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The MHC-II antigen presentation machinery and B7 checkpoint ligands display distinctive patterns correlated with acute myeloid leukaemias blast cells HLA-DR expression

Ion Antohe [a,b,1], Mariana Pavel Tanasa [c,1], Angela Dăscălescu [a,b], Cătălin Dănăilă [a,b], Amalia Titieanu [a,b], Mihaela Zlei [d], Iuliu Ivanov [e], Adriana Sireteanu [e], Petru Cianga [c,*]

[a] Haematology Department, "Grigore T. Popa" University of Medicine and Pharmacy, Iaşi, Romania
[b] Haematology Department, Regional Oncology Institute, Iaşi, Romania
[c] Immunology Department, "Grigore T. Popa" University of Medicine and Pharmacy, Iaşi, Romania
[d] Immunophenotyping Department, Regional Oncology Institute, Iaşi, Romania
[e] Molecular Diagnostic Department, Regional Oncology Institute, Iaşi, Romania

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ABSTRACT

Acute Myeloid Leukaemia (AML) is a neoplasia characterised by rapid proliferation and an increased rate of relapses. The AML blasts display features of antigen-presenting cells (APC), and thus can directly modulate the anti-tumour T cell responses. The bone marrow of a group consisting of 30 newly diagnosed patients and four healthy donors (HD) was investigated for the expression of HLA-DR, several molecules involved in MHC-II antigen-presentation and MHC-II groove editing, like HLA-DM, CD74 and CLIP, as well as a set of immune checkpoint ligands, like ICOS-L, B7.2, PD-L2 and B7-H3. The patients were further characterised for their genetic anomalies and distributed to favourable, intermediate and adverse ELN risk categories. We were able to show that while 23% of our patients displayed a low level of HLA-DR surface expression, all patients displayed higher HLA-DM and CD74 expression compared to HD. However, a higher CLIP expression was noticed only in the HLA-DR low patients. The co-inhibitory PD-L2 and B7-H3 molecules were increased in the cases with normal HLA-DR expression; oppositely, the co-stimulatory ICOS-L and the dual function B7.2 were significantly increased in the cases with HLA-DR low expression. Furthermore, no favourable ELN risk cases were found within the HLA-DR low group. All in all, these data show that the AML with low *versus* normal HLA-DR expression display different profiles of MHC class II machinery molecules and B7 ligands, which are correlated with distinct ELN stratification. Furthermore, as our study included healthy individuals, it offers valuable information about the expression levels that should be considered as normal for these markers known to cause differences in peptide repertoires, reflected further in distinct T-cells polarisation pathways.

1. Introduction

Recent advances in understanding the cancer-immune interaction have led to spectacular progress in the field of cancer immunotherapy. In 2013, Chen and Mellman described this interaction as a continuous, stepwise process, in which immune effectors are primed and eliminate tumour cells, referred to as the cancer-immunity cycle (Chen and Mellman, 2013). Its first crucial step involves the MHC (Major Histocompatibility Complex)-mediated presentation of tumour-derived antigens and the priming of anti-tumour T-cells. The "classical" human MHC (named HLA, Human Leukocyte Antigen)-II proteins (HLA-DP, HLA-DQ, HLA-DR) are expressed on the cell surface, while the "non-classical" molecules (HLA-DN, HLA-DO, HLA-DM) mostly play intracellular regulatory roles (Muers, 2011; Horton et al., 2004). MHC-II expression is restricted to a category of cells functionally characterised as antigen-presenting cells (APC). Their physiological role of presenting MHC groove-bound peptides to CD4+ T-cells is of paramount importance for the maintenance of immune homeostasis (Wang, 2001; Lim and Elenitoba-Johnson, 2004).

However, tumour cells can function themselves as APCs if they

* Corresponding author.
E-mail address: petru.cianga@umfiasi.ro (P. Cianga).
[1] Contributed equally.

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express MHC-II and appropriate co-stimulatory apparatus (Barrett and Le Blanc, 2010). Acute myeloid leukaemia (AML) is a rapidly proliferating haematologic malignancy, in which the high relapse rates following conventional chemotherapy have directed the search for novel therapies, including immunotherapy. The immune biology of AML is strikingly different from that of solid tumours: 1) it originates in the immune privileged, Treg enriched milieu of the bone marrow (BM); 2) it has a low neoantigen fitness, resulting in relatively few leukaemia-specific antigens that can elicit immune responses; 3) it expresses nonetheless both MHC-II and B7 co-signalling molecules (Curran et al., 2017). Further studies have thus demonstrated that AML blasts can not only function as APCs, but can also fine tune the activity of CD4+ and CD8+ T-cells by upregulating their expression of checkpoint-ligands such as the Programmed-Death Ligand 1 (PD-L1), PD-L2, and B7.2 (Antohe et al., 2020) under the influence of interferon-gamma (IFN-γ) (Kondo et al., 2010; Berthon et al., 2010; Kronig et al., 2014). Moreover, the expression levels of the MHC-II antigen-presenting machinery (Chamuleau et al., 2004) and B7 checkpoint-ligands (Tamura et al., 2005; Chen et al., 2008; Greaves and Gribben, 2013; Hu et al., 2015; Guery, et al., 2015) hold prognostic relevance in AML.

Two distinct, MHC-II antigen-presentation pathways are presently acknowledged. In the first one, considered as a "classical" pathway, MHC-II molecules are associated with the Invariant chain (Ii, CD74), that protects the peptide binding groove and drives the complex from the endoplasmic reticulum via trans-Golgi network into the late-endosomal MHC-II compartments (MIIC), where CD74 is being cleaved. Its MHC groove-bound fragment, namely CLIP (Class II-associated invariant chain peptide) evades degradation. The removal of CLIP and the stable binding of a high-affinity peptide are modulated by HLA-DM and its negative regulator, HLA-DO. HLA-DM is thus critical in ensuring the optimal peptide repertoire of MHC-II molecules. Hence, MHC-II ligands can be categorised as: 1) DM-sensitive, whose presentation is abolished in the presence of HLA-DM, and 2) DM-resistant, which can be presented in the presence of HLA-DM, as reviewed in (Unanue et al., 2016; Kremer et al., 2012).

The idea of an "alternative" MHC-II presentation pathway emerged due to growing evidence that roughly 30% of MHC-II-bound peptides are endogenous in nature and that peptide loading is not restricted to the MIIC compartment (Munz, 2011). Thus, Moldenhaeuer et al. brought evidence that HLA-DR molecules can be internalised and re-routed towards the early-endosomes in cells co-expressing surface Ii chain (Moldenhauer et al., 1999). Dani et al. showed that the MIIC vesicles can be reached by the endogenous antigens via a distinctive perinuclear cathepsin-D positive lysosomal-compartment (Dani et al., 2004), while van Luijn et al., using a myeloid leukaemic-cell line, demonstrated that MHC-II expressed by tumour APCs can present endogenous antigens in a proteasome-and TAP-dependent, but Ii-independent manner (van Luijn et al., 2010a; 2010b). Furthermore, cytosolic and nuclear content can be delivered for degradation in the endo-lysosomal compartment by autophagy (Dengjel et al., 2005; Paludan et al., 2005; Zhou et al., 2005). All in all, these various alternatives translate in additional enzymatic setups that offer adequate conditions to generate a wider set of peptides offered for MHC-II presentation. These complex mechanisms are impacting CD4+ T lymphocytes activation, as they explain the less optimal leukaemia-specific peptides presentation with MHC-II to CD4+ T-cells (Thibodeau et al., 2012). Deficient MHC-II antigen-presentation might undermine the first crucial step of the cancer-immunity cycle in AML.

