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ORIGINAL ARTICLE

Cytotoxic activity of gemtuzumab ozogamicin (Mylotarg) in acute myeloid leukemia correlates with the expression of protein kinase Syk

L Balaian and ED Ball

Blood and Marrow Transplantation Division, Department of Medicine and Moores UCSD Cancer Center, University of California, San Diego, La Jolla, CA, USA

Acute myeloid leukemia (AML) cells express the cell surface antigen CD33 that, upon ligation with a monoclonal antibody (mAb), is a downregulator of cell growth in a Syk-dependent manner. An anti-CD33 mAb coupled to a toxin, gemtuzumab ozogamicin (GO), is used for the treatment of AML (Mylotarg). Therefore, we investigated whether the response of AML cells to GO treatment also depends on Syk expression. Forty primary AML samples (25 Syk-positive and 15 Syk-negative) were tested for their response to the anti-proliferative effects of GO and unmodified anti-CD33 mAb. A correlation between Syk expression and the response of leukemia cells to GO and anti-CD33 mAb was found. 'Blocking' of Syk by small interfering RNA resulted in unresponsiveness of AML cells to both GO and anti-CD33 mAb-mediated cytotoxicity. Syk upregulation by the demethylating agent 5-azacytidine (5-aza) induced re-expression of Syk in some cases, resulting in enhanced GO and anti-CD33-mediated inhibition of leukemia cell growth. Thus, the cytotoxicity of both GO and anti-CD33 in primary AML samples was associated with Syk expression. 5-Aza restored Syk and increased the sensitivity of originally Syk-negative, non-responsive cells to CD33 ligation to levels of Syk-positive cells. These data have clinical significance for predicting response to GO and designing clinical trials.
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Introduction

CD33 is a cell surface glycoprotein specifically expressed on myeloid cells including myeloid leukemia cells.[1] Monoclonal antibodies (mAbs) against CD33 have been used in the diagnosis and therapy of acute myeloid leukemia (AML) for many years.[2] CD33, a member of the siglec family, is engaged in sialic acid-dependent cell interactions and adhesion of myeloid cells.[3] The cytoplasmic tail of CD33 contains two immune tyrosine-based inhibitory motifs (ITIMs) and therefore, may serve as a potential inhibitory receptor.[4–6] Engagement of CD33 induced apoptosis and inhibition of proliferation in leukemia cells from AML and chronic myeloid leukemia patients.[7–10] However, little is known about the molecular mechanisms of the CD33 signaling events leading to inhibition of cell growth and apoptosis.

The toxin (calicheamycin)-conjugated anti-CD33 mAb gemtuzumab ozogamicin (GO) is now established as a useful component in the therapy of AML.[11] GO induces remissions in about 30% of patients with relapsed AML. GO, a humanized immunoglobulin G (IgG) 4 mAb, contains human sequences whereas the complementarity-determining regions are derived from a murine antibody that binds CD33. The antibody is linked to *N*-acetyl-gamma calicheamycin via a bifunctional linker and is 50% loaded with 4–6 mol of calicheamycin per mole of antibody. Notably, the remaining 50% of the antibody is not linked to calicheamycin. Therefore, we examined whether the effects of GO depend on CD33-coupled molecules such as Syk. As GO is effective in only a minority of patients, the signaling activity of CD33 may be relevant to the responses achieved, in addition to the effect of the toxin calicheamycin.

The protein kinase Syk is an essential element in many antigen receptor (B-cell receptor, T-cell receptor, Fc receptors) downstream signaling cascades, resulting in cell responses such as adhesion, phagocytosis, proliferation and differentiation.[12–17] During early stages of antigen ligation, Syk binds to the receptor, becomes activated and phosphorylated and then phosphorylates specific substrates such as phospholipase C-gamma with consecutive calcium influx.[18] Late signaling events such as the activation of transcriptional factor nuclear factor-kappa B couple Syk activation with cell proliferation and differentiation.[19]

