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ORIGINAL ARTICLE

Expression of ERp5 and GRP78 on the membrane of chronic lymphocytic leukemia cells: association with soluble MICA shedding

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Abstract MICA is a ligand of the activating receptor NKG2D, expressed by NK and T cells. MICA expression is induced in cancer cells favoring their elimination by the immune system; however, many advanced tumors shed soluble MICA (sMICA), which impairs NKG2D-mediated cytotoxicity. ERp5 and GRP78 are endoplasmic reticulum-resident proteins that are translocated to the surface of epithelial tumor cells where they interact with MICA and are involved in sMICA shedding. In this study, we analyze the role of ERp5 and GRP78 in sMICA shedding in chronic lymphocytic leukemia (CLL). Immunofluorescence and flow cytometry analyses showed that ERp5 and GRP78 were significantly expressed on the surface of B cells and leukemia cells, but they were not expressed on T cells. The expression of ERp5 and GRP78 was significantly higher in leukemia cells than in B cells from controls. ERp5 and GRP78 co-localized with MICA on the surface of leukemia cells and the levels of expression of ERp5 and GRP78 correlated with the level of expression of membrane-bound MICA in CLL patients. Associated with higher expression of membrane-bound ERp5 and GRP78, serum sMICA levels were approximately threefold higher in patients than in controls. Elevated sMICA levels in CLL patients were associated with the down-modulation of NKG2D surface expression on CD8 T cells. Finally, pharmacological inhibition of B cell lines and stimulated leukemia cells showed that ERp5 activity is involved in sMICA shedding in CLL. In conclusion, these results uncover a molecular mechanism which regulates MICA protein shedding and immune evasion in CLL.

Keywords Chronic lymphocytic leukemia · NKG2D · MICA · ERp5 · GRP78 · NK cells

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Introduction

NKG2D is an activating receptor expressed by NK cells and T cells [1–3]. Expression of NKG2D ligands is restricted under physiological conditions, but they are up-regulated in stressed, infected, and transformed cells, which become susceptible to NK cell-mediated killing [4–10]. Although NKG2D may lead to effective rejection of early stage tumors, advanced cancers develop several mechanisms to evade the NKG2D-mediated response [11–15]. MICA is a tumor-associated ligand of NKG2D receptor that is involved in the elimination of cancer cells; however, advanced cancers frequently counter the antitumor activity of the immune system by shedding soluble MICA (sMICA) [12–15]. This soluble ligand impairs the elimination of

tumor cells by down-regulating NKG2D expression on immune cells and by promoting the evasion of the immune system [12, 13]. Elevated levels of sMICA correlated with advanced stage tumors, metastasis and poor prognosis. sMICA is an independent prognostic factor in multiple myeloma patients [15]. It is also significantly increased in chronic lymphocytic leukemia (CLL) patients and is associated with poor treatment-free survival [16].

Thiol isomerases are endoplasmic reticulum enzymes capable of formation and rearrangement of disulfide-bonding patterns of nascent proteins. Recent studies have suggested additional functions for thiol isomerase enzymes on the surface of cells, where they participate in receptor activation and remodeling, substrate processing and platelet function [17–19]. ERp5 (also called PDIA6 or P5) is a thiol isomerase that has been associated with sMICA shedding in epithelial tumors [20]. Glucose-regulated protein 78 kDa (GRP78, also known as BiP) is a prototype endoplasmic reticulum-resident protein that regulates protein folding by the protein disulfide isomerase (PDI) family of proteins, including ERp5 [21]. ERp5 and GRP78 are translocated to the surface of tumor cells where ERp5 forms a transitory disulfide bond with MICA from which sMICA is released after proteolytic cleavage [20]. ERp5 is expressed on the surface of myeloma cells where it has been associated with sMICA shedding [22].

The clinical relevance of ERp5 expression on the surface of tumor cells is highlighted by the association of anti-ERp5 antibodies with the response to immunotherapy in diverse solid tumors and myeloid leukemia patients [23]. This raises the possibility that ERp5 might serve as a target for therapeutic monoclonal antibodies therapies. In this report, we analyze the expression of ERp5 and GRP78 on the surface of leukemia cells of CLL patients and their association with sMICA shedding.

Materials and methods

Patient and CLL samples

One hundred CLL patients satisfying the diagnostic criteria for CLL and 23 subjects matched for sex and age were analyzed in this study [24]. The median age at diagnosis of the patients was 71.6 years old and 58.9% of patients were male. Patients were either untreated or had not received cytoreductive treatment within 6 months of the investigation. Patients were classified as having stable (39%) or progressive disease (61%), as previously described [25]. Peripheral blood mononuclear cells (PBMCs) were purified from freshly isolated blood obtained from patients and controls by Ficoll-Paque (Pharmacia) density centrifugation. For functional experiments, B cells were further purified using EasySep® Human B Cell Enrichment Kit without CD43 Depletion (Stemcell Technologies). This study was approved by the Ethics Committee of our Institution, and informed consent was obtained from all patients and controls.

