

# Class II-associated invariant chain peptide down-modulation enhances the immunogenicity of myeloid leukemic blasts resulting in increased CD4<sup>+</sup> T-cell responses

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*The online version of this article has a Supplementary Appendix.*

## ABSTRACT

### Background

Disease recurrence in patients with acute myeloid leukemia may be partially explained by the escape of leukemic blasts from CD4<sup>+</sup> T-cell recognition. The current study investigates the role of aberrant HLA class II antigen presentation on leukemic blasts by determining both the clinical and functional impact of the class II-associated invariant chain peptide (CLIP).

### Design and Methods

The levels of expression of CLIP and HLA-DR on blood and bone marrow samples from 207 patients with acute myeloid leukemia were correlated with clinical outcome. Irradiated CLIP<sup>-</sup> and CLIP<sup>+</sup> leukemic blasts were compared for their ability to induce CD4<sup>+</sup> T cells during mixed leukocyte reactions. To discriminate between these blasts, we down-modulated CLIP expression on myeloid leukemic cell lines by RNA interference of the invariant chain, a chaperone protein critically involved in HLA-DR processing, and performed flow cytometric sorting for their isolation from primary acute myeloid leukemia samples.

### Results

We found that patients with leukemic blasts characterized by a high amount of HLA-DR occupied by CLIP (relative amount of CLIP) had a significantly shortened disease-free survival. The clear reductions in amount of HLA-DR occupied by CLIP on blasts of the THP-1 and Kasumi-1 myeloid leukemic cell lines after treatment with invariant chain short interfering RNA resulted in enhanced rates of allogeneic CD4<sup>+</sup> T-cell proliferation. Similar findings were obtained in an autologous setting, in which there were strong increases in proliferation of remission CD4<sup>+</sup> T cells stimulated with CLIP<sup>-</sup>-sorted leukemic blasts from HLA-DR<sup>+</sup> acute myeloid leukemia patients, in contrast to CLIP<sup>+</sup>-sorted leukemic blasts from the same patients.

### Conclusions

These data highlight the relevance of CLIP expression on leukemic blasts and the potential of CLIP as a target for immunomodulatory strategies to enhance HLA class II antigen presentation and CD4<sup>+</sup> T-cell reactivity in acute myeloid leukemia.

Key words: acute myeloid leukemia, CLIP expression, leukemic blasts.

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## 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. Manipulation of this immune escape phenomenon could, therefore, have important implications for the development of additional immunotherapeutic strategies aimed at eradicating minimal residual disease in AML patients.<sup>3</sup> The immunotherapeutic strategies most commonly used, including allogeneic hematopoietic stem cell transplantation and donor lymphocyte infusions, are based on the generation of specific CD8<sup>+</sup> cytotoxic lymphocyte (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 CTL upon activation by antigen-presenting cells (APC).<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 were significantly reduced when donor APC 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 presentation pathway in APC 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 and associate with 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 endoplasmic reticulum to lysosomal antigen-loading compartments called MIIC (MHC class II antigen-loading compartments).<sup>11</sup> During transport from the endoplasmic reticulum to the MIIC, 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 MIIC, HLA-DO, is able to down-regulate the catalytic function of HLA-DM, resulting in limited presentation of exogenous antigens at the plasma membrane.<sup>14</sup>

In tumors lacking HLA class II expression, tumor-specific CD4<sup>+</sup> T-cell activation can only be induced by professional APC 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 at the plasma membrane 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-mediated presentation of endogenously synthesized tumor peptides,

leading to activation of tumor-specific CD4<sup>+</sup> T cells.

In the case of AML, leukemic blasts have an intrinsic property of expressing both HLA class II and co-stimulatory molecules at the plasma membrane.<sup>20,21</sup> For this reason, it could be hypothesized that leukemic blasts expressing Ii intracellularly, as well as HLA-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 amount of CLIP (the relative amount of HLA-DR occupied by CLIP) on leukemic blasts is significantly correlated with a poor clinical outcome. In addition, we report that a low relative amount of CLIP on leukemic blasts of HLA-DR<sup>+</sup> myeloid cell lines and from AML patients clearly improves CD4<sup>+</sup> T-cell recognition, indicating that CLIP down-modulation is a promising immunomodulatory strategy to optimize HLA class II antigen presentation and tumor immunogenicity in AML patients.

