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Dual-targeting triplebody 33-16-123 (SPM-2) mediates effective redirected lysis of primary blasts from patients with a broad range of AML subtypes in combination with natural killer cells

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ABSTRACT

A number of agents designed for immunotherapy of Acute Myeloid Leukemia (AML) are in pre-clinical and early clinical development. Most of them target a single antigen on the surface of AML cells. Here we describe the development and key biological properties of a tri-specific agent, the dual-targeting triplebody SPM-2, with binding sites for target antigens CD33 and CD123, and for CD16 to engage NK cells as cytolytic effectors. Primary blasts of nearly all AML patients carry at least one of these target antigens and the pair is particularly promising for the elimination of blasts and leukemia stem cells (LSCs) from a majority of AML patients by dual-targeting agents. The cytolytic activity of NK cells mediated by SPM-2 was analyzed *in vitro* for primary leukemic cells from 29 patients with a broad range of AML-subtypes. Blasts from all 29 patients, including patients with genomic alterations associated with an unfavorable genetic subtype, were lysed at nanomolar concentrations of SPM-2. Maximum susceptibility was observed for cells with a combined density of CD33 and CD123 above 10,000 copies/cell. Cell populations enriched for AML-LSCs (CD34pos and CD34pos CD38neg cells) from 2 AML patients carried an increased combined antigen density and were lysed at correspondingly lower concentrations of SPM-2 than unsorted blasts. These initial findings raise the expectation that SPM-2 may also be capable of eliminating AML-LSCs and thus of prolonging survival. In the future, patients with a broad range of AML subtypes may benefit from treatment with SPM-2.

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Introduction

Current chemotherapeutic treatments (CT) induce a complete remission (CR) for 60–80% of AML patients, but more than 50% of the initial responders experience relapse within 5 years after CR.[1,2] Prognosis for patients with relapsed disease is generally poor, and therefore, new treatment options are urgently needed. Recurrence of the disease is thought to be caused by Leukemia Stem Cells (LSCs) and leukemic progenitor cells which survived CT.[3–7] New treatment strategies attempting to reach prolonged and deeper remissions therefore need to aim at an improved elimination of AML LSCs. Immuno-therapeutic approaches are promising for this purpose. One possibility is to direct cytolytic effector cells specifically towards AML cells through the identification of suitable target antigens expressed on their surface and the development of antibodies and antibody-derived agents with specificity for these targets. This approach, termed "redirected lysis" (RDL), is pursued by several agents currently under development and in clinical testing.

Suitable target antigens for RDL of both AML blasts and LSCs include CD33 and CD123. Blasts from 85–90% of AML patients as well as normal myeloid progenitors and myelocytes express CD33.[8,9] Expression is restricted to normal and malignant hematopoietic cells, including AML-LSCs,[10–13] and therefore, CD33 is a promising target for immunotherapies of AML.[8,10,13]

Gemtuzumab-Ozogamycin (GO, Mylotarg®), a CD33-directed antibody-drug conjugate (ADC), is an approved drug for the treatment of AML.[14,15] The agent has clinical efficacy for certain subtypes of AML, but its commercial availability was suspended due to safety concerns.[15] However, in spite of these limitations, the agent produced clear clinical benefits, including long-lasting treatment successes in particular after administration in fractionated doses to limit toxicity, probably due to its ability to eliminate some of the relapse-initiating LSCs.[16,17] Another CD33-

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directed antibody-drug conjugate, SGN-CD33A (Vadustuximab Talirine), is in clinical development,[18,19] as well as bispecific T cell engagers (BiTEs; AMG 330 and others),[20–24] and tetravalent tandabs targeting CD33 and engaging T cells as cytolytic effectors (AMV 564).[25] Finally, genetically engineered T cells equipped with transgenic chimeric antigen receptors (CARs) specific for CD33 showed anti-leukemic efficacy in xeno-transplanted mice and were tested in an AML-patient.[26–31] Taken together, these developments establish CD33 as a clinically validated target of proven usefulness for the treatment of AML.

To further increase selectivity and efficacy of immunotherapeutic agents, it is desirable to include additional antigens into the spectrum of targets, which can help to discriminate further between normal hematopoietic stem cells (HSCs) and leukemia stem- and progenitor cells. A number of antigens potentially useful towards this goal have been reported, including CLL-1 (also called hMICL), Tim-3, CD96, CD44, CD45RA, CD47, CD32, CD25, CD123 and CD157.[32–39] Among these, CD123 has particularly favorable properties for the development of immunotherapeutic agents.

CD123, the α-chain of the interleukin-3 receptor, is expressed on normal myeloid cells and their progenitors, and on blasts and AML-LSCs from 75–89% of AML-patients.-[8,40–45] In a landmark study, fewer than 1% of CD34pos CD38neg progenitor cells from a normal human bone marrow (BM) expressed CD123, while 99% of the corresponding cells from a patient with CD34-positive AML showed high grade expression. CD34pos CD38neg cells, which are enriched in AML-LSCs for patients with CD34-positive AML, from 16 of 18 primary AML samples showed strong expression of CD123.[40] Another prominent study also reported CD123 expressed on the surface of CD34pos CD38neg AML cells, but in this study the antigen was also present on the corresponding cellular subsets from healthy bone marrow (BM) and cord blood (CB), although expression levels on the enriched normal BM cells were at least 10-fold lower than on on the corresponding AML cells.[12] CD123 is also expressed on normal HSCs from AML patients in follow-up after induction therapy.[46] On aggregate, the published reports agree that CD123 is expressed with far greater frequency and surface densities on AML-LSCs and progenitor cells than on normal HSCs.[7] A fraction of normal HSCs may therefore survive therapies targeting CD123.[27] Finally, CD123 is also expressed on cells from other hematologic malignancies including Acute Lymphoblastic Leukemia (ALL), Chronic Myeloid Leukemia (CML), Myelo-Dysplastic Syndromes (MDS), Hodgkin Lymphoma (HL), Hairy Cell Leukemia (HCL) and others.[44,45]

CD123 therefore is an attractive target for the design of immunotherapies against AML. A number of therapeutic agents with similar molecular formats as those described for CD33 have been developed, including immunoglobulins,[47–49] a radioimmunoconjugate,[50] ADCs and a fusion protein between the cytokine IL-3 and a fragment of diphtheria toxin,[51–54] bi-specific T cell-recruiting agents (BiTEs) and Dual-Antigen Recruiting T-cell engagers (DARTs),[55–58] dual-targeting triplebodies,[59,60] and CAR-transfected T cells.[61–64] Expression of CD123 is largely restricted to hematopoietic cells, but expression on endothelial cells has been reported.[65] Importantly, CD123 shows low expression on megakaryocytic progenitors.[66].

Most of the agents developed to date for the treatment of AML are mono-targeting. However, under treatment of other malignancies with mono-targeting agents, escape variants have often emerged. An example is the CD20 antibody Rituximab. After long-term treatment with this agent, escape variants occurred in almost one third of lymphoma patients.[67] Similarly, after treatment of Acute Lymphoblastic Leukemia (ALL) patients with CAR-transfected T-cells specific for CD19, escape variants were frequently observed.

