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A recombinant trispecific single-chain Fv derivative directed against CD123 and CD33 mediates effective elimination of acute myeloid leukaemia cells by dual targeting

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3 **A recombinant trispecific single-chain Fv derivative directed against CD123**
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6 **and CD33 mediates effective elimination of acute myeloid leukaemia cells**
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9 **by dual targeting**
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52 **Running title:** CD123-, CD33- and CD16-directed trispecific single-chain Fv fragment
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54 **Keywords:** CD33, CD123, dual targeting, leukaemia stem cell, single-chain Fv triplebody
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Summary

Two trivalent constructs consisting of single-chain Fv antibody fragments (scFvs) specific for the interleukin-3 receptor α chain (CD123), CD33 and the Fc γ -receptor III (CD16) were designed and characterized for the elimination of acute myeloid leukaemia (AML) cells. The dual targeting single-chain Fv triplebody (sctb) [123 x ds16 x 33] and the mono targeting sctb [123 x ds16 x 123] both specifically bound their respective target antigens and were stable in human serum at 37°C for at least 5 days. Both constructs induced potent antibody-dependent cellular cytotoxicity (ADCC) of two different AML-derived CD33- and CD123 double-positive cell lines in the low picomolar range using isolated mononuclear cells (MNCs) as effector cells. In these experiments the dual targeting molecule produced significantly stronger lysis than the mono targeting agent. In addition, the sctbs showed a high potency in mediating ADCC of primary leukaemia cells isolated from peripheral blood or bone marrow of seven AML patients. Hence, these novel molecules displayed potent anti-leukaemic effects against AML cells *in vitro* and represent attractive candidates for further preclinical development.

Introduction

Acute myeloid leukaemia (AML) is a haematopoietic malignancy characterized by a block in cell maturation and an accumulation of undifferentiated blasts in the bone marrow and peripheral blood (Lowenberg *et al*, 2003, Hiddemann *et al*, 2005). AML is commonly treated with an induction chemotherapy, followed by a consolidation therapy and sometimes maintenance therapy (Buchner *et al*, 2005, Robak and Wierzbowska 2009). As a postremission therapy, autologous or allogeneic bone marrow transplantation can be performed in selected patients (Shipley and Butera 2009). For patients in first relapse older than 60 years and for patients resistant to standard chemotherapy, the immunoconjugate Gemtuzumab Ozogamicin (GO) is approved by the Food and Drug Administration (FDA) (Bross *et al*, 2001, Sievers 2001). GO is a CD33-specific antibody chemically coupled to the cytotoxic drug calicheamicin (Sievers *et al*, 1999, Stasi 2008). CD33, a 67-kDa transmembrane cell surface glycoprotein specific for the myeloid lineage, is not expressed on non-haematopoietic tissue but is expressed on a subset of normal haematopoietic stem cells (HSCs) and on AML leukaemia stem cells (AML-LSCs; Freeman *et al*, 1995, Taussig *et al*, 2005, Hauswirth *et al*, 2007). In a phase II clinical trial, 30% of relapsed AML patients responded to GO, but severe side-effects were observed (Sievers 2001, Larson *et al*, 2002). In a recent European multicenter phase III study, GO was found to provide no benefit when administered as a post-remission therapeutic agent to older patients (Lowenberg *et al*, 2010), and the drug is not approved in Europe. Furthermore, the agent displayed antigen-independent cytotoxicities towards CD33 negative cell lines (Bross *et al*, 2001, Jedema *et al*, 2004, Schwemmlin *et al*, 2006). Despite of these problems GO clearly produces clinical benefits for a subgroup of AML patients, and CD33 is a validated target antigen. Still, new therapeutic modalities with fewer toxicities are urgently needed for the treatment of AML (Estey and Dohner 2006). Another important AML-associated antigen is CD123, the alpha subunit of the interleukin-3 receptor (IL-3R α), which is predominantly expressed on myeloid cells and on a

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3 subpopulation of B-lymphocytes. It is not expressed on platelets, red blood cells, natural killer
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5 cells (NK cells), peripheral T-cells (Moretti *et al*, 2001) and only in low density on HSCs
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7 (Huang *et al*, 1999, Jin *et al*, 2009). However, CD123 has been detected in a variety of
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9 haematopoietic malignancies and on AML-LSCs (Jordan *et al*, 2000, Munoz *et al*, 2001,
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11 Testa *et al*, 2002, Taussig *et al*, 2005). Strikingly, CD123 shows a 4-fold increased expression
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13 on AML-LSCs over normal HSCs (Jin *et al*, 2009). This finding together with the clinical
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15 observations, that elevated expression of CD123 in AML is associated with higher blast
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17 counts at diagnosis and a lower complete remission rate resulting in poorer prognosis (Testa
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19 *et al*, 2002, Graf *et al*, 2004, Testa *et al*, 2004) make CD123 a particularly interesting target
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21 for antibody-derived therapeutics (Jin *et al*, 2009).
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27 AML-LSCs are known to possess several remarkable properties including self-renewal
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29 potential and increased resistance against chemotherapeutics and DNA damage (Lapidot *et al*,
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31 1994, Bonnet and Dick 1997, Guan and Hogge 2000, Guzman *et al*, 2001, Hope *et al*, 2004,
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33 Ishikawa *et al*, 2007, Dick 2008). This unique cell population is thought to play a key role in
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35 the frequent occurrence of minimal residual disease (MRD) in relapsed AML patients after
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37 conventional chemotherapy (van Rhenen *et al*, 2005, Ravandi and Estrov 2006). Hence, after
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39 the discovery of AML-LSCs a great amount of effort has been spent to investigate the surface
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41 antigen profile of this clinically relevant cell population to identify suitable markers for their
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43 targeted elimination (Jordan *et al*, 2000, Taussig *et al*, 2005, Jin *et al*, 2006, Hosen *et al*,
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45 2007, Jin *et al*, 2009). The fact that both antigens, CD33 and CD123, are expressed on AML-
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47 LSCs offers novel prospects for the treatment of AML. So far an immunotoxin (Du *et al*,
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49 2007), a fusion of IL-3 with a truncated version of diphtheria toxin (Feuring-Buske *et al*,
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51 2002, Frankel *et al*, 2008), a neutralizing full-length antibody (7G3; Jin *et al*, 2009), and a
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53 **bispecific single-chain Fv (bscFv; Stein *et al*, 2010)** directed against CD123 have been
54
55 described, and the monoclonal antibody 7G3 has been tested in a phase I clinical study
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60 (Roberts *et al*, 2008; <http://clinicaltrials.gov/ct2/show/NCT00401739?term=CSL360&rank=1>).

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3 Well-studied antibody-derived agents directed against CD33 include apart from GO an
4 immunotoxin (Schwemmlin *et al*, 2006), a fusion protein between a single chain Fv antibody
5 fragment (scFv) and the sTRAIL death receptor ligand (ten Cate *et al*, 2009), and siRNA-
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10 loaded liposomes coated with CD33-directed scFvs (Rothdiener *et al*, 2010).

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12 **BsscFvs** have advantageous properties over full-length bispecific antibodies and chemically
13 coupled bispecific Fragment antibody binding (Fab) fragments, and have recently produced
14 convincing therapeutic effects in clinical trials (Bargou *et al*, 2008). To overcome some of the
15 remaining weaknesses of this format, such as an unfavorable plasma retention, the format has
16 been further improved by expanding it to tandem diabodies (Kipriyanov *et al*, 1999) and
17 single-chain triplebodies (sctbs; Kellner *et al*, 2008). The incorporation of a second scFv-
18 binding site for tumour-antigens in these extended formats has led to increased avidity for the
19 tumour cell, increased anti-tumour activity in antibody dependent cellular cytotoxicity
20 (ADCC) reactions, and improved plasma retention *in vivo* (Kellner *et al*, 2008). An example
21 for a sctb with improved ADCC activity for AML cells is the sctb [33 x ds16 x 33] (Singer *et*
22 *al*, 2010) with two scFv antigen binding sites for CD33 on AML cells and one for CD16, the
23 low affinity Fcγ RIII receptor for IgG on NK cells and macrophages (Ravetch and Perussia
24 1989, Daeron 1997). In a direct comparison, this sctb showed increased anti-leukaemic
25 activity for AML cells over the corresponding bsscFv [33 x ds16] with only one binding site
26 each for CD33 and CD16. Before these trivalent single-polypeptide formats, other trispecific
27 antibody derivatives had been explored. One example was a trispecific Fab construct,
28 consisting of three chemically coupled Fab-fragments (Somasundaram *et al*, 1999). A second
29 trispecific construct was called a "tri-body", a two-chain polypeptide based on a Fab fragment
30 as scaffold (Schoonjans *et al*, 2001). Two scFvs were fused to the C-termini of the L- and Fd-
31 chains of this Fab, respectively. Finally, a trispecific molecule based on a VH domain has
32 been reported, which carried two scFvs fused to a VH-domain (Song *et al*, 2003).
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3 The key molecule presented here, the sctb [123 x ds16 x 33], differs significantly in its design
4 from all of these examples. It is a single-chain polypeptide with only one binding site for an
5 effector cell and two for two different tumour antigens, CD123 and CD33. The goal of this
6 design was to achieve improved anti-leukaemic activity by "dual targeting" of the tumour cell,
7 i.e. by simultaneous binding to two different antigens on the same tumour cell. We expected
8 this molecule to bind with greater avidity to CD123 and CD33 on double-positive AML cells
9 than the corresponding control sctbs [123 x ds16 x 123] and [33 x ds16 x 33], because it
10 would take advantage of two populations of target antigens rather than only one. Based on
11 theoretical arguments, the avidity of such proteins was expected to be proportional to the
12 combined target antigen density on the tumour cell (Mattes 1997, Pluckthun and Pack 1997).
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27 The prediction of added benefits by dual- over mono-specific targeting of tumour cells is
28 intuitively appealing, and has been pursued for a number of years (Robinson *et al*, 2008), but
29 has not yet been conclusively proven for scFv-derived agents recruiting effector cells. A
30 definitive proof of this prediction is experimentally demanding and is still under investigation.
31 However, indirect evidence in favour of the proposition has been obtained for scFv-
32 immunotoxins, and therefore, the concept may also extend to agents recruiting effector cells
33 (Vallera *et al*, 2005). The molecules presented here provide an opportunity to directly
34 evaluate the benefits of dual over mono-specific targeting for AML cells. Finally, they
35 provide new means to test the prediction that dual targeting a combination of two antigens,
36 which are more highly expressed on AML-LSCs than on HSCs, may permit the preferential
37 elimination of AML-LSCs. It would be important to test this prediction, because if confirmed
38 it would provide a possibility for a deliberate attack on the LSCs with the intent to eliminate
39 MRD cells and prevent relapse, while preserving some HSCs for haematopoietic recon-
40 stitution after the end of treatment, without the need for autologous stem cell transplantation.
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60 The molecules presented here provide first steps towards these important goals of rational
leukaemia therapy.