## Design and Methods

### Patients' samples

Blood and bone marrow samples were collected, in accordance with the declaration of Helsinki, from 207 patients with previously untreated AML between 1992 and 2007. Informed consent was obtained from all patients for the research use of their specimens, with approval of the institutional review board. The diagnosis of AML and its subtypes was made according to the French-American-British (FAB) criteria.<sup>23</sup> Patients with acute promyelocytic leukemia (FAB-M3), whose leukemic blasts lacked HLA-DR expression, were excluded. All patients received remission induction and consolidation therapy according to the 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 was defined as the period from inclusion to death or last date of follow-up. Disease-free survival was defined as the period between achievement of complete remission 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 cultures

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 fetal bovine serum (FBS; Greiner, Alphen a/d Rijn, The Netherlands) at a cell density of  $5 \times 10^5$  cells/mL. Similar culture conditions were used for both the Kasumi-1 and ME-1 cell lines, except that in these cases the medium contained 15% and 20% FBS, respectively. The KG-1 cell line was cultured at a density of  $3 \times 10^5$  cells/mL in Iscove's modified Dulbecco's medium (Gibco) supple-

mented 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: phycoerythrin (PE)-labeled cerCLIP1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-HLA-DM (BD PharMingen, San Diego, CA, USA), CD27 and IgG1 isotype control (BD, San Jose, CA, USA); fluorescein isothiocyanate (FITC)-labeled anti-HLA-DR (L243 isotype; BD), anti-HLA-DO (BD PharMingen), CD45RA (Sanquin, Amsterdam, The Netherlands), CD45RO (Dako, Glostrup, Denmark), CD4 (BD), IgG2a (Dako) and IgG2b (BD PharMingen) isotype controls; peridin chlorophyll (PerCP)-labeled CD45, CD8 and IgG1 isotype control (BD); allophycocyanin (APC)-labeled CD3 and IgG1 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, USA).<sup>24</sup> Anti-HLA-DR blocking antibodies L243 were prepared from supernatants of the HB-55 hybridoma (ATCC). Immunofluorescence staining was performed as indicated in the *Online Supplementary Appendix*.

### Cell lysate preparation and western blotting

For western blot analysis, 10 $\times$ 10<sup>6</sup> myeloid leukemic blasts were washed three times with sterile phosphate-buffered saline (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 sodium dodecyl-sulfate (SDS) and transferred onto a methanol-activated polyvinylidene fluoride 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 horseradish peroxidase-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

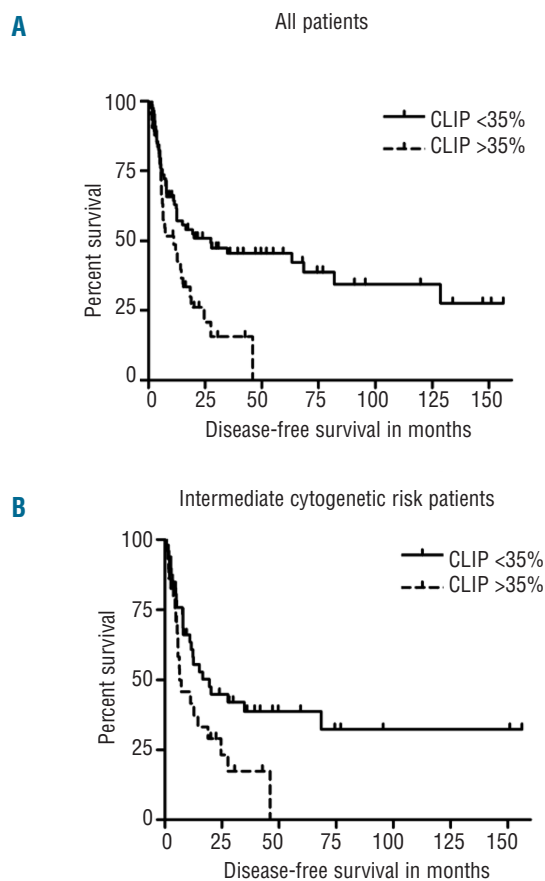
A pSIREN-RetroQ vector (Clontech) containing a puromycin resistance gene and a specifically prepared Ii short interfering RNA (siRNA) insert (sequence 53) was used to silence Ii expression. Retrovirus production and transduction were performed as described earlier,<sup>18,26</sup> and also stated in the *Online Supplementary Appendix*.

After generation of stable Ii siRNA-transduced myeloid leukemic cells, no differences in cell growth were observed during culture between these and wild type blasts. As demonstrated in a previous study,<sup>26</sup> the process of retroviral transduction and siRNA formation does not influence Ii expression and CD4<sup>+</sup> T-cell induction.

### Allogeneic T-cell proliferation assays

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

Myeloid leukemic blasts were irradiated at 30 Gy and added, with or without HLA-DR blocking antibody (L243; 11.5  $\mu$ g/mL), to a fixed concentration of 1 $\times$ 10<sup>5</sup> responder cells per well. After co-culturing at different stimulator-to-responder ratios for 5 days, each well was pulsed with 0.4 mCi [<sup>3</sup>H]-thymidine (Amersham Pharmacia Biotech, Buckinghamshire, UK) for 18 h at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. Co-cultures were harvested onto a fiberglass filter and analyzed for [<sup>3</sup>H]-thymidine incorporation as a measure of cell proliferation using a liquid scintillation counter (Wallac, Turku, Finland).



