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Large Molecule Therapeutics

Molecular Cancer Therapeutics

Preclinical Characterization of AMG 330, a CD3/CD33-Bispecific T-Cell–Engaging Antibody with Potential for Treatment of Acute Myelogenous Leukemia

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Abstract

There is high demand for novel therapeutic options for patients with acute myelogenous leukemia (AML). One possible approach is the bispecific T-cell–engaging (BiTE, a registered trademark of Amgen) antibody AMG 330 with dual specificity for CD3 and the sialic acid–binding lectin CD33 (SIGLEC-3), which is frequently expressed on the surface of AML blasts and leukemic stem cells. AMG 330 binds with low nanomolar affinity to CD33 and CD3ε of both human and cynomolgus monkey origin. Eleven human AML cell lines expressing between 14,400 and 56,700 CD33 molecules per cell were all potently lysed with EC_{50} values ranging between 0.4 pmol/L and 3 pmol/L (18–149 pg/mL) by previously resting, AMG 330–redirected T cells. Complete lysis was achieved after 40 hours of incubation. In the presence of AML cells, AMG 330 specifically induced expression of CD69 and CD25 as well as release of IFN-γ, TNF, interleukin (IL)-2, IL-10, and IL-6. *Ex vivo*, AMG 330 mediated autologous depletion of CD33-positive cells from cynomolgous monkey bone marrow aspirates. Soluble CD33 at concentrations found in bone marrow of patients with AML did not significantly affect activities of AMG 330. Neoexpression of CD33 on newly activated T cells was negligible as it was limited to 6% of T cells in only three out of ten human donors tested. Daily intravenous administration with as low as 0.002 mg/kg AMG 330 significantly prolonged survival of immunodeficient mice adoptively transferred with human MOLM-13 AML cells and human T cells. AMG 330 warrants further development as a potential therapy for AML. *Mol Cancer Ther; 13(6); 1549–57. ©2014 AACR.*

Introduction

Acute myelogenous leukemia (AML) accounts for approximately one third of all leukemias in the United States and is on the rise in industrialized nations due to an ageing population and little improvement of standard of care over the last 20 years (1–3). Hence, there is a strong demand for novel therapeutics to extend disease-free survival, effectively treat patients in remission, and for establishment of second- and third-line treatment regimens. Such novel treatments should come with an improved risk/benefit profile, provide better quality of life, and the potential to cure.

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The huge impact of antibody-based therapies seen in the treatment of B-cell malignancies has not yet reached AML therapy. With the withdrawal from the market of anti-CD33 antibody drug conjugate (ADC) gemtuzumab ozogamicin (Mylotarg, Pfizer), and failure of anti-CD33 immunoglobulin G (IgG)-1 lintuzumab (Seattle Genetics) and anti-CD33 ADC AVE9633 (Sanofi-Aventis) in clinical studies, no antibody-based therapy is currently commercially available for therapy of AML. Issues with novel targeted therapies may come from the wide range of genetic factors contributing to AML, the absence or low expression of available targets on leukemic stem cells, shortcomings of the drug's mode of action, and severe immunosuppression of patients.

Most clinical experience was gained with CD33 as target antigen (4, 5). It is predominantly expressed on myeloid-derived cells and found overexpressed on bone marrow cells from patients with AML, chronic myelogenous leukemia, and myelodysplastic syndrome (6). Importantly, CD33 has been found expressed on leukemic stem cells, and only $CD33^+$ leukemic stem cells gave rise to leukemia in mice (7, 8). The level of shed CD33 was recently described as marker for AML disease progression (9, 10). A novel modality to use CD33 for targeting AML blasts and leukemic stem cells are T-cell–engaging antibodies of

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the bispecific T-cell–engaging (BiTE, a registered trademark of Amgen) format (11–13). The CD19/CD3-bispecific BiTE antibody blinatumomab (AMG 103; Amgen) has shown high clinical activity against CD19-expressing non-Hodgkin lymphoma (NHL; refs. 14, 15) and acute lymphoblastic leukemia (ALL; refs. 16, 17).

Following the paradigm of blinatumomab in B-cell malignancies, we have generated a novel BiTE antibody called AMG 330 for treatment of AML by redirecting polyclonal T cells against CD33-expressing cells. AMG 330 has previously shown robust *ex vivo* activity in peripheral blood mononuclear cell (PBMC) samples from patients with AML by redirecting autologous T cells for lysis of AML blasts at low endogenous effector-to-target (E:T) ratios (18). Here, we have characterized AMG 330 in detail for its dual binding activity, CD33 epitope recognition, stability in serum, cross-reactivity with a non-human primate species, potency of redirected lysis of multiple human AML cell lines, potency at reduced E:T ratios, sensitivity toward shed CD33, T-cell activation, and impact of intravenously (i.v.) and subcutaneously (s.c.) administered AMG 330 on mouse survival in a xenograft model. From all these analysis, AMG 330 emerged as a suitable and highly potent BiTE antibody with potential for treatment of AML by engaging previously resting, polyclonal T cells for redirected lysis of $CD33^+$ blasts.

