

ORIGINAL ARTICLE

T lymphocytes can be effectively recruited for *ex vivo* and *in vivo* lysis of AML blasts by a novel CD33/CD3-bispecific BiTE antibody construct

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Patients with acute myelogenous leukemia (AML) are in high need of novel targeted therapies. Here we explored the *ex vivo* activity of AMG330, a novel T-cell-engaging BiTE (bi-specific T-cell engagers) antibody (Ab) construct, that is bispecific for the myeloid differentiation antigen, CD33 and CD3, in primary samples from AML patients ($N = 23$) and AML cell lines. KG-1 and U937 cells were lysed in co-culture with healthy donor T-cells at AMG330 concentrations as low as 0.1 ng/ml (1.8 pM). T-cells derived from AML patient samples were found to be as active in redirected lysis by AMG330 as T-cells from healthy donors. In an autologous setting, AMG330 could activate and expand T-cells in primary AML patient samples, and effectively mediated the redirected lysis of AML blasts and normal myeloid cells. A deficiency in target-cell lysis was only observed in samples with very low initial effector-to-target (E:T) ratio. However, this could be overcome if previously stimulated autologous T-cells were tested in patient samples at a higher E:T ratio. *In vivo* experiments in immunodeficient mice demonstrated significant inhibition of tumor growth by AMG330 and an inducible infiltration of human T-cells into subcutaneous HL60 tumors. The activities of the CD33/CD3-bispecific BiTE Ab construct AMG330 warrant further development for the treatment of AML.

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INTRODUCTION

The search for new agents and new treatment modalities in acute myeloid leukemia (AML) has been stimulated by the lack of significant improvements in outcome over the last 10 years.¹ Among the several new agents that offer potential therapeutic use in AML, considerable attention has been focused on monoclonal antibodies (mAbs), which target antigens expressed primarily on myeloid and derived-malignant cells including CD33, CD123 and CD135. The frequent expression of CD33 on both AML blasts and AML stem cells has made it a target antigen of choice for developing mAb-based approaches in the past.^{2,3} However, all three major developments of CD33-targeting Abs in AML faced problems. The CD33-specific mAb drug conjugate (ADC), gemtuzumab ozogamicin (Mylotarg, Wyeth, NJ, USA), recently lost market approval in the United States owing to an inferior risk/benefit profile.⁴ Recent studies, however, showed potential for beneficial use in distinct patient subpopulations.^{5,6} The humanized anti-CD33 IgG1 mAb, lintuzumab, showed insufficient efficacy in a phase-3 trial in combination with cytarabine,⁷ as did monotherapy with the CD33-specific ADC, AVE9633, in a phase-1 study.⁸ Therefore, for the treatment of AML, the target antigen CD33 requires to be used with drug formats that have a more potent or safer mode of action than IgG1 or ADCs, respectively. This may be achieved by mAb-based constructs that selectively engage cytotoxic T- or natural killer (NK)-cells. A number of bi- and tri-specific mAb constructs with this property are currently in early development.^{9–12}

A clinically validated format of bispecific Abs are the so called BiTE (bi-specific T-cell engagers) molecules, which are based on two fused single-chain mAbs.⁴ A CD19/CD3-bispecific BiTE construct called blinatumomab has demonstrated high anti-lymphoma and -leukemia activity at very low dose levels in clinical studies.^{13–15} BiTE Ab constructs specific for solid tumor targets EpCAM and CEA are currently under early clinical investigation, and many others have been shown to have potent activity in co-culture experiments and xenograft models.^{16–18} By transiently tethering effector T-cells with target cells, BiTE Abs elicit T-cell-mediated cellular cytotoxicity independent of the specificity of the T-cell receptor or the presence of MHC/peptide complexes on target cells. The mechanism of BiTE action has been shown to rely on the activity of perforin, granzymes and caspases in target cells.¹⁷ Other immunotherapeutic strategies for AML patients such as adoptive T-cell transfer and vaccination may prove difficult as many immunological defects are known to occur in AML. For instance, loss of MHC class-I expression is frequent in AML cells, and some studies indicate that virtually all AML blasts have defects in their antigen processing and presenting machinery.^{19,20} As BiTE Ab constructs are known to induce strong proliferation and activation of T-cells in a polyclonal fashion and lyse target cells independently of the antigen processing and presenting machinery, a BiTE Ab construct specific for a myeloid-specific target antigen could provide for an alternative mode of action as needed for a successful targeted therapy of AML. Based on the

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positive experience with blinatumomab in the treatment of NHL and ALL patients, we were prompted to apply this novel therapeutic principle to the treatment of AML by developing a CD33/CD3-bispecific BiTE Ab construct called AMG330.

Here we characterized the biological activities of AMG330 *in vitro* with human AML cell lines, *ex vivo* with peripheral-blood mononuclear cell samples from AML patients, and *in vivo* in an immunodeficient mouse xenograft model.

MATERIALS AND METHODS

Cell lines

CD33-expressing cell lines, U937 and KG-1, were obtained from ATCC. Cells were cultured in RPMI 1640 (PAN Biotech, Aidenbach, Germany) supplemented with 10% FCS (PAA, Pasching, Austria) at 37 °C and 5% CO₂.

Primary cells

Mononuclear cells (MNCs) collected from healthy volunteers' leukapheresis products or from AML patients' peripheral blood or bone marrow were separated by density gradient centrifugation (PAN-Biotech, Aidenbach, Germany). Informed consent was obtained from patients according to the Declaration of Helsinki. The study was approved by the local ethics committee. Cells were cryopreserved in medium containing 90% FCS (PAA, Pasching, Austria) and 10% DMSO (Sigma, Munich, Germany). Characteristics of AML patients are shown in Supplementary Table 1.

Cell cultures

All *in vitro* cultures were performed in 96-well plates (Falcon, BD, Heidelberg, Germany), containing RPMI 1640 (PAN-Biotech, Aidenbach, Germany) supplemented with 10% human serum (PAN-Biotech, Aidenbach, Germany) at 37 °C and 5% CO₂. BiTEs were added only once at the beginning of the culture at the concentrations indicated.