To alleviate this problem of escape variants our team has developed a new class of dual-targeting agents designed for simultaneous binding to one copy each of 2 different targets on the surface of the same cancer cell. We anticipated that this molecular design should permit us to reach 2 main objectives: a) a significant reduction of the probability for emergence of "double-escape variants", simultaneously unresponsive to agents targeting both antigens; and b) an "enhanced selectivity of lysis". This term signifies that in a mixed population of normal and malignant cells, as it is typically present in a tumor environment, the agent should preferentially bind and destroy the malignant cells with the help of the cytolytic effectors it engages. We hypothesized that "enhanced selectivity of lysis" can be achieved by dual-targeting triplebodies directed against a suitably chosen pair of target antigens, which are present on the cancer cells in a greater combined surface density than on the corresponding normal cells.[68–70] We have searched for a pair of target antigens on AML cells and AML-LSCs fulfilling this condition, and the pair of CD33 and CD123 emerged a promising candidate.

Published studies so far have mostly addressed the expression of CD33 and CD123 individually in patient samples, and the cohorts analyzed in clinical studies were small.[8] Larger studies in patients with many different subtypes of the disease are needed to investigate, whether new dual-targeting agents simultaneously addressing both antigens on the same cell [59,60] or combinations of corresponding mono-targeting agents are promising for clinical applications for a broad group of AML patients. In a first large study pursuing this goal, the expression of CD33 and CD123 alone and in combination was investigated on cellular samples from 319 AML patients.[8] Samples from 88% of the patients expressed CD33, 9 % expressed CD123 without concomitant expression of CD33, and 69% expressed both antigens simultaneously. Blasts from patients with mutations in the NPM-1 gene showed elevated expression of CD33 and CD123, suggesting that Measurable Residual Disease (MRD)-guided and MRD-directed interventions with immunotherapeutic agents simultaneously targeting CD33 and CD123 may become feasible for these patients.[8]

Our team has designed the dual-targeting triplebody 123–16-33 with specificity for CD123 and CD33.[60] Here we describe the generation of the clinical candidate SPM-2, an optimized variant carrying humanized and disulfide-stabilized single-chain variable fragments (scFvs) as antigen binding sites, plus additional mutations favoring its clinical development. We anticipate that SPM-2 will be able to discriminate at least to a degree between AML-LSCs and remaining normal HSCs of a patient, due to its dual-

targeting capacity and the greater combined surface density of CD33 and CD123 on AML-LSCs than on normal HSCs.[11,12,24,27,40,46] A preferential elimination of AML-LSCs over normal HSCs would be a distinct advantage, if it could be reached, because the surviving HSCs may be able to reconstitute the patient's hematopoietic system after the end of therapy at least in part, possibly even without the need for a stem cell transplant. Here we have studied, whether SPM-2 in conjunction with activated human NK cells was capable of eliminating blasts from patients with a very broad range of AML subtypes, as predicted,[8] and found the prediction to be fulfilled.

Finally, as a first step towards a test of our hypothesis claiming that SPM-2 should be able to mediate the elimination also of AML-LSCs by NK cells, we asked, whether the subsets of CD34-positive and (CD34-positive, CD38-negative) cells from patients with CD34-positive AML, which are enriched in AML-LSCs, were susceptible to lysis by NK cells plus SPM-2. The initial still incomplete results reported here are consistent with this hypothesis.

Results

Design, production and protein-chemical characterization of SPM-2

Expression vectors for SPM-2 were constructed from pre-existing cDNA-bearing vectors encoding scFv-domains specific for CD33, CD123 and CD16.[59] The original scFv domains were of murine origin[59] and were humanized, disulfide-stabilized and stability-engineered for incorporation into SPM-2. A final clinical candidate named SPM-2 was identified, and a production process in stably transfected human Freestyle™ 293F-cells, as well as a downstream purification process following industry standard procedures was developed. The protein had a molecular mass of 82.5 kDa and expression yields were in the range of 2 – 5 mg/L of purified protein, sufficient for production in the quantities needed for late preclinical development and clinical use. The final product was highly pure and contained neither abundant breakdown products nor detectable aggregates (Figure 1). A suitable formulation buffer with industry standard composition has been developed, and in long-term stability studies the protein was stable in this buffer at 4°C for 12 months and longer. Only marginal degradation was observed after 12 months of storage (Figure 1B). The catalytic activity after storage for 12 months at 4°C was still in the picomolar range (EC_{50} values of approx. 50 pM, where EC_{50} is the concentration, for which half-maximum lysis is obtained) as judged by *in vitro* RDL assays with the human AML-derived target cell line MOLM-13. This line carries elevated surface densities of CD33 and CD123 and is highly susceptible to lysis by SPM-2 plus NK cells (Figure 2; Supplement Figure 1, Supplement Table 1). The protein had good thermostability, and the monovalent binding affinities (equilibrium dissociation constants; K_D) of the individual binding sites for CD33 and CD123 were in the low nanomolar range. Early preclinical development of the agent is advanced, and the agent is ready for late preclinical development and advancement to first-in-human (FIH) clinical studies.

Lysis of primary blasts from patients with different subtypes of AML by SPM-2 plus NK cells

To test the prediction that agents capable of bivalent binding to one copy each of CD33 and CD123 on the same AML blast should be able to eliminate blasts from almost all AML patients,[8] RDL experiments were performed with primary cells from a panel of 29 patients with a broad range of AML subtypes. The panel included patients with AML belonging to all genetic risk groups according to the ELN (European Leukemia Network) classification,[2] (Table 1). For *in vitro* cytolysis assays the target cells were labeled with calcein.[60,68] Peripheral blood mononuclear cells (PBMCs) from an unrelated healthy donor were expanded in culture for 20 d in the presence of IL-2. These cells, called lymphokine-activated killer cells (LAK cells), consisted of approx. 25% NK cells, 70% T cells and a small fraction of NKT cells[69,71] and were used at an effector-to-target cell (E: T) ratio of NK cells: targets of 2: 1. After a 4 hr reaction the extent of specific lysis mediated by the agent (beyond the spontaneous lysis achieved by the LAK cells alone, in the absence of added triplebody) was measured by calcein release, and dose-response curves were plotted (Figure 2). From these curves, half-maximum effector concentrations (EC_{50} values) were computed. Target cells were MNCs enriched by density centrifugation from bone marrow (BM) or peripheral blood (PB) samples (BMMCs; PBMCs), either freshly drawn or stored under liquid nitrogen.

Cells from all 29 patients responded to lysis by SPM-2 plus NK (LAK) cells (Figure 2A-E). First cytolytic effects were seen at 10 pM concentrations of SPM-2 for many samples, and all samples responded at 100 pM and above. For some samples, such as the sample from patient #25, the extent of specific lysis was small, but was still clearly measurable above background within the sensitivity of the assay. MOLM-13 cells were carried along as a positive control (standard of susceptibility), and the triplebody Her2-16-Her2 was used as a negative control. This is a triplebody in the same molecular format as SPM-2, but with binding sites for Her2, a tumor antigen expressed on human breast cancer cells and other carcinoma cells, but not on AML blasts. The Her2-directed triplebody was biologically active in separate control experiments with Her2-positive target cells. In another control, carried along in all experiments, the LAK cells were omitted but the triplebody was present. This control permitted us to evaluate any potential contribution of autologous CD16-bearing effector cells (NK cells, gamma-delta T cells, etc.) contained in the MNC target cell population to the lytic effect. These controls revealed no detectable contributions of autologous effector cells contained in the MNC population. A final control were target cells lacking the expression of CD33 and CD123. For this control (Suppl. Figure1) MNCs prepared from peripheral blood of a healthy donor (MNCs HD) were used as targets, and no measurable specific lysis was observed, although the

