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Research paper

Development of a novel fully-human anti-CD123 antibody to target acute myeloid leukemia

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ABSTRACT

Monoclonal antibodies are being considered as biopharmaceuticals for the *in vivo* targeting of acute myeloid leukemia. Here we describe the generation and characterization of a fully-human monoclonal antibody specific to CD123, a surface marker which is overexpressed in a variety of hematological disorders, including acute myeloid leukemia. The cloning and expression of the extracellular portion of CD123 as recombinant Fc fusion allowed the selection and affinity maturation of a human antibody, called H9, which specifically recognized the cognate antigen in biochemical assays and on leukemic cells. The H9 antibody and a previously-described anti-CD123 antibody (CSL362) were reformatted into full immunoglobulin human IgG1 formats, including a variant bearing S293D and I332E mutations to enhance antibody-dependent cell-mediated cytotoxicity (ADCC). The two antibodies recognized different epitopes on the surface of the N-terminal domain of CD123, as revealed by crystallography and SPOT analysis. Both H9 and CSL362 in full immunoglobulin format were able to selectively kill leukemic cells in *in vitro* ADCC assays, performed both with cell lines and with patient-derived AML blasts. Further, the two antibodies, when reformatted as bispecific BiTE™ reagents by fusion with the anti-CD3 scFv (OKT3) antibody fragment, induced selective killing of AML blasts by patient-derived, autologous T-cells in an *in vitro* setting, but BiTE(CSL362/OKT3) exhibited a 10-fold higher potency compared to BiTE(H9/OKT3). The availability of two classes of CD123-specific biopharmaceuticals, capable of redirecting the cytolytic activity of NK cells and T cells against AML blasts, may enable novel interventional strategies and combination opportunities for the treatment of AML.

1. Introduction

In spite of progress in the treatment of acute myeloid leukemia (AML), overall disease mortality remains high for young patients who do not benefit from allogeneic hematopoietic stem cell transplantation (allo-HSCT) and for the elderly population, not eligible for intensive chemotherapy and allo-HSCT [1]. Moreover, conventional chemotherapeutic agents are often unable to promote durable complete remissions [2]. Therefore, there is an urgent need to develop new therapeutic agents that display a biocidal activity against AML cells, while ideally sparing hematopoietic stem cells (HSCs).

Monoclonal antibodies have been considered as biopharmaceuticals for the treatment of AML. Immunoglobulins directed against CD33, CD123 or FLT3 have been studied for antibody-dependent cell-mediated cytotoxicity (ADCC) applications [3–5], given the differential expression of the cognate antigen on blasts and hematopoietic stem cells (HSCs). Moreover, products based on “armed” antibody have been investigated in advanced clinical trials for AML treatment, including antibody-drug conjugates (ADCs) [6,7], bispecific antibodies [8,9] and Chimeric Antigen Receptor-T cells (CAR-Ts) [10,11]. For example, Mylotarg™ is an ADC product which has been approved for the treatment of relapsed or refractory CD33 + AML [6], while several other antibody therapeutics are currently being studied in clinical trials in AML patients.

The α chain of interleukin 3 receptor (IL3Rα or CD123) is overexpressed in a variety of hematological disorders including AML, chronic myeloid leukemia, B-lymphoid leukemia, blastic plasmocytoid dendritic neoplasms and hairy cell leukemia [12]. It was firstly reported as unique surface marker for CD34 + CD38- acute myeloid leukemia stem cells (LSCs) [13] and was later shown that CD123 was also overexpressed on AML blasts compared to normal HSCs and myeloid progenitors [14,15]. Additionally, high levels of CD123 correlate with

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an adverse prognosis [16,17]. For these reasons, pharmaceutical companies have considered CD123 as a target for the development of biopharmaceuticals. Clinical-stage ADCs directed against CD123 include SGN-CD123A and IMGN632 [18,19]. In addition, bispecific antibody products (*e.g.*, XmAB14045, MGD006, JNJ63709178) [20–22], CAR-T cells (*e.g.*, MB-102, UCART123) [11,23], as well as "naked" IgGs with enhanced ADCC activity (CSL362, KHK2823) have started clinical development programs [24,25].

CSL360 was the first anti-CD123 antibody to be tested in patients but failed to show anti-leukemic activity in a phase I clinical trial (clinicaltrial.gov identifier NCT00401739) [26]. A second-generation anti-CD123 antibody (CSL362, Talacotuzumab) was derived from CSL360 after humanization, affinity-maturation and ADCC-potentiation by the insertion of S293D and I332E substitutions in the Fc region [27]. However, the industrial development of CSL362 has been discontinued for unreleased reasons [28], possibly due to unfavorable risk/benefit profiles *in vivo*. It may therefore be desirable to generate CD123-targeting products, with improved activity and tolerability.

CD123 is composed of three extracellular domains [termed N-terminal (NTD), extracellular domain 2 (ED2) and 3 (ED3)], followed by a transmembrane and cytoplasmic region [29].

Upon binding to the NTD – which is the most distal from the cell membrane – CSL362 is able to inhibit IL-3 binding and signaling [29]. While NTD is not essential to form a functional receptor, its presence increases the binding avidity towards IL3, thus contributing to an optimal ligand recognition and signaling [30,31]. The precise position of the antigen's epitope influences biocidal activity mediated by antibody therapeutics working through antibody-directed cell cytotoxicity (ADCC) and by bispecific antibodies [32–34].

In this article, we describe the generation, characterization and *in vitro* profiling of a fully human anti-CD123 antibody which has been affinity matured and engineered for enhanced antibody dependent cellular cytotoxicity [termed $IgG1^{pot}$(H9)]. The previously-described clinical-stage CSL362 anti-CD123 antibody [27] was used as benchmark. H9 and CSL362 recognized different epitopes on the N-terminal domain of CD123 and were able to induce selective AML killing, when reformatted as intact human IgG1 immunoglobulins or as bispecific T cell engagers (BiTEs™).