Material and Methods

Affinity determination by surface plasmon resonance

Biacore analysis experiments were performed using the recombinant extracellular domain of human or macaque CD33 fused to murine antibody Fc-γ to determine affinity of AMG 330 to CD33. For CD3 affinity measurements, recombinant fusion proteins containing the N-terminal 27 amino acids of human or macaque CD3ε fused to human antibody Fc portion were used.

CM5 Sensor Chips (GE Healthcare) were immobilized with approximately 100 RU of the respective recombinant antigen using acetate buffer pH4.5 according to the manufacturer's manual. The BiTE antibody samples were loaded in five concentrations: 50 nmol/L, 25 nmol/L, 12.5 nmol/L, 6.25 nmol/L, and 3.13 nmol/L diluted in HBS-EP running buffer (GE Healthcare). Flow rate was 30 to 35 μL/minute for 3 minutes, then HBS-EP running buffer was applied for 7 to 8 minutes again at a flow rate of 30 to 35 μL/minute. Regeneration of the chip was performed using 100 mmol/L glycine and 0.5 mol/L NaCl pH 2.0. Datasets were analyzed using BiaEval Software. In general, two independent experiments were performed.

Epitope determination using linear peptide mapping

The CD33-binding scFv of AMG 330 was subjected to an epitope mapping approach based on peptide scanning (Pepscan). Two hundred and twenty-three different 20-mer peptides were synthesized that span the entire extracellular amino acid sequence of human CD33 and overlap with each neighboring 20-mer peptide by 19 amino acids. These peptides were coated to ELISA wells in a 384-well plate format. For this experiment, the anti-CD33 scFv of the BiTE antibody AMG 330 was produced in Escherichia *coli* and crude periplasmic extracts were used for ELISA. The scFv was incubated with the peptides and specific binding detected using an anti-hexahistidine antibody. Binding signals were measured in a 384-well ELISA reader (19, 20).

Cell lines and cell culture

U-937, EOL-1, HL-60, KASUMI-1, KG-1, M-07e, MOLM-13, NB-4, NOMO-1, SKM-1, THP-1, and NALM-6 were purchased from the "Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures" (DSMZ, Braunschweig, Germany). The cell lines were obtained before the experiment series and analyzed for authenticity by the respective cell bank (DNA-fingerprinting techniques such as short tandem repeat profiling). No additional identification was conducted in-house. All cells were cultured in RPMI-1640 (Invitrogen) and 10% fetal calf serum (Invitrogen). Cells were cultured at 37°C in a 5% CO_2 chamber.

Quantification of CD33 cell surface expression, cytotoxicity, T-cell activation, and cytokine release

The number of CD33 surface molecules on different human AML cell lines was determined by saturation binding using the monoclonal anti-CD33 antibody clone WM-53 (BD Biosciences) and the Dako QIFIKIT according to the manufacturer's instructions.

Redirected T-cell cytotoxicity was assayed by flow cytometry using human PBMC, which had been separated from autologous $CD33^+$ cells, as effector cells and various $CD33^+$ human AML cell lines as targets. PBMCs were isolated by Ficoll density gradient centrifugation. Separation from autologous $CD33^+$ cells was performed by magnetic cell sorting using CD33 MicroBeads (Miltenyi Biotech) according to the manufacturer's instructions. Increasing concentrations of AMG 330 or AMG 103 were incubated with Vybrant DiO (Invitrogen)-labeled target cells as well as effector cells at a PBMC E:T cell ratios of 5:1 or 10:1. Cell lysis was assessed by flow cytometry as loss of target-cell membrane integrity, which is reflected by nuclear uptake of propidium iodide (21).

Activation of $CD4^+$ and $CD8^+$ T cells was determined by CD25 surface expression using an allophycocyanine (APC)-conjugated anti-CD25 antibody in combination with either phycoerythrin (PE)-labeled anti-CD4 or an APC-Cy7-labeled anti-CD8 antibody (BD Biosciences). CD33 on T cells was determined with PE-labeled anti-CD33 (clone WM-53), APC-Cy7-labeled anti-CD8, and PE-Cy7-conjugated anti-CD4 antibodies (BD Biosciences).

Cells were incubated with the appropriate antibody mixtures for 20 minutes at 4°C. Samples were analyzed by flow cytometry on a FACSCanto II flow cytometer (Becton Dickinson) and data were evaluated using the BD FACS Diva software version 6.1.2.

Single-cell preparations from cynomolgus monkey bone marrow aspirates were prepared by filtration of the

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bone marrow cells through a 70-μm filter followed by erythrocyte lysis.

