CD7-restricted activation of Fas-mediated apoptosis: a novel therapeutic approach for acute T-cell leukemia.

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Abstract
Agonistic anti-FAS antibodies and multimeric recombinant FASL preparations show highly potent anti-leukemia activity, but are not suitable for clinical application due to unacceptable systemic toxicity. Consequently, new anti-leukemia strategies based on FAS activation have to meet the criterion of strictly localized action at the tumour cell surface. Recent insight into the FASL/FAS system has revealed that soluble homotrimeric FASL (sFASL) is in fact non-toxic to normal cells, but also that it lacks tumoricidal activity. We report on a novel fusion protein, designated scFvCD7:sFASL, designed to have leukemia-restricted activity. ScFvCD7:sFASL consists of sFASL genetically linked to a high affinity scFv antibody fragment specific for the T-cell leukemia-associated antigen CD7. Soluble homotrimeric scFvCD7:sFASL is inactive and acquires tumoricidal activity only after specific binding to tumour cell surface-expressed CD7. Treatment of T-ALL cell lines and patient-derived T-ALL, PTCL, and CD7-positive AML cells with homotrimeric scFvCD7:sFASL revealed potent and CD7-restricted apoptosis induction that could be augmented by various conventional drugs, farnesyl transferase inhibitor L-744,832, and proteasome inhibitor Velcade. Importantly, identical treatment did not affect normal human PBLs and endothelial cells, with only moderate apoptosis induction in IL-2/CD3-activated T-cells. The leukemia-restricted activation of FAS by homotrimeric scFvCD7:sFASL revitalizes the applicability of FAS signalling in leukemia therapy.

Introduction
Despite advances in T-cell leukemia therapy, only a minority of patients achieves long term tumour-free survival with conventional chemotherapy at the cost of significant and often irreversible toxic side effects. Therefore, new therapeutic approaches with enhanced tumour selectivity and more favourable toxicity profiles are urgently needed. Several promising targeted approaches have been developed, including naked antibodies, immunotoxins, and various cancer-selective small inhibitory molecules. Furthermore, certain members of the TNF-superfamily show promising pro-apoptotic activity towards various human leukemias and lymphomas. FASL, a prominent member of the TNF-superfamily, shows superior anti-leukemia activity. FASL is present on lymphocytes and monocytes/macrophages as a type II transmembrane protein, hereafter referred to as memFASL. FAS, the cognate receptor for FASL, belongs to the growing family of transmembrane proteins known as death receptors. Death receptors can detect the presence of specific extracellular death signals and rapidly trigger cellular destruction by apoptosis. FAS expression at the cell surface is observed in biopsies and cell lines derived from a variety of tumours. Moreover, the anti-tumoral effects of various chemotherapeutic drugs have been attributed partly to p53-mediated up-regulation of FAS and FASL. FAS signalling is also known to be a key element in the effector phase
of a CTL response against tumour cells. Like other members of the TNF-superfamily, the extra-cellular domain of FASL can be proteolytically cleaved into a soluble homotrimeric form\textsuperscript{13-15}, hereafter referred to as sFASL.

Early attempts to exploit FAS agonists such as anti-FAS antibodies and multimeric recombinant FASL preparations for therapy revealed extremely potent tumoricidal effects towards isolated primary tumour cells and cell lines\textsuperscript{13,16-19}. However, \textit{in vivo} application of most FAS antagonists was associated with acute lethality in mice\textsuperscript{20-22}, thereby excluding therapeutic evaluation in humans. Nevertheless, the principal feasibility of therapeutic FAS activation in cancer therapy was clearly demonstrated in mice that lack a functional FASL/FAS system (lpr/gld mice)\textsuperscript{23} and by treatment of xenografted tumours with human FAS-specific antibodies\textsuperscript{24}.

Recent studies have revealed that certain recombinant sFASL preparations contain oligomeric, multimeric, and even aggregated sFASL forms and that these forms are responsible for the observed systemic toxicity\textsuperscript{25}. In contrast, homotrimeric sFASL is not toxic to normal cells and may even antagonize the function of membrane bound FAS\textsuperscript{25-27}. Importantly, homotrimeric sFASL also lacks tumoricidal activity. However, inactive homotrimeric sFASL can rapidly be re-activated by applying secondary crosslinking antibodies.

Recently, we demonstrated that the leukemia selectivity of homotrimeric TRAIL, another TNF-superfamily-member, can be strongly enhanced by genetically fusing it to a CD7-selective antibody fragment\textsuperscript{28}. Human CD7 is a lineage-specific antigen that is highly expressed on acute T cell leukemia and \~10\% of acute myeloid leukemia\textsuperscript{29-32}. The function of CD7 is not yet fully understood. In normal cells CD7 expression is limited to T- and myeloid cells in early hematopoietic cell ontogeny, thymocytes, NK cells, and to a distinct subset of peripheral blood T-cells\textsuperscript{33-37}. Human CD7 has been used for the targeted delivery of several MAb-toxin conjugates in both pre-clinical studies and clinical trials\textsuperscript{4, 5,38,39}.