Beads and normalization

To normalize the cell numbers between wells and over time during *in vitro* culture, 2 µl Cytometer Setup and Tracking Beads (BD, Heidelberg, Germany) were added to each well of the culture directly before cell harvest. For flow cytometric analysis, beads were identified as events that were positive in all the used fluorescence channels. Numbers of cells were normalized to cells/100 beads, thus allowing the analysis of decrease or increase of cell numbers over time.

Flow cytometry

Flow cytometry analysis was performed with a FACS Canto II (BD, Heidelberg, Germany). For detection of specific surface markers of AML and healthy donor MNCs, the following fluorochrome-conjugated Abs were used: CD14-FITC, CD34-PE, CD33-PerCP, CD13-PeCy7, CD3-APCCy7, CD56-V450 and CD45-V500. Activation of T-cells was detected with CD69-FITC, CD54-FITC, CD71-APC, CD4-PE, CD25-PeCy7, CD8-APCCy7, CD3-V450 and CD45-V500. For phenotyping of T-cell subpopulation, the following Abs were used: CCR7-FITC, CD57-PE, CD8-PerCP, TCRαβ-APC, CD45RO-PeCy7, CD3-APCCy7, CD27-V450 and CD4-V500. All Abs were from BD (Heidelberg, Germany) and used according to the manufacturer's instructions. Proliferation of CFSE- (Sigma, Munich, Germany) stained T-cells was determined by flow cytometry.

Cell sorting

Flow cytometric cell sorting. Isolation of specific cellular subsets from MNCs of healthy donors or AML patients was performed by FACS Sorting on a MoFlow sorter (Beckmann-Coulter, Krefeld, Germany). For purification of T-cells, the following Abs were used: CD4-FITC, CD8-PE (BD, Heidelberg, Germany), TCRαβ-APC (Miltenyi Biotec, Bergisch-Gladbach, Germany); all Abs were used according to the manufacturer's instructions. Re-analysis showed that the purities of the specifically enriched population was routinely > 95%.

Magnetic cell sorting. CD8⁺ T-cells were enriched from MNC of healthy donors or AML patients with the human CD8⁺ T-cell Isolation Kit II from Miltenyi Biotec (Bergisch-Gladbach, Germany) according to the manufacturer's instructions, by depletion of CD4⁺, CD11b⁺, CD16⁺, CD19⁺,

CD20⁺ and CD56⁺ cells. The resulting population consisted of > 90% CD8⁺ T-cells, and was used as effector cell population.

Chromium release assay

The cytotoxic activity of T-cells was measured by a 19-h ⁵¹Cr release assay as described.¹¹ Effector-to-target (E:T) ratios were calculated based on the number of T-cells in the effector population in order to accurately compare different T-cell populations. ⁵¹Cr-labeled target cells were CD33⁺ cell lines.

BiTE Ab constructs

AMG330 is a fusion protein consisting of an N-terminal single-chain Ab specific for CD33, and a C-terminally fused single-chain Ab specific for the epsilon chain of CD3. AMG330 was constructed by recombinant DNA technologies as described in detail for several other BiTE Ab constructs.^{16,21} Binding affinities for CD33 and CD3 are in the low nM-range, and the apparent molecular weight is 55 kDa. AMG330 was selected from a larger panel of CD33-specific BiTE Ab constructs based on its superior pharmacological and pharmaceutical properties (to be reported elsewhere). BiTE Ab constructs were produced by a stably transfected Chinese hamster ovary cell clone, and the monomeric form in cell culture supernatants purified to homogeneity by a three-step purification. The Control BiTE consisted of the same human anti-CD3 single-chain Ab as AMG330, but recognized with the second binding site a herbicide instead of CD33. It was also produced by a stably transfected CHO cell clone and purified to homogeneity by a three-step procedure.

Animal tumor studies

Seven to eight-week-old NOD/SCID mice were obtained from Charles River (L'Arbresle Cedex, France) and housed in sterilized cages. All mice were fed ssniff R/M-H food pellets (ssniff, Soest, Germany) sterilized by 25 kGy irradiation, and autoclaved drinking water *ad libitum*. Animals were maintained on a 12-h/12-h light/dark cycle at 22 ± 2 °C, and a relative humidity between 34 and 73%. All procedures were performed in accordance with the German Animal Protection Law and with permission from the responsible local authorities. Animals were irradiated with a single dose (2 Gy) before subcutaneous (s.c.) injection of 2 × 10⁶ HL-60 human AML cells. When tumors had reached a volume of ~100–200 mm³, animals were treated with a single intravenous (i.v.) bolus injection of anti-asialo GM1 rabbit Ab (WAKO, Neuss, Germany) one day before human T-cell injection to deplete murine NK cells. *In vitro* activated and expanded human T-cells isolated from the blood of a single healthy donor (Miltenyi Biotec, Bergisch-Gladbach, Germany) were injected (1.6 × 10⁷ per mouse) into the peritoneal cavity. Three days after T-cell injection, when tumors had reached a volume of ~300 mm³, mice received BiTE or vehicle once daily by either s.c. or i.v. bolus injection for a total of 9 days. Treatment efficacy in terms of tumor growth was determined by external caliper measurements, and tumor volumes were calculated using a standard hemi-ellipsoid formula: (length (mm) × width (mm)²)/2.

Characterization of tumor-infiltrating immune cells by flow cytometry

Two tumors from each group were used for single cell preparation and FACS analysis. Briefly, tumors were dissected; tissue was cut into small pieces prior to digestion for 1 h in TDE buffer (collagenase A, dispase grade II, DNase I, 15% FCS, in RPMI medium 1640) at 37 °C. After erythrocyte lysis, cells were stained with a mouse anti-human CD8 Ab (BD, Heidelberg, Germany, clone SK-1) and 1 mg/ml propidium iodide (Sigma-Aldrich, Munich, Germany).

Statistics and software

Flow cytometric data were analyzed with Kalzua Software v1.1 (Coulter, Krefeld, Germany). Statistical analysis was done with Mann-Whitney-U tests. P-values of < 0.05 were considered significant and are indicated by an asterisk. Graphical representations of data were generated with Prism5 Software from Graphpad (San Diego, USA). Data represent mean values of several independent experiments (numbers given in figure legends) ± s.d.

