongest expression, and its positivity on activated T cells enables to use these cells as an internal positive control for the detection of endothelial cells (Fig. 1b). We used the multiparameter flow cytometry method to identify EPCs and CECs. Our 7-color panel included leukocyte marker CD45; endothelial markers CD146 and CD31 (PECAM-1); hematopoietic progenitor markers CD34, CD117, and CD133; and the T-cell marker CD3 serving as an internal control. Our measurements confirmed that CECs are CD45dim/–, CD34+, CD31+, CD146+, and CD133– and could be reliably quantified according to this immunophenotype. In comparison with the CECs, EPCs additionally express the hematopoietic progenitor marker CD133; nevertheless their quantification is very difficult due to very low numbers in the peripheral blood close to the limit of sensitivity of flow cytometry.

CECs and EPCs are very rare cell populations [1], and therefore their quantification is very difficult and depends on precise and standardized preparation of the biological material and a well-defined determination of their immunophenotype. Using our standardized method for peripheral blood preparation and an optimized panel of monoclonal antibodies, we could quantify the number of CECs in the whole peripheral blood. However, the number of CECs measured in patients with thrombophilia was very low (median, 17.7 cells/mL) and did not statistically differ ($p = 0.7605$) from CECs found in healthy controls (median, 14.2 cells/mL). Increased CEC levels were confirmed in patients with hematological malignancies after high-dose chemotherapy and patients with acute myocardial infarction (median, 82.9 cells/mL) compared to healthy controls ($p < 0.0001$). The number of EPCs was not above the limit of detection in all cohorts apart from 4 hematological patients after autologous stem cell transplantation.

Alessio et al. [19] showed significantly and slightly increased CEC numbers in patients with acute and chronic deep venous thrombosis, respectively. However, our study did neither show significantly increased numbers of CECs and EPCs in thrombophilia patients with or without thrombosis nor in patients with acute thrombosis. On the contrary, our results support published data showing elevated CEC levels in patients with cardiovascular disease [18] and posttransplantation status [16].

In conclusion, our study provides a multicolor flow-cytometric protocol for rapid and unambiguous identification and quantification of both CECs and EPCs in the peripheral blood. No significant changes in EPC or CEC numbers were detected in patients with known thrombophilia compared to healthy controls. However, we have shown significantly increased numbers of CECs in selected patients with hematological malignancies and in patients with acute myocardial infarction. Hence, our data

suggest that CECs might be used as a marker of endothelial damage and dysfunction. Sensitive multicolor flow cytometry using specific endothelial lineage markers should be the preferred method of identifying and enumerating these cells in further studies.

Statement of Ethics

The present study was approved by the local ethics committee, and all patients gave their written informed consent.

Disclosure Statement

The authors have no conflicts of interest to declare.

Author Contributions

M.Ř., E.B., and M.Š. collected the data, M.Ř., E.B., M.Š., T.K., and J.K. designed the study and wrote the manuscript, and M.Ř. performed the statistical analyses.

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Original Article

PD-1, PD-L1 and PD-L2 Expression in Mantle Cell Lymphoma and Healthy Population

(mantle cell lymphoma / PD-1 / PD-L1 / PD-L2 / B cells / T cells/ immunosenescence)

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Abstract: Cell surface expression of PD-1, PD-L1 and PD-L2 immune checkpoints on B and T cells obtained from patients with mantle cell lymphoma shows ambiguous results across many studies and creates obstacles for the implementation of immune checkpoint inhibitors into the therapy of mantle cell lymphoma. Using multiparameter flow cytometry we analysed surface expression of PD-1, PD-L1 and PD-L2 molecules on B and T cells of 31 newly diagnosed mantle cell lymphomas and compared it with the results of 26 newly diagnosed chronic lymphocytic leukaemias and 20 healthy volunteers. To gain insight into the age-dependent changes of surface expression of these immune checkpoints, flow cytometric subanalysis of 30 healthy volunteers of 25–93 years of age was conducted. Overall, we demonstrated weak surface expression of PD-1, PD-L1 and PD-L2 on B and T cells of mantle cell lymphoma patients (< 10 % when compared to healthy individuals). A significant age-dependent increase in the expression of PD-1 and its ligand PD-L2 was observed in healthy volunteers. Our results suggest that neither PD-1 nor its ligands represent relevant druggable targets for the therapy of mantle cell lymphoma. The observed age-dependent changes in healthy population could impact efficiency of immune checkpoint inhibitors and could be at least partly connected with increased incidence of cancer with age.

