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To cite this version:
Markus Kügler, Christoph Stein, Christian Kellner, Kristin Mentz, Domenica Saul, et al.. A recombinant trispecific single-chain Fv derivative directed against CD123 and CD33 mediates effective elimination of acute myeloid leukaemia cells by dual targeting. British Journal of Haematology, Wiley, 2010, 150 (5), pp.574. 10.1111/j.1365-2141.2010.08300.x. hal-00556388

HAL Id: hal-00556388
https://hal.archives-ouvertes.fr/hal-00556388
Submitted on 16 Jan 2011
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| Journal: | British Journal of Haematology |
|---|---|
| Manuscript ID: | BJH-2010-00662.R1 |
| Manuscript Type: | Ordinary Papers |
| Date Submitted by the Author: | 28-May-2010 |
| Complete List of Authors: | Kügler, Markus; University of Erlangen-Nuremberg, Chair of Genetics Stein, Christoph; University of Erlangen-Nuremberg, Chair of Genetics Kellner, Christian; University of Schleswig-Holstein, Section of Stem cell Transplantation and Immunotherapy, University Medical Center Mentrz, Kristin; University of Erlangen-Nuremberg, Chair of Genetics Saul, Domenica; University of Erlangen-Nuremberg, Chair of Genetics Schwenkert, Michael; University of Erlangen-Nuremberg, Chair of Genetics Schubert, Ingo; University of Erlangen-Nuremberg, Chair of Genetics Singer, Heiko; University of Erlangen-Nuremberg, Chair of Genetics Oduncu, Fuat; University Hospital Munich, Campus Innenstadt - Medizinische Klinik, Division Hematology/Oncology Stockmeyer, Bernard; University Hospital Erlangen, Department of Medicine 5, Division Of Haematology/Oncology Mackensen, Anreas; University Hospital Erlangen, Department of Medicine 5, Division Of Haematology/Oncology Fey, G; University of Erlangen-Nuremberg, Chair of Genetics |
| Key Words: | CD33, CD123, dual targeting, leukaemia stem cell, single-chain Fv triplebody |
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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This research was supported by grants from the DFG (Deutsche Forschungsgemeinschaft; German Research Community) to GHF and AM, a student fellowship from Bayerische Eliteförderung (Bavarian Scholarship Foundation) to CS, postdoctoral/PhD fellowships from the German José-Carreras Leukemia-Foundation to MS and IS, a research grant No. 2007.049.1 from the Wilhelm Sander Foundation, Neustadt, Germany to GHF and BS, and support from the Stiftung Deutshe Krebshilfe, the Beitlich Foundation, Tübingen, and the Association “Kaminkehrer helfen krebskranken Kindern” (Chimney Sweeps support children with cancer) to GHF. Part of this work was funded by an intramural grant from the ELAN fond and the Training Grant GK592 from the German Research Community (DFG).

Running title: CD123-, CD33- and CD16-directed trispecific single-chain Fv fragment

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 Fcγ-receptor III (CD16) were designed and characterized for the elimination of acute myeloid leukaemia (AML) cells. The dual targeting single-chain Fv triplebody (sctb) \([123 \times ds16 \times 33]\) and the mono targeting sctb \([123 \times ds16 \times 123]\) 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, Schwemmlein 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-3Rα), which is predominantly expressed on myeloid cells and on a
subpopulation of B-lymphocytes. It is not expressed on platelets, red blood cells, natural killer cells (NK cells), peripheral T-cells (Moretti et al, 2001) and only in low density on HSCs (Huang et al, 1999, Jin et al, 2009). However, CD123 has been detected in a variety of haematopoietic malignancies and on AML-LSCs (Jordan et al, 2000, Munoz et al, 2001, Testa et al, 2002, Taussig et al, 2005). Strikingly, CD123 shows a 4-fold increased expression on AML-LSCs over normal HSCs (Jin et al, 2009). This finding together with the clinical observations, that elevated expression of CD123 in AML is associated with higher blast counts at diagnosis and a lower complete remission rate resulting in poorer prognosis (Testa et al, 2002, Graf et al, 2004, Testa et al, 2004) make CD123 a particularly interesting target for antibody-derived therapeutics (Jin et al, 2009).

