# Granzyme B-H22(scFv), a human immunotoxin targeting CD64 in acute myeloid leukemia of monocytic subtypes

**Bettina Stahnke,[4] Theo Thepen,[4] Michael Stöcker,[2] Reinhard Rosinke,[4] Edgar Jost,[3] Rainer Fischer,[2,4] Mehmet K. Tur,[1] and Stefan Barth[1,4]**

[1]Helmholtz-Institute for Biomedical Engineering, Department of Experimental Medicine and Immunotherapy, [2]Institute for Molecular Biotechnology, and [3]University Hospital, Medical Clinic IV, RWTH Aachen University; [4]Fraunhofer IME, Department of Pharmaceutical Product Development, Aachen, Germany

## Abstract

**Acute myeloid leukemia (AML) cells of subtypes M4 and M5 show enhanced expression of CD64 (FcγRI), the high-affinity receptor for IgG, which is normally expressed at high levels only on activated cells of the myeloid lineage. CD64 is therefore a prime target for the specific delivery of cytotoxic agents. A promising toxin candidate is granzyme B, a human serine protease originating from cytotoxic granules of $CD8^+$ T lymphocytes and natural killer cells. After evaluating the sensitivity of the AML-related cell line U937 toward cytosolic granzyme B, we genetically fused granzyme B to H22, a humanized single-chain antibody fragment (scFv) specific for CD64, to obtain Gb-H22(scFv), a fusion protein lacking the immunogenic properties of nonhuman immunofusions. Gb-H22(scFv) was successfully expressed in human 293T cells, secreted, and purified from cell culture supernatants. The purified protein bound specifically to $CD64^+$ U937 cells. Despite linkage to the binding domain, the proteolytic activity of functional Gb-H22(scFv) was identical to that of free granzyme B. Target cell-specific cytotoxicity was observed with a half-maximal inhibitory concentration ($IC_{50}$) between 1.7 and 17 nmol/L. In addition, the induction of apoptosis in U937 cells was confirmed by Annexin A5 staining and the detection of activated caspase-3 in the cytosol. Finally, apoptosis was observed in primary $CD64^+$ AML cells, whereas $CD64^-$ AML cells were unaffected. This is the first report of a completely human granzyme B-based immunotoxin directed against CD64, with activity against an AML-related cell line and primary AML cells. [Mol Cancer Ther 2008;7(9):2924–32]**

## Introduction

Acute myeloid leukemia (AML) is the most common form of acute leukemia in adults and is estimated to have affected 13,410 people and accounts for 8,990 deaths in 2007 in the United States (1). It is a heterogeneous group of malignancies having in common the overproliferation of hematopoietic stem cells and their failure to differentiate, leading to uncontrolled accumulation of myeloblasts in bone marrow and blood (2). Current treatment strategies for AML generally comprise two phases. First, induction chemotherapy eradicates all hematopoietic precursor cells from the bone marrow, allowing repopulation of the blood with normal cells. After remission, a second therapeutic phase is applied to remove residual cells that are likely to account for a relapse. Current approved postremission therapeutic strategies include intensive consolidation chemotherapy, high-dose chemotherapy with or without radiation therapy, low-dose maintenance therapy, and autologous or allogenic hematopoietic stem cell transplantation (3, 4). For both induction and postremission therapy, the risks and benefits of the therapeutic approach have to be evaluated and stratified according to the patient's age and general condition, disease progression, cytogenetic aberrations of leukemic cells, and expression of proteins that confer multidrug resistance or that are pivotal to the regulation of cell proliferation and survival (3, 4).

In contrast to the therapies described above, which are directed at hematopoietic cells in general and thus can cause severe side effects, the specific targeting and elimination of malignant cells only is to be preferred. This can be achieved using immunoconjugates based on antibodies or natural ligands that are directed to cell-specific surface receptors. Indeed, AML cells have been successfully targeted via the receptors for interleukin-3 (5) and granulocyte-macrophage colony-stimulating factor (6, 7) as well as via CD33 (8). One of these immunoconjugates is gemtuzumab ozogamicin (Mylotarg), an anti-CD33 antibody chemically coupled to the organic bacterial drug calicheamicin, which was approved by the Food and Drug Administration in 2000 (8). However, severe hematologic and hepatotoxic side effects caused at least in part by insufficient targeting have limited its therapeutic application (9, 10). Similar issues have been reported for

Received 2/22/08; revised 6/13/08; accepted 6/22/08.

**Grant support:** German Cancer Aid Association grant 106521 and MAVO-IMHOTEP of the Fraunhofer-Gesellschaft (S. Barth).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

**Requests for reprints:** Stefan Barth, Fraunhofer IME, Department of Pharmaceutical Product Development, Forckenbeckstr. 6, 52074 Aachen, Germany. E-mail: stefan.barth@ime.fraunhofer.de

Copyright © 2008 American Association for Cancer Research.

doi:10.1158/1535-7163.MCT-08-0554

Downloaded from http://aacrjournals.org/mct/article-pdf/7/9/2924/1881828/2924.pdf by guest on 28 September 2022

DT388-GM-CSF (11). We therefore decided to target AML cells via another cell surface receptor, CD64, whose expression is enhanced on AML subtypes M4 and M5 but not restricted to these subtypes (12).

The 72-kDa glycoprotein CD64 (FcγRI) is the only IgG receptor that binds monomeric IgG with high affinity (13). It is expressed on cells of the myeloid lineage (monocytes, macrophages, and dendritic cells). These cells can be activated by several cytokines that up-regulate CD64, including IFN-γ, which plays a crucial role in inflammatory responses and causes a 5- to 10-fold increase of CD64 expression as well as enhanced receptor activity (14). CD64 mediates endocytosis/phagocytosis, antibody-dependent cellular cytotoxicity, and production of cytokines and superoxide (15). It also influences the severity of various inflammatory diseases such as rheumatoid arthritis (16), allergic asthma (17), and atopic dermatitis (18).