2. Materials and methods

2.1. Cell lines

The TF-1 (CRL-2003) and the Kasumi-1 (CRL-2724) cell lines were obtained from the American Type Culture Collection (ATTC) and CHO-S cells from Invitrogen. The OCI-AML3 cell line (ACC-247) was obtained from the DSMZ. Cell lines were expanded, and stored as cryopreserved aliquots in liquid nitrogen. Cells were grown according the supplier's protocol and kept in culture for no longer than 2 weeks. Authentication of the cell line also included check of post-freeze viability, growth properties, morphology, test for mycoplasma contamination, isoenzyme assay, and sterility test were performed by the cell bank before shipment. The Kasumi-1 cell line was stably transfected with a lentivirus containing the vector with the GFP sequence (lentilab.unige.ch) [35].

2.2. Cloning and expression of recombinant CD123

The cDNA of the extracellular domains of human CD123 were obtained from Genscript and cloned into the pcDNA3.1(+) expression vector with a C-terminal 6-His tag (CD123-His) or a Fragment crystallizable (Fc) tag (CD123-Fc). The fusion proteins were expressed using polyethyleneimine (PEI)-mediated transient gene expression in CHO-S cells as described [36]. The proteins were purified from the culture medium *via* protein A affinity chromatography or Ni-NTA agarose resin (ThermoFisher) and analyzed using SDS-PAGE and size exclusion chromatography (Superdex 200 Increase, 10/300, GE Healthcare).

2.3. Generation of a stable CD123 monoclonal cell line

CHO-S cells were stably transfected with a gene coding for the whole CD123 and a second gene conferring antibiotic resistance against neomycin. After antibiotic selection and limiting dilution to generate monoclonal cell lines, the cells were screened for expression of CD123 by flow cytometry using an anti-human CD123 antibody (clone 6H6, BioLegend).

2.4. Selection of antibodies from the ETH-2 Gold library by phage display and affinity maturation

Human monoclonal antibodies specific to the CD123 antigen were isolated by three rounds of biopanning from the ETH-2 Gold antibody phage display library as described [37]. Bacterial supernatants containing recombinant scFv antibody fragments were screened by ELISA. Those with positive signal in ELISA were analyzed using a BIAcore 200 instrument (GE Healthcare) and sequenced. The affinity maturation library was cloned by introducing sequence variability in the CDR1 of both heavy and light chain of the best clone using partially degenerate primers as already shown in [38]. The library was electroporated into fresh electrocompetent TG-1 cells. Bacterial supernatants of individual colonies were screened by ELISA and BIAcore. The best clone was sequenced and reformatted into the fully human IgG format by cloning VH and VL into pcDNA3.1 (+) resulting in the H9 antibody.

2.5. Cloning of BiTE(H9/OKT3), BiTE (CSL362/OKT3) and the CSL362 antibody

The sequence of CSL362 and the OKT3 was retrieved from the corresponding patents (US 8,569,461 B2 and US 7, 635, 472 B2). Vectors containing the heavy (without the ADCC engineered mutations) or light chain sequence and the OKT3 sequence were ordered from Genscript. BiTE(H9/OKT3) was assembled by PCR in the following format using specific primers: N-terminus- signal peptide-variable light chain of the H9-variable heavy chain of the H9- variable heavy chain of OTK3- variable light of OTK3- C-terminus. BiTE(CSL263/OKT3) was assembled in the same format, but a His-tag was added at the C-terminus.

2.6. Expression of antibodies and antibody fragments

The vectors containing the antibody, antibody fragment and BiTE sequences were used for transient gene expression using polyethyleneimine as described before. The proteins were purified by protein A affinity chromatography or Ni-NTA agarose resin (ThermoFisher).

2.7. Epitope mapping using peptide array

In order to characterize the binding of the H9 antibody, we identified the epitope on CD123 extracellular domain using a peptide array (PepSpot, JPT).

The synthesis of the target antigen as an overlapping linear series of peptides covalently attached to a cellulose support was performed by SPOT technique at JPT.

A consecutive series of 15-mer with 3 residues shift each resulted in 92 peptides, spanning the 287 amino acid sequence of CD123. The assay was performed according to manufacturer's instructions and the binding spots were detected by a chemiluminescence imager (Agfa Curix 60, Agfa Healthcare) after the incubation of the membrane with IgG(H9) and then with protein A-HRP (GE Healthcare).