The expression of CD74 on leukaemic cells was not only described, but was also associated with an increased risk of relapse of AML (van den Ancker et al., 2014), while the lack of CD74 expression on these cells favours the activation of CD4+ T lymphocytes targeting leukaemic cells (van Luijn et al., 2011). The surface CLIP expression, regarded as a marker of deficient antigen-presentation, has been thus linked to poor prognosis in AML, at diagnosis, as well as in remission patients displaying CLIP+ minimal residual disease (van Luijn et al., 2014). The variations in the expression of CLIP are linked to the presence of the peptide editors, HLA-DM and -DO. Thus, a DM/DO ratio in favour of the latter promotes CLIP retention in the MHC-II groove, compromising the presentation of leukaemia-derived peptides (Chamuleau et al., 2004).

In the current study we have investigated the surface expression of HLA-DR, CLIP and the intracellular expression of CD74 and HLA-DM on leukaemic blasts of newly diagnosed AML patients. These data were further correlated with the expression of B7 checkpoint-ligands on AML cells, as these molecules are of paramount importance for the anti-tumour T-cell priming.

2. Materials and method

2.1. Patient selection

This study included 30 patients diagnosed between 2016 and 2019 at the Iași Regional Oncology Institute from Romania with *de novo*, non-promyelocytic AML, as well as a group of four healthy volunteers, that were all extensively characterised previously (Antohe et al., 2020). Sample collection was performed based on an informed consent. This study has been approved by the institutional ethics committee. BM samples were collected at diagnosis. The diagnosis of AML was established according to the WHO diagnostic criteria (Arber et al., 2016). The cytogenetic risk of the patients was evaluated in accordance with the 2017 European Leukaemia Net (ELN) recommendations (Dohner et al., 2017), as favourable, intermediate, or adverse (Table 1).

2.2. Flow cytometry

AML blasts were analysed by multiparameter flow-cytometry (MFC) on erythrocyte-lysed fresh BM samples, as previously described (Antohe et al., 2020). We first confirmed the AML diagnosis using protocols based on the EuroFlow recommendations (Antohe et al., 2020; van Dongen et al., 2012). AML blasts gating was performed on CD45+/CD34+/CD117+ events. The HLA-DR levels were assessed based on the expression of this marker on BM monocytes. Thus, cases with reduced leukaemic blast HLA-DR expression when compared to monocytes were categorised as HLA-DR low. Subsequently, the BM leukaemic blast cells surface expression level of CLIP, ICOS-L, B7-1, B7.2, PD-L1, PD-L2, B7-H3 and the intracellular expression levels of HLA-DM and CD74 were assessed.

T cell gating was performed on CD3+/CD4+ and CD3+/CD8+ events. The following T cell maturation subpopulations were defined based on their differential expression of CD28, CD27, and CD45RA: naive (N), central memory (CM), intermediate effector memory (iEM), late effector memory (late EM) (Antohe et al., 2020). Data acquisition was performed on a BD FACS ARIA III cytometer and data was interpreted using the FACS DIVA v6.1.3 software. An identical investigation protocol was applied for the CD45+/CD34+/CD117+ BM cells of all four healthy subjects. The monoclonal antibodies (MoAbs) used in this study are detailed in Supplementary Tables 1–2.

Statistical analysis. Statistical analyses were performed using the IBM® SPSS Statistics 21.0 Software. The Chi square, Mann Whitney test and Wald test were used to analyse the associations between different variables. The Pearson correlation coefficient was calculated to investigate the relationships between numerical variables. The two-way ANOVA test was used to analyse the differences among multiple variables. A p value <0.05 was considered as statistically significant.

3. Results

Comparison of clinical characteristics of patients. Among the AML patients, but not in the healthy donors (HD) group, we have identified 7 cases (23.3%) with low HLA-DR expression of BM CD45+/CD34+/CD117+ AML cells (with lower HLA-DR expression than the one identified on BM monocytes) (Table 1), in accord with literature data (Feller

Table 1
Patterns of expression of HLA-II molecules and B7 ligands relative to WHO AML type and ELN risk.

WHO AML Type	Baseline AML characteristics				HLA-DR surface expr.	HLA-DM % of ic. expr.	CD74 % of ic. expr.	CLIP % of surface expr.	B7 Checkpoint ligands % of surface expression					
	Age	AML	Karyotype	ELN Risk	low *vs.* normal				B7.1	B7.2	ICOS-L	PD-L1	PD-L2	B7-H3
AML with recurrent genetic anomalies	83	*NPM1*	NK	Fav		1.8	3.1	0.1	4.4	13.2	0.1	1.3	27.4	62.4
	70	*NPM1*	NK	Fav		47.1	98.3	5.3	1.8	26.3	7.1	20.5	7.1	16.7
	34	*NPM1*	CK	Fav		13.1	99.9	14.1	1.5	2.5	0.4	4.9	2.4	4.6
	60	t(8;21)	t(8;21)	Fav		52.4	98.1	2.8	2.4	5.7	0.2	2.4	1.5	1.5
	66	t(8;21)	t(8;21)	Fav		90.7	99.9	0.3	0.8	1.3	0.5	5.6	6.5	0.9
	48	t(8;21)	t(8;21)	Fav		61.6	99.1	0.8	0.9	0.9	0.5	0.7	4.1	0.8
	44	t(8;21)	t(8;21)	Fav		39.0	99.0	0.1	0.3	3.2	0.1	0.6	1.3	0.3
	27	t(9;11)	t(9;11)	Int		11.2	99.0	5.4	2.7	19.8	2.5	2.7	1.8	2.9
AML NOS	43	NOS 5	NK	Int		53.7	97.1	1.7	1.8	34.7	1.8	25.0	12.0	2.5
	36	NOS 5	NK, *FLT 3*	Int		84.4	99.9	0.1	0.1	22.0	0.6	8.4	4.6	0.4
	74	NOS 5	NK	Int		32.4	93.5	5.2	3.7	17.0	2.9	9.9	9.8	5.9
	66	NOS 5	NK	Int		65.6	100.0	0.4	0.4	43.6	0.1	1.0	0.5	0.4
	51	NOS 5	NK	Int		58.6	99.8	1.6	1.5	20.0	15.2	23.5	6.7	2.0
	66	NOS 4	NK	Int		46.1	97.7	2.1	1.6	26.1	22.9	21.1	4.8	0.8
	71	NOS 4	NK	Int		27.7	98.7	5.7	4.7	20.9	3.7	52.3	52.9	5.5
	41	NOS 4	NK	Int		63.5	99.1	0.3	0.3	37.2	0.2	8.3	2.4	0.5
	42	NOS 4	NK	Int		27.4	72.4	1.4	1.1	4.5	1.0	5.3	6.7	1.0
	48	NOS 1	NK	Int		53.0	99.9	4.3	2.7	8.2	1.8	14.1	21.2	3.5
	67	NOS 1	NK	Int		63.7	99.8	0.3	1.2	9.2	0.2	1.6	16.0	2.2
	56	NOS 1	NK	Int		83.8	100.0	1.8	0.4	1.4	0.1	8.3	0.6	1.2
	59	NOS 0	NK	Int		19.8	98.2	8.0	2.4	7.7	0.4	13.1	3.1	34.4
	71	NOS 0	NK	Int		90.6	97.5	1.7	0.4	0.8	0.4	1.4	2.0	0.3
	64	NOS 5	t(1;11)	Adv		67.6	97.7	12.0	0.5	10.9	0.1	4.4	0.9	0.4
	45	NOS 4	CK	Adv		8.1	94.3	1.6	0.2	3.3	0.1	9.3	1.8	0.3
	53	NOS 4	CK	Adv		33.7	97.5	0.6	0.2	80.5	14.9	67.6	0.5	0.8
	51	NOS 4	CK, *FLT 3*	Adv		28.9	98.4	0.3	0.6	48.4	0.7	4.7	2.4	1.0
	69	NOS 2	CK	Adv		8.8	99.5	6.9	0.9	23.1	19.0	19.6	1.0	1.2
	65	NOS 1	CK	Adv		14.2	95.5	6.0	0.7	8.2	1.4	8.9	6.2	6.2
	59	NOS 1	CK	Adv		2.2	39.9	1.2	0.0	16.3	0.0	0.1	0.0	0.0
	67	NOS7	Del 7	Adv		47.3	98.4	0.0	0.2	1.6	0.9	1.4	0.5	9.8
HD1						0.3	96.2	1.8	1.1	9.3	1.8	13.9	0.8	0.9
HD2						19.7	96.2	9.1	1.3	13.1	4.8	3.2	1.0	0.5
HD3						7.2	59.8	0.2	0.4	7.7	0.4	0.4	5.6	0.8
HD4						15.7	97.2	3.6	4.2	10.3	2.8	2.8	2.4	2.1