Introduction

Mantle cell lymphoma (MCL) is an aggressive type of B-cell non-Hodgkin lymphoma (B-NHL) characterized by overexpression of cyclin D1 as a result of translocation t(11; 14). MCL represents approximately 7 % of newly diagnosed B-NHL and is a highly heterogeneous disease with clinical behaviour ranging from indolent cases to aggressive blastoid forms. Despite a number of therapeutic options including stem cell transplantation, relapsed and refractory MCL is still considered incurable by currently available treatment options (Klener, 2019). Immunotherapy with immune checkpoint inhibitors emerged as an innovative, highly effective anti-tumour approach in many solid tumours and haematological malignancies. While Hodgkin lymphoma became a flagship of successful usage of immune checkpoint inhibitors in the clinical grounds, potential implementation of this type of T cell-based immunotherapy in other lymphoid neoplasms is still a matter of investigation (Xu-Monette et al., 2018). Programmed cell death 1 (PD-1) and its ligands (PD-L1, PD-L2) play key roles in shutting down the activity of cytotoxic

T cells during inflammation. Disruption of their signalling can result in development of autoimmune diseases, collapse of peripheral tolerance, and fading of anti-infectious and anti-tumour immunity. Ligation of PD-1 on the surface of tumour-infiltrating T lymphocytes results in their exhaustion and ensuing tumour-induced immune suppression. PD-1 and its ligands PD-L1 and PD-L2 can be expressed by tumour cells, or by non-malignant cells of the tumour microenvironment including B cells, T cells or macrophages (Francisco et al., 2010; Jin et al., 2011; Xu-Monette et al., 2018).

Cell surface expression of PD-1 and its ligands as well as clinical usage of immune checkpoint inhibitors in the treatment of MCL patients remains a matter of investigation. In this study, cell surface expression of PD-1 and its ligands PD-L1 and PD-L2 on malignant and nonmalignant B cells, T cells and T-cell subpopulations obtained from peripheral blood of 31 patients with treatment-naïve MCL was evaluated. A panel of 26 patients with newly diagnosed chronic lymphocytic leukaemias (CLL) and 20 healthy controls was analysed and compared with the MCL cohort.

The incidence of cancer including lymphoproliferative disorders increases with age, and immunosenescence is one of the suspects responsible for this phenomenon. Age-related changes of the immune system and deterioration of its reactivity during aging may affect the efficacy of immune checkpoint inhibitors. Increasing PD-1 expression with age in mouse models and humans has already been reported (Elias et al., 2017). To gain insight into the age-dependent changes of cell surface expression of immune checkpoints on B and T cells of healthy individuals, separate subanalysis of additional 30 healthy volunteers was conducted.

Material and Methods

Biological samples

Peripheral blood (PB) samples were obtained from 31 patients (65 % male) with newly diagnosed MCL (see Supplementary Material, Table S1a) and from 26 patients (70 % male) with newly diagnosed CLL (see Supplementary Material, Table S1b). A total of 50 samples were obtained from healthy volunteers. Of these, 20 samples (50 % male) were used for age-matched comparison to MCL, while 30 samples were used for analysis of age-dependent cell surface expression profiles of PD-1, PD-L1 and PD-L2 on non-malignant circulating B and T cells. All peripheral blood samples were collected after informed consent and in accordance with the Institutional Guidelines of General University Hospital in Prague and the Declaration of Helsinki. This study was approved by the Ethics Committee of General University Hospital (Prague, Czech Republic).

Flow cytometry analyses

After the collection of blood samples, full blood cell and differential counts were immediately measured by a Sysmex XN 3000 haematology analyser (Sysmex, Kobe, Japan). Then, six-colour or seven-colour flow cytometry (Supplementary Material, Table S2a, S2b respectively) was performed using a Navios flow cytometer (Beckman Coulter, Miami, FL). Samples were processed according to standardized EuroFlow sample preparation and instrument set-up protocols (Kalina et al., 2012). Data analysis was done with Kaluza software version 2.1. (Beckman Coulter). The expression of CD279 (PD-1), CD274 (PD-L1) and CD273 (PD-L2) was evaluated based on the parameter of median fluorescence intensity (MFI) and proportion of positive cells (%); the corresponding isotype controls were used as negative controls.

Statistical analyses

Statistical analyses were done using GraphPad version 5 (GraphPad Prism version 5.00 for Windows, GraphPad Software, La Jolla, CA, www.graphpad.com). Detailed statistical approaches together with complex descriptive statistics were performed to show differences in distributions between the analysed data sets (i.e., CTRL vs CLL, CTRL vs MCL and CLL vs MCL) and are described in supplementary materials (see Supplementary Material, Table S3).