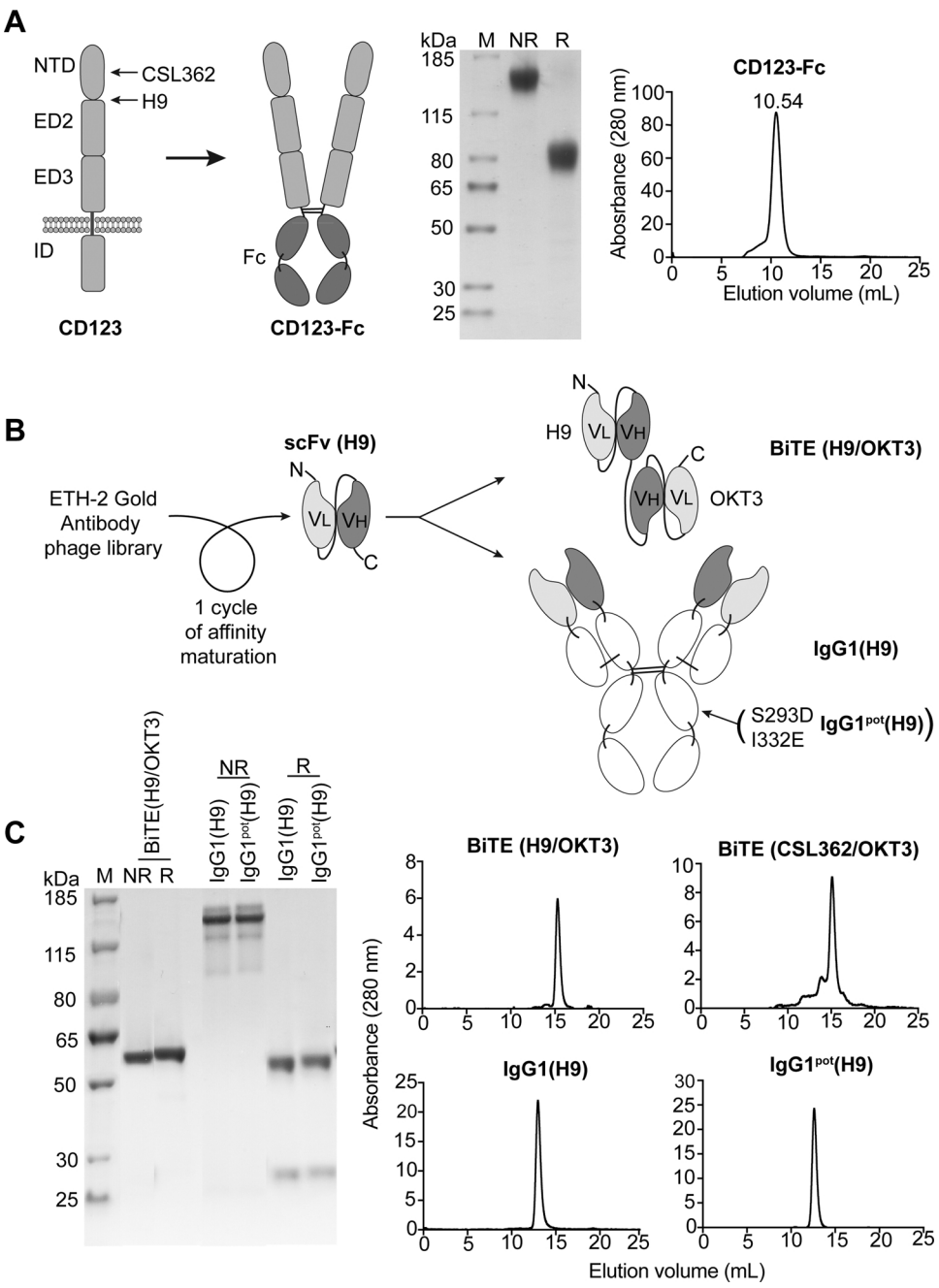

Fig. 1. Generation and expression of fully human antibodies against CD123. (A) CD123 is composed of three extracellular domains: the N-terminal domain (NTD) and extracellular domains 2 and 3 (ED2 and ED3). ED3 is followed by a transmembrane domain and an intracellular domain (ID). CSL362 and H9 bind to different epitopes on NTD. The extracellular domains of CD123 were fused to a Fc tag, expressed in mammalian cells and analyzed using SDS-PAGE and size exclusion chromatography. (B) Schematic illustration of the workflow to generate anti CD123 therapeutics. A large synthetic human antibody library was used to isolate anti-CD123 antibodies. The clone in scFv format with the best signal in the ELISA was selected and affinity matured to yield the scFv(H9). The VL and VH domains of H9 were used to generate a BiTE(H9/OKT3) and a full IgG (H9). Point mutations were inserted into the Fc domain to obtain ADCC potentiated mutants. (C) SDS-PAGE analysis under non-reducing (NR) and reducing (R) conditions and (D) size exclusion chromatography profile of BiTE(H9/OKT3), BiTE (CSL362/OKT3), IgG1(H9) and IgG1^{pot}(H9). M = Marker. The SDS-PAGE analysis and size exclusion profile of IgG1(CSL362), IgG1^{pot}(CSL362) and BiTE (CSL362/OKT3) can be found in Supplementary Fig. 1.

2.8. Flow cytometry-based binding of H9 and CSL362 to stable CD123 monoclonal cell line

Stably transfected CD123 expressing CHO-S cells were centrifuged and washed in FACS buffer (2% FBS in PBS). Cells were stained with primary antibodies H9, CSL362 and a negative control antibody L19 (targeting the extracellular domain A of fibronectin). After washing, an AF647 goat anti human IgG (ThermoFisher) was used to detect the primary antibodies. The cells were washed again and analyzed on a 2-L CytoFLEX Flow Cytometer (Beckman Coulter).

2.9. Surface plasmon resonance

CD123-Fc was immobilized on a CM-5 sensor chip with a density of 1600 RU using a BIAcore 200 system. Serial dilutions of antibody fractions or bacterial supernatants were investigated. The binding curves were analyzed with the BIAevaluation 3.2 software.

2.10. ADCC potentiation

To enhance the antibody dependent cellular cytotoxicity of the antibody, two amino-acid mutations S293D and I332E were introduced into the Fc region by PCR as it has been done for other antibodies including the CSL362 antibody [27,39]. This yielded the IgG1^{pot}(H9) and the IgG1^{pot}(CSL362) antibody which were expressed by transient gene expression as described before.

2.11. Isolation of human peripheral blood mononuclear cells (PBMCs)

Peripheral blood samples were obtained from healthy donors from the Blutspendedienst SRK, Zurich. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation on Ficoll Paque Plus (GE Healthcare) according to the manufacturer's instructions.