Cell lines, cell cultures and cellular cytotoxicity assay

The Burkitt's lymphoma cell line Raji and HT29 cells were obtained from ATCC. The B-lineage-derived cell lines, MEC-I (derived from chronic lymphocytic leukemia patient), Karpas-1718 (splenic lymphoma), SUDHL-6 (follicular lymphoma), and Jeko-I (a mantle cell lymphoma) were provided by Dr. Ocio (Servicio de Hematología, Hospital Universitario y Centro de Investigación del Cáncer, University of Salamanca, Spain). Cell lines and leukemia cells were grown in RPMI-1640. Raji cells were supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine and gentamicin. B-lineage cell lines and leukemia cells were supplemented with 10% human AB serum, 2 mM L-glutamine and gentamicin. Cells were exposed to DMSO, bacitracin, phenylarsine oxide (PAO), or Phorbol-12-myristate-13-acetate (PMA) (Sigma) in the indicated experiments.

Flow cytometry

Each patient's diagnosis of CLL was confirmed by flow cytometry which revealed a typical CD19+, CD20+, CD5+, CD23+, and Ig light chain (κ or λ)-restricted phenotype [26]. The expression of MICA, ERp5, and GRP78 on the surface of freshly isolated leukemia cells was analyzed by flow cytometry. For staining, 10^6 cells were labeled using the following primary antibodies: MICA (6G6), ERp5 (a rabbit polyclonal antibody anti-P5, Affinity Bioreagents), and GRP78/BiP (the rabbit polyclonal antibody GL-19, Sigma). The surface expression of these molecules was also confirmed using the following antibodies: anti-MICA (clone 159227) and anti-MICB (clone 841621) from R&D; anti-MICA (AMO1) from Axxora; anti-ERp5 (SAB1406928) from Sigma; anti-ERp5 (rabbit polyclonal antibody) from Thermo Scientific Pierce Antibodies; and anti-ERp5 (sc-107533) and anti-GRP78 (sc-166490) from Santa Cruz Biotechnology. After washing, cells were stained with a fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Serotec). In the case of primary leukemia samples, cells were washed again and stained with anti-human phycoerythrin-conjugated CD19, anti-human APC-conjugated CD5, and anti-human PerCP-conjugated CD3 antibodies (all from Becton–Dickinson).

NK and T cells were stained with the following antibody conjugates: anti-CD3-FITC, anti-CD4-PerCP, anti-CD8-Pacific Blue, anti-CD56-APC (all from Immunostep), and anti-NKG2D-PE (Miltenyi Biotec). The populations of cells were defined as follows: CD4 T cells were defined as

CD3+CD4+, CD8 T cells were defined as CD3+CD8+, and NK cells were defined as CD3−CD56+. Cells were analyzed in a BD Biosciences FACSCalibur cytometer and data were acquired and analyzed by CellQuest software (Beckton Dickinson).

Confocal microscopy

For ERp5 and GRP78 staining, 10^6 cells from CLL patients were incubated with the anti-ERp5 or anti-GRP78 antibodies at a dilution of 1:200 for 45 min at 4°C. After washing, cells were incubated for 45 min with a Cy3-conjugated anti-rabbit IgG secondary antibody (GE Healthcare). For MICA staining, 10^6 cells were incubated with the antibody to MICA (6G6) for 45 min at 4°C. After washing, cells were incubated for 45 min with a Cy5-conjugated anti-mouse IgG secondary antibody (GE Healthcare). After washing, B and T cells were detected using anti-human FITC-labeled CD3 or CD19 antibodies (Beckton Dickinson). After incubation, the cells were washed again, resuspended in fresh 4% paraformaldehyde. Cells were analyzed using a Leica TCS-SP2-AOBS confocal microscope.

Western blotting

Cells were lysed with 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 50 mM NaF, 1 mM dithiothreitol, complete inhibitor mixture (Roche Applied Science), and phosphatase inhibitor cocktails I and II (Sigma). Cell lysates supernatants were quantified and 15 μg of protein was loaded on 10% SDS-PAGE gels and transferred to nitrocellulose membranes (Amersham). Blots were blocked with 3% nonfat dry milk and incubated overnight at 4°C with the corresponding antibodies against ERp5 (Affinity Bioreagents), GRP78 (Sigma-Aldrich), and β-actin (Santa Cruz Biotechnology). Immunoreactive proteins were visualized using goat anti-mouse-horseradish peroxidase (HRP) secondary antibody (Santa Cruz Biotechnology), goat anti-rabbit-horseradish peroxidase (HRP) secondary antibody (Sigma-Aldrich), and the chemiluminescent detection kit (Millipore).

ELISA for measurement of sMICA

sMICA was measured in the sera of CLL patients and controls by ELISA with the commercial DuoSet ELISA development kit (R&D Systems) following the manufacturer's instructions.

Statistical analysis

The Kolmogorov–Smirnov test was used to evaluate the normality of the data. After testing for Gaussian distribution, continuous variables were compared with Mann–Whitney U test or t Student test. Spearman correlation was used to correlate the level of expression of ERp5, GRP78, and MICA with clinical and laboratory parameters.