Here we report on a novel homotrimeric sFASL fusion protein, designated scFvCD7:sFASL with enhanced and leukemia-restricted activity towards T-ALL cell lines and patient-derived T-ALL, PTCL, and CD7-positive AML cancer cells. We provide evidence that homotrimeric scFvCD7:sFASL is bioactive only after specific binding to cell surface-expressed CD7 with no toxicity towards CD7-negative cells and only moderate activity towards IL-2/CD3-activated CD7-positive T-cells.

**Materials & Methods**

\textit{Monoclonal antibodies and scFv antibody fragment}

MAb TH69 is a murine IgG1 with specificity for human CD7\textsuperscript{3} and was kindly provided by Prof. Dr. Martin Gramatzki, Division of Stem Cell and Immunotherapy, 2nd
Medical Department, University Clinic Schleswig-Holstein, Kiel, Germany. Phagemid pCANTAB5E/scFv3A1F encoding anti-CD7 antibody fragment 3A1F was kindly provided by Dr. Chris Pennell, Department of Laboratory Medicine and Pathology, University of Minnesota. MAb TH69 and scFv-3A1F compete for binding to the same or overlapping epitope on the extracellular domain of human CD7. FASL-neutralizing MAb Alf2.1 was purchased from Sigma (Sigma-Aldrich Chemie B.V. Zwijndrecht, The Netherlands).

Chemotherapeutics
The cytostatic drugs used are Vincristin (stock; 1 mg/ml in PBS), Amsacrine (stock; 1mM in PBS), and Actinomycin D (stock; 2 mg/ml in ethanol). Farnesyl Transferase inhibitor L-744,832 was purchased from Merck (Darmstadt, Germany) and was dissolved at 10 mM in DMSO. The proteasome inhibitor Velcade (Millennium pharmaceuticals, Cambridge, MA) was dissolved at 10mM in dH2O. All final concentrations were prepared by serial dilutions in serum free medium.

Cell lines
Human CD7-positive T-ALL cell lines Jurkat, CEM, and the CD7-negative human B-cell lymphoma cell lines Ramos and Raji were purchased from the ATCC (Manassas, USA). T-cell lines MOLT-16, HuT-78 were a kind gift of Prof. Dr. Martin Gramatzki. A CD7-positive transfectant of the Ramos cell line (Ramos.CD7) was generated as previously described. All cell lines were cultured in RPMI (Cambrex, New Jersey, New Hampshire, USA) supplemented with 13% FCS, at 37ºC in humidified 5% CO2 atmosphere.

Leukocytes, PBLs, activated T-cells, and HUVECs
Leukocytes were isolated from whole blood of healthy donors by a standard Ammonium Chloride method. Peripheral blood lymphocytes (PBLs) were isolated from whole blood of healthy donors by standard density gradient centrifugation procedures (Lymphoprep, Axis-Shield PoC As., Oslo, Norway). Freshly isolated PBLs were resuspended at 2-106 cell/ml in RPMI, supplemented with 10% Human Pooled Serum. Activated T-cells were obtained by incubation of freshly isolated PBLs with anti-CD3 MAb WT32 (0.5 µg/ml) for 72 h, followed by IL-2 stimulation (100 ng/ml) for 48 h. HUVECs were isolated as previously described. HUVEC cells were used before culture passage number four and, for experiments, were pre-cultured in 6 well plates at 60% confluency. HUVEC cells were activated with TNF-α or IFN-γ at a final concentration of 100ng/ml.

Construction of scFvCD7:sFASL
Previously, we constructed the eukaryotic expression plasmid pEE14scFv:sTRAIL for the rapid construction, evaluation and stable expression of scFv:sTRAIL fusion proteins in
CHO-K1 cells. Important features of this vector are the presence of the murine kappa light-chain leader peptide encoded upstream of 2 multiple cloning sites (MCSs) that are separated by a 26 residue in-frame linker sequence, and the glutamine synthetase selectable marker gene, which allows for amplified expression of the recombinant protein in the established industrial production cell line CHO-K1. The vector exploits the strong CMV promoter to drive recombinant protein expression, while the leader peptide directs secretion of the fusion protein into the culture supernatant. In the first MCS, a 745 bp DNA fragment encoding anti-CD7 scFv3A1F derived from Phagemid pCANTAB5E/scFv3A1F was directionally inserted using the unique SfiI and NotI restriction enzyme sites. In the second MCS, sTRAIL encoding cDNA was swapped for a PCR-truncated 539 bp DNA fragment encoding the extracellular domain of human sFASL using restriction enzymes XhoI and HindIII and standard DNA manipulation procedures. FASL cDNA truncation was performed by PCR using proofread DNA polymerase according to standard protocol using primers: T1: 5’-ATCCCTCGAGTCTAGTGGGAGCGATCTACCAGCCAGATGCACACA-3’ (XhoI site is underlined) and T2: 5’-CCCAAGCTTTGCTTCTCTTAGA-3’ (HindIII site is underlined).