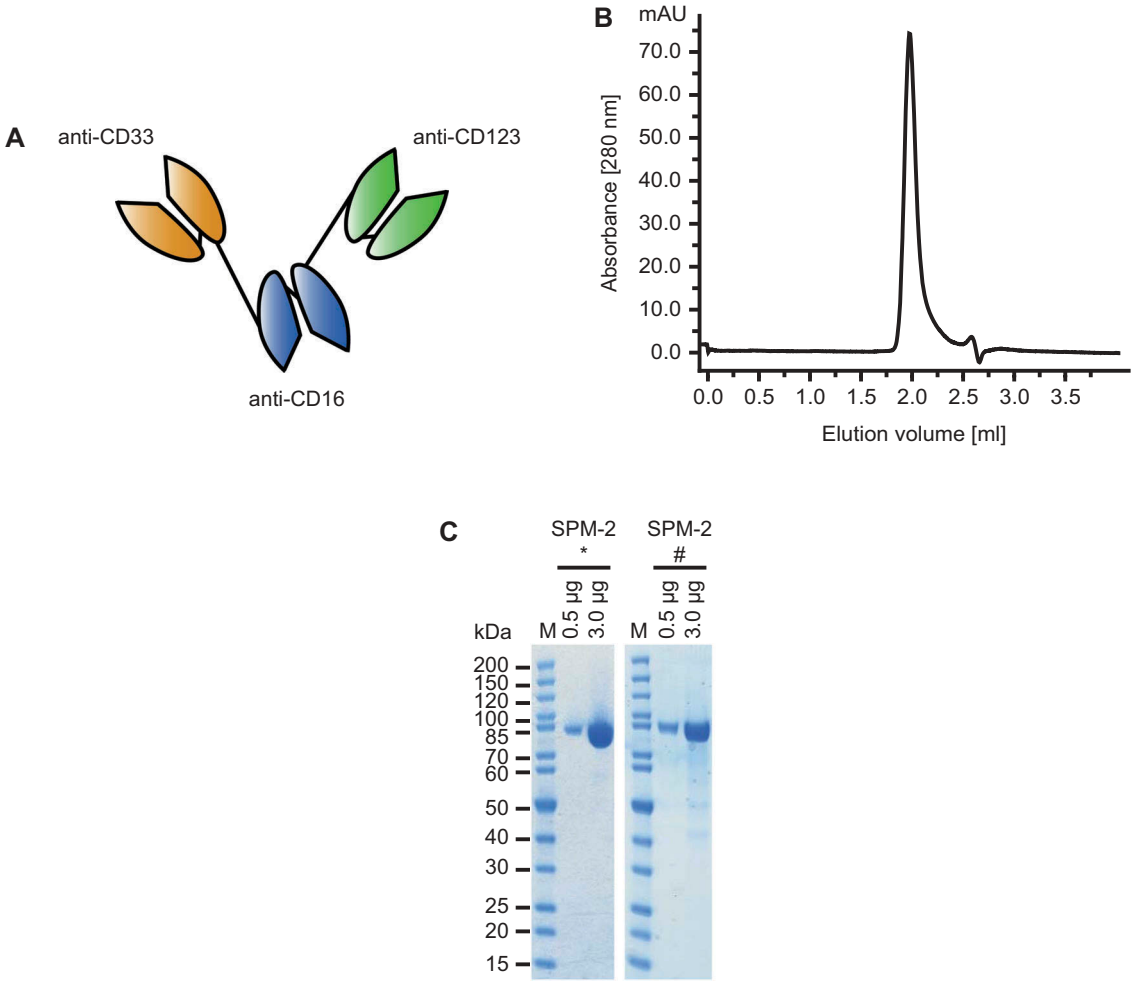

Figure 1. Design and properties of purified SPM-2. A. Molecular structure of triplebody SPM-2 (schematic). Single chain Fragment variable (scFv) domains, consisting of one VH domain joined to one VL domain, are connected into a single polypeptide chain. Black lines: flexible (Gly4Ser) linkers between the domains. One of the two distal scFv domains (orange) is specific for CD33 and the other (green) for CD123. The central scFv domain (blue) binds to CD16 and permits recruitment of various CD16-bearing types of effector cells, including NK and gamma delta T cells. B. After capture with a metal-ion affinity reagent and chromatographic purification the protein appeared as monomeric peak by size exclusion chromatography. C. SMP-2 was analyzed by SDS-PAGE directly after purification (left) and after storage for 12 months at 4°C (right). Only very small quantities of degradation products were observed. *: protein analyzed directly after purification, #: protein analyzed after 12 months of storage at 4°C; M: molecular weight marker in kDa.

sample contained approx. 10% of myeloid cells expressing CD33 and CD123. However, averaged over the entire MNC population the densities amounted to only a few hundred copies per cell (Suppl. Table 1), and specific lysis of this entire population remained below the detection limit.

Only one sample (patient 12) manifested a greater extent of lysis than the MOLM-13 control (Figure 2C). Cells from patients # 5, 14, 18, 23, 26 and 28 with AML subtype FAB M1 (Table 2) responded with EC_{50} values of 242, 229, 221, 475, 58 and 560 pM, respectively (Figure 2B, E). Blasts from patients with a FAB M1 subtype often display a very immature maturation state and express only low levels of CD33, or are CD33 negative, and are often difficult to treat with agents mono-specific for CD33.[60] Cells from patients 2 to 9, 11 to 16, 19, 20, 22, 23 and 25 to 28 with intermediate and adverse ELN genetic subtypes (Table 2) were also lysed efficiently by SPM-2 with EC_{50} values ranging from 10.3 – 1078 pM (Figure 2B-D). These data strongly support the prediction[8] that a dual-targeting agent with specificity for CD33 and CD123 acting together with NK cells should be able to eliminate blasts from almost all AML patients with a very broad range of disease subtypes.

For comparison, the susceptibility of myeloid cells from healthy donors to RDL by SPM-2 or the control triplebody Her2-16-Her2 plus NK (LAK) cells was also examined (Figure 2F). When the total population of PBMCs from healthy donors was used as targets, only marginal lysis was observed (≤ 5% specific lysis; Suppl. Figure 2). This was to be expected, because typically only a small fraction of normal PBMCs (15 – 20%) are myeloid cells. To investigate the effect of SPM-2 plus NK cells on healthy myeloid cells, PBMCs of healthy donors were enriched for CD11b-bearing cells by preparative immuno-magnetic sorting with commercially available beads coated with a CD11b-specific antibody. This resulted in an enrichment of CD11b-bearing myeloid cells to ≥ 90%. These cells were then tested as targets in RDL experiments (Suppl. Table 3). Myeloid cells from normal donors enriched in this manner now demonstrated similar antigen densities and similar susceptibility to lysis by SPM-2 plus NK (LAK) cells as those from the non-