Results

Cell surface expression of PD-1 and its ligands PD-L1 and PD-L2 in patients with newly diagnosed MCL shows minimal changes when compared to age-matched healthy controls and is significantly different when compared to patients with newly diagnosed CLL

Cell surface expression of the PD-1 molecule and its ligands PD-L1 and PD-L2 on B and T cells of 31 newly diagnosed MCLs was analysed and compared with 26 newly diagnosed CLLs and 20 healthy age-matched controls (CTRL). The expression of PD-1, PD-L1 and PD-L2 was evaluated based on the parameter of median fluorescent intensity (MFI) and percentage of PD-1, PD-L1 and PD-L2 positive B and T cells (%). We performed complex descriptive statistics to show distributions (i.e., minimums, maximums, deciles, means, etc.) in the analysed cohorts (MCL vs CTRL, CLL vs CTRL, and MCL vs CLL, see Supplementary Material, Table S3).

Cell surface expression of the PD-1 molecule on B cells of MCL patients evaluated by MFI and by percentage of positive cells was significantly increased when compared to CTRL (P = 0.0004) and CLL cohorts (P < 0.0001). In case of cell surface expression of PD-L1 on B cells of MCL patients, a significant increase was observed when compared to both, CTRL and CLL cohorts (P < 0,0001, P = 0.0001, respectively). However, despite the statistical significance, both PD-1 and PD-L1 levels of expression on MCL B cells did not reach more

than 10 % cell positivity when compared with healthy individuals. No statistically significant changes were observed in cell surface expression of PD-L2 on B cells of MCL when compared with the CTRL cohort. The expression of PD-L2 on B and T cells in the CLL cohort showed a statistically significant increase in expression compared to CTRL based on MFI results, but not on the percentage of positive cells (P = 0.0002, P < 0.0001, respectively). Similarly as other studies already performed (Brusa et al., 2013; Harrington et al., 2019), we observed strong statistically significant PD-1 expression (evaluated by MFI and by percentage of positive cells) both on T cells (P = 0.0002, P < 0.0001, respectively) and B cells (P < 0.0001, P < 0.0001, respectively) of CLL samples with the median reaching more than 30 % cell positivity. The expression of PD-L1 on T cells of the CLL cohort was not statistically significantly changed (see Table 1).

Separate analysis of 11 leukaemized and 20 non-leukaemized patients with MCL was performed to observe whether the above-mentioned changes in surface expression on B cells of MCL are affected by the presence of MCL malignant B cells in peripheral blood. Almost identical changes in the cell surface expression of PD-1, PD-L1 and PD-L2 were found on B cells, regardless of whether the patient was leukaemized or non-leukaemized (data not shown).

No significant changes in the expression of the above-mentioned molecules were found on T cells in the MCL cohort when compared with CTRL and CLL cohorts (see Table 1, Supplementary Material, Fig. S1).

Cell surface expression of PD-1 on T-cell subsets in newly diagnosed MCL patients shows minimal changes when compared with newly diagnosed CLL patients and healthy controls

We further tested cell surface PD-1 expression on CD3$^+$ T-cell subsets obtained from patients with MCL and CLL and compared them to healthy controls. T-cell subsets were divided based on the surface expression of CD45RA and CD197 (Monteiro et al. 2007). This enabled analysis of expression of PD-1 on the surface of naïve (N) T cells (CD45RA$^+$CD197$^+$), central memory

(CM) T cells (CD45RA$^-$CD197$^+$), effector memory T cells (CD45RA$^-$CD197) and terminally differentiated (TD) T cells (CD45RA$^+$CD197$^-$). No significant changes were observed in the MCL cohort when compared to CTRL and CLL cohorts. In contrast, the CLL cohort showed a significant increase of surface PD-1 expression on naïve CD4$^+$ and CD8$^+$ T cells compared to the CTRL cohort (see Table 2).

Detailed descriptive statistics of PD-1, PD-L1 and PD-L2 cell surface expression on B and T cells of MCL, CLL and healthy controls together with descriptive statistics of PD-1 cell surface expression on T-cell subsets was performed, showing particular differences in distributions between the analysed cohorts (CTRL vs CLL, CTRL vs MCL, and CLL vs MCL, for details see Supplementary Material, Table S3).