Production of scFvCD7:sFASL
ScFvCD7:sFASL was expressed in CHO-K1 cells with the glutamine synthetase selection/amplification system essentially as described previously. Briefly, CHO-K1 cells were transfected with pEE14scFvCD7:sFASL using Fugene-6 reagent (Roche Diagnostics, Almere, The Netherlands). Stable transfectants with amplified expression were isolated and single cell sorted with a high-speed cell sorter (Cytomation, Fort Collins, USA). Individual clones were assessed for stable and high secretion of scFvCD7:sFASL in the absence of the MSX selection reagent by a FASL ELISA according to manufacturer’s recommendations (Alexis, 10P’s BVBA, Breda, The Netherlands). This procedure identified CHO-K1 production cell line 100B2, which stably secreted scFvCD7:sFASL (1.34 µg/ml) into the medium. ScFvCD7:sFASL containing supernatant was harvested (10,000xg; 10 min) and stored at -80°C until use.

Solution behaviour of scFvCD7:sFASL
Size-exclusion FPLC: The solution behaviour of scFvCD7:sFASL was analyzed by size-exclusion (SE) FPLC with a calibrated HiLoad 16/60 Superdex 200 Prep-grade column (Amersham Biosciences, Uppsala, Sweden) of a bed volume of 120 ml; 5 ml supernatant derived from CHO-K1 cell line 100B2 was loaded onto the column, after which individual samples were collected at 3-min intervals. Individual samples were analyzed for their capacity to induce apoptosis in CD7-positive FASL-sensitive MOLT-16 cells.
CD7-restricted activation of Fas in T-cell leukemia

**CD7-specific binding of scFvCD7:sFASL**

CD7-specific binding of scFvCD7:sFASL was assessed by incubation of $1.0 \times 10^6$ CEM cells with scFvCD7:sFASL containing medium (1.34 µg/ml) in the presence or absence of CD7-blocking MAb TH69 (5 µg/ml). CD7-specific binding was analyzed by flow cytometry with PE-conjugated anti-FASL MAb (Diaclone SAS, Besancon, France). Incubations were carried out for 45 min at 0°C and were followed by two washes with serum free medium.

**CD7-restricted apoptosis induction by scFvCD7:sFASL**

Tumour cells were seeded at $0.25 \times 10^6$ cells/well in a 48-well plate and treated for 16 h with the indicated concentrations of scFvCD7:sFASL in the presence or absence of MAb TH69 (5 µg/ml) or MAb ALF2.1 (1 µg/ml). Apoptosis was assessed by one of the below-described assays. Percentage of specific killing was calculated using the following formula: (experimental apoptosis - spontaneous apoptosis)/(100 - spontaneous apoptosis) x 100%. Assays employed to assess apoptosis: PS exposure to the outer cell membrane; flow cytometric analysis of exposure of phosphatidyl serine (PS) on the outer membrane with an AnnexinV-FITC/PI kit (NeXins Research, Kattendijke, The Netherlands) according to manufacturer’s instructions. Loss of mitochondrial membrane potential ($\Delta \psi$); $\Delta \psi$ was analyzed with the cell-permeant green-fluorescent lipophilic dye DiOC6 (Molecular Probes, Eugene, USA). After treatment, cells were harvested by centrifugation (300xg; 5 min), incubated for 20 min at 37°C with 0.1 µM DiOC6 in fresh medium, washed once with PBS, and analyzed by flow cytometry. Fluorescence microscopy of activated caspase-3; After treatment, cells were spotted on microscope slides and fixed in acetone. Active caspase 3 staining was performed with MAb 5A1 (Cell Signalling) and secondary FITC-conjugated antibody (DAKO). DAPI was used to stain all nuclei. Specific staining was evaluated using a Quantimed 600S fluorescence microscope (Leica Camera Ag, Solms, Germany). Detection of apoptotic DNA fragmentation; DNA-fragmentation was analyzed using MAb F7-26 (Alexis) according to manufacturer’s recommendations. MAb F7-26 specifically detects DNA fragmented by apoptosis without reactivity for otherwise fragmented double-stranded DNA.