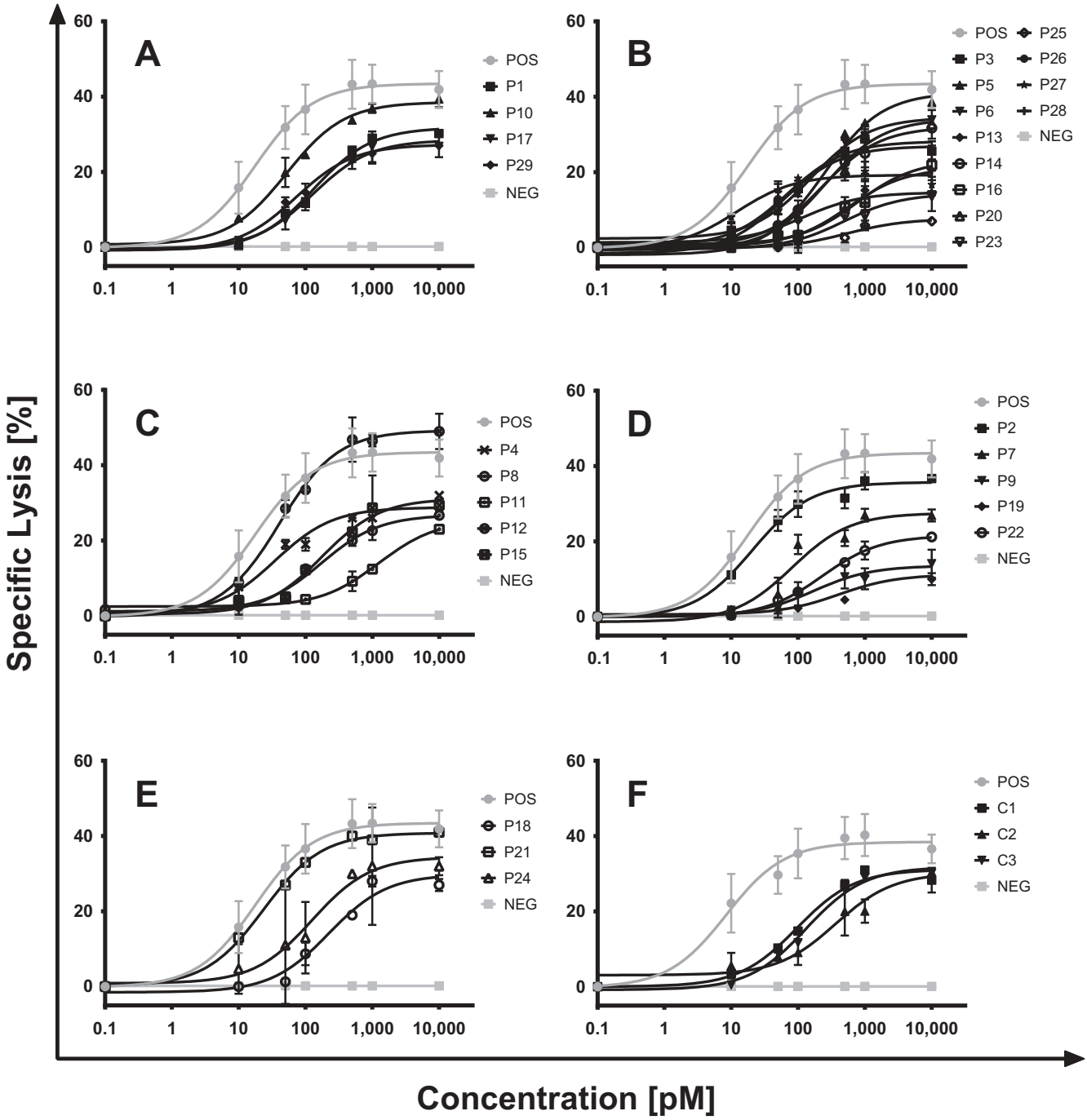

Figure 2. SPM-2-mediated redirected lysis of blasts from patients with different AML subtypes in combination with NK cells from a healthy unrelated donor. Blasts contained in the PBMC or BMMC populations of AML patients were obtained at the disease stages (diagnosis, remission, relapse) specified in Table 1. Blasts were labeled with calcein and used as targets in RDL reactions mediated by SPM-2 or control proteins in combination with *ex vivo* expanded, IL-2 stimulated NK cells from an unrelated healthy donor. NK cells were part of a population of LAK cells, consisting to 70% of T cells, 25% of NK cells, and 5% of NKT cells, after *ex vivo* expansion for 20 d in the presence of IL-2 (Material & Methods). The LAK cells were added in a 10: 1 effector to target cell ratio, corresponding to an effective E: T ratio of NK: targets of 2: 1. SPM-2 triplebody was present in the reactions at the concentrations shown in pM. A) Samples from patients with favorable AML subtype according to the ELN (European Leukemia Network) classification[2]. B) AML with intermediate-I ELN risk subtype. C) Samples from patients with ELN intermediate-II risk subtype. D) samples from patients with adverse ELN risk disease. E) samples from patients with an unclassified disease subtype. F) Myeloid cells from healthy donors (C1, C2), preparatively enriched by immuno-magnetic sorting with CD11b beads show similar susceptibility to SPM-2 mediated lysis as non-enriched blasts from a representative patient sample (C3; patient P1 in Table 1). In all experiments, MOLM-13 cells were carried along as a positive control, and triplebody Her2-16-Her2 as a negative control. Additional controls have previously been performed and reported, showing that target cells devoid of CD33 and/or CD123, such as HEK 293 and CHO cells, failed to bind triplebodies with specificity for CD33 and CD123.[58] Specific lysis was computed as outlined in Materials & Methods. Error bars represent the standard error of the mean (SEM) computed for triplicate samples of each measurement point.

enriched blasts of patient 1 (Figure 2F). They showed no greater susceptibility than the malignant cells from AML patients.

Finally, BMMCs from patients with non-leukemic disorders showing no alterations in their hematopoietic system (donors with non-AML disorders; Suppl. Figure 2 and

Table 1. Patient data and characterization of primary cell samples.

ID	M/F	Age	Diagnosis	Source of material	Blast Count [%]	Cytogenetics	NPM1 mut	FLT3-ITD	ELN genetic group
P1	M	71	AML M2 from MDS	BM	54	46, xy; t(8;21) Runx1-Runx1T1 (AML-ETO)	ND	ND	favorable
P2	M	43	relapsed bi-pheno-typic AL	BM	96	46, xy; complex aberrant MLL rearranged	-	-	adverse
P3	M	61	AML M4	PB	91	46, xy MLL-PTD	+ (Type D)	+ (Flt3-TKD)	intermediate-I
P4	M	24	AML M4	PB	ND	47, xy; +8	-	+	intermediate-II
P5	M	74	AML M1	BM	93	46, xy	-	-	intermediate-I
P6	F	22	AML M5b	BM	83	46, xx	+	+	intermediate-I
P7	M	77	AML M6	BM	24	complex aberrant del(5q31); del(ETV6); del(Nup98)(11p15)	-	ND	adverse
P8	M	81	AML M2	PB	46	46, xy, t(1;21) (p36;q22)	-	ND	intermediate-II
P9	F	74	AML M2	PB	54	complex aberrant RUNX1 amplification	-	ND	adverse
P10	F	72	AML M4	PB	93	46, xx	+	-	favorable
P11	M	61	AML M4	BM	92	del(12)(p12) (partial, 2/8)	-	-	intermediate-II
P12	F	55	AML ND	PB	ND	trisomy 4	+	+	intermediate-II
P13	F	75	AML ND	BM	75	46, xx	ND	+	intermediate-I
P14	M	20	AML M1	BM	96	46, xy	-	-	intermediate-I
P15	M	72	AML ND	PB	93	46, xy; t(6;9) (p22;q34)	-	+	intermediate-II
P16	F	85	AML ND	PB	92	46, xx	+	+	intermediate-I
P17	M	82	AML ND	PB	92	46, xy	+	-	favorable
P18	M	85	AML M1	BM	ND	ND	+	-	unclassifiable
P19	M	46	AML M5	PB	87	47, xy; +8; t(9;11)(p22;923)	-	-	adverse
P20	F	57	AML ND	PB	97	46, xx	+	+	intermediate-I
P21	M	23	AML M3 V	PB	86	46, xy; t(15;17)(q22;q12)	+	+	unclassifiable
P22	M	66	AML M2	PB	ND	44, xy; complex aberrant	-	-	adverse
P23	F	64	AML M1	PB	85	46, xx	+	+	intermediate-I
P24	F	69	AML ND	PB	ND	46, xx	ND	ND	unclassifiable
P25	M	76	AML M5 (from CMML)	PB	94	46, xy	-	-	intermediate-I
P26	F	50	AML M1	BM	ND	46, xx	-	-	intermediate-I
P27	M	69	AML M4	PB	91	46, xy	+	+	intermediate-I
P28	M	42	AML M1 (refractory)	PB	86	46, xy	+	+	intermediate-I
P29	M	61	AML M4	BM	97	46, xy	+	-	favorable