The peripheral blood was three-fold diluted in PBS solution

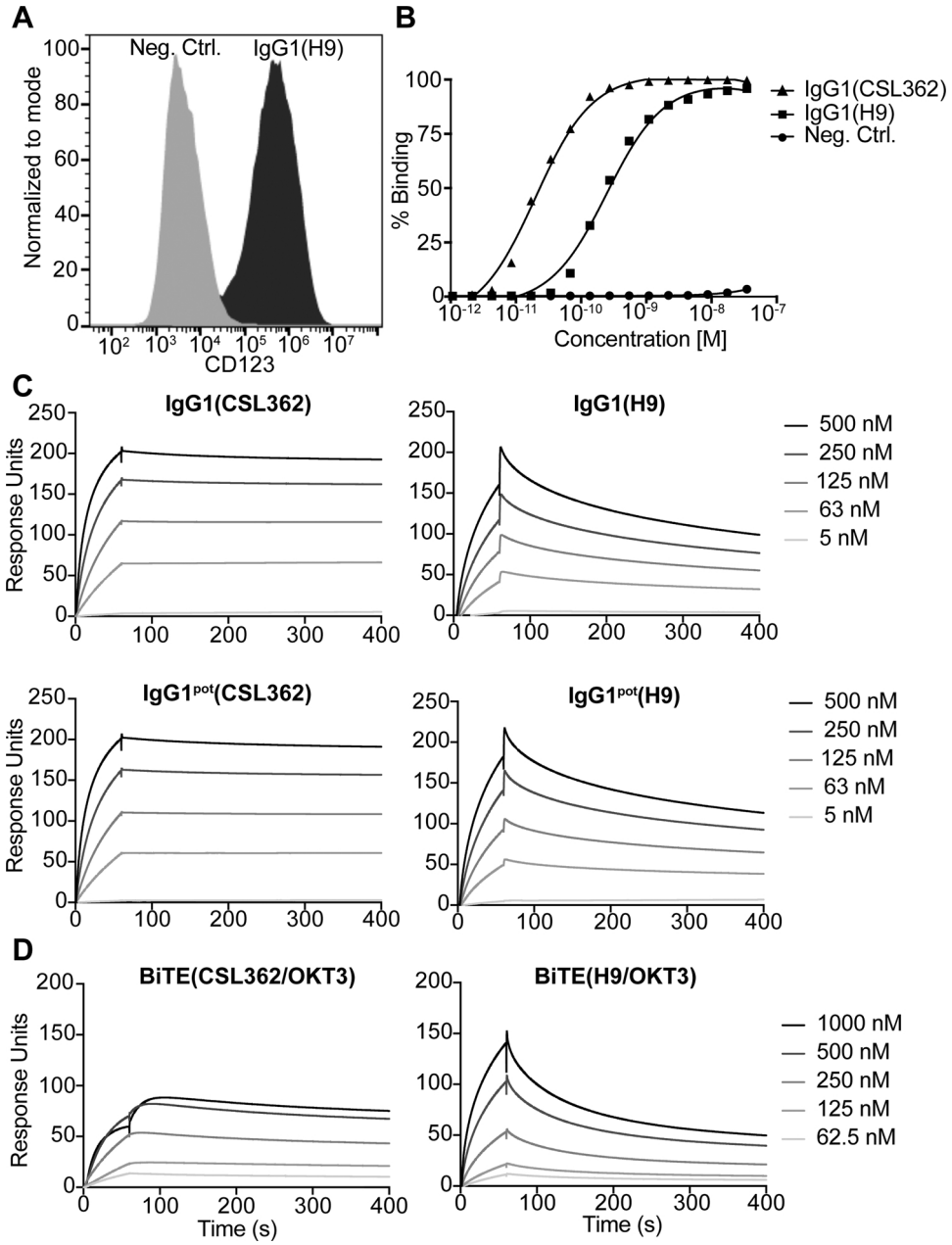

Fig. 2. Binding analysis. (A) Representative histogram display of IgG1(H9) (black shaded) and a negative control (neg. Ctrl.) antibody IgG1(L19) to visualize binding of CD123 on CD123+ CHO-S cells. (B) Dose titration of IgG1(CSL362), IgG1(H9), and a negative control IgG (neg. Ctrl.) IgG1(L19) to determine the binding affinity of the antibodies to CD123 expressing CHO-S cells. (C) and (D) Surface plasmon resonance analysis of IgG1(CSL362), IgG1^{pot}(CSL362), IgG1(H9), IgG1^{pot}(H9), BiTE (CSL362/OKT3), and BiTE(H9/OKT3) on a CD123-Fc coated CM-5 chip.

containing 2 mM EDTA. Then, 30 ml of diluted peripheral blood were layered on 12.9 ml Ficoll and centrifuged at 400 x g for 40 min at room temperature. PBMCs were collected, and then washed 2 times with PBS/EDTA. After washing, the cells were immediately subjected to the *in vitro* killing assay.

2.12. ADCC assay on human cell lines

Antibody-dependent cellular cytotoxicity was analyzed using flow cytometry as described [40]. For this, target cells were labelled with CFSE (Invitrogen) according to the manufacturer's instructions and cultured with PBMC at an effector to target cell ratio (E:T) of 50:1 and the antibodies for 16 h at 37 °C in RPMI supplemented with 5% or 10% FBS. The cells were harvested, and stained with Fixable Viability Dye (Invitrogen) which labels dead cells. After 30 min of incubation, the cells were washed with PBS containing 2% FBS and then subjected to FACS analysis using a 2-L CytoFLEX cytometer (Beckman Coulter), and data were processed using FlowJo (v.10, Tree Star). The percentage of specific lysis was calculated as follows: (1-number of alive target cells/number of alive target cells without antibody)*100.