Color code:

	AML blasts with HLA-DR low expression
	AML blasts with high HLA-DM, CD74 and CLIP expression
	Expression of ICOS-L and/or B7.2 B7 checkpoint ligands on HLA-DR low AML blasts
	Expression of ICOS-L and/or B7.2 B7 checkpoint ligands on normal HLA-DR AML blasts
	Expression of co-inhibitory PD-L1, PD-L2, B7-H3 B7 checkpoint ligands on HLA-DR low AML blasts
	Expression of co-inhibitory PD-L1, PD-L2, B7-H3 B7 checkpoint ligands on normal HLA-DR AML blasts

FLT3= *FLT3-ITD* mutated, *NPM1*= NPM1 mutated, NK= normal karyotype, CK= complex karyotype, Fav = favourable, Int = intermediate, Adv = adverse, HD= healthy donor, ic. = intracellular, expr. = expression.

et al., 2013). Based on this observation, we have divided the AML cases in 2 distinct categories: low ($n = 7$) and normal ($n = 23$) HLA-DR groups. The patients included in the two HLA-DR groups were of similar age and male to female ratio. All favourable ELN risk patients had normal HLA-DR levels, summing for 34.8% of the patients ($P = 0.068$), and the percentage of patients presenting an adverse ELN risk was lower in the normal HLA-DR group (21.8%) compared to HLA-DR low (42.9%) (Table 2). The HLA-DR MFI values of AML blasts from the HLA-DR low group were significantly lower than those of the HLA-DR normal group ($P = 0.0009$, ratio MFI HLA-DR low/ MFI HLA-DR normal = 0.45, Supplementary Fig. 1A).

3.1. HLA-DM expression in AML patients and HD

The percentage of CD34+/CD117+/HLA-DM+ cells was compared between the HD control group ($n = 4$) and the patients with low ($n = 7$) and normal HLA-DR ($n = 23$) expression. While no statistical differences were seen in terms of HLA-DM intracellular expression between the two patient groups (Fig. 1A), we have noticed a remarkable 4 times higher expression when compared to the control healthy group (Fig. 1B). In terms of HLA-DM MFI values, no significant differences were seen among the three groups.

3.2. CD74 and CLIP expression in AML patients and HD

To further characterise the HLA-DM and Ii/CLIP interconnection, we have investigated the intracellular CD74 and surface CLIP expression in the CD45+/CD34+/CD117+ BM cells. A total of 80.5% of AML patients expressed higher percentages of CD74+ cells than the HD ($P = 0.001$) despite overall high CD74 expression levels (Table 1, Fig. 2A). This observation suggests that among the molecules of the AML exogenous antigen-presenting system, CD74 along with HLA-DM seem to be

Table 2
Comparison of clinical features between low- and normal-HLA-DR expressing groups.

Parameter	HLA-DR low group (n = 7)	HLA-DR normal group (n = 23)	*P*-value
Age (years)	55 ± 14.5	57 ± 13.6	0.693
Sex, Male (n, %)	3 (42.9)	13 (56.5)	0.526
WHO AML type			0.212
AML with recurrent genetic abnormalities (n, %)	1 (14.3) t(9,11)	7 (30.4) t(8,21), *NPM1*	0.398
AML NOS (n, %)			
NK (n, %)	6 (85.7)	16 (69.6)	
CK (n, %)	3 (42.85)	13 (56.6)	0.525
	3 (42.85)	3 (13.0)	0.084
ELN Risk		8 (34.8)	0.169
Favourable	0 (0)	10 (43.4)	0.068
Intermediate	4 (57.1)	5 (21.8)	0.526
Adverse	3 (42.9)		0.269

NPM1 = NPM1 mutated, NK = normal karyotype, CK = complex karyotype.

overexpressed. Indeed, HLA-DM expression strongly correlates with CD74: $R = 0.45$, $P = 0.012$ (Fig. 2B).

The CD74 expression in AML cases displayed a left skewed distribution with values ranging from 3.1% to 100% and high median, 25th and 75th percentiles values of 98.4%, 97.4% and 99.8%, respectively. Among AML, all HLA-DR low cases showed medium (above the 25th percentile and less than the 75th percentile) and high (above the 75th percentile) Ii intracellular expression, while the normal HLA-DR group included 30.4% of cases with low Ii expression. All HD cases showed low percentages values for CD74+ cells (Fig. 2C). Interestingly, the HLA-DR low group expressed CD74 in 98.8% of the BM cells (with 97.5% and 100% as minimum and maximum values, respectively), significantly higher than the normal HLA-DR (mean = 90.4%, min = 3.1%, max = 100%; $P = 0.026$) or HD (mean = 87.4%, min = 59.8%, max = 97.2%; $P = 0.049$) groups (Fig. 2D). The CD74 MFI values further confirmed these observations: reduced values by 25% and 50% in the HLA-DR normal group and HD, respectively (Supplementary Fig. 1B-C). In two normal HLA-DR AML cases, we identified extremely low levels of CD74 expressing BM cells: 3.1% in one AML patient with NPM1 (nucleophosmin 1) mutation and normal karyotype, and 39.9% in another AML patient with a complex karyotype comprising various chromosomal monosomies (7, 12, 20) and del(5q) (Fig. 2D).

The overall CLIP expression was very low (bellow 10%) in AML cases and HD, with two AML exceptions: 12% in one patient with t(1;11)-associated AML and 14.1% in a patient with *NPM1*-mutated AML with complex karyotype (Table 1).