In myeloid cells, Syk involvement in proximal signaling mediated by activated Fc receptor family members containing the tyrosine-based activation motif (ITAM) is well documented.[19–23] However, recent discoveries revealed that Syk is also involved in signaling of the ITIM-bearing CD22 receptor in B cells.[24–27] We extended these findings by demonstrating that Syk (or ZAP-70) play an important role in CD33 signaling.[28,29] Upon CD33 ligation, Syk becomes phosphorylated and creates complexes with phosphorylated forms of the CD33 molecule itself and protein phosphatase Src homology phosphatase-1 (SHP-1). Moreover, we showed that the anti-proliferative response of AML cells to CD33 ligation correlates with the level of Syk expression. Thirty percent of primary AML samples demonstrate no detectable Syk expression. Significantly greater numbers of Syk/Zap-70-positive samples respond to anti-CD33 mAb treatment.[29]

Recent discoveries established Syk as a tumor suppressor and linked deficient Syk expression to a variety of human hematopoietic[30–33] and solid tumors.[33–35] In breast cancer, Syk kinase is a potent modulator of malignant growth and a potential tumor suppressor, presumably by controlling cell division.[33] Moreover, it was demonstrated that loss of Syk expression in breast cancer and T-cell acute lymphoblastic leukemia cells occurs at the transcriptional level, and is a result of DNA hypermethylation.[31,36,37] Treatment of these cells with 5-azacytidine (5-aza), a methylation inhibitor, restored Syk expression and function.[36,37]

Correspondence: Dr ED Ball, Blood and Marrow Transplantation Division, Department of Medicine and Moores UCSD Cancer Center, University of California, San Diego, 3855 Health Sciences Drive, La Jolla, CA 92093-0960, USA.
E-mail: tball@ucsd.edu
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5-Aza is a nonspecific Syk DNA methyltransferase inhibitor that is used for the treatment of myelodysplasia[38] and possibly AML.[39,40] However, the effects of 5-aza on leukemia cell growth are not well defined. As expression levels of Syk are likely to play an important role for the competency of the immune system and may also play a role in oncogenesis, we examined a panel of 40 primary AML samples for possible correlations between Syk expression and their response to the anti-proliferative effects of unmodified or immunotoxin-bound anti-CD33 mAb.

Materials and methods

Antibodies

The anti-CD33 mAb was obtained from Medarex Inc. (Princeton, NJ, USA). Anti-Syk, anti-SHP-1, anti-CD33 rabbit or goat polyclonal antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The anti-CD13 mAb was purchased from Cell Sciences Inc. (Norwood, MA, USA). A horseradish peroxidase-conjugated anti-phosphotyrosine mAb, 4G10, was obtained from Upstate Biotechnology Inc. (Lake Placid, NY, USA).

Materials

All chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Cells

The human AML cell line Hodgkin's lymphoma (HL)-60 was obtained from ATCC (Manassas, VA, USA). Cells from AML patients were collected from peripheral blood after informed consent under the auspices of the University of California San Diego Institutional Review Board. Mononuclear cells were isolated on a Ficoll-Hypaque gradient and cultured under standard conditions in Rosewell Park Memorial Institute medium (RPMI)-1640 containing 10% fetal calf serum (FCS) and 100 ng/ml of granulocyte-monocyte colony-stimulating factor.

Proliferation assay

AML cells were cultured in triplicate wells in 96-well round bottom plates, with or without various amounts of anti-CD33 or control anti-CD13 mAb in a final volume of 200 μl of RPMI-10 at 37°C in humidified 5% CO_2 in air, at 10^5 cells per well. The cultures were pulsed with 1 microCi [^{3}H]-thymidine per well during the last 3 h of the 48 h culture for cell lines. Primary AML cells were pulsed during the last 16 h of culture. The amount of ^{3}H-thymidine incorporated into acid-precipitable DNA was assessed via liquid scintillation counting.

Flow cytometry analysis

Leukemia cells were washed and then suspended in staining media (SM), containing RPMI-1640, 3% FCS, 0.01% NaN_3 and 1 μg/ml propidium iodide (Calbiochem, La Jolla, CA, USA), plus saturating amounts of fluorescein isothiocyanate-conjugated anti-CD33, anti-CD64 mAbs, or an isotype-matched control mAb of irrelevant specificity. After 30 min at 4°C, the cells were washed in SM twice and then analyzed on a FACScan (Becton Dickinson, San Jose, CA, USA). Dead cells and debris were excluded from analysis by characteristic forward and side-scatter profiles and propidium iodide staining. The immunophenotype of primary AML peripheral blood mononuclear cells was determined by flow cytometry. CD2, CD3, CD7, CD10, CD13, CD14, CD15, CD16, CD19, CD20, CD33, CD34, CD64, CD95, human leukocyte antigen-DR, TdT expression were analyzed. All antibodies were obtained from BD Pharmingen (San Jose, CA, USA).