PD-1 and PD-L2 molecules in healthy individuals show an age-dependent increase in expression

There is limited information on the correlation of surface expression of PD-1, PD-L1 and PD-L2 molecules on healthy circulating T and B cells with age. Potential age-dependent changes in the cell surface expression of the analysed antigens were investigated using a cohort of 30 healthy volunteers from six different age groups (5 volunteers per each group): 25 to 34 years, 35 to 44 years, 45 to 54 years, 55 to 64 years, 65 to 74 years, and over 75 years. The oldest healthy volunteer was 93 years old. Significant positive correlation was observed between age and expression of PD-1 and PD-L2 on the surface of both circulating B lymphocytes (P = 0.005, P < 0.001, respectively) and T lymphocytes (P = 0.001, P < 0.001, respectively). In contrast, the cell surface expression of PD-L1 on B and T lymphocytes remained virtually unchanged with increasing age (see Table 3, Fig. 1).

Discussion

Several studies have reported increased surface expression of PD-1 and PD-L1, PD-L2 molecules on B and T cells obtained from patients with CLL compared

Table 1. Expression of PD-1, PD-L1 and PD-L2 molecules on B and T cells in MCL and CLL cohorts

			CLL	MCL	MCL vs CCL				CLL	MCL	MCL vs CCL
MFI	**B cells**	**PD-1**	< 0.0001*	**0.0004***	< 0.0001*	**%**	**B cells**	**PD-1**	< 0.0001*	**< 0.0001***	< 0.0001*
		PD-L1	0.002	**< 0.0001***	0.002			**PD-L1**	0.419	**0.0001***	0.006
		PD-L2	0.0002*	**0.369**	0.0007*			**PD-L2**	0.003	**0.0009**	0.226
	T cells	**PD-1**	0.0002*	**0.493**	0.01		**T cells**	**PD-1**	< 0.0001*	**0.016**	0.035
		PD-L1	0.011	**0.802**	0.092			**PD-L1**	0.061	**0.035**	0.423
		PD-L2	< 0.0001*	**0.034**	0.441			**PD-L2**	0.005	**0.543**	0.003

P values of Mann-Whitney tests on identical distributions in studied populations (statistical significance: * = 5% simultaneous significance level, % = proportion of positive cells).

Table 2. Expression of PD-1 molecule on the cell surface of specific subpopulations of T lymphocytes in MCL and CLL cohorts

PD-1	CD3+ T cells	CLL	MCL	MCL vs CLL	PD-1	CD3+ T cells	CLL	MCL	MCL vs CLL
MFI	**all**	0.006	**0.065**	0.453	**%**	**all**	<0.0001*	**0.140**	0.086
	CD4+ T cells	<0.0001*	**0.039**	0.248		**CD4+ T cells**	<0.0001*	**0.044**	0.037
	CD3+8+ T cells	0.763	**0.787**	0.644		**CD8+ T cells**	0.072	**0.243**	0.012
	CD4+ NT	<0.0001*	**0.025**	0.241		**CD4+ NT**	<0.0001*	**0.006**	0.017
	CD4+ CMT	0.099	**0.163**	0.714		**CD4+ CMT**	0.072	**0.259**	0.749
	CD4+ EMT	0.568	**0.417**	0.590		**CD4+ EMT**	0.511	**0.450**	0.950
	CD4+ TDT	0.027	**0.615**	0.040		**CD4+ TDT**	0.010	**0.664**	0.067
	CD8+ NT	0.069	**0.985**	0.121		**CD8+ NT**	<0.0001*	**0.213**	0.003
	CD8+ CMT	0.228	**0.710**	0.570		**CD8+ CMT**	0.763	**0.923**	0.869
	CD8+ EMT	0.079	**0.279**	0.344		**CD8+ EMT**	0.062	**0.259**	0.258
	CD8+ TDT	0.706	**0.779**	0.857		**CD8+ TDT**	0.095	**0.582**	0.262

P values of Mann-Whitney tests on identical distributions in studied populations (statistical significance: * 5% simultaneous significance level).

