Targeting of Acute Myeloid Leukemia (AML) Stem Cells by scFv:Death Ligand fusion proteins with designed specificity for C-type Lectin-Like Molecule-1 (CLL-1)

B. ten Cate¹, E. Bremer¹, M. de Bruyn¹, G. Huls² and W. Helfrich¹

¹Department of Surgery, Surgical Research Laboratories and ²Department of Hematology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands.

Manuscript in Preparation
ABSTRACT

Currently, the majorities of AML patients eventually relapse and develop refractory disease indicating that novel and preferably targeted approaches are urgently needed. Recently, C-type Lectin-Like Molecule-1 (CLL-1) has been identified as a cell surface marker expressed in ~90% of the AML patients. Importantly, CLL-1 is expressed on AML blasts and CD34+/CD38- AML stem cells, but not on normal hematopoietic stem cells. Moreover, this expression profile is retained under all conditions of treatment and disease. This prompted us to develop a novel approach for the targeted elimination of CLL-1+ AML and AML stem cells. To this end, a CLL-1-specific antibody fragment (scFvCLL-1) was genetically fused to soluble forms of the pro-apoptotic proteins TRAIL (sTRAIL) and FasL (sFasL), yielding fusion proteins scFvCLL-1:sTRAIL and scFvCLL-1:sFasL, respectively. Both fusion proteins potently induced CLL-1-restricted apoptosis in AML cell lines and primary AML cells. Moreover, CD34+/CD38- AML stem cells were sensitive to treatment with scFvCLL-1:sTRAIL and scFvCLL-1:sFasL. In contrast, human CLL-1- hepatocytes were fully resistant to treatment, whereas CLL-1+ monocytes showed moderate sensitivity to scFvCLL-1:sFasL. In conclusion, treatment with scFvCLL-1:sTRAIL and scFvCLL-1:sFasL results in AML-restricted apoptosis including the CD34+/CD38- AML stem cells. This novel approach may be of value for the treatment of AML.

INTRODUCTION

Currently, the majority of Acute Myeloid Leukemia (AML) patients succumb within five years due to therapy-related morbidity and mortality and the frequent occurrence of therapy-resistant relapses [1;2]. These relapses are considered to result from drug-resistant AML stem and progenitor cells present in the bone marrow. The use of antibody-based therapeutics appears to be a promising approach for the treatment of relapsed AML as is exemplified by the CD33-targeted immunotoxin Gemtuzumab Ozogamicin (GO). CD33 is a myeloid cell surface marker absent on normal hematopoietic stem cells but expressed in about 90% of AML patients [3].

Currently, GO is indicated for the treatment of a small subset of patients with CD33+ AML in first relapse who are 61 years of age or older and who are not considered candidates for standard cytotoxic chemotherapy [4]. Unfortunately, GO treatment is frequently associated with significant side-effects including severe hepatotoxicity and myelosuppression [5;6]. This and other observed toxicities may be attributable to the untimely off-target release of calicheamicin, the highly cytotoxic agent that is chemically coupled to the anti-CD33 antibody domain of GO via a rather unstable hydrolysable linker [7].

Overall, only a minority of AML patients benefits from GO treatment suggesting that AML stem cells are heterogeneous in their CD33 expression. Indeed various reports indicate that the CD34+/CD38- AML stem cells express variable amounts of CD33 [8;9]. Therefore, the curative elimination of AML may require novel agents that more effectively target and eliminate AML stem and progenitor cells with a more favorable toxicity profile. In this respect, C-type Lectin-Like Molecule-1 (CLL-1) appears to be attractive alternative...
target antigen to CD33. CLL-1 is expressed in ~90% of the AML patients [10] in which expression appears to be retained under all conditions of treatment and disease [11]. In peripheral blood, both monocytes and granulocytes show moderate CLL-1 expression, while CLL-1 is completely absent in other tissues [10]. Importantly, CLL-1 is expressed on the CD34+/CD38- AML stem cells and the CD34+/CD38+ AML progenitor cells, whereas it is not expressed on normal hematopoietic stem cells [11;12]. Interestingly, CLL-1 and CD33 appear to complement each other as therapeutic targets, since 33% of the CLL-1- AML samples express CD33, and 67% of the CD33- samples express CLL-1 [10].