Cell transfection

SMARTpool small interfering RNAs (siRNAs) (collection of at least four individual siRNA, catalog No. 60–047) that target human Syk (Gen Bank Accession No. NM_003177) as well as control RNA (nonspecific pool) were purchased at Upstate Biotechnology Inc. (Lake Placid, NY, USA). Leukemia cells were transfected according to the manufacturer's instructions. Briefly, cells (3–4 $\times 10^6$) were transfected by 5 μg of Syk siRNA or control naked siRNA mix using 6 μl Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Seventy-two hours after transfection, the cells were used for proliferation assays and Western blot analysis.

Cell activation and immunoprecipitation

Mononuclear cells from AML patients or AML cell lines (5–6 $\times 10^6$) were activated by 10 μg/ml anti-CD33 or anti-CD13 mAb of similar isotype for 20 min at room temperature, followed by addition of polyclonal anti-mouse IgG at 20 μg/ml for variable times (1–60 min). The reaction was stopped by adding ice-cold phosphate-buffered saline (PBS). After three washes in ice-cold PBS, the cells were lysed in lysis buffer, containing 1% (v/v) Triton X-100, 0.15 M NaCl, 50 mM Tris-HCl (pH = 7.2), 0,1% sodium dodecyl sulfate (SDS), 1 mM Na-orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1% (v/v) ethylenediaminetetraacetic acid, 1% (v/v) Aprotinin and 0.03 mM Leupeptin. After 30 min on ice, the nuclear debris was removed by centrifugation for 15 min at 13 000 $\times$ g. Lysates were equalized with respect to the amount of protein as assessed by optical density at 280 nM. Specific immunoprecipitation was performed for 2 h to overnight in the presence of 30% (vol./vol.) 'Protein A/G', conjugated with agarose (Santa Cruz Biotech., Santa Cruz, CA, USA). Immunoprecipitates were washed three times in lysis buffer and then suspended in equal volume of Laemli sample buffer for SDS-polyacrylamide gel electrophoresis (PAGE).

SDS-PAGE and Western Blotting

Total cell lysates or immunoprecipitates were added to separate wells (8 μg/well) of SDS-PAGE (7.5–10% acrylamide) gel, electrophoretically size-separated under reducing conditions, and then transferred onto nitrocellulose for immunoblotting. The filters were first incubated for 1 h in 5% non-fat dry milk in PBS-T (PBS plus 0.01% Tween 20), and then incubated with the primary antibody for 2 h. After washing in PBS-T, the filters were incubated for 1 h in horseradish peroxidase-conjugated matching secondary antibodies. The filters were then washed in PBS-T, incubated with the enhanced chemoluminescence detection reagents (Pierce, Rockford, IL, USA), and exposed to X-ray film (Fuji Film, Fischer, Tustin, CA, USA). In all figures, samples were run in parallel gels.

Statistical analyses

S.e.m. calculation, graph production and statistical evaluation were performed using Sigma-Plot 8.0 version (Systat Software Inc., Point Richmond, CA, USA) and Microsoft Excel (Microsoft, Seattle, WA, USA). Statistical significance of the difference

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t-test and was defined as a *P*-value less than or equal to 0.05. The significance of observed differences in proportions was tested using the χ^2 test and was defined as a $P \leqslant 0.05$.