Materials and Methods

Cell lines and hybridomas

The human AML cell lines MOLM-13, THP-1 (t(9;11)(p22;q23), expressing *MLL-MLLT3*) and U937 were from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Chinese hamster ovary (CHO) cells, stably transfected with a human CD16 cDNA expression vector, were provided by Dr. J. van de Winkel (University of Utrecht, the Netherlands). The hybridoma 3G8; FcγRIII, CD16; IgG1, (Fleit *et al*, 1982) and the human 293T and 293 cells were from the American Type Cell Culture Collection (ATCC, Manassas, VA, USA). The 4G7 hybridoma (CD19; IgG1) was from Dr R. Levy (Stanford University, Palo Alto, CA;(Meeker *et al*, 1984). CHO, THP-1, U937 and the 3G8 hybridoma cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 Glutamax-I medium (Invitrogen, Karlsruhe, Germany) containing 10% fetal calf serum (FCS; Invitrogen), 100 units/ml penicillin (Invitrogen), and 100 µg/ml streptomycin (Invitrogen). MOLM-13 cells were cultured in RPMI 1640 Glutamax-I medium containing 20% FCS, 100 units/ml penicillin and 100 µg/ml streptomycin (Invitrogen). Human 293, 293T and 293 cells stably transfected with CD123 (Stein *et al.*, 2010) were maintained in DMEM (Invitrogen) Glutamax-I medium containing 10% FCS, 100 units/ml penicillin and 100 µg/ml streptomycin (Invitrogen). The medium for the CD123 expressing cells further contained 400 µg/ml Geneticin (Invitrogen).

Construction of scTbs [123 x ds16 x 33] and [123 x ds16 x 123]

Escherichia coli strain XL-1 blue (Stratagene, Amsterdam, The Netherlands) was used as host for the amplification of plasmids and cloning. For the construction of the scTb [123 x ds16 x 33] expression vector the sequence coding for the CD123-specific scFv was excised from the vector pAK400-CD123scFv (Stein *et al*, 2010) and cloned as a SfiI-cassette into the existing vector pSecTag2HygroC-STREP-His-CD33xdsCD16xCD33 (Singer *et al*, 2010) replacing

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3 the coding sequence for the N-terminal CD33-specific scFv. Thereby the vector
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5 pSecTag2HygroC-STREP-His-CD123xdsCD16xCD33 was produced. In these constructs, the
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7 index ds designates the disulfide-stabilized variants (Bruenke *et al*, 2004). To generate the
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9 vector pSecTag2HygroC-STREP-His-CD123xdsCD16xCD123, the sequence coding for the
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11 CD123-specific scFv was amplified by PCR from the vector pAK400-CD123scFv and ligated
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13 into pSecTag2HygroC-STREP-His-CD123xdsCD16xCD33, using XhoI/EcoRV restriction
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15 sites, and replacing the coding sequence for the C-terminal CD33-specific scFv. For
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17 construction of the expression vector pSecTag2HygroC-hCD123ex-DsRed, cDNA coding for
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19 the extracellular domain of CD123 was amplified by PCR using the existing vector
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21 pSecTag2HygroC-hCD123ex-Fc (Stein *et al*, 2010) and ligated into the vector
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23 pSecTag2HygroC-CD33ex-DsRed (Singer *et al*, 2010), replacing the extracellular domain of
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25 CD33. Correct construction of the final constructs was confirmed by DNA sequence analysis
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27 on an AppliedBiosystems automated DNA sequencer (ABI Prism 310 Genetic Analyzer;
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29 Perkin-Elmer, Ueberlingen, Germany).

38 **Expression and purification of sctbs [123 x ds16 x 33] and [123 x ds16 x 123]**

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40 For expression of the recombinant sctbs [123 x ds16 x 33] and [123 x ds16 x 123], and the
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42 control sctbs ds[19 x 16 x 19] and [7 x ds16 x 7] specific for the cell surface antigens CD 19
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44 and CD7, respectively, 293T cells were stably transfected with the respective expression
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46 vectors (Kellner *et al*, 2008). Cells were cultured under permanent selection with hygromycin
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48 C in a mini-PERM bioreactor (Greiner Bio-One, Frickenhausen, Germany) with a dialysis
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50 membrane with a 12.5 kDa cutoff following manufacturer's instructions. The culture
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52 supernatants containing the recombinant protein were collected 4 times in a period of 2
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54 weeks. For expression of the control sctb [33 x 64 x 33] specific for the cell surface antigens
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56 CD33 and CD64 (C. Stein, unpublished data), 293T cells were transiently transfected with the
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58 expression vector using the calcium phosphate technique including 5 mM chloroquine
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3 (Sambrook *et al.*, 1989). The transfection medium was replaced by fresh culture medium after
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5 10 h and for 5 d supernatants were collected every day and combined. Supernatants were
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7 analyzed for the presence of antibody fragments by flow cytometry. The recombinant His-
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9 tagged proteins were enriched by affinity chromatography using nickel-nitrilotriacetic acid
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11 agarose (NTA) beads (Qiagen, Hilden, Germany) and dialyzed against phosphate-buffered
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13 saline (PBS). Fusion proteins with green or red fluorescence protein were transiently
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15 expressed in 293T cells and purified as described above.
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22 **Sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blot analysis**

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24 Eluted proteins were analyzed by reducing sodium dodecyl sulphate polyacrylamide gel
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26 electrophoresis (SDS-PAGE) using standard procedures (Laemmli, 1970). Gels were stained
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28 with Coomassie brilliant blue R250 (Sigma-Aldrich, Taufkirchen, Germany). In Western blot
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30 experiments, recombinant proteins were detected with an unconjugated penta-His antibody
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32 (Qiagen) and a secondary horseradish peroxidase-coupled goat anti-mouse IgG antibody
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34 (Dianova, Hamburg, Germany). Western Blots were developed using enhanced
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36 chemiluminescence reagents (Amersham Pharmacia Biotech, Freiburg, Germany).
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43 **Flow cytometry analysis**

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45 Immunofluorescence analysis was performed on a FACSCalibur instrument using CellQuest
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47 software (Becton Dickinson, Heidelberg, Germany) as described (Schwemmlein *et al.*, 2006).
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49 Briefly, 1×10^4 events were collected for each sample, and whole cells were analyzed using
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51 appropriate scatter gates to exclude cellular debris and aggregates. The recombinant scfb
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53 proteins were detected using a penta-His antibody and a PE-conjugated goat anti-mouse-IgG
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55 F(ab')₂ (DAKO Diagnostica GmbH, Hamburg, Germany), unless otherwise stated.
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Determination of equilibrium binding constants (K_D)

K_D values were determined by calibrated flow cytometry as described (Benedict *et al*, 1997). The highest mean fluorescence value was set to 100%, and all data points were normalized to this value. The experiments were repeated 6 times. The K_D values were calculated using a nonlinear regression curve fit.

Measurement of *in vitro* serum stability

To determine the *in vitro* serum stability of scTbs [123 x ds16 x 33] and [123 x ds16 x 123], aliquots of the recombinant proteins were incubated at a sub-saturating concentration of 2.5 $\mu\text{g/ml}$ at 37°C (day 5) in human serum or stored at -20°C and thawed at defined time points (day 0-4). The residual binding activity was measured by flow cytometry on day 0 (as described above). The experiment was repeated at least 4 times and results were fitted to a monoexponential decay.

Isolation of mononuclear cells (MNCs) and AML cells from human donors

Citrate buffered or heparinized peripheral blood from both healthy volunteers and AML patients or bone marrow from AML patients were obtained after receiving informed consent and with the approval of the Ethics Committee of the University of Erlangen-Nuremberg. MNCs were enriched by Lymphoflot (Biotest, Dreieich, Germany) Ficoll density centrifugation in Leukosep tubes (Greiner, Frickenhausen, Germany) according to manufacturer's instructions, and suspended in RPMI 1640 Glutamax-I medium containing 10% FCS, 100 units/mL penicillin and 100 mg/mL streptomycin. Viability was verified by Trypan blue exclusion and exceeded 95%.

ADCC reactions

ADCC assays, using MNCs from healthy donors as effector cells, were performed in triplicates using a 3-hour ^{51}Cr release assay as described (Elsasser *et al*, 1996). For blocking experiments, the parental antibody 3G8, the IgG1 isotype control, the CD123- and CD33-specific scFvs and the control scFv were added at a 125-fold and 500-fold molar excess, respectively. Dose-response curves were recorded using several equimolar 5-fold serial dilutions of the respective antibody fragments at a constant effector-to-target-cell (E:T) ratio of 40:1 MNCs to target cells. Background lysis induced by MNCs alone was subtracted from each data point, and EC_{50} values (concentration of an antibody fragment producing 50% of maximum specific lysis) and maximal lysis were calculated by using a sigmoidal dose-response curve fit. The experiments were repeated 6 times and mean values are reported, unless otherwise stated.