Previously, we reported on an alternative CD33-targeted anti-AML agent, designated scFvCD33:sTRAIL, in which an anti-CD33 scFv antibody fragment is genetically linked to sTRAIL [7]. TRAIL is a homotrimeric transmembrane protein that belongs to the TNF-family of death ligands that also includes death ligand FasL. In vivo, TRAIL and FasL can be proteolytically processed into soluble molecules (sTRAIL and sFasL, respectively). Several recombinant forms of sTRAIL and sFasL have been described that show promising pro-apoptotic activity against various forms of cancer including AML [10;11]. In this respect, TRAIL is of particular interest since it exclusively induces apoptosis in malignant cell types, while fully sparing normal cells. Our experiments with fusion protein scFvCD33:sTRAIL indicated that it is superior to GO in terms of in vitro selectivity, activity and stability, without any toxicity towards normal CD33+ monocytes [7]. The favorable expression profile of CLL-1 and the promising AML-selective activity of the scFvCD33:sTRAIL fusion protein prompted us to construct and pre-clinically evaluate two new fusion proteins with engineered specificity for CLL-1, designated scFvCLL-1:sTRAIL and scFvCLL-1:sFasL, respectively. Here, we present pre-clinical data indicating that these novel fusion proteins have promising tumor-restricted activity towards AML cell lines and primary AML cells, including the CD34+/CD38- AML stem cells and the CD34+/CD38+ AML progenitor cells.

MATERIAL AND METHODS

Antibodies and reagents

Antibodies and reagents used in this study are: PE-conjugated anti-TRAIL (Diaclonne SAS, Besancon, France), PE-conjugated anti-FasL (Santa Cruz Biotechnology, Heidelberg, Germany), TRAIL-neutralizing mAb2E5 (Alexis, Kordia Life Sciences, Leiden, The Netherlands), FasL-neutralizing mAblf2.1 (Santa Cruz, Heidelberg, Germany), anti-activated caspase-3 (asp175; Cell Signaling Technology, Leiden, the Netherlands), Goat-anti-Rabbit-TRITC (Jackson ImmunoResearch Europe Ltd, Suffolk, England), magnetic bead-labeled anti-CD14 (Miltenyi Biotec, Bergisch Gladbach, Germany), anti-CD34-APC and anti-CD38-PE (IQ-products, Groningen, The Netherlands).

A stock solution (10mM) of 4-benzyl, 2-methyl, 1,2,4-thiadiazolidine, 3,5dione (TDZD-8, Sigma-Aldrich, Zwijndrecht, The Netherlands) was prepared in DMSO and stored -20°C. TDZD-8 is a non-ATP competitive inhibitor of GSK-3β originally designed for the treatment of Alzheimer’s disease [13] that shows potent tumoricidal activity towards AML cells, including the CD34+/CD38- stem cell population [14].
Monocytes, hepatocytes, cell lines and primary AML cells

All cells were cultured and maintained in RPMI 1640 (Cambrex, New Jersey, New Hampshire, USA) supplemented with 10% FCS, at 37°C in humidified 5% CO2 containing atmosphere. Leukemia cell lines U-937 (AML, CLL-1+), HL-60 (AML, CLL-1+) and Ramos (B-CLL, CLL-1-) were purchased from the ATCC (Manassas, USA). CLL-1+ transfectant Ramos cells (Ramos.CLL-1) were generated by electroporation with the eukaryotic expression plasmid pCLL-1-IRIS2-EGFP, containing a cDNA encoding CLL-1 and EGFP separated by an internal ribosome entry site (IRIS) sequence. Monocytes were isolated from healthy donors by standard density gradient centrifugation and subsequent magnetic bead separation of CD14+ cells. Primary hepatocytes were purchased from Tebu-Bio (Heerhugowaard, The Netherlands) and cultured according to the manufacturer’s recommendations. Primary AML tumor cells were obtained after informed consent from AML patients by isolation of peripheral blood mononuclear cells using standard density-gradient centrifugation (GE Healthcare, Diegem, Belgium).

Construction and production of fusion proteins scFvCLL-1:sTRAIL, scFvCLL-1:sFasL and chimeric minibody scFvCLL-1:Fc