2.13. ADCC assay on AML blasts

The primary human cells used in this study were selected from the biobank of the department of Medical Oncology and Hematology, University Hospital Zurich, Zurich, Switzerland. All AML patient bone marrow and blood samples were obtained with written informed consent. The study was approved by the Ethics Board of the Canton of Zurich (2009-0062). CD3/CD19 depleted AML patient bone marrow cells were thawed and incubated for 4 days in IMDM medium

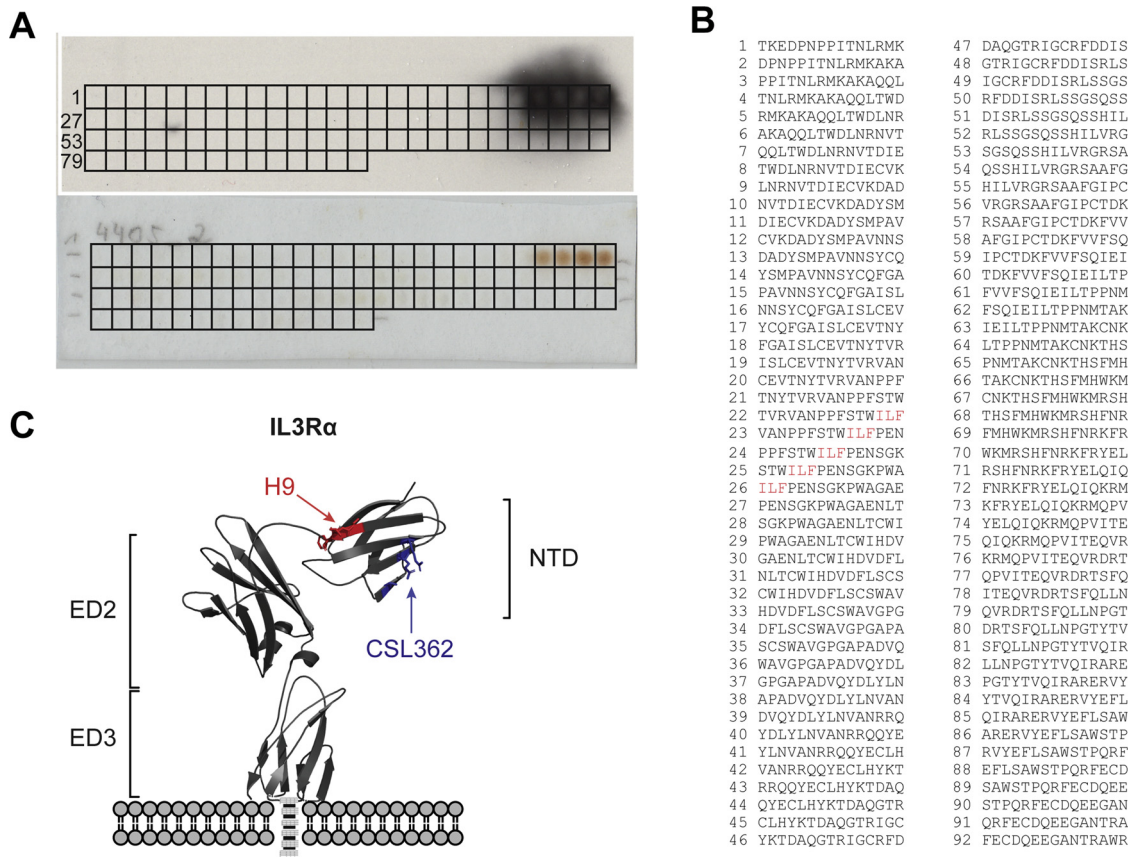

Fig. 3. Epitope mapping of antibody binding to the NTD of CD123. (A) Autoradiographic results of the SPOT analysis performed using the H9 antibody. Portions of the extracellular domain of CD123 were synthetized as a consecutive series of 15 amino acid-long peptides [listed in (B)], spanning the protein sequence with 3 residues shift each.(C) Three dimensional model of CD123, indicating both the binding sites of H9 (red) and of CSL362 (blue), was built on the basis of the 4JZJ pdb file [29].The binding sites of IgG(H9) and of IgG(CSL362) both lie on the NTD domain. The epitope of H9 is centered around the I94-L95-F96 sequence, while the epitope of CSL362 is centered around E51, S59, and R84 [30]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

supplemented with 20% FBS and 1% penicillin/streptomycin (GIBCO, Thermo Fischer Scientific) as well as 50 µM mercaptoethanol, 10 ng/mL recombinant SCF, 50 ng/mL recombinant TPO and 10 ng/mL FLT3 ligand (Peprotech). To determine CD123 expression on leukemic blast surface, CD33-BV711 (Biolegend), CD34-PE (Thermo Fischer), CD45-e450/PB (Biolegend) were co-stained with CD123-APC (Biolegend). Dead cells were excluded from analysis by staining cells with Aqua Live/Dead cell viability dye (Biolegend). After the isolation of healthy PBMCs by Ficoll centrifugation (GE Healthcare), NK cells were enriched by EasySep™ Human NK cell Isolation Kit (STEMCELL) following the manufacturer's instructions.

Allogeneic NK cells and cultured CD3/CD19 depleted AML patient bone marrow cells were mixed at an effector to target ratio of 10:1 in IMDM medium supplemented with 20% FBS and 1% penicillin/streptomycin. A dilution series of IgG(L19), IgG^{pot}(CSL362) and IgG^{pot}(H9) was subsequently added to the cell culture which was then incubated for 24 h at 37 °C. The specific lysis was analyzed by flow cytometry using a LSR II Fortessa cell analyzer (BD Biosciences) and quantified using the formula described before.

2.14. In vitro BiTE-mediated T-cell killing of primary AML blast

T cells were thawed 1 day before the assay and cultured overnight in advanced RPMI supplemented with 1x Glutamax (Gibco), 10% FBS and 1% penicillin/streptomycin (T cell medium). Effector and target cells were co-cultured in T cell medium (E:T = 10:1). A dilution series of BiTE was added to the cell solution and incubated for 24 h at 37 °C. The specific lysis of AML blasts ($CD45^{dim}$) was analyzed by flow cytometry using a LSR II Fortessa cell analyzer (BD Biosciences) and quantified using the formula described before.