**Figure 1.** Kaplan-Meier curves for disease-free survival of AML patients. A significant difference was observed between patients with HLA-DR<sup>+</sup>CLIP<sup>-</sup> leukemic blasts and patients with HLA-DR<sup>+</sup>CLIP<sup>+</sup> leukemic blasts, both in the total (panel A;  $P=0.013$ , log-rank) and intermediate cytogenetic risk (panel B;  $P=0.025$ , log-rank) groups of patients. Cut-off levels of 45% of blasts positive for HLA-DR and 35% of blasts positive for CLIP were used.

### Autologous T-cell proliferation assays

CLIP-CD45<sup>dim</sup> and CLIP-CD45<sup>dim</sup> myeloid leukemic blasts were sorted from thawed *de novo* AML patients' samples by using a FACSAria flow cytometer (BD). For this flow cytometric sorting, cells were stained with the PerCP-labeled CD45 (BD) and PE-labeled cerCLIP.1 (Santa Cruz Biotechnology) monoclonal antibodies. To select for CD4<sup>+</sup> T cells, we collected peripheral blood from patients in complete remission for more than 6 months and isolated the mononuclear fractions as described above. Peripheral blood mononuclear cells were negatively selected for CD14 and positively selected for CD4 with microbeads using MACS (Miltenyi Biotec). Remission CD4<sup>+</sup> T cells ( $5 \times 10^5$ ) were cultured together with irradiated blasts from the same patient at a 1:1 ratio in RPMI 1640 medium (Gibco) containing 10% human AB serum (ICN Biochemicals, Aurora, OH, USA), 1% penicillin/streptomycin, 50  $\mu$ M 2-ME and interleukin-7 (10 ng/mL; Miltenyi Biotec). CD4<sup>+</sup> T cells were re-stimulated once a week at least twice with blasts and cultured again in fresh medium. Interleukin-2 (50 U/mL; Miltenyi Biotec) was added 2 days after each stimulation with blasts. CD4<sup>+</sup> T-cell proliferation was determined by trypan blue dye exclusion.