Bone marrow cells were incubated with AMG 330 at 37°C in a 5% CO_2 chamber, and viable $CD33^+$ cells in bone marrow cultures were determined using a PE-labeled anti-CD33 antibody (Miltenyi Biotec) and exclusion of propidium $iodide^+$ cells by flow cytometry. $CD4^+$ and $CD8^+$ T cells were detected with APC-conjugated anti-CD4 and anti-CD8 antibodies (BD Biosciences). For staining of cell surface proteins, cells were incubated with the appropriate antibody mixtures for 30 minutes at 4°C and samples were analyzed by flow cytometry.

To quantify BiTE antibody-induced cytokine release, cytokine concentrations were measured in supernatants of the cytotoxicity assays using the human Th1/Th2 or non-human primate Th1/Th2 cytometric bead arrays (CBA-Kit, BD Bioscience) in accordance to the manufacturer's protocol.

Animal tumor studies

Antitumor activity was evaluated in 7- to 8-week-old NOD.CB17-Prkdcscid/J mice purchased from Harlan (Harlan Laboratories). This strain carries a double mutation that results in a lack of T and B cells as well as impaired natural killer cell function. Animals were group housed in individually ventilated cages on Lignocel dust-free bedding (ssniff). Animals had *ad libitum* access to pelleted feed (ssniff diet # V1534/727) and autoclaved tap water via water bottles.

Animals were maintained on a 12:12 hour light-dark cycle in rooms at 22 ±2°C, and a relative humidity range of 45%–65%) and had access to enrichment opportunities. All animals were determined specific pathogen free for (i.e., mouse parvovirus, Helicobacter). All experiments were performed according to the German Animal Welfare Law with permission from the responsible local authorities.

Female NOD/SCID mice were irradiated with a single dose (2 Gy) of ^{137}Cs γ-ray before intravenous injection of MOLM-13 (1×10^7) tumor cells. To deplete murine natural killer (NK) cells, animals received a single intravenous bolus injection of anti-asialo GM1 rabbit antibody (WAKO) one day before human T-cell injection. The human T cells originated from a single healthy donor, and they were activated and expanded using a T-cell activation/expansion kit (Miltenyi Biotech). With the exception of a vehicle-treated control group ($n = 5$), all animals ($n = 10$ per group) were intraperitoneally injected with T cells on day 3 after tumor cell injection. AMG 330 or vehicle was administered by intravenous bolus injection into the lateral tail vein once daily for 26 consecutive days starting on day 6 after tumor cell injection.

Statistical analysis

One-way ANOVA analysis was performed to compare *in vitro* cytotoxicity assays. Survival was statistically analyzed with the log-rank test using GraphPad Prism version 5.04 for Windows (GraphPad software). A (two-tailed) P value of <0.05 was considered to be statistically significant.

Results

Generation and production of AMG 330

AMG 330 was assembled from four variable immunoglobulin domains by recombinant DNA technology as described in the Supplementary Methods and shown in Supplementary Fig. S1A. The single-chain antibody (scFv) binding to CD33 is positioned N-terminally, and the scFv binding to CD3ε C-terminally followed by a hexahistidine sequence. AMG 330 was stably expressed and produced in a Chinese hamster ovary (CHO) cell clone at levels >0.8 g/L of cell culture. Capture and affinity purification of the BiTE antibody from cell culture supernatants was executed by immobilized metal affinity chromatography followed by size exclusion chromatography. As demonstrated by SDS-PAGE (Supplementary Fig. S1B) and cation exchange chromatography (Supplementary Fig. S1C), AMG 330 was produced as a nonglycosylated, highly homogenous monomer with a mass spectroscopy confirmed molecular size of approximately 54 kDa.

Cross-species binding of AMG 330 to CD33 and CD3ε

As analyzed by fluorescence-activated cell sorting (FACS), AMG 330 bound with similar intensity to CHO cell clones stably expressing human or cynomolgus monkey CD33, and to human T-cell line HPB-ALL or cynomolgus PBMC, respectively. Biacore analysis using immobilized human and macaque CD33 and CD3 proteins, respectively, revealed dissociation rate constants for binding of AMG 330 to human CD3ε of $K_D = 5.1$ nmol/L, for cynomolgus CD3ε of $K_D = 8.0$ nmol/L, for human CD33 of $K_D = 8.0$ nmol/L, and for cynomolgus CD33 of $K_D = 8.6$ nmol/L. These results attest to a cross-species reactivity of AMG 330 and relatively strong binding affinity of monomeric BiTE antibody to target antigens.

Using PepScan analysis, a string of overlapping 20-mer peptides derived from human CD33 was identified that was strongly bound by the CD33-binding scFv of AMG 330. This indicates that AMG 330 recognizes a linear epitope located in the V-set domain of CD33 with the core sequence IPYYDKN. As seen in the crystal structure of human CD33 (22), the presumed epitope is part of a loop structure on the surface that is facing away from the membrane-anchoring part of CD33.