Results

The growth inhibitory effects of anti-CD33 mAb: correlation with Syk expression

The cell surface CD33 receptor on myeloid leukemia cells functions as a negative regulator of cell growth. Anti-CD33 mAb-mediated growth arrest occurs in a dose-dependent manner, at concentrations greater than 0.1 μg/ml.[28,29] At optimal concentrations (0.1 μg/ml), anti-CD33 mAb inhibited colony formation of HL-60 cells by >50% and DNA synthesis in primary AML cells up to 40%. We previously found that CD33 ligation induced tyrosine phosphorylation of the tyrosine kinase Syk, but not of src-family tyrosine kinases (Fyn, Lyn).[28,29]

As these results suggested an important role of Syk in CD33 signaling, we tested a panel of 40 primary AML samples from different French-American-British (FAB) types for Syk expression (Table 1). All samples had high blast counts (data not shown), and the majority (36 of 40) contained high percentages (>60%) of surface CD33. By Western blotting (data not shown), Syk expression was undetectable in about 37% (15 of 40) of primary AML samples. We did not detect any correlation between Syk expression and FAB type or CD33 expression.

We previously reported that anti-CD33 mAb induced dose-dependent growth inhibition more effectively in primary AML cells that expressed Syk.[29] Here, in order to amplify the representative groups, a panel of 40 primary AML samples was tested for their response to the optimal concentration (0.1 μg/ml) of anti-CD33 mAb (Figure 1a). The level of inhibition in Syk-positive samples was considerably higher (mean value >35%) than in Syk-negative samples (mean value <20%). This difference was statistically significant ($P<0.05$; Student's *t*-test). Moreover, among Syk-positive samples ($n=25$), 68% demonstrated significant growth inhibition in response to CD33 ligation (responders, as defined by >25% inhibition of ^{3}H-thymidine uptake by anti-CD33 mAb) whereas 32% had no significant response (non-responders, defined by <25% growth inhibition) (Figure 1b). In contrast, only 20% of the Syk-negative

Table 1 Syk expression on primary AML samples

FAB	*Total no. of samples*	*CD33 expression (median) (%)*	*No. of Syk-positive samples*	*No. of Syk-negative samples*
MO	1	92	1	0
M1	5	81	2	3
M2	7	85	5	2
M3	1	73	1	0
M4	10	86	5	5
M5	9	94	5	4
M6	2	85	2	0
M7	1	92	1	0
Unknown	4	83	3	1

Abbreviations: AML, acute myeloid leukemia; FAB, French-American-British.