AML-LSCs are known to possess several remarkable properties including self-renewal potential and increased resistance against chemotherapeutics and DNA damage (Lapidot et al, 1994, Bonnet and Dick 1997, Guan and Hogge 2000, Guzman et al, 2001, Hope et al, 2004, Ishikawa et al, 2007, Dick 2008). This unique cell population is thought to play a key role in the frequent occurrence of minimal residual disease (MRD) in relapsed AML patients after conventional chemotherapy (van Rhenen et al, 2005, Ravandi and Estrov 2006). Hence, after the discovery of AML-LSCs a great amount of effort has been spent to investigate the surface antigen profile of this clinically relevant cell population to identify suitable markers for their targeted elimination (Jordan et al, 2000, Taussig et al, 2005, Jin et al, 2006, Hosen et al, 2007, Jin et al, 2009). The fact that both antigens, CD33 and CD123, are expressed on AML-LSCs offers novel prospects for the treatment of AML. So far an immunotoxin (Du et al, 2007), a fusion of IL-3 with a truncated version of diphtheria toxin (Feuring-Buske et al, 2002, Frankel et al, 2008), a neutralizing full-length antibody (7G3; Jin et al, 2009), and a bispecific single-chain Fv (bsscFv; Stein et al, 2010) directed against CD123 have been described, and the monoclonal antibody 7G3 has been tested in a phase I clinical study (Roberts et al, 2008; http://clinicaltrials.gov/ct2/show/NCT00401739?term=CSL360&rank=1).
Well-studied antibody-derived agents directed against CD33 include apart from GO an immunotoxin (Schwemmlein et al, 2006), a fusion protein between a single chain Fv antibody fragment (scFv) and the sTRAIL death receptor ligand (ten Cate et al, 2009), and siRNA-loaded liposomes coated with CD33-directed scFvs (Rothdiener et al, 2010).

**BsscFvs** have advantageous properties over full-length bispecific antibodies and chemically coupled bispecific Fragment antibody binding (Fab) fragments, and have recently produced convincing therapeutic effects in clinical trials (Bargou et al, 2008). To overcome some of the remaining weaknesses of this format, such as an unfavorable plasma retention, the format has been further improved by expanding it to tandem diabodies (Kipriyanov et al, 1999) and single-chain triplebodies (sctbs; Kellner et al, 2008). The incorporation of a second scFv-binding site for tumour-antigens in these extended formats has led to increased avidity for the tumour cell, increased anti-tumour activity in antibody dependent cellular cytotoxicity (ADCC) reactions, and improved plasma retention in vivo (Kellner et al, 2008). An example for a setb with improved ADCC activity for AML cells is the setb [33 x ds16 x 33] (Singer et al, 2010) with two scFv antigen binding sites for CD33 on AML cells and one for CD16, the low affinity Fcγ RIII receptor for IgG on NK cells and macrophages (Ravetch and Perussia 1989, Daeron 1997). In a direct comparison, this setb showed increased anti-leukaemic activity for AML cells over the corresponding bsscFv [33 x ds16] with only one binding site each for CD33 and CD16. Before these trivalent single-polypeptide formats, other trispecific antibody derivatives had been explored. One example was a trispecific Fab construct, consisting of three chemically coupled Fab-fragments (Somasundaram et al, 1999). A second trispecific construct was called a "tri-body", a two-chain polypeptide based on a Fab fragment as scaffold (Schoonjans et al, 2001). Two scFvs were fused to the C-termini of the L- and Fd-chains of this Fab, respectively. Finally, a trispecific molecule based on a VH domain has been reported, which carried two scFvs fused to a VH-domain (Song et al, 2003).
The key molecule presented here, the sctb [123 x ds16 x 33], differs significantly in its design from all of these examples. It is a single-chain polypeptide with only one binding site for an effector cell and two for two different tumour antigens, CD123 and CD33. The goal of this design was to achieve improved anti-leukaemic activity by "dual targeting" of the tumour cell, i.e. by simultaneous binding to two different antigens on the same tumour cell. We expected this molecule to bind with greater avidity to CD123 and CD33 on double-positive AML cells than the corresponding control sctb [123 x ds16 x 123] and [33 x ds16 x 33], because it would take advantage of two populations of target antigens rather than only one. Based on theoretical arguments, the avidity of such proteins was expected to be proportional to the combined target antigen density on the tumour cell (Mattes 1997, Pluckthun and Pack 1997).