ADCC reactions with enriched NK cells

NK cells were enriched from the MNC fraction using a NK cell isolation Kit (Miltenyi, Bergisch Gladbach Germany) and immunomagnetic bead (MACS) technology following manufacturer's protocols. Purities ranged from 83 to 98% as judged by 2-colour flow cytometry using FITC-coupled CD16-specific antibodies (Beckman Coulter) and PC7-conjugated CD56-specific antibodies (BD Biosciences, Heidelberg, Germany) according to the manufacturer's instructions. Flow cytometric analysis was performed on a FC 500 flow cytometer (Brea, CA). Isolated NK cells were cultured overnight at a density of 2×10^6 cells/ml in RPMI 1640 Glutamax-I medium (Invitrogen) containing 10% FCS (Invitrogen), 100 units/ml penicillin (Invitrogen), and 100 $\mu\text{g}/\text{ml}$ streptomycin (Invitrogen). Cytotoxicity reactions were performed in an E:T cell ratio of 10:1 in a standard 4 h ^{51}Cr as described above. After 4 hours of incubation, 25 μl of supernatant was mixed with scintillation solution

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3 Supermix (Applied Biosystems, Darmstadt, Germany) and incubated for 15 min with
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5 agitation. ^{51}Cr -release from triplicates was measured in counts per minute (cpm) using the
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7 scintillation and luminescence counter 1450 Micro Beta TriLux (Perkin Elmer). Maximal ^{51}Cr
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9 release was determined by adding Triton X-100 (1% final concentration) to target cells.
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12 13 14 15 **Graphical and statistical analysis**

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17 Graphical and statistical analyses were performed using Graph Pad Prism Software (Graph
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19 Pad Software Inc, San Diego, CA) and Microsoft EXCEL. Group data were reported as
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21 means \pm standard error of the mean (SEM). Differences between groups were analyzed using
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23 paired Student *t* test. *P* values < 0.05 were considered significant.
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Results

Generation and binding characteristics of sctbs [123 x ds16 x 33] and [123 x ds16 x 123]

Sctbs [123 x ds16 x 33] and [123 x ds16 x 123] were constructed from previously published scFv components with specificities for CD123, CD33 and CD16 (Singer *et al*, 2010, Stein *et al*, 2010) (Fig 1A). Both constructs were expressed in stably transfected human 293T cells with yields ranging from 1.2 - 7.4 mg (sctb [123 x ds 16 x 33]) and 1.7 - 2.8 mg (sctb [123 x ds16 x 123]) per liter of cell culture medium. The electrophoretic mobilities of sctbs [123 x ds16 x 33] and [123 x ds16 x 123] corresponded to the molecular masses calculated from their amino acid sequences of approximately 89 and 90 kDa, respectively, and no breakdown products were detected (Fig 1B/C). The sctb [123 x ds16 x 33] specifically reacted with CD33⁺/CD123⁻/CD16⁻ U937 cells and stably transfected CD123⁺ or CD16⁺, 293 and CHO cells, respectively, but not with untransfected 293 or CHO cells (Fig 2A). Equilibrium binding constants of the dual targeting sctb were 18.8 ± 0.9 , 17.8 ± 2.2 , and 21.7 ± 1.8 nM for the reactions with CD123, CD33 and CD16 on single-positive cells, respectively (Table II). On double-positive cells, the sctb bound to CD123 and CD33 with a K_D -value of 13.5 ± 0.6 nM, demonstrating simultaneous binding with both terminal scFvs and a small avidity effect. The mono targeting sctb [123 x ds16 x 123] reacted specifically with CD123⁺ 293 and CD16⁺ CHO cells but not with untransfected 293 and CHO cells (Fig 2B). This mono targeting sctb bound CD123 and CD16 with K_D -values of 6.4 ± 1.1 and 24.7 ± 3.3 nM, respectively. The higher avidity for CD123 of the mono targeting agent compared to the affinity of 18.8 nM for the dual targeting agent (sctb [123 x ds16 x 33]) with only one binding site for CD123 also gave evidence that both terminal scFvs of the mono targeting agent contributed to the overall reaction with CD123.

In competition experiments binding of sctb [123 x ds16 x 33] was completely blocked by pre-incubation of antigen single-positive U937, CD123⁺ 293, and CD16⁺ CHO cells with a 50-fold molar excess of CD33- or CD123-specific scFv, or a 20-fold molar excess of mAb

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3 3G8, respectively (Fig 3A). Therefore, all three scFvs within the sctb [123 x ds16 x 33]
4
5 specifically reacted with their respective antigens.
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8 To test whether sctb [123 x ds16 x 33] was able to bind to more than one antigen
9
10 simultaneously, CD33 single-positive U937 cells were incubated with the sctb and stained in
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12 parallel with the recombinant proteins CD123ex-RFP and CD16ex-GFP (Fig 3B). These
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14 proteins contained the extracellular domains of the surface proteins CD123 and CD16,
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16 genetically fused to the red and green fluorescence proteins, respectively. As a result, the
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18 target cells showed green and red fluorescence, indicating that the sctb mediated binding of
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20 both CD123ex-RFP and CD16ex-GFP to the same target cell.
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24 To determine the *in vitro* serum stability of sctbs [123 x ds16 x 33] and [123 x ds16 x 123], the
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26 recombinant proteins were incubated in human serum at 37°C for different lengths of time.
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28 Residual binding activity was measured by flow cytometry and ranged between 81% and 94%
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30 on day 5 for each scFv on respective single-positive cell lines. Therefore, stability for both
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32 molecules for at least 5 days was confirmed (Table I).
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39 *ADCC of established AML-derived cell lines mediated by sctbs [123 x ds16 x 33] and*
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41 *[123 x ds16 x 123]*
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43 To test the ability of both sctbs to mediate ADCC *in vitro*, ADCC reactions with 2 different
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45 AML-derived cell lines were performed. Both, the sctbs [123 x ds16 x 33] and
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47 [123 x ds16 x 123] mediated potent lysis of the CD123⁺/CD33⁺ double-positive cell lines
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49 MOLM-13 and THP-1 in a dose-dependent manner (Fig 4). Samples were incubated for 3 hrs
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51 in a chromium release assay using unstimulated MNCs from unrelated healthy donors at an
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53 effector-to-target-cell ratio of 40:1. For MOLM-13 cells the sctbs [123 x ds16 x 33] and
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55 [123 x ds16 x 123] gave rise to EC₅₀ values in the low picomolar range of 21 and 50 pM,
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57 respectively. Maximum specific lysis was ~46% and ~37%, respectively (Fig 4A). For THP-1
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59 cells, EC₅₀ values ranged from 118 pM for the sctb [123 x ds16 x 33] to 212 pM for sctb
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3 [123 x ds16 x 123], with maximum specific lysis of ~32% and ~19%, respectively (Fig 4B).
4
5 In these experiments the dual targeting scfb [123 x ds16 x 33] showed significantly greater
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7 lysis at several concentrations than the mono targeting scfb [123 x ds16 x 123]. To test
8
9 whether target cell lysis was antigen-specific, competition ADCC experiments were
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11 performed with scfb [123 x ds16 x 33] at a concentration of 1 nM (Fig 5). Co-incubation with
12
13 a simultaneous 500-fold molar excess of both CD33- and CD123-specific scFvs completely
14
15 blocked lysis of MOLM-13 cells, whereas competition with either scFv alone reduced target
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17 cell lysis only to about half-maximum value. Therefore, both scFvs specific for CD33 and
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19 CD123 contained within the triplebody must have contributed to the ADCC reaction. Also,
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21 co-incubation with a 125-fold molar excess of the parental CD16-specific monoclonal
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23 antibody 3G8 but not with an IgG1 isotype control antibody (4G7), significantly reduced lysis
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25 of MOLM-13 cells, highlighting the specificity in triggering CD16⁺ effector cells.
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34 *ADCC of the established AML-derived cell line MOLM-13 mediated by scfbs*
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36 *[123 x ds16 x 33] and [123 x ds16 x 123] and enriched NK cells*
37

38 To investigate whether the scfbs were able to activate CD16⁺ effector cells not only within the
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40 MNC fraction but also to directly activate purified CD16⁺ NK cells, NK cells sorted with
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42 immunomagnetic beads were used in ADCC reactions (Fig 4C). Purified NK cells and
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44 MOLM-13 cells were used at an effector-to-target-cell ratio of 10:1 with triplebody
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46 concentrations of 1 nM in 3 independent experiments. Both scfbs mediated similar significant
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48 lysis (~60%) of AML cells compared to a control scfb. No specific lysis was observed when
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50 samples were incubated in the absence of NK cells. These results show first, that the scfbs
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52 were able to directly activate purified CD16⁺ NK cells in ADCC reactions; second, that the
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54 proteins alone without effector cells had no effect on the target cells; and third, that the
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56 protein preparations were therefore free of cytotoxic contaminants.
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ADCC of primary leukaemia cells from patients mediated by scpbs [123 x ds16 x 33], [123 x ds16 x 123] and [33 x ds16 x 33]

Finally, both scpbs were tested for their ability to mediate ADCC of freshly isolated primary leukaemia cells from AML patients. For these experiments, isolated MNCs from either peripheral blood or bone marrow of seven patients were used in ADCC reactions. Overall, six peripheral blood and two bone marrow samples were studied. MNCs from one unrelated healthy donor per AML patient sample were used as effector cells (Fig 6; Table II). No deliberate effort was made to match the histocompatibility type and killer cell Ig-like receptor (KIR) patterns of tumour cells and donors. The AML FAB- and World-Health-Organization- (WHO; Vardiman *et al*, 2009) classifications for each patient were known and are listed in Table II. For direct comparison of the potencies of the dual targeting and mono targeting scpbs in ADCC of primary leukaemia cells, the previously published scpb [33 x ds16 x 33] (Singer *et al*, 2010) was also carried along. All three scpbs showed potent lysis of primary AML cells in a concentration-dependent manner (Fig 6). Four representative ADCC reactions of samples from patients 1 and 2 (peripheral blood; Fig 6A+B) and patients 6 and 7 (bone marrow; Fig 6C+D) are depicted. At a concentration of 5 nM, all three recombinant proteins produced potent specific lysis (Fig 6E). In 4/8 samples, the dual targeting scpb [123 x ds16 x 33] reached the highest extent of lysis. This finding was confirmed when ADCC data from all six peripheral blood samples were combined (Fig 6F). The EC₅₀ values derived from this data set were ~250, ~130, and ~250 pM for the scpbs [123 x ds16 x 33], [123 x ds16 x 123] and [33 x ds16 x 33], respectively. Maximum specific lysis was ~25%, ~19% and ~19% of input cells, respectively. Although the differences in maximum specific lysis between the dual targeting scpb [123 x ds16 x 33] and the mono targeting scpbs [123 x ds16 x 123] and [33 x ds16 x 33] **did not yet reach statistical significance**, the dual targeting molecule showed a remarkable tendency to mediate higher specific lysis of primary AML cells than its mono targeting relatives. These results clearly demonstrate that the scpbs can induce potent

ADCC of primary AML cells of different AML types *in vitro*, and point to small but distinct advantages for the dual targeting agent over the corresponding mono targeting agents.