The cDNA sequence of scFvCLL-1 was synthesized using standard splice-by-overhang-extension PCR technology according to published VH and VL sequence data. The cDNA encoding scFvCD7 was removed from the previously described eukaryotic plasmids pEE14-scFvCD7:sTRAIL [15] and pEE14-scFvCD7:sFasL [16], using unique SfiI and NotI restriction enzyme sites. Standard recombinant DNA technology was used to insert cDNA encoding scFvCLL-1 yielding plasmids pEE14-scFvCLL-1:sTRAIL and pEE14-scFvCLL-1:sFasL. Plasmids were transfected in CHO-K1 cells using the Fugene-6 reagent (Roche Diagnostics, Almere, The Netherlands). Stable transfectants were generated by the glutamine synthetase selection method [17]. This procedure generated CHO-K1 production cell lines A17 and D58 that stably secreted the respective fusion protein into the culture medium (5.5μg/ml scFvCLL-1:sTRAIL and 260ng/ml scFvCLL-1:sFasL, respectively). A chimeric scFvCLL-1:Fc minibody was constructed by removing the cDNA encoding sTRAIL from plasmid pEE14-scFvCLL-1:sTRAIL using unique XhoI and HindIII restriction enzyme sites and subsequent replacement by a cDNA encoding the Fc domain of human IgG1 using standard recombinant DNA technology.

CLL-1 selective binding of scFvCLL-1:sTRAIL and scFvCLL-1:sFasL

CLL-1 selective binding activity of scFvCLL-1:sTRAIL and scFvCLL-1:sFasL was analyzed by flow cytometry (FACSCalibur, BD biosciences). In short, 1.0x10⁶ Ramos.CLL-1 were incubate with either scFvCLL-1:sTRAIL (550ng/ml) or scFvCLL-1:sFasL (26ng/ml) in the absence or presence of the competing minibody scFvCLL-1:Fc (2.4μg/ml). Binding of the respective fusion proteins was detected by using either a PE-conjugated anti-TRAIL or a PE-conjugated anti-FasL mAb. In control experiments non-specific binding of fusion proteins was assessed using wild-type Ramos cells (CLL-1) Incubations were performed for 45 minutes at 0°C and were followed by 2 washes with serum-free medium.

CLL-1 restricted apoptosis induction by scFvCLL-1:sTRAIL and scFvCLL-1:sFasL

Various leukemia cells were seeded at 1.0x10⁶ cells/ml in a 48-wells plate and treated for 24 hours with the indicated concentrations of scFvCLL-1:sTRAIL or scFvCLL-1:sFasL,
in the presence or absence of the competing minibody scFvCLL-1:Fc (2.4µg/ml) or the death ligand neutralizing mAb2E5 or mAbAlf2.1 (1µg/ml). Apoptosis was assessed as described below.

Assessment of apoptosis induction
The leukemia cells were seeded at density of 1.0x10^6 cells/ml in a 48-wells plate and subsequently treated for 24hours. Monocytes and hepatocytes were plated at a density of 3,5 x10^5 cell/ml in 96 wells plate and allowed to adhere for 24h. Subsequent adhered monocytes and hepatocytes were treated for 24h with scFvCLL-1:sTRAIL (550ng/ml) or scFvCLL-1:sFasL (26ng/ml). Induction of apoptosis was assessed using one of the following apoptosis assays: Exposure of phosphatidylserine (PS); The early apoptotic feature of exposure of phosphatidyl serine on the outer membrane was analyzed by flow cytometry using an AnnexinV-FITC (Nexins research, Kattendijke, The Netherlands) according to manufacturer’s instructions. Loss of mitochondrial membrane potential (∆Ψ); ∆Ψ was analyzed by flow cytometry using the cell-permeant green-fluorescent lipophilic dye DiOC6 (Molecular Probes, Eugene, USA) essentially as described previously [15]. Caspase-3/7 activation; The hallmark apoptotic feature of caspase-3/7 activation, was measured using the caspase-Glo™ 3/7 assay kit (Promega, Mannheim, Germany) according to manufacturer’s protocol. Loss of viability; was determined with the MTS assay kit (Promega) according the manufacturer’s recommendations. The formation of formazan was analyzed by measuring the absorbance at 490nm using a Victor3 multilabel plate reader (Perkin Elmer, Groningen, The Netherlands).

Percentage of experimental apoptosis was calculated using the following formula: specific apoptosis = (experimental apoptosis-spontaneous apoptosis)/(100-spontaneous apoptosis) x 100%.