Results

ERp5 and GRP78 are expressed on the surface of leukemia cells

The expression of ERp5 and GRP78 on the surface of leukemia cells was initially analyzed by confocal microscopy. ERp5 and GRP78 were expressed on the surface of B cells of CLL patients, but they were not expressed on T cells (Fig. 1a). The specificity of of ERp5 and GRP78 surface expression was confirmed using antibodies which recognize different parts of these proteins and by western blotting analysis (Fig. 1b). B cells from healthy individuals also displayed a significant surface expression of ERp5 and GRP78 (not shown). Surface expression of both molecules was further analyzed in freshly isolated PBMCs obtained from 100 CLL patients and 23 healthy donors by flow cytometry. Leukemia cells from CLL patients (CD19+CD5+) showed a significant surface expression of ERp5 and GRP78, whereas these molecules were not expressed on the surface of T cells (Fig. 1c). A marked protein expression of both molecules was also detected in B cells obtained from healthy individuals and in five B-lineage cell lines analyzed (not shown).

These results clearly indicate that ERp5 and GRP78 are expressed on the surface of cells of B cell origin. Nevertheless, leukemia cells displayed higher mean fluorescence intensities (MFI) of ERp5 (median 82.7 ± 69.4 vs. 16.5 ± 9.3, $P < 0.001$) and GRP78 (median 98.8 ± 76.1 vs. 17 ± 6; $P < 0.001$) than B cells from controls (Fig. 2a). ERp5 surface expression in plasma cells has been associated with progression of multiple myeloma [22]. To analyze whether ERp5 and GRP78 may be associated with progression, CLL patients were classified into progressive (39%) and stable (61%) [25]. ERp5 and GRP78 expressions were significantly higher in progressive than in stable patients (median 112.7 ± 69.2 vs. 63.5 ± 67.5, $P < 0.01$, and 118.2 ± 69.2 vs. 86.8 ± 79, $P < 0.01$, respectively) (Fig. 2a). Of note, there was a significant correlation between the MFI of ERp5 and GRP78 of CLL patients ($r = 0.76$, $P < 0.001$), suggesting a potential association of these molecules on the surface of leukemia cells (Fig. 2b).

Analysis of MICA and sMICA in chronic lymphocytic leukemia

The expression of MICA on the surface of leukemia cells was analyzed by flow cytometry. Leukemia cells had low expression of MICA (ranging from 0 to 39.9 of MFI). No