The prediction of added benefits by dual- over mono-specific targeting of tumour cells is intuitively appealing, and has been pursued for a number of years (Robinson et al, 2008), but has not yet been conclusively proven for scFv-derived agents recruiting effector cells. A definitive proof of this prediction is experimentally demanding and is still under investigation.

However, indirect evidence in favour of the proposition has been obtained for scFv-immunotoxins, and therefore, the concept may also extend to agents recruiting effector cells (Vallera et al, 2005). The molecules presented here provide an opportunity to directly evaluate the benefits of dual over mono-specific targeting for AML cells. Finally, they provide new means to test the prediction that dual targeting a combination of two antigens, which are more highly expressed on AML-LSCs than on HSCs, may permit the preferential elimination of AML-LSCs. It would be important to test this prediction, because if confirmed it would provide a possibility for a deliberate attack on the LSCs with the intent to eliminate MRD cells and prevent relapse, while preserving some HSCs for haematopoietic reconstitution after the end of treatment, without the need for autologous stem cell transplantation.

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-CD33dsCD16xCD33 (Singer et al, 2010) replacing
the coding sequence for the N-terminal CD33-specific scFv. Thereby the vector 
pSecTag2HygroC-STREP-His-CD123xdsCD16xCD33 was produced. In these constructs, the 
index ds designates the disulfide-stabilized variants (Bruenke et al, 2004). To generate the 
vector pSecTag2HygroC-STREP-His-CD123xdsCD16xCD123, the sequence coding for the 
CD123-specific scFv was amplified by PCR from the vector pAK400-CD123scFv and ligated 
into pSecTag2HygroC-STREP-His-CD123xdsCD16xCD33, using XhoI/EcoRV restriction 
sites, and replacing the coding sequence for the C-terminal CD33-specific scFv. For 
construction of the expression vector pSecTag2HygroC-hCD123ex-DsRed, cDNA coding for 
the extracellular domain of CD123 was amplified by PCR using the existing vector 
pSecTag2HygroC-hCD123ex-Fc (Stein et al, 2010) and ligated into the vector 
pSecTag2HygroC-CD33ex-DsRed (Singer et al, 2010), replacing the extracellular domain of 
CD33. Correct construction of the final constructs was confirmed by DNA sequence analysis 
on an AppliedBiosystems automated DNA sequencer (ABI Prism 310 Genetic Analyzer; 
Perkin-Elmer, Ueberlingen, Germany).

Expression and purification of scfvs [123 x ds16 x 33] and [123 x ds16 x 123]

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

**Sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blot analysis**

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

**Flow cytometry analysis**

Immunofluorescence analysis was performed on a FACSCalibur instrument using CellQuest software (Becton Dickinson, Heidelberg, Germany) as described (Schwemmlein et al., 2006). Briefly, 1 x 10⁴ events were collected for each sample, and whole cells were analyzed using appropriate scatter gates to exclude cellular debris and aggregates. The recombinant sctb proteins were detected using a penta-His antibody and a PE-conjugated goat anti-mouse-IgG F(ab’)₂ (DAKO Diagnostica GmbH, Hamburg, Germany), unless otherwise stated.
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 µg/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 x 10$^6$ cells/ml in RPMI 1640 Glutamax-I medium (Invitrogen) containing 10% FCS (Invitrogen), 100 units/ml penicillin (Invitrogen), and 100 µg/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
Supermix (Applied Biosystems, Darmstadt, Germany) and incubated for 15 min with agitation. $^{51}$Cr-release from triplicates was measured in counts per minute (cpm) using the scintillation and luminescence counter 1450 Micro Beta TriLux (Perkin Elmer). Maximal $^{51}$Cr release was determined by adding Triton X-100 (1% final concentration) to target cells.

**Graphical and statistical analysis**

Graphical and statistical analyses were performed using Graph Pad Prism Software (Graph Pad Software Inc, San Diego, CA) and Microsoft EXCEL. Group data were reported as means ± standard error of the mean (SEM). Differences between groups were analyzed using paired Student $t$ test. $P$ values < 0.05 were considered significant.
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<sub>D</sub>-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<sub>D</sub>-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
3G8, respectively (Fig 3A). Therefore, all three scFvs within the scFv [123 x ds16 x 33] specifically reacted with their respective antigens.