**Differential quantification of apoptosis in target and bystander cells in mixed culture experiments**

For mixed culture experiments, CD7-positive target cells were labeled with the red fluorescent dye DiI (Molecular probes). Briefly, cells ($1.0 \times 10^6$ ml$^{-1}$) were incubated for 5 min at 37°C in serum free medium containing 5 µM DiI, followed by three washes with standard medium. DiI-labeled target and non-labeled bystander cells were mixed at indicated ratios with a final cell concentration of $0.5 \times 10^6$ cells/well in a 48-well plate. After treatment, differential fluorescent characteristics of target cells and bystander cells were
used to separately evaluate apoptosis by PS exposure to the outer cell membrane or by $\Delta \psi$ as described above.

**Additive induction of apoptosis by scFvCD7:sFASL, chemotherapeutics and small inhibitory molecules**
Additive apoptotic effects of treatment of cells with scFvCD7:sFASL and various chemotherapeutics or small inhibitory molecules was determined using the cooperativity index (CI), in which the sum of apoptosis induced by single-agent treatment is divided by apoptosis induced by combination-treatment. CI<1, treatment was termed synergistic; CI=1, treatment was termed additive; CI>1, treatment was termed antagonistic.

**CD7-restricted apoptosis induction in patient-derived leukemic cells**
Blood cells derived from four individual T-ALL patients (patients #1 to #4), one PTCL patient (patient #5), and one CD7-positive AML patient (patient #6), were treated for 16 h with scFvCD7:sFASL (150 ng/ml) in the presence or absence of MAb TH69, where indicated. Additionally, blood cells derived from patient #6 (AML) were co-treated with either Vincristin or Amsacrine. Apoptosis induction was assessed by PS exposure to the outer cell membrane and staining for active caspase 3 as described above.

**Results**

**Fractionation and stability of homotrimeric scFvCD7:sFASL**
Fractionation of crude supernatant containing scFvCD7:sFASL by SE-FPLC and subsequent assessment of the apoptotic activity of each separate fraction revealed two peaks of apoptotic activity (Fig.1A). One peak corresponded to a molecular weight (MW) of approximately 160 kDa, which closely resembles that of the calculated MW of 158 kDa for homotrimeric scFvCD7:sFASL. Samples taken from the 160 kDa peak showed strong and CD7-restricted apoptosis induction towards MOLT-16 cells that was completely abrogated when cells were pretreated for 3 hours with CD7-blocking MAb TH69. Samples taken from the 700 kDa peak showed strong apoptosis induction towards both CD7-positive MOLT-16 cells and CD7-negative Ramos cells, while apoptosis induction could not be inhibited by pre-treatment with MAb TH69. Samples taken from the 700 kDa peak were discarded. Fractions from the 160 kDa peak containing homotrimeric scFvCD7:sFASL were pooled and used for further experimental procedures and analyses. Subsequently, we analyzed for the secondary formation of scFvCD7:sFASL multimers or aggregates. This analysis indicated that homotrimeric scFvCD7:sFASL is stable for up to 7 days, with no detectable secondary formation of multimers or aggregates (data not shown). Furthermore, scFvCD7:sFASL incubated for up to 9 days at 37°C in the presence of serum retained potent and CD7-restricted apoptotic activity towards FASL-sensitive MOLT-16 cells (Fig.1B).
CD7-restricted induction of apoptosis by homotrimeric scFvCD7:sFASL

Incubation of CEM cells with homotrimeric scFvCD7:sFASL resulted in strong and specific binding to the cell surface (Fig.2A, solid line), which was inhibited by pre-incubation with MAb TH69 (Fig.2A, dotted line). Treatment of a series of CD7-positive leukemic T cell lines with serially increasing concentrations of homotrimeric scFvCD7:sFASL (1–110 ng/ml) resulted in potent and dose-dependent increase in apoptosis induction in all CD7-positive T cell lines tested (Fig.2B; Jurkat, 82%; CEM, 90%; HuT-78, 70%; MOLT-16, 98%). CD7-negative FASL-sensitive Raji cells were fully resistant to apoptosis induction even at the highest concentrations tested (Fig.2B; 2.0%). Apoptosis induction by homotrimeric scFvCD7:sFASL was associated with apoptotic DNA-fragmentation that was inhibited by pre-treatment with MAb TH69 (Fig.2C). Similarly, co-incubation with FASL-neutralizing MAb Alf2.1 completely abrogated apoptotic DNA-fragmentation (Fig.2C).