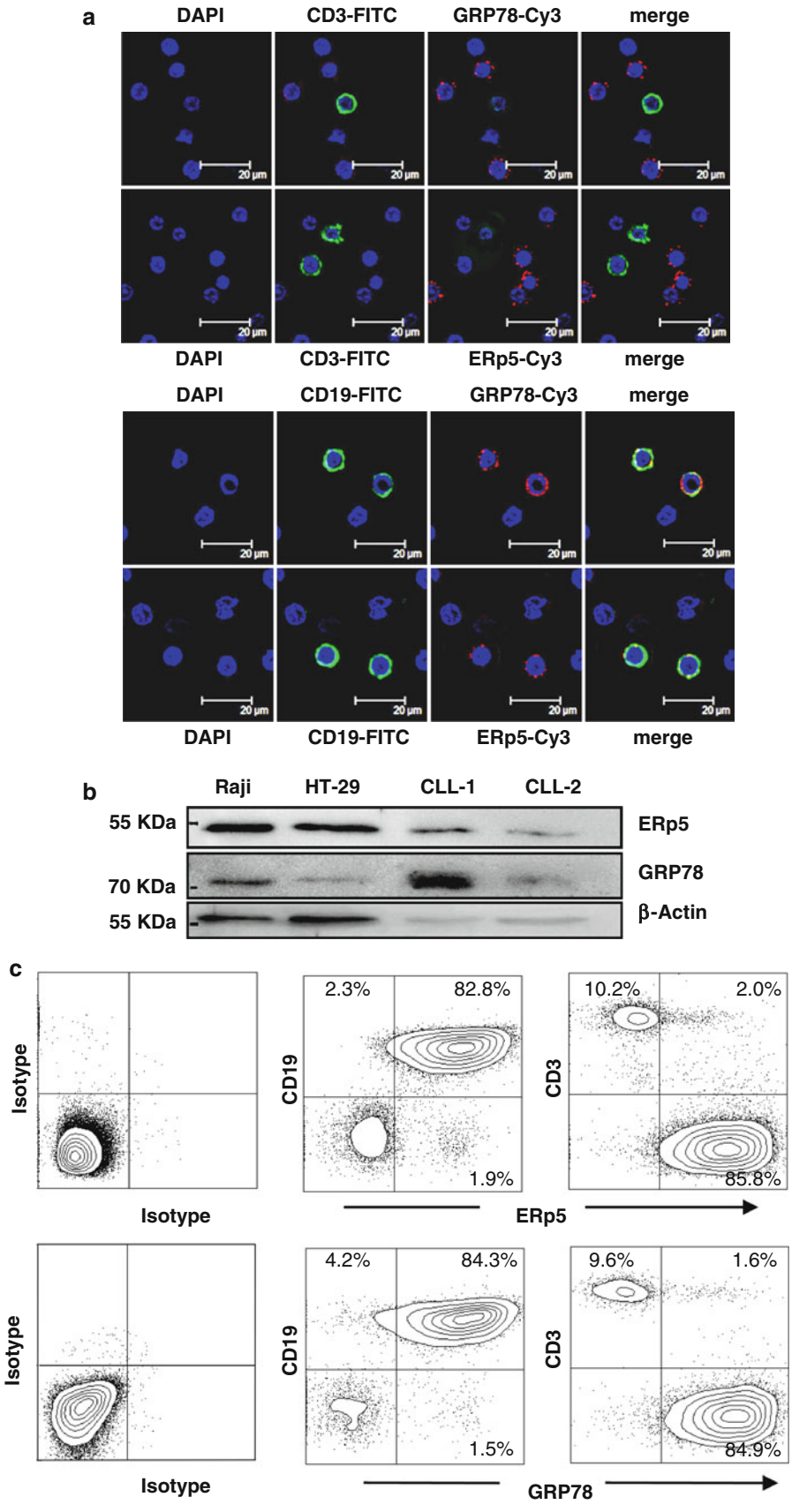

Fig. 1 Analysis of ERp5 and GRP78 expression on the surface of leukemia cells. **a** Confocal microscopy analysis of surface staining of ERp5 and GRP78 with respect to CD3 and CD19. CLL cells were stained with Cy3-labeled ERp5 and GRP78 antibodies combined with FITC-conjugated CD3 and CD19 fluorescent antibodies. Stained cells were analyzed using a Leica TCS-SP2-AOBS confocal microscope. A representative patient is shown. **b** Western blotting analyses were performed to monitor ERp5 and GRP78 expression in HT29 cells, Raji cells and leukemia cells purified from two CLL patients. ERp5 staining corresponded with a major protein band of 50 kDa and GRP78 staining corresponded with a single protein band of 76–78 kDa [20]. B-actin was used as control. **c** Peripheral blood mononuclear cells obtained from CLL patients were stained with fluorescein (FITC)-labeled ERp5 or GRP78 and with anti-CD19, -CD5 and -CD3 antibodies. Flow cytometry analysis of one representative patient is shown

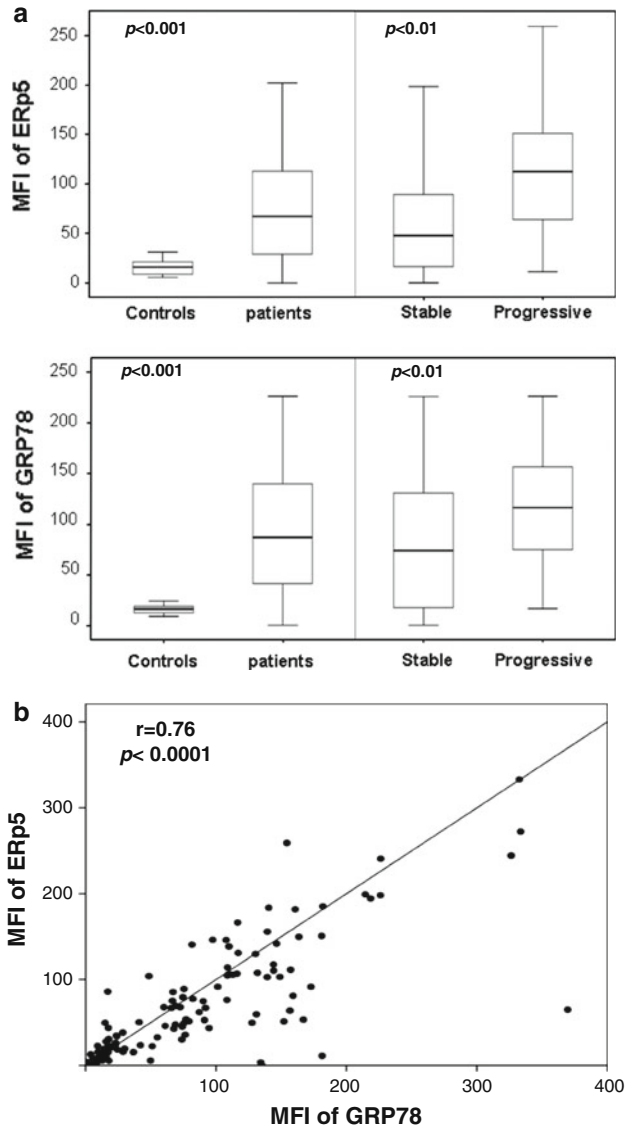

Fig. 2 Surface expression of ERp5 and GRP78 in CLL patients and controls. **a** The figure shows the comparison of the mean fluorescence intensities (MFI) of ERp5 and GRP78 staining on B cells between patients and controls. The expression of ERp5 and GRP78 on B cells was analyzed by flow cytometry. Patients were also stratified by stable (39%) or progressive disease (61%) [25]. **b** Correlation between the MFI of ERp5 and GRP78 staining on leukemia cells of CLL patients. The correlation between the MFI of ERp5 and GRP78 was analyzed by the Spearman's correlation test

significant expression of MICB was detected. No significant expression of MICA and MICB was detected on B cells from controls (Table 1). Sera of healthy donors contained a median of 9 pg/mL sMICA which was close to the detection limit of the ELISA assay, but CLL patients exhibited a broad range of sMICA levels (0–553.5 pg/mL). The median of sMICA levels of CLL patients was approximately threefold higher than controls (29.6 vs. 9 pg/mL; $P = 0.03$).