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Discussion

Here we describe the generation and characterization of two new antibody derivatives designed for use against AML cells. Although both sctbs are trivalent, the first, [123 x ds16 x 33] represents a “dual targeting” molecule, whereas the second, [123 x ds16 x 123], is a bispecific molecule with only one specificity for the tumour cell which we refer to as a “mono specific” or “mono targeting” agent. To our knowledge, the sctb [123 x ds16 x 33] is the first “dual targeting” single-chain triplebody simultaneously addressing two different antigens on a tumour cell to be reported. The sctb [123 x ds16 x 123] is an improvement over the existing bsscFv [123 x ds16] (Stein *et al*, 2010), in which the recently gained knowledge, that addition of a second scFv binding site for a tumour antigen leads to increased anti-tumour activity (Kellner *et al*, 2008), has been incorporated.

Both recombinant proteins were produced in stably transfected eukaryotic cells with expression yields ranging from 1.2 - 7.4 mg (sctb [123 x ds 16 x 33]) and 1.7 - 2.8 mg (sctb [123 x ds16 x 123]) per liter of cell culture medium. Both molecules bound specifically to antigen-positive cells. Furthermore, the sctb [123 x ds16 x 33] was able to bind to more than one antigen simultaneously, an essential prerequisite for the recruitment of CD16-positive effector cells. This also highlights the fact that three scFvs in tandem in one polypeptide-chain can produce a functional protein. Equilibrium binding constants on single-positive cells were approximately 22 nM (CD16), 18 nM (CD33), 19 nM (CD123) for the individual components carried in the sctbs, and 14 nM for the overall molecule on CD33/CD123 double-positive cells. The gain in affinity of approximately 1.4-fold on CD33/CD123 double-positive cells was less than one might have expected, based on our previous experience with the sctbs ds[19 x 16 x 19] and [33 x ds16 x 33] (Kellner *et al*, 2008, Singer *et al*, 2010). This may be due to differences in antigen density for CD33 and CD123 on double-positive cells, to differences in accessibility of both antigens or their spatial segregation into different subdomains. However, the result clearly indicates that both scFvs

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2
3 against CD33 and CD123 carried by the dual targeting agent participate in binding to the
4
5 respective antigens on double-positive cells. The K_D values of the mono targeting
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7 scfb [123 x ds16 x 123] were approximately 25 nM and 6 nM for the CD16 and CD123 scFvs,
8
9 respectively. This highlights a 3-fold gain in affinity for CD123 compared to the dual
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11 targeting scfb [123 x ds16 x 33], which must be due to an avidity effect caused by the addition
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13 of a second CD123-specific scFv in the mono targeting protein. This result is a further clear
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15 indication that both antigens actively participate in binding to the target cell, confirming
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17 earlier findings for this molecular format (Kellner *et al*, 2008, Singer *et al*, 2010). Another
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19 important parameter for antibody-derivatives is stability. When administered into the blood
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21 stream as therapeutic agents, such molecules need to be stable against degradation and
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23 unfolding (Carter 2006). Both proteins have been tested and were stable in human serum at
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25 37°C for at least 5 days. **Stability tests in immunocompetent mice similar to those**
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27 **previously reported for the scfb ds[19 x 16 x 19] (Kellner *et al*, 2008) are planned.**

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29 Both scfbs induced potent ADCC of the AML-derived, CD123/CD33 double-positive cell
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31 lines MOLM-13 and THP-1. For several concentrations the dual targeting
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33 scfb [123 x ds16 x 33] revealed a significantly higher lysis than the mono targeting
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35 scfb [123 x ds16 x 123]. This interesting result is probably due to the fact that a greater
36
37 combined antigen density was accessible to the dual targeting agent **and hence the molecule**
38
39 **showed a prolonged opsonisation of the tumour cell. This probably leads to an increased**
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41 **probability to recruit effector cells.** Competition experiments revealed that cell lysis by
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43 scfb [123 x ds16 x 33] was strictly antigen-specific. Co-incubation of the scfb with an excess
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45 of either anti-CD33 or anti-CD123 scFvs separately reduced cell lysis to about 50-60%.
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47 Remarkably, simultaneous co-incubation with both scFvs completely abolished cell lysis,
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49 indicating that both binding moieties played an active role not only in binding to the tumour
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51 cell but also in mediating ADCC. The same is true for CD16. Co-incubation with the CD16
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53 monoclonal antibody 3G8 reduced the measured cell lysis to such an extent that it was no
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3 longer statistically significant compared to the lysis obtained with a control scbt. In addition,
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5 both scbts were able to induce potent ADCC with purified CD16⁺ NK cells as effector cells at
6
7 an effector-to-target ratio of 10:1. Furthermore, cell lysis due to binding of the proteins to the
8
9 target cells alone in the absence of effector cells and lysis due to toxic contaminants in the
10
11 protein preparations were ruled out in these experiments by omission of the effector cells.
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14
15 Finally, the scbts also induced potent ADCC of primary cells from peripheral blood or bone
16
17 marrow of AML patients. For direct comparison of the ability to mediate ADCC of primary
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19 cells, the existing mono targeting scbt [33 x ds16 x 33] (Singer *et al*, 2010) was included in
20
21 these experiments. Although not yet statistically significant, the dual targeting
22
23 scbt [123 x ds16 x 33] induced the highest maximum lysis averaged over all patients. The
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25 isolated cells originated from a variety of AML subtypes according to FAB- and WHO-
26
27 classifications. These samples showed a high variability in the response to the scbts (Figure
28
29 6A-E). This is most likely due to the highly heterogenous nature of the AML, with many
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31 subtypes characterized by different genomic alterations, different disease phenotypes, disease
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33 progression and responses to treatment (Hiddemann *et al*, 2005). Therefore, a broad
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35 variability both in the fraction of total cells expressing CD33 and CD123 and cells susceptible
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37 to ADCC lysis mediated by our recombinant proteins was to be expected and had also been
38
39 previously observed for the susceptibility to the CD33-ETA' immunotoxin (Schwemmlin *et*
40
41 *al*, 2006). **It would be interesting in the future to study, whether the CD34⁺CD38⁻**
42
43 **compartment of primary AML-cells, obtained by preparative sorting, which contains**
44
45 **the AML-LSCs, shows a stronger extent of ADCC-lysis than other compartments devoid**
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47 **of AML-LSCs. Until now, these experiments could not be performed due to limited**
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49 **availability of primary patient cells.**
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55 Recent studies gave rise to the concept that CD16-positive monocytes/macrophages may play
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57 a vital role as effector cells *in vivo* mediating anti-tumour effects of therapeutic antibodies
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59 (Uchida *et al*, 2004, Tedder *et al*, 2006). In particular, their role in the elimination of AML
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3 cells by phagocytosis has been extensively studied (Jaiswal *et al*, 2009, Majeti *et al*, 2009). In
4
5 our experiments, CD16-positive NK cells represented the main effector cell population in
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7 purified MNCs, a leukocyte fraction devoid of monocytes/macrophages. It will be an
8
9 important question for the future to determine, whether our triplebodies are able to activate
10
11 not only NK cells by monovalent binding to CD16 but also CD16-positive macrophages.
12
13 Current studies in our group address this question.
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17 The novel scTbs [123 x ds16 x 33] and [123 x ds16 x 123] described in this study represent
18
19 promising therapeutic molecules for the treatment of AML in the future. Especially the dual-
20
21 targeting scTb [123 x ds16 x 33] might become valuable for the targeted elimination of AML-
22
23 LSCs. Its novel combination of two scFvs directed against antigens present on AML-LSCs at
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25 greater densities than on normal HSCs offers the potential for a preferential targeting of this
26
27 important cell type *in vivo*. Future studies will need to answer the question whether
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29 preferential targeting indeed is possible *in vivo*.
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Figure legends

Figure 1: Design and purification of scpbs [123 x ds16 x 33] and [123 x ds16 x 123]. (A) Structure of scpbs [123 x ds16 x 33] and [123 x ds16 x 123]. κ , secretion leader sequence from the murine Ig κ light chain; V_L and V_H, variable region light and heavy chains; L, (G₄S)₄ linker; S-S, disulphide bond; S, H, M, STREP-, hexahistidine- and myc-tags. (B) Purity of scpbs eluted from nickel-NTA, evaluated by Coomassie blue staining. (C) Western blot analysis using anti-His antibodies. Left lane: scpb [123 x ds16 x 33]; right lane: scpb [123 x ds16 x 123].

Figure 2: Antigen-specific binding of scpbs [123 x ds16 x 33] and [123 x ds16 x 123]. (A) Specific binding of the dual targeting scpb [123 x ds16 x 33] to CD33 single-positive U937 cells (I), CD123-stably transfected 293 (II), CD16-stably transfected CHO (III), untransfected 293 (IV), and untransfected CHO cells (V), respectively, analyzed by flow cytometry. White: control scpb; black: scpb [123 x ds16 x 33]. (B) Specific binding of the mono targeting scpb [123 x ds16 x 123] to single-positive CD123-stably transfected 293 (I), single-positive CD16-stably transfected CHO (II), untransfected 293 (III), and untransfected CHO cells (IV), respectively. White: control scpb; black: scpb [123 x ds16 x 123].