Table 3. Statistical analysis of PD-1, PD-L1, PD-L2 surface expression on B and T cells of healthy volunteers

	Molecule	Pearson r	Bootstrap estimate of P	Bootstrap 95% CI
B cells	PD-1	0.501	0.005	from 0.199 to 0.699
	PD-L1	0.371	0.047	from −0.016 to 0.659
	PD-L2	0.600	< 0.001	from 0.329 to 0.792
T cells	PD-1	0.574	0.001	from 0.134 to 0.814
	PD-L1	0.275	0.142	from −0.319 to 0.695
	PD-L2	0.645	< 0.001	from 0.402 to 0.817

Slightly negative CI for PD-L1 surface expression is presumably caused by a small sample size rather than the absence of an association between the analysed variables. CI = confidence interval.

to healthy controls (Xerri et al., 2008; Grzywnowicz et al., 2012, 2015; Nunes et al., 2012; Brusa et al., 2013; Riches et al., 2013). In contrast, studies of MCL yielded conflicting results, the majority of them showing no or weak PD-1 or PD-L1, PD-L2 expression (Muenst et al., 2010; Andorsky et al., 2011; Wang et al., 2013; Gatalica et al., 2015; Menter et al., 2016; Vranic et al., 2016). Five out of six studies conducted with MCL patients were primarily based on immunohistochemistry analysis of tumour PD-1, PD-L1, PD-L2 expression on infiltrating T cells or neoplastic cells, while only a few of them studied expression of PD-1 and its ligands on circulating cells using multiparameter flow cytometry.

Recently, Harrington et al. (2019) demonstrated strong PD-L1 surface antigen and mRNA expression in MCL. However, only four out of 16 analysed patients were newly diagnosed and treatment-naïve (Harrington et al., 2019). The increase in mRNA expression of the PD-L1 molecule in lymphoid malignancies in comparison with healthy controls (12/92 MCL samples) was also demonstrated by Yang and Hu (2019), who also observed a decrease in expression of PD-L1 after treatment initiation. In contrast, our own results are in conflict with those mentioned above, as we demonstrated only weak sur-

face expression of PD-1 and PD-L1 on B and T cell populations of MCL patients. Using multiparameter flow cytometry, we analysed a homogenous cohort of 31 treatment-naïve MCL patients and compared them to 26 newly diagnosed CLL patients, because the expression profile in CLL is relatively well described (Monteiro et al., 2007; Xerri et al., 2008; Grzywnowicz et al., 2012, 2015; Nunes et al., 2012; Brusa et al., 2013). In concordance with the recently published studies (Brusa et al., 2013; Riches et al., 2013), we confirmed significant surface expression of PD-1 on B and T cells of patients with CLL. The biological impact of the increased expression of PD-1 molecule compared to healthy controls remains to be elucidated and requires further research.

