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Advances in Brief

Class II-Associated Invariant Chain Peptide Expression on Myeloid Leukemic Blasts Predicts Poor Clinical Outcome

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Abstract

Effective antitumor responses need the activation of CD4+ T cells. MHC class II antigen presentation requires the release of class II-associated invariant chain peptide (CLIP) from the antigen-binding site. In antigen-presenting cells, human leukocyte antigen DM (HLA-DM; abbreviated DM in this article) catalyzes CLIP dissociation. In B cells, HLA-DO (DO) down-modulates DM function. Cell surface CLIP:HLA-DR (DR) ratio correlates to DO:DM ratio and the efficacy of antigen presentation. We examined 111 blood and bone marrow samples of patients with newly diagnosed acute myeloid leukemia (AML) for the expression of CLIP, DR, DM, and DO by flow cytometry. Patients with DR+/CLIP− blasts had a significant longer disease-free survival than patients with DR+/CLIP+ blasts. DO, until now believed to be restricted to lymphoid cells, could be demonstrated at protein level as well as by reverse transcription-PCR. DO:DM ratio correlated to CLIP:DR ratio, suggesting that, unlike in other antigen-presenting cells of the nonlymphoid cell type, both DO and DM mediate regulation of CLIP expression in AML blasts. We hypothesize that DR+/CLIP− AML blasts are able to present leukemia-specific antigens to CD4+ T helper cells initiating an effective and long-lasting antitumor response resulting in a prolonged disease-free survival.

Introduction

The role of MHC class II molecules presenting tumor antigens to CD4+ T helper cells in antitumor responses is well established (1). The major MHC class II molecule, DR, consists of an α and β chain that, after translocation to the endoplasmic reticulum, form a complex with the invariant chain (Ii; ref. 2). Ii serves as a chaperone for newly synthesized class II molecules and prevents the binding of undesired antigen in the endoplasmic reticulum. The Ii is cleaved in the endosomal/lysosomal pathway until only a small remnant, called class II-associated invariant chain peptide (CLIP), remains associated with the antigen-binding groove (3). Release of CLIP is necessary for replacement with antigens, a process catalyzed by DM, a nonclassical MHC class II molecule (4). In mice lacking DM, CLIP is not released from the antigen-binding site and severely injures antigen presentation (5).

In B cells and thymic epithelial cells, DO, another nonclassical MHC class II molecule, associates with DM. DO down-modulates the catalytic activity of DM in a pH-dependent manner, thus altering the repertoire of presented antigens (6). By expression of different levels of DM and DO, B cells regulate the antigen presentation capacity of their MHC class II molecules (7). High cell surface expression of CLIP correlates with a high DO:DM ratio and can be viewed as an indicator for low effectiveness of antigen presentation (8).

In MHC class II negative tumors, the activation of CD4+ T cells relies on presentation of tumor antigens by professional antigen-presenting cells (APCs). MHC class II transfection studies in mice have shown that, upon expression of MHC class II molecules, tumor cells can present their tumor antigens directly to CD4+ T cells, thus bypassing the need for professional APCs (9). Moreover, simultaneous Ii suppression by antisense therapy revealed that these cells present endogenous tumor antigens and can mount a tumor-specific immune response (10). DR+/Ii− tumor cells, which are believed to present an optimal range of endogenous tumor antigens, are the predominant APC *in vivo* and have been demonstrated to be potent vaccines for tumor-bearing mice (11). Moreover, as recently described (12), human DR+/Ii−/CD80+-transfected tumor cell lines were demonstrated to elicit tumor-specific T-cell responses.

In AML, acquired mutations of the hematopoietic stem cells block differentiation. The result is accumulation of immature cells in the bone marrow and peripheral blood, often accompanied by suppression of the normal blood cells. With chemotherapy and stem cell transplantation, ~70% of patients achieve complete remission, but approximately one half of these patients relapse (13). Although AML blasts generally express MHC class II molecules and costimulatory molecules (14), they must have escaped the initial immune response in acute disease status. We hypothesize that in the situation of minimal tumor burden, *i.e.*, after achieving complete remission, AML blasts with a functional MHC class II complex could evoke effective immunosurveillance. In this study, we report that CLIP expression could be detected on AML blasts. Furthermore, we found a strong correlation between a high level of CLIP-positive AML blasts and a shortened disease-free survival. Also, the regulation of CLIP dissociation was studied. Strikingly, DO expression, until now demonstrated only in lymphoid cells (15), was demonstrated in AML blasts. This indicates that, in these myeloid cells, both DO and DM contribute to the efficacy of CLIP-antigenic peptide exchange.