ND: not determined; BM: bone marrow; PB: peripheral blood; genetic risk groups assigned according to European Leukemia Net (ELN) classification [3, 4]; M,F: male, female; TKD = Tyrosine Kinase Domain; blast counts were determined after Ficoll density enrichment of BM MNCs (mononuclear cells) by cytofluorimetry; gates were set in an SSC vs. CD45 plot as illustrated in Supplementary Figure 3

Suppl. Table 2) were tested by RDL, and only low levels of lysis were found. These cells were not enriched for myeloid cells by preparative sorting with CD11b immuno-magnetic beads and therefore showed similar susceptibility as non-enriched PBMCs from unrelated healthy donors. This finding suggests that the hematotoxic damage in the bone marrow that has to be expected for the normal hematopoietic cells of patients with AML under treatment with SPM-2 will be limited relative to the therapeutic effect on the malignant cells.

Surface antigen densities of CD33 and CD123 on primary cells from patients with different subtypes of AML and from patients with non-hematologic disorders

A loose correlation between cytolytic and cytotoxic activity and target antigen density has been reported for the CD33-specific agents AMG330 [9] and GO.[72] Therefore, we studied here, whether a similar correlation also existed for SPM-2 plus NK cells. To this effect, mean antigen densities of CD33 and CD123 on the patient-derived cells and on myeloid cells from healthy donors were determined with precision by calibrated cytofluorimetry and expressed in copy numbers per cell (Table 2; Suppl. Table 1–3). All samples showed measurable expression of CD33 and CD123, but with considerable variability between samples. The sample from patient 12 had the highest density of CD33 with approx. 17,600 copies/cell, and the sample from patient 3 had the highest density of CD123 with approx. 25,800 copies/cell. The sample from patient 3 also had the highest combined antigen density of CD33 plus CD123 cell with approx. 38,780 copies/cell (Table 2). This combined density is roughly comparable with the value for MOLM-13 cells, which was approximately 46,000 copies/cell (Suppl. Table 1). A weak correlation between the EC_{50} values and the combined antigen densities of CD33 plus CD123 was detected (Figure 3). A threshold for lysis by SPM-2 appeared to exist at a combined density of approximately 10,000 copies/cell of (CD33 plus CD123). However, this apparent correlation must be interpreted with caution, because

Table 2. Target antigen densities and susceptibility to SPM-2-mediated cytolysis of cell samples.

ID	% CD33+	% CD123+	# 33	# 123	Σ # (33 + 123)	EC_{50} (pM)	ELN genetic group
P1	73	30	2,781	5,349	8,130	131	favorable
P2	100	74	9,765	1,388	11,153	20	adverse
P3	100	100	12,974	25,812	38,786	67	intermediate-I
P4	100	97	9,045	25,424	34,469	32	intermediate-II
P5	85	99	1,459	5,852	7,311	242	intermediate-I
P6	99	80	9,489	3,769	13,258	155	intermediate-I
P7	70	36	6,405	2,668	9,073	79	adverse
P8	57	30	1,197	6,411	7,608	133	intermediate-II
P9	94	80	5,214	2,138	7,352	166	adverse
P10	84	ND	15,414	8,122	22,536	51	favorable
P11	47	ND	1,192	2,391	3,583	1,078	intermediate-II
P12	84	ND	17,635	13,101	30,736	38	intermediate-II
P13	65	ND	4,987	4,994	9,481	245	intermediate-I
P14	52	ND	1,010	7,123	8,133	229	intermediate-I
P15	95	ND	4,797	8,206	13,003	177	intermediate-II
P16	90	ND	1,877	8,257	10,134	758	intermediate-I
P17	95	ND	6,463	7,448	13,911	125	favorable
P18	79	ND	4,544	6,408	10,952	221	unclassifiable
P19	82	ND	2,876	766	3,642	406	adverse
P20	96	ND	16,377	9,072	25,449	251	intermediate-I
P21	87	ND	11,891	7,657	19,548	22	unclassifiable
P22	80	ND	1,250	1,892	3,142	225	adverse
P23	75	ND	1,245	6,479	7,724	476	intermediate-I
P24	70	ND	6,790	3,639	10,429	127	unclassifiable
P25	75	ND	5,193	2,051	7,244	541	intermediate-I
P26	92	ND	3,71	2,389	5,560	58	intermediate-I
P27	80	ND	5,458	6,892	12,350	10	intermediate-I
P28	84	ND	7,399	5,237	12,636	560	intermediate-I
P29	88	ND	4,896	7,438	12,334	70	favorable

ND: not determined, genetic risk groups assigned according to European Leukemia Net (ELN) classification [3, 4], % CD33+: fraction of MNCs scoring positive for CD33; # 33: antigen copies per cell determined by calibrated cytofluorimetry.

the number of samples analyzed is still small and statistical significance has not yet been reached.

Subsets of patient blasts enriched for CD34-bearing cells show increased expression of CD123 and increased susceptibility to lysis by SPM-2 plus NK cells

Our hypothesis is that AML-LSCs should be susceptible to lysis by SPM-2 plus NK cells, because they have been reported to

Table 3. Antigen expression and susceptibility to redirected lysis of patient blasts and analysis after preparative sorting for CD34-bearing cells.

ID	Cellular Subset	Antigen Density (# molecules/cell) CD33	CD123	CD33 + CD123	EC50 (pM)
P9 (AML-M2)	Bulk ($CD45^{dim}$ SSC^{low})	5,500	2,500	8,500	166
	CD34-enriched MNCs	13,000	9,100	22,100	43
P11 (AML-M4)	Bulk ($CD45^{dim}$ SSC^{low})	1,200	2,400	3,600	1,078
	CD34-enriched MNCs	500	14,500	15,000	681
	LSC-enriched fraction ($CD34^+$ $CD38^-$ $CD123^+$)	300	18,000	18,300	ND

ND: not determined

display high surface densities of CD33 and CD123.[7,10–12,40,46] We are not aware of any published reports indicating that they should differ from AML blasts in their intrinsic susceptibility to RDL by antibodies or antibody-derived agents in combination with NK cells. For most patients with CD34-positive AML, the relapse-relevant AML-LSCs are contained predominantly in the compartments of CD34-positive and (CD34-positive CD38-negative) cells.[12,40,43–46,73] Therefore, we have first enriched CD34-bearing cells from 2 patients with CD34-positive AML, for whom sufficient cell numbers were available (patients 9 & 11; Table 3), by immuno-magnetic sorting with a commercial kit. Both samples carried intermediate combined densities of CD33 plus CD123 on their blasts (MNCs; approx. 8,000 and 3,600 copies/cell, respectively; Tables 2 and 3). Both blast (MNC) samples were lysed by (SPM-2 plus NK cells) in RDL experiments with EC_{50} values of 166 and 1,078 pM, respectively, prior to enrichment for CD34-positive cells (Tables 2, 3). After the enrichment, CD33 and CD123 densities and susceptibility to RDL were determined again. The CD33 density was now reduced for cells from patient 11 to 500 copies/cell, and the CD123 density was increased to 14,500 copies/cell (Table 3). The EC_{50} value fell to 681 pM, indicating an approximately 2-fold increased susceptibility for lysis relative to the value for non-enriched cells. For patient 9 the CD33 density on the enriched cells was 13,000 copies/cell, the density