Figure 3: Simultaneous and specific binding of scpb [123 x ds16 x 33]. (A) The fluorescence signal on CD33 single-positive U937 cells incubated with scpb [123 x ds16 x 33] and stained with a fusion protein consisting of the extracellular domain of CD123 and red fluorescence protein (CD123ex-RFP) was abolished after preincubation of the cells with a 50-fold molar excess of CD33-specific scFv, whereas a control scFv did not reduce the signal (I). The fluorescence signal on CD123 single-positive 293 cells incubated with scpb [123 x ds16 x 33] and stained with a fusion protein consisting of CD16ex and green fluorescence protein

(CD16ex-GFP) was abolished after preincubation with a 50-fold molar excess of CD123-specific scFv, whereas a control scFv did not reduce the signal (II). (I) + (II) White: control sctb; black: sctb [123 x ds16 x 33]; light grey: sctb [123 x ds16 x 33] + control scFv; dark grey: sctb [123 x ds16 x 33] + CD33 specific scFv (I) + CD123-specific scFv (II). The fluorescence signal on CD16 single-positive CHO cells incubated with sctb [123 x ds16 x 33] and stained with CD33ex-RFP was abolished after preincubation with a 20-fold molar excess of anti-CD16 mAb 3G8, whereas a control mAb did not reduce the signal. White: control sctb; black sctb [123 x ds16 x 33]; light grey: sctb [123 x ds16 x 33] + control mAb; dark grey: sctb [123 x ds16 x 33] + 3G8 (III). (B) Simultaneous binding of sctb [123 x ds16 x 33] to CD33, CD123 and CD16. CD33 single-positive U937 cells were incubated with the sctb and stained simultaneously with CD123ex-RFP (I) and CD16ex-GFP (II). White: control sctb; black: sctb [123 x ds16 x 33].

Figure 4: Sctbs [123 x ds16 x 33] and [123 x ds16 x 123] induce ADCC of double-positive AML cell lines. Sctbs [123 x ds16 x 33] (closed triangle) and [123 x ds16 x 123] (open circle) mediated dose-dependent ADCC of double-positive MOLM-13 (A) and THP-1 (B) cells, whereas a control sctb (closed square) did not induce cell lysis. Data points represent mean percentage of specific lysis obtained with isolated MNCs from six different healthy donors at an effector-to-target-cell (E:T) ratio of 40:1. Specific lysis is total lysis minus spontaneous lysis. *Statistically significant differences ($P < 0.05$) in ADCC relative to the control sctb. #Statistically significant differences in mediating ADCC by sctb [123 x ds16 x 33] relative to sctb [123 x ds16 x 123]. (C) Specific lysis with purified NK cells mediated by sctbs [123 x ds16 x 33] and [123 x ds16 x 123]. In ADCC-reactions MOLM-13 and purified NK cells were used at an E:T ratio of 10:1 and incubated with the sctb at 1 nM. Three independent experiments with NK cells from three different donors were performed in triplicates. *Statistically significant differences ($P < 0.05$) in ADCC relative to the control sctb. Dark

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3 grey: no scfb; white: control scfb; black: scfb [123 x ds16 x 33]; light grey: scfb [123 x ds16 x
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10 **Figure 5:** Antigen-specific induction of ADCC in the double-positive AML cell-line MOLM-
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12 13 by scfb [123 x ds16 x 33]. Scfb [123 x ds16 x 33] induced potent ADCC of MOLM-13
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15 cells at a concentration of 1 nM. Simultaneous addition of a 500-fold molar excess of CD33-
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17 and CD123-specific scFvs each completely blocked the ADCC reaction, but not the addition
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19 of either CD33- or CD123-specific scFv alone, or a control scFv. Simultaneous incubation
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21 with a 125-fold molar excess of 3G8 antibody, but not with a control IgG1 (4G7),
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23 significantly reduced ADCC. Data points represent mean percentage of relative specific lysis
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25 obtained with isolated MNCs from six different healthy donors at an E:T ratio of 40:1.
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27 Specific lysis measured for the scfb [123 x ds16 x 33] was defined as 100%, lysis with no Ab
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29 was defined as 0%. Specific lysis is total lysis minus spontaneous lysis. *Statistically
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31 significant differences ($P < 0.05$) in ADCC relative to the control scfb.
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39 **Figure 6:** Lysis of primary AML cells by scfbs [123 x ds16 x 33], [123 x ds16 x 123] and [33
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41 x ds16 x 33]. Scfbs [123 x ds16 x 33] (closed triangle), [123 x ds16 x 123] (open circle) and
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43 [33 x ds16 x 33] (open triangle) mediated dose-dependent ADCC of primary AML cells,
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45 whereas a control scfb (closed square) failed to induce cellular lysis. (A and B) Induction of
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47 ADCC by scfbs of purified primary AML cells isolated from peripheral blood (patient 1 and
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49 2). (C and D) Induction of ADCC by scfbs of purified primary AML cells isolated from bone
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51 marrow (patient 6 and 7). Data points represent percentage of specific lysis obtained with
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53 isolated MNCs from one healthy donor at an E:T ratio of 40:1. (E) Induction of ADCC by
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55 scfbs at a concentration of 5 nM for all 8 samples. For patients 1 to 6 the cells analysed were
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57 MNCs isolated from peripheral blood. For patients 6 and 7, bone marrow-derived cells were
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59 studied. White bars: control scfb; black bars: scfb [123 x ds16 x 33]; light grey bars: scfb
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3 [123 x ds16 x 123]; dark grey bars: sctb [33 x ds16 x 33]. (F) Induction of ADCC by sctbs of
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5 purified AML cells from peripheral blood of 6 different patients (patient 1-6), combined data.
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7 Data points represent mean percentage of specific lysis averaged over the 6 patients obtained
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9 with isolated MNCs from one healthy donor per patient sample at an E:T ratio of 40:1.
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12 Specific lysis is total lysis minus spontaneous lysis.
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For Peer Review