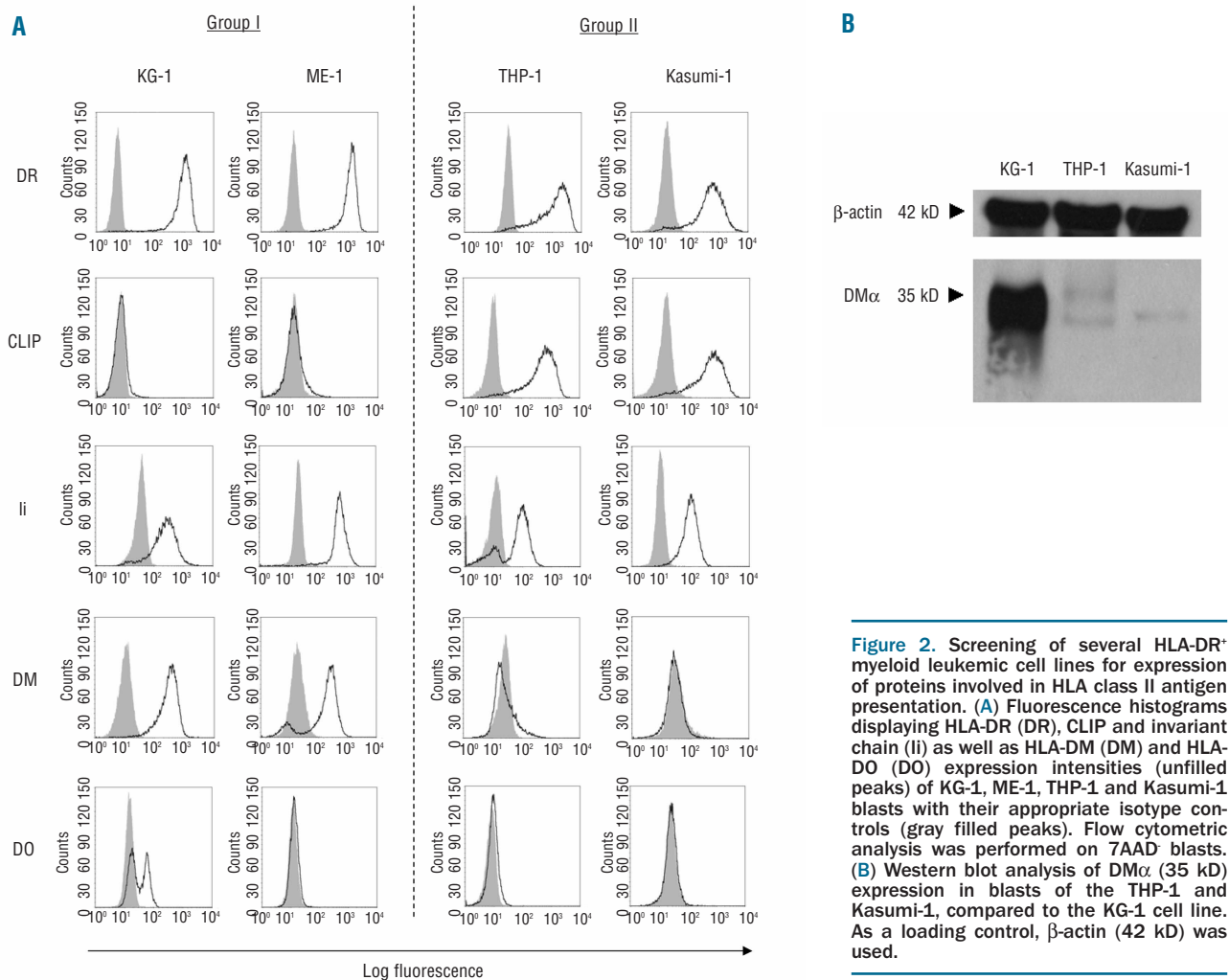
### Statistical analysis

Statistical analyses were conducted using SPSS 15.0 software. To determine associations between variables, Spearman's correlation coefficient was used. Differences between patients' characteristics were analyzed with the 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 amount of CLIP on leukemic blasts is correlated with poor clinical outcome

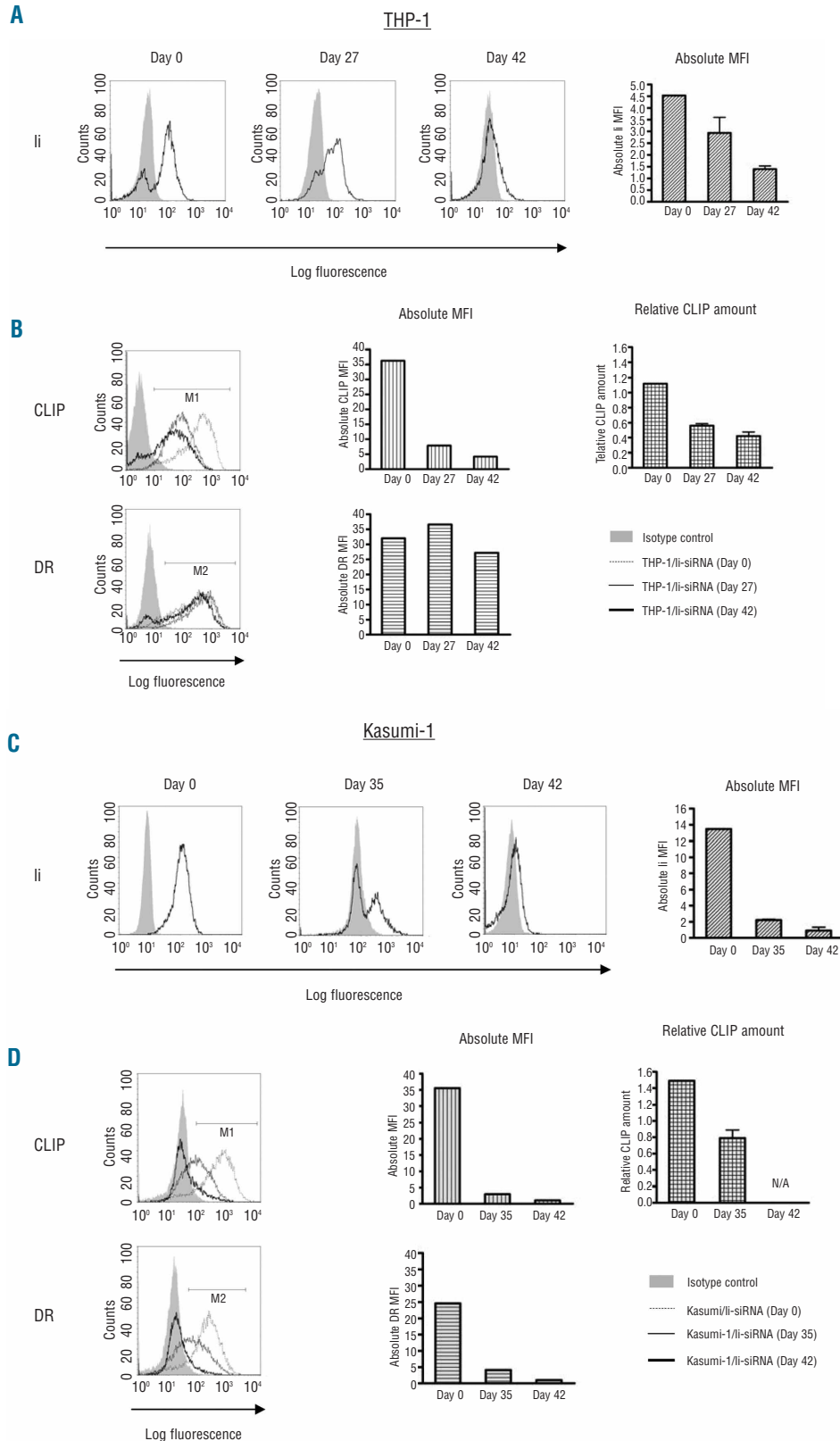
We previously demonstrated that high CLIP expression on leukemic blasts was correlated with poor clinical outcome in a cohort of 111 AML patients (1992-2003).<sup>22</sup> We have now expanded this cohort to 207 patients (1992-2007). These patients' characteristics are presented in the *Online Supplementary Table S1*, showing that the cohort is a representative group of AML patients.