To test whether scFv [123 x ds16 x 33] was able to bind to more than one antigen simultaneously, CD33 single-positive U937 cells were incubated with the scFv and stained in parallel with the recombinant proteins CD123ex-RFP and CD16ex-GFP (Fig 3B). These proteins contained the extracellular domains of the surface proteins CD123 and CD16, genetically fused to the red and green fluorescence proteins, respectively. As a result, the target cells showed green and red fluorescence, indicating that the scFv mediated binding of both CD123ex-RFP and CD16ex-GFP to the same target cell.

To determine the in vitro serum stability of scFvs [123 x ds16 x 33] and [123 x ds16 x 123], the recombinant proteins were incubated in human serum at 37°C for different lengths of time. Residual binding activity was measured by flow cytometry and ranged between 81% and 94% on day 5 for each scFv on respective single-positive cell lines. Therefore, stability for both molecules for at least 5 days was confirmed (Table I).

**ADCC of established AML-derived cell lines mediated by scFvs [123 x ds16 x 33] and [123 x ds16 x 123]**

To test the ability of both scFvs to mediate ADCC in vitro, ADCC reactions with 2 different AML-derived cell lines were performed. Both, the scFvs [123 x ds16 x 33] and [123 x ds16 x 123] mediated potent lysis of the CD123⁺/CD33⁺ double-positive cell lines MOLM-13 and THP-1 in a dose-dependent manner (Fig 4). Samples were incubated for 3 hrs in a chromium release assay using unstimulated MNCs from unrelated healthy donors at an effector-to-target-cell ratio of 40:1. For MOLM-13 cells the scFvs [123 x ds16 x 33] and [123 x ds16 x 123] gave rise to EC₅₀ values in the low picomolar range of 21 and 50 pM, respectively. Maximum specific lysis was ~46% and ~37%, respectively (Fig 4A). For THP-1 cells, EC₅₀ values ranged from 118 pM for the scFv [123 x ds16 x 33] to 212 pM for scFv
[123 x ds16 x 123], with maximum specific lysis of ~32% and ~19%, respectively (Fig 4B). In these experiments the dual targeting sc tb [123 x ds16 x 33] showed significantly greater lysis at several concentrations than the mono targeting sc tb [123 x ds16 x 123]. To test whether target cell lysis was antigen-specific, competition ADCC experiments were performed with sc tb [123 x ds16 x 33] at a concentration of 1 nM (Fig 5). Co-incubation with a simultaneous 500-fold molar excess of both CD33- and CD123-specific scFvs completely blocked lysis of MOLM-13 cells, whereas competition with either scFv alone reduced target cell lysis only to about half-maximum value. Therefore, both scFvs specific for CD33 and CD123 contained within the triplebody must have contributed to the ADCC reaction. Also, co-incubation with a 125-fold molar excess of the parental CD16-specific monoclonal antibody 3G8 but not with an IgG1 isotype control antibody (4G7), significantly reduced lysis of MOLM-13 cells, highlighting the specificity in triggering CD16+ effector cells.

**ADCC of the established AML-derived cell line MOLM-13 mediated by sc tbs [123 x ds16 x 33] and [123 x ds16 x 123] and enriched NK cells**

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

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

Both scFvs induced potent ADCC of the AML-derived, CD123/CD33 double-positive cell lines MOLM-13 and THP-1. For several concentrations the dual targeting scFv [123 x ds16 x 33] revealed a significantly higher lysis than the mono targeting scFv [123 x ds16 x 123]. This interesting result is probably due to the fact that a greater combined antigen density was accessible to the dual targeting agent and hence the molecule showed a prolonged opsonisation of the tumour cell. This probably leads to an increased probability to recruit effector cells. Competition experiments revealed that cell lysis by scFv [123 x ds16 x 33] was strictly antigen-specific. Co-incubation of the scFv with an excess of either anti-CD33 or anti-CD123 scFvs separately reduced cell lysis to about 50-60%. Remarkably, simultaneous co-incubation with both scFvs completely abolished cell lysis, indicating that both binding moieties played an active role not only in binding to the tumour cell but also in mediating ADCC. The same is true for CD16. Co-incubation with the CD16 monoclonal antibody 3G8 reduced the measured cell lysis to such an extent that it was no
longer statistically significant compared to the lysis obtained with a control sc tb. In addition, both sc tbs were able to induce potent ADCC with purified CD16+ NK cells as effector cells at an effector-to-target ratio of 10:1. Furthermore, cell lysis due to binding of the proteins to the target cells alone in the absence of effector cells and lysis due to toxic contaminants in the protein preparations were ruled out in these experiments by omission of the effector cells.