Previous studies focusing on the targeting of dysregulated $CD64^+$ cells in cutaneous inflammation and arthritis have shown that anti-CD64 immunotoxin treatment successfully eliminated $CD64^+$ activated macrophages but left resting macrophages with low CD64 expression unaffected (19, 20). The key element here is not the degree of CD64 expression but rather the actual state of activation of these cells. Therefore, CD64 might also be an effective target in the therapy of AML. Indeed, anti-CD64 immunofusions with Ricin A as well as with *Pseudomonas* exotoxin A (ETA′) proved to be potent triggers of apoptosis in $CD64^+$ AML cells (21, 22). However, protein domains of nonhuman origin can provoke undesirable immune responses. To avoid this, we decided to use human granzyme B as the toxic moiety.

The serine protease granzyme B is an essential component of the granules released by cytotoxic T cells and natural killer cells (23, 24). The cosecreted protein perforin delivers granzyme B to the cytosol of target cells (25), where its proteolytic activity induces apoptosis. Known substrates of granzyme B include several caspase zymogens, among which procaspase-3 was described to be processed as the first and most important substrate (26). Granzyme B also cleaves several caspase substrates as well as caspase-independent proteins such as Bid, providing multiple routes to initiate apoptosis (26). For granzyme B activity, a free $NH_2$ terminus was described as crucial (27).

To study the cytotoxicity of an anti-CD64-granzyme B immunoconjugate, we chose the AML-related lymphoma cell line U937, a model cell line of the myeloid lineage that can be activated with IFN-γ resulting in enhanced CD64 expression. After first evaluating the sensitivity of U937 cells toward human granzyme B by expressing the enzyme in the cytosol (28), we genetically fused human granzyme B to H22, a humanized CD64-specific scFv that has been used in previous studies (29, 30). An enterokinase cleavage site directly preceding granzyme B allows the $NH_2$ terminus of the enzyme to be exposed. Here, we describe the successful expression of EGb-H22(scFv) in human 293T cells, its purification, its activation by enterokinase cleavage to obtain Gb-H22(scFv) featuring a free $NH_2$ terminus of granzyme B, and its functional characterization in terms of binding, proteolytic, and cytotoxic properties.

## Materials and Methods

### Construction of Plasmids

*Escherichia coli* strain XL1-Blue (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*$^q$Z$\Delta$*M15* Tn*10* (Tet$^r$)]) (Stratagene) was used for all cloning procedures. Primers were supplied by MWG Biotech. Restriction and ligation enzymes were delivered by New England Biolabs. Plasmids or plasmid fragments were prepared and purified using the QIAprep Spin Miniprep Kit and QIAquick Gel Extraction Kit (Qiagen). Cloning was done according to standard techniques (31).

The sequence encoding mature granzyme B was amplified from human blood-derived RNA and cloned into a pMS vector (32, 33) to obtain pMS-LKGb-II, a plasmid encoding Ki4(scFv)-granzyme B, as described elsewhere (32).

To obtain a plasmid encoding active granzyme B for cytosolic expression, the sequence of mature granzyme B was amplified from pMS-LKGb-II (32) using the primers Gbfor (5′-attaca**gctagc**caccatg-ATCATCGGGGGACATGAGG-3′) and Gb+*Blp*I+*Eco*RIrev (5′-attaca**gaattc**tcagt**gctcagc**GTAGCGTTTC-ATGGTTTTCTTTATC-3′), introducing restriction sites for *Nhe*I, *Blp*I, and *Eco*RI (granzyme B sequences in uppercase, additional sequences in lowercase, restriction sites in bold). The PCR product was inserted into the backbone of pMS-LKGb-II via *Nhe*I and *Eco*RI to yield pMS-Gb (Fig. 1A). A control pMS vector encoding H22(scFv) was constructed in a similar manner using pMT-H22-ETA′ as the template (21) but lacking a *Blp*I restriction site [H22+*Nhe*Ifor (5′-attaca**gctagc**caccatgGCCCAGGTGCAG-CTGGTGG-3′) and H22+*Eco*RIrev (5′-attaca**gaattc**tcaTTT-TGATCTCCAGCTTG-3′)].

The pMS vector encoding the Gb-H22(scFv) fusion protein was constructed by amplifying granzyme B from pMS-LKGb-II (32) with primers adding an enterokinase cleavage site (shown in italic) to the $NH_2$ terminus of granzyme B [*Xba*I-Entero-Gb (5′-gc**tctaga**catatc*gacgac-gacgacaag*ATCATCGGGGGACATGAGG-3′) and His6 (5′-ttaaactcaATGATGATGATGATGATG-3′)]. The PCR product was inserted into the backbone of pMS-NAngK-III/G (33) using *Xba*I and *Blp*I. The Igκ leader sequence (isolated from pMS-LKGb-II) was inserted following *Nhe*I/*Xba*I digestion. The resulting plasmid pMS-LEGbK-IV was digested with *Sfi*I/*Not*I to remove the binding domain Ki4(scFv) and replace it with H22(scFv), which was obtained from pMT-H22-ETA′ (21). The final plasmid was named pMS-LEGbH22-IV (Fig. 2A).

### Cell Lines and Primary Cells

293T cells (CRL-11268; American Type Culture Collection) as well as $CD64^+$ cell line U937 (ACC5; German Collection of Microorganisms and Cell Cultures) and $CD64^-$ cell line Raji (ACC319; German Collection of

Downloaded from http://aacrjournals.org/mct/article-pdf/7/9/2924/1881828/2924.pdf by guest on 28 September 2022

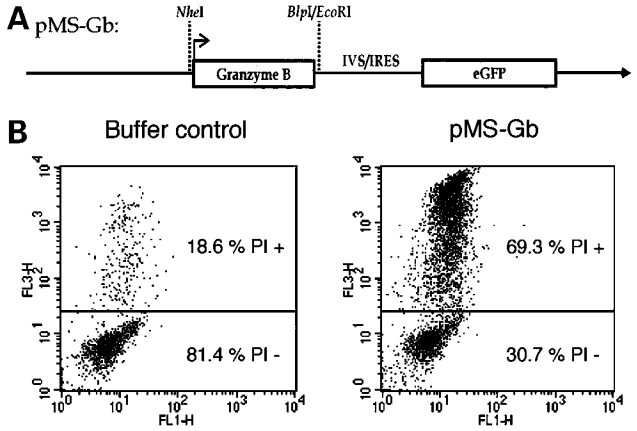


**Figure 1.** Sensitivity of U937 cells toward granzyme B. **A,** schematic structure of pMS-Gb, a vector lacking a leader sequence to allow cytosolic expression of granzyme B. IVS/IRES, synthetic intron/internal ribosome entry site. **B,** flow cytometry of PI-stained U937 cells 24 h after transfection. *Left,* cells transfected without DNA; *right,* cells transfected with pMS-Gb.