CLIP expression in this cohort was comparable to that in the 1992-2003 cohort and ranged from 1-99% (mean 30.5%, median 24%). The relative amount of CLIP ranged from 0.0006-5.3 (mean 0.23, median 0.03). As already previously shown in a smaller subset of patients, both high CLIP expression and a high relative amount of CLIP were significantly

correlated with a shortened overall survival ( $P=0.007$  and  $P=0.027$ , respectively, Spearman's correlation). Furthermore, a cut-off level of 35% for CLIP expression resulted in a significant difference in Kaplan-Meier survival curves for disease-free survival of AML patients (Figure 1A;  $P=0.013$ , log-rank test). Multivariate analysis revealed that the relative amount



**Figure 3.** The effect of invariant chain (Ii) silencing in myeloid leukemic cell lines on the relative amount of CLIP. THP-1 and Kasumi-1 blasts were analyzed for the expression of intracellular Ii (panel A and C, respectively) and extracellular HLA-DR (DR) and CLIP (panel 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 day 27 and 35, respectively, after transduction. Absolute MFI values and relative CLIP amounts were defined as described in the *Design and Methods* section. 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 (± SD) of two independent Ii siRNA transductants; N/A, not applicable.

of CLIP is a significant predictor of disease-free survival, in contrast to white blood cell count and age ( $P=0.023$ ,  $P=0.36$  and  $P=0.37$ , respectively, Cox regression). No significant association was found between each of the FAB subtypes and disease-free survival.

As the numbers of patients in the good and poor cytogenetic risk groups were too small to enable reliable multivariate analysis, we determined the predictive value of CLIP in patients with an intermediate cytogenetic risk profile. When analyzing this intermediate risk patient group, the largest group in our cohort, again significant differences in survival curves were found between HLA-DR<sup>+</sup>CLIP<sup>-</sup> and HLA-DR<sup>+</sup>CLIP<sup>+</sup> groups of patients (Figure 1B;  $P=0.025$ , log-rank).

In conclusion, in this expanded group of patients, we were able to confirm that a high relative amount of CLIP on leukemic blasts predicts a poor clinical outcome for AML patients, indicating that impaired HLA class II antigen presentation on leukemic blasts is functionally involved in disease progression.

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

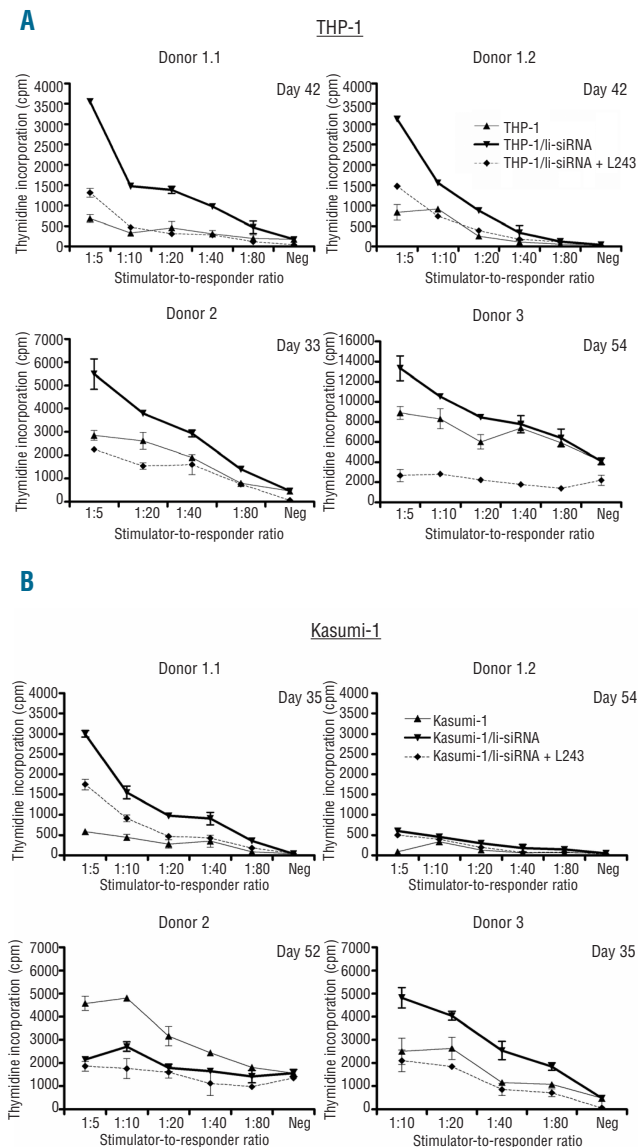
To explore the functional role of HLA class II antigen presentation on leukemic blasts, we screened six myeloid leukemic cell lines for the expression of the key proteins involved in the HLA class II presentation pathway by flow cytometry. Plasma membrane HLA-DR and CLIP as well as intracellular Ii,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