Quantification of bystander activity of scFvCLL-1:sTRAIL and scFvCLL-1:sFasL using mixed culture experiments
Tumor progression and/or tumor heterogeneity frequently result in the appearance of tumor cells that no longer express the relevant target antigen. Consequently, these tumor cells are able to escape from conventional antibody-based approaches [18;19].
Previously, we have demonstrated that our scFv:sTRAIL and scFv:sFasL fusion protein approach not only eliminates target antigen expressing tumor cells, but also exploits them to relay the pro-apoptotic activity towards neighboring tumor cells, including tumor cells that lack the relevant target antigen [7;16;20;21]. This anti-tumor activity is usually indicated by the term bystander activity. To quantify the bystander activity of scFvCLL-1:sTRAIL and scFvCLL-1:sFasL we treated mixtures of CLL-1\(^+\) tumor cells and CLL-1\(^-\) bystander tumor cells and subsequently evaluated apoptosis induction in both tumor cell populations separately. To this end Ramos.CLL-1 tumor cells and CLL-1\(^-\) Ramos bystander tumor cells were differentially labeled using the Vybrant cell labeling solution (Invitrogen, Breda, The Netherlands). Ramos.CLL-1 (1.0x10\(^6\) cells/ml) cells were labeled with 5µM DiO (Invitrogen, Breda, The Netherlands) for 5min at 37\(^\circ\)C in serum free medium, followed by three washes with culture medium. Next, labeled Ramos.CLL-1 cells were mixed at 1:1 ratio with unlabelled Ramos bystander cells at a density of 0.5x10\(^6\) cells/well of a 48-wellsplate and treated with scFvCLL-1:sTRAIL. After incubation the cells were spotted on microscope slides and incubated with an antibody specific for activated caspase-3, followed by incubation with Goat-anti-Rabbit-TRITC. Cell nuclei were counterstained with DAPI (Sigma-Aldrich). Computer-coded fluorescent images were obtained using a standard fluorescence microscope (Leica DM-RXA, Leica Camera, Solms, Germany) equipped with a CCD camera (Leica DC350 FX) and utilizing dedicated acquisition software (Leica QwinPro).

For flowcytometric quantification of bystander activity, DiI-labeled (Invitrogen) Ramos. CLL-1 target cells were mixed at a 1:1 ratio with unlabeled Ramos bystander cell and treated with either fusion protein. After treatment, the differential fluorescent characteristics of the two cell populations were used to separately assess the induction of apoptosis (ΔΨ) using flowcytometric analysis.

**Treatment of primary AML cells with fusion protein scFvCLL-1:sTRAIL or scFvCLL-1:sFasL**

From 10 AML patients, leukemic cells were freshly isolated and plated at a density of 1.0x10\(^6\) cells/ml of a 48-wellsplate. Primary AML cells were treated for 24h with either scFvCLL-1:sTRAIL (550ng/ml) or scFvCLL-1:sFasL (26ng/ml) alone or in combination with TDZD-8 (5µM). AnnexinV-FITC staining was performed to assess apoptosis induction in the total AML cell population. Apoptosis induction in CD34\(^+\)/CD38\(^-\) AML stem cell and CD34\(^+\)/CD38\(^+\) AML progenitor cell populations was assessed by flowcytometric analysis using anti-CD34-APC, anti-CD38-PE and AnnexinV-FITC.

**Assessment co-treatment with TDZD-8**

The effect of co-treatment of the respective fusion proteins with TDZD-8 (5µM) was evaluated by calculating the cooperativity index (CI), in which the sum of the average apoptosis induction by single-agent treatment is divided by the average apoptosis induction upon co-treatment. CI was qualified as follows: CI <0.9, synergy; 0.9< CI <1.1, additive; CI >1.1, antagonistic.

**RESULTS**

**CLL-1-specific cell surface binding of scFvCLL-1:sTRAIL and scFvCLL-1:sFasL.**

The CLL-1-specific binding of scFvCLL-1:sTRAIL (Fig. 1A) and scFvCLL-1:sFasL(Fig. 1B)
was assessed by flowcytometry. Analysis of this data demonstrated CLL-1-specific accretion of scFvCLL-1:sTRAIL and scFvCLL-1:sFasL at the cell surface of Ramos.CLL-1 cells. Cell surface binding was inhibited by pre-incubation with the competing minibody scFvCLL-1:Fc. No binding of scFvCLL-1:sTRAIL and scFvCLL-1:sFasL to Ramos cells (CLL-1-) was observed. These data indicate that both scFvCLL-1:sTRAIL and scFvCLL-1:sFasL specifically bind to cell surface expressed CLL-1.