Microorganisms and Cell Cultures) were cultured in RPMI complex medium [RPMI 1640 plus Gluta-MAX-I (Life Technologies/Invitrogen) supplemented with 10% (v/v) FCS, 100 units/mL penicillin, and 100 μg/mL streptomycin] and passaged to $1 \times 10^5$/mL twice a week (U937 and Raji) or 1:20 once a week (293T).

Primary cells from a $CD64^+$ AML patient [~80% blasts, AML subtype M5 developed secondarily from a myelodysplatic syndrome, cytogenetic aberrations: add(7)(q36)] and a $CD64^-$ AML patient [~80% blasts, AML subtype M1, cytogenetic aberrations: t(1;3)(p36;q21)] were obtained before treatment and after informed consent and with the approval of the Clinical Research Ethics Board of the University of Aachen. Mononuclear cells were isolated by density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare) and cultured with RPMI complex medium.

For activation, U937 cells as well as $CD64^+$ AML cells (and for control purposes also $CD64^-$ AML cells) were stimulated 1 day before use with 500 units/mL IFN-γ (Sigma). U937 cells were passaged to a cell density of $1 \times 10^5$/mL before stimulation.

All cells were cultured at 37°C in a 100% humidified 5% $CO_2$ atmosphere.

### Nucleofection

U937 cells were transfected using the Cell Line Nucleofector Kit V (Amaxa) according to the manufacturer's instructions. Briefly, cells were centrifuged at 90 × *g* for 10 min and resuspended in Cell Line Nucleofector Solution V to a final concentration of $1 \times 10^6$ per 100 μL. Then, 100 μL cell suspension was combined with 1 μg plasmid DNA, transferred to the cuvette, and nucleofected using the program V-01. After nucleofection, cells were seeded out in 12-well plates containing prewarmed RPMI complex medium. Following incubation at 37°C, 100% humidity, and 5% $CO_2$, cells were analyzed by propidium iodide (PI) staining (c = 5 μg/mL) and flow cytometry.

### Expression, Purification, and Activation of Gb-H22(scFv)

293T cells were transfected using 1 μg plasmid DNA and 3 μL Roti-Fect (Roth), which were added to 300 μL serum-free RPMI 1640 plus GlutaMAX-I (Life Technologies/Invitrogen) and incubated for 20 min at room temperature before the transfection mixture was added to the cells. After 1 h incubation at 37°C, 100% humidity, and 5% $CO_2$, 1 mL RPMI complex medium was added followed by incubation for 2 to 3 days before supernatants were checked for expression of the target protein. Subcloning was carried out in RPMI complex medium containing 100 μg/mL zeocin (Invitrogen). Further cultivation was carried out either in RPMI complex medium or in serum-free HL-1 medium (BioWhittaker/Cambrex/Lonza) supplemented 1:100 with GlutaMAX (Invitrogen). Both media contained 100 μg/mL zeocin for selection purposes.

Gb-H22(scFv) was enriched from cell culture supernatants by IMAC/FPLC. After centrifugation, cleared supernatants were blended with 4× incubation buffer (pH 7.4) as described previously (33), filter sterilized, and applied to an XK16 column (Amersham/GE Healthcare) containing 5 mL Talon Superflow resin (Clontech/Takara) pre-equilibrated with 1× incubation buffer (pH 7.4). Washing and elution were also done with the buffers described by Stocker et al. (33) modified to pH 7.4. Eluted protein was rebuffered in PBS containing 20 mmol/L Tris using a HiPrep 26/10 desalting column (Amersham/GE Healthcare) and concentrated by centrifugation at 3,800 × *g* with a Vivaspin 6 ultrafiltration spin column 5 kDa MWCO (Sartorius). Activation of the protein was achieved by overnight incubation with recombinant enterokinase (Novagen/Merck) according to the manufacturer's instructions. Protein extracted from serum-containing supernatants was further purified via cation exchange chromatography using a HiTrap SP HP 1 ml column (Amersham/GE Healthcare). Washing was carried out with PBS containing 300 mmol/L NaCl, and the protein was eluted in PBS containing 500 mmol/L NaCl. Desalting and concentration were repeated as above and the protein was filter sterilized and stored in aliquots at -80°C. The protein concentration was determined with Roti-Nanoquant (Roth). Purity was calculated after SDS-PAGE analysis and Coomassie staining using AIDA Image Analyzer Software (Raytest Isotopenmessgeräte).

### SDS-PAGE and Western Blot

Protein samples were analyzed by SDS-PAGE using a 12% SDS gel and reducing conditions followed by Coomassie staining or Western blotting according to standard techniques (31). The protein was detected with rabbit polyclonal anti-enterokinase cleavage site antibody (Novus Biologicals) and goat anti-rabbit IgG Fc alkaline phosphatase conjugate (Dianova). Blots were developed using NBT/BCIP [3.33% (w/v) NBT, 1.67% (w/v) BCIP in dimethylformamide], all supplied by Roth, added to the 100× volume of alkaline phosphatase buffer [100 mmol/L Tris, 100 mmol/L NaCl, 5 mmol/L $MgCl_2$ (pH 9.6)].