Materials and Methods

Patients. After informed consent, blood and bone marrow samples were collected from 111 patients with previously untreated AML between 1992 and 2003. Patients were classified according to the French-American-British (FAB) classification (13). Patients with promyelocytic leukemia (FAB-M3), whose leukemic blasts were DR negative, were excluded. Patients received remission induction and consolidation therapy according to HOVON (Dutch-Belgian Hematology-Oncology Cooperative Group) protocols. Cytogenetic risk group was defined as favorable [t(8;21), or inversion(16)], standard (neither favorable nor adverse), or adverse [complex karyotype, −5 or −7, deletion(5q), abnormality 3q or 11q] (13). Human leukocyte antigen (HLA) allotype was diagnosed with a serological microcytotoxic assay (Sanquin Research at CLB, Amsterdam). Disease-free survival was defined as the time period between achievement of complete remission and the moment of relapse

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or the last date of follow-up in nonrelapsed patients. Patient characteristics are shown in Table 1 and reflect a representative AML patient group.

Mononuclear Cell Isolation. Bone marrow mononuclear cells and peripheral blood mononuclear cells, withdrawn before the start of therapy, were collected through density-gradient centrifugation (Ficoll-PaquePLUS, Amersham Biosciences). Samples were analyzed immediately or cryopreserved in liquid nitrogen until analysis. For mRNA detection, cryopreserved samples were thawed and the CD45dim/CD19-/7AAD-population (representing living myeloid leukemic, non-B cells) was isolated by sorting [FACS Vantage, Becton Dickinson (BD)]. Purity was always >99%.

Antibodies and Flow Cytometry Analysis. The following mouse antibodies were used: FITC-labeled anti-HLA-ABC (Dako), anti-DR (BD), CD86 (BD), anti-DO (BD PharMingen), CD22 (BD); phycoerythrin-labeled anti-DM (BD PharMingen), CD19 (Dako), CD20 (BD), CD14 (BD); peridinin chlorophyll protein-labeled CD45 (Coulter); allophycocyanin-labeled CD34 (BD); and 7-AAD (Via-Probe, BD PharMingen). CerCLIP.1 was kindly provided by P. Cresswell (Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, CT; Ref. 4).

Mononuclear cell fractions were preincubated with 10% human gammaglobulin (6 mg/ml; Sanquin, Amsterdam), followed by incubation with directly labeled antibodies. For CLIP detection, cells were incubated with CerCLIP and subsequently with phycoerythrin-conjugated rabbit-antimouse immunoglobulin (Dako). A mixture of nonrelevant mouse antibodies of different isotypes was added to avoid aspecific binding of subsequently directly labeled antibodies. For intracellular staining with DO or DM antibodies, cells were, after plasma membrane staining, fixed with PBS-1% paraformaldehyde and permeabilized with PBS-0.05% saponine. All of the incubations were performed at room temperature during 15 min for extracellular and 30 min for intracellular staining. Cells were washed after every incubation step with PBS-0.1% BSA-0.05% sodiumazide and analyzed on a FACSCalibur (BD). 25,000 living cells on a forward scatter were analyzed using CellQuest software (BD). Blasts were defined as CD45dim/SSClow. Mean fluorescence intensity index (MFI) was defined by the following formula:

$$\frac{\textit{Mean fluorescence intensity (total population)} - \textit{mean fluorescence intensity (isotype control)}}{\textit{Mean fluorescence intensity (isotype control)}}$$

The capability of eliciting effective antitumor immune responses is likely to depend on the total number of DR molecules that are not occupied by CLIP. To take both the number of DR- and CLIP-positive cells, as well as the amount of DR and CLIP molecules per cell, into consideration, we defined CLIP expression in respect to clinical data as follows:

$$\textit{Relative CLIP amount} = \frac{\textit{percentage CLIP positive cells}}{\textit{percentage DR positive cells}} \times \frac{\textit{MFI CLIP}}{\textit{MFI DR}}$$