Potent anti-tumour bystander apoptosis induction by homotrimeric scFvCD7:sFASL

Cell surface accretion of homotrimeric scFvCD7:sFASL on CD7-positive target cells can be exploited to crosslink FAS on neighbouring tumour cells that are devoid of CD7-expression, a principle known as the bystander effect. The pro-apoptotic bystander effect of homotrimeric scFvCD7:sFASL was assessed using mixed culture experiments in which Ramos.CD7+ target cells were mixed with CD7-negative Raji bystander cells (ratio...
In the Raji bystander cells, a bystander apoptotic effect of up to 31% was detected (Fig. 3), which was specifically inhibited in the presence of MAb TH69 (7%) or MAb ALF2.1 (0.2%). No apoptosis induction was observed when parental CD7-negative Ramos cells were used in this experiment (data not shown).

Absence of apoptotic activity of homotrimeric scFvCD7:sFASL towards PBLs and leukocytes
A subpopulation of normal PBLs is CD7-positive and might sustain collateral damage during treatment. Therefore, we investigated the apoptotic activity of homotrimeric
CD7-restricted activation of Fas in T-cell leukemia

**Fig. 3. Potent induction of apoptosis in CD7-negative bystander tumor cells.** Mixed cultures of Ramos.CD7 target cells and Raji bystander cells (ratio 1:1) were treated for 16 h with scFvCD7:sFASL (100 ng/ml) in the presence or absence of MAb TH69 or MAb Alf2.1. The differential fluorescent labeling of target and bystander cell populations was used to separately evaluate apoptosis induction in the bystander population by $\Delta\psi$. Indicated values are representatives of three independent experiments.

scFvCD7:sFASL towards normal PBLs. To this end, PBLs were treated with excess amounts of scFvCD7:sFASL (325 ng/ml) for up to 9 days (Fig. 4A). This treatment revealed no increase in apoptosis compared to medium control (Fig. 4A). Next, we assessed a possible 'innocent' bystander apoptotic effect towards normal human leukocytes when homotrimeric scFvCD7:sFASL is present in a membrane bound state at the cell surface of CD7-positive tumour cells (Jurkat). Treatment of mixed cultures of Jurkat cells and freshly isolated leukocytes (ratio 4:1) with homotrimeric scFvCD7:sFASL revealed no increase in apoptosis in leukocytes (Fig. 4B; PBLs, 8%; Jurkat, 72%).

**Moderate apoptotic activity of homotrimeric scFvCD7:sFASL towards activated T-cells**

Treatment of antiCD3/IL-2 activated T-cells with excess amounts of homotrimeric scFvCD7:sFASL (325 ng/ml) induced apoptosis in approximately 35% of activated T-cells at day 1 (Fig. 4C). Up to day 9, the level of apoptosis in scFvCD7:sFASL treated cells was consistently higher (~10%) than in cells incubated with medium only.

**No apoptotic activity towards resting and activated HUVECs**

To simulate the effect of scFvCD7:sFASL on human endothelial cells, resting and TNF-α or IFN-γ activated HUVECs were treated with excess amounts of homotrimeric scFvCD7:sFASL. No apoptosis was detected after 24h treatment with homotrimeric scFvCD7:sFASL in either resting - or activated HUVECs (Fig. 4D), whereas treatment with excess amount of Actinomycin D resulted in significant induction of apoptosis (53%, 55%, and 56%, respectively).

When homotrimeric scFvCD7:sFASL is bound to the cell surface of circulating leukemic
Fig. 4. Treatment of normal human leukocytes, activated T-cells and HUVECs with scFvCD7:sFASL. 

A: Resting PBLs were subjected to treatment with scFvCD7:sFASL (325 ng/ml) or unconditioned medium for up to 9 days, after which apoptosis was assessed by AnnexinV/PI staining. Indicated values are representatives of three independent experiments. 

B: Isolated PBLs were mixed at a ratio of 1:10 with DiI-labeled Jurkat cells. Mixed cultures were treated for 24h with scFvCD7:sFASL (100 ng/ml). Differential fluorescent labeling of Jurkat target cells and PBLs was used to separately evaluate apoptosis induction by AnnexinV staining. Indicated values are representatives of three independent experiments. 

C: Activated T-cells were subjected to treatment with scFvCD7:sFASL (325 ng/ml) or unconditioned medium for up to 9 days, after which apoptosis was assessed by AnnexinV/PI staining. Indicated values are representatives of three independent experiments. 

D: Resting, TNF-α or IFN-γ activated HUVEC cells were treated for 24 h with medium, scFvCD7:sFASL (100 ng/ml) or Actinomycin D (2 µg/ml). Apoptosis was assessed by ∆ψ. 