**Figure 2. CLL-1-restricted apoptosis induction by scFvCLL-1:sTRAIL and scFvCLL-1:sFasL.**

Dose-dependent apoptosis induction in wild-type Ramos cells by (A) scFvCLL-1:sTRAIL (22ng/ml – 550ng/ml) or (B) scFvCLL-1:sFasL (0.52ng/ml – 26ng/ml). (C) Apoptosis induction in U-937 cells by scFvCLL-1:sTRAIL (550ng/ml) and scFvCLL-1:sFasL (26ng/ml) is strongly inhibited by either pre-incubation with the chimeric scFvCLL-1:Fc minibody or by incubation with a TRAIL or FasL neutralizing mAb (2E5 or Alf2.1, respectively). (D) Apoptosis induction in U-937 cells by scFvCLL-1:sTRAIL (550ng/ml) and scFvCLL-1:sFasL (26ng/ml) is characterized by caspases-3/7activation. This caspase 3/7 activation is inhibited by pre-incubation with the chimeric scFvCLL-1:Fc minibody or by incubation with a TRAIL or FasL neutralizing mAb. In A, B and C apoptosis was assessed by analysis of Δψ, indicated values are means ± SEM of at least three independent experiments.

**CLL-1-restricted apoptosis induction by scFvCLL-1:sTRAIL and scFvCLL-1:sFasL.**

Next, the apoptotic activity of scFvCLL-1:sTRAIL and scFvCLL-1:sFasL towards CLL-1+ cell lines U-937, HL-60 and Ramos.CLL-1 cells was assessed. Both, scFvCLL-1:sTRAIL (Fig. 2A) and scFvCLL-1:sFasL (Fig. 2B) dose-dependently (range 0-550ng/ml and 0-26ng/ml, respectively) induced apoptosis in the CLL-1+ leukemia cells only. The anti-tumor activity of scFvCLL-1:sTRAIL was strongly inhibited after pre-incubation with the competing minibody scFvCLL-1:Fc or the TRAIL-neutralizing mAb 2E5 (data shown for U-937 cells.
only). Likewise, the anti-tumor activity of scFvCLL-1:sFasL towards U-937 cells was strongly inhibited after pre-incubation with the competing minibody scFvCLL-1:Fc or the FasL-neutralizing mAb Alf2.1 (Fig. 2C). Additionally, the activation of caspases-3/7 was assessed as one of the hallmark features of the death ligand induced apoptotic pathway. Both, treatment with scFvCLL-1:sTRAIL or scFvCLL-1:sFasL resulted in a potent activation of caspase-3/7. This activation was inhibited when the cells were pre-treated with either minibody scFvCLL-1:Fc or the respective death ligand-neutralizing mAb (Fig. 2D). Taken together, these data demonstrate that both scFvCLL-1:sTRAIL and scFvCLL-1:sFasL potently induce target antigen-restricted apoptosis in CLL-1+ tumor cells.

Figure 3. scFvCLL-1:sTRAIL and scFvCLL-1:sFasL convey apoptotic bystander activity towards CLL-1+ tumor cells. (A) Immunofluorescence analysis of activated caspase-3 (red fluorescence) in mixed cultures of Ramos.CLL-1 target cells (green fluorescence) and wild-type (CLL-1−) Ramos bystander cells. All cells were counterstained with the nuclear dye DAPI (blue fluorescence). In the left panel: absence of activated caspase-3 in untreated cells. In the middle panel: strong activation of caspases-3 in both the target cells and the bystander cells upon treatment with scFvCLL-1:sTRAIL (550ng/ml). In the right panel: activation of caspases-3 by scFvCLL-1:sTRAIL is inhibited by incubation with the TRAIL neutralizing mAb 2E5. (B) Flowcytometric analysis of the apoptosis induction by scFvCLL-1:sTRAIL (550ng/ml) or scFvCLL-1:sFasL (26ng/ml) in mixed cultures containing Ramos.CLL-1 target cells and wild-type Ramos bystander cells. The bystander apoptosis induction by the fusion proteins is inhibited by incubation with the TRAIL and FasL neutralizing mAb’s (2E5 and Alf2.1, respectively). Apoptosis was assessed by analyzing ΔΨ. Indicated values are means ± SEM of at least three independent experiments.
Bystander apoptosis by scFvCLL-1:sTRAIL and scFvCLL-1:sFasL towards CLL-1+ leukemia cells.

The bystander effect of scFvCLL-1:sTRAIL and scFvCLL-1:sFasL is based on the principle that targeted CLL-1+ tumor cells are not only eliminated, but are also exploited to convey a therapeutic effect towards neighboring tumor cells that are devoid of CLL-1 expression. Using a caspase-activatable fluorescent peptide and fluorescence microscopy strong activation of caspases-3 was visualized in CLL-1+ bystander tumor cells (Fig. 3A) after treatment with scFvCLL-1:sTRAIL. Activation of caspase-3 was abrogated in both the target and bystander tumor cells when pre-treated with minibody scFvCLL-1:sTRAIL or the TRAIL-neutralizing mAb2E5.