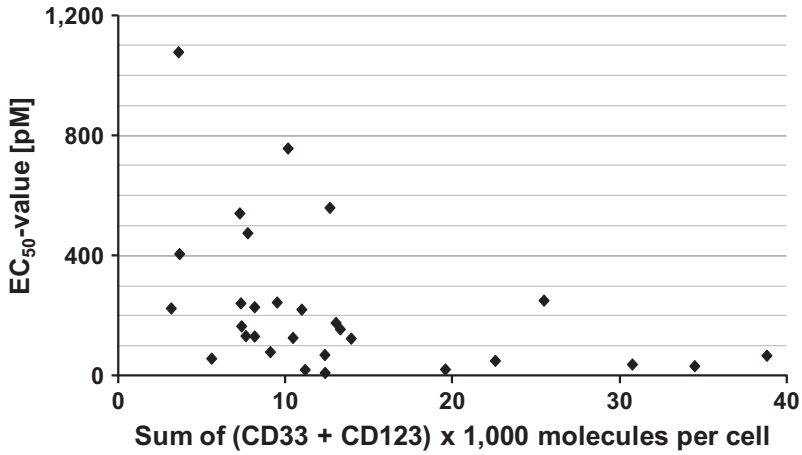

Figure 3. Correlation between susceptibility to lysis by SPM-2 plus NK cells (EC_{50} values) and combined surface antigen density of (CD33 + CD123) on patient blasts. Mean surface densities of CD33 and CD123 were determined for each patient sample. EC_{50} values were computed for each sample from the dose response curves shown in Figure2. Numerical values were the same as those shown in Table 3.

of CD123 was 9,100 copies/cell, and the combined density was 22,100 copies/cell. The EC_{50} value for the enriched cells of this patient was reduced by about 4-fold to 43 pM. The increase in the combined density of CD33 plus CD123 and the decrease in EC_{50} values in both cases are consistent with our hypothesis positing that cellular compartments encompassing the AML-LSCs more closely may indeed have greater susceptibility to lysis by SPM-2 than bulk AML blasts (MNCs). Similar initial data were also obtained for SPM-2-mediated RDL of cells from the (CD34-positive, CD38-negative) subset of 2 AML patients. From the small number of cases tested we cannot yet draw statistically valid conclusions, but the initial results obtained so far are consistent with our hypothesis, although the data set is still very small.

Discussion

The main result of this study is that primary blasts from AML patients with a very broad range of disease subtypes responded strongly to treatment with SPM-2 plus NK cells in RDL assays *in vitro*. Cells from all 29 patients showed a clear response, although the response varied for different patients and was weak for some patients. In addition, we have studied a number of AML-derived cell lines (Suppl. Table 1, Suppl. Figure 1 and others), and all lines expressing either CD123 or CD33 or both on their surface showed a clear RDL response to SPM-2 plus NK cells. These data support the recent prediction that malignant cells from almost all AML patients should be lysed by agents jointly binding CD33 and CD123.[8]

We expect that this prediction will be further confirmed, when lysis of AML cells by the patients' autologous NK cells, mediated by SPM-2, will be studied. Such studies are planned for the future, but have not yet been performed so far. They will likely be informative for the question, whether the combination of the new agent with the patients autologous cells is beneficial for patients with a broad range of AML subtypes. With the present study we tried to answer a different question. We tried to isolate the AML subtype as the sole variable and wanted to determine, whether the same molecular agent was effective for samples from patients with different disease subtypes in combination with a constant source of human NK cells. The results obtained here are remarkable, because for other antibody therapeutics in clinical use against AML, such as GO, typically only less than half of the patients with antigen-positive disease responded to the treatment.[10,15,72]

Correlation between combined surface antigen density of CD33 and CD123 and susceptibility to RDL lysis by SPM-2 plus NK cells

A loose correlation between these two properties was observed for bulk AML cells (Figure 3), and similar correlations have been reported for the CD33-specific BiTE AMG330,[9] the CD123 antibody Talacotuzumab (CSL 362),[48,49] and the CD3 x CD123 DART agent MGD006.[56] While the target antigen density likely dictates the strength of binding of these agents to the target cells and the subsequent engagement of killer cells, it is unlikely that binding strength alone will translate 1:1 into an enhanced lytic activity. Other relevant processes independent of target antigen density will influence the cytolytic outcome. Therefore, it would be unrealistic to expect a very tight correlation between target antigen density and the susceptibility of the patients blasts to lysis by SPM-2 plus NK-cells (LAK cells) in our cell culture RDL assays.

Our findings are consistent with a recent study of the CD33-directed agent AMG330.[9] In this study surface densities of CD33 were determined for samples from 621 patients and CD33 was strongly expressed on the blasts from 99% of the patients. However, the surface expression level of CD33 was not the sole determinant of lytic activity, although the degree of lysis differed between strongly and weakly expressing cells. Interestingly, monocytes and dendritic cells (DCs) showed strong expression of CD33, but no lysis of DCs by AMG 330 plus T cells was observed in RDL assays.[9] Data about lysis of monocytes and DCs by SPM-2 plus NK cells are not yet available.

Effect of SPM-2 on cellular subfractions enriched in AML LSCs

Our hypothesis is that in concert with NK cells SPM-2 will mediate the elimination not only of bulk AML cells, but also of AML-LSCs. As a first approximation to a test of this hypothesis, cellular subsets enriched for CD34-bearing cells by immuno-magnetic sorting were prepared from primary cells of 2 AML patients with CD34-positive AML. These populations displayed increased surface antigen densities of (CD33 plus CD123) and correspondingly an enhanced susceptibility to lysis by SPM-2 plus NK cells (Table 3). The data are consistent with the hypothesis but do not yet provide direct evidence in its favor. Even though the AML-LSCs are enriched in the populations of CD34-bearing and (CD34-positive, CD38-negative) cells, the LSCs probably still account for only a small fraction of cells in these populations. Therefore, the data presented in Table 3, which represent properties averaged over all cells of the enriched populations, probably do not yet reflect the properties of the far narrower subset of true LSCs.

To test our hypothesis, clinical studies are needed. A suitable flow cytometric method permitting to monitor the effect of a treatment with SPM-2 plus NK-cells on AML LSCs has recently been published. This assay was tested and validated for the detection of LSCs from a majority of patients with CD34-positive AML, which account for approximately 80% of all AML cases.[46] We intend to use this and other methods (quantitative polymerase chain reaction, qPCR, where applicable) in the future, to monitor the effect of SPM-2 plus autologous NK cells on the subset of AML-LSCs.