Finally, the sc tbs also induced potent ADCC of primary cells from peripheral blood or bone marrow of AML patients. For direct comparison of the ability to mediate ADCC of primary cells, the existing mono targeting sc tb [33 x ds16 x 33] (Singer et al, 2010) was included in these experiments. Although not yet statistically significant, the dual targeting sc tb [123 x ds16 x 33] induced the highest maximum lysis averaged over all patients. The isolated cells originated from a variety of AML subtypes according to FAB- and WHO-classifications. These samples showed a high variability in the response to the sc tbs (Figure 6A-E). This is most likely due to the highly heterogenous nature of the AML, with many subtypes characterized by different genomic alterations, different disease phenotypes, disease progression and responses to treatment (Hiddemann et al, 2005). Therefore, a broad variability both in the fraction of total cells expressing CD33 and CD123 and cells susceptible to ADCC lysis mediated by our recombinant proteins was to be expected and had also been previously observed for the susceptibility to the CD33-ETA' immunotoxin (Schwemmlein et al, 2006). It would be interesting in the future to study, whether the CD34+CD38- compartment of primary AML-cells, obtained by preparative sorting, which contains the AML-LSCs, shows a stronger extent of ADCC-lysis than other compartments devoid of AML-LSCs. Until now, these experiments could not be performed due to limited availability of primary patient cells.

Recent studies gave rise to the concept that CD16-positive monocytes/macrophages may play a vital role as effector cells in vivo mediating anti-tumour effects of therapeutic antibodies (Uchida et al, 2004, Tedder et al, 2006). In particular, their role in the elimination of AML
cells by phagocytosis has been extensively studied (Jaiswal et al, 2009, Majeti et al, 2009). In our experiments, CD16-positive NK cells represented the main effector cell population in purified MNCs, a leukocyte fraction devoid of monocytes/macrophages. It will be an important question for the future to determine, whether our triplebodies are able to activate not only NK cells by monovalent binding to CD16 but also CD16-positive macrophages. Current studies in our group address this question.

The novel sctbs [123 x ds16 x 33] and [123 x ds16 x 123] described in this study represent promising therapeutic molecules for the treatment of AML in the future. Especially the dual-targeting sctb [123 x ds16 x 33] might become valuable for the targeted elimination of AML-LSCs. Its novel combination of two scFvs directed against antigens present on AML-LSCs at greater densities than on normal HSCs offers the potential for a preferential targeting of this important cell type in vivo. Future studies will need to answer the question whether preferential targeting indeed is possible in vivo.

Acknowledgements

The authors thank Prof. R. Levy from Stanford University for making the 4G7 hybridoma available, Dr. J. G. van de Winkel for the CD16 transfected CHO cells, and Prof. J. Dick from Toronto for valuable scientific advice. We are grateful to B. Bock and S. Standar for excellent technical assistance and Th. Lange for administrative assistance.
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**Figure legends**

**Figure 1:** Design and purification of sctbs [123 x ds16 x 33] and [123 x ds16 x 123]. (A) Structure of sctbs [123 x ds16 x 33] and [123 x ds16 x 123]. κ, secretion leader sequence from the murine Igκ light chain; VL and VH, 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 sctbs eluted from nickel-NTA, evaluated by Coomassie blue staining. (C) Western blot analysis using anti-His antibodies. Left lane: sctb [123 x ds16 x 33]; right lane: sctb [123 x ds16 x 123].

**Figure 2:** Antigen-specific binding of sctbs [123 x ds16 x 33] and [123 x ds16 x 123]. (A) Specific binding of the dual targeting sctb [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 sctb; black: sctb [123 x ds16 x 33]. (B) Specific binding of the mono targeting sctb [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 sctb; black: sctb [123 x ds16 x 123].