RESULTS

Lysis of human AML cell lines by CD8⁺ T-cells redirected by the CD33/CD3-bispecific BiTE Ab construct AMG330

To establish a BiTE concentration for effective *in vitro* activity, two myeloid leukemia cell lines were tested for CD33 expression and redirected lysis. KG-1 and U937 cells revealed significant AMG330-specific lysis *in vitro* at concentrations as low as 0.1 ng/ml (1.8 pM), and reached maximal lysis at a concentration of 1 ng/ml (Supplementary Figure 1). No lysis was observed in the presence of the T-cell-binding Control BiTE.

As later experiments with patient samples would not allow specific labeling of target cells as it is practised for ⁵¹Cr release assays, we established target cell identification by *ex post* FACS staining (Figure 1). Monocytes from healthy donors were identified as CD45⁺CD3⁻CD13⁺CD14^{high} cells, donor T-cells as CD45⁺CD3⁺CD14⁻ cells and U937 cells as being positive for CD45⁺CD13⁺CD56⁺CD95⁺. After co-culture for 24 h in the presence of AMG330, an almost-complete elimination of the U937 cell population (orange) as well as a reduction of the normal monocytic cell population (blue) was observed, demonstrating a high activity of AMG330 and the suitability of the FACS-based assay for analysis of patient-derived samples. An increase in the sideward scatter of T-cells in co-cultures with AMG330 may be indicative of T-cell activation. In co-cultures with the Control BiTE Ab construct that is solely binding to CD3, the U937 cell signal increased during the 24-h-incubation period, apparently due to proliferation, whereas the signal for the CD14^{high} monocytic population decreased possibly due to adherence of monocytes to the plastic surface. For further experiments, calibration beads were therefore added before cell

harvest in order to quantify absolute gain and loss of cell populations.

AMG330-mediated lysis of primary AML blasts by healthy donor T-cells

To determine if primary blasts from AML patients can be as effectively lysed by AMG330-engaged T-cells as an AML cell line, MNC from AML patients presenting with initial diagnosis or relapse of AML and containing leukemic blasts were utilized. CD33 expression was examined by flow cytometry (Supplementary Figure 2). Monocytes and the remaining myeloid cells in patient samples showed a higher level of CD33 expression than AML blasts.

MNCs from AML patients were incubated for 72 h with MNCs from healthy donors at an E:T ratio of 1:1 at 1 ng/ml of AMG330 or Control BiTE, or in the absence of both. Total myeloid cells were analyzed daily and gated as single (exclusion of cell aggregates by forward and sideward scatter) CD45⁺CD3⁻CD13⁺ cells. Within this cell population, CD13⁺CD34⁺ or CD13⁺CD117⁺ cells were considered to be AML blasts, whereas CD13⁺CD14⁺ cells were considered to be monocytes (Supplementary Figure 3).

In co-cultures without BiTE Ab constructs, a moderate loss of myeloid cells and AML blasts was observed, whereas CD14⁺ monocytes steadily decreased over time (Figure 2). In the presence of AMG330, a marked reduction of all CD13⁺ cells was observed. Between 48 and 72 h, a significant reduction of leukemic blasts by AMG330 compared to the Control BiTE, and a complete depletion of monocytes was evident. These data suggest that AMG330 can engage allogeneic T-cells for lysis of blasts and other CD33⁺ myeloid cell populations as contained in blood and bone marrow from AML patients.

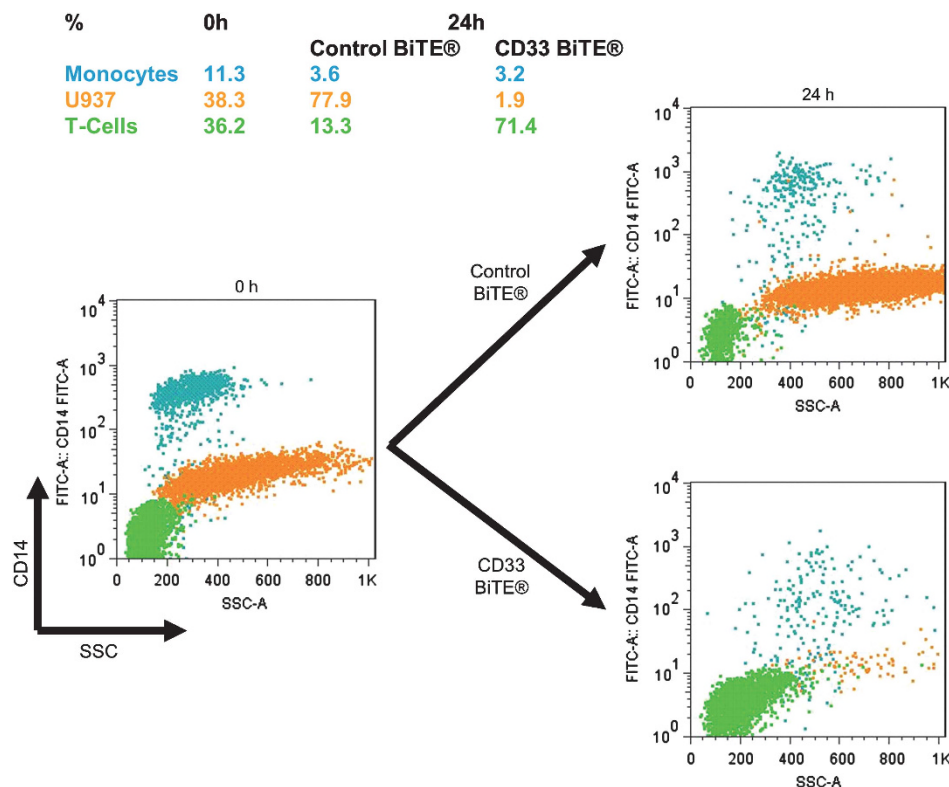


Figure 1. Flow cytometry-based cytotoxicity assay for quantification of AMG330-specific lysis of myeloid cells. The CD33⁺ cell line U937 was co-incubated for 24 h with MNC from a healthy donor at an E:T ratio of 1:1 (referred to CD3⁺ T-cell) in the presence of AMG330 (CD33 BiTE) or Control BiTE Ab constructs. Dot plots are gated on live, single, CD45⁺ cells and show CD13⁺CD14⁺ monocytes in blue, donor CD3⁺ T-cells in green and CD56⁺CD14⁺ U937 target cells in orange. The percentage of respective populations in culture at baseline and after 24 h is given in the table.