Downloaded from http://aacrjournals.org/mct/article-pdf/7/9/2924/1881828/2924.pdf by guest on 28 September 2022

### Granzyme B Activity Assays

The proteolytic activity of the granzyme B fusion protein was compared with that of free granzyme B (Calbiochem/Merck) by cleavage of Ac-IETD-*p*NA (granzyme B Substrate I, colorimetric; Calbiochem/Merck), a synthetic substrate mimicking the cleavage site of procaspase-3. The protein solution and assay buffer [100 mmol/L NaCl, 50 mmol/L HEPES, 10 mmol/L DTT, 1 mmol/L EDTA, 10% (v/v) glycerol, 0.1% (w/v) CHAPS (pH 7.4)] were combined to a volume of 100 μL/well in a 96-well plate. Samples were pipetted in triplicate. The reaction was started by adding Ac-IETD-*p*NA to a final concentration of 200 μmol/L. Absorbance at 405 nm was monitored in a microplate reader at 5 min intervals for 1 h.

### Binding Analyses/Flow Cytometry

To evaluate the cell binding activity of Gb-H22(scFv) by flow cytometry, $2 \times 10^5$ to $4 \times 10^5$ cells were washed with PBS in a cell washer (Dade Serocent) and incubated for 30 min on ice with 1 μg Gb-H22(scFv) in 50 μL PBS. After another washing step, cells were treated with anti-Gb-PE (ImmunoTools) for 30 min on ice. After removing nonspecifically bound antibodies by washing, cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson).

### Cell Viability Assays

The cytotoxic effect of Gb-H22(scFv) was monitored by bioreduction of MTS to a colored formazan product by reduction equivalents generated by dehydrogenase enzymes of metabolically active cells. The quantity of formazan product as measured by absorbance at 490 nm is directly proportional to the number of living cells. 1:5 dilutions of Gb-H22(scFv) samples in RPMI complex medium were pipetted into a 96-well plate in triplicates. Wells containing zeocin (Invitrogen; final concentration 2 mg/mL) or PBS were used as positive and negative controls, respectively; 5,000 cells were seeded out to each well. Following an incubation period of 72 h at 37°C, 100% humidity, and 5% $CO_2$, 20 μL Cell Titer $AQ_{ueous}$ One Solution (Promega) was added per well. Measurement was carried out after 4 h at

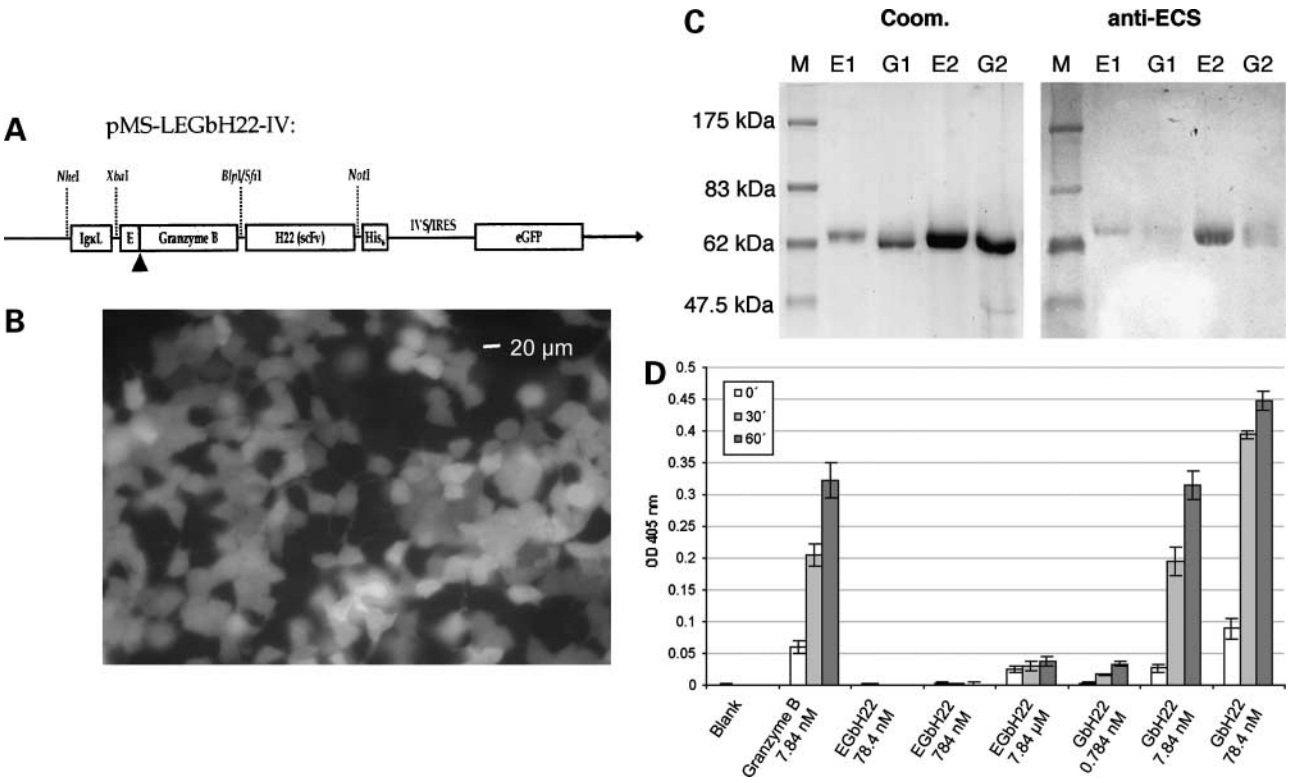


**Figure 2.** Expression of Gb-H22(scFv) in 293T cells. **A,** schematic structure of pMS-LEGbH22-IV. The Igκ leader sequence (*Igκl*) preceding granzyme B facilitates secretion of the protein into the cell culture supernatant. An enterokinase cleavage site (*E*) allows the generation of a free $NH_2$ terminus of granzyme B. Granzyme B was linked to the $NH_2$ terminus of H22(scFv). A COOH-terminal 6×-histidine tag (His) facilitates purification via IMAC. A synthetic intron sequence and an internal ribosome entry site followed by the gene coding *egfp* allow for coexpression of EGFP via a bicistronic mRNA and thus permit the detection of transfected 293T cells under a fluorescence microscope (**B**). **C,** SDS-PAGE and Western blot of the purified fusion proteins. Enterokinase cleavage was detected by an antibody targeting the enterokinase cleavage site. *M,* prestained protein marker (New England Biolabs); *E1/G1,* EGb-H22(scFv) and Gb-H22(scFv) purified from serum-containing cell culture supernatants; *E2/G2,* EGb-H22(scFv) and Gb-H22(scFv) purified from serum-free cell culture supernatants. **D,** cleavage of the synthetic granzyme B substrate Ac-IETD-*p*NA by EGb-H22(scFv), Gb-H22(scFv), or free granzyme B during a 60 min incubation. The release of *p*NA was monitored every 5 min by absorbance at 405 nm. Data show the results after 0, 30, and 60 min. Measurements were obtained in triplicate. *Bars,* SD.