3. Results

3.1. Generation, affinity maturation and reformatting of fully human antibodies against CD123

A fusion protein consisting of the extracellular domains of human CD123 (NTD, ED2 and ED3) and a human Fragment crystallizable (Fc) tag (CD123-Fc) was cloned, expressed in mammalian cells and purified to homogeneity [Fig. 1A]. The protein was glycosylated and migrated as a disulfide-linked homodimer in SDS-PAGE analysis performed in non-reducing conditions, while a monomeric band of the expected size was visible in reducing conditions [Fig. 1A]. Size-exclusion chromatography analysis confirmed that CD123-Fc was eluted at a volume consistent with a disulfide-linked structure [Fig. 1A].

The fusion protein CD123-Fc was used to isolate a first low-affinity anti-CD123 antibody from a large synthetic human antibody library (ETH-2 Gold library) using established protocols [Fig. 1B] [37,38]. In order to affinity-mature this clone, we generated and screened a phage display library of 6 million antibody variants, obtained by combinatorial mutagenesis of 6 residues in the CDR1 loop of the VH and VL domains, using previously described procedures [17] [Fig. 1B]. The antibody clone (termed "H9″) which gave the strongest ELISA signal was further characterized and used for reformatting activities. The VL and VH domains of H9 were used to clone a BiTE(H9/OKT3) and a full IgG1(H9) [Fig. 1B]. Moreover, two amino acid mutations (S239D and

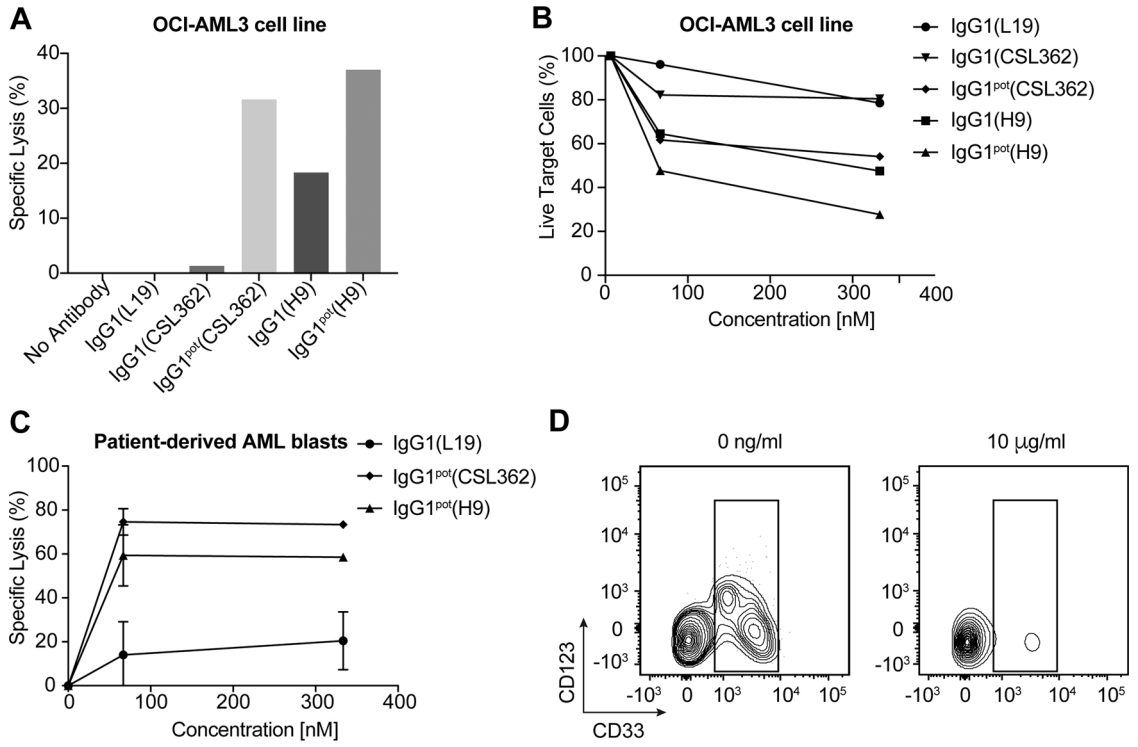

Fig. 4. *In vitro* ADCC killing of human AML cell lines and of primary AML blast cells. (A) Percentage of specific lysis of OCI-AML3 cells 24 h after co-culture with healthy donor PBMCs only or with PBMCs and 10 µg/ml of either anti-EDB IgG(L19), anti-CD123 IgG(CSL362), IgG^{pot}(CSL362), IgG(H9) or IgG^{pot}(H9) antibodies. The anti-EDB IgG1(L19) antibody was used as negative control. (B) The percentage live target cells (OCI-AML3 cells) after 24 h of co-culture with healthy donor PBMCs only or with PBMCs and with indicated concentrations of either IgG(L19), IgG(CSL362), IgG^{pot}(CSL362), IgG(H9) or IgG^{pot}(H9) antibodies. (C) Percentage specific lysis of CD45^{dim} CD33 + AML blast cells after 24 h of co-culture with purified healthy donor NK cells and either anti-EDB IgG(L19), IgG^{pot}(CSL362) or IgG^{pot}(H9) antibodies at the indicated concentrations. The effector to target ratio was set to 10:1. (D) Representative flow cytometry plots of CD45^{dim} CD33 + AML blast cells incubated with healthy donor NK cells and different concentration of IgG^{pot}(CSL362) after 24 h.

I332E) were introduced into the Fc region in order to enhance ADCC activity, as previously described for IgG1(CSL362) [27]. Similar IgG1 and BiTE products were generated for CSL362, starting from a synthetic gene of the antibody sequence [27,39]. The resulting proteins were expressed in mammalian cells and characterized using standard biochemical assays, such as SDS-PAGE analysis and size exclusion chromatography [Fig. 1C and Supplementary Fig. 1]. The complete amino acid sequence of these constructs can be found in Supplementary Fig. 2.