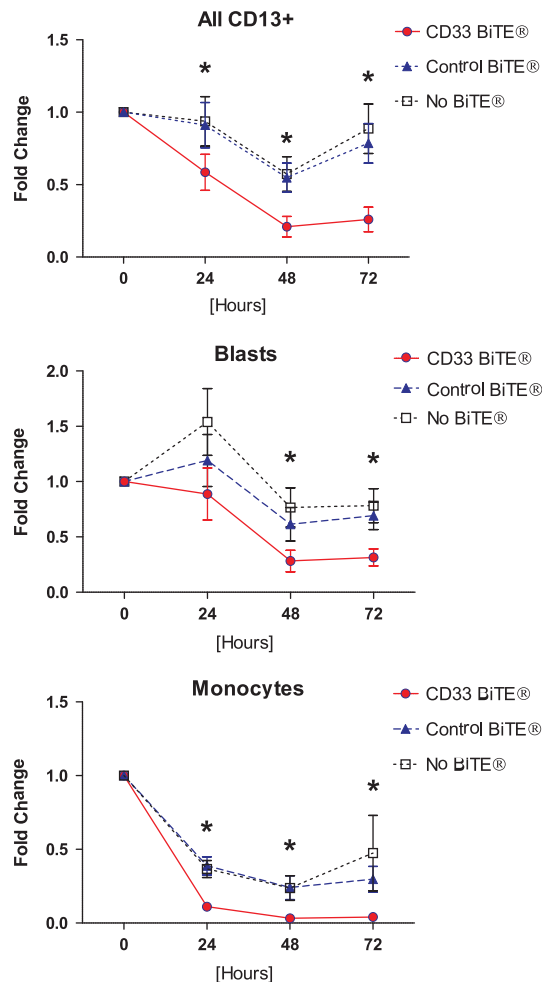


Figure 2. The effect of AMG330 on redirected lysis of primary AML cells by healthy donor MNCs. Primary cells from AML patients were incubated for up to 72 h with healthy donor MNC at an E:T ratio of 1:1. Cultures contained either 1 ng/ml AMG330 (CD33 BiTE), 1 ng/ml Control BiTE, or no BiTE. Data show mean values for fold change in population cell numbers (standardized to $d_0 = 1$) ($\pm$ s.d.) of eight different AML samples. Asterisks indicate significant differences between cultures containing Control BiTE and CD33 BiTE.

T-cells from AML patients can be effectively engaged by AMG330 for lysis of CD33-expressing target cells

We next compared the performance of healthy donor T-cells with T-cells derived from AML patient samples. T-cells were isolated with $>90\%$ purity from peripheral blood of healthy donors and AML patients with active disease, and co-cultured with the ^{51}Cr -labeled AML cell line U937 at an E:T ratio of 1:1 and 1 ng/ml AMG330 or Control BiTE, or no BiTE. No significant difference in redirected lysis of U937 cells by T-cells from seven AML patients and seven healthy donors was observed ($P = 0.4487$) (Figure 3). Both T-cell populations caused a 40% specific lysis of CD33⁺ target cells after 19 h, demonstrating that T-cells from blood or bone marrow of patients with AML can be effectively recruited for AMG330-specific lysis. No unspecific lysis could be observed.

AMG330 can potentially activate and expand autologous T-cells in primary AML patient samples

We next investigated the activity of AMG330 in a completely autologous setting of AML patient samples. As shown in Figure 4a, AMG330 could induce a high expression of T-cell activation markers CD25, CD54, CD69 and CD71 on almost all CD8⁺ T-cells

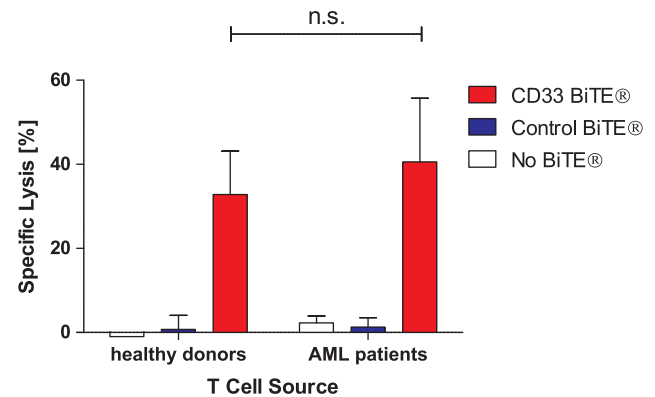


Figure 3. Performance of T-cells from patients with AML compared to T-cells from healthy donors with respect to redirected lysis of U937 AML target cells. Resting CD8⁺ T-cells derived from peripheral blood of healthy donors or from AML patients with active disease were isolated. ^{51}Cr -labeled, CD33⁺ positive U937 target cells were co-incubated for 19 h with T-cells at an E:T ratio of 1:1 in the presence or absence of AMG330 (CD33 BiTE), Control BiTE Ab constructs, or no BiTE. Data show mean ($\pm$ s.d.) percentages of redirected lysis with seven healthy and seven AML T-cell donors.

after 24 h. The increase of activation markers on CD4⁺ T-cells in response to AMG330 was comparable to that of CD8⁺ cells (data not shown). Proliferation of T-cells, as demonstrated by CFSE dilution, could be observed in AMG330-treated AML co-cultures as early as 72 h after the start of BiTE incubation with a clear increase after 144 h (Figure 4b). Analysis of secreted cytokines revealed high levels of IL-1 β , IL-2, IFN- γ and TNF only in the presence of the specific BiTE Ab construct (Supplementary Figure 4). In contrast, even after long-term culture, no unspecific T-cell activation or secretion of cytokines was observed in the controls.

To further characterize the AMG330-mediated *ex vivo* T-cell response, we analyzed the various CD8⁺ T-cell subsets of AML patient samples ($N = 10$) before and after incubation with AMG330, Control BiTE and in the absence of any BiTE Ab construct (see Supplementary Figure 5). We found that at baseline, T-cells from AML patients contained lower levels of naïve (CD45RO⁻CCR7⁺) and central memory (CD45RO⁺CCR7⁻) CD8⁺ T-cells compared to those from healthy individuals, whereas TemRA cells (CD45RO⁻CCR7⁻CD45RA⁺) were increased compared to healthy individuals. Only in the presence of 1 ng/ml AMG330 a marked change of subpopulations was observed after 144 h, indicating a substantial and selective expansion of the effector memory T-cell subset by AMG330.