Figure 1

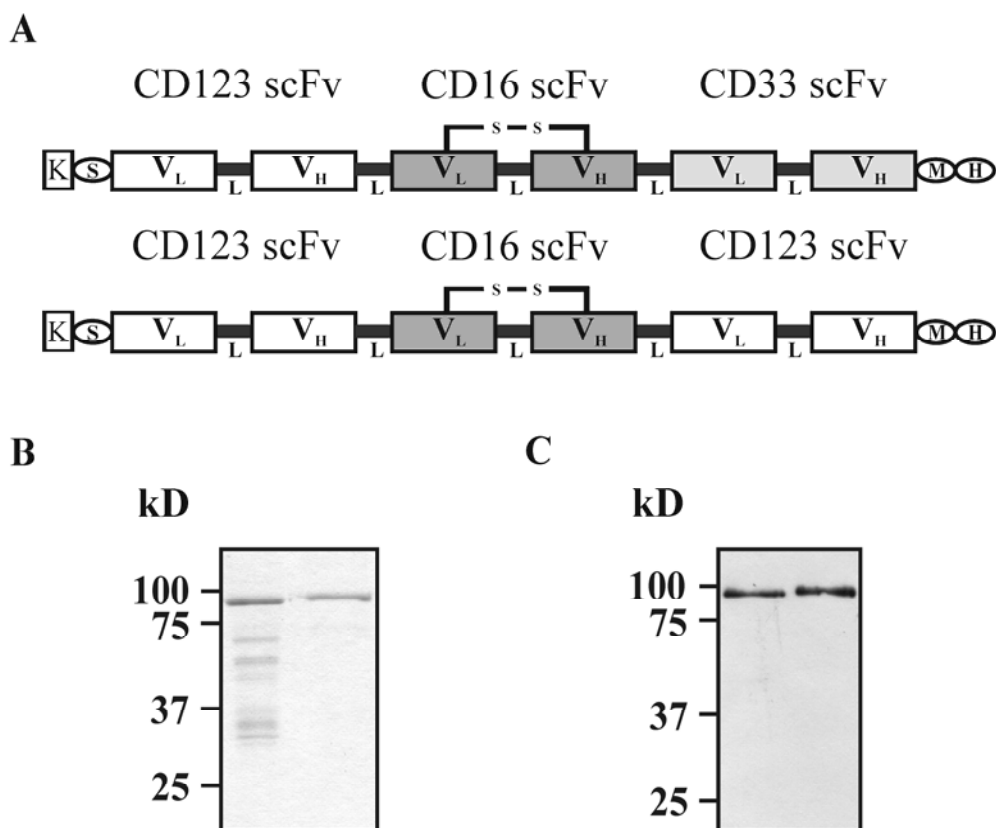


Figure 2

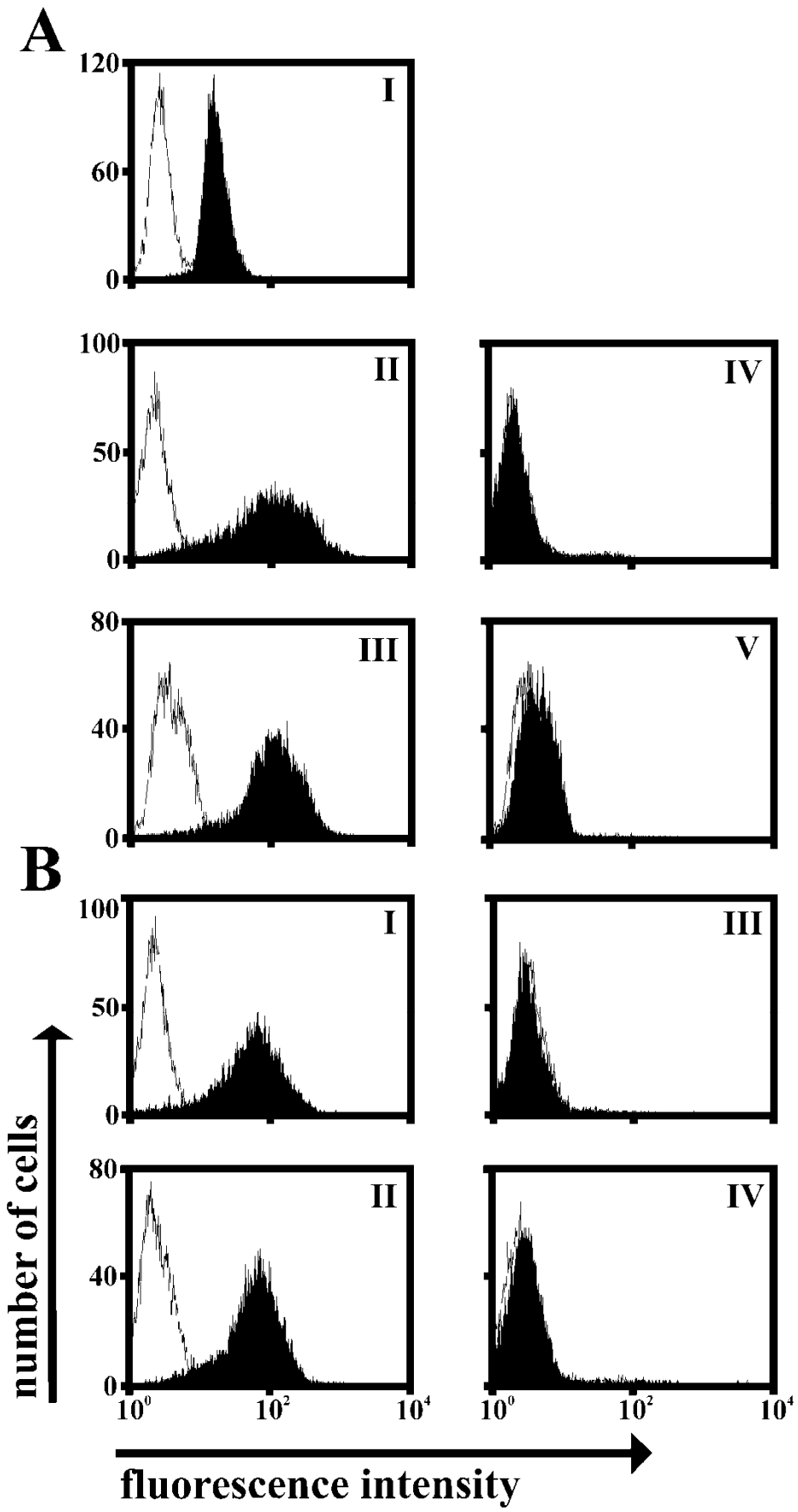


Figure 3

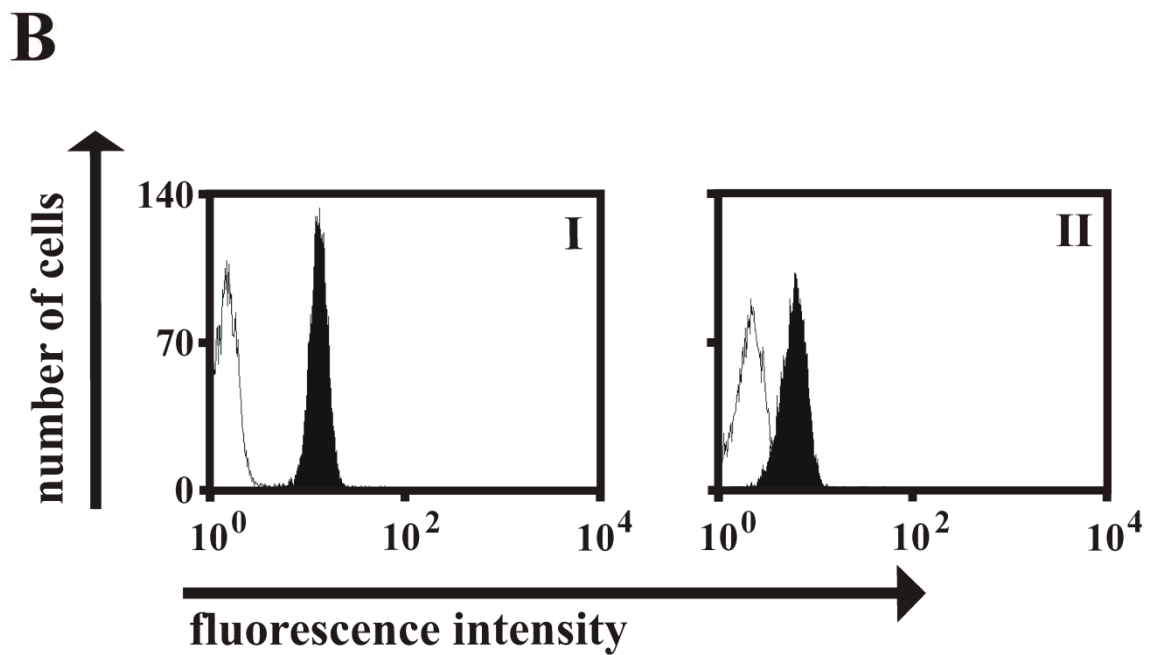
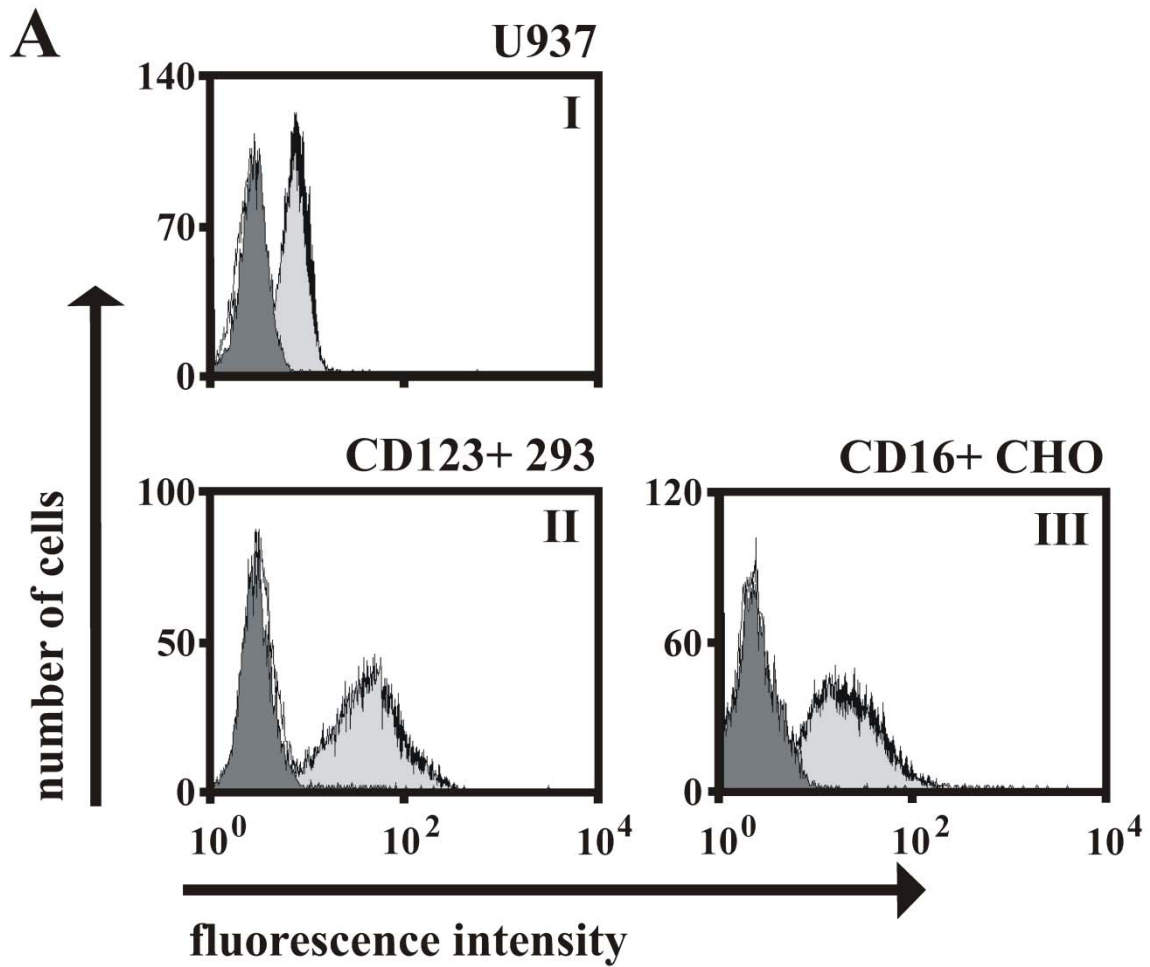


Figure 4

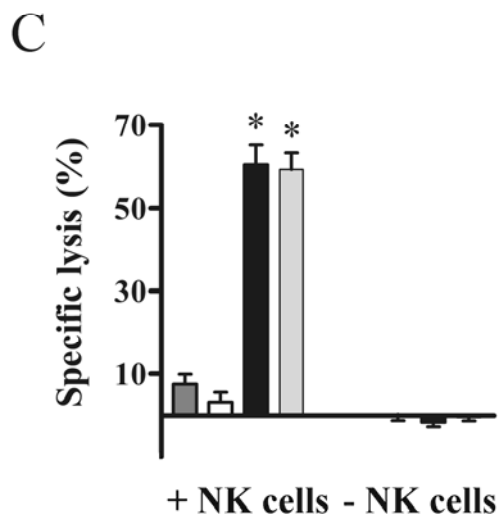
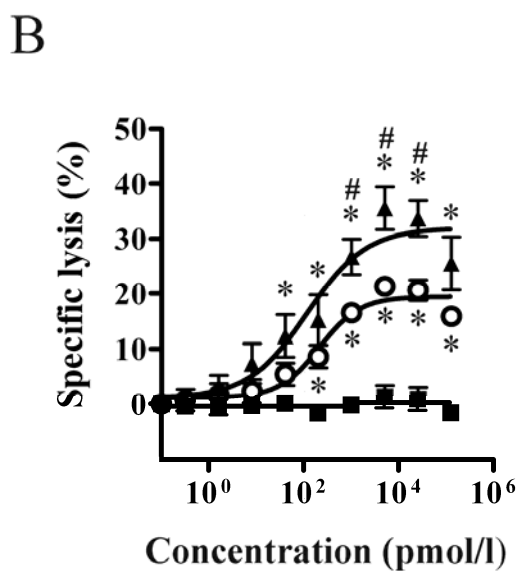
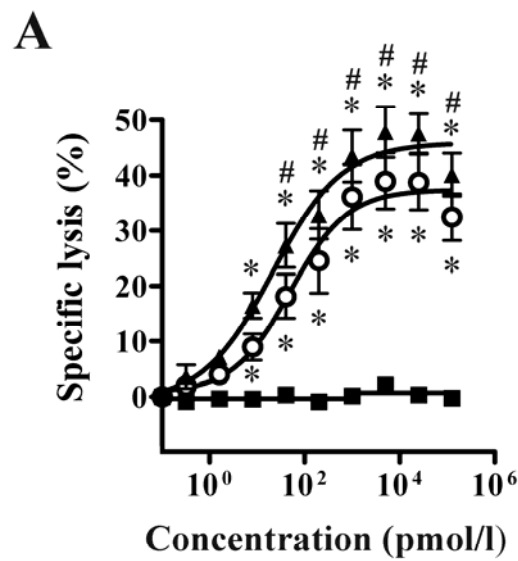