Downloaded from http://aacrjournals.org/mct/article-pdf/7/9/2924/1881828/2924.pdf by guest on 28 September 2022

490 nm against a reference wavelength of 630 nm using a microplate reader. Gb-H22(scFv) concentration-dependent viability of cells in relation to PBS and zeocin controls was calculated with GraphPad Prism software and displayed by a nonlinear regression sigmoidal dose-response curve with variable slope.

### Activation of Endogenous Procaspase-3

The generation of active caspase-3 by cleavage of the procaspase-3 zymogen by granzyme B was monitored using Human Active Caspase-3 CBA (BD Biosciences). Briefly, $5 \times 10^5$ cells in 1 mL RPMI complex medium were incubated with 150 nmol/L Gb-H22(scFv) for 24 h at 37°C, 100% humidity, and 5% $CO_2$. After two washes with PBS, the cells were incubated on ice in 250 μL lysis buffer for 30 min while vortexing every 10 min. Cellular debris was pelleted by centrifugation. Clear cell lysate (50 μL) was added to 50 μL human caspase-3 capture beads and 50 μL human active caspase-3 PE detection reagent and incubated for 4 h at room temperature in the dark. After another washing step, the bead pellet was resuspended in 300 μL wash buffer and analyzed by flow cytometry.

### Apoptosis Assays

Apoptosis in target cells was documented by Annexin A5/PI staining. Cells ($5 \times 10^5$) were seeded out per well of a 12-well plate and incubated with 150 to 240 nmol/L Gb-H22(scFv) at 37°C, 100% humidity, and 5% $CO_2$. Aliquots of cells were taken at different time points and washed with PBS in the cell washer (Dade Serocent). The cell pellet was resuspended in 450 μL cell-free culture supernatants from 293T cells transfected with a pMS vector encoding Annexin A5-enhanced green fluorescent protein (EGFP; ref. 34), freshly supplemented with 5 μg/mL PI, and 50 μL of 10× Annexin A5 binding buffer (100 mmol/L HEPES, 1.5 mol/L NaCl, 50 mmol/L KCl, and 20 mmol/L $CaCl_2$), incubated at 4°C for 10 to 15 min, and analyzed by flow cytometry.

## Results

### Pretesting of the CD64$^+$ Cell Line U937 to Determine Sensitivity to Granzyme B-Induced Apoptosis

To determine whether granzyme B could induce apoptosis in U937 cells, we constructed a eukaryotic plasmid designed for cytosolic granzyme B expression (Fig. 1A). Following nucleofection of U937 cells and an incubation period of 24 h, PI staining and fluorescence-activated cell sorting analysis revealed 69% mortality in granzyme B-transfected cells, whereas negative control cells transfected without DNA showed only 19% mortality (Fig. 1B). Control plasmids encoding H22(scFv) or GFP (pMAXGFP; Amaxa) each caused 28% mortality (data not shown).

### Construction and Expression of Gb-H22(scFv)

Having shown that granzyme B could kill U937 cells, we genetically fused granzyme B to H22(scFv) and introduced an enterokinase cleavage site preceding the granzyme B $NH_2$ terminus using the eukaryotic pMS vector system (ref. 33; Fig. 2A). To obtain correct post-translational modification, EGb-H22(scFv) was expressed in human 293T cells and successfully secreted in both serum-containing and serum-free medium. DNA transfer and ongoing expression was confirmed by monitoring the green fluorescence resulting from the coexpression of cytosolic EGFP via a bicistronic mRNA (Fig. 2B). Purification from cell culture supernatants was carried out solely by $Co^{2+}$-based IMAC in the case of serum-free medium or using a combination of $Co^{2+}$-based IMAC and cation exchange