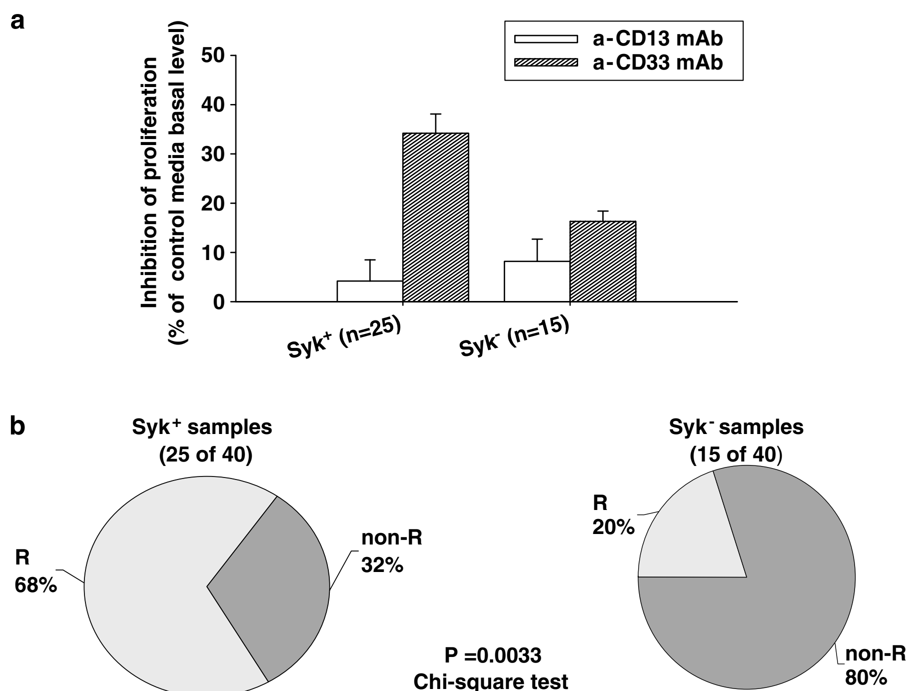

Figure 1 Correlation between Syk expression and the anti-proliferative effect of CD33 ligation in primary AML cells. (**a**) Proliferation assay. Primary AML cells were cultured for 48 h in the presence or absence of 0.1 μg/ml of anti-CD33 mAb or control anti-CD13 mAb. ^{3}H-thymidine incorporation was measured during the last 18 h of culture. Basal proliferation in the absence of antibody (c.p.m.>5500 c.p.m.) was considered 0, and results are expressed as % change for each condition. Samples were grouped according to the expression of Syk/Zap70 and their response to CD33 ligation. Responders (R) are defined as samples with >25% inhibition of ^{3}H-thymidine uptake in the presence of anti-CD33 mAb, whereas non-responders (NR) are defined as samples with <25% inhibition. Error bars indicate the s.e.m. (**b**) Correlation between Syk expression and the

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samples ($n=15$) responded to anti-CD33 mAb. These results suggest a correlation between Syk expression and responsiveness of AML cells to CD33 ligation ($P=0.0033$ by χ^2 test).

The growth inhibitory effects of GO (immunotoxin-conjugated anti-CD33): correlation with Syk expression

As Syk$^+$ AML cells were more likely to respond to CD33 ligation with growth inhibition compared to Syk$^-$ AML samples, we asked whether the anti-proliferative effect of GO might also depend on the presence of Syk. GO inhibited DNA synthesis in a dose-dependent manner in both Syk-positive and Syk-negative samples, but the level of inhibition was significantly higher in the Syk$^+$ compared to Syk samples at all concentrations tested ($P<0.003$, Student's *t*-test) (Figure 2a). The difference was more prominent at low doses of GO and diminished at higher doses, probably owing to free toxin activity.

Response to GO was defined as >50% inhibition of ^{3}H-thymidine uptake at 10 ng/ml, whereas non-responders demonstrated <50% inhibition at this concentration. In Syk-positive AML samples, the number of responsive samples was significantly higher (73%) compared to Syk-negative samples (17%) (Figure 2b). These data show a correlation between Syk expression in primary AML cells and the inhibitory activity of GO ($P=0.02$; χ^2 test).

siRNA-mediated silencing of Syk expression largely prevents the anti-proliferative response of AML cells to anti-CD33 mAb and GO

To determine whether Syk expression was required for CD33-mediated inhibition of DNA synthesis, we used a siRNA strategy to downregulate Syk expression. We transfected HL-60 cells with Syk-specific siRNA or control siRNA using Lipofectamine 2000 (Figure 3). Seventy-two hours post-transfection, Syk was undetectable by Western blotting in cells treated with the Syk-siRNA, the level of SHP-1 was unchanged in these cells, demonstrating specificity of the effect (Figure 3c). Downregulation of Syk expression largely prevented the anti-proliferative effects of the naked anti-CD33 mAb (Figure 3a) as well as the immunotoxin-conjugated mAb GO (Figure 3b). Thus, the Syk-specific siRNA converted initially Syk-positive and CD33-responsive AML cells into Syk-negative non-responsive cells. These results show that Syk plays a central role in CD33 signaling.

5-Aza treatment of Syk-negative primary AML cells increases their response to GO and anti-CD33 mAb

Syk is an important tyrosine kinase, which appears to function as a tumor suppressor that is silenced by hypermethylation in many cancer cells. As we found a correlation between Syk expression and the response of primary AML samples to GO and anti-CD33 m Ab, the DNA methylase inhibitor 5-aza was used in a panel of 40 primary AML samples alone (Figure 4) and in combination with GO or anti-CD33 mAb (Figure 5).

First, we analyzed dose- and time-dependent anti-proliferative effects of 5-aza on human leukemia cell line HL-60 (Figure 4a). Treatment conditions: dose of 100 nM for 48 h was considered as suboptimal, and was used in consequent experiments with primary AML cells. Based on the level of inhibition mediated by 5-aza, we distinguished three groups of primary AML samples (Figure 4b). The majority (21 of 40 samples) demonstrated low response (inhibition of proliferation <25%). In nine of 40 samples, 5-aza treatment induced medium (25–50% inhibition) and in 10 of 40 samples this treatment mediated high (>50% inhibition) responses. Response to 5-aza was defined as >25% inhibition of ^{3}H-thymidine uptake at 100 nM after 48 h treatment, whereas non-responders demonstrated <25% inhibition at these treatment conditions. In Syk-positive AML samples, the number of responsive samples was significantly higher (60%) compared to Syk-negative samples (26%) (Figure 4c). These data suggest a correlation between Syk expression in primary AML cells and the inhibitory activity of 5-aza ($P=0.04$; χ^2 test).