AMG330 mediates redirected lysis of primary AML blasts by autologous T-cells

Next, we examined the lysis of myeloid cell populations within autologous unmanipulated MNC cultures from untreated AML patients at their physiological E:T cell ratios. After up to 3 days of culture, only minor cytotoxic effects towards autologous myeloid cells were seen, probably due to the low numbers of effector T-cells present in primary AML samples. After 144 h, 16 out of the 20 AML samples (80%) showed a specific reduction of myeloid cells including blasts of $>20\%$ in the presence of the CD33-specific BiTE Ab construct. In 8 out of the 20 samples (40%), a specific lysis of 50–93% of all myeloid target cells was seen in primary cultures (example shown in Figure 5a). In only 4 out of the 20 samples (20%), no significant killing of myeloid cells with an expansion of blasts could be observed (example shown in Figure 5b).

In an attempt to understand these differences in the *ex vivo* responsiveness of AML patient samples, we tried to correlate the extent of myeloid cell lysis with the E:T ratios as observed at the

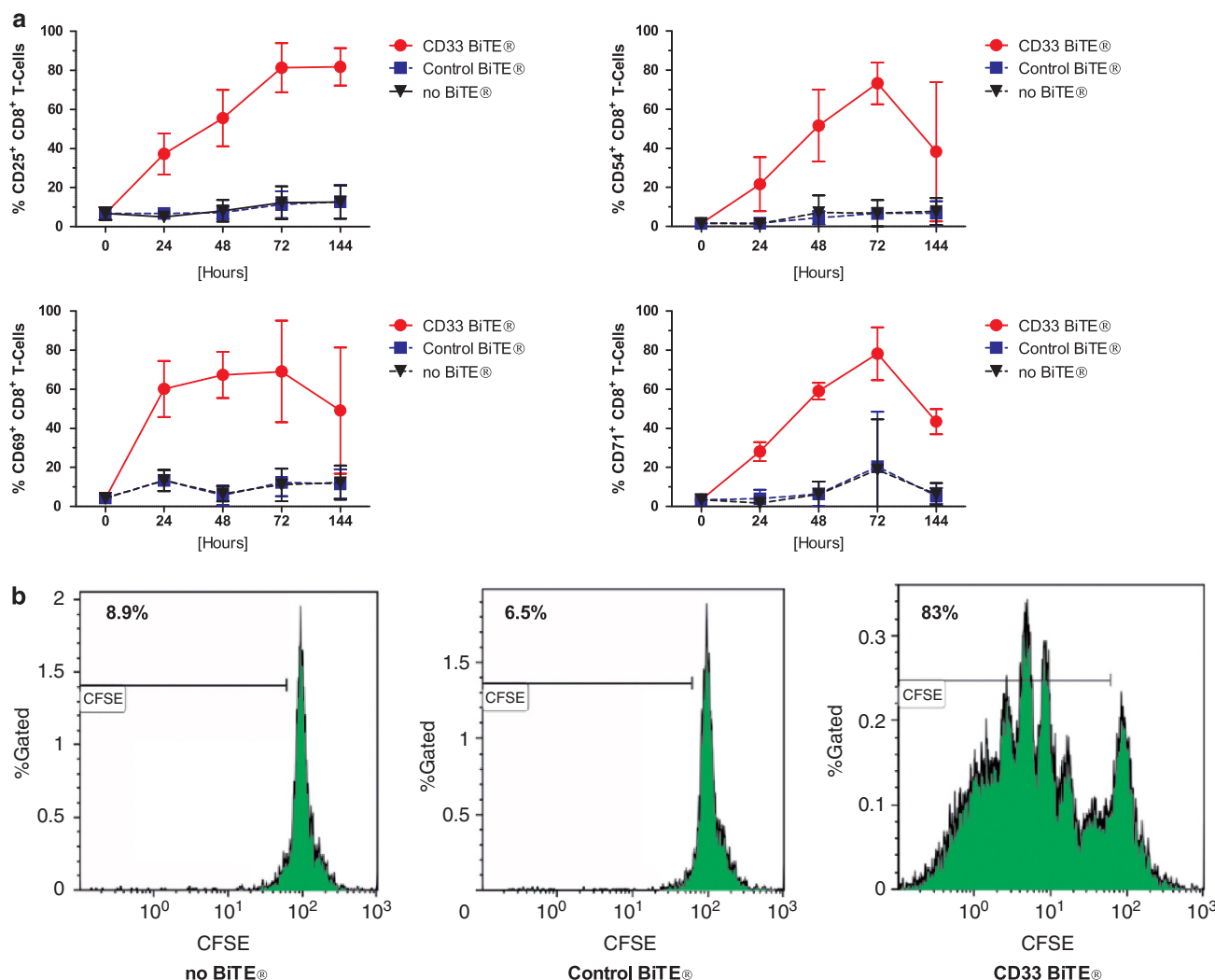


Figure 4. The effect of AMG330 on autologous T-cell activation and proliferation in primary AML cultures. MNCs from patients with active AML were incubated for 144 h in the presence of 1 ng/ml AMG330 (CD33 BiTE) or 1 ng/ml Control BiTE, or no-BiTE Ab constructs. **(a)** Expression of activation markers CD25, CD54, CD69 and CD71 on CD8⁺ T-cells. **(b)** Proliferation of total T-cells. Proliferation of CFSE-labeled T-cells was quantified by flow cytometry. Histograms show CFSE staining of CD3⁺-gated T-cells.

beginning of the *ex vivo* cultures. For calculation of E:T ratios, all CD13⁺ cells were regarded as potential target cells, whereas all CD3⁺ T-cells were considered effector cells. We found that AMG330 was active in more than 90% of all AML samples with an E:T ratio of > 1:20 leading to blast lysis up to 95%. In contrast, in the subgroup of six AML samples with E:T ratios of < 1:20, lysis of myeloid target cells was marginal in two cases, and in 3 cases could not be observed at all.