Real-Time Reverse Transcription-PCR. Six samples of leukemic myeloid blasts with different DO and DM protein expression were selected with the purpose of correlating protein expression with mRNA expression. Sorted cells (see above) were lysed in peqGOLD Trifast (PeQlab, Erlangen, Germany). GlycoBlue (Ambion, Austin, TX) was added as a carrier, and total RNA was extracted according to the manufacturer's instructions. First-strand cDNA was reverse transcribed using random hexamers [pd$(N)_6$; Amersham Biosciences, Piscataway, NJ] and a SuperScript II, RNase H-reverse transcriptase kit (Invitrogen, Breda, the Netherlands). Gene expression was measured in the

Table 1 *Patients' characteristics and flow cytometric expression of different markers (percentage of positive cells) on myeloid blasts of total group and different subgroups (DR− defined as <10% of cells positive; CLIP+ defined as >35% of cells positive)*

	Total	Patients with DR− blasts	Patients with DR+/CLIP− blasts	Patients with DR+/CLIP+ blasts
A. Clinical characteristics of patients				
No. of patients	111	11	78	22
M/F	56/55	5/6	39/39	11/11
Age at diagnosis, y, mean (range)	52 (16–79)	46 (23–77)	52 (16–77)	52 (22–79)
WBCs at diagnosis	67 (1–300)	73 (1–300)	70 (1–282)	58 (1–246)
Follow-up, mo, mean (range)	21 (0.03–113)	21 (0.46–58)	23 (0.03–113)	14 (0.7–80)
CR rate, *n* (%)	85 (75)	9 (82)	59 (76)	15 (68)
DFS, mean (95% CI)*	41 (30–53)	32 (16–50)	48 (34–62)	11 (5–18)
FAB classification, *n* (%)				
AML M0	6 (5)	2 (18)	2 (3)	2 (10)
AML M1	17 (15)	5 (46)	8 (10)	4 (18)
AML M2	21 (19)	3 (27)	15 (19)	3 (14)
AML M4	29 (26)	0	25 (32)	4 (18)
AML M5	29 (26)	0	20 (26)	9 (41)
AML M6	4 (4)	1 (9)	3 (4)	0
RAEB-t	4 (4)	0	4 (5)	0
Not classified	1 (1)	0	1 (1)	0
Cytogenetic risk group, *n* (%)				
Favorable	9 (8)	0	9 (11.5)	0
Standard	65 (59)	8 (73)	45 (58)	12 (55)
Adverse	17 (9)	2 (18)	11 (14)	4 (18)
No metaphase	14 (13)	0	11 (14)	3 (13.5)
Not done	6 (5)	1 (9)	2 (2.5)	3 (13.5)
B. Flow cytometric analysis of myeloid leukemic blasts				
CD40, %†	25.7	2	28.8	26.6
CD80, %	1.3	0.6	1.6	0.8
CD86, %‡	22.4	4.4	24.2	25.5
MHC class I, %	98.8	98	98.6	99.6
MHC class II, %	66.2	1.18	70.7	83
CLIP, %	19.8	4.1	12.3	54.5
HLA-DM, %§	55.6	0.62	61.1	59.5
HLA-DO, %	16.5	26.5	15.3	16.4

Abbreviations: WBC, white blood cells; CR, complete remission; DFS, disease-free survival; CI, confidence interval; RAEB-t, refractory anemia with excess blasts in transformation.

* Significant differences were seen in DFS between DR+/CLIP+ and DR+/CLIP− patients ($P = 0.015$, log-rank). Differences in other characteristics were not significant.

† Significant differences were seen in CD40 expression between DR+ and DR− patients, ($P = 0.001$, Mann-Whitney *U* test). Differences in other characteristics were not significant.

‡ Significant differences were seen in CD86 expression between DR+ and DR− patients ($P = 0.003$, Mann-Whitney *U* test). Differences in other characteristics were not significant.