E: Resting, TNF-α or IFN-γ-activated HUVEC cells were mixed with fluorescently labeled Jurkat cells (ratio 1:1) and treated with scFvCD7:sFASL (100 ng/ml) or Actinomycin D (2 µg/ml) for 24 h. Differential fluorescent labeling of Jurkat target cells and HUVEC bystander cells was used to separately evaluate apoptosis by ∆ψ.
T-cells there might be a possible innocent bystander effect towards endothelial cells. We simulated this situation using mixed culture experiments in which HUVECs were cocultured with Jurkat cells (ratio 1:1) in the presence of homotrimeric scFvCD7:sFASL. In this mixed culture experiment, resting and activated HUVECs proved to be fully resistant to a possible innocent bystander effect of homotrimeric scFvCD7:sFASL treatment (Fig.4E; resting HUVEC, 2%; TNF-α activated HUVEC, 3%; Jurkat, 77%).

Fig.5. Additive tumoricidal effect of scFvCD7:sFASL with several classes of anti-leukemia agents. Jurkat cells were treated for 16 h either alone or with scFvCD7:sFASL at the indicated concentrations combined with A: Vincristin (0.1 ng/ml), B: farnesyl transferase inhibitor L-744,832 (25 µM) and C: proteasome inhibitor Velcade (10 nM). Apoptosis induction was assessed by ∆ψ. Synergy was determined using the cooperativity index as described in materials and methods section.
Additive tumoricidal effects of scFvCD7:sFASL with chemotherapeutics and small inhibitory molecules

Sensitivity to FASL-induced apoptosis has previously been shown to be augmented by pre- or co-treatment with various chemotherapeutics and small inhibitory molecules. To establish whether scFvCD7:sFASL had similar properties, Jurkat cells were simultaneously treated with homotrimeric scFvCD7:sFASL, the microtubule inhibitor vincristin, the farnesyl transferase inhibitor L-744,832, or the proteasome inhibitor Velcade. Co-treatment with homotrimeric scFvCD7:sFASL and Vincristin resulted in a synergistic increase in apoptosis compared to either agent alone (Fig.5A; CI=0.9). Co-treatment with L-744,832 or with Velcade similarly increased apoptosis in a synergistic manner (Fig.5B and C, CI=0.7 and 0.85, respectively). Identical treatment of PBLs, activated T-cells or resting and activated HUVECs did not result in a significant increase in apoptosis compared to single agent treatment with the respective chemotherapeutic (Fig.6A, B, and C).

Treatment in vitro of T-ALL, PTCL, and CD7-positive AML patient-derived leukemic blood samples

Blood samples derived from 6 patients suffering from various forms of CD7-positive leukemia were treated in vitro with homotrimeric scFvCD7:sFASL. The samples included T-ALL (patients #1 to #4), PTCL (patient #5), and CD7-positive AML (patient #6), all containing >90% leukemic cells (Fig.7A). In three out of four T-ALL patient samples, treatment with homotrimeric scFvCD7:sFASL resulted in a marked increase in apoptosis induction (34%, 56%, and 53% for patients #1, #3, and #4, respectively). Tumour cells from T-ALL patient #2 were refractory to treatment (7% apoptosis). Tumour cells derived from patient #5 (PTCL) showed a moderate response of 14%, while treatment of tumour cells derived from patient #6 (CD7-positive AML) resulted in a 25% increase in apoptosis. Co-incubation with MAb TH69 inhibited apoptosis induction by homotrimeric scFvCD7:sFASL, as exemplified for patient #6 (CD7-positive AML) (Fig.7B).

After treatment, cells from patient #4 were analyzed for the formation of active caspase 3 using MAb 5A1 and evaluated by fluorescence microscopy. Specific staining indicated the activation of caspase 3, whereas untreated cells showed no formation of active caspase 3 (Fig.7C).

Additive apoptotic effect of chemotherapy on primary leukemic cells

Blood samples derived from patient #6 (CD7-positive AML) were treated simultaneously with scFvCD7:sFASL and either vincristin (1 ng/ml) or amsacrine (1 µM) (Fig.7D). In both cases, combination treatment resulted in an additive induction of apoptosis (Fig.7D; vincristin, CI=1.0; amsacrine, CI=1.0).
Agonistic anti-FAS antibodies and multimeric recombinant FASL preparations show highly potent anti-leukemia activity. However, attempts to exploit these conventional FAS agonists for cancer therapy have met with unacceptable systemic toxicity, largely excluding FAS signalling as a therapeutic strategy for treatment of human malignancies. The toxicity observed for sFASL preparations appears to be directly related to presence...
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Fig. 7. Treatment of T-ALL, PTCL, and CD7-positive AML patient-derived blood samples in vitro. A; Blood cells derived from four T-ALL patients, one PTCL patient, and one CD7-positive AML patient were subjected to treatment with scFvCD7:sFASL (150 ng/ml) and analyzed for apoptosis induction using AnnexinV/PI staining. B; Primary CD7-positive AML cells were subjected to treatment with scFvCD7:sFASL alone or in the presence of MAb TH69 for 16 h, after which apoptosis was assessed by AnnexinV/PI staining. C; Primary T-ALL cells were subjected to scFvCD7:sFASL (150 ng/ml) and then assessed for the presence of active caspase-3 using fluorescent microscopy. D; Primary CD7-positive AML cells were subjected to single agent treatment with scFvCD7:sFASL (100 ng/ml), vincristin (10 ng/ml), Amsacrine (1 µM) or to combination treatment. Apoptosis was assessed by AnnexinV/PI staining. Additive induction of apoptosis was determined using the cooperativity index.