For technical reasons, our *in vitro* assay was limited to 144 h of incubation. Therefore, we mimicked a continued T-cell activation and expansion *in vitro* as it would occur upon longer term administration of AMG330. To this end, we investigated whether addition of pre-stimulated autologous T-cells at higher E:T ratios could improve the outcome of *ex vivo* blast lysis in patient samples. T-cells from non-responsive AML samples were isolated after 144 h in presence of AMG330 by FACS sorting. When those autologous T-cells were added back to autologous AML patient samples at an E:T ratio of 1:1, a fast and almost-complete elimination of all CD13⁺ target cells including CD34⁺ AML blasts was observed within only 24 h (Figure 6). This rapid depletion was specific as it was not observed with the Control BiTE, or in the absence of any BiTE Ab construct.

AMG330 engages human T-cells and inhibits AML tumor growth in immunodeficient mice

The CD33⁺ human AML cell line HL-60 was subcutaneously injected into immunodeficient mice, and after tumors had reached a volume of 100–200 mm³ animals received a single i.v. injection of anti-asialo GM1 rabbit Ab to deplete residual NK cells.

Human T-cells from a single healthy donor were expanded *in vitro* for 18 days and injected in the peritoneal cavity of tumor-bearing mice. Three days after T-cell injection, when tumors had reached a volume of ~300 mm³, animals were treated once daily with either vehicle or AMG330 (0.05 or 0.5 mg/kg/d) by i.v. injection or with 0.25 mg/kg/d by s.c. injection for 9 consecutive days. A significant reduction of tumor growth was observed following either i.v. or s.c. AMG330 administration when compared to the vehicle control group. In all AMG330-treated groups, the effect was found to be independent of the route of administration or dose level used in this study (Figure 7a). After single-cell preparation of two tumors from each group, FACS analysis revealed the presence of intra-tumoral T-cells in all AMG330-treated animals, whereas no T-cell infiltration was observed in tumors from vehicle-treated animals (Figure 7b). Of interest, mice receiving the highest i.v. dose showed even higher T-cell

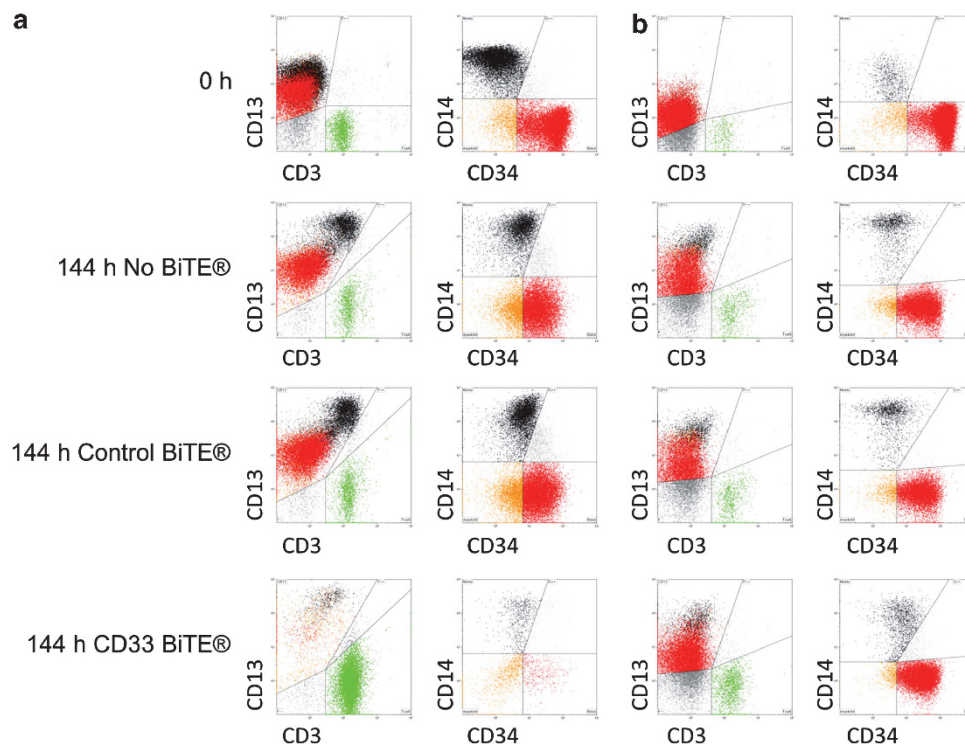


Figure 5. The effect of AMG330 on redirected lysis of primary AML blasts by autologous MNCs in patient samples. Primary MNCs from AML patients were incubated for up to 144 h. Cultures contained either 1 ng/ml AMG330 (CD33 BiTE), 1 ng/ml Control BiTE or no BiTE. T-cells are shown in green, CD34⁺ blasts in red, CD14⁺ monocytic cells in black, and remaining myeloid cells in orange. (a) AML sample with high initial E:T cell ratio. (b) AML sample with low initial E:T cell ratio.

infiltration when compared to both other groups, thus implicating a potential dose dependency of T-cell infiltration.

DISCUSSION

Although current conventional chemotherapies for AML are very successful in eliminating the bulk of malignant cells, they rarely provide long-term success.¹ Therefore, alternative and/or complementing treatment modalities are highly needed. Allogeneic stem cell transplantation is a viable option for improved results in younger patients, albeit at the price of high treatment-related morbidity and mortality. In elderly patients, in whom the highest AML incidence occurs, treatment options are more limited.^{22,23}

In this study, we examined the anti-leukemic properties of a novel CD33/CD3-bispecific Ab-construct of the BiTE format against primary human AML cells by a combination of *ex vivo*, *in vitro* and *in vivo* approaches. We decided not to add any growth factors or cytokines to primary cell cultures to avoid artificial myeloid cell stimulation. As a consequence, we observed a steady decrease in the viability of myeloid cells throughout the culture period, whereby on average most cultures lost >50% of vital cells within one week. This limited our maximal assay period to 6 days. As this technical limitation will not allow very low initial T-cell numbers in patient samples to sufficiently expand and exert significant effector functions against malignant target cells, in the first set of experiments we used healthy donor MNCs to provide sufficient numbers of effector cells.