**Figure 3:** Simultaneous and specific binding of sctb [123 x ds16 x 33]. (A) The fluorescence signal on CD33 single-positive U937 cells incubated with sctb [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 sctb [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
grey: no scFv; white: control scFv; black: scFv [123 x ds16 x 33]; light grey: scFv [123 x ds16 x 123].

**Figure 5:** Antigen-specific induction of ADCC in the double-positive AML cell-line MOLM-13 by scFv [123 x ds16 x 33]. ScFv [123 x ds16 x 33] induced potent ADCC of MOLM-13 cells at a concentration of 1 nM. Simultaneous addition of a 500-fold molar excess of CD33- and CD123-specific scFvs each completely blocked the ADCC reaction, but not the addition of either CD33- or CD123-specific scFv alone, or a control scFv. Simultaneous incubation with a 125-fold molar excess of 3G8 antibody, but not with a control IgG1 (4G7), significantly reduced ADCC. Data points represent mean percentage of relative specific lysis obtained with isolated MNCs from six different healthy donors at an E:T ratio of 40:1. Specific lysis measured for the scFv [123 x ds16 x 33] was defined as 100%, lysis with no Ab was defined as 0%. Specific lysis is total lysis minus spontaneous lysis. *Statistically significant differences (P < 0.05) in ADCC relative to the control scFv.

**Figure 6:** Lysis of primary AML cells by scFvs [123 x ds16 x 33], [123 x ds16 x 123] and [33 x ds16 x 33]. ScFvs [123 x ds16 x 33] (closed triangle), [123 x ds16 x 123] (open circle) and [33 x ds16 x 33] (open triangle) mediated dose-dependent ADCC of primary AML cells, whereas a control scFv (closed square) failed to induce cellular lysis. (A and B) Induction of ADCC by scFvs of purified primary AML cells isolated from peripheral blood (patient 1 and 2). (C and D) Induction of ADCC by scFvs of purified primary AML cells isolated from bone marrow (patient 6 and 7). Data points represent percentage of specific lysis obtained with isolated MNCs from one healthy donor at an E:T ratio of 40:1. (E) Induction of ADCC by scFvs at a concentration of 5 nM for all 8 samples. For patients 1 to 6 the cells analysed were MNCs isolated from peripheral blood. For patients 6 and 7, bone marrow-derived cells were studied. White bars: control scFv; black bars: scFv [123 x ds16 x 33]; light grey bars: scFv
[123 x ds16 x 123]; dark grey bars: sctb [33 x ds16 x 33]. (F) Induction of ADCC by sctbs of purified AML cells from peripheral blood of 6 different patients (patient 1-6), combined data. Data points represent mean percentage of specific lysis averaged over the 6 patients obtained with isolated MNCs from one healthy donor per patient sample at an E:T ratio of 40:1. Specific lysis is total lysis minus spontaneous lysis.
Figure 1

A

CD123 scFv  CD16 scFv  CD33 scFv

CD123 scFv  CD16 scFv  CD123 scFv

B

kD

100  75  37  25

C

kD

100  75  37  25
Figure 2

A

I

II

IV

III

V

B

I

III

II

IV

number of cells

fluorescence intensity
Figure 3

A

U937

CD123+ 293

CD16+ CHO

number of cells

10^0 10^2 10^4

fluorescence intensity

B

number of cells

10^0 10^2 10^4

fluorescence intensity
Figure 4

A

B

C

Specific lysis (%)

10^0 10^2 10^4 10^6

Concentration (pmol/l)

Specific lysis (%)

Specific lysis (%)

+ NK cells - NK cells
Figure 5

[Graph showing relative-specific lysis (%)]

setb [123 x ds16 x 33]
Figure 6

A

B

C

D

E

F
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 setbs $[123 \times ds16 \times 33]$ and $[123 \times ds16 \times 123]$.

| scFv       | CD16          | CD33          | CD123         | CD33/CD123*   |
|------------|---------------|---------------|---------------|--------------|
| $[123 \times ds16 \times 33]$ |
| $K_D$ (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 |
| $[123 \times ds16 \times 123]$ |
| $K_D$ (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
|                  | CD16  | CD33  | 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 | - |
| 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       | puncto 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     |