of multimeric - and aggregated sFASL species that are frequently formed during or after overexpression in primitive host cells such as *Escherichia coli*. Moreover, it was shown
that as few as two adjacent trimeric (hexameric) sFASL molecules are already sufficient for FAS signalling in Raji, Jurkat, and activated T-cells, as was evidenced by the formation of a death-inducing signalling complex (DISC) and subsequent induction of apoptosis. We produced scFvCD7:sFASL in CHO-K1 cells, a currently favoured industrial host cell line for the production of therapeutic recombinant proteins. We employed a murine kappa light-chain leader peptide to direct the produced fusion protein through the ER and Golgi complex taking advantage of the associated stringent quality control mechanisms that facilitates the secretion of correctly folded and non-aggregated fusion protein into the culture medium. Using SE-chromatography we removed most, if not all, unwanted multimeric forms of scFvCD7:sFASL. Importantly, homotrimeric scFvCD7:sFASL did not display any detectable secondary aggregate formation even after prolonged storage at 37°C in the presence of serum, whereas the apoptotic activity was retained up to this time-point. From this we conclude that scFvCD7:sFASL can be produced as soluble homogeneous homotrimers with no or only minimal secondary aggregate formation. These features opened the possibility to specifically target biologically inactive homotrimeric scFvCD7:sFASL to CD7-positive cells after which sFASL activation is induced by immobilization and multimerization on the target cell surface. Treatment of a number of CD7-positive leukemia T-cell lines with scFvCD7:sFASL showed potent and dose-dependent induction of apoptosis, which was specifically inhibited when cells were pre-treated with MAb TH69 or co-incubated with FAS-neutralizing MAb Alf2.1. Flowcytometric analysis identified that binding of scFvCD7:sFASL to FAS was barely detectable (data not shown). This can be explained by the fact that antibody-based proteins typically have fast-on and slow off-binding rates (for review see), whereas the binding of FASL to FAS (both trimers) is characterized by fast on/fast off rates, typical for cytokine/cytokine receptor interaction. Moreover, in homotrimeric scFvCD7:sFASL binding via the antibody domains substantially benefits from the associated avidity effect of the presence of three scFv reading heads. From this it can be concluded that biologically inactive homotrimeric scFvCD7:sFASL acquires full bioactivity only upon binding to cell surface-expressed CD7 and that apoptosis is commenced by reciprocal crosslinking of FAS in an autocrine or paracrine manner. Using conventional MAb-based therapies target antigen-negative tumour cells can readily escape from therapy. The paracrine activation of FAS by CD7-immobilized scFvCD7:sFASL opens the possibility to induce apoptosis in neighbouring FAS-sensitive leukemia cells that, for some reason, have lost CD7 expression. Previously, we reported on an exceptionally strong anti-tumour bystander effect for an analogous scFv:sTRAIL fusion protein. We assessed this mode of action of scFvCD7:sFASL using mixed culture experiments in which CD7-positive target cells were co-cultured with CD7-negative bystander tumour cells. In the bystander tumour cell population a
strong apoptotic effect was detected, which was specifically inhibited in the presence of MAb TH69 or MAb ALF2.1. Taken together, this indicates that the bystander effect of scFvCD7:sFASL predominantly depends on activation of scFvCD7:sFASL on the cell surface of CD7-positive tumour cells. In contrast, only moderate bystander effects have been reported for immunotoxin-based strategies, most likely because these strategies usually depend on a number of consecutive features, including internalization, inter-cellular gap junction communication, and enzymatic conversion\textsuperscript{57,58}.

Previously, Samel et al provided proof of principle of targeted FAS signalling by fusing sFASL to a scFv specific for the fibroblast activation protein FAP, a tumour-associated stroma marker. The intravenous application of this novel FAS-reagent in mice revealed no signs of systemic toxicity and prevented growth of xenotransplanted FAP-positive, but not FAP-negative, tumour cells\textsuperscript{52}. Nevertheless, from these elegant experiments it can not be concluded that in humans a similar favourable toxicity profile will be observed.