Planned studies of antileukemic efficacy of SPM-2 in xenotransplanted animals and in comparison with corresponding mono-targeting agents in cell culture studies

Efficacy studies with SPM-2 in mice xenotransplanted with human AML cells have so far not been performed but are planned for the near future. These studies were postponed until SPM-2 protein in GMP or GMP-like purity grade

became available, so that the data are admissible by the regulatory authorities. Also, the scFv binding domains carried in SPM-2 are uniquely specific for the human target antigens and do not cross-react with their murine homologues. Therefore, both the human target cells and human NK cells need to be transplanted into the mice, and these experiments are difficult and expensive. However, they will be performed with high priority as soon as the first batch of SPM-2 protein in GLP purity grade becomes available. An alternative is to use a mouse model with a xenotransplanted fully human haematopoietic system, and then to transplant only the human AML target cells. This model is available on a collaborative basis, but is very labor-intensive and expensive, and therefore, we need to wait for a first batch of SPM-2 protein in GLP purity grade to become available. Another set of important studies needed to be postponed for the same reason. This was the investigation, whether the final clinical candidate SPM-2 mediates not only enhanced selectivity of lysis in a mixture of two target cell populations, one highly double-positive and the other lowly double-positive for CD33 and CD123 as predicted. We also urgently wish to determine, whether the dual-targeting triplebody mediates more potent lysis at lower EC50 doses in RDL experiments than the mono-targeting related proteins CD33-16 and CD123–16, either individually or in combination, similar to the studies performed earlier with the triplebodies 123-16-33, HLAII-16–19 and 33–3-19.[59,68–70]

NK cells vs. T cells as cytolytic effectors

When SPM-2 was designed, several studies had reported that T cells engaged by immuno-therapeutic agents caused serious CRS (Cytokine Release Syndrome). If NK cells should turn out to be sufficiently strong killers when engaged by our agent, then we would prefer to promote an agent engaging NK cells into clinical development, because it would likely produce less severe CRS and may be better tolerated by the patients.

Our earlier study [60] and others quoted therein reported that NK cells collected at the time of diagnosis were greatly reduced in numbers and specific cytolytic activity in the BM of AML patients, but that they frequently recovered in both regards by the end of induction CT, when the leukemic burden was greatly reduced. Therefore, SPM-2 was designed for use in a post-induction setting with the objective to improve survival by eliminating the relapse-inducing LSCs, and for this purpose NK cells appeared to be suited in the single case studied so far.[60]

In a recent publication of data obtained for the CD157-specific antibody MEN1112,[39] the authors reported, that autologous NK cells from a sizable cohort of AML patients showed only marginal cytolytic RDL activity in assays with this antibody. However, in this study, NK cells obtained from the patients at the time of diagnosis were used, a time point, when NK cells are typically reduced in numbers and cytolytic activity. We have not yet tested the effects of SPM-2 in combination with a similar set of autologous NK cells from a large cohort of AML patients. We only tested NK cells from a single AML patient so far, prepared from a BM sample obtained in a first remission after standard CT. These NK cells were highly active for lysis of the autologous AML blasts.[60] In the study of MEN1112,[39] the RDL reaction also was mediated by NK cells binding with their CD16 receptor to the Fc domain of the CD157 antibody, and thereby triggering RDL. In our case, the NK cells were actively engaged by an scFv binding domain with high affinity for CD16 contained in the triplebody. This is an important difference, and therefore, the strong binding of our agent to CD16 on NK cells may conceivably cause a stronger activation of the NK cells than binding of CD16 on NK cells to the Fc-domain of the CD157 antibody. NK cells from the patients studied by Krupka and colleagues[39] might still have shown a sufficiently strong response towards autologous AML blasts in concert with SPM-2, if they had been drawn in remission.

Finally, a number of methods have recently been developed, which appear suited to optimize NK cell function in human AML patients either by pharmaceutical agents [74–77] or by adoptive transfer of *ex vivo* expanded NK cells.[71,77] The combination of SPM-2 with some of these approaches may contribute to the elimination of MRD and thus lead to favorable therapeutic outcomes in the future.

The results reported here for SPM-2 are very encouraging, and this agent may therefore become useful for the therapy of AML in a post-induction, MRD-directed setting, even without an adoptive transfer of NK cells. This agent clearly deserves to be advanced into clinical development.

Materials and methods

Patients, healthy donors, and donors with non-aml disorders

Written informed consent was obtained from patients in accordance with the Declaration of Helsinki. After approval by the Institutional Review Boards (IRBs) of the Ludwig-Maximilians-Universität (LMU) Munich (patients 1–9; Table 1) and the Friedrich Alexander Universität (FAU) Erlangen (patients 10–29; Table 1) was obtained, PB and BM samples were collected from healthy donors (HDs) and patients with AML at diagnosis or relapse. HDs were adults free of known disease, and "donors with non-AML disorders" were patients with disorders other than acute leukemias and without symptoms of their hematopoietic system, which were treated at the LMU Medical Center in the context of other clinical studies. Their cells were made available after their use for the present study was approved by the LMU's IRB.

Expression in mammalian cells, purification and protein-chemical characterization of triplebody SPM-2

The DNA construct coding for SPM-2 was synthesized by a commercial provider (Eurofins/MWG-Operon). To create this construct, the scFv subunits were disulfide-stabilized[78-80] humanized[81-83] and stability-engineered[84] by standard procedures. The TransIT®-LT1 transfection reagent (Mirus Bio LLC, catalog # MIR 2300) was used for transfection according to the manufacturer´s protocol to generate a stable cell pool of Freestyle™ 293F cells (ThermoFisher Scientific, cat. #

R79007) for protein expression. Cells were then cultured under continuous selection with hygromycin C. SPM-2 was captured from cell culture supernatants via its C-terminal hexahistidine tag by immobilized zinc ion affinity chromatography followed by anion exchange chromatography using a 1 ml (bed volume) HiTrap Q Sepharose HP column (GE Healthcare). The third purification step was a cation exchange (CEX) chromatography. Here, a 1 ml HiTrap SP Sepharose HP column (GE Healthcare) was used and connected to an Äkta liquid chromatography system (GE Healthcare). SPM-2 preparations were analyzed by size exclusion chromatography (Superdex S200 5/150 GL column, GE Healthcare) for quality control and by SDS polyacrylamide gel electrophoresis (SDS-PAGE) after the final purification step. The protein concentration was determined by UV absorption at 280 nm using a NanoDrop spectrometer (PeqLab). The theoretical extinction coefficient was calculated from the primary amino acid sequence with the computer program ProtParam (www.expasy.ch). A final yield after purification of approx. 2.5 to 5 mg SPM-2 per L of culture medium was achieved in several independent experiments.

Preparation of primary cells from blood and bone marrow of human donors

PB and BM samples were drawn from subjects into either EDTA or heparin solution. Leukemia-derived cells and Mononuclear Cells (MNCs) from healthy donors were enriched by density centrifugation using the Lymphoflot reagent (Bio-Rad, cat. # 824012) according to manufacturer's instructions. Peripheral Blood Mononuclear Cells (PBMCs) were then either suspended in RPMI medium 1640 with GlutaMAX™ Supplement (ThermoFisher Scientific, cat. # 61870–044) containing 10% Gibco™ fetal bovine serum (FBS; ThermoFisher Scientific, cat. # 10082–147) with Gibco™ penicillin-streptomycin (PS; ThermoFisher Scientific, cat. # 15140–122) at 100 units/ml and 100 µg/ml, respectively, for immediate use, or stored frozen in a solution containing 90% FBS and 10% DMSO. Cell viability was assessed by Trypan blue exclusion before use.