§ Significant differences were seen in HLA-DM expression between DR+ and DR− patients ($P = 0.001$, Mann-Whitney *U* test). Differences in other characteristics were not significant.

ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA). Selection and specificity of the primers (B&G Biotech, Freiburg, Germany) have been described previously (16). The sequences of the primers are as follows: DOα, forward 5′-GAGCCATCAACGTGCCTC-3′, reverse 5′-AGTGACAGTTTGGCCGTTG-3′; DOβ, forward 5′-GGAGAAAGAT-GCTGAGTGGC-3′, reverse 5′-AGGGAGCAGAACAGCTCTTG-3′; and DMβ, forward 5′- CCAGCCCAATGGAGACTG-3′, reverse 5′- CAGC-CCAGGTGTCCAGTC-3′. As endogenous control, primers specific for human 18S rRNA were used, forward 5′-CGGCTACCACATCCAAGGAA-3′ and reverse 5′-GCTGGAATTACCGCGGCT-3′. Relative quantitation of gene expression was determined using the comparative threshold cycle method as suggested by the manufacturers. All of the results were normalized with respect to the internal control 18S rRNA and are expressed relative to CD19-positive B cells from healthy donors. We used the T2 cell line as a negative control, in this study transfected with the *HLA-B27* allele (*T2-B27*), as described previously (17).

Statistical Analysis. Statistical analyses were conducted with a SPSS 9.0 software program. To analyze associations between variables, Spearman's correlation coefficient was used. Differences between patient characteristics were analyzed with the Mann-Whitney *U* and the χ^2 test. For survival data, Kaplan-Meier curves were constructed and compared by the log-rank test. To explore the simultaneous effect of several variables on disease-free survival, the Cox regression model was used. Relative CLIP amount was log transformed, yielding a normally distributed variable.

Results

Flow Cytometry Studies. We analyzed the cell surface expression of HLA-ABC, DR, CLIP, CD80, CD86, and CD40 expression on 111 samples of patients with AML. In line with previous observations (14), we found a consistently high percentage of cells expressing HLA-ABC, a low percentage expressing CD80, and a variable percentage expressing DR, CD86, and CD40. The percentage of cells with CLIP expression was variable (Table 1). All of the samples allowed clear analysis of these markers (Fig. 1). Double labeling of CLIP and DR was not possible because of steric hindrance of both monoclonals.

CLIP expression did not differ between peripheral blood and bone marrow samples, nor did it change after freezing and thawing ($n = 4$; data not shown). CLIP expression did not vary significantly among several FAB classes. Because cell surface expression of CLIP on blood and bone marrow cells was described only on mature B cells and monocytes (7), we investigated the cross-lineage expression of CD19, CD20, and CD22 (as B-cell markers) and CD14 (monocytoid marker) on AML blasts. No correlation between relative CLIP amount and these markers was observed (neither for MFI nor for percentage; $P = 0.37$, 0.32, 0.56, 0.28, and 0.53, 0.27, 0.81, 0.21, respectively). Finally, we tested whether differences in CLIP expression were a reflection of DR allotype. We did not observe however, any relationship between DR allotype and CLIP expression ($n = 19$; data not shown) as was suggested previously (18).

Correlation of Relative CLIP Expression with Clinical Data. To test the hypothesis that a functional MHC II complex (DR+/CLIP−) would result in higher antigen-presenting capacity and, hence, a survival benefit, we excluded 11 DR- patients. We observed no differences in relative CLIP amount between patients that achieved complete remission and those that did not ($P = 0.15$). In AML patients, most relapses occur within 2 years. Therefore, we compared the relative CLIP amount between patients who survived at least 24 months after complete remission and those who suffered relapse within this period. Significantly lower relative CLIP amount was observed in patients with prolonged remission compared with that in patients who relapsed before 24 months ($P = 0.04$). In clinical practice, the percentage of positive cells of different markers is used to determine the phenotype of leukemic blasts. AML patients without relapse had a maximum level of 33% CLIP-positive AML blasts. A cutoff level of 35% resulted in strongly deviating Kaplan-Meier curves ($P = 0.015$) that clearly demonstrated the survival advantage for DR+/CLIP− patients (Fig. 2). To exclude good- and poor-risk patients based on cytogenetic risk profile, a similar Kaplan-Meier curve including only patients with an intermediate cytogenetic risk profile was constructed. The *P* value was slightly increased (0.08) because of the smaller group size (Fig. 2).