Next, flowcytometric analysis was used to quantify the bystander activity of scFvCLL-1:sTRAIL and scFvCLL-1:sFasL. A mixed tumor cell culture containing Ramos.CLL-1 target cells and Ramos bystander cell was treated with scFvCLL-1:sTRAIL (550ng/ml) or scFvCLL-1:sFasL (26ng/ml). This analysis demonstrated potent bystander activity for both scFvCLL-1:sTRAIL and scFvCLL-1:sFasL fusion proteins towards CLL-1- leukemia cells (26.9±1.1% and 47.7±6.8% respectively). Furthermore, treatment in the presence of the relevant death ligand-neutralizing mAb inhibited bystander activity down to 1.7±0.8% and 3.0±0.5% respectively (Fig. 3B).

Treatment of CLL-1+ normal monocytes and CLL-1- normal hepatocytes
Flowcytometric analysis demonstrated that normal resting CD14+ monocytes express significant levels of CLL-1 (Fig. 4A). However, treatment with scFvCLL-1:sTRAIL resulted in a minimal reduction in cell viability of the normal monocytes (3.5±6.0%) and a moderate reduction in viability by scFvCLL-1:sFasL (23.1±7.1%) (Fig. 4B). Flowcytometric analysis indicated that normal human hepatocytes do not express CLL-1 (Fig. 4C), Treatment with scFvCLL-1:sTRAIL and scFvCLL-1:sFasL did not reduce the viability of the hepatocytes (-11.9±6.0% and -4.0±1.8%, respectively) (Fig. 4D).

Apoptosis induction in primary AML cells
The anti-tumor activity of scFvCLL-1:sTRAIL (550ng/ml) and scFvCLL-1:sFasL (26ng/ml) was assessed towards primary AML samples that were freshly derived from 10 individual AML patients. The average apoptosis induction in these 10 samples by scFvCLL-1:sTRAIL was 11.1±4.5% and 36.9±7.3% by scFvCLL-1:sFasL. In addition, we combined the scFvCLL-1:sTRAIL or scFvCLL-1:sFasL treatment of the 10 AML samples with 5µM TDZD-8. This co-treatment synergized the apoptosis induction of both fusion proteins For scFvCLL-1:sTRAIL this resulted in an average apoptosis induction of 41.3±14.0% (CI=0.65) and for scFvCLL-1:sFasL in 44.0±8.4% (CI=0.89) (Fig. 5A).

Furthermore, since CLL-1 is also expressed on CD34+/CD38- AML stem cells and CD34+/CD38+ AML progenitor cells we analyzed apoptosis induction in these populations of 3 AML samples. In the CD34+/CD38- AML stem cell population, treatment with scFvCLL-1:sTRAIL resulted in an average apoptosis in 30.9±10.1% of the cells. The activity of scFvCLL-1:sFasL resulted in apoptosis in 22.4±8.4% of the CD34+/CD38- AML cells. Moreover, we also assessed the apoptosis induction in these CD34+/CD38- AML cells upon co-treatment with TDZD-8. Surprisingly, the apoptotic activity of both scFvCLL-1:sTRAIL and scFvCLL-1:sFasL was antagonized when combined with 5µM TDZD-8. For scFvCLL-
1:sTRAIL this resulted in apoptosis in 31.4±13.6% (CI=1.39) of the CD34+/CD38- AML cells and 22.4±8.4% (CI=1.55) for scFvCLL-1:sFasL (Fig. 5B).

In the CD34+/CD38+ progenitor cell population, the average apoptosis induction by scFvCLL-1:sTRAIL (550ng/ml) was 24.9±11.2% and 9.6±8.5% by scFvCLL-1:sFasL (26ng/ml). In the CD34+/CD38+ AML progenitor cell population co-treatment with 5µM TDZD-8...
synergized the apoptotic activity of the fusion proteins. This synergistic activity resulted in an average apoptosis induction in 31.9±13.8% (CI =0.82) of the CD34+/CD38+ cells by scFvCLL-1:sTRAIL. The activity of scFvCLL-1:sFasL in combination with TDZD-8 resulted in apoptosis in 20.7±7.0% (CI =0.53) of the CD34+/CD38+ cells (Fig 5C).

DISCUSSION

In order to achieve curative treatment of AML novel targeted approaches are needed that effectively eliminate all malignant cell types that are present in AML, including AML stem cells, AML progenitor cells, and target-antigen negative mutant AML cells, while sparing normal cells.