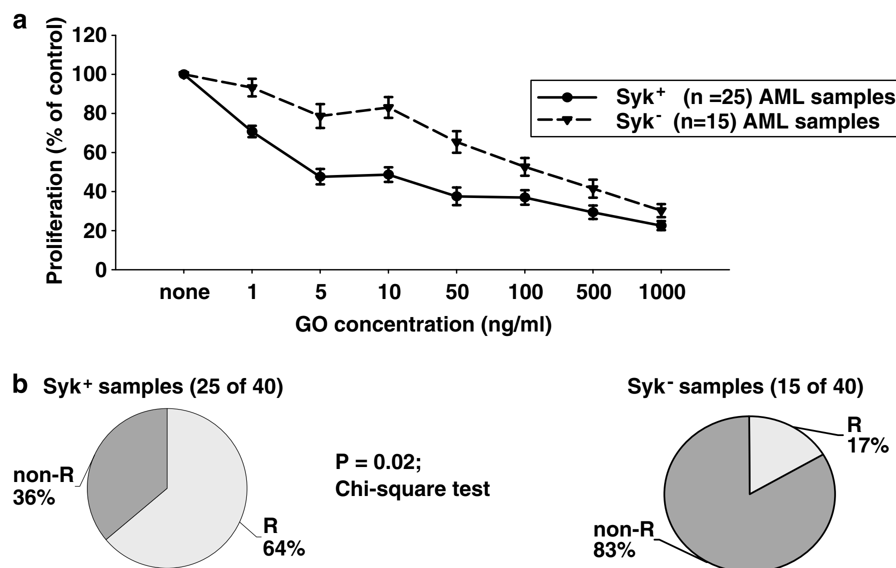

Figure 2 The effect of GO on growth of AML cells is dependent on Syk expression. (**a**) Dose-dependent inhibition of proliferation induced by GO treatment. Proliferation assays were performed with primary AML cells as described in Figure 1a. GO was added for 48 h at the indicated concentration. Basal cell proliferation of untreated cells (>10 000 c.p.m. of ^{3}H-thymidine incorporation) was considered to be 100%. (**b**) Correlation between Syk expression and the response of primary AML cells to GO treatment. Summarized data (mean) for 25 Syk-positive and 15 Syk-negative samples are presented. Error bars indicate the s.e.m.

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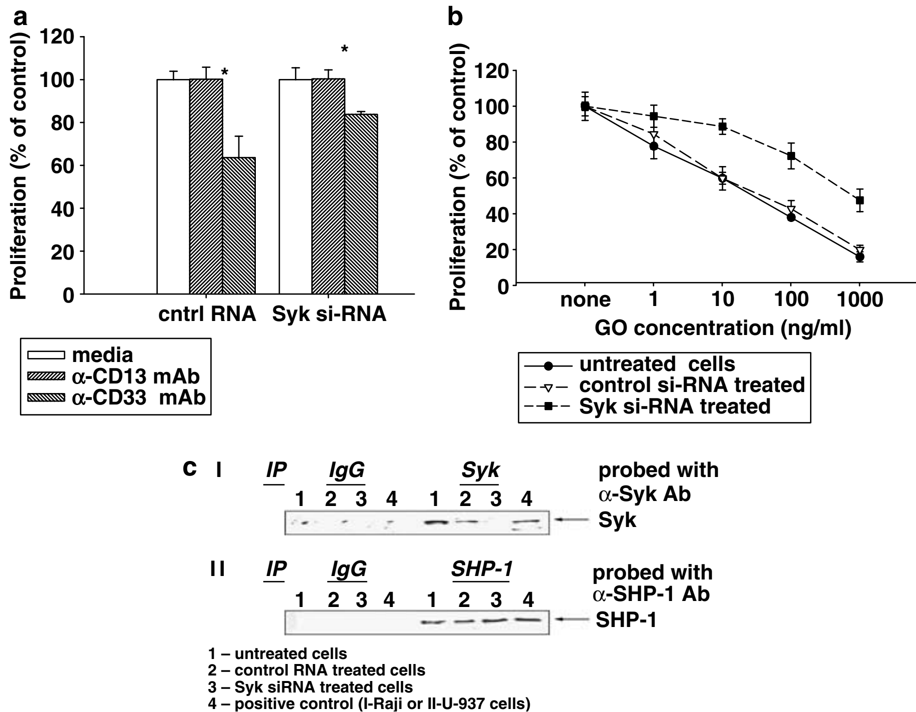