To address the functional consequence of sMICA release, the expression of NKG2D receptor on NK and CD8

Table 1 Surface expression of MICA and serum levels of sMICA in CLL patients and controls

	Controls ($n = 23$)		Patients ($n = 100$)		P
	Median	95% CI	Median	95% CI	
MICA (MFI)	0	0–0	5.9	4.6–7.2	<0.001
sMICA (pg/mL)	8	0–15	27.7	13.6–41.8	0.03

Expression of MICA on B cells (stained with CD19) was analyzed by flow cytometry. The mean fluorescence intensities (MFI) of MICA staining in patients and controls are shown. Serum sMICA was analyzed by ELISA

T cells was analyzed by flow cytometry in CLL patients who released significant amounts of sMICA (Fig. 3a). NKG2D surface expression on CD8 T cells showed a negative correlation with serum sMICA levels ($r = -0.47$, $P = 0.05$) (Fig. 3b). No significant correlation was observed between NKG2D expression on NK cells and sMICA levels (not shown). It is also noteworthy that serum sMICA levels correlated with the number of CD8 T cells ($r = 0.29$; $P < 0.01$), CD4 T cells ($r = 0.22$; $P < 0.05$), and NK cells ($r = 0.25$; $P < 0.01$) (Fig. 3c). All data together suggest that sMICA may have a significant immunomodulatory role in CLL.

Functional association of ERp5 and GRP78 with sMICA shedding in CLL

Flow cytometry analyses of leukemia cells of CLL patients showed a correlation of the expression of MICA with both ERp5 ($r = 0.26$, $P < 0.01$) and GRP78 ($r = 0.21$, $P = 0.03$), suggesting a potential association of these molecules on the surface of leukemia cells (Fig. 4a). To further explore the functional relationship between ERp5, GRP78, and MICA, the co-localization of these molecules on the surface of CLL cells was analyzed by confocal microscopy. MICA staining on the surface of leukemia cells co-localized with ERp5 or GRP78 staining (Fig. 4b).

Bacitracin is a strong inhibitor of PDI activity of CLL cells, causing a dramatic increase in surface protein thiol expression [18]. The potential role of ERp5 in sMICA shedding was further analyzed by inhibition of ERp5 activity. Thus, treatment of five B-lineage cell lines with a non-toxic dose of bacitracin (1 mM) for 72 h significantly inhibited the shedding of sMICA (ranging from 25 to 75%) (Fig. 5). Of note, treatment of MEC-I, a CLL-derived cell line, resulted in an inhibition of 75%.

Next, purified leukemia cells obtained from seven CLL patients, who showed variable levels of sMICA in their sera, were cultured in the presence of bacitracin. As reported, we could not detect sMICA in the supernatant of cultured leukemia cells obtained from any of the seven patients [16]. Thus, we analyzed whether B cell stimulation may increase the release of sMICA in primary leukemia cells. Treatment of