In the current study we explored the feasibility and safety of scFv-targeted FAS signalling \textit{in vitro} by treating various CD7-positive human leukemia types in the absence or presence of normal human blood cells and endothelial cells (HUVEC). Treatment of PBLs with scFvCD7:sFASL did not induce apoptosis in any of the normal cell types present, including resting CD7-positive T-cells and CD7-positive NK-cells. This is a remarkable finding since specific binding of scFvCD7:sFASL to CD7 on these cells results in the local activation of scFvCD7:sFASL, that is subsequently able to perform autocrine or paracrine FAS signalling. Apparently, normal cell types are relatively resistant to this form of FAS signalling. In contrast, treatment of antiCD3/IL-2 activated T-cells with homotrimeric scFvCD7:sFASL induced apoptosis in approximately 35\% of activated T-cells at day 1 (Fig.4C). Up to day 9, the level of apoptosis in scFvCD7:sFASL treated cells was consistently higher (~10\%) than in cells incubated with medium only. It is well established that fraticidal FAS/FASL interactions between activated T-cells are paramount to the effective resolution of an immune response\textsuperscript{53}. Although not studied here, we speculate that fusion proteins such as scFvCD7:sFASL, or those that target activation markers such as CD69, can be applied to treat T cell-mediated autoimmunity. We established that resting HUVEC cells are fully resistant to scFvCD7:sFASL treatment. Importantly, also activated HUVEC cells, known to express FAS at the cell surface\textsuperscript{51,52}, proved to be resistant to homotrimeric scFvCD7:sFASL.

Upon \textit{in vivo} application in leukemia patients many different cell types will simultaneously encounter either free or cell-bound scFvCD7:sFASL. Binding of homotrimeric scFvCD7:sFASL to the cell surface of abundantly circulating leukemic T-cells might lead to a potentially harmful innocent bystander effect towards normal cells e.g. endothelial cells. We simulated this situation using mixed culture experiments in which HUVECs were co-cultured with CD7-positive Jurkat cells (ratio 1:1) in the presence of homotrimeric
scFvCD7:sFASL. In this experiment both resting and activated HUVECs proved to be fully resistant to a possible innocent bystander effect of scFvCD7:sFASL treatment (Fig.4E).

Subsequently, we treated blood samples derived from 6 patients suffering from various forms of CD7-positive leukemia with homotrimeric scFvCD7:sFASL. Samples, all containing >90% leukemic, were derived from four T-ALL patients, one PTCL patient and one CD7-positive AML patient, respectively. Three out of four T-ALL patient samples showed a marked increase in apoptosis induction (32%, 56%, and 53% for patients #1, #3, and #4 respectively). Tumour cells from T-ALL patient #2 (7% apoptosis) were refractory to treatment. Tumour cells derived from patient #5 (PTCL) showed a moderate response of 14%, while treatment of tumour cells derived from patient #6 (CD7-positive AML) resulted in a 25% increase in apoptosis. At first glance, the therapeutic effect of scFvCD7:sFASL towards these primary tumour cells might seem rather moderate. However, *ex vivo* primary tumour cells typically grow in a non-synchronized way at relatively low cell division rates. Previously, it was shown that leukemia cells are only sensitive to FASL-induced apoptosis in the G1 phase of the cell cycle. Consequently, the effect observed *ex vivo* may be an underestimation of the therapeutic effect of scFvCD7:sFASL when applied *in vivo*. Moreover, repeated rounds of treatment, as well as treatment with synchronizing agents might help to overcome cell cycle-related resistance.

Various chemotherapeutic agents are known to sensitize tumour cells to FAS-mediated apoptosis at distinct levels, including receptor-proximal, mitochondrial, and/or effector-caspase level. We subjected blood samples derived from patient #6 (CD7-positive AML) to co-treatment with scFvCD7:sFASL and sub-optimal concentrations of vincristin and amsacrine, both chemotherapeutic agents that are already part of clinical practice. Co-treatment resulted in a promising additive effect on apoptosis induction (Fig.7D). Importantly, identical treatment of normal PBLs, activated T-cells, and resting/activated HUVEC did not result in significant induction of apoptosis compared to chemotherapy alone.

In conclusion, we describe a novel and promising anti-T cell leukemia agent that shows strong and CD7-restricted tumoricidal activity towards various CD7-positive leukemic cell types that can be augmented with various chemotherapeutic agents and small inhibitory molecules, such as velcade. Toxicity towards normal cells appears to be restricted to a sub-set of activated T cells. Obviously, more research is needed to evaluate toxicity towards other cells and tissues with an emphasis on liver toxicity. *New in vitro* technologies including the use of human liver organ slices appear to be appropriate for this purpose. Alternatively, scFvCD7:sFASL might be an excellent candidate for the purging of bone marrow from malignant CD7-positive cells. In each case further pre-clinical evaluation for scFvCD7:sFASL is warranted.
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Chapter 7

CD7-restricted activation of Fas in T-cell leukemia


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