Strong HLA-DR expression was observed on blasts of both the KG-1 and ME-1 AML cell lines (mean fluorescence intensity, MFI=199.7 and 96.5, respectively), while no CLIP expression was detected (group I). The THP-1 and Kasumi-1 AML cell lines not only expressed high levels of HLA-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 HLA-DM and/or HLA-DO in the MIIC. When comparing HLA-DM and HLA-DO expression levels between the AML cell lines of groups I and II, HLA-DM expression was found in CLIP<sup>-</sup> KG-1 and ME-1 blasts (MFI=30.8 and 13.6, respectively), whereas its expression was very low in CLIP<sup>+</sup> THP-1 and Kasumi-1 blasts. This low HLA-DM expression in THP-1 and Kasumi-1 blasts was confirmed by western blot analysis (Figure 2B). HLA-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 HLA-DM (and the relative absence of HLA-DO expression) probably causes an exchange of CLIP for antigenic peptides in the MIIC, which results in a HLA-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, regarding the low amount of HLA-DM, CLIP may poorly dissociate from HLA-DR in the MIIC, leading to a DR<sup>+</sup>CLIP<sup>+</sup> phenotype with consequent aberrant HLA class II antigen presentation.

### Invariant chain silencing in HLA-DR<sup>+</sup>CLIP<sup>+</sup> THP-1 and Kasumi-1 blasts contributes to reduced relative amounts of CLIP at the plasma membrane

Since a high relative amount of HLA-DR molecules occupied by CLIP interferes with antigen presentation, we used Ii as a target in HLA-DR<sup>+</sup>CLIP<sup>+</sup> THP-1 and Kasumi-1 blasts to lower the relative amount of CLIP and thereby increase HLA-DR-mediated antigen presentation at the plasma membrane. For this purpose, we transduced both cell lines with retro-



**Figure 4.** Proliferation assays of allogeneic CD4<sup>+</sup> T cells stimulated with CLIP down-modulated blasts of myeloid leukemic cell lines. The ability of CLIP<sup>+</sup> (wild type) and CLIP<sup>-</sup> (Ii-silenced) THP-1 blasts (panel A) and Kasumi-1 blasts (panel B) to induce allogeneic CD4<sup>+</sup> T-cell proliferation was compared in different MLR. 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; 'Neg'). MLR were carried out in triplicate at each stimulator-to-responder ratio. MLR 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-silenced 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 an indicator of CD4<sup>+</sup> T-cell proliferation.

viruses encoding for specific Ii siRNA and subsequently followed intracellular Ii as well as HLA-DR and CLIP expression at the plasma membrane for 6 weeks of cell culture by using flow cytometry. Wild type blasts exhibited stable expression of Ii, HLA-DR and CLIP during this period (*data not shown*).

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

Immunofluorescence staining 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 points, decreased amounts of CLIP were observed on THP-1/Ii-siRNA blasts, as MFI values dropped from 36.2 to 7.8 and 4.2, respectively. Interestingly, in spite of the strong decline in Ii expression, HLA-DR levels remained relatively constant at the plasma membrane (MFI=36.5 and 27.1). Due to these different effects of Ii silencing on CLIP and HLA-DR expression, the relative amounts of CLIP on THP-1/Ii-siRNA blasts decreased 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 HLA-DR expression was also markedly influenced (MFI dropped from 24.6 to, respectively, 4.1 and 1.0). Still, a stronger decline was observed in expression levels of CLIP compared to those of HLA-DR at day 35 after transduction, which resulted in a decrease of the relative amount of CLIP from 1.5 to 0.8 ( $\pm 0.09$ ) (Figure 3D). At day 42 after transduction, HLA-DR expression levels on Kasumi-1/Ii-siRNA blasts were too low to determine the relative amount of CLIP. These data reveal that Ii silencing with specific siRNA affects HLA-DR expression differently in THP-1 and Kasumi-1 blasts. However, relative amounts of CLIP were strongly decreased at the plasma membrane, which may cause altered HLA class II antigen presentation, either stable or transient, on Ii-silenced blasts of both cell lines.

#### **Reduced relative CLIP amounts on invariant chain-silenced THP-1 and Kasumi-1 blasts increases their ability to induce allogeneic CD4<sup>+</sup> T-cell proliferation**

To assess the functional impact of CLIP variation, we tested Ii-silenced THP-1 and Kasumi-1 blasts with reduced relative amounts of CLIP for their ability to induce allogeneic CD4<sup>+</sup> T cells as compared to wild type blasts. We isolated CD4<sup>+</sup> T cells from three different healthy donors and analyzed their proliferative responses to these blasts in MLR at various stimulator-to-responder ratios.