Figure 5

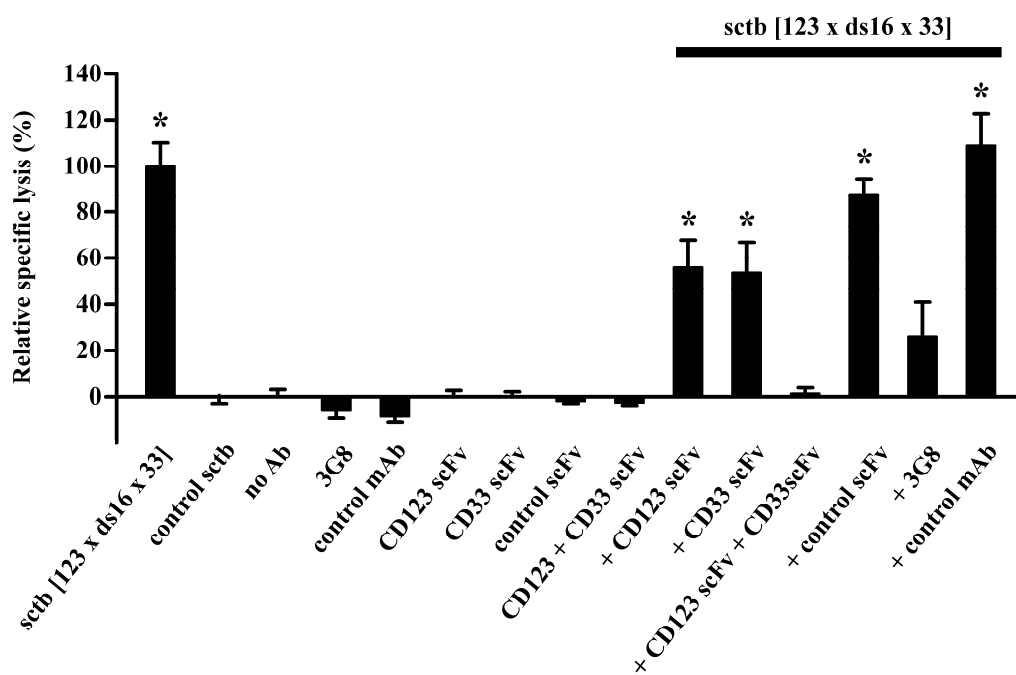


Figure 6

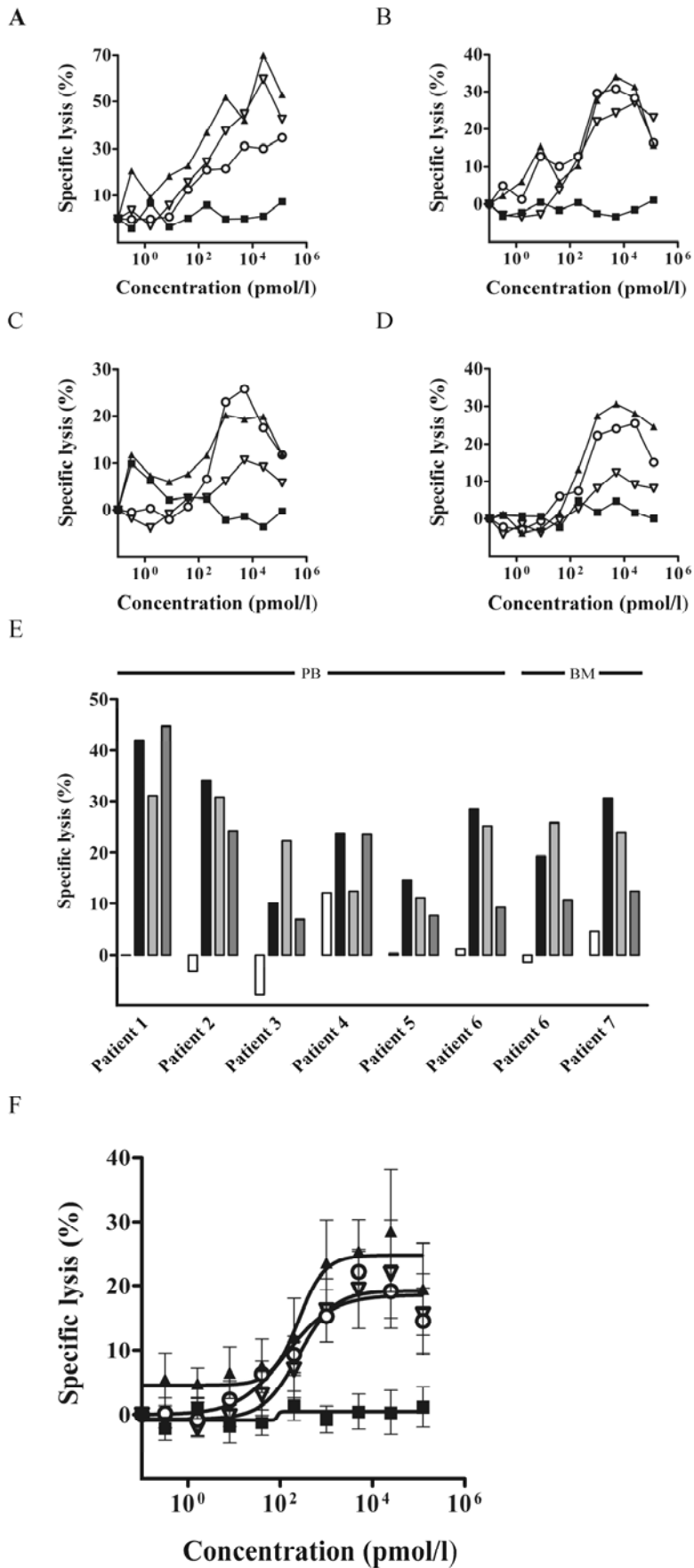


Table I: Equilibrium binding constants (K_D) and stability in human serum at 37°C for 5 days of the scFvs specific for CD123, CD33 and CD16 contained in the sctbs [123x ds16 x 33] and [123 x ds16 x 123].

	scFv			
	CD16	CD33	CD123	CD33/CD123*
sctb [123 x ds16 x 33]				
K_D (nM)	21.7 ± 1.8	17.8 ± 2.2	18.8 ± 0.9	13.5 ± 0.6
stable in human serum at 37°C	yes	yes	yes	yes
sctb [123 x ds16 x 123]				
K_D (nM)	24.7 ± 3.3	-	6.4 ± 1.1	-
stable in human serum at 37°C	yes	-	yes	-

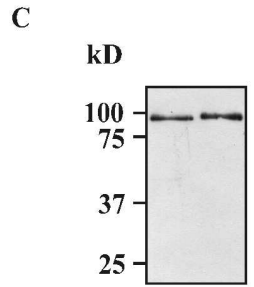
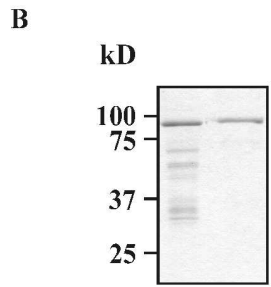
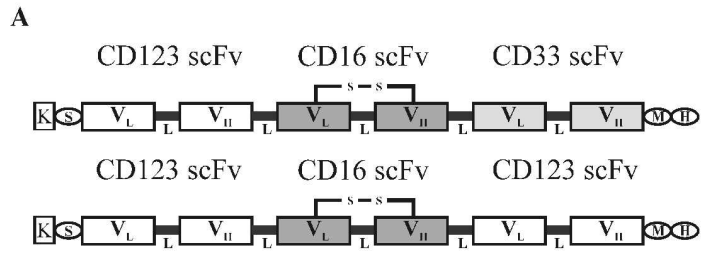
* avidity of the entire triplebody for the antigen-combination on double-positive cells

Table II. FAB/WHO classifications and source of primary AML material.

Patient	FAB	WHO	Source
1	M1	AML with mutated NPM1	PB
2	M2	AML with inv(16)	PB
3	M2	AML with Trisomy 14	PB
4	M4	AML with myelodysplasia-related changes	PB
5	punctio sicca	AML with myelodysplasia-related changes with mutated NPM1	PB
6	M2	AML with t(8;21)(q22;q22)	PB/BM
7	M2	Therapy related myeloid leukemia; t(8;21), Chr. 3 abn.	BM

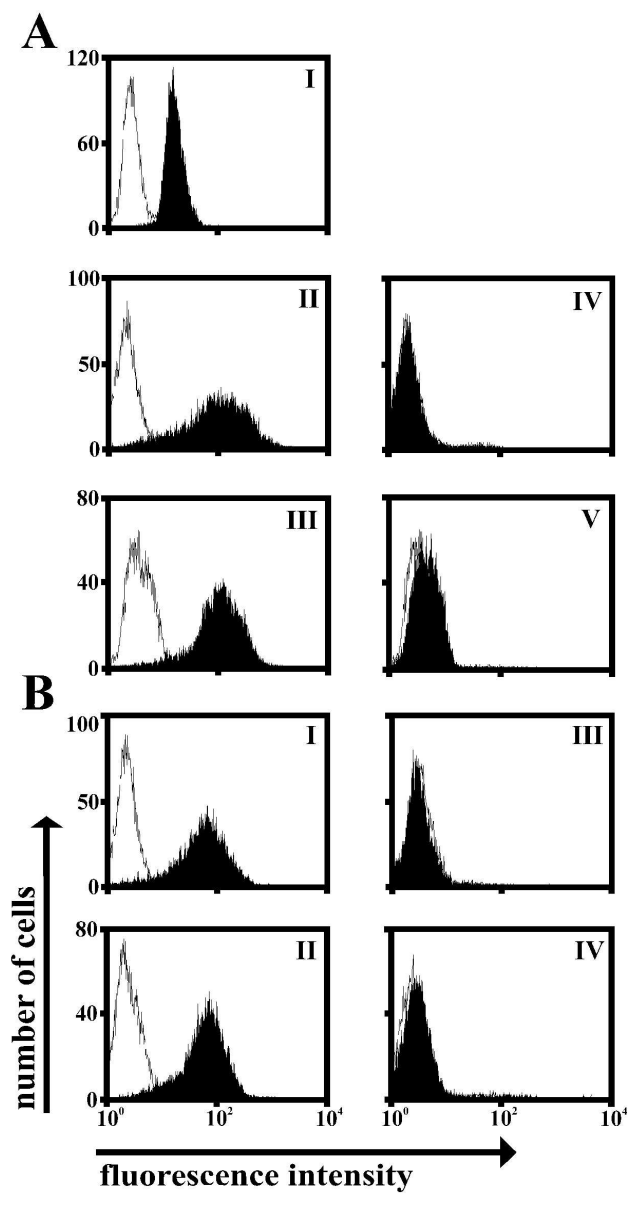
FAB, French-American-British classification; WHO, World Health Organization classification (Vardiman *et al*, 2009); BM, bone marrow; PB, peripheral blood; *punctio sicca*, sterna puncture produced no bone marrow material