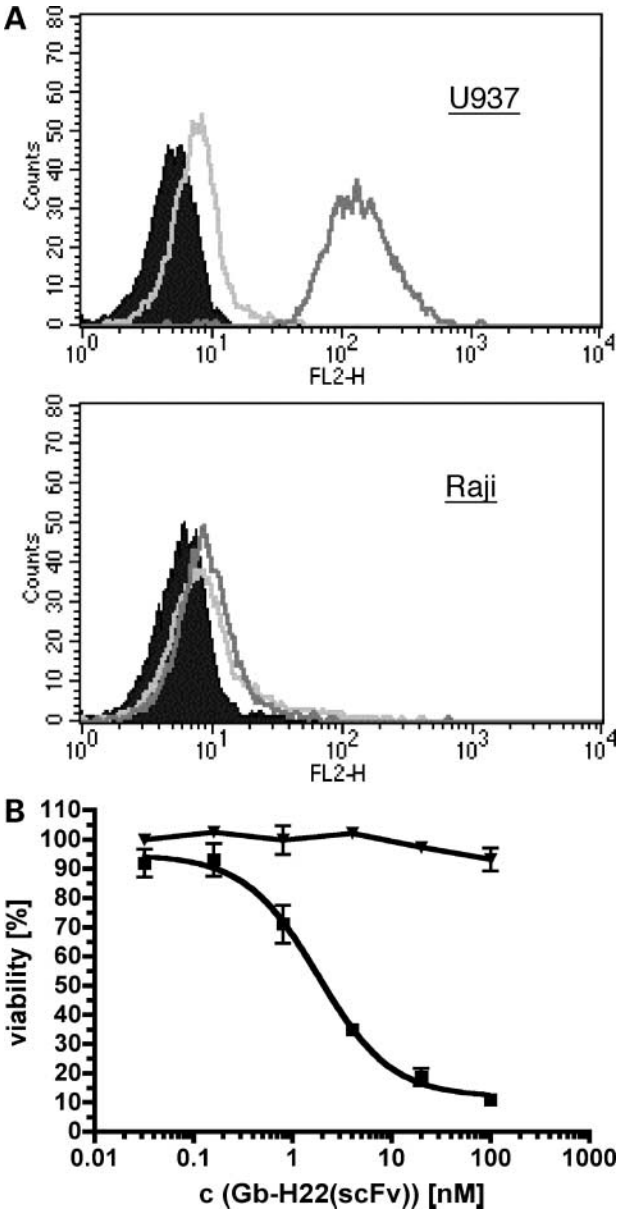


**Figure 3.** Analysis of binding and cytotoxicity of Gb-H22(scFv) toward CD64$^+$ U937 and CD64$^-$ Raji cells. **A,** fluorescence-activated cell sorting analysis. *Filled black curve,* untreated cells; *light gray curve,* background control (cells + aGb-PE); *medium gray curve,* protein sample [cells + Gb-H22(scFv) + aGb-PE]. **B,** cytotoxic activity of GbH22(scFv). U937 cells (*squares*) and Raji cells (*triangles*) were incubated with increasing concentrations of Gb-H22(scFv) for 72 h in a humidified 37°C, 5% $CO_2$ atmosphere. Cell viability in relation to a 100% PBS control and a 0% zeocin control was determined with a colorimetric MTS-based cell viability assay. Samples were measured in triplicate wells. *Bars,* SD.

Downloaded from http://aacrjournals.org/mct/article-pdf/7/9/2924/1881828/2924.pdf by guest on 28 September 2022

chromatography in the case of serum-containing medium. In both cases, activation of granzyme B by enterokinase cleavage to obtain Gb-H22(scFv) was carried out after IMAC. SDS-PAGE analysis and Western blotting indicated the high purity of the purified protein and confirmed successful enterokinase cleavage (Fig. 2C).

### Proteolytic Activity of EGb-H22(scFv)

The synthetic granzyme B substrate Ac-IETD-*p*NA, which mimics the cleavage and activation site of human procaspase-3, was used to determine the proteolytic activity of enterokinase-cleaved Gb-H22(scFv) compared with the uncleaved EGb-H22(scFv). Equimolar amounts of Gb-H22(scFv) and commercially available granzyme B showed the same proteolytic activity, whereas the uncleaved EGb-H22(scFv) showed no reaction even when applied in 1,000-fold molar excess (Fig. 2D). Therefore, Gb-H22(scFv) was chosen for the subsequent assays.

### Binding Properties of Gb-H22(scFv)

Binding of Gb-H22(scFv) to the human $CD64^+$ cell line U937 confirmed the anticipated functionality of the single-chain antibody. In contrast, no binding was observed to $CD64^-$ Raji cells, showing the specificity of the interaction (Fig. 3A).

### *In vitro* Cytotoxic Activity

The concentration-dependent cytotoxic effect of Gb-H22(scFv) was evaluated in a MTS-based colorimetric cell proliferation assay using $CD64^+$ U937 and $CD64^-$ Raji cells. After a 3-day incubation period with Gb-H22(scFv), the viability of U937 cells was dramatically reduced in a concentration-dependent manner, whereas Raji cells remained unaffected (Fig. 3B). $IC_{50}$ values were between 1.7 and 17 nmol/L ($n$ = 9 samples in 6 experiments). Unconjugated granzyme B showed no toxicity (data not shown).

### Activation of Caspase-3

The potential of Gb-H22(scFv) to initiate the proteolytic cascade culminating in apoptosis in U937 cells was investigated via the detection of active caspase-3 in the cytosol. Cell lysates were incubated with caspase-3 capture beads. Bound caspase-3 was detected with a PE-labeled antibody specific for active caspase-3 but not procaspase-3. Significant activation of caspase-3 was observed in lysates of the cells treated with Gb-H22(scFv) compared with the untreated control (Fig. 4), indicating that the induction of apoptosis by Gb-H22(scFv) was mediated by caspase-3.

### Induction of Apoptosis in U937 Cells

Apoptosis was detected in U937 cells incubated for 72 h with 150 nmol/L Gb-H22(scFv) by Annexin A5/PI staining. Whereas Raji cells remained viable during the whole incubation period, the number of viable U937 cells decreased to 3% in Gb-H22(scFv)-treated samples compared with 79% in the PBS control. Among the cells treated with Gb-H22(scFv), 18% were early apoptotic (Annexin A5+/PI-), whereas 79% were late apoptotic or dead (Annexin A5+/PI+; Fig. 5).

### Induction of Apoptosis in Primary $CD64^+$ AML Cells

Primary $CD64^+$ and $CD64^-$ AML cells were incubated for 48 h with 238 nmol/L Gb-H22(scFv). Again, apoptosis was detected via Annexin A5/PI staining. Gb-H22(scFv) induced apoptosis in $CD64^+$ AML cells as indicated by reduction of the viable population (Annexin A5-/PI-; *bottom left quadrant*) combined with an increase in the populations of early apoptotic (Annexin A5+/PI-; *bottom right quadrant*) and late apoptotic/dead cells (Annexin A5+/PI+; *top right quadrant*; Fig. 6). Cells from a patient with $CD64^-$ AML were left unaffected by Gb-H22(scFv).

## Discussion

CD64 (FcγRI), the high-affinity receptor for IgG, is expressed in several types of AML cells even without monocytic differentiation (12, 35). However, receptor numbers are highest in AML subtypes M4 and M5 of the older French-American-British classification, which is part of the revised classification of the WHO.