Chronically activated or exhausted T cells are generally characterized by increased surface expression of PD-1 together with the shift to more differentiated subsets of T cells such as effector memory or terminally differentiated T cells (Shimatani et al., 2009; Francisco et al., 2010; Adekambi et al., 2012). In our study, we observed homogeneous expression of PD-1 across all T-cell populations of MCL patients including naïve subsets, which is comparable with healthy controls. In the CLL cohort, we confirmed significantly increased PD-1
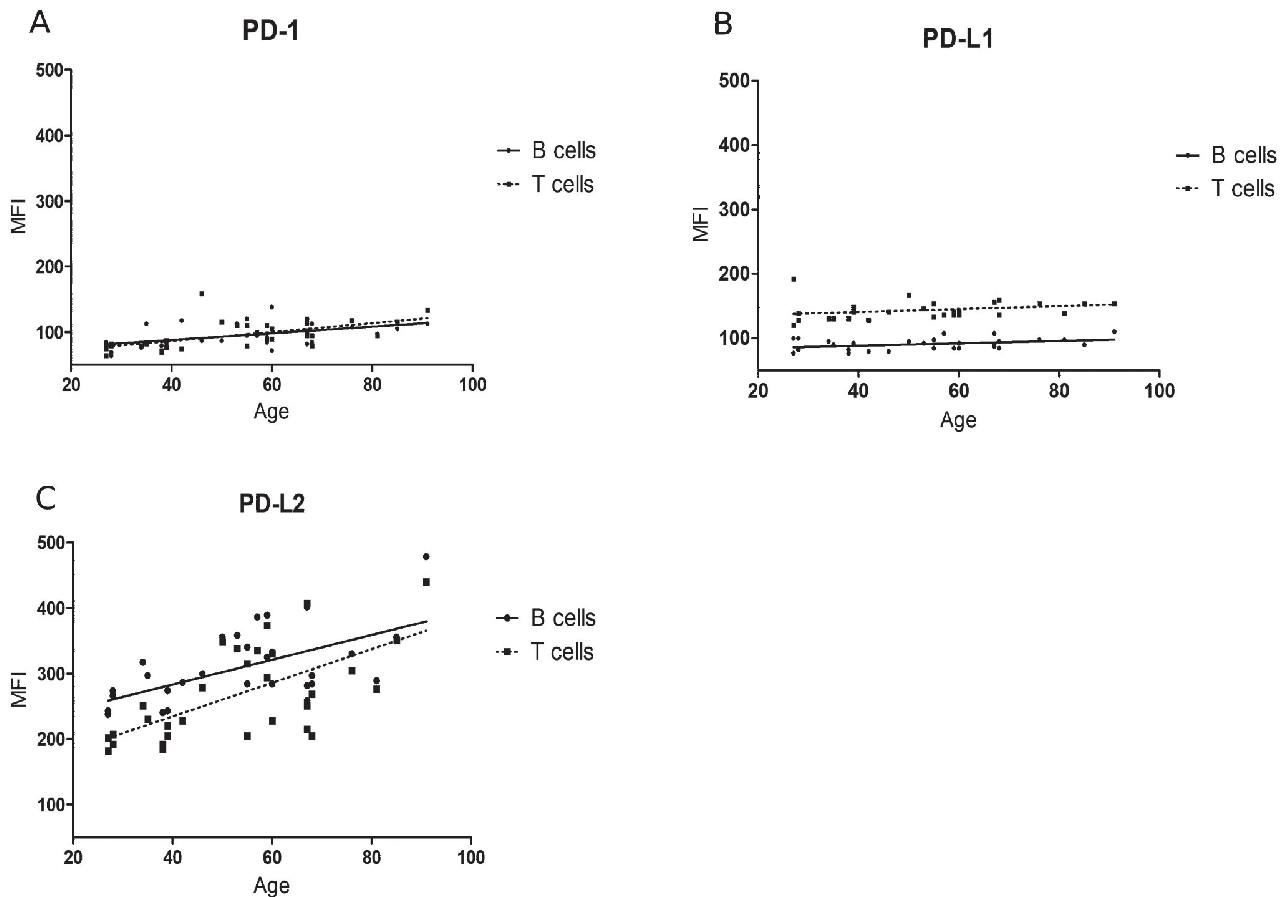
Fig. 1. Age-dependent surface expression of PD-1 (**A**) and its ligands PD-L1 (**B**), PD-L2 (**C**). Age-dependent surface expression (evaluated by mean fluorescent intensity = MFI) in six different age cohorts showing correlation between surface expression of PD-1, PD-L1 and PD-L2 with age.

expression on T cells when compared with healthy controls. The largest cell surface expression was detected on CD4+ naïve T-cell and CD8+ naïve T-cell subset.

Analysis of age-dependent changes in the cohort of 30 healthy volunteers aged 25 to 93 years demonstrated significant changes in the surface expression of PD-1 and its ligands on circulating B and T lymphocytes. We confirmed that PD-1 and PD-L2 molecules in healthy volunteers show an age-dependent increase in expression, while expression of PD-L1 remains virtually unchanged. Our results thus support the current concept of impaired tumour immune surveillance in the elderly and can at least partially contribute to the observed increased incidence of cancer with increasing age (Elias et al., 2017). Changes in the expression of PD-1, PD-L1 and PD-L2 may also theoretically contribute to the high activity of immune checkpoint inhibitors in the elderly patients (Elias et al., 2018). Other studies dedicated to the usage of immune checkpoint inhibitors should be performed to see the benefits of this treatment in elderly patients.

In conclusion, our results demonstrate overall weak surface expression of PD-1 and its ligands on circulating neoplastic and normal B and T cells obtained from pa-

tients with newly diagnosed MCL. In contrast, high, statistically significant expression of PD-1 on both B and T cells was observed in samples obtained from patients with CLL.

Our data thus do not suggest a major role for PD-1 and its ligands in the pathogenesis of MCL, or clinical usage of immune checkpoint inhibitors in the therapy of newly diagnosed MCL patients. The potential usage of immune checkpoint inhibitors in combination with targeted agents such as ibrutinib in the treatment of relapsed/refractory MCL remains a matter of investigation. Concerning the age-dependent changes of PD-1, PD-L1 and PD-L2 molecules, we demonstrated positive correlation of PD-1 and PD-L2 expression with increasing age on circulating B and T cells. To what extent the observed age-related changes of PD-1 and PD-L2 may impact the incidence of cancer or efficacy of immune checkpoint inhibitors remains to be elucidated.

RM and SM participated in flow cytometry analysis and statistical analysis. All authors read and approved the final manuscript.

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