Using these effector cells, we found that even low concentrations of 1 ng/ml AMG330 (18 pM) were sufficient to induce fast, efficient and specific lysis of all CD13⁺ myeloid cell populations, including AML blasts after 24–48 h of culture. Of note, the monocytic CD14⁺ population seemed even more sensitive for

BiTE-mediated killing than other CD33⁺ cell populations. This could be due to the higher surface expression of CD33 on those cells or different susceptibility against apoptosis induction in different cell types, but could also result from an unspecific reduction of these cells due to adherence to the plastic surface as documented elsewhere.²⁴

A question with major importance for a clinical utility of AMG330 is whether autologous T-cells as present in AML patients can be efficiently redirected for lysis of AML blasts. Previous publications have described specific defects of T-cells from AML patients, for instance, with respect to the formation of immunological synapses or effector functions by the expression of inhibitory molecules of the PD-1/PD-L1 axis.^{25–28} We observed that the cytolytic performance of AMG330-engaged T-cells derived from AML patients was not any different from that of healthy donor T-cells against a human AML cell line. As the BiTE Ab construct solely relies on expression of CD33 on target cells and of CD3 on effector T-cells a multitude of immune-escape-mechanisms affecting TCR-MHC-peptide-complex formation, the differentiation of effector T-cell clones from naïve T-cells, or immune-modulatory receptors are unlikely to have an impact on redirected lysis. The high *ex vivo* response rate observed in this study further supports this notion.

Having demonstrated that T-cells from AML patient samples were efficiently engaged by AMG330 and that primary malignant cells were sensitive to BiTE-mediated killing by allogeneic T-cells from healthy donors, we here also studied the redirected lysis of leukemic blasts in a completely autologous setting at natural E:T ratios. We therefore incubated MNCs from patients with untreated, full-blown AML in the absence of additional effector T-cells for the activity of AMG330 and respective controls. These MNCs typically contained high counts of malignant leukemic cells and low numbers of T-cells. Whereas activation of T-cells, as demonstrated

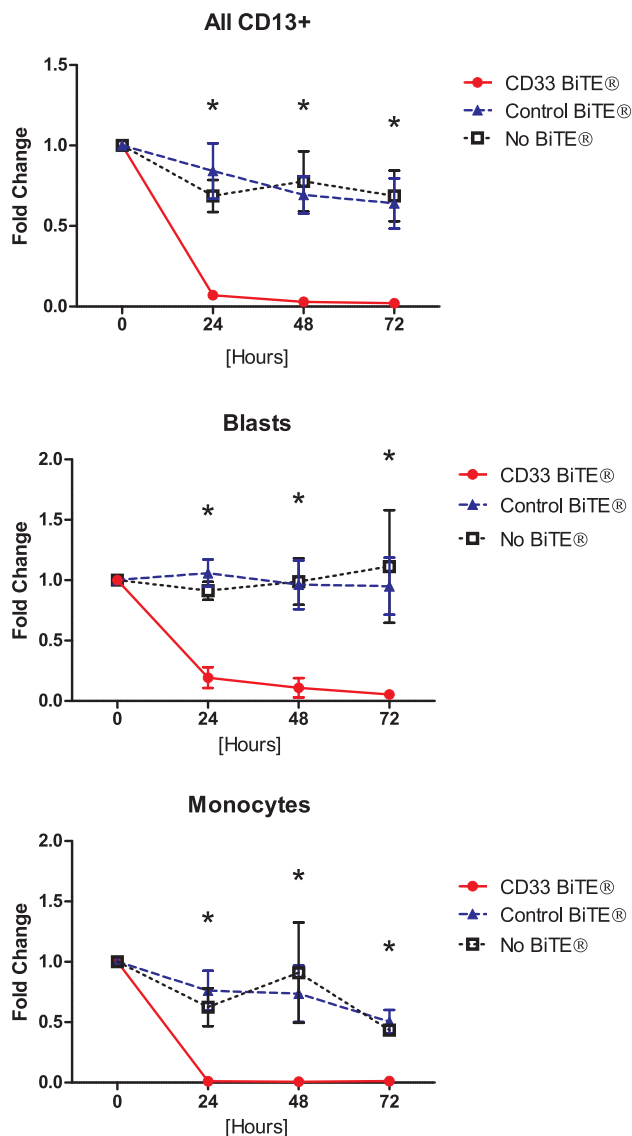


Figure 6. The effect of pre-stimulation of autologous T-cells on AMG330-mediated redirected lysis of primary autologous AML blasts. AML samples were co-cultured for 144 h in the presence of 1 ng/ml AMG330 (CD33 BiTE), 1 ng/ml Control BiTE, or no BiTE. CD8⁺ T-cells were harvested from these cultures and incubated for the indicated time points together with a second MNC sample from the same patient at an E:T ratio of 1:1. A mean of four independent experiments from four different AML patients $\pm$ s.d. is shown.

by expression of activation markers and secretion of cytokines, and T-cell proliferation was evident in almost all samples studied, a specific reduction of myeloid cell populations including AML blasts was restricted to those 80% of the patient samples that had sufficiently high E:T ratios at the start of *ex vivo* treatment. However, T-cells from the small number of non-responsive samples led to fast and complete lysis of blasts and myeloid cells when they were re-introduced after pre-stimulation at a higher E:T ratio to an AML blast sample of the same patient. This may indicate that prolonged treatment of AML patients with BiTE Ab construct AMG330 can overcome initially low E:T ratios, and lead to potent AML blast lysis once T-cells have been sufficiently activated and expanded. Induction of T-cell memory using the BiTE approach might be possible, but no memory T-cell responses