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

MLR 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 amount of CLIP on THP-1/Ii-siRNA blasts at day 42 after transduction (Figure 3B). On the other hand, low proliferative

responses were noted in MLR 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 (Figure 4A). Although mild alloreactivity of CD4<sup>+</sup> T cells from donors 2 and 3 was observed against THP-1 wild type blasts, their reactivity against THP-1/Ii-siRNA blasts was increased much more 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 HLA-DR blocking monoclonal antibody L243, which confirmed HLA-DR restriction of these proliferative responses in MLR.

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

In accordance with the MLR with THP-1/Ii-siRNA blasts (day 42), MLR 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 compared to MLR 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 MLR 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 MLR 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, MLR 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 HLA-DR expression observed on Kasumi-1/Ii-siRNA blasts at day 42 after transduction (Figure 3D).

Overall, in contrast to wild type blasts, Ii-silenced THP-1 and Kasumi-1 blasts that retained their HLA-DR expression were able to induce marked allogeneic CD4<sup>+</sup> T-cell responses in a HLA-DR-restricted manner, indicating that a reduced relative amount of CLIP on myeloid leukemic blasts leads to an enhancement of tumor immunogenicity.

#### **CLIP<sup>-</sup> leukemic blasts derived from patients with acute myeloid leukemia are able to stimulate autologous remission CD4<sup>+</sup> T cells in contrast to CLIP<sup>+</sup> leukemic blasts**

We further evaluated the role of a low relative amount of CLIP in tumor immunogenicity by using primary leukemic blasts of HLA-DR<sup>+</sup> AML patients and CD4<sup>+</sup> T cells isolated from the same patients in complete remission. CLIP<sup>-</sup> and CLIP<sup>+</sup> leukemic blasts were flow cytometrically sorted from AML patients' samples and cultured with autologous remission CD4<sup>+</sup> T cells several times during culture. For two different AML patients, we found a strong increase in proliferation rate of CD4<sup>+</sup> T cells that were stimulated with CLIP<sup>-</sup> sorted blasts (*Online Supplementary Figure S1*). CD4<sup>+</sup> T cells from patient 1 already showed high proliferative responses at day 12 (after one re-stimulation) and the proliferation of CD4<sup>+</sup> T cells from patient 2 markedly increased at day 28 (after three re-stimulations). No increase in proliferation was observed



with CD4<sup>+</sup> T cells that were equally stimulated with CLIP<sup>+</sup>-sorted blasts from the same patient. Pre-treatment of CLIP<sup>-</sup>-sorted blasts with the HLA-DR blocking monoclonal antibody L243 abolished the blasts' ability to induce CD4<sup>+</sup> T-cell proliferation, indicating that the increase in proliferation was restricted to HLA-DR expression on these blasts.

From these experiments we conclude that the absence of CLIP on leukemic blasts of AML patients results in increased recognition by autologous remission CD4<sup>+</sup> T cells, which may imply that the relative amount of CLIP is a critical factor for the immunogenicity of leukemic blasts.

## 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 showing that the relative amount of CLIP on leukemic blasts is a strong predictor of disease-free survival (Figure 1A). This underlines our hypothesis that the presence of CLIP on leukemic blasts acts as an important immune escape mechanism in these patients after achieving complete remission. It has already become clear that the use of modulated leukemic blasts as APC is an emerging approach to activate the immune system in AML patients.<sup>27,28</sup> It is, therefore, a challenge to develop immunotherapeutic strategies that down-modulate the relative amount of CLIP on leukemic blasts, resulting in enhanced HLA class II antigen presentation and immune recognition.

Immunophenotypic screening of several human myeloid leukemic cell lines revealed large differences in relative amounts of CLIP (Figure 2A), which is in line with the results from a study performed by Harris *et al.*<sup>29</sup> CLIP<sup>+</sup> KG-1 and ME-1 blasts abundantly expressed HLA-DM while CLIP<sup>+</sup> THP-1 and Kasumi-1 blasts did not (Figure 2A-B), indicating that the catalytic function of HLA-DM is important for HLA-DR-restricted peptide loading in these cell lines. This agrees with the association between CLIP/HLA-DR and HLA-DO/HLA-DM expression that was found previously for B cells,<sup>30</sup> as well as leukemic blasts.<sup>22</sup> We observed low levels of HLA-DO expression in the HLA-DM<sup>low</sup> THP-1 and Kasumi-1 cell lines (Figure 2A), which contrasts with the high levels of expression that were recently demonstrated in two other HLA-DM<sup>low</sup> leukemic cell lines, the HL-60 and K562.<sup>31</sup> The HLA-DM<sup>low</sup>HLA-DO<sup>-</sup> immunophenotype of the THP-1 and Kasumi-1 cells implies an acquired ineffectiveness of blasts to regulate HLA-DR-restricted peptide loading in the MIIC. Our data on the expression patterns for KG-1 (early myeloblast; CLIP<sup>+</sup>HLA-DM<sup>+</sup>HLA-DO<sup>+</sup>), ME-1 (myelomonocyte with eosinophilia; CLIP<sup>+</sup>HLA-DM<sup>+</sup>HLA-DO<sup>+</sup>), THP-1 (monocyte; CLIP<sup>+</sup>HLA-DM<sup>low</sup>HLA-DO<sup>-</sup>) and Kasumi-1 (late myeloblast; CLIP<sup>+</sup>HLA-DM<sup>low</sup>HLA-DO<sup>-</sup>) blasts further indicates a potential relationship with the degree of maturation. We hypothesize that, during differentiation, the ability of myeloid blasts to regulate HLA-DR-restricted peptide loading in the MIIC is impaired and, hence, CLIP expression at the plasma mem-