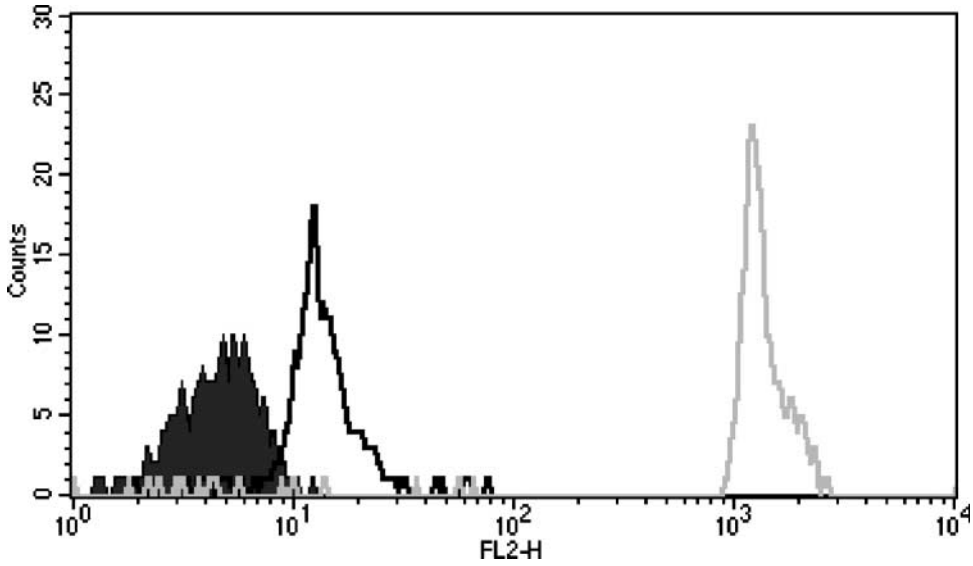


**Figure 4.** Caspase-3 activation. After 24 h incubation of U937 cells with Gb-H22(scFv), cytosolic cleavage of procaspase-3 was monitored by incubation of cell lysates with active caspase-3 capture beads and detection with a PE-labeled anti-active caspase-3 antibody. *Black filled curve,* beads lacking cell lysate; *black open curve,* beads incubated with lysate from PBS-treated cells; *gray curve,* beads incubated with lysate from Gb-H22(scFv)-treated cells.



Downloaded from http://aacrjournals.org/mct/article-pdf/7/9/2924/1881828/2924.pdf by guest on 28 September 2022

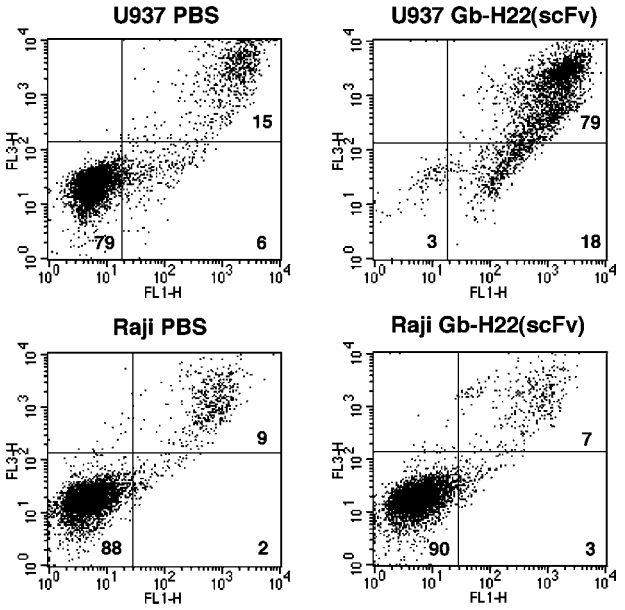


**Figure 5.** Induction of apoptosis in CD64$^+$ U937 cells. U937 and Raji cells were incubated with 150 nmol/L Gb-H22(scFv) for 72 h in a humidified 37°C, 5% $CO_2$ atmosphere. Apoptosis was detected by Annexin A5-EGFP/PI staining. *Bottom left,* viable cells; *bottom right,* early apoptosis; *top right,* late apoptosis/dead cells. *Numbers in the quadrants,* percentage of cells of each category.

A main function of CD64 is internalization, which makes it a useful target for immunotoxins. In case of the immunoconjugates targeting CD33 (8), which is a cell adhesion molecule, or receptors such as interleukin-3 receptor (5) and granulocyte-macrophage colony-stimulating factor receptor (6, 7), which are signal transduction receptors, presumably only a small portion of the administered immunotoxin becomes internalized, whereas CD64 internalizes very efficiently (36). Furthermore, the expression pattern of the receptor is limited, and normal, nonactivated CD64-expressing cells are not affected by immunoconjugates (19, 22, 37). Therefore, CD64 can be considered as beneficial for the targeted delivery of cytotoxic or immunomodulating drugs.

The murine CD64-specific monoclonal antibody M22 (38) was humanized to obtain H22 (30) and converted to a single-chain format (29). H22 and its derivatives have been used in a variety of applications including as fusion proteins with antibodies (39–41) and toxic components such as calicheamicin (42), *Pseudomonas* ETA′ (21) and Ricin A (19, 20, 22, 37). Both Ricin A and ETA′ fusions to H22 have been used as therapeutic agents against AML cells (21, 22). Although both fusion proteins proved to be effective, the nonhuman fusion partners could prevent repetitive administration due to the likelihood of an undesired immune response. To avoid such immunogenicity, we chose human granzyme B as the toxic component of our H22-based immunotoxin. Granzyme B is an apoptosis-inducing serine protease naturally expressed and secreted by cytotoxic T lymphocytes and natural killer cells (23, 24).

First, we tested the sensitivity of the AML-related cell line U937 toward granzyme B by transfection with a plasmid allowing cytosolic expression of granzyme B. These experiments showed that granzyme B killed the cells efficiently, whereas control constructs and transfection procedures had no significant effect. We then genetically fused an enterokinase cleavage site to the $NH_2$ terminus of granzyme B and H22(scFv) to its COOH terminus and expressed the construct in human 293T cells. After IMAC enrichment, overnight incubation with or without enterokinase, and final purification by cation exchange chromatography, cleaved Gb-H22(scFv) was compared with uncleaved EGb-H22(scFv) in a proteolysis assay with a synthetic granzyme B substrate. Only the activated Gb-H22(scFv) showed the same proteolytic activity as free granzyme B, whereas the uncleaved EGb-H22(scFv) failed to cleave the synthetic substrate (Fig. 2D). We therefore decided to focus further experiments on Gb-H22(scFv).