Recently, it was uncovered that C-type Lectin-Like Molecule-1 (CLL-1) is expressed at the tumor cell surface in ~90% of the AML patients [10;12]. Unlike CD33, the CLL-1 expression appears to be retained under all conditions of treatment and disease [11]. Importantly, CLL-1 is expressed on CD34+/CD38- AML stem cells and CD34+/CD38+ AML progenitor cells but not on normal hematopoietic stem cells [11].

This unique expression profile of CLL-1 prompted us to develop a novel targeted approach that aims for the safe and selective elimination of CLL-1+ malignant cells in AML. To this end, a CLL-1-specific antibody fragment (scFvCLL-1) was genetically fused to soluble forms of the pro-apoptotic proteins TRAIL (sTRAIL) and FasL (sFasL), yielding fusion proteins scFvCLL-1:sTRAIL and scFvCLL-1:sFasL, respectively.

We selected pro-apoptotic proteins sTRAIL and sFasL for this purpose because of their potent intrinsic anti-tumor activity. In this respect, sTRAIL appears to be a particularly promising since it has potent pro-apoptotic activity towards AML cells, while fully sparing normal cells.

Flowcytometric analysis demonstrated strong and specific binding for both scFvCLL-1:sTRAIL and scFvCLL-1:sFasL to cell surface-expressed CLL-1 on AML cells that could be inhibited by the competing minibody scFvCLL-1:Fc. Moreover, both scFvCLL-1:sTRAIL and scFvCLL-1:sFasL showed strong CLL-1-restricted pro-apoptotic activity towards CLL-1+ AML cell lines and to Ramos tumor B cells that ectopically expressed CLL-1. The anti-tumor activity of scFvCLL-1:sTRAIL was strongly inhibited after pre-treatment with the competing minibody scFvCLL-1:Fc or the TRAIL-neutralizing mAb2E5. Likewise, the anti-tumor activity of scFvCLL-1:sFasL was strongly inhibited after pre-treatment with scFvCLL-1:Fc or the FasL-neutralizing mAbAlf2.1. Both treatment with scFvCLL-1:sTRAIL or scFvCLL-1:sFasL of AML cells resulted in a potent activation of caspase-3/7, one of the hallmark features of the death ligand induced apoptotic pathway. Taken together, these data demonstrate that both scFvCLL-1:sTRAIL and scFvCLL-1:sFasL potently induce target antigen-restricted apoptosis in CLL-1+ tumor cells.

It has been reported that target antigen-negative leukemia cells can escape from conventional antibody-based therapeutic approaches [18;19]. Previously, we have demonstrated that scFv:sTRAIL and scFv:sFasL fusion proteins have potent anti-tumor bystander activity [7;16;20;21]. This anti-tumor bystander activity is based on the fact that these fusion proteins not only eliminate the respective target antigen-positive tumor cells, but also convey potent apoptotic signals towards neighboring tumor cells that are devoid of this target antigen. Here, we demonstrate similar promising bystander activities for fusion proteins scFvCLL-1:sTRAIL and scFvCLL-1:sFasL. In mixed culture experiments,
in which CLL-1\(^+\) target tumor cells and CLL-1\(^-\) bystander tumor cells were mixed at a ratio of 1:1, fusion protein scFvCLL-1:sTRAIL eliminated up to 27% of the CLL-1\(^-\) bystander tumor cells.

At the same target to bystander tumor cells ratio a bystander effect of up to 48% was observed for fusion protein scFvCLL-1:sFasL. The potent bystander activity observed for both fusion proteins may help to prevent the escape of CLL-1-negative tumor cells from CLL-1-targeted therapy.

![Figure 5. Apoptosis induction in the total AML cell population and in CD34+/CD38- and CD34+/CD38+ AML cells.](image)

Subsequently, we assessed the activity of scFvCLL-1:sTRAIL and scFvCLL-1:sFasL towards normal human cells. In contrast to GO, normal human CLL-1\(^+\) monocytes proved to be fully resistant to treatment with scFvCLL-1:sTRAIL and only moderately sensitive to treatment with scFvCLL-1:sFasL (23.1±7.1%). In addition, primary normal human hepatocytes appeared to be fully resistant to both scFvCLL-1:sTRAIL and scFvCLL-1:sFasL. The absence of activity towards CLL-1\(^+\) monocytes and CLL-1\(^-\) hepatocytes indicates a favorable toxicity profile for both scFvCLL-1:sTRAIL and scFvCLL-1:sFasL.