brane is increased. This contrasts with the proposal by Harris *et al.* that exogenous antigen processing is a developmentally acquired characteristic for myeloid blasts.<sup>29</sup>

Despite the similar immunophenotype of THP-1 and Kasumi-1 blasts (Figure 2), Li silencing had different effects on surface expression of HLA-DR (Figure 3B, D). According to the conventional pathway of HLA class II antigen presentation, Li is required for the transport of HLA class II molecules from the endoplasmic reticulum to the MIIC. In monocytic THP-1 blasts however, we found that upon silencing of Li, HLA-DR molecules were still able to be expressed extracellularly. As Li also prevents binding of endogenous peptides to newly synthesized HLA class II molecules in the endoplasmic reticulum,<sup>32</sup> this might indicate that HLA-DR molecules in THP-1/Li-siRNA blasts are loaded with endogenous peptides, including leukemia-associated peptides, thereby bypassing the Li-dependent routing to the MIIC. This is in agreement with the ability of monocyte-derived dendritic cells to present endogenous antigens to CD4<sup>+</sup> T cells.<sup>33</sup> On the other hand, Kasumi-1/Li-siRNA blasts exhibited strongly reduced levels of extracellular HLA-DR, resulting in a decrease of the blasts' capacity to stimulate allogeneic CD4<sup>+</sup> T cells. Nevertheless, during the Li silencing process, a clear decrease in relative amount of CLIP was observed at day 35 after transduction (Figure 3D), which may allow an altered, although transient, HLA-DR-mediated antigen presentation around these time points. These results may imply a difference in Li dependency of HLA-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 a low relative amount of CLIP on leukemic blasts resulted in increased CD4<sup>+</sup> T-cell induction, both in allogeneic and autologous settings. THP-1/Li-siRNA and Kasumi-1/Li-siRNA blasts (35 days after transduction) were found to strongly induce allogeneic CD4<sup>+</sup> T-cell proliferation (Figure 4A-B), in a manner inversely correlated to their relative amounts of CLIP. These observations are in agreement with those of previous studies, in which it was shown that HLA class II<sup>+</sup> tumor cells lacking Li expression were able to enhance tumor-reactive CD4<sup>+</sup> T-cell activation.<sup>18,19</sup> In MLR performed with CD4<sup>+</sup> T cells from donors 2 and 3, some alloreactivity was observed against THP-1 and Kasumi-1 wild type blasts. The allogeneic CD4<sup>+</sup> T-cell responses to both THP-1/Li-siRNA and Kasumi-1/Li-siRNA blasts at day 35 after transduction were, however, much higher, indicating that these responses were, at least partly, peptide-specific.

Recent studies show that alloreactive T cells can indeed specifically react against HLA-peptide ligands.<sup>34</sup> In addition, CLIP<sup>-</sup> leukemic blasts from AML patients were also able to stimulate proliferation of autologous remission CD4<sup>+</sup> T cells, in contrast to CLIP<sup>+</sup> leukemic blasts (*Online Supplementary Figure S1*). As a low relative amount of CLIP is likely related to increased tumor peptide presentation, this increase in CD4<sup>+</sup> T-cell induction might functionally explain the improved clinical outcome of AML patients with less than 35% of leukemic blasts expressing CLIP (Figure 1).

Taken together, our study demonstrates that CLIP expression on myeloid leukemic blasts affects the blasts' recognition by CD4<sup>+</sup> T cells and could be a mechanism for escaping from immune surveillance. We hypothesize that leukemic



blasts with a HLA-DR<sup>+</sup>CLIP<sup>-</sup> phenotype at their plasma membrane are able to present endogenous antigens by HLA-DR, thereby evoking CD4<sup>+</sup> T-cell induction as observed in our proliferation assays. Since the immunological escape of leukemic blasts is a major obstacle to the development of immunotherapy in AML, molecular interventions that down-modulate the relative amount of CLIP might be used as an additional strategy in AML dendritic cell and whole-cell vaccination protocols to activate leukemia-specific CD4<sup>+</sup> T cells.

## Authorship and Disclosures

MMvL performed experiments, analyzed data and wrote the paper; MEDC analyzed clinical data, performed the statistical analysis and discussed results; JAT and SO provided valuable reagents and discussed results; TMW and YS discussed results; and GJO, SMvH and AAvdL designed the research and discussed results.

The authors declare no competing financial interests.

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