3.2. *In vitro characterization of anti-leukemic activity of H9- and CSL362-based therapeutics*

First, we generated a stable CD123 expressing CHO-S cell line, which was used to analyze the binding affinity of the antibody products. IgG1(H9) exhibited a large shift in FACS analysis, compared to an antibody of irrelevant specificity in this setting [IgG1(L19), an anti-EDB antibody [41], which was used as negative control [Fig. 2A]. Analysis of a titration series for the FACS experiment revealed that IgG1(CSL362) exhibited a high functional affinity (K_d^{app} = 20 pM), which was ~10-fold higher than the one of IgG1(H9) (K_d^{app} = 250 pM) [Fig. 2B]. Next, we analyzed the kinetic binding properties of the two antibodies in different formats. As expected, IgG1(CSL362) exhibited an extremely low kinetic dissociation profile from the antigen coated on a BIAcore microsensor chip, while a measurable kinetic dissociation profile was visible for IgG1(H9) [Fig. 2C]. Similarly, BiTE(CSL362/OKT3), featuring a monovalent binding to the cognate CD123 antigen, displayed a flat dissociation profile from the antigen, while BiTE(H9/OKT3) exhibited a k_{off} value of 2×10^{-3} s^{-1} [Fig. 2D].

The binding site of CSL362 on the N-terminal domain of CD123 has previously been characterized by X-ray crystallography [29]. In order to identify the binding site of H9 on the surface of CD123, we used SPOT technology [42] and immunodetection of antibody binding events to peptides spanning the antigen sequence, synthesized on cellulose. Fig. 3 shows that CSL362 and H9 recognize different epitopes, located on opposite faces of the N-terminal domain of CD123.

We then tested the antibodies in conventional IgG1 format and in the corresponding ADCC-potentiated version (IgG1^{pot}) for their ability to mediate ADCC against a human AML cell line *in vitro*. In this assay, both ADCC-potentiated antibodies induced ~30% specific lysis of the OCI-AML3 cell line when used at 10 µg/ml, in keeping with previously published reports for CSL362 [27] [Fig. 4A]. We repeated the ADCC assay using three different concentrations, confirming that IgG1^{pot}(H9) was the product with the most potent ADCC activity [Fig. 4B]. Similar experiments, performed using patient-derived AML blasts and purified allogeneic NK cells at an effector to target (E:T) ratio of 10:1, confirmed the ability of the two antibodies to selectively kill leukemic cells *in vitro* [Fig. 4C]. Over 75% of CD45^{dim} CD33 + AML blasts were efficiently depleted by 10 µg/ml IgG1^{pot}(CSL362) after 24 h of co-culture with allogeneic NK cells [Fig. 4D]. Interestingly, also CD45^{dim}CD33 + CD123^{low} AML blasts were killed in this assay.

3.3. *In vitro analysis of BiTE biocidal activity*

Fig. 5A shows a FACS gating analysis of patient-derived AML blasts (left panel) prior to *in vitro* cytotoxicity assay and evaluation (right panel). Virtually all leukemic blasts exhibited CD123 cell surface expression in the CD45^{dim} CD33+ cell population. We tested the anti-leukemic activity of BiTE(H9/OKT3) and of BiTE(CSL362/OKT3) in an *in vitro* assay, using patient's derived AML blasts and autologous T cells as effectors. Using an E:T ratio of 10:1, we observed a concentration-dependent AML killing. BiTE(CSL362/OKT3), with an IC_{50} value of 4 ng/ml (corresponding to 7.3 pM), was approximately 10-times more

