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

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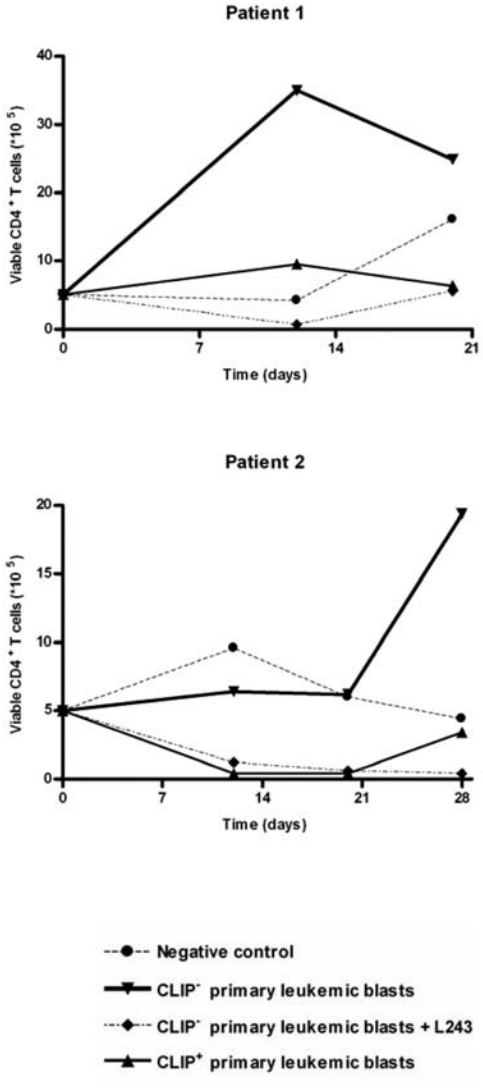
Immunofluoresence stains

With regard to the immunofluorescence stainings, 1×10^5 cells were pre-incubated with 10% human gamma-globulin (60 mg/mL; Sanquin) for 10 min. Mouse monoclonal antibodies were added for 15 min for extracellular staining and for 30 min for intracellular staining of cells. For intracellular staining, cells were fixed with phosphate-buffered saline (PBS)-1% paraformaldehyde and permeabilized using lysing solution (BD) for Ii and PBS-0.1% saponin (Sigma-Aldrich) for both HLA-DM and HLA-DO detection. Intracellular Ii was determined with PIN1.1 followed by a second incubation step of 20 min with rabbit anti-mouse immunoglobulin conjugated to phycoerythrin (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). The myeloid leukemic blasts from patients' samples were defined by $CD45^{dim}/SSC^{low}$ expression. Absolute mean fluorescence intensity (MFI) values were determined by using the median values of all gated cells. We defined CLIP expression as the percentage of $CLIP^+$-stained cells above isotype control staining. To compare the total number of plasma membrane-expressed HLA-DR molecules occupied by CLIP, the relative amount of CLIP was calculated as previously reported,[22] using the following formula:

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

Retrovirus production and transduction

293T cells (obtained from the Harvard Gene Therapy Institute, Boston, MA, USA) were plated in 6-cm dishes at 2.3×10^5 cells/mL in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% FBS and cultured for 20 h at 37 °C. After replacing medium with Iscove's modified Dulbecco's medium consisting of 10% fetal bovine serum (FBS), 293T cells were transfected with 8 μg pSIREN-RetroQ-Ii-siRNA53 plus 6 μg pMD.MLV gag.pol and 2 μg pMD.G using $CaPO_4$. After approximately 16 h, the 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-μm filter (Millipore) and eventually stored at -80°C. Green fluorescent protein (GFP) expression of 293T cells simultaneously transfected with the pEGFP-N1 vector (Clontech, Palo Alto, CA, USA) was monitored with UV light to evaluate the virus titer. For retroviral transduction, 5×10^5 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 μg polybrene and 10 mM Hepes (Sigma-Aldrich). Retroviral supernatant was thawed and added drop-wise to reach a final polybrene concentration of 4 μg/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 μg/mL puromycin for two weeks and then increasing the dose gradually to a final concentration of 1.0 μg/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.



Online Supplementary Figure S1. Proliferation assays of autologous remission CD4$^+$ T cells stimulated with CLIP$^-$-sorted leukemic blasts from AML patients. CLIP$^-$CD45^{dim} and CLIP$^+$CD45^{dim} blasts were flow cytometrically sorted from thawed samples from two different AML patients and used for stimulation of autologous remission CD4$^+$ T cells during co-cultures. CD4$^+$ T cells from patient 1 were re-stimulated twice and CD4$^+$ T cells from patient 2 were re-stimulated three times with either CLIP$^-$- or CLIP$^+$-sorted leukemic blasts at 7 days after each stimulation. Trypan blue dye exclusion was used to determine the number of viable CD4$^+$ T cells. In the negative control situation, no primary leukemic blasts were added.

Online Supplementary Table S1. Patients' characteristics.

	Total	Patients with HLA-DR$^+$CLIP$^-$ blasts	Patients with HLA-DR$^+$CLIP$^+$ 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 (10^9/L)	43 (0-388)	40 (0-280)	50 (1-388)
CR rate, number (%)	160 (77)	99 (72)	42 (85)
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)

HLA-DR+ defined as >45% and CLIP+ defined as >35% of cells positive. WBC: white blood cells; CR: complete remission; FAB: French-American-British; RAEB-t: refractory anemia with excess blasts in transformation. Baseline characteristics were not significantly different between HAL-DR$^+$CLIP$^-$ and HAL-DR$^+$CLIP$^+$ groups of patients (Mann-Whitney U test).