Next, we assessed the ex vivo activity of scFvCLL-1:sTRAIL and scFvCLL-1:sFasL towards primary patient-derived AML cells. Treatment with either scFvCLL-1:sTRAIL or scFvCLL-1:sFasL resulted in an induction of apoptosis in 10 out of 10 primary AML samples (11.1±4.5% and 36.9±7.3%, respectively).
Previously, the GSK-3β inhibitor TDZD-8 has been shown to induce apoptosis in AML cells, including the CD34+/CD38- AML stem cells[14]. In all patient-derived AML samples tested in our study the anti-tumor activity of both scFvCLL-1:sTRAIL and scFvCLL-1:sFasL was synergistically enhanced when treatment was performed in the presence of TDZD-8 (41.3±14.0%; CI=0.65 and 44.0±8.4%; CI=0.89, respectively). This suggests that the anti-AML activities of both scFvCLL-1:sTRAIL and scFvCLL-1:sFasL may be significantly enhanced when used in combination with other anti-AML drugs.

Currently, the concept is generally accepted that CD34+/CD38- AML stem cells and CD34+/CD38+ AML progenitor cells are the root of recurrences in AML patients. Therefore, the selective elimination of these cells is imperative in order to achieve curative therapy [22;23]. Unfortunately, AML stem cell populations appear to be particularly resistant to conventional therapeutic approaches. To address this issue we assessed the activity of scFvCLL-1:sTRAIL and scFvCLL-1:sFasL towards CD34+/CD38- AML stem cells and CD34+/CD38+ AML progenitor cells in three patient-derived AML samples. Both scFvCLL-1:sTRAIL and scFvCLL-1:sFasL proved to have marked anti-tumor activity towards CD34+/CD38- AML stem cells (30.9±10.1% and 22.4±8.4%, respectively) and the CD34+/CD38+ AML progenitor cells (24.9±11.2% and 9.6±8.5%, respectively). These results indicate that AML blasts are more sensitive to treatment with scFvCLL-1:sFasL than CD34+/CD38- AML stem cells (36.9±7.3% and 22.4±8.4%, respectively). Conversely, CD34+/CD38- AML stem cells appeared to be more sensitive to treatment with scFvCLL-1:sTRAIL than AML blast cells (30.9±10.1% and 11.1±4.5%, respectively). The observed differential sensitivity of CD34+/CD38- AML stem cells for scFvCLL-1:sTRAIL may be explained by the reported upregulated expression of TRAIL-R1 and TRAIL-R2 on the CD34+/CD38- AML stem cells [24].

Taken together our data indicates that in single agent treatment schemes both scFvCLL-1:sTRAIL and scFvCLL-1:sFasL show promising activity towards patient-derived CD34+/CD38- AML stem cells and CD34+/CD38+ AML progenitor cells. Previously, Guzman et al. reported that treatment with TDZD-8 induced rapid and selective death in both CD34+/CD38- AML stem cells and CD34+/CD38+ AML progenitor cells [14]. Therefore, we assessed whether the activity of scFvCLL-1:sTRAIL and scFvCLL-1:sFasL towards the AML stem cells and AML progenitor cells could be synergized by TDZD-8 co-treatment. Our data indicate that CD34+/CD38+ AML progenitor cells are indeed more sensitive when treatment with scFvCLL-1:sTRAIL or scFvCLL-1:sFasL is combined with TDZD-8 (31.9±13.8%; CI=0.82 and 20.7±7.0%; CI=0.53, respectively). Intriguingly, for unknown reasons, no enhanced effect of combination treatment with TDZD-8 was observed towards CD34+/CD38- AML stem cells.

In conclusion, we devised a novel CLL-1 targeted approach that may be of value for the effective elimination of the major malignant cell types that are present in AML, including AML stem cells and AML progenitor cells, while sparing normal cells such as CLL-1+ monocytes and CLL-1 normal hepatocytes. Moreover, the anti-tumor bystander activity of these fusion proteins may help to prevent the emergence of CLL-1 AML mutant cells. Furthermore, the single agent activity of scFvCLL-1:sTRAIL and scFvCLL-1:sFasL may be enhanced by combination treatment with novel anti-AML small inhibitory molecules such as TDZD-8. Further pre-clinical development of both scFvCLL-1:sTRAIL and scFvCLL-1:sFasL based approaches for the treatment of AML appears warranted.
ACKNOWLEDGMENTS

Supported by grants from the Dutch Cancer Society (2005-3358 and 2007-3784) to W.H. The authors would like to thank T. Bijma, J. Dokter-Fokkens, G. Mesander, D. Samplonius and D. Jager for their excellent technical assistance.

REFERENCES