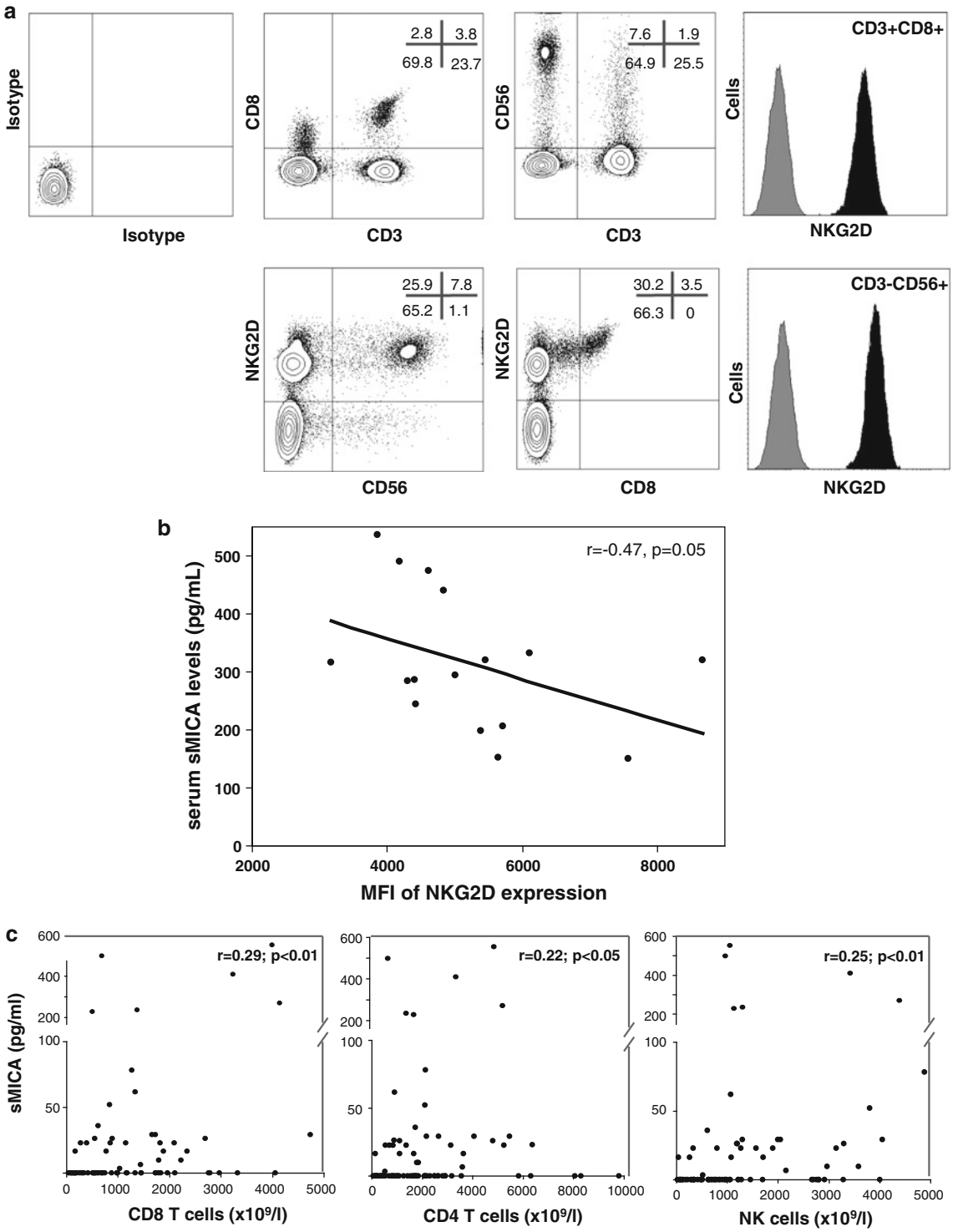

Fig. 3 Correlation between serum sMICA levels and NKG2D expression. **a** NKG2D surface expression on CD8 T cells (CD3+CD8+) and NK cells (CD3−CD56+) was analyzed in CLL patients by flow cytometry. One representative patient is shown. **b** Correlation between the mean fluorescence intensity (MFI) of NKG2D staining on CD8 T cells of CLL patients and serum sMICA levels. **c** Correlation between the number of CD8 T cells, CD4 T cells (CD3+CD4+) and NK cells of CLL patients and serum sMICA levels. The correlations were analyzed by the Spearman's correlation test

leukemia cells obtained from five patients, who expressed low levels of sMICA in their sera, with PMA led to an increase of surface MICA expression (2.5-fold) and a marked increase of sMICA levels (Fig. 6a, b). Further, treatment of PMA-stimulated leukemia cells with bacitracin resulted in a decrease of 81% of sMICA levels and a concomitant increase of MICA