Redirected lysis of human AML cell lines by AMG 330–activated T cells

We have procured eleven different human AML cell lines and determined their level of CD33 surface expression, which ranged from 14,400 to 56,700 molecules per cell (Table 1). As exemplified with $CD33^+$ AML line HL-60, redirected lysis and upregulation of T-cell activation marker CD25 were only observed when both human T cells and AMG 330 were present, but not in the absence of T cells or with an inactive analogue compound,

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Table 1. CD33 surface expression and EC_{50} values for AMG 330–mediated redirected lysis

Cell line	CD33 surface molecules per cell	EC_{50} (pg/mL)
U-937	14,400	23.3
M-07e	15,400	117.8
KASUMI-1	16,200	59.1
NB-4	16,600	76.5
NOMO-1	20,300	132.4
EOL-1	21,200	148.8
SKM-1	29,500	102.9
KG-1	35,900	18.5
THP-1	37,400	33.8
HL-60	45,500	27.4
MOLM-13	56,700	31.4

NOTE: Number of CD33 surface molecules on human AML cell lines was determined using the DAKO QUIFIKIT and the anti-CD33 monoclonal antibody clone WM53. EC_{50} values for redirected lysis of human AML cell lines after 48 hours at an E:T-cell ratio of 5:1 were calculated from dose–response curves using GraphPad Prism software.

which solely binds to CD3ε (Fig. 1A). Similar results were obtained with activation marker CD69, which showed faster kinetics of appearance and disappearance compared with CD25 (data not shown). During redirected lysis, AMG 330 induced a robust release of interleukin (IL)-2, IL-6, IL-10 (data not shown), IFN-γ, and TNF-α (TNF) cytokines into cell culture medium with different kinetics. Highest levels were determined for IFN-γ followed by TNF (Fig. 1B).

To assess the potency of redirected lysis by AMG 330, HL-60 target cells were incubated in the presence of increasing concentrations of AMG 330 with a 5-fold excess of unstimulated PBMC as effector cells, and lysis was measured after 48 hours via FACS by nuclear uptake of propidium iodide (Fig. 1C). Redirected lysis of HL-60 cells was only observed when both human PBMC and AMG 330 were present, but not in the absence of T cells or with an inactive analogue compound that solely binds to CD3ε. AMG 330 dose–response curves for redirected lysis for all eleven AML lines were quite comparable with EC_{50} values ranging between 0.4 pmol/L and 3 pmol/L (18–149 pg/mL) AMG 330 (Table 1).

Both, the dose and time dependency of redirected lysis was analyzed for AML lines HL-60, SKM-1, and KG-1 lines using resting human PBMC as effector cells (Fig. 1D). Unlike preactivated T cells (data not shown), resting T cells required a several-hour lag phase before lysis was detectable. For all three cell lines, complete lysis was achieved after 40 hours of incubation at a PBMC E:T cell ratio of 5:1. In cocultures incubated for prolonged periods of time, EC_{50} values for redirected lysis significantly dropped with KG-1 and SKM-1 target cells, indicating that, even with these more slowly lysed cell lines, T cells became fully activated by AMG 330 over time.

Redirected lysis at lower E:T cell ratios was studied with AML line HL-60 (Fig. 1E). As expected, the extent of cell lysis after 72 hours of incubation was lower at E:T ratios of 1:1 and 1:2 compared with 5:1 and 10:1.

AMG 330 mediates autologous *ex vivo* depletion of $CD33^+$ cells from cynomolgus monkey bone marrow aspirates

Cross-reactivity of AMG 330 with CD33 and CD3 antigens of cynomolgus monkey origin was further investigated in bone marrow aspirates of the nonhuman primate species. Complete *ex vivo* depletion of $CD33^+$ cells by autologous T cells was observed in bone marrow samples at 50 ng/mL AMG 330 (Fig. 2A). Depletion of target cells was accompanied by a robust expansion of $CD4^+$and $CD8^+$ T cells (Fig. 2B). This T-cell expansion increased over time and was maintained for at least 5 days.

Relative insensitivity of AMG 330 activities to soluble CD33

CD33 can be shed and was found in bone marrow plasma of patients with AML at concentrations ranging from 0.4 to 29.6 ng/mL (10). We have investigated whether a recombinantly produced extracellular domain of CD33 (sCD33) can influence target-cell lysis and T-cell activation by AMG 330. Lysis of HL-60 target cells (Fig. 3A) and concomitant upregulation of CD25 (Fig. 3B) by AMG 330 were barely affected by up to 100 ng/mL of sCD33.

The EC_{50} value for lysis marginally increased from 30 pg/mL under control conditions to 55 pg/mL in the presence of 100 ng/mL sCD33, while the overall extent of lysis was not affected.

Neoexpression of CD33 on BiTE-activated T cells is modest

Activated human NK and T cells were shown to newly express CD33 (23). Using ten different healthy donor PBMCs, we have investigated what percentage of $CD4^+$ and $CD8^+$ T cells becomes $CD33^+$ after 48, 72, and 144 hours of T-cell stimulation with anti-CD19 BiTE antibody AMG 103 in the presence of $CD19^+$ NALM-6 target cells. The unrelated BiTE antibody AMG 103 was used to produce a CD33-independent T-cell activation signal. Even at the highest concentration of AMG 103 of 1 μg/mL, PBMC of only three of 10 donors developed 3% to 6% $CD33^+$ T cells after 144 hours of incubation (Fig. 4). At 0.731 ng/mL AMG 103, a serum concentration with high clinical activity (24), only a background frequency of 2% or less of $CD33^+$ T cells was detected after 144 hours of incubation.

AMG 330 prolongs survival in MOLM-13 xenograft model

A survival model was established in NOD/SCID mice using intravenously transferred human AML line MOLM-13 and resting human T cells as effector cells. With the vehicle control alone, mice did not survive a

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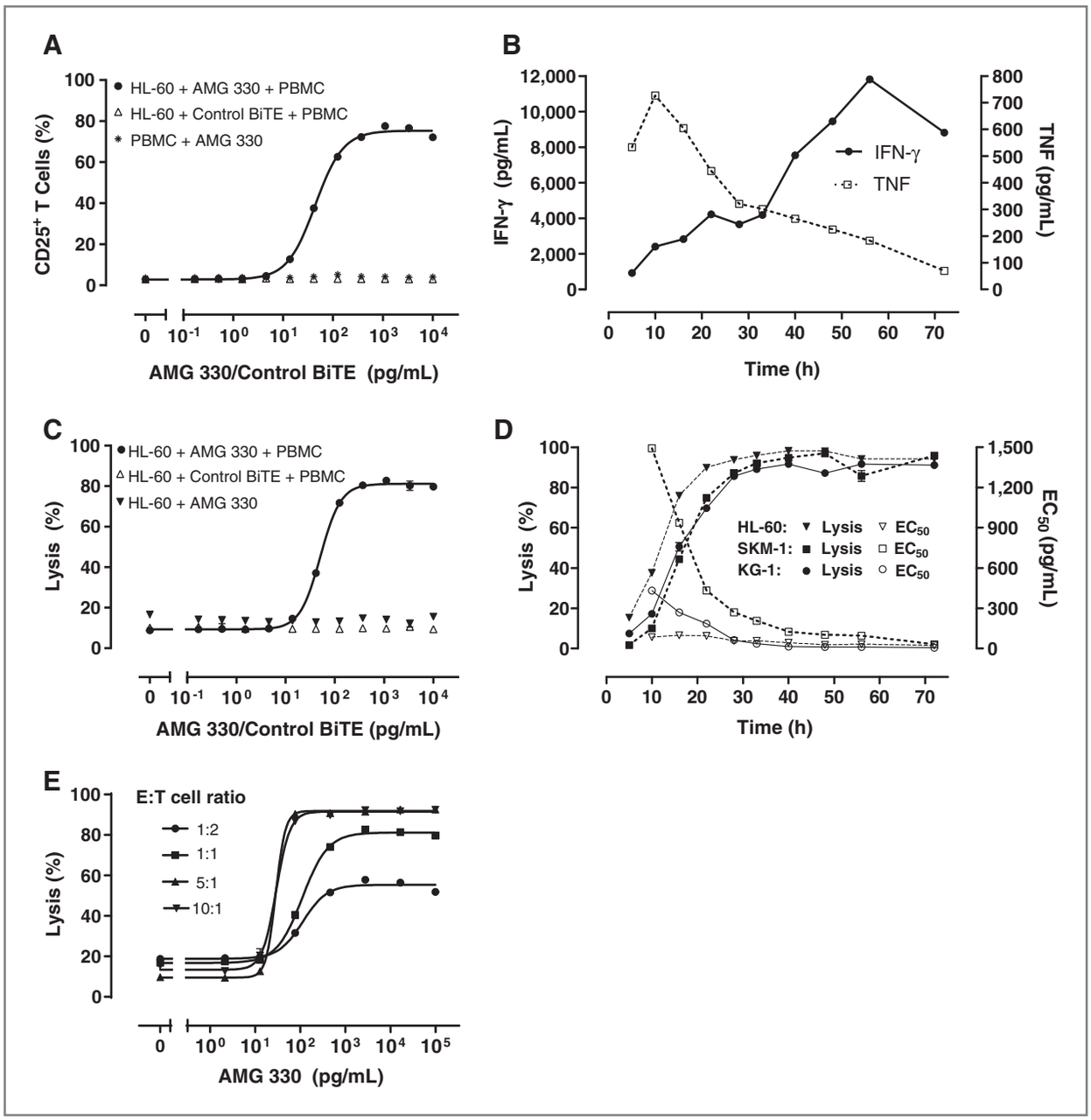

Figure 1. Effects of AMG 330 on T-cell activation, cytokine release, and redirected lysis in coculture experiments. HL-60 human AML target cells were incubated with or without human PBMC effector cells at an E:T-cell ratio of 5:1 and increasing concentrations of AMG 330. An inactive analogue compound solely binding to CD3 served as a control. A, T-cell activation was analyzed by determination of CD25 on $CD4^+$ and $CD8^+$ cells after 48 hours by flow cytometry. Significant differences in CD25 expression were observed between cocultures without target cells or with control BiTE compared with cocultures of HL-60, AMG 330, and effector cells ($P < 0.05$). Each data point represents the mean of triplicate measures and error bars represent SEM. B, human PBMC were coincubated with HL-60 cells at a PBMC to target-cell ratio of 5:1 and 2.8 ng/mL AMG 330 for 48 hours. Concentrations of TNF and IFN-γ in the cell culture supernatants were determined using the BD Cytometric Bead Array (CBA) Human Th1/Th2 Cytokine Kit II by flow cytometry. C, HL-60 cells were incubated with or without human PBMC effector cells at an E:T-cell ratio of 5:1 and increasing concentrations of AMG 330. An inactive analogue compound solely binding to CD3 served as a control. Target-cell lysis was determined by monitoring of propidium iodide uptake into nuclei of lysed cells by flow-cytometric determination after 48 hours. Significant differences in target cell lysis were observed between cocultures without effector cells or with control BiTE compared with cocultures of HL-60, AMG 330, and effector cells ($P < 0.05$). Each data point represents the mean of triplicate measures and error bars represent SEM. D, EC_{50} values (right axis; open symbols) or maximum lysis at 2.8 ng/mL AMG 330 (left axis; filled symbols) were plotted versus time for the target cell lines HL-60, SKM-1, and KG-1. Each data point for target cell lysis represents the mean of triplicate measures and error bars represent SEM. EC_{50} values were calculated from dose–response curves based on triplicate measurements of redirected lysis. E, HL-60 cells were cocultured with PBMC at various ratios and incubated with increasing AMG 330 concentrations. Target-cell lysis was measured by propidium iodide uptake after 72 hours using flow cytometry. Each data point represents the mean of triplicates, and error bars represent SEM.

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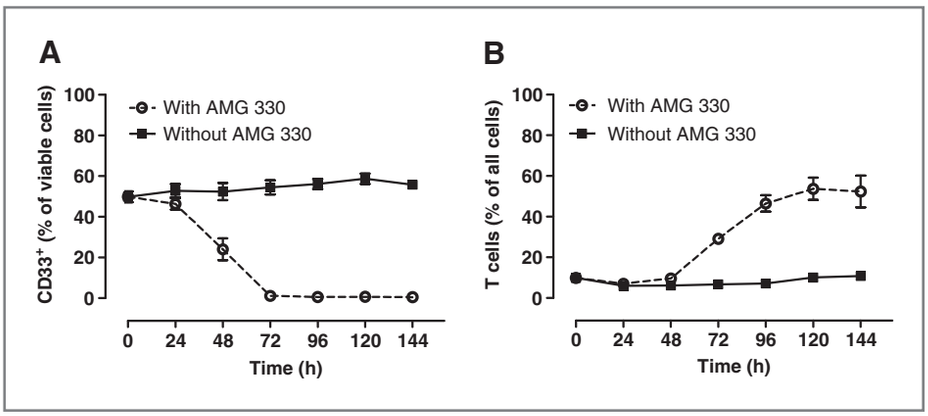

Figure 2. AMG 330–mediated autologous *ex vivo* depletion of $CD33^+$ cells from cynomolgous monkey bone marrow grafts. A, cynomolgus monkey bone marrow cells were cocultivated with or without 50 ng/mL AMG 330 and the percentage of viable $CD33^+$ cells was determined at the indicated times by flow cytometry. A significant decrease of CD33 was observed from 72 hours onwards ($P < 0.05$). B, bone marrow cells from healthy cynomolgus monkeys were coincubated with or without 100 ng/mL AMG 330 and the percentage of $CD4^+$ and $CD8^+$ T cells was determined at the indicated times by flow cytometry. A significant increase of T cells was observed from 72 hours onwards ($P < 0.05$). Data points represent means of four different donors. Error bars represent SEM.

dose of 1×10^7 MOLM-13 AML cells beyond day 23 (Fig. 5), and coadministration of T cells did not significantly impact survival. However, even the lowest AMG 330 dose of 0.002 mg/kg administered daily i.v. significantly extended survival with mice surviving up to 44 days ($P < 0.001$). AMG 330 doses of 0.02 and 0.2 mg/kg were equally potent with 30% to 50% of mice still being alive after 111 days ($P < 0.001$). No human $CD33^+$ cells were detected in the bone marrow of these animals at the end of the study (data not shown).

Discussion

With AMG 330, we have produced a novel prototypic BiTE antibody with characteristics very similar to those previously described for blinatumomab (25, 26). Like the CD19/CD3-bispecific BiTE antibody, the CD33/CD3-bispecific AMG 330 showed a dose-dependent activation of T cells as was evident by upregulation of CD69 and CD25 at low picomolar concentrations, which was dependent on the presence of target cells, and not seen with an inactive analogue solely binding to CD3 on T cells. T-cell activation by AMG 330 also involved transient release of inflammatory cytokines with individual kinetics. Likewise, redirected target cell lysis was dependent on dual binding and showed EC_{50} values for lysis in the same concentration range reported for blinatumomab. Lysis of $CD33^+$ cells in coculture experiments reached completion after 40 hours. With some AML lines, like SKM-1, the potency of AMG 330 was low at the beginning but dramatically increased during the first day of coculture, as seen by a reduction in EC_{50} value. The lag in time to maximal activity suggests that T cells may require some time to ramp up their level of granzymes and perforin or that there is a time-dependent increase in the ability of T cells to form cytolytic synapses. Intrinsic resistance mechanisms of some AML cell lines may create a higher

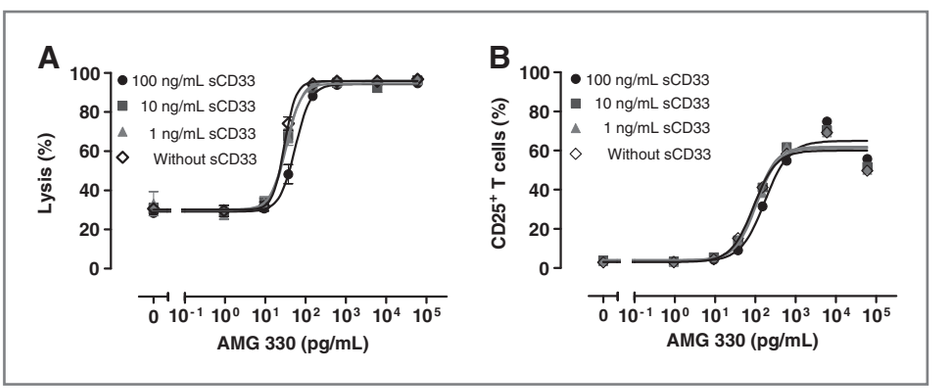

Figure 3. Impact of sCD33 on AMG 330-mediated redirected lysis and T-cell activation. Various concentrations of recombinant sCD33 were coincubated for 48 hours with PBMC, HL-60 target cells, and increasing concentrations of AMG 330. A, AMG 330–mediated redirected target cell lysis was determined by flow cytometry. B, T-cell activation was analyzed by determination of CD25 expression on $CD4^+$ and $CD8^+$ cells by flow cytometry. Data points represent means of duplicate samples. Error bars represent SEM.

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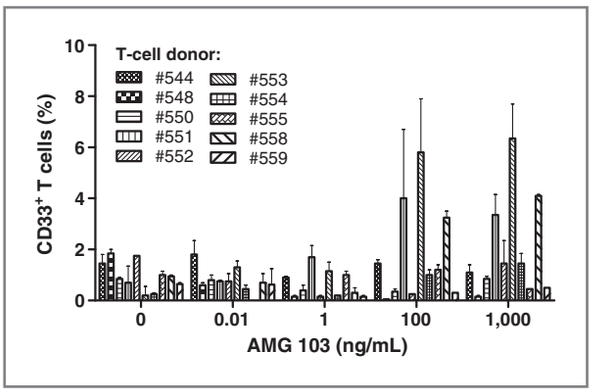

Figure 4. Neoexpression of CD33 on BiTE-activated T cells. Human PBMCs from ten donors were cocultured with BiTE antibody AMG 103 and the target cells NALM-6 at an E:T-cell ratio of 10:1 for 144 hours. Expression of CD33 on $CD4^+$ and $CD8^+$ cells was determined by immunostaining and flow cytometry. Bars represent means of duplicate samples. Error bars represent SEM.

bar for initial activity that is overcome with additional exposure. Differences in response behavior may also occur among blasts from patients with AML. This indicates that longer term exposure to AMG 330 may be required to fully engage T cells for lysis of certain AML lines and blasts. An *ex vivo* study indeed suggested that complete lysis of AML blasts by AMG 330 at the low

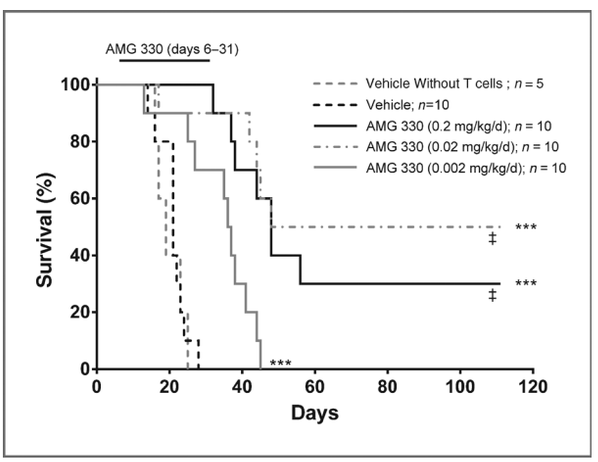

Figure 5. Antitumor activity of AMG 330 in a MOLM-13 xenograft model in NOD/SCID mice. MOLM-13 (1×10^7) human AML cells were administered intravenously to female NOD/SCID mice and animals were allocated to treatment groups ($n = 5$ or 10). *In vitro* expanded and activated human T cells were transplanted into the peritoneal cavity except for five animals that served as control on day 3. Five animals, which had not been injected with human T cells, served as a control to monitor the impact of T cells on tumor growth. A second control group ($n = 10$) injected with T cells was treated with vehicle. Finally, all AMG 330–treated groups contained 10 animals each. Animals were treated from day 6 onwards with the indicated doses of AMG 330 or vehicle once daily for 26 days by intravenous bolus injection into the lateral tail vein. Asterisks denote statistically significant differences (log-rank test; ***, $P < 0.001$) between vehicle- and AMG 330–treated groups. ‡, no $CD33^+$ cells were detected at necropsy in the bone marrow of the animals euthanized on day 111.

frequency of autologous T cells present in patients' PBMC samples required incubation periods of at least 5 days or longer (18). We here confirmed with human AML lines that AMG 330 has considerable cytolytic activity at an E:T ratio of 5:1.

Recent studies have demonstrated that AMG 330 can effectively recruit autologous T cells, induce their proliferation, and mediate redirected lysis of AML blasts in a time-, dose-, and E:T-cell ratio-dependent manner (27, 28). EC_{50} values for redirected lysis were found to correlate with the number of surface-expressed CD33 (28).

An important feature of AMG 330 is its cross-reactivity with CD33 and CD3 of nonhuman primates, which may give it an advantage over other CD33/CD3-bispecific antibodies in early development (29). This feature allows investigation of toxicology and pharmacodynamics in a relevant species, the cynomolgus monkey, and allows determination of a safe starting dose of the BiTE antibody for first-in-human trials. We have here observed that AMG 330 was able to *ex vivo* deplete $CD33^+$ target cells and expand T cells in bone marrow samples from monkeys by the endogenous T cells thereby confirming the validity of the nonhuman primate species.

Recent studies have revealed two characteristics of the CD33 target that needed to be explored in this study for their impact on a CD33-specific BiTE antibody. One is shedding of CD33 (9, 10), which could create a sink for AMG 330 in plasma or bone marrow inhibiting its target binding. The other is neoexpression of CD33 on activated T cells (23), which would turn newly activated T cells into $CD33^+$ target cells for lysis. As previously reported for BiTE antibodies binding to CEA (30) and EpCAM (31), we could not observe a significant impact of soluble CD33 at physiologically relevant concentrations on redirected lysis or T-cell activation by AMG 330. Neoexpression of CD33 on blinatumomab-activated T cells did not exceed 6% of all T cells even after very long incubation periods. This is in contrast with CD69 or CD25 where typically >50% of all T cells become within short time positive for the activation markers following BiTE stimulation (21, 32, 33). We therefore do not consider shedding or CD33 neoexpression on a low percentage of activated T cells a particular issue for the efficacy of AMG 330.

Potency and efficacy in xenograft models can also provide key decision parameter to further develop a drug candidate. We here observed that daily intravenous dosing of AMG 330 for 26 days significantly impacted survival of NOD/SCID mice with leukemia caused by human MOLM-13 AML cells. Even the lowest dose of 2 μg AMG 330/kg per day prolonged survival, while it required at least 20 μg/kg to cure >30% of mice. This shows an outstanding *in vivo* potency of AMG 330 in line with the high *in vitro* cytotoxicity observed in coculture assays.

The exquisite cytotoxic potential of T cells has thus far not been leveraged for treatment of AML, although it has been very successful for treating patients with NHL and ALL as exemplified by both blinatumomab (14, 34, 35),

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and T cells expressing a CD19-specific chimeric antigen receptor (36). A T-cell–based therapy of AML may now become possible with the novel CD33/CD3-bispecific BiTE antibody AMG 330.

Disclosure of Potential Conflicts of Interest

T. Raum has ownership interest in a patent. P. Hoffmann has ownership interest in patents. R. Kischel has ownership interest in Inventorship in Amgen-held patents. P. Kufer has ownership interest in a patent. No potential conflicts of interest were disclosed by the other authors.

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Molecular Cancer Therapeutics

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