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 surface 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
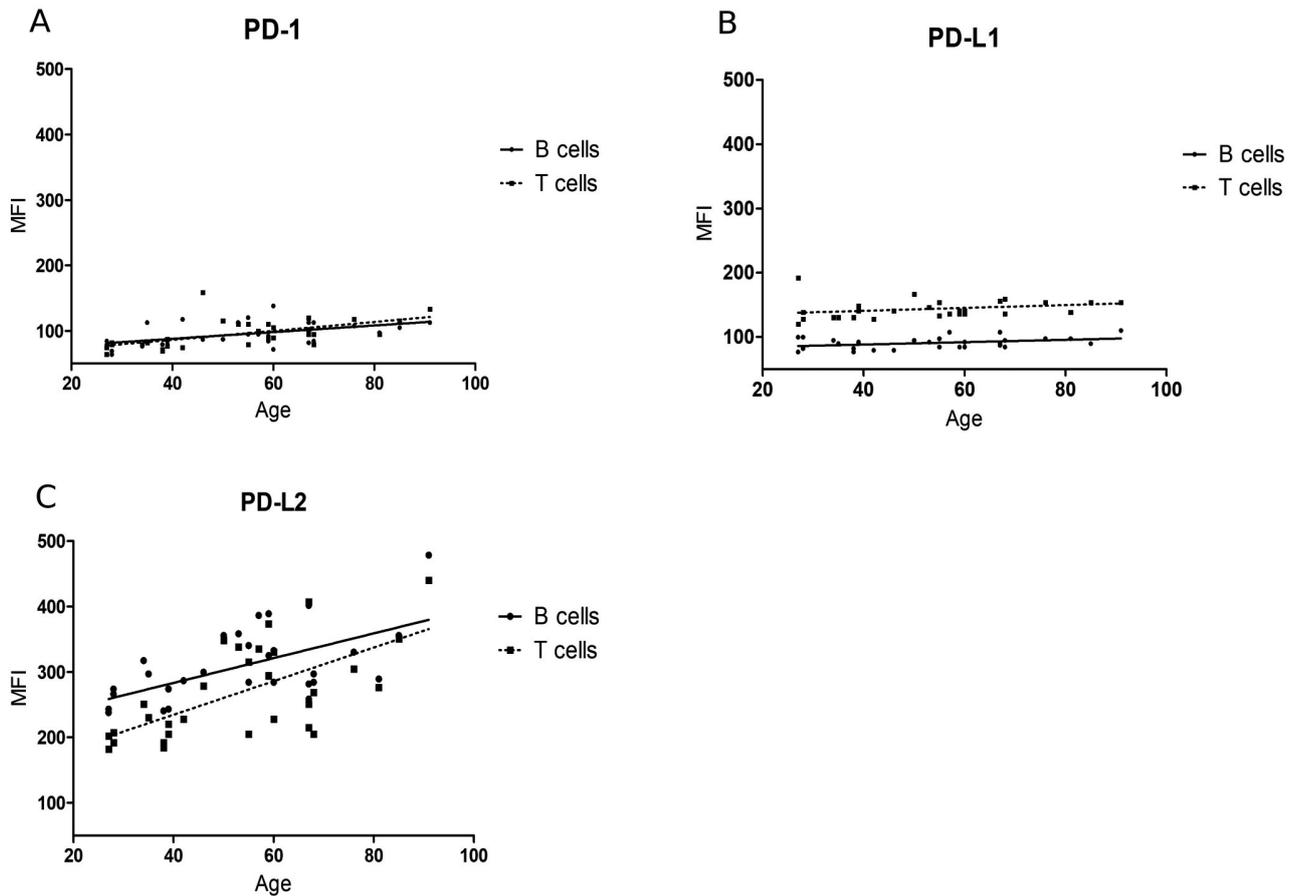
Fig. 1. Age-dependent surface expression of PD-1 (**A**) and its ligands PD-L1 (**B**), PD-L2 (**C**). Age-dependent surface expression (evaluated by mean fluorescent intensity = MFI) in six different age cohorts showing correlation between surface expression of PD-1, PD-L1 and PD-L2 with age.

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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