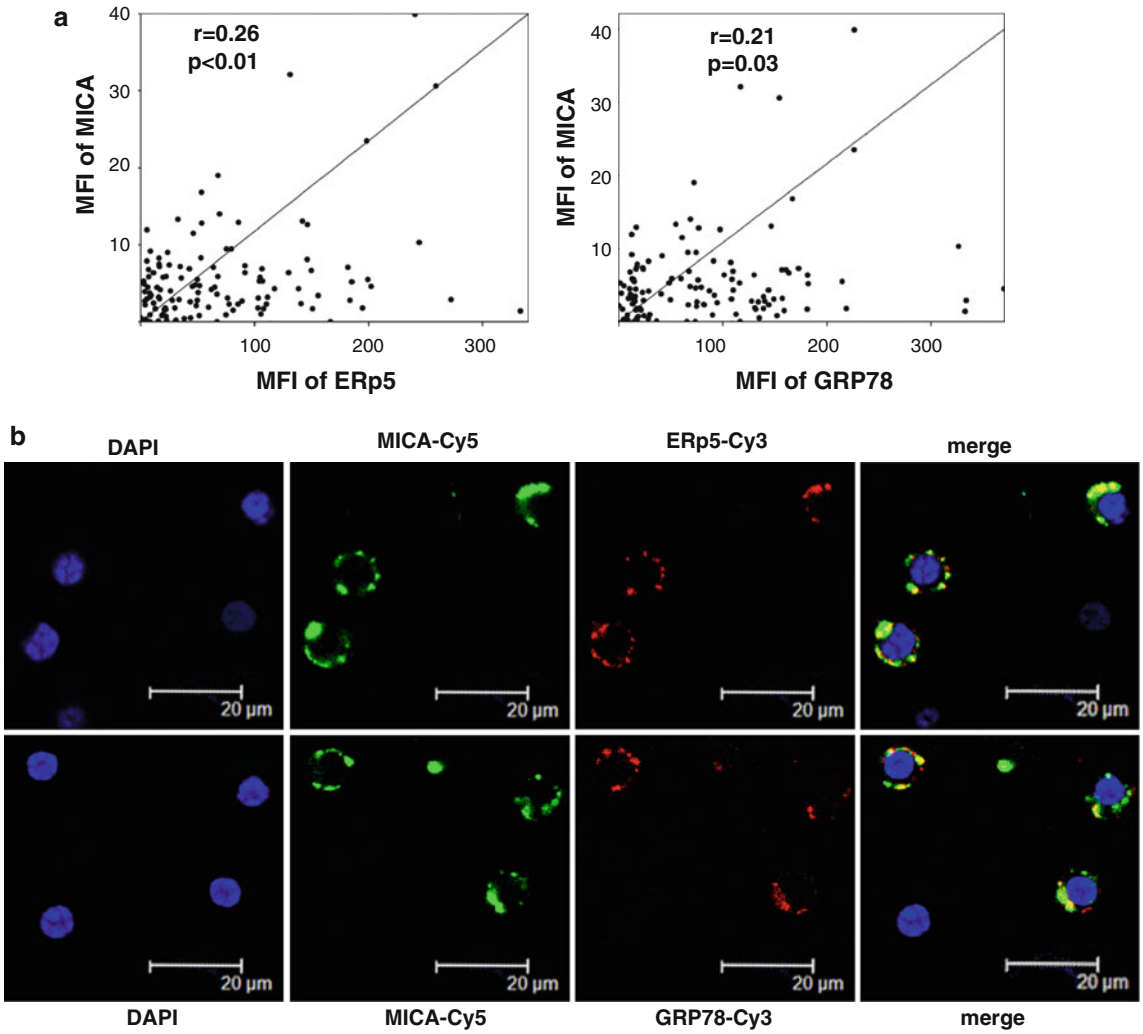

Fig. 4 Correlation between the surface expression of ERp5, GRP78 and MICA on leukemia cells of CLL patients. **a** ERp5, GRP78 and MICA expression was analyzed by flow cytometry. The correlation between the mean fluorescent intensities (MFI) of MICA, ERp5 and GRP78 staining was analyzed by the Spearman's correlation test. **b** Co-localization of the expression of MICA and ERp5 and GRP78 on the surface of leukemia cells. Leukemia cells obtained from a CLL patient were stained with Cy3-labeled anti-ERp5 or anti-GRP78 antibodies and Cy5-labeled anti-MICA antibodies and were analyzed using a Leica TCS-SP2-AOBS confocal microscope. MICA staining (*red*) was observed on the surface of leukemia cells and appeared completely yellow when merged with ERp5 or GRP78 staining. A representative patient is shown

surface expression (Fig. 6b). Treatment of leukemia cells with non-toxic doses of PAO, another inhibitor of ERp5 enzymatic activity, significantly reduced the levels of sMICA shedded from PMA-stimulated leukemia cells (~66%) (Fig. 6c, d). All data together indicate that ERp5 may be functionally involved in the shedding of sMICA in CLL.

Discussion

Thiol isomerases have been found on the surface of several cell types, which suggest a wider role for these enzymes in cell function [17–19]. In this study, we show for the first time that ERp5 and GRP78 are expressed on the surface of normal B cells and primary leukemia cells. The expression of ERp5 and GRP78 was higher in leukemia cells than in B cells obtained from healthy individuals, and it was also higher in progressive than in stable CLL patients. These data are in agreement with earlier studies, which also showed that CLL cells expressed up to tenfold higher PDI activity than controls [18]. Surface ERp5 expression was also higher in multiple myeloma patients than in patients with monoclonal gammopathy of undetermined significance [22]. These data and the association of cytoplasmic ERp5 and GRP78 with tumor proliferation, survival, invasion, metastasis, and resistance to a wide variety of therapies suggest that the activity of ERp5 and GRP78 could be associated with transformation in CLL.

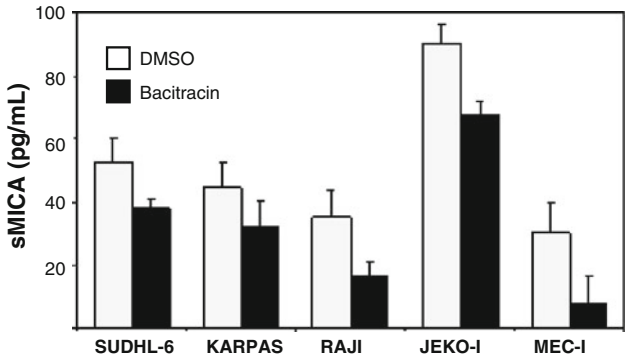

Fig. 5 Pharmacological inhibition of ERp5 significantly reduced sMICA shedding in B-lineage cell lines. B-lineage cell lines were treated with 1 mM bacitracin (*black bars*) or DMSO (*white bars*) for 72 h and sMICA was measured in the supernatant of the cultured cells by ELISA. The average and SD of three different experiments are shown

The well-known functions of ERp5 and GRP78 in the regulation of the protein folding and formation of disulfide bonds support the hypothesis that membrane-bound ERp5 and GRP78 in leukemia cells may also regulate protein folding. However, the potential ligands and functions of membrane-bound ERp5 and GRP78 in B cells and leukemia cells have not yet been analyzed, and further studies are clearly warranted. Nevertheless, we previously showed that ERp5 forms a transitory disulfide bond with MICA on the surface of epithelial tumor cells from which sMICA is released after proteolytic cleavage [20]. In this study, we observed that the levels of sMICA were approximately threefold higher in patients than in controls. Further, the expression of MICA on the surface of leukemia cells significantly correlated with the expression of membrane-bound ERp5 and GRP78 and both molecules co-localized with MICA on the surface of leukemia cells. We also show that the inhibition of ERp5 activity in several B-lineage cell lines and in stimulated primary leukemia cells significantly decreased sMICA shedding. These results clearly suggest that ERp5 and GRP78 are involved in sMICA shedding in CLL cells. The fact that PAO impairs ERp5 function by interacting with its catalytic sites suggests that ERp5 catalytic activity is required for sMICA shedding [20].