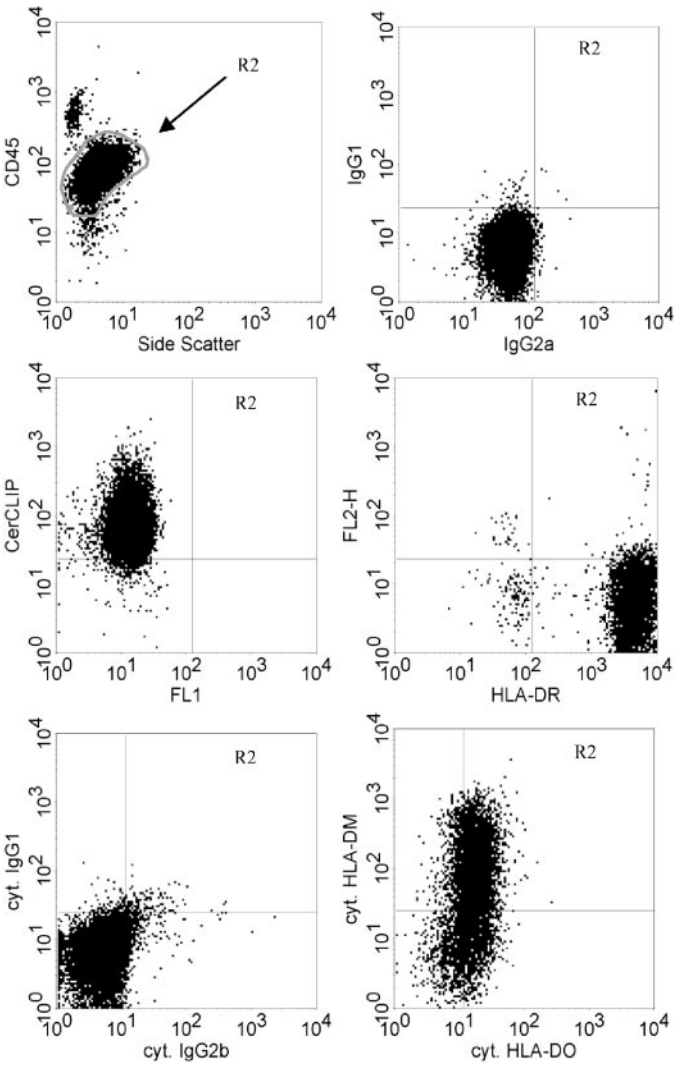

Fig. 1. Example of CLIP, DR, DO, and DM expression on myeloid leukemic blasts (*gate R2*: CD45dim/SSClow) with appropriate isotype controls (gated on 99% of the population). Double labeling of CLIP and DR was not possible because of steric hindrance of both monoclonals. (*cyt.*, cytoplasmatic (intracellular)

Because the relative CLIP amount was not related to the capability of patients to achieve complete remission, the cytogenetic risk profile, which is currently the best predictive variable for outcome for AML patients, remained a stronger predictor for overall survival than did the relative CLIP amount ($P = 0.06$ *versus* $P = 0.08$, Cox regression). However, after achievement of complete remission, when the immune surveillance is supposed to control disease status, relative CLIP amount was a better predictor than cytogenetic risk profile ($P = 0.06$ *versus* $P = 0.36$, Cox regression) for the duration of the disease-free survival.

Antigen presentation in the absence of adequate costimulatory signals leads to anergy. DR+/CLIP− blasts do express costimulatory molecules like CD86 and CD40. DR+ cells showed significantly higher expression levels of CD86 and CD40 as compared with DR−

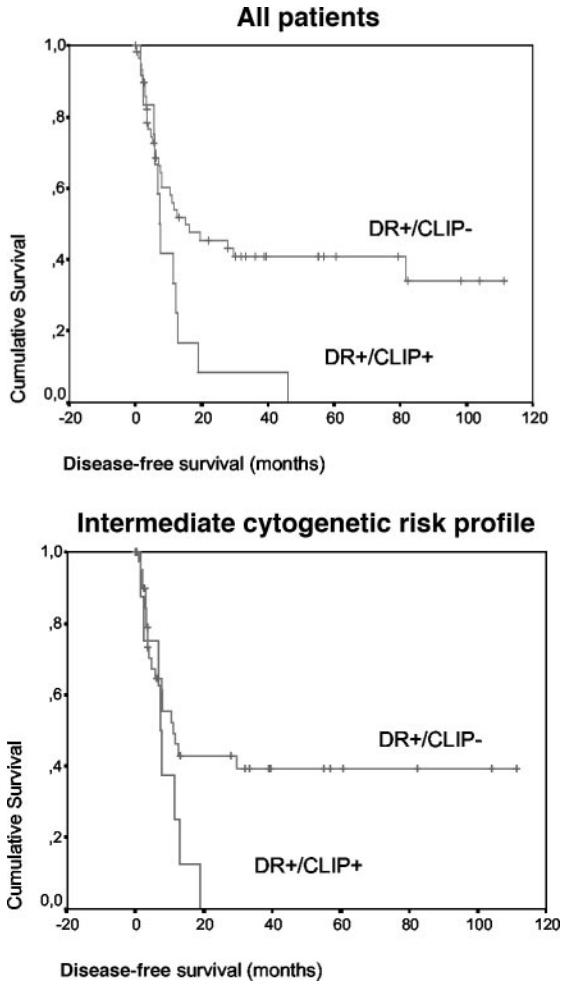

Fig. 2. Kaplan-Meier analyses for disease-free survival. *Top panel, All patients*, significant differences were seen between DR+/CLIP− and DR+/CLIP+ (cutoff 35% of cells CLIP positive), $P = 0.015$ (log-rank). *Bottom panel*, including only patients with an intermediate cytogenetic risk profile resulted in a survival curve with a similar shape ($P = 0.08$, log-rank).

cells ($P = 0.003$ and 0.001, respectively), apparently above the minimal threshold required for effective costimulation.

Regulation of CLIP Expression Level by HLA-DO. To elucidate the mechanism underlying the regulation of CLIP expression in AML blasts, we analyzed 89 samples for intracellular DO and DM expression by flow cytometry (Fig. 1). Surprisingly, DO was readily detectable in myeloid blasts. In B cells, the intracellular DO:DM ratio correlates with the cell surface CLIP:DR ratio. In AML blasts, we also could demonstrate a correlation [Fig. 3*A*; $P = 0.001$, correlation coefficient (R) = 0.46], indicating a functional role of DO and DM for cell surface CLIP expression in AML blasts similar to that in B cells. In B cells, reduced DO:DM ratio levels could not be explained by similar changes in transcriptional regulation (8). We assessed six samples of blasts with different DO and DM protein expression levels for DOα, DOβ, and DMβ that were readily detectable (Fig. 3*B*). As in B cells, however, a significant correlation between transcription level and protein level of DO and DM could not be demonstrated (data not shown).

Discussion

Aberrant MHC class I antigen presentation can function as a mechanism of tumor immune escape. The importance of antigen presentation via the MHC class II pathway for establishing effective antitumor immunity via proper CD4+ helper activation is still an emerging topic of research. Here, we report that AML blasts consistently express high levels of MHC class I molecules, whereas MHC class II is variably expressed. Moreover, we show for the first time that differences in class II antigen presentation are associated with the clinical outcome of disease in humans.

The immune system as surveillant in AML is not likely to play a role at the moment of diagnosis (when an enormous tumor burden exists), but rather during the period of minimal residual disease (when the patient has achieved complete remission). Indeed, we demonstrated in this study that the level of relative CLIP amount does not influence the probability of patients to achieve complete remission but that patients in complete remission with a high percentage DR+/CLIP− AML blasts have a significantly better disease-free survival rate compared with patients with DR+/CLIP+ blasts. Our finding strongly indicates that, analogous to mouse MHC class II+/Ii–tumor cells, the release of CLIP from the DR-binding site, and subsequent presentation of a broad panel of tumor antigens to CD4+ helper cells, is a prerequisite for an effective and long-lasting antitumor response. Because of the lack of effective methods for identifying MHC class II-restricted tumor antigens in small samples of patient material, proof of this hypothesis will be difficult, but functional studies to demon-

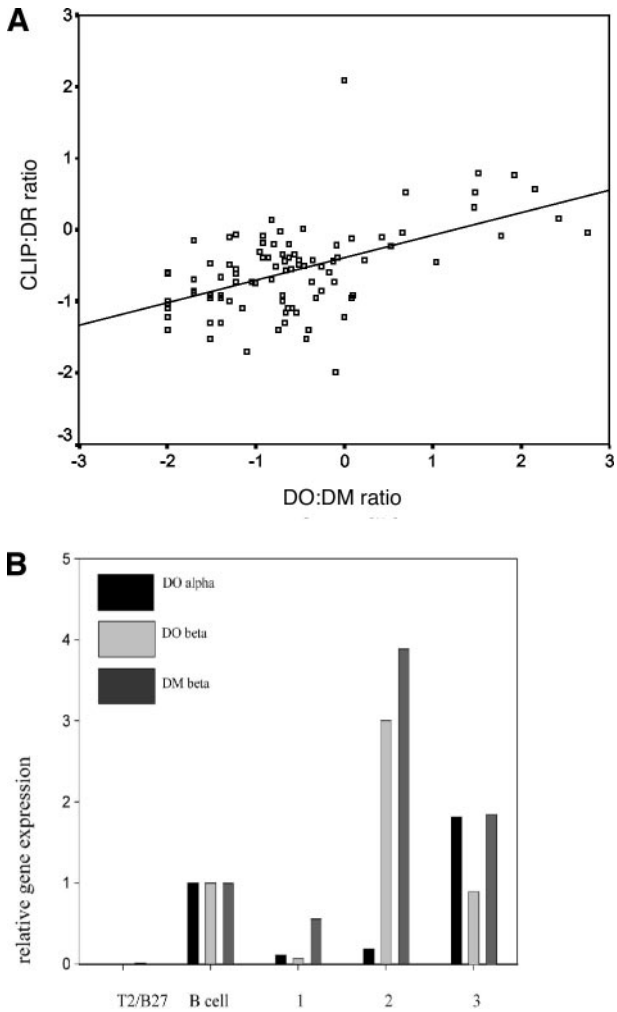

Fig. 3. *A*, ratio of percentage of CLIP+ cells to percentage of DR+ cells (CLIP:DR ratio) correlated to the ratio of percentage DO+ cells to percentage of DM+ cells (DO:DM ratio) (Spearman, $P < 0.001$, $r = 0.46$; 10 log values yielding normal values). *B*, relative gene expression levels of DOα, DOβ, and DMβ of negative (T2/B27) and positive (B cells) control and three representative AML samples. Results are expressed relative to the positive control.

strate the higher immunogenicity of DR+/CLIP− blasts are currently being undertaken in our laboratory.

At first sight, our finding seems to contradict the fact that the patients with the FAB-M3 subtype, which is DR negative, have a better prognosis than patients with the other subtypes. However, FAB-M3 blasts present a highly immunogenic fusion protein (PML-RARα), via their MHC class I molecule or via professional APCs, to the immune system (19).

Until now, expression of DO as a coregulator of antigen-presenting capacity was observed only in B cells that have acquired B-cell receptor expression. B cells regulate their antigen-presenting capacity during differentiation by differing levels of DO, compared with less varying DM expression (8). Loss of DO favors more efficient peptide loading on removal of CLIP. We were also able to demonstrate the presence of DO in myeloid blasts and to relate a low DO:DM ratio to a low cell surface CLIP:DR ratio. As in B cells, the absence of relationship between mRNA and protein levels of DO, which could be due to protein degradation (20), is an issue that needs further investigation.

We conclude that cell surface CLIP expression on AML blasts seems to be regulated by the balance of both DO and DM and shows a striking correlation with disease-free survival, pointing to the active involvement of the MHC class II presentation pathway. Ineffective MHC class II antigen presentation seems to be an immune escape mechanism of AML blasts that offers opportunities for developing immunotherapy for AML patients based on manipulation of the MHC class II antigen-processing pathway.

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