The HLA-DR low patients showed also a higher CLIP expression (Fig. 2E), which correlated with adverse or intermediate ELN risk when compared to the normal HLA-DR or HD groups (Table 2). In contrast, most cases with normal HLA-DR levels and high HLA-DM intracellular expression (above 75th percentile – 63.5%) associated low CLIP levels (Fig. 2F). Interestingly, in the case of the unprocessed Ii chain, only the intermediate and low HLA-DM cases showed less CD74 expression when compared to the HLA-DR low group (Fig. 2G, grey coloured bars).

3.3. Expression of checkpoint-ligands: co-stimulatory vs. co-inhibitory

The CLIP enriched cancer cells (HLA-DR low) showed a significant increase in the expression of co-stimulatory ICOS-L (HLA-DR low *vs.* HD: $P = 0.03$, Fig. 3A) and dual function (according to the ligand involved) B7.2 (HLA-DR low *vs.* HD: $P = 0.014$, Fig. 3B) molecules: 10.75% *vs.* 2.45% and 35.93% *vs.* 10.1%, respectively. No statistical differences were seen between the normal HLA-DR AML cells and HD cells regarding ICOS-L and B7.2 expression. The ICOS-L and B7.2 MFI values followed similar patterns as the cellular positivity percentages (HLA-DR low *vs.* HD: $P = 0.024$ (ICOS-L MFI) and $P = 0.048$ (B7.2 MFI), Supplementary Fig. 2A-B).

On the opposite side, the cases with normal HLA-DR expression had remarkably increased co-inhibitory PD-L2 (normal HLA-DR *vs.* HD: $P = 0.021$, Fig. 3C) and B7-H3 (normal HLA-DR *vs.* HD: $P = 0.029$, Fig. 3D) molecules: 8.3% *vs.* 2.45% and 7.01% *vs.* 1.08%. As expected, the expression of co-inhibitory PD-L2 and B7-H3 molecules was not affected in the group of HLA-DR low cases. Interestingly, the PD-L2 MFI values were reduced in the HLA-DR low, but not in the HLA-DR normal group (HLA-DR low *vs.* HD: $P = 0.0008$, Supplementary Fig. 2C). On the contrary, the B7-H3 MFI values followed a similar pattern as the above presented cellular percentages: a significant three times increase in the HLA-DR normal group compared to HD (HLA-DR normal *vs.* HD: $P = 0.028$, Supplementary Fig. 2D). Indeed HLA-DM and CD74 levels inversely correlated with the expression of B7-H3 ($R = -0.43$ and $R = -0.63$, respectively (Fig. 4A-B), while CLIP MFI values positively correlated with PD-L2 ($R = 0.41$, Supplementary Fig. 3A-B). Oppositely, the CD74 and CLIP expression (both cell percentages and MFI values) inversely correlated with the dual function B7.2 molecule in HLA-DR low cases ($R = -0.53$ for cell percentages – Fig. 4B, and $R = -0.54$ for MFI values – Supplementary Fig. 3B), probably suggesting a bias of the immune system towards Th2 responses in the case of DM-sensitive peptides, most likely of endogenous tumour origin.

3.4. BM CD4+ and CD8+ effector memory T cell subpopulations in AML cases and HD.

When compared to HD, HLA-DR normal patients only displayed significantly lower percentages of CD8+ T cells ($P = 0.019$) – Supplementary Fig. 4A). Although no differences were seen regarding the

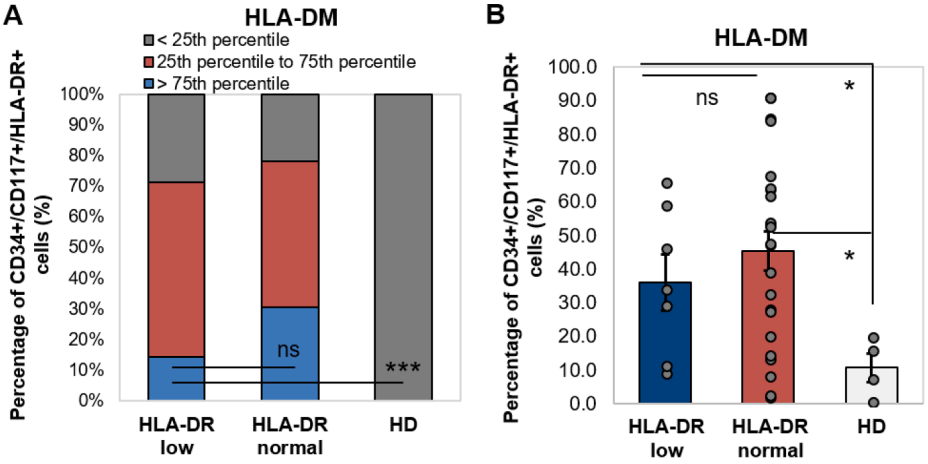

Fig. 1. HLA-DM expression in AML patients and HD. (A) Percentage of CD34+/CD117+/HLA-DR+ cells expressing HLA-DM in AML patients with low ($n = 7$) and normal ($n = 23$) HLA-DR expression and HD ($n = 4$). The percentages of cells were categorised in three intervals based on the 25th (19.7%) and 75th (63.5%) percentile values of HLA-DM in AML patients: below 25th percentile, between 25th to 75th percentile and above 75th percentile (***$P < 0.001$, ns – not significant; Wald test). Overall chi-squared test: $P = 0.006$. (B) Percentage of CD34+/CD117+/HLA-DR+ cells expressing HLA-DM in AML patients with low ($n = 7$) and normal ($n = 23$) HLA-DR expression and HD ($n = 4$). Individual values are represented as points. Bars represent the mean ± SEM (*$P < 0.05$, ns – not significant; one-way ANOVA test).

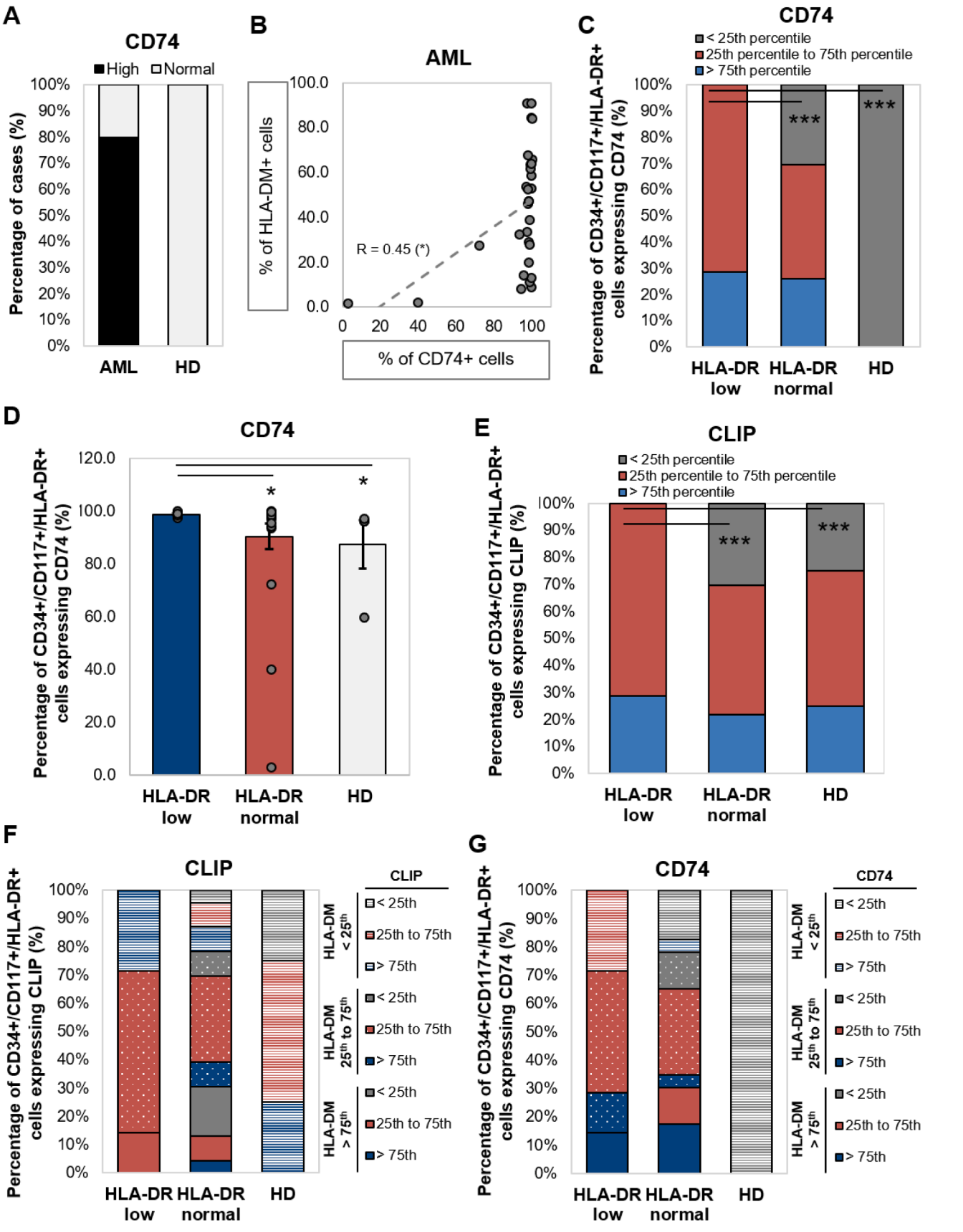

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Fig. 2. CD74 and CLIP expression in AML patients and HD. (A) Percentage of AML cases with higher CD74 expression values than HD. Chi-squared test: $P = 0.001$. (B) CD74 and HLA-DM correlation in AML patients (*$P < 0.05$; ANOVA test). (C) Percentage of CD34+/CD117+/HLA-DR+ cells expressing CD74 in AML patients with low ($n = 7$) and normal ($n = 23$) HLA-DR expression and HD ($n = 4$). The percentages of cells were categorised in three intervals based on the 25th (97.4%) and 75th (99.8%) percentile values of CD74 in AML patients: below 25th percentile, between 25th to 75th percentile and above 75th percentile (***$P < 0.001$; Wald test). Overall chi-squared test: $P = 0.003$. (D) Percentage of CD34+/CD117+/HLA-DR+ cells expressing CD74 in AML patients with low ($n = 7$) and normal ($n = 23$) HLA-DR expression and HD ($n = 4$). Individual values are represented as points. Bars represent the mean ± SEM (*$P < 0.05$; one-way ANOVA test). (E) Percentage of CD34+/CD117+/HLA-DR+ cells expressing CLIP in AML patients with low ($n = 7$) and normal ($n = 23$) HLA-DR expression and HD ($n = 4$). The percentages of cells were categorised in three intervals based on the 25th (0.31%) and 75th (5.32%) percentile values of CLIP in AML patients: below 25th percentile, between 25th to 75th percentile and above 75th percentile (***$P < 0.001$; Wald test). Overall chi-squared test: $P = 0.246$. (F) Percentage of CD34+/CD117+/HLA-DR+ cells expressing CLIP and HLA-DM in AML patients with low ($n = 7$) and normal ($n = 23$) HLA-DR expression and HD ($n = 4$). The percentages of cells were categorised in 9 intervals based on the 25th and 75th percentile values of HLA-DM and CLIP in AML patients. (G) Percentage of CD34+/CD117+/HLA-DR+ cells expressing CD74 and HLA-DM in AML patients with low ($n = 7$) and normal ($n = 23$) HLA-DR expression and HD ($n = 4$). The percentages of cells were categorised in 9 intervals based on the 25th and 75th percentile values of HLA-DM and CD74 in AML patients.

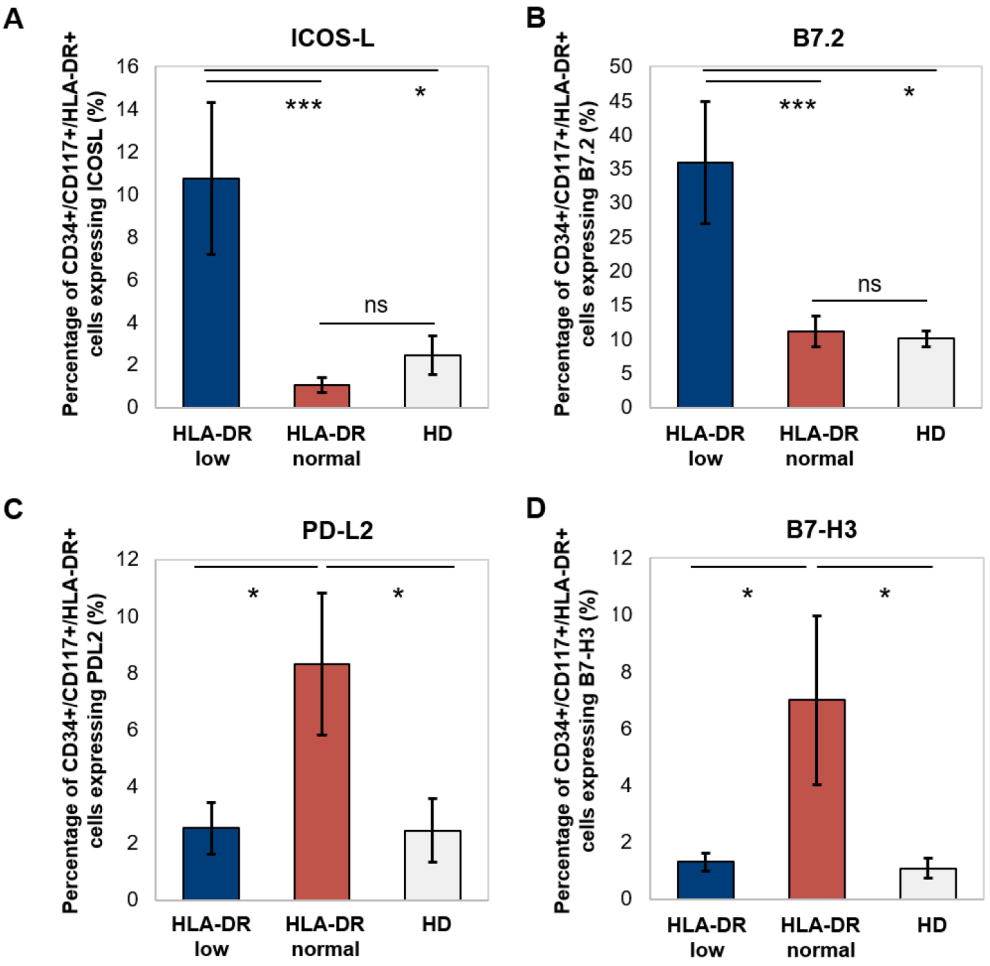

Fig. 3. Expression of co-stimulatory (ICOS-L), dual function (B7.2) and co-inhibitory (PD-L2, B7-H3) B7 ligands in AML patients and HD. (A) Percentage of CD34+/CD117+/HLA-DR+ cells expressing ICOS-L in AML patients with low ($n = 7$) and normal ($n = 23$) HLA-DR expression and HD ($n = 4$). Bars represent the mean ± SEM (***$P < 0.001$, *$P < 0.05$, ns – not significant; ANOVA test). (B) Percentage of CD34+/CD117+/HLA-DR+ cells expressing B7.2 in AML patients with low ($n = 7$) and normal ($n = 23$) HLA-DR expression and HD ($n = 4$). Bars represent the mean ± SEM (***$P < 0.001$, *$P < 0.05$, ns – not significant; ANOVA test). (C) Percentage of CD34+/CD117+/HLA-DR+ cells expressing PD-L2 in AML patients with low ($n = 7$) and normal ($n = 23$) HLA-DR expression and HD ($n = 4$). Bars represent the mean ± SEM (*$P < 0.05$; ANOVA test). (D) Percentage of CD34+/CD117+/HLA-DR+ cells expressing B7-H3 in AML patients with low ($n = 7$) and normal ($n = 23$) HLA-DR expression and HD ($n = 4$). Bars represent the mean ± SEM (*$P < 0.05$; ANOVA test).

percentage of naïve T cells among HLA-DR low, normal and HD cases (Supplementary Fig. 4B), the effector memory T cell subpopulations showed distinct patterns: (1) the majority of the BM CD4+ and CD8+ T cells were CM cells in HLA-DR low cases, while the majority of BM CD8+ T cells were late EM in the HLA-DR normal and HD cases ($P < 0.001$, chi-square test); (2) lower percentages of CD4+ lateEM (HLA-DR low *vs.* HLA-DR normal: $P = 0.04$, HLA-DR low *vs.* HD: $P = 0.005$) and CD8+ iEM (HLA-DR low *vs.* HLA-DR normal: $P = 0.009$, HLA-DR low *vs.* HD: $P = 0.04$) T cells in HLA-DR low AML patients when compared to HLA-DR normal and HD cases (Fig. 5A). Additionally, CLIP values strongly correlated with naïve T cell percentages and showed inversed correlation with effector memory T cell subsets (CD4+ iEM and late EM, C8+ lateEM) in HLA-DR low cases (Supplementary Fig. 4C). These last results suggest a less efficient effector memory T cell differentiation in HLA-DR low cases.

4. Discussion

Immunotherapy is emerging as a promising alternative in the treatment of AML, and thus, the immune gene expression profiling of the leukaemic microenvironment is establishing itself as a useful biomarker in guiding and personalizing this approach (Masarova et al., 2017; Davidson-Moncada et al., 2018). Although AML is regarded as a poorly immunogenic cancer (Curran et al., 2017), leukaemic cells are nonetheless capable of presenting a large range of leukaemia-derived immunogenic peptides, such as NPM1, PML-RARA or BCR-ABL (Biernacki and Bleakley, 2020; Makita et al., 2002; Greiner et al., 2017) to T-cells, and to elicit anti-leukaemic immune responses. Given its constitutive surface expression of MHC-II molecules, AML is a tumour type capable of presenting its own antigens (van Luijn et al., 2012). Thus, antigen-presentation is a first vital step of the cancer-immunity cycle

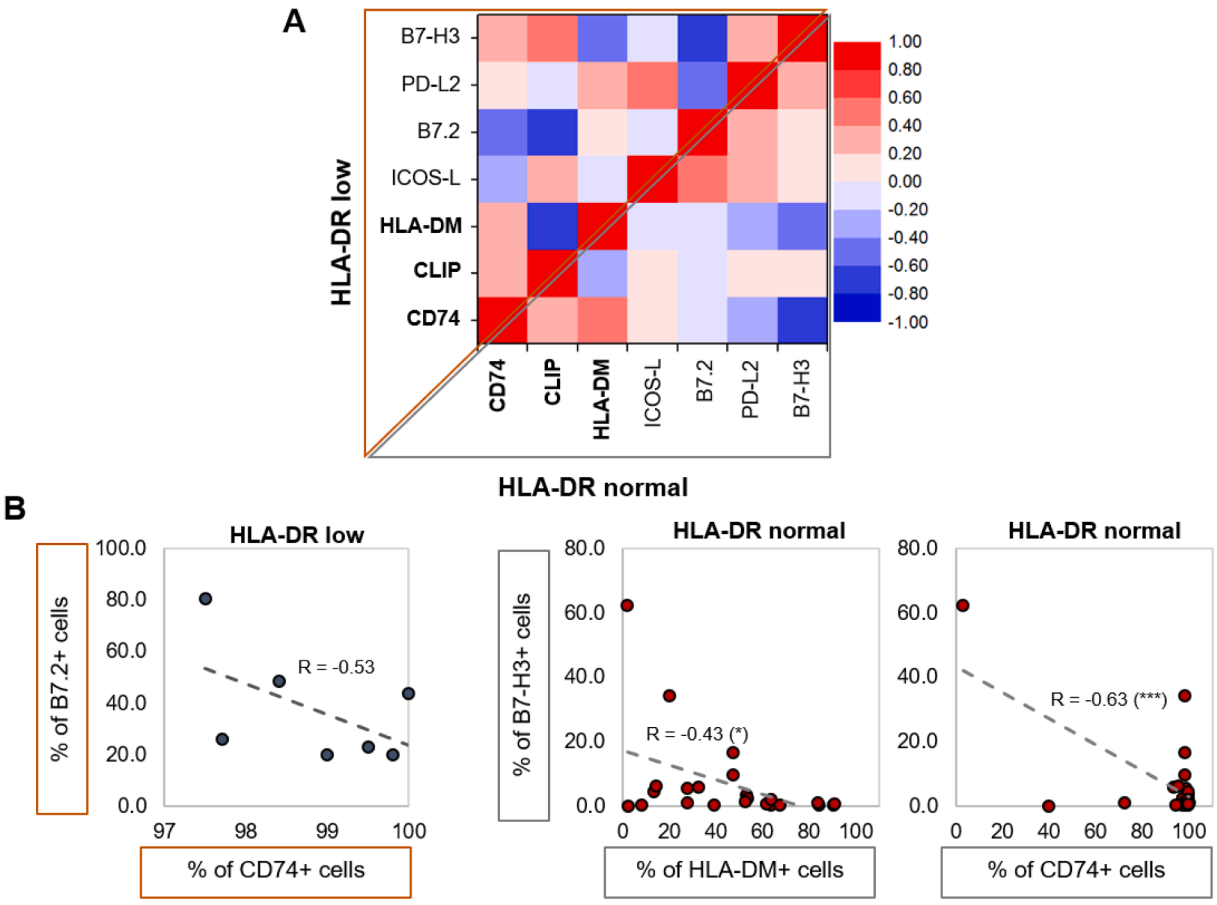

Fig. 4. CD74/HLA-DM and B7 ligands co-expression in AML patients. (A) Heat map of CD74/HLA-DM and B7 ligands co-expressions. (B) Pearson correlation coefficients (R) were computed, and ANOVA test was used to analyse the significance of the identified correlations (***$P < 0.001$; *$P < 0.05$).

that can be compromised by leukaemic cells in order to achieve immune evasion. Antigen-presentation is influenced by the MHC-II surface expression levels. A reduced HLA-DR expression, as we were able to show for a group of our AML patients, might be explained by three mechanisms: i) reduced transcription of MHC-II molecules ii) intra-endosomal/lysosomal sequestration iii) increased proteolysis and turnover (Roche and Furuta, 2015).

However, there is much more to antigen-presentation within the MHC-II groove than this. In two consecutive papers, Santambrogio et al. showed that immature DCs not only express high levels of empty MHC-II on their surfaces, but they can also take advantage of extracellular processing to fill the grooves (van Luijn et al., 2010a; 2010b; Santambrogio et al., 1999a, 1990b). Over the years, several other research groups offered compelling evidence about "alternative" antigen processing and presentation pathways, that offered us the possibility to refine our understanding on the roles that molecules like CD74/ CLIP and HLA-DM play in defining the MHC-II repertoire (Moldenhauer et al., 1999; van Luijn et al., 2010a; 2010b; Dengjel et al., 2005; Paludan et al., 2005; Zhou et al., 2005). Surface expression of CD74 in professional APCs is drastically diminished due to its enzymatic cleavage within the endosomal compartment. Conversely, surface expression of CLIP is largely regarded as a marker for defective antigen-presentation and improper peptide loading into the MHC-II groove (Sherman et al., 1995; Denzin and Cresswell, 1995), while low or absent expression of CLIP is associated with the presentation of leukaemic antigens by MHC-II molecules on the cell surface and a more favourable disease prognostic (Chamuleau et al., 2004). The peptide-editor HLA-DM allows the expression of DM-resistant antigens and abolishes the antigen-presentation of peptides displaying a CLIP-like behaviour (DM-sensitive). Thus, MHC-II ligands can be categorised as DM-sensitive, whose presentation are abolished in the presence of HLA-DM, and DM-resistant, which can be presented in the presence of HLA-DM (Kremer et al., 2012). In fact, the entire process depends on the delicate balance between HLA-DM, its natural inhibitor HLA-DO, and CD74.

Here we analysed the interlink between the cellular expression of the MHC class II - related machinery molecules (the classical HLA-DR molecule, the non-classical HLA-DM, CLIP, CD74) and B7 ligands (ICOS-L, B7.2, PD-L1, B7-H3). Thus, we have shown that AML blasts have a significantly increased HLA-DM expression compared to the normal HD value of 10.7%. This information is extremely relevant, as the identified HLA-DM expression levels of BM cells from HD may establish a normal reference value for this marker. Further, 23.3% of AML cases displayed low HLA-DR expression on leukaemic blasts, forming a distinctive group of patients. Despite similar HLA-DM levels between the two differentially expressed HLA-DR groups (HLA-DR low *vs.* normal), only the HLA-DR low group had significant increased CD74 and CLIP levels. The high intracellular CD74 levels, corroborated with the increased HLA-DM levels, suggest a reinforced mechanism of the cancer cells to protect the MHC-II groove from loading endogenous derived peptides in the endoplasmic reticulum or endosomal compartments, thus preventing cross-presentation of tumour-specific DM-sensitive antigens to CD4+ T-cells (Kremer et al., 2014). The increased expression of surface CLIP within the HLA-DR low group might be due to an additional HLA-DO higher co-expression, as CLIP/HLA-DR ratio was shown to strongly correlate with HLA-DO/HLA-DM ratio in AML blasts (Berthon et al., 2010). This phenotype preferentially assists the presentation of a broad DM-sensitive peptide repertoire exclusively intracellularly processed (Kremer et al., 2014). Since the T-cell responses against endogenous antigens rather allow the recognition of DM-sensitive antigens than the targeting of DM-resistant long-lived HLA-