NKG2D plays an important role in the immune surveillance of cancer. In advanced tumors, sMICA promotes the evasion of the immune system and immunosuppression [11–15]. In fact, the down-modulation of NKG2D expression and the impairment of NKG2D-mediated immune response have been described in tumors releasing substantial amounts of sMICA [12, 13, 27]. Our experiments suggest that a functional consequence of sMICA release in CLL patients may be the down-modulation of NKG2D expression on CD8 T cells, which provides a potential underlying mechanism responsible for the association of

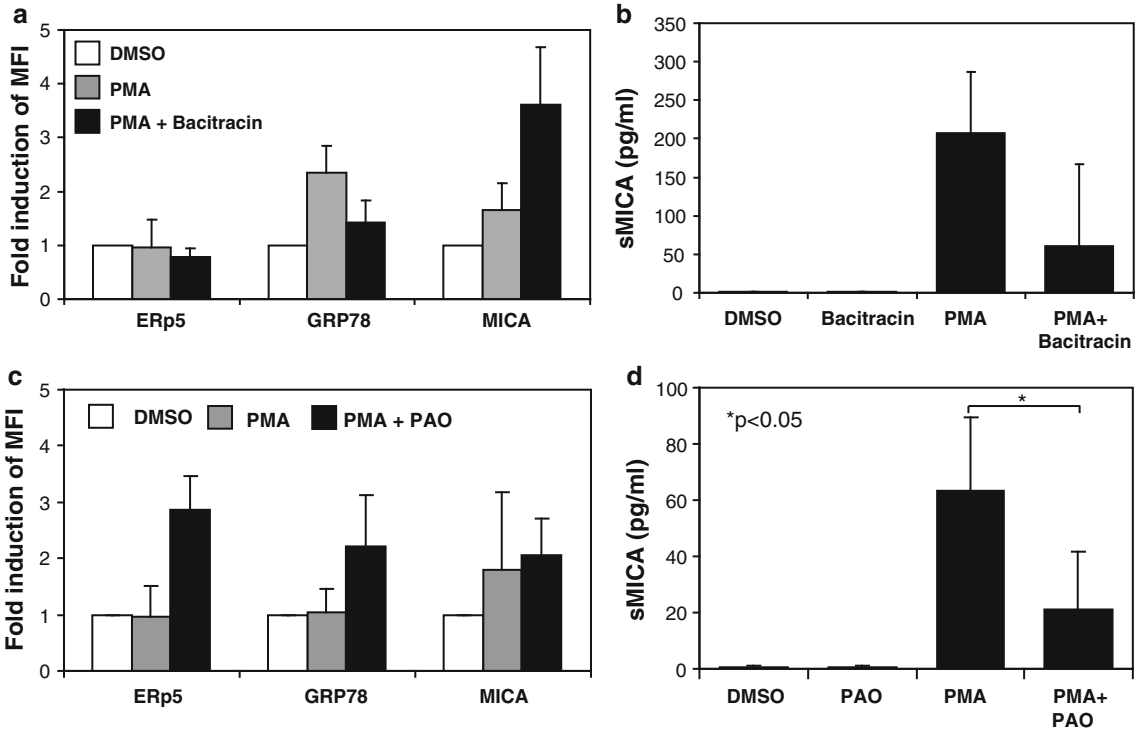

Fig. 6 Pharmacological inhibition of ERp5 significantly reduced sMICA shedding in stimulated leukemia cells. Freshly isolated leukemia cells obtained from CLL patients were treated with DMSO (*white bars*) or 10 ng/mL of PMA in absence (*gray bars*) or in presence of 1 mM of bacitracin or 10 μM PAO (*black bars*). The surface expression of ERp5, GRP78 and MICA was analyzed by flow cytometry at 48 h (**a**, **c**) and sMICA was measured in the supernatant at 72 h (**b**, **d**)

sMICA levels with poor prognosis in CLL patients [16]. Furthermore, a slight correlation between serum sMICA levels and the number of T cells and NK cells of CLL patients was observed, which may suggest a potential additional effect of sMICA on survival or proliferation of immune cells.

Several proteases have been involved in sMICA shedding in different types of tumors, but little is known about the potential proteases involved in CLL [28–30]. Nevertheless, the involvement of ERp5 in sMICA shedding in this disease and other types of tumors may have important clinical relevance. Thus, sMICA is associated with tumor progression in many cancer types and neutralizing anti-MICA antibodies are associated with beneficial effects on the innate and adaptive immune responses [22]. Additionally, anti-ERp5 antibodies are induced by diverse solid and myeloid leukemia patients who responded to GM-CSF-secreting tumor vaccines. These antibodies were associated with the response to the immunotherapy [23]. This raises the possibility that ERp5 could serve as a target for therapeutic monoclonal antibodies in CLL.

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Conflict of interest The authors declare that they have no conflict of interest.

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