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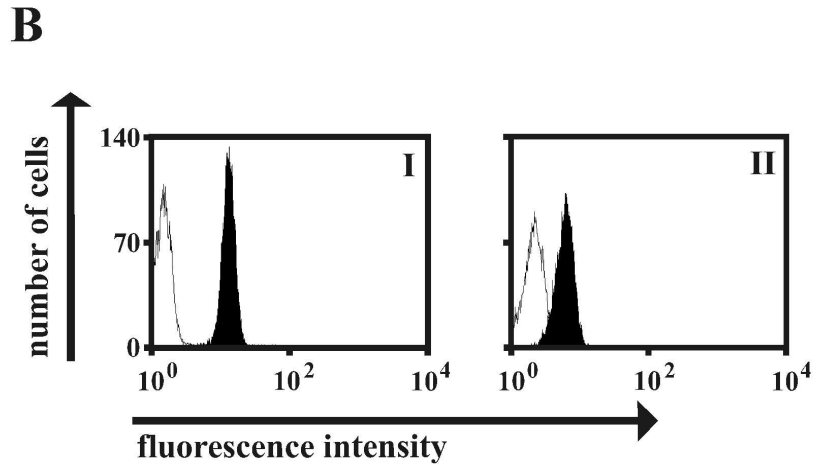
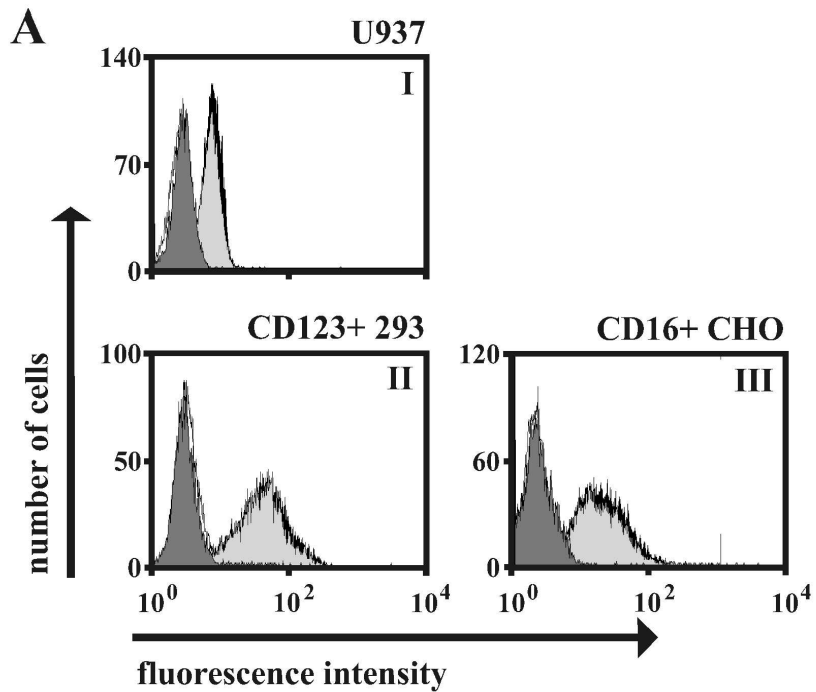


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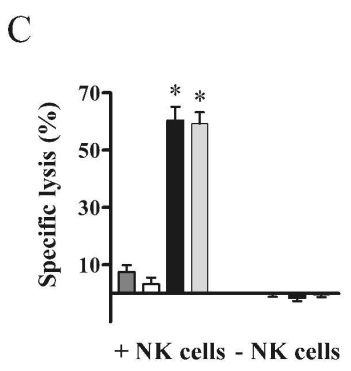
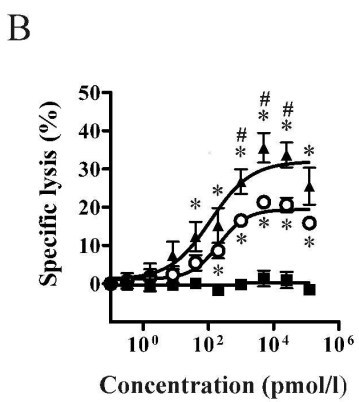
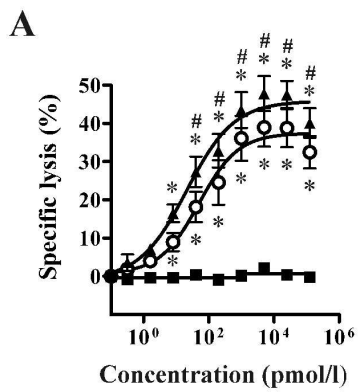
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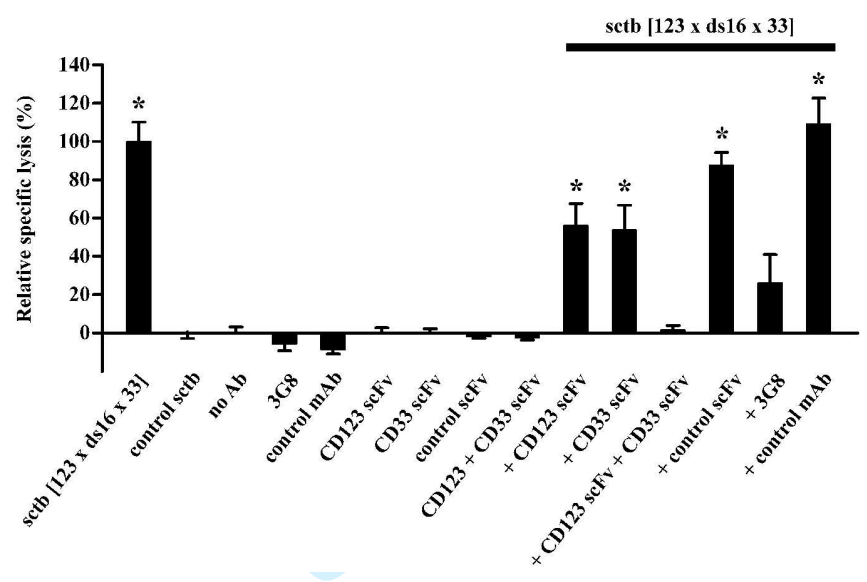
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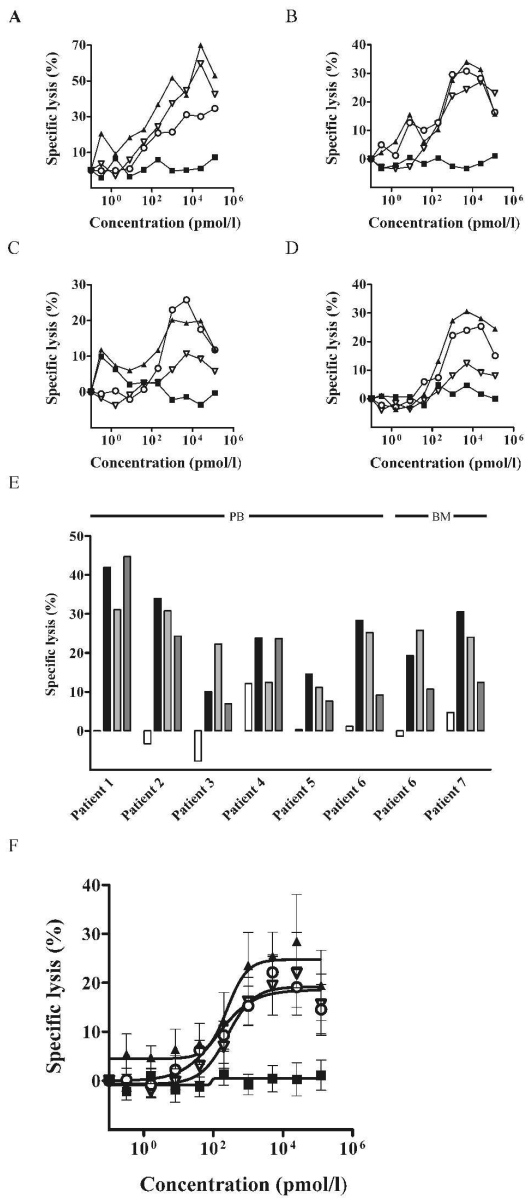


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Peer Review

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	CD16	CD33	scFv CD123	CD33/CD123
setb [123 x ds16 x 33]				
K_D (nM)	21.7 ± 1.8	17.8 ± 2.2	18.8 ± 0.9	13.5 ± 0.6
stable in human serum at 37°C	yes	yes	yes	yes
setb [123 x ds16 x 123]				
K_D (nM)	24.7 ± 3.3	-	6.4 ± 1.1	-
stable in human serum at 37°C	yes	-	yes	-

For Peer Review

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Patient	FAB	WHO	Source
1	M1	AML with mutated NPM1	PB
2	M2	AML with inv(16)	PB
3	M2	AML with Trisomy 14	PB
4	M4	AML with myelodysplasia-related changes	PB
5	punctio sicca	AML with myelodysplasia-related changes with mutated NPM1	PB
6	M2	AML with t(8;21)(q22;q22)	PB/BM
7	M2	Therapy related myeloid leukemia; t(8;21), Chr. 3 abn.	BM

For Peer Review

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