Gb-H22(scFv) bound to CD64$^+$ U937 cells, but not CD64$^-$ Raji cells, and exhibited potent cytotoxicity toward U937 cells alone. $IC_{50}$ values ranging from 1.7 to 17 nmol/L were observed. This is in accordance with the published $IC_{50}$ values of other granzyme B-based immunotoxins (43–46). However, toxicity was also observed with uncleaved EGb-H22(scFv), albeit with slightly higher $IC_{50}$ values (data not shown). This points to the cytosolic modes of action of granzyme B apart from procaspase-3 cleavage (cleavage of other caspases, downstream caspase substrates such as PARP, DNA-PK, ICAD, NuMa, and lamin B, and

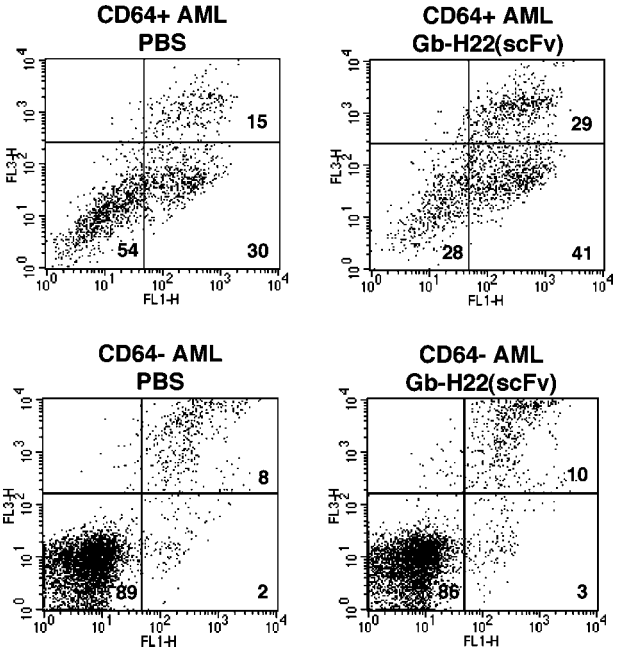


**Figure 6.** Detection of apoptosis in primary CD64$^+$ and CD64$^-$ AML cells. Cells were incubated with 238 nmol/L Gb-H22(scFv) for 48 h in a humidified 37°C, 5% $CO_2$ atmosphere. Apoptosis was detected by Annexin A5-EGFP/PI staining. *Bottom left,* viable cells; *bottom right,* early apoptosis; *top right,* late apoptosis/dead cells. *Numbers in the quadrants,* percentage of cells of each category.

Downloaded from http://aacrjournals.org/mct/article-pdf/7/9/2924/1881828/2924.pdf by guest on 28 September 2022

Bid and other caspase-independent substrates; ref. 47). This implies that granzyme B has additional benefits as a toxic component, because the development of avoidance strategies by tumor cells is therefore much more difficult than with toxins acting by a single apoptosis-inducing mechanism.

Additionally, based on the work of Zhong et al. (22), who worked with H22 coupled to Ricin A, we could show that Gb-H22(scFv) induced apoptosis in primary $CD64^+$ AML cells, whereas $CD64^-$ AML cells were unaffected. However, the susceptibility of cancer cells toward a granzyme B-based immunotoxin is likely to vary between patients and cell types. Therefore, with an aim to develop individualized therapies, the cytosolic expression approach could provide a rapid prescreening method to evaluate the sensitivity of malignant cells from particular patients toward granzyme B *ex vivo*.

A known bottleneck in the application of all immunotoxins is the translocation of the immunotoxin to the cytosol. In its natural function, granzyme B is secreted from cytotoxic T cells and natural killer cells along with perforin, which serves as endosomolytic agent to release granzyme B to the cytosol of target cells (48). Nevertheless, cytosolic translocation has been achieved for granzyme B immunotoxins without coadministration of any endosomolytic agent as shown herein and in previous reports (28, 44–46). However, Dalken et al. (43) worked with granzyme B immunotoxins targeting EGFR and ErbB2, respectively, and showed that granzyme B-induced cell death could be achieved within 14 h if chloroquine was coadministered. We also tested chloroquine but found that cell death occurred in the presence of this reagent alone, even at concentrations fivefold lower than previously used (ref. 43; data not shown). Whether an increase in cytotoxic efficacy of our constructs can be accomplished with other endosomolytic agents or adapter sequences mediating translocation (49) is currently being investigated in our group. Animal studies with disseminated tumor models similar to those carried out by Barth et al. (50) will further elucidate the applicability of Gb-H22(scFv) *in vivo*.

In the clinic, Gb-H22(scFv) could be useful for both induction and postremission therapy. When used in combination therapy with conventional chemotherapeutics, it might be possible to reduce the dose of the chemotherapeutic or its frequency of administration, thus limiting side effects and promoting long-term remission. Furthermore, the treatment of inflammatory diseases that are characterized by enhanced CD64 expression on dysregulated macrophages (16–18) lies within the range of possible applications.

In summary, we were able to produce recombinant Gb-H22(scFv), the first granzyme B-based human immunotoxin targeting CD64, in human 293T cells. Following purification and activation of the protein, we showed specific binding to and elimination of $CD64^+$ U937 cells as well as patient-derived $CD64^+$ AML cells *in vitro*.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

### Acknowledgments

We thank Dr. Richard M. Twyman for critical reading of the article.

### References

1. American Cancer Society. Cancer facts and figures 2007. Available from: http://www.cancer.org/downloads/STT/CAFF2007PWSecured.pdf; 2007.

2. Stone RM, O'Donnell MR, Sekeres MA. Acute myeloid leukemia. Hematology (Am Soc Hematol Educ Program) 2004;2004:98–117.

3. National Cancer Institute. Adult acute myeloid leukemia (PDQ®): treatment. Health Professional Version. Available from: http://www.cancer.gov/cancertopics/pdq/treatment/adultAML/healthprