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# Chapter IV

Invariant Chain and CLIP down-modulation enhances immunogenicity of leukemic blasts resulting in increased CD4<sup>+</sup> T cell responses

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## Abstract

In acute myeloid leukemia (AML), disease development might be partially explained by the ability of leukemic blasts to escape immune surveillance. Since CD4<sup>+</sup> T cells are essential for anti-leukemic immunity, we examined the clinical relevance of aberrant HLA class II antigen presentation on leukemic blasts and whether this could be improved to enhance tumor immunogenicity. Blood and bone marrow samples from a cohort of 207 AML patients were analyzed for plasma membrane expression of the class II-associated invariant chain self peptide (CLIP) and HLA-DR (DR) by flow cytometry. Significantly shortened disease-free and overall survival rates were found for patients with leukemic blasts exhibiting a high relative CLIP amount *i.e.* the relative amount of DR occupied by CLIP. Interestingly, the relative CLIP amount on blasts of the THP-1 and Kasumi-1 AML cell line was clearly reduced after down-modulating the expression of the Invariant Chain (Ii), a protein mediating transport and preventing endogenous peptide loading of HLA class II. Furthermore, Ii down-modulated THP-1 and Kasumi-1 blasts were able to induce strong increases in allogeneic CD4<sup>+</sup> T cell proliferation in a dose-dependent manner. These data introduce Ii down-modulation as a potential immunotherapeutic strategy to activate leukemia-reactive CD4<sup>+</sup> T cells in AML patients.

## Introduction

Although intensive chemotherapy induces complete remission in 70-80% of younger and 40-60% of elderly patients with acute myeloid leukemia (AML), relapses still occur in the majority of cases<sup>1</sup>. It is hypothesized that leukemic blasts, which survive induction and consolidation treatment, have acquired a non-immunogenic phenotype during the process of immune editing<sup>2</sup>, resulting in the outgrowth of minimal residual disease. Therefore, manipulation of this immune escape phenomenon could have important implications for the development of additional immunotherapeutic strategies that intend to eradicate minimal residual disease in AML patients<sup>3</sup>.

The immunotherapeutic strategies most commonly used, including allogeneic hematopoietic stem cell transplantations and donor lymphocyte infusions, are based on the generation of specific CD8<sup>+</sup> CTL responses against leukemic blasts *in vivo*<sup>4;5</sup>. In order to induce an effective and long-lasting anti-leukemic T cell response, however, CD4<sup>+</sup> T cells are necessary to provide help to CTLs upon activation by APCs<sup>6;7</sup>. Also, in mice suffering from myeloid leukemia, CTL-mediated graft-versus-leukemia responses following delayed donor lymphocyte infusion were totally dependent on CD4<sup>+</sup> T cell help and reduced significantly when donor APCs lacked MHC class II expression<sup>8</sup>.

Since tumor-specific activation of CD4<sup>+</sup> T cells relies on optimal presentation of tumor antigens by HLA class II molecules, the HLA class II antigen presentation pathway in APCs is likely to play an important role in anti-tumor immunity. During HLA class II synthesis, class II  $\alpha$  and  $\beta$  chains dimerize in the endoplasmic reticulum (ER) and associate to the Invariant Chain (Ii) to form a nonameric complex. This interaction with Ii contributes to proper folding and prevents premature peptide loading of the HLA class II molecule<sup>9;10</sup>. Furthermore, Ii plays an important role in HLA class II trafficking from the ER to lysosomal antigen-loading compartments called MIICs (MHC class II antigen-loading compartments)<sup>11</sup>. During transport from the ER to the MIICs, Ii is proteolytically cleaved and a small peptide remnant, called the class II-associated invariant chain peptide (CLIP), remains associated with the antigen-binding groove<sup>12</sup>. For binding of exogenously derived antigenic peptides, which are processed in the endosomal/lysosomal pathway, CLIP has to be released from the antigen-binding groove by a specialized HLA-like chaperone, termed HLA-DM<sup>13</sup>. In B cells, another molecule residing in the MIICs, HLA-DO, is able to down-regulate the catalytic function of HLA-DM, resulting in limited presentation of exogenous antigens on the cell surface<sup>14</sup>.

In tumors lacking HLA class II expression, tumor-specific CD4<sup>+</sup> T cell activation can only be induced by professional APCs that are loaded with tumor antigens<sup>15</sup>. We and others demonstrated in mice that tumor cells transfected with HLA class II genes are able to present tumor antigens on the cell surface and can induce

strong CD4<sup>+</sup> T cell responses<sup>16;17</sup>. Interestingly, these and other studies further revealed that Ii expression in HLA class II-transfected tumor cells is negatively involved in activating tumor-reactive CD4<sup>+</sup> T cells<sup>18;19</sup>. This indicates that the absence of Ii in HLA class II-expressing tumor cells contributes to efficient HLA class II presentation of endogenous *i.e.* tumor-associated antigens, leading to activation of tumor-specific CD4<sup>+</sup> T cells.

In the case of AML, leukemic blasts have an intrinsic property to express both HLA class II and costimulatory molecules at the plasma membrane<sup>20;21</sup>. For that reason, it could be hypothesized that leukemic blasts expressing Ii intracellularly, as well as HLA-DR (DR) and CLIP at the plasma membrane, are able to escape immune surveillance by circumventing leukemia-specific CD4<sup>+</sup> T cell recognition. In line with this hypothesis, we previously showed that a high expression of CLIP on leukemic blasts of AML patients predicts a shortened disease-free survival<sup>22</sup>. In the current study, we expanded our cohort of newly diagnosed AML patients and confirmed that a high relative CLIP amount (the relative amount of DR occupied by CLIP) on leukemic blasts significantly correlates to a poor clinical outcome. In addition, we report that Ii RNA interference could be used as a strategy to increase DR-mediated antigen presentation, enhance immunogenicity and potentially circumvent the immunologic escape of Ii<sup>+</sup>DR<sup>+</sup>CLIP<sup>+</sup> leukemic blasts to improve clinical outcome for AML patients.

## Materials and Methods

### Patient samples

After informed consent and according to the Helsinki declaration, blood and bone marrow samples were collected from 207 patients with previously untreated AML between 1992 and 2007. Patients were classified according to the French-American-British (FAB) classification<sup>23</sup>. Patients with promyelocytic leukemia (FAB-M3), whose leukemic blasts lacked DR expression, were excluded. Patients received remission induction and consolidation therapy according to HOVON (Dutch-Belgian Hematology-Oncology Cooperative Group) protocols, available at [www.hovon.nl](http://www.hovon.nl). 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 11q23]<sup>1</sup>. Overall survival (OS) was defined as the time period from inclusion to death or last date of follow-up. Disease-free survival (DFS) was defined as the time period between achievement of complete remission (CR) and the moment of relapse or the last date of follow-up in non-relapsed patients.

Bone marrow and peripheral blood mononuclear cells, withdrawn before the start of therapy, were collected through density-gradient centrifugation

(Ficoll-PaquePLUS; Amersham Biosciences, Freiburg, Germany). Samples were analyzed by flow cytometry immediately or cryopreserved in liquid nitrogen until analysis.

### Cell lines and cell culturing

Human myeloid leukemic cell lines HL-60 (FAB-M2), THP-1 (FAB-M5), U-937 (FAB-M5), Kasumi-1 (FAB-M2), ME-1 (FAB-M4eo) and KG-1 (FAB-M0/1) were purchased from the American Type Culture Collection (ATCC) and frozen in aliquots at low cell passage. HL-60, THP-1 and U-937 cell lines were maintained in RPMI 1640 medium (Gibco, Paisley, UK) supplemented with 1% L-glutamine and 10% heat-inactivated FBS (Greiner, Alphen a/d Rijn, The Netherlands) with a cell density of  $5 \times 10^5$  cells/ml. Similar culture conditions were used for both the Kasumi-1 and ME-1 cell line, only in these cases medium contained 15% and 20% FBS, respectively. The KG-1 cell line was cultured at  $0.3 \times 10^6$  cells/ml in IMDM (Gibco) supplemented with 20% FBS, 1% L-glutamine (Gibco), 25 mM Hepes (Sigma-Aldrich, St Louis, MO, USA) and 50  $\mu$ M 2-ME (Gibco). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> and culture medium was refreshed every 2-3 days.

### Antibodies and immunofluorescence staining

The following mouse monoclonal antibodies were used: PE-labeled anti-cerCLIP.1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-HLA-DM (BD PharMingen, San Diego, CA, USA), CD27 and IgG<sub>1</sub> isotype control (BD, San Jose, CA, USA); FITC-labeled anti-HLA-DR (L243 isotype; BD), anti-HLA-DO (BD PharMingen), CD45RA (Sanquin, Amsterdam, The Netherlands), CD45RO (Dako, Glostrup, Denmark), CD4 (BD), IgG<sub>2a</sub> (Dako) and IgG<sub>2b</sub> (BD PharMingen) isotype controls; PerCP-labeled CD45, CD8 and IgG<sub>1</sub> isotype control (BD); APC-labeled CD3 and IgG<sub>1</sub> isotype control (BD); and 7-amino-actinomycin D (7AAD; Via-Probe, BD). PIN1.1 antibody (anti-Ii) was kindly provided by Peter Cresswell (Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, CT)<sup>24</sup>. Anti-HLA-DR blocking antibodies L243 were prepared from supernatants of the HB-55 hybridoma (ATCC).

Regarding immunofluorescence stainings,  $1 \times 10^5$  cells were pre-incubated with 10% human gamma-globulin (60 mg/ml; Sanquin) for 10 min. Mouse monoclonal antibodies were added during 15 min for extracellular and 30 min for intracellular staining of cells. For intracellular staining, cells were fixed with PBS-1% paraformaldehyde and permeabilized using lysing solution (BD) for Ii and PBS-0.1% saponin (Sigma-Aldrich) for both HLA-DM and -DO detection. Intracellular Ii was determined with PIN1.1 followed by a second incubation step of 20 min with rabbit anti-mouse (RAM) immunoglobulin conjugated to PE (Dako). All incubations were performed at room temperature and after each

incubation step cells were washed twice with PBS-0.1% HSA-0.05% sodium azide. Cells were measured on a FACSCalibur flow cytometer (BD) and analyzed with CellQuest software (BD). In patient samples, myeloid blasts were defined by CD45<sup>dim</sup>/SSC<sup>low</sup> expression.

Absolute mean fluorescence intensity (MFI) values were determined by relating median values of all gated cells to those of isotype controls. To compare the total number of plasma membrane expressed HLA-DR (DR) molecules occupied by CLIP, relative CLIP amount was defined as previously reported<sup>22</sup>:

$$\text{Relative CLIP amount} = \frac{\text{Percentage CLIP}^+ \text{ cells}}{\text{Percentage DR}^+ \text{ cells}} \times \frac{\text{MFI CLIP}}{\text{MFI DR}}$$

### Cell lysate preparation and Western blotting

For Western blot analysis,  $10 \times 10^6$  myeloid leukemic blasts were washed three times with sterile PBS (pH 7.4). Cell lysates were prepared by snap-freezing the samples in liquid nitrogen and incubating them for 45 min in 250  $\mu$ l ice-cold lysis buffer, which consisted of PBS supplemented with 1% Ipegal and 15% protease inhibitor cocktail (Complete; 1 tablet per 7.5 ml H<sub>2</sub>O; Boehringer Mannheim Biochemica, Mannheim, Germany). After centrifugation in an Eppendorf micro-centrifuge (5 min, 10,000 rpm), the protein content of the supernatant was determined by the Bio-Rad protein assay (Biorad Laboratories, Hercules, CA, USA). Then, proteins from 10  $\mu$ g of total cell lysates were separated on a 12.5% polyacrylamide gel containing SDS and transferred onto a methanol-activated PVDF membrane. The membranes were pre-incubated for 1 h at 4°C in blocking buffer (5% Marvel milk powder in TBS-T; 10 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 0.1% Tween-20) to prevent non-specific antibody binding. After blocking, the membranes were incubated overnight at room temperature with mouse anti-DM $\alpha$  (1:1000; 5C1)<sup>25</sup> or  $\beta$ -actin (1:3000; Chemicon, Billerica, MA, USA) antibody. After four washing steps with TBS-T, the membranes were incubated for 1 h with HRP-labeled goat anti-mouse (1:2500; Dako, Glostrup, Denmark) secondary antibody and protein complexes were visualized by Amersham ECL Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, UK).

### Retrovirus production, transductions and drug selection

A pSIREN-RetroQ vector (Clontech) containing a puromycin resistance gene and a specifically prepared Ii siRNA insert (sequence 53) was used to silence Ii expression and retrovirus production was performed as described earlier<sup>19;26</sup>. In short, 293T cells (obtained from the Harvard Gene Therapy Institute, Boston, MA, USA) were plated in 6-cm dishes at  $2.3 \times 10^5$  cells/ml DMEM (Gibco) supplemented with 10% FBS and cultured for 20 h at 37°C. After replacing medium with IMDM consisting of 10% FBS, 293T cells were transfected with 8  $\mu$ g pSIREN-RetroQ-

Ii-siRNA53 plus 6  $\mu\text{g}$  pMD.MLV gag.pol and 2  $\mu\text{g}$  pMD.G using  $\text{CaPO}_4$ . After approximately 16 h, medium was replaced with DMEM/10% FBS and transfected cells were incubated for another 24 h at 37°C. Retrovirus-containing supernatant was harvested, filtered through a 0.22- $\mu\text{m}$  filter (Millipore) and eventually stored at -80°C. GFP expression of 293T cells simultaneously transfected with the pEGFP-N1 vector (Clontech, Palo Alto, CA, USA) was monitored with UV light to evaluate virus titer.

For retroviral transduction,  $5 \times 10^5$  myeloid leukemic blasts were cultured in 0.5 ml culture medium per well using 6-well plates. When about 40% confluency was achieved, cells were washed with PBS and resuspended in 0.5 ml DMEM containing 10% FBS, 4  $\mu\text{g}$  polybrene and 10 mM Hepes (Sigma-Aldrich). Retroviral supernatant was thawed and added drop-wise to reach a final polybrene concentration of 4  $\mu\text{g}/\text{ml}$  per well. Following 6 h incubation, transduced cells were washed three times with excess PBS and kept in culture medium for three more days before adding selective drugs. Ii-siRNA53-transduced cells were selected by first adding 0.5  $\mu\text{g}/\text{ml}$  puromycin for two weeks and then increasing the dose gradually to a final concentration of 1.0  $\mu\text{g}/\text{ml}$ . Transduction efficiency was assessed by simultaneously transducing myeloid leukemic blasts with GFP retrovirus, resulting in about 20% of the cells displaying GFP expression as determined by flow cytometry.

### Allogeneic T cell proliferation assays

MLRs were performed in 96-well round-bottomed plates (Costar) with myeloid leukemic blasts acting as stimulator and allogeneic  $\text{CD4}^+$  T cells as responder cells. To obtain  $\text{CD4}^+$  T cells, PBMCs were isolated from buffy coats of different healthy donors by density gradient centrifugation (Ficoll-Paque; Amersham Biosciences). Subsequently, cells were negatively selected for  $\text{CD14}$  and  $\text{CD8}$  with microbeads using magnetic cell separation columns (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of the resulting  $\text{CD4}^+$  cell population exceeded 95%, as confirmed by flow cytometric analysis (data not shown).

Myeloid leukemic blasts were irradiated at 30 Gy and added with and without DR blocking antibody (L243; 11.5  $\mu\text{g}/\text{ml}$ ) to a fixed concentration of  $1 \times 10^5$  stimulator cells per well. After co-culturing at different stimulator-to-responder ratios for 5 days, each well was pulsed with 0.4  $\mu\text{Ci}$  [ $^3\text{H}$ ]thymidine (Amersham Pharmacia Biotech, Buckinghamshire, UK) for 18 h at 37°C in a humidified  $\text{CO}_2$  (5%) atmosphere. Co-cultures were harvested onto a fibre glass filter and analyzed for [ $^3\text{H}$ ]-thymidine incorporation as a measure of cell proliferation using a liquid scintillation counter (Wallac, Turku, Finland).



## Statistical analysis

Statistical analyses were conducted using SPSS 15.0 software. To determine associations between variables, Spearman's correlation coefficient was used. Differences between patient characteristics were analyzed with Mann-Whitney *U* test. For survival data, Kaplan-Meier curves were compared by means of the log-rank test. Multivariate Cox regression analysis was performed to evaluate the predictive value of several variables on survival.

## Results

### High relative CLIP amount on leukemic blasts predicts poor clinical outcome

We have previously demonstrated that high CLIP expression on leukemic blasts is correlated to poor clinical outcome in a cohort of 111 AML patients (1992-2003)<sup>22</sup>. We now have expanded this cohort to 207 patients (1992-2007). Patient characteristics are shown in Table 1 and reflect a representative AML patient group.

CLIP expression was comparable to the cohort of 1992-2003 and ranged from 1-99% (mean 30.5%, median 24%). Relative CLIP amount ranged from 0.0006-5.3 (mean 0.23, median 0.03). As already in a smaller subset of patients previously shown, high CLIP expression and high relative CLIP amount were both significantly correlated to a shortened overall survival ( $p=0.007$  and  $p=0.027$  (Spearman), respectively). A cut-off level of 35% for CLIP expression resulted in a significant difference in overall survival (Table I;  $p=0.021$ , Mann-Whitney *U* test) and again in significantly different Kaplan-Meier survival curves for disease-free survival of AML patients (Figure 1;  $p=0.013$ , log-rank).

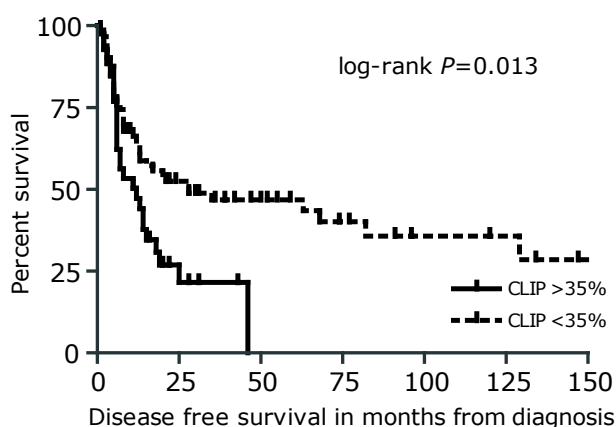
Multivariate analysis revealed that the relative CLIP amount is a significant predictor of DFS, in contrast to WBC count and age ( $p=0.023$  versus  $p=0.36$  and  $p=0.37$  respectively, Cox regression). No significant association was found between each of the FAB subtypes and DFS.

As the number of patients with a good and poor cytogenetic risk group was too small to produce reliable multivariate analysis, we determined the predictive value of CLIP in the patient group with an intermediate risk profile. When analyzing this intermediate risk patient group, concerning the largest group in our cohort, again significant differences in survival curves between DR<sup>+</sup>CLIP<sup>-</sup> and DR<sup>+</sup>CLIP<sup>+</sup> patient groups were observed ( $p=0.025$ , log-rank). In conclusion, in this expanded patient group, we are able to confirm that a high relative CLIP amount on leukemic blasts predicts an adverse clinical outcome of AML patients.

**Table 1.** Patient characteristics.

	Total	Patients with DR <sup>+</sup> CLIP <sup>-</sup> blasts	Patients with DR <sup>+</sup> CLIP <sup>+</sup> blasts
Number of patients	207	138	69
Male/female	112/95	75/63	37/32
Age in years at diagnosis, mean (range)	54 (16-82)	55 (16-82)	53 (21-81)
WBC at diagnosis (10 <sup>9</sup> /l )	43 (0-388)	40 (0-280)	50 (1-388)
CR rate, number (%)	160 (77)	99 (72)	42 (85)
OS in months, mean (range)*	21 (0-158)	25 (0-158)	13 (0-114)
DFS in months, mean (range)	21 (0-157)	25 (0-156)	11 (2-46)
FAB classification, number (%)			
AML M0	6 (3)	5 (4)	1 (1)
AML M1	25 (12)	13 (9)	12 (17)
AML M2	37 (18)	26 (19)	11 (16)
AML M4	33 (16)	27 (20)	6 (9)
AML M5	41 (20)	19 (14)	22 (31)
AML M6	14 (7)	11 (8)	3 (4)
RAEB-t	33 (16)	25 (18)	8 (12)
AML (not otherwise classified)	18 (9)	12 (9)	6 (9)
Cytogenetic risk group, number (%)			
Favorable	17 (8)	15 (11)	2 (3)
Standard	126 (61)	78 (57)	48 (69)
Adverse	27 (13)	21 (15)	6 (9)
No metaphases	31 (15)	19 (14)	12 (17)
Not done	6 (3)	5 (4)	1 (1)

Abbreviations: WBC, white blood cells; CR, complete remission; OS, overall survival; DFS, disease-free survival; FAB, French-American-British; RAEB-t, refractory anemia with excess blasts in transformation. DR<sup>+</sup> defined as >45% of cells positive, CLIP<sup>+</sup> defined as >35% positive. \*Significant difference between DR<sup>+</sup>/CLIP<sup>-</sup> and DR<sup>+</sup>/CLIP<sup>+</sup> patients was seen in overall survival ( $p=0.021$ , Mann Whitney U test). Other characteristics were not significantly different between DR<sup>+</sup>/CLIP<sup>+</sup> and DR<sup>+</sup>/CLIP<sup>-</sup> patient groups.



**Figure 1.** Kaplan-Meier curve for disease-free survival of AML patients. A significant difference was observed between patients with DR<sup>+</sup>CLIP<sup>-</sup> leukemic blasts and patients with DR<sup>+</sup>CLIP<sup>+</sup> leukemic blasts ( $n=207$ ;  $p=0.013$ , log-rank). Cut-off levels of 45% of blasts positive for DR and 35% of blasts positive for CLIP were used.

## Immunophenotypic characterization of blasts from several myeloid leukemic cell lines reveals different expression patterns of proteins involved in the HLA class II antigen presentation pathway

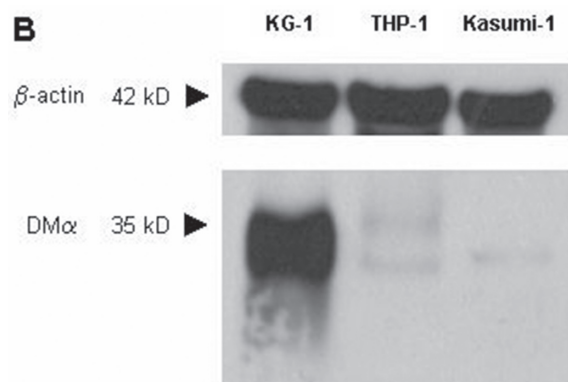
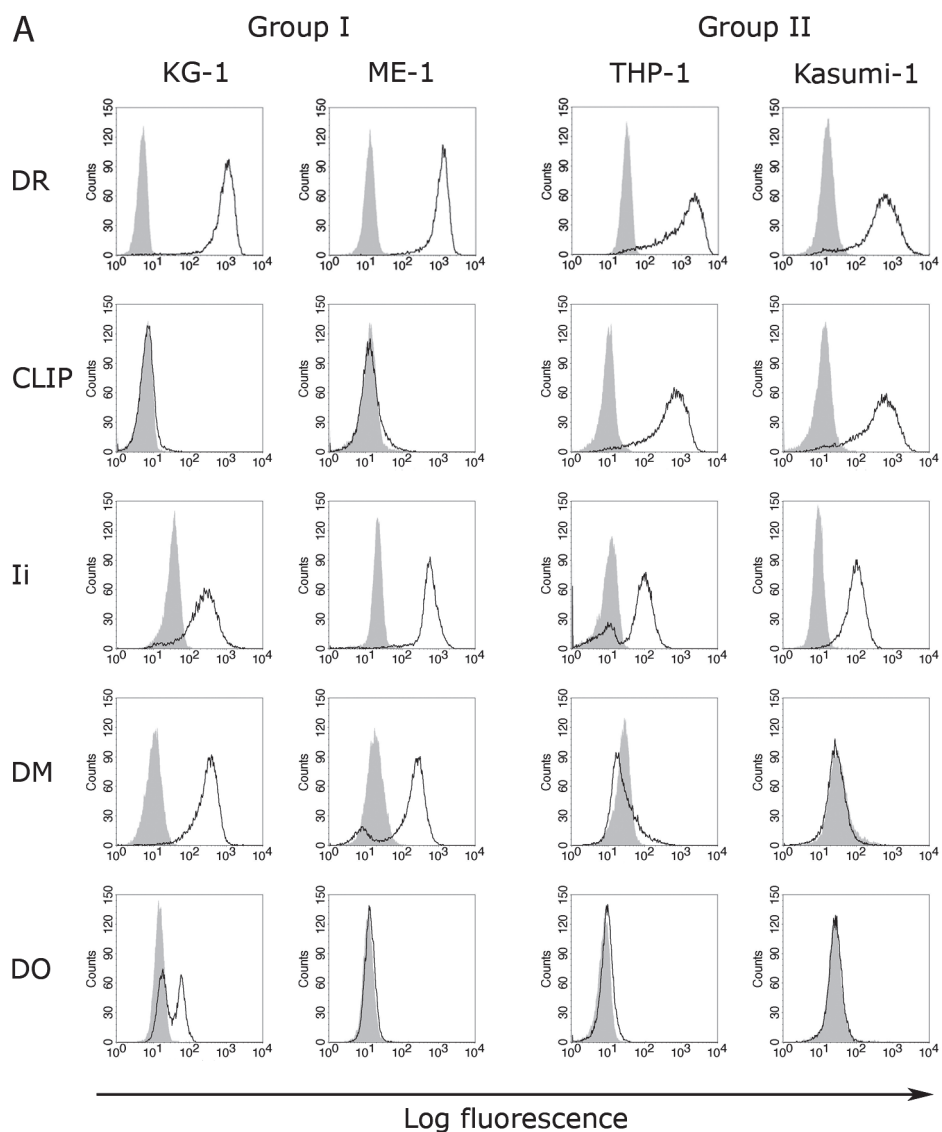
To be able to functionally explore the role of HLA class II antigen presentation on leukemic blasts, we screened six myeloid leukemic cell lines for the expression of the key proteins involved in the HLA class II antigen presentation pathway by flow cytometry. In Figure 2A, plasma membrane HLA-DR (DR) and CLIP as well as intracellular Ii, HLA-DM (DM) and HLA-DO (DO) expression levels are shown for four of these cell lines. Blasts of the HL-60 and U-937 AML cell line did not express DR and CLIP at all (data not shown).

Strong DR expression was observed on blasts of both the KG-1 and ME-1 AML cell line (MFI=199.7 and 96.5, respectively), while no CLIP expression was detected (group I). The THP-1 and Kasumi-1 AML cell line not only expressed high DR levels (MFI=47.0 and 34.6, respectively), but also CLIP (MFI=55.7 and 36.2, respectively) was clearly present at the plasma membrane (group II). Ii was abundantly expressed in the AML cell lines of both groups, which indicates that the observed differences in plasma membrane expressed CLIP are induced by DM and/or DO in the MIICs. When comparing DM and DO expression levels between the AML cell lines of group I and II, DM expression was found in CLIP<sup>-</sup> KG-1 and ME-1 blasts (MFI=30.8 and 13.6), whereas its expression was very low in CLIP<sup>+</sup> THP-1 and Kasumi-1 blasts. This low DM expression in THP-1 and Kasumi-1 blasts was confirmed by Western blot analysis (Figure 2B). DO expression was only detected in 42% of KG-1 blasts (MFI=4.0), while blasts of the other AML cell lines from group I and II were negative.

In conclusion, these findings demonstrate that, in the AML cell lines of group I, the presence of DM (and the relative absence of DO expression) probably causes an exchange of CLIP for antigenic peptides in the MIICs, which results in a DR<sup>+</sup>CLIP<sup>-</sup> phenotype of blasts. These blasts presumably have the ability to present leukemia-associated antigens. In the AML cell lines of group II, as a consequence of low amount of DM, CLIP is poorly dissociated from DR in the MIICs, leading to a DR<sup>+</sup>CLIP<sup>+</sup> phenotype and consequently having aberrant HLA class II antigen presentation.

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**Figure 2.** Screening of several HLA-DR<sup>+</sup> myeloid leukemic cell lines for the expression of proteins involved in HLA class II antigen presentation. A; Fluorescence histograms displaying DR, CLIP and Ii as well as DM and DO expression intensities (unfilled peaks) of KG-1, ME-1, THP-1 and Kasumi-1 blasts with their appropriate isotype controls (grey filled peaks). Flow cytometric analysis was performed on 7AAD<sup>-</sup> blasts. B; Western blot analysis of DM $\alpha$  (35 kD) expression in blasts of the THP-1 and Kasumi-1, compared to the KG-1 cell line. As a loading control,  $\beta$ -actin (42 kD) was used.



## **Ii down-modulation in Ii<sup>+</sup>DR<sup>+</sup>CLIP<sup>+</sup> THP-1 and Kasumi-1 blasts contributes to decreased relative CLIP amounts at the plasma membrane**

Since a high relative amount of DR molecules occupied by CLIP (relative CLIP amount) interferes with antigen presentation, we used Ii as a target in Ii<sup>+</sup>DR<sup>+</sup>CLIP<sup>+</sup> THP-1 and Kasumi-1 blasts to lower the relative CLIP amount and thereby increase DR-mediated antigen presentation on the cell surface. For this purpose, we transduced both cell lines with retroviruses encoding for specific Ii siRNAs and subsequently followed intracellular Ii as well as DR and CLIP expression at the plasma membrane for six weeks of cell culture by using flow cytometry. Wild type blasts exhibited stable expression of Ii, DR and CLIP during this period (data not shown).

### **THP-1/Ii-siRNA transductants**

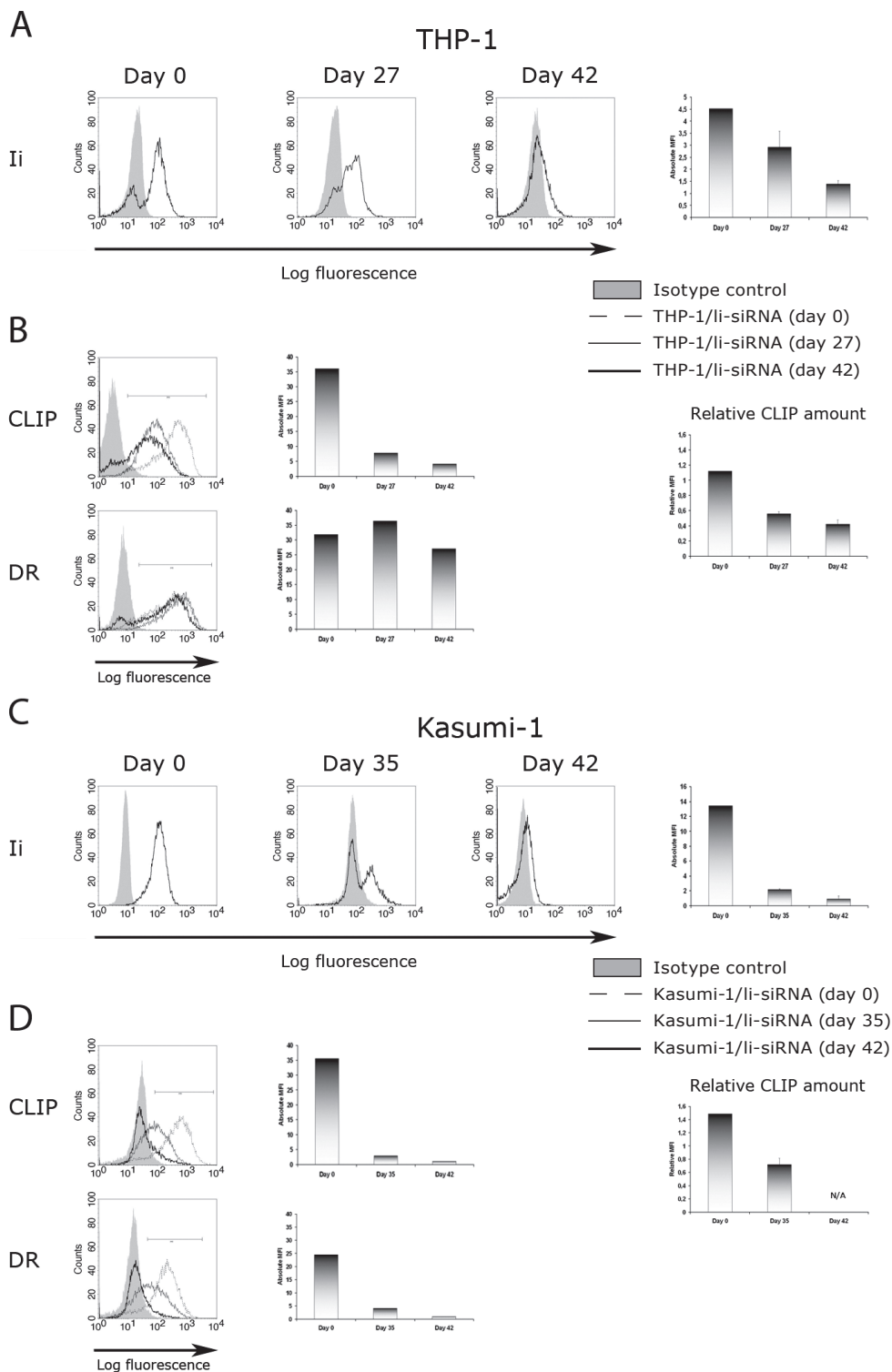
Immunofluorescence stainings of Ii siRNA-transduced THP-1 (THP-1/Ii-siRNA) blasts showed a clear reduction in Ii expression level at day 27 (MFI=2.9) and day 42 (MFI=1.4) after transduction, compared to that of wild type blasts (MFI=4.5) (Figure 3A). At the same time point after transduction, decreased amounts of CLIP were observed on THP-1/Ii-siRNA blasts, as MFI values dropped from 36.2 to respectively 7.8 and 4.2. Interestingly, in spite of the strong decline in Ii expression, DR levels remained relatively constant at the plasma membrane (MFI=36.5 and 27.1). Due to these different effects of Ii down-modulation on CLIP and DR expression, relative CLIP amounts on THP-1/Ii-siRNA blasts reduced from 1.24 to 0.56 ( $\pm 0.03$ ) and 0.42 ( $\pm 0.06$ ), respectively (Figure 3B).

### **Kasumi-1/Ii-siRNA transductants**

Similar Ii expression decreases were noted in Ii-siRNA-transduced Kasumi-1 (Kasumi-1/Ii-siRNA) blasts, with MFI values declining from 13.5 to 2.2 at day 35 and 0.9 at day 42 after transduction (Figure 3C). In contrast to THP-1/Ii-siRNA blasts, Kasumi-1/Ii-siRNA blasts not only displayed reduced amounts of CLIP (MFI dropped from 35.5 to respectively 3.0 and 1.1), but also DR expression was markedly influenced (MFI dropped from 24.6 to respectively 4.1 and 1.0). Still,

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**Figure 3.** The effect of Invariant Chain down-modulation in leukemic blasts on the relative CLIP amount. THP-1 and Kasumi-1 blasts were analysed for the expression of intracellular Ii (A and C, respectively) and extracellular DR and CLIP (B and D, respectively) by flow cytometry. Due to variable sensitivity for the retroviral transduction and selection procedure of THP-1 and Kasumi-1 blasts, measurements on viable blasts could not be performed until respectively day 27 and 35 after transduction. Absolute MFI values and relative CLIP amounts were defined as described in Materials and Methods. As an example, DR and CLIP levels of one of two independent Ii siRNA transductants are depicted. The M1 and M2 regions indicate the percentages of CLIP<sup>+</sup> and DR<sup>+</sup> blasts, respectively. Ii expression and relative CLIP amounts represent the means ( $\pm$  SD) of two independent Ii siRNA transductants.



a stronger decline was observed in expression levels of CLIP compared to DR at day 35 after transduction, which resulted in a decrease of relative CLIP amount from 1.5 to 0.8 ( $\pm 0.09$ ) (Figure 3D). At day 42 after transduction, DR expression levels on Kasumi-1/Ii-siRNA blasts were too low for determining the relative CLIP amount.

These data reveal that Ii down-modulation with specific siRNAs affects DR expression differently in THP-1 and Kasumi-1 blasts. However, Ii down modulation strongly decreased relative CLIP amounts at the plasma membrane, which may alter HLA class II antigen presentation, either stable or transient, in Ii down-modulated blasts of both cell lines.

### **Ii down-modulation in Ii<sup>+</sup>DR<sup>+</sup>CLIP<sup>+</sup> THP-1 and Kasumi-1 blasts increases their ability to induce allogeneic CD4<sup>+</sup> T cell proliferation**

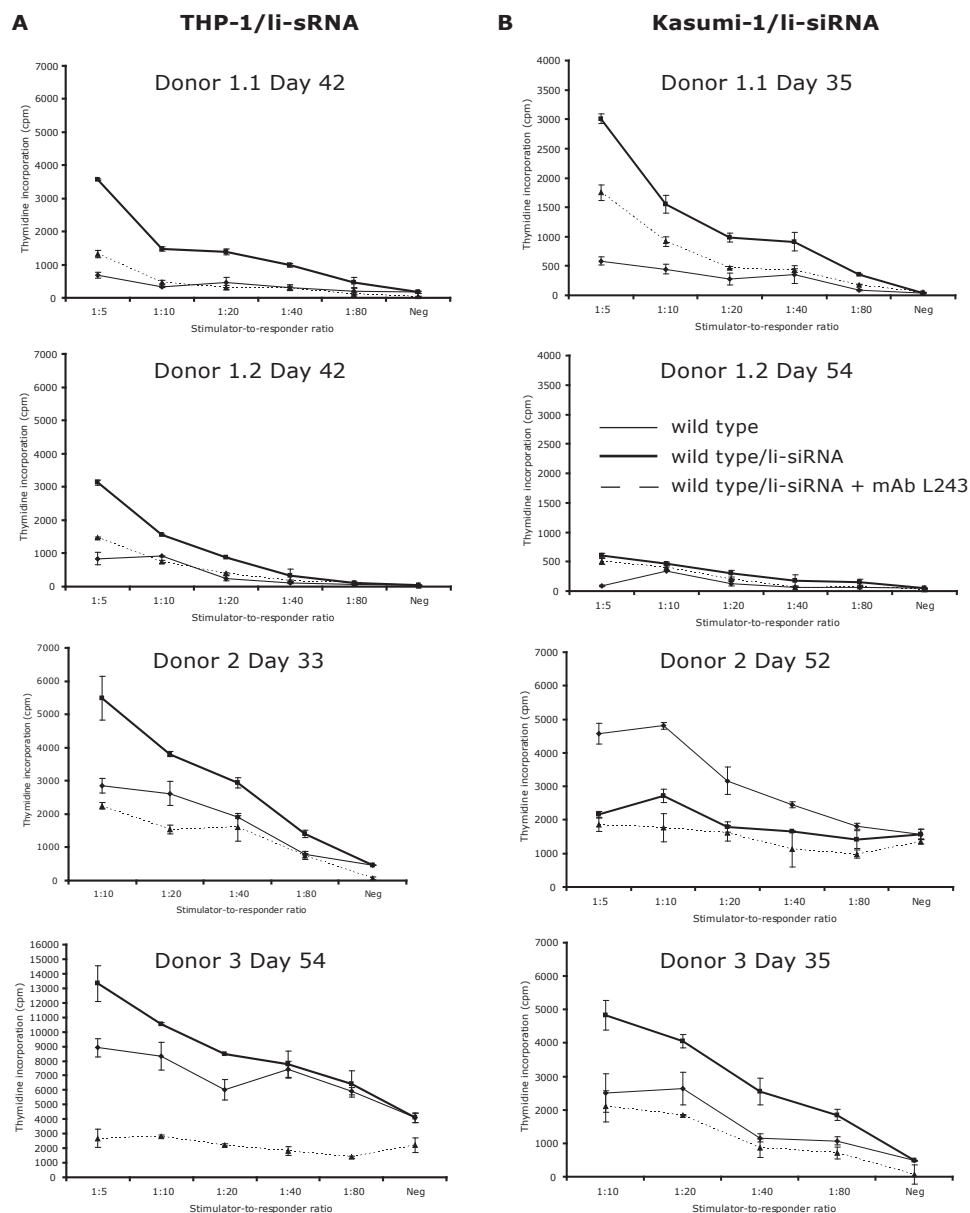
To assess the functional impact of Ii down-modulation in THP-1 and Kasumi-1, we tested Ii down-modulated blasts for their ability to induce allogeneic CD4<sup>+</sup> T cells. Therefore, we used CD4<sup>+</sup> cells obtained from three different healthy donors and analyzed their proliferative responses to these blasts in MLRs at various stimulator-to-responder ratios.

#### **THP-1/Ii-siRNA transductants**

MLRs performed with THP-1/Ii-siRNA blasts (day 42) revealed strong increases in proliferation of CD4<sup>+</sup> T cells from donor 1, in a dose-dependent manner, as shown in Figure 4A. This was in agreement with the decrease in relative CLIP amount of THP-1/Ii-siRNA blasts at day 42 after transduction (Figure 3B). On the other hand, low proliferative responses were noted in MLRs with THP-1 wild type blasts and CD4<sup>+</sup> T cells obtained from the same donor. This resulted in a maximum mean increase in CD4<sup>+</sup> T cell proliferation of 4.5( $\pm 1.0$ )-fold when comparing the two experiments of THP-1/Ii-siRNA blasts to those of THP-1 wild type blasts, as depicted in Figure 4A. Although mild alloreactivity of CD4<sup>+</sup> T cells from donor 2 and 3 was observed against THP-1 wild type blasts, their reactivity against THP-1/Ii-siRNA blasts was much more increased at the highest stimulator-to-responder ratios (Figure 4A). Importantly, the induction of CD4<sup>+</sup> T cell proliferation by THP-1/Ii-siRNA blasts was prevented in the presence of the DR blocking mAb L243, confirming DR restriction of these proliferative responses in MLRs.

#### **Kasumi-1/Ii-siRNA transductants**

In accordance with the MLRs with THP-1/Ii-siRNA blasts (day 42), MLRs performed with Kasumi-1/Ii-siRNA blasts (day 35) showed high proliferative responses of CD4<sup>+</sup> T cells from donor 1, also in a dose-dependent manner (Figure 4B). When



**Figure 4.** Proliferation assays of allogeneic CD4<sup>+</sup> T cells co-cultured with Ii down-modulated leukemic blasts. The ability of wild type and Ii down-modulated THP-1 (A) and Kasumi-1 (B) blasts to induce CD4<sup>+</sup> T cell proliferation was compared in different MLRs. CD4<sup>+</sup> T cells were obtained from three independent healthy donors. Stimulator-to-responder ratios of 1:5, 1:10, 1:20, 1:40 and 1:80 were used together with a negative control (only stimulator cells). MLRs were carried out in triplicate at each stimulator-to-responder ratio. MLRs with CD4<sup>+</sup> T cells from donor 1 were performed twice (donor 1.1 and 1.2) for both cell lines to confirm reproducibility. In each graph, the days after Ii-siRNA transduction is noted at which Ii down-modulated blasts were tested in the MLR. Results show the means ( $\pm$  SEM) of [<sup>3</sup>H]-thymidine incorporation in counts per minute (cpm), as indicator for CD4<sup>+</sup> T cell proliferation.



compared to MLRs with Kasumi-1 wild type blasts, a 5.1-fold increase in CD4<sup>+</sup> T cell proliferation was found at the highest stimulator-to-responder ratio. However, in MLRs performed with Kasumi-1/Ii-siRNA blasts at day 54 after transduction, no proliferation was observed of CD4<sup>+</sup> T cells obtained from the same donor. When Kasumi-1/Ii-siRNA blasts at day 35 after transduction were co-cultured in MLRs with CD4<sup>+</sup> T cells from donor 3, strong increases with respect to CD4<sup>+</sup> T cell proliferation were observed again, as compared to wild type blasts (Figure 4B). In addition, MLRs between Kasumi-1/Ii-siRNA blasts (day 52) and CD4<sup>+</sup> T cells from donor 2 showed no induction of proliferation (Figure 4B), in contrast to THP-1/Ii-siRNA blasts (day 33) and CD4<sup>+</sup> T cells from the same donor (Figure 4A). These results were in line with the total absence of DR expression observed on Kasumi-1/Ii-siRNA blasts at day 42 after transduction (Figure 3D). Overall, in contrast to wild type blasts, Ii down-modulated blasts of both the THP-1 and Kasumi-1 cell line were able to induce marked allogeneic CD4<sup>+</sup> T cell responses in a DR restricted manner. This indicates that the altered HLA class II-mediated antigen presentation on THP-1 and Kasumi-1 blasts due to Ii down-modulation leads to an enhancement of tumor immunogenicity.

## Discussion

The ultimate goal in cancer immunotherapy is to generate effective and long-lasting immune responses against tumor cells *in vivo*. In AML, leukemic blasts might have specific characteristics that allow them to escape immune surveillance. We previously showed that increased HLA class II-mediated antigen presentation of the self peptide CLIP on leukemic blasts from AML patients is significantly associated with a poor clinical outcome<sup>22</sup>. Statistical analysis on an expanded cohort of newly diagnosed AML patients (n=207) confirmed our finding by demonstrating that relative CLIP amount on leukemic blasts is a strong predictor of disease-free and overall survival (Figure 1 and Table 1). It has already become clear that the use of modulated leukemic blasts as APCs is an emerging approach to activate the immune system in AML patients<sup>27;28</sup>. This makes it very challenging to develop immunotherapeutic strategies that can induce anti-leukemic immunity via modulation of HLA class II antigen presentation on leukemic blasts.

Immunophenotypic screening of several human myeloid leukemic cell lines revealed large differences in relative CLIP amount (Figure 2A), which is in agreement with the results from a study performed by Harris et al.<sup>29</sup>. CLIP<sup>-</sup> KG-1 and ME-1 blasts in contrast to CLIP<sup>+</sup> THP-1 and Kasumi-1 blasts abundantly expressed DM (Figure 2A and B), indicating that the catalytic function of DM is important for antigen loading of DR molecules in these cell lines. This is in line with the correlation between CLIP/DR and DO/DM expression that was

found previously for B cells<sup>30</sup>, as well as for leukemic blasts obtained from AML patients. We did not observe DO expression in the DM<sup>low</sup> THP-1 and Kasumi-1 cell line (Figure 2A), which is in contrast to the high DO levels that were recently demonstrated in two other DM<sup>low</sup> leukemic cell lines, the HL-60 and K562<sup>31</sup>. The DM<sup>low</sup>DO<sup>-</sup> immunophenotype of both cell lines might implicate an acquired ineffectiveness of blasts to regulate exogenous antigen loading of DR molecules in the MIICs.

Our data on the expression patterns for the KG-1 (early myeloblast; CLIP<sup>-</sup>DM<sup>+</sup>DO<sup>+</sup>), ME-1 (myelomonocyte with eosinophilia; CLIP<sup>-</sup>DM<sup>+</sup>DO<sup>-</sup>), THP-1 (monocyte; CLIP<sup>+</sup>DM<sup>low</sup>DO<sup>-</sup>) and Kasumi-1 (late myeloblast; CLIP<sup>+</sup>DM<sup>low</sup>DO<sup>-</sup>) cell line further implicate a potential relation to the degree of maturation. We hypothesize that, during differentiation, the ability of myeloid blasts to regulate exogenous antigen loading of DR molecules in the MIICs is reduced and, hence, CLIP expression at the plasma membrane is increased. This is in contrast to the proposal by Harris et al. that exogenous antigen processing of myeloid blasts is a developmentally acquired characteristic<sup>29</sup>. In accordance with the observed CLIP expression on monocytic THP-1 blasts, most patients with DR<sup>+</sup>CLIP<sup>+</sup> blasts from our cohort (31%) were classified as acute monocytic leukemia (FAB-M5; Table 1). Amongst this particular subgroup, however, the amount of patients with DR<sup>+</sup>CLIP<sup>+</sup> blasts was similar to those with DR<sup>+</sup>CLIP<sup>-</sup> blasts.

Despite the similar immunophenotype of THP-1 and Kasumi-1 blasts (Figure 2), different effects of Ii down-modulation were observed on surface expression of DR (Figure 3B and 3D). According to the conventional pathway of HLA class II antigen presentation, Ii is required for the transport of HLA class II molecules from the ER to the MIICs. In monocytic THP-1 blasts however, we found that upon down-modulation of Ii, DR molecules were still able to be expressed extracellularly. As Ii also prevents binding of endogenous peptides to newly synthesized HLA class II molecules in the ER<sup>32</sup>, this might indicate that DR molecules in THP-1/Ii-siRNA blasts are loaded with endogenous peptides, including leukemia-associated peptides, thereby bypassing the Ii-dependent routing to the MIICs. This is in agreement with the ability of monocyte-derived dendritic cells (moDCs) to present endogenous antigens to CD4<sup>+</sup> T cells<sup>33</sup>. On the other hand, Kasumi-1/Ii-siRNA blasts exhibited strongly reduced levels of extracellular DR. Still, during the Ii down-modulation process, a clear decrease in relative CLIP amount was observed at day 35 after transduction (Figure 3D), which may allow an altered, although transient, DR-mediated antigen presentation around these time points. These results may implicate a difference in Ii dependency of DR-mediated antigen presentation between blasts from the monocytic lineage (THP-1) and the granulocytic lineage (Kasumi-1) of myeloid differentiation.

We further demonstrated that down-modulation of Ii expression in two myeloid leukemic cell lines not only altered HLA class II antigen presentation, but also CD4<sup>+</sup> T cell activation. Both THP-1/Ii-siRNA and Kasumi-1/Ii-siRNA blasts

(35 days after transduction) were found to strongly induce CD4<sup>+</sup> T cells from different donors (Figure 4A and B). It revealed strong inverse correlations between relative CLIP amounts and the induction of allogeneic CD4<sup>+</sup> T cell proliferation, indicating enhanced tumor immunogenicity. These observations are in agreement with the results from previous studies, in which HLA class II<sup>+</sup> tumor cells that lacked Ii expression were able to enhance tumor-reactive CD4<sup>+</sup> Th<sub>1</sub> cell activation<sup>18;19</sup>. In MLRs performed with CD4<sup>+</sup> T cells from donor 2 and 3 some CD4<sup>+</sup> T cell alloreactivity was found against THP-1 and Kasumi-1 wild type blasts. Still, the allogeneic CD4<sup>+</sup> T responses to both THP-1/Ii-siRNA and Kasumi-1/Ii-siRNA blasts (at day 35 after transduction) were much higher, showing that these responses were, at least partly, peptide-specific. Although the underlying molecular mechanism of T cell alloreactivity is still a major point of discussion, recent studies show that alloreactive T cells indeed can specifically react against HLA-peptide ligands<sup>34</sup>.

Altogether, our study shows that altering the HLA class II antigen presentation pathway affects CD4<sup>+</sup> T cell activation and thus could be a mechanism of leukemic blasts to escape immune surveillance. We provide evidence that in different myeloid leukemic cell lines, in which down-modulation of Ii results in a DR<sup>+</sup>CLIP<sup>-</sup> phenotype at the plasma membrane, endogenous antigens are presented by DR, thereby evoking CD4<sup>+</sup> T cell responses as demonstrated in MLRs. Since the immunologic escape of leukemic blasts is a major obstacle for the development of immunotherapy in AML, Ii down-modulation could be used as an additional strategy in AML DC and whole-cell vaccination protocols to activate CD4<sup>+</sup> T cells specific for a broad range of leukemia-associated antigens.

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