Figure 3 siRNA silencing of Syk expression decreases the anti-proliferative response of primary AML cells to anti-CD33 mAb including GO. (**a** and **b**) Proliferation assay. HL-60 cells were transfected with Syk siRNA or control siRNA. Seventy-two hours post-transfection, cells were treated with either naked anti-CD33 mAb or control anti-CD13 mAb (**a**) or they received the indicated concentrations of GO (**b**). Proliferation assays were performed as described in Figure 1a. The basal ^{3}H-thymidine incorporation of untreated cells was >10 000 c.p.m. (considered as 100%). Data represent the mean±s.e.m. of three independent experiments. The asterisks indicate statistically significant difference between groups of data. (**c**) Western blots of HL-60 cells transfected with Syk siRNA or control siRNA. After 72 h of culture, cell lysates were subjected to immunoprecipitation and Western blotting with the indicated mAb. Shown results represent one of three independent experiments.

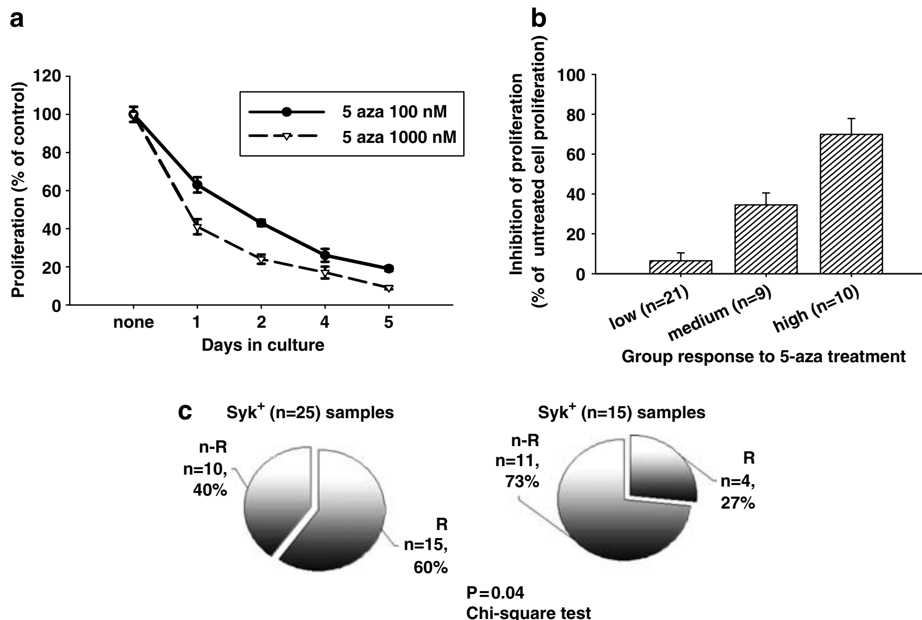

Figure 4 The effect of 5-aza on growth of AML cells is dependent on Syk expression. (**a**) Proliferation assay. HL-60 cells were treated with indicated concentrations of 5-aza for 1–5 days. After two washes, proliferation assays were performed as described in Figure 1a. The basal ^{3}H-thymidine incorporation of untreated cells was >10 000 c.p.m. (considered as 100%). Data represent the mean±s.e.m. of three independent experiments. (**b**) Proliferation assay. Primary AML cells were cultured for 48 h in the presence of 100 nM of 5-aza and then after two washes, proliferation assays were performed as described in Figure 1b. (**c**) Correlation between Syk expression and the response of primary AML cells to 5-aza treatment. Summary of primary AML cell proliferation

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