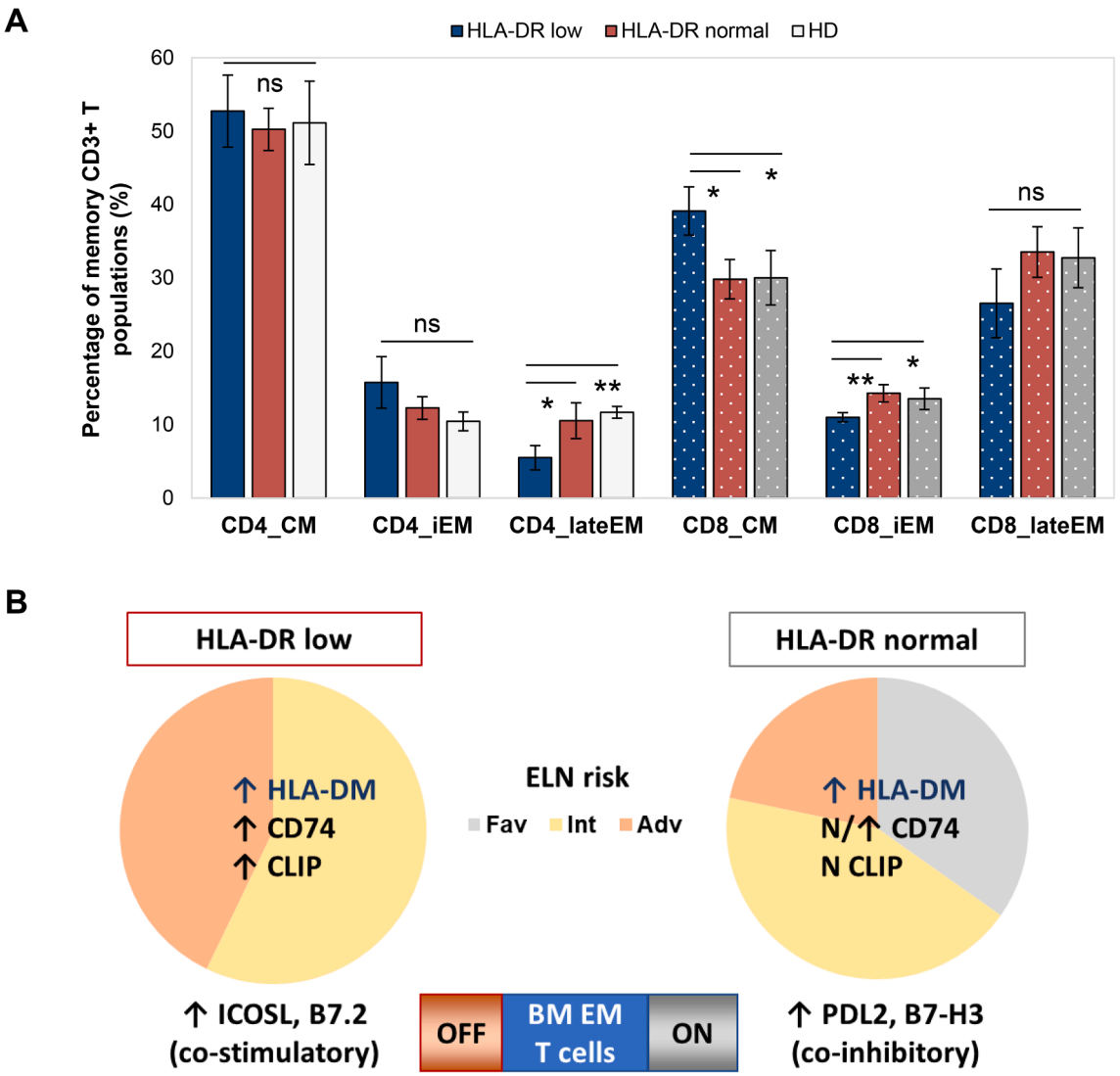

Fig. 5. BM effector memory T cell subsets in AML patients. (A) Percentage of CD4+ or CD8+ T cell populations – central memory (CM), intermediate effector memory (iEM), and late effector memory (late EM) – in AML patients with low ($n = 7$) and normal ($n = 23$) HLA-DR expression and HD ($n = 4$). Bars represent the mean ± SEM (**$P < 0.01$, *$P < 0.05$, ns – not significant; ANOVA test). (B) Proposed model for the interaction of HLA-II molecules and B7 ligands in AML cases (N = normal, ↑ = increased, Fav = favourable, Int = intermediate, Adv = adverse).

peptide complexes (Kremer et al., 2012, 2014; van den Ancker et al., 2014), it is highly probable that the HLA-DR low AML blasts tend to present CLIP-like endogenous antigens in T-cell responses. As expected, for those cases with normal HLA-DR expression, the high HLA-DM intracellular expression (above 75th percentile – 63.4%) probably led to the removal of CLIP from the HLA-DR grooves, with the subsequent degradation of CLIP, as its surface expression did not significantly differ from the reference value corresponding to HD (3.7%). We could thus conclude that the AML blasts with an HLA-DR expression like that encountered in normal cells would promote the display of DM-resistant exogenous antigens, and these cancer cells generally tend to suppress the endogenous presentation to T-helper cells. This mechanism should be regarded as highly relevant not only for AML, but for malignant cells in general. Other classical MHC–II molecules, such as HLA-DQ, are also known to be related to HLA-DM sensitivity, and thus, further studies are required for an in-depth understanding of those phenomena.

The emerging relevance for studying the co-expression of MHC class-II machinery molecules and B7 ligands on AML blasts is directly related to the identification of strategies meant to improve leukaemia specific T-cell immunity in AML patients. There are already relevant data pointing out that increased CLIP levels are associated with shortened disease-free survival, while its downregulation on AML blasts stimulates the rate of allogeneic CD4+ T cell proliferation and CD4+ and CD8+ T cell priming (van Luijn et al., 2010). On the other hand, lower CLIP levels direct the Th cell polarisation towards Th1 (increased IFN-γ and HLA-DR expression) and favour efficient proliferation of effector memory CD4+ T cells (van Luijn et al., 2011). Our results indirectly support the above observation as our data show that HLA-DR low cases associate a high CLIP expression, pointing towards a potential less effective Th1 response in tumour cell removal. Furthermore, HLA-DR low cases had less BM CD4+ lateEM T cells and BM CD8+ iEM T cells as compared to the HLA-DR normal and HD cases, suggesting the deployment of a less efficient immune response in terms of effector memory differentiation.

Our study has also limitations, including the relatively small size of the AML group. Importantly, this group is exclusively made of newly diagnosed AML cases which reflect the AML heterogeneity and our observations complement the previous work of Antohe et al (Antohe et al., 2020) and support the conclusions rising from the study of Williams et al (Williams et al., 2019) with similar number of cases. The relatively small size of the HLA-DR low ($n = 7$) compared to the HLA-DR normal ($n = 23$) subgroup of AML cases actually reflect the lower frequency of the first subgroup of only 23%, in accord with literature data (Feller et al., 2013).

Since our study mainly aimed to investigate the potential interlink between the MHC class II machinery and B7 ligands, additional factors contributing to the leukaemia immune biology such as T helper subsets, NK cells, APC and suppressor cells were kept out from the present analysis.

Overall this data shows that AML patients, based on the HLA-DR expression levels on BM blasts, are categorised in two groups with distinct HLA-DM and Ii/CLIP phenotypes: i) the HLA-DR low cases, characterised by high intracellular HLA-DM, high CD74 and high surface CLIP expression and ii) the HLA-DR normal cases, characterised by high intracellular HLA-DM, normal or high CD74 and lower surface CLIP expression (Fig. 5B). Further, these two distinct groups also differ in terms of ELN risk profiles: the HLA-DR low group lacks favourable ELN risk cases, while the normal HLA-DR group has less adverse risk patients and comprises all the AML favourable cases. Interestingly, the normal HLA-DR cases had increased expression of co-inhibitory PD-L2 and B7-H3 molecules, which rather suggests an inhibition of T-cell responses, probably to compensate the increased presentation of DM-resistant peptides induced by upregulated HLA-DM. In contrast, the HLA-DR low group had significantly higher levels of expression of B7 ligands ICOS-L and B7.2 which would favour CD4+ T-cells responses. More precisely, B7.2 preferentially induces the differentiation of naïve T-helper (Th) cells towards Th2 while ICOS-L primarily sustains the humoral responses mediated by Th2 cells (Tamura et al., 2005). As Th1/CTL responses would be preferred for a better prognostic in AML, an amplified Th2 signature would divert the immune system away from a functional status. In other AML cases, a B7.2 and ICOS-L signature was shown to provoke an initial suboptimal CD4+ T-cell activation, proliferation and differentiation towards Th1 and/or Th17, marked by IFNγ, TNFα or IL-17 secretion (Dolen and Esendagli, 2013). In turn, the Th1 and Th17 effector cells downregulated the co-stimulatory B7.2 molecule and upregulated the co-inhibitory PD-L1 ligand on AML blasts, inducing immunosuppressive functions to AML cells by favouring a PD1-dependent Treg differentiation (Dolen and Esendagli, 2013). Indeed, one HLA-DR low case co-expressed high B7.2 and PD-L1 levels (Table 1). However, despite the initially chosen differentiation pathway, either Th1/Th17 or Th2, the HLA-DR low AML blasts would mount an ineffective CD4+ T response. This observation might contribute in understanding the lack of association of HLA-DR low cases with favourable ELN risk (Table 1).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Availability of data and materials

All the datasets generated in this study are available from the corresponding author on reasonable request.

Authors' contributions

IA, MZ, and PC are responsible for the study design. IA, AD, and CD were involved in the clinical management of the patients. MZ, II, and AS performed the immunophenotypic, molecular, and cytogenetic analyses. MO, IA, MZ and PC were involved in data analysis. MP performed the statistical analysis and contributed to the graphical illustrations. MP, IA, and PC wrote the manuscript. All the authors were involved in the revision of the manuscript.

Ethics approval and consent to participate

The studies involving human participants were reviewed and approved by Ethics Committee, Grigore T. Popa University of Medicine and Pharmacy, Iasi, Romania. The patients/participants provided their written informed consent for participation in this study.

Patient consent for publication

Not applicable.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.imbio.2020.152049.

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