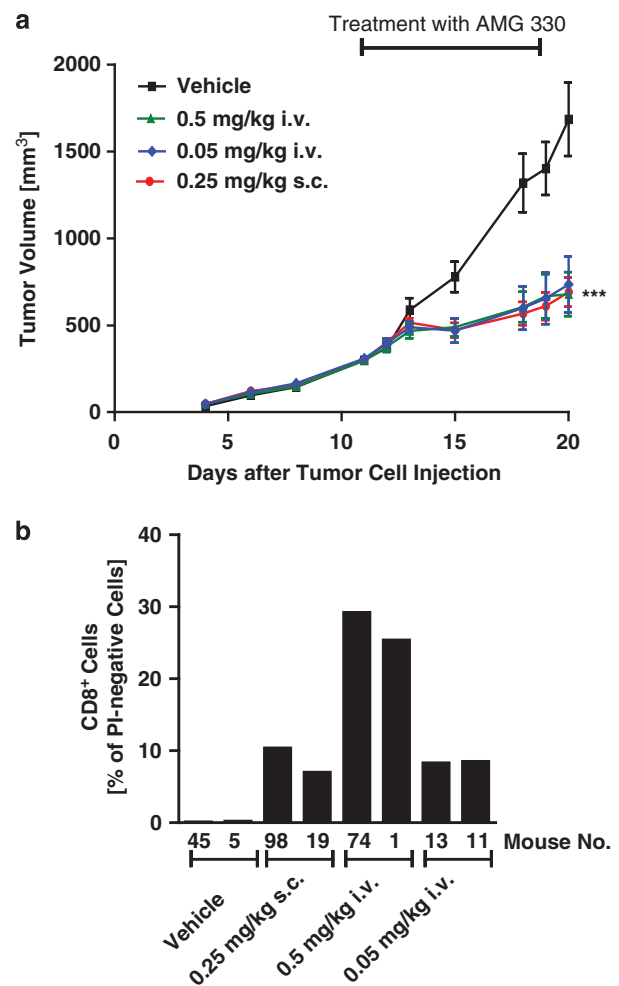


Figure 7. Anti-tumor activity of AMG330 in mouse AML xenograft model. Irradiated NOD/SCID mice were injected with of 2×10^6 HL-60 human AML cells. After depletion of murine NK cells, *in vitro* activated and expanded human 1.6×10^7 T-cells were injected into the peritoneal cavity. When tumors had reached a volume of ~ 300 mm³ animals received AMG330 or vehicle once daily by either s.c. or i.v. bolus injection into the lateral tail vein for a total of 9 days. (a) Tumor volume progression over period of treatment. (b) FACS analysis of infiltration of T-cells into tumors from two mice of each group at day 20.

against tumor-associated antigens have thus far been reported in clinical trials with the BiTE Ab blinatumomab.

Lastly, we observed that AMG330 can inhibit growth of established subcutaneous HL60 tumors. Most importantly, tumors were grown s.c. to an average size of 100–200 mm³ in NOD/SCID mice in the absence of human T-cells. *Ex vivo* expanded human T-cells were then injected into the peritoneal space of mice from where they had to enter the peripheral blood compartment in order to reach the s.c.-implanted tumor. Three days following adoptive T-cell transfer, dosing of AMG330 was started that triggered a rapid inhibition of further tumor growth compared to vehicle treated animals. This rapid response suggests that both AMG330 given i.v. or s.c., and T-cells delivered intraperitoneally readily gained access to the s.c. tumor. A novel observation was that residual tumor tissue in AMG330-treated mice became heavily infiltrated by peripheral T-cells. This may indicate that the initial status of T-cell infiltration of tumor tissue may not matter for the efficacy of BiTE Ab constructs as they are capable of driving T-cell infiltration.

CD33 has been most frequently selected as target for Ab-directed treatment of AML based on its wide expression in patients with AML and restricted expression to normal cells of the myeloid lineage.²⁹ It is expressed on 90% of AML cases,^{30,31} although to varying levels, which is in accordance with the CD33 expression levels of our AML population studied here. Two reports suggest that also the population of leukemic AML stem cell expresses CD33.^{2,32} We have observed that AMG330 was not only active against AML blasts but also depleted other myeloid cells in patient samples expressing the differentiation antigen CD33. Monocytes were found to have higher expression levels of CD33 compared to AML blasts and slightly higher levels than remaining myeloid cells. Of note, all AML blasts studied expressed CD33 and had similar surface levels by flow cytometry. Future studies in non-human primates will investigate the tolerability of depleting normal CD33-expressing myeloid cells. As pluripotent hematopoietic stem cells in the bone marrow do not express CD33, a potential regrowth of normal myeloid cells is possible from those stem cells after complete elimination of the CD33⁺ target compartment by AMG330-engaged T-cells. From CD20-specific mAbs that efficiently deplete B-cells in NHL and rheumatoid arthritis patients, it is known that a total compartment eradication is tolerated by patients and that normal B-cell counts can recover after treatment.³³

By engaging T-cells rather than Fc γ -receptor positive immune cells, the BiTE format may have advantages in terms of potency over regular Abs-mediated antibody-dependent cell-mediated cytotoxicity. This became evident in recent clinical studies with the CD19/CD3-bispecific BiTE Ab construct blinatumomab.^{14,34–36} In particular, high activity of blinatumomab was observed in bone marrow of ALL and NHL patients, which is the same site where AML blasts reside. We therefore expect that AML is an indication that is well suited for treatment with a novel BiTE Ab construct. AMG330 also shares with blinatumomab a relatively small size and high activity level where specific target cell lysis is observed at low picomolar concentrations.³⁷ Other CD33-targeting Ab constructs are under early development that have dual or triple specificity, and induce half-maximal lysis at concentrations of $\sim 2 \mu\text{g/ml}$. AMG330 here showed effective lysis at 0.1 ng/ml, that is, at a $5000 \times$ times lower concentration.^{38,39}

In summary, we were able to establish a completely autologous setting for *ex vivo* analysis of BiTE-mediated anti-AML responses. AMG330 emerged as an appropriate BiTE candidate to activate and redirect AML patient-derived T-cells for lysis of blasts. The BiTE Ab construct is a very promising candidate for further preclinical studies and for testing its therapeutic potential in future clinical trials.

CONFLICT OF INTEREST

MA, JF, AM, SWK: research funding from Amgen Research (Munich) GmbH. RK, PK, PAB, KS, AH, BR and MF are employed by Amgen Research (Munich) GmbH and have an ownership interest in Amgen, Inc.

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AUTHOR CONTRIBUTIONS

MA and SWK developed the flow cytometric evaluation strategy. MA analyzed the data from primary cell cultures. JF performed and analyzed the experiments on primary AML cells. SS analyzed the T-cell activation data. SWK analyzed patient-related data. RK produced and characterized AMG330. PK spearheaded development of AMG330 for treatment of AML. KS, AH, BR and MF performed and analyzed the mouse experiments. PAB initiated the study and revised the paper. MA, AM and SWK designed the study and wrote the paper.

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Supplementary Information accompanies the paper on the Leukemia website (<http://www.nature.com/leu>)