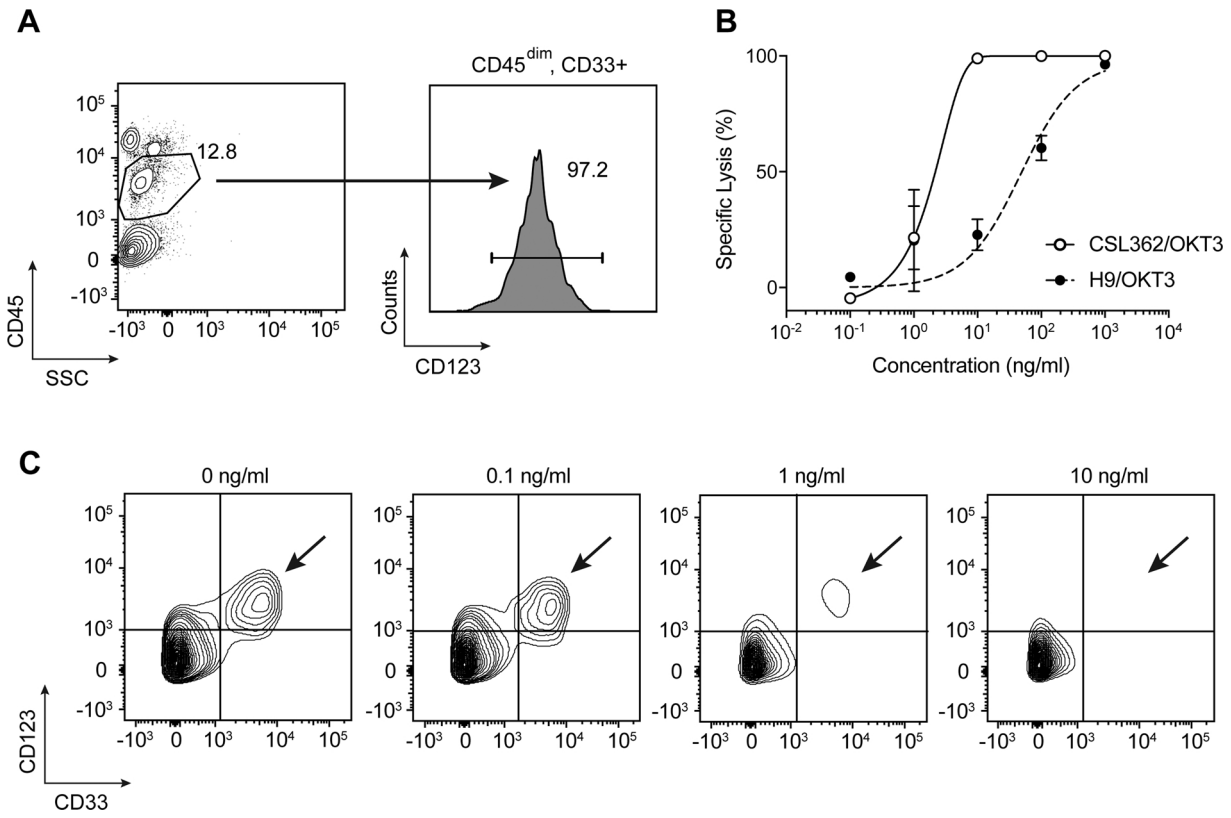

Fig. 5. *In vitro* BiTE mediated depletion of primary AML blast cells. (A) Flow cytometry plot showing the percentage of CD45^{dim} AML blast cells from a CD3/CD19 depleted AML patient bone marrow sample (left panel). Almost all CD45^{dim} CD33 + AML blast cells (97.2%) were also positive for CD123 (right panel). (B) Specific lysis of CD123+ CD45^{dim} CD33 + AML blast cells after 24 h of co-culture with autologous T cells (E:T = 10:1), at the indicated concentrations of CSL/OKT3 or H9/OKT3 BiTEs. (C) Representative flow cytometry plots of patient-derived CD45^{dim} CD33+ CD123 + AML blast cells after treatment with various concentrations of CSL/OKT3 BiTE with autologous T cells (E:T = 10:1). The arrow indicates the population of AML blasts that disappears as a result of the activity of the bispecific antibody.

potent than BiTE(H9/OKT3) [Fig. 5B and C].

4. Discussion

We have reported the generation of a novel fully-human anti-CD123 antibody (named H9) and we have compared its properties to the ones of a previously described clinical-stage antibody (named CSL362). The two antibodies recognized different epitopes on the same domain of CD123, allowing an initial comparison of anti-cancer activity for different types of therapeutic products. CD123 has been considered for many years as a target for antibody-based anti-AML approaches [12] and its differential expression on AML cell, compared to normal leukocytes and hematopoietic stem cells, has been studied by FACS and by surface proteomics methodologies [43–45].

The use of Fc-engineered antibodies in IgG1 format led to the best anti-AML activity results in *in vitro* ADCC assays. The simultaneous substitution of two amino acid residues in the C_H2 domain of the heavy chain (S293D and I332E) led to a potentiation of ADCC activity. These mutations have previously been shown to increase Fc binding to human V158 and F148 FcγRIIIa receptors of several antibodies such as trastuzumab, rituximab and cetuximab [39]. Other antibodies harboring the S293D-I332E amino acid modifications have previously displayed a substantial improvement of ADCC activity *in vitro* compared to their native IgG molecules. MOR208, an anti-CD19 antibody, carries the same amino acid mutations and is currently tested in clinical studies in Non-Hodgkin lymphoma, large B cell lymphoma, chronic lymphocytic leukemia and adult acute lymphoblastic leukemia (clinicaltrials.gov identifier: NCT02763319, NCT01161511, NCT01685021, NCT01685008, NCT02639910, NCT02399085, NCT02005289) [46].

It is likely that the therapeutic activity of Fc-potentiated anti-CD123 products may be enhanced by combination with certain antibody-cytokine fusions. We have previously reported that rituximab completely eradicated human B-cell lymphoma xenografts in combination with the tumor targeting immunocytokine L19-IL2 [47]. Additionally, we have shown that the antibody-based delivery of interleukin-2 (IL-2) to the tumor neovasculature potently increased the activity of the chemotherapeutic cytarabine [48]. A clinical trial, featuring the combination of the F16-IL2 fusion protein [49] with an Fc-engineered anti-CD33 antibody in patients with AML is currently on-going (clinicaltrials.gov identifier: NCT03207191).

The anti-CD123 antibodies exhibited promising results also when reformatted as bispecific antibodies. BiTE(CSL362/OKT3) was superior to BiTE(H9/OKT3) for the *in vitro* killing of patient-derived AML blasts in an autologous setting, but both products were able to kill AML cell lines at concentrations in the picomolar range. There have been recent research reports on the fact that epitopes closer to the cell membrane may lead to more efficient tumor cell killing with bispecific antibodies [34]. In this context, it may be attractive in the future to study novel antibody therapeutics, which recognize epitopes on CD123 which are closer to the lipid bilayer.

In summary, we have generated novel anti-CD123 antibodies and biopharmaceuticals, which have shown initial signs of activity in *in vitro* models of AML. IgG1^{pot}(H9) may be the most promising product for ADCC applications, while BiTE(CSL362/OKT3) would be a good candidate for bispecific antibody-based therapeutic purposes.

CD123-targeting products in IgG format leaded to recruitment of NK cell biocidal activity (ADCC), while in BiTE format leaded to a redirection of T cell killing. These procedures typically require an

antibody concentration in the 0.1–1 μM range. By contrast, T cell-mediated killing may occur at very low BiTE concentrations (*i.e.*, in the subnanomolar range) in *in vitro* experiments.

More than 15 formats have been described for the engineering of bispecific antibodies [50]. While the only bispecific antibody on the market (Blinatumomab, approved for patients with B-cell precursor acute lymphoblastic leukemia) is in BiTE format, it is still not clear whether biopharmaceuticals with a longer circulatory half-life may be preferable. In all cases, the antibodies described in this article may represent useful tools for the implementation of novel anti-AML strategies. We do not know, at this moment in time, whether selective tumor cell killing can be achieved while sparing hematopoietic stem cells.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.leukres.2019.106178.

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