Ex vivo expansion of MNCs from a healthy donor in the presence of IL-2 (LAK-cells)

PBMCs from an adult healthy donor were expanded *ex vivo* in RPMI medium containing Interleukin-2 (IL-2) (ThermoFisher Scientific, cat. # PHC0023) plus 5% human serum (ThermoFisher Scientific, cat. # 34005–100, discontinued) for 20 days as described.[68,71] At the end of this expansion period, the LAK (Lymphokine Activated Killer) cell population consisted of approx. 25% NK cells, 70% T cells and 5% NKT cells. LAK cells were then frozen in aliquots for subsequent use. Prior to use in cytolysis experiments, the cells were thawed and cultured overnight in RPMI medium containing 5% human serum (Invitrogen) plus 50 units/ml and 50 µg/ml PS, respectively, but no additional IL-2.

Enrichment of human CD34-positive cells by preparative sorting with immuno-magnetic beads

CD34-positive cells were enriched by positive selection using the commercial human $CD34^+$ Multisort Kit (Miltenyi Biotec MACS sorting kit, cat. #130–058-701) according to manufacturer's instructions. Starting material were purified PBMCs from PB or BMMCs from donor BM samples. The enriched CD34-positive cells were then either suspended in RPMI medium containing 10% FBS with PS at 100 units/ml and 100 µg/ml, respectively, for use in RDL assays, or placed in Dulbecco's phosphate buffered saline (PBS) solution (ThermoFisher Scientific, cat. # 14190–250) containing 1% bovine serum albumin (BSA) (Sigma-Aldrich Co., LLC, cat. # A9418) for flow cytometric analysis.

Flow cytometry

Flow cytometric analysis was performed with an Accuri C6 flow cytometer (BD Biosciences). A typical staining reaction used 300,000 cells per FACS tube containing the specific monoclonal antibody (mAb) and proceeded for 20 min on ice. Wash steps were performed with a PBA solution (PBS; 0.1% Bovine Serum Albumin and 0.02% Sodium Azide). AML blasts were defined as the population of $CD45^{DIM}$ x SSC^{LOW} cells detected among the patient's PBMCs or BMMCs with a Fluorescein-isothiocyanate (FITC)-conjugated CD45-specific mAb (Beckman Coulter, Immunotest, cat. # IM0782U). To determine the content of CD34-positive cells after enrichment by immuno-magnetic (MACS) beads, staining was performed with a phycoerythrin (PE)-labelled CD34-specific mAb and a FITC-labelled CD45-specific mAb.

Flow cytometric measurement of CD33 and CD123 surface antigen densities

Initial analysis of patient samples was performed in the laboratories of the participating hospitals. The fractions of CD33- and for some samples of CD123-expressing leukocytes were determined by FACS analysis (FACS Navios, Beckman Coulter). Cell surface densities of CD33 and CD123 were measured by using a calibrated cytofluorimetric procedure as described.[60,68,85] For this purpose, a kit of fluorescent beads with known numbers of fluorescent chromophores per bead (QIFIKIT®; Agilent Technologies, cat. # K0078) was used for calibration purposes. The procedure allows the investigator to express the measured fluorescence intensity of antibodies bound to a cell surface in terms of average number of antigen copies per cell.[60,68,85] PE-labeled mAbs specific for CD33 and CD123, calibrated for QIFI kit measurements of antigen densities on MOLM-13 AML cells, were used as a substitute for the less economic complete QIFI kits. MOLM-13 cells were used as standards because surface densities of CD33 and CD123 on these cells remained sufficiently constant over time to make these cells useful for calibration purposes. To obtain absolute numbers for the calibration-batch of MOLM-13 cells, CD33 and CD123 densities were first measured with the QIFI kit, and then this batch of cells was used as a standard for secondary calibration for the next 20–30 generations of MOLM-13 cells. Antigen densities on patient- or

donor-derived "test cells" were then measured by calibrating them against this standard batch of MOLM-13 cells.

PE-labeled CD33 and CD123 mAbs (CD33; Beckmann Coulter Immunotest cat # A07775 and CD123; ebioscience cat # 12–1239-42) were used to stain the standard batch of MOLM-13 cells and the test samples of patient- and donor-derived cells. Mean Fluorescence Intensities (MFI) were measured with an Accuri C6 flow cytometer (BD Biosciences). In the Accuri C6 instrument the laser- and optical alignments are pre-set and locked, and detector voltages are not adjustable, as opposed to other cytometers. This feature permitted reproducible measurements of Mean Fluorescence Intensity (MFI) values for CD33 and CD123 on MOLM-13 cells. MFI data recorded in this manner gave rise to numerically similar values expressed in MFI units as the number of antigen copies per cell (SABC values) measured with the QIFI kit. The close similarity of numerical values obtained with both methods allowed us to use the direct staining protocol as a surrogate for the QIFI kit for routine measurements of antigen copy numbers per cell (T. Braciak; unpublished data).

Re-directed lysis (RDL) assays using calcein release

Cytolysis assays based on the release of calcein from target cells prelabeled with calcein AM (ThermoFisher Scientific, cat. # C3100MP) were performed as described.[60,68] In the final cytolytic reaction, no calf or human serum was present. The cytolytic activity of SPM-2 was measured in RDL reactions using NK cells expanded for 20 d in culture in the presence of IL-2 (LAK cells) as mediators of lysis with AML patient's blasts as targets. The reactions with cells from all 29 patients, AML cell lines and controls (Figure 2; Suppl. Figure1) were performed with LAK cells from the same large standard batch of *ex vivo* expanded cells that were frozen in aliquots stored under liquid nitrogen. Similarly, all reactions were performed with SPM-2 from the same batch of protein, also frozen in aliquots, to assure that all experimental variables were kept constant and that the sole variable, for which we wanted to test, was the origin of the target cells. The reproducibility of the assay was assessed by using MOLM-13 cells from the same standard batch as calibration standards together with the standard batch of LAK cells. The maximum degree of specific lysis of the MOLM-13 cells varied by less than 10% in different experiments using separate frozen and thawed aliquots of the standard batch of LAK cells, and the EC_{50} values varied by less than a factor 2. This amounts to a high degree of reproducibility of the assay, given the fact, that the EC_{50} values were derived from plots, in which the dose of the agent was plotted on an exponential scale. Therefore, the differences seen for samples from different patients truly reflect the contribution of the cellular origin as the main variable. Control reactions using the Her2-specific triplebody Her2-16-Her2 as a negative control were run side by side in each experiment. The Her2-specific triplebody contained 2 scFv (antibody-derived single chain Fragment variable) binding domains for EGFR-2 and the same CD16-specific trigger binding domain as SPM-2. In addition, reactions with MOLM-13 cells as targets were included in each experiment as a positive control. Specific lysis was measured by quantitating the release of calcein from target cells using a fluorimeter/ELISA plate reader and expressed in Relative Light Units (RLU) at 485/535 nm. Calcein release was measured at the 4 hr time point. "Specific lysis" was defined as overall lysis minus the background of spontaneous lysis mediated by the NK cells alone, in the absence of added antibody-reagents, expressed as a fraction of net maximum release. "Specific lysis" was therefore computed by the formula:

% specific lysis = 100 x (Experimental RLU-Background RLU)/(Maximal RLU-Background RLU)

EC_{50} values and estimates of MABEL doses

EC_{50} values (concentration of triplebody protein producing 50% of maximum specific lysis) were calculated by using a nonlinear regression curve fit (variable slope) analysis using Graph Pad Prism Software (Graph Pad Software Inc.). MABEL (Minimum Anticipated Biologically Effective Levels) concentrations were deduced from (Figure 2), panels A-E, by visual inspection as the levels, for which a clear cytolytic effect beyond background was detected for more than 90% of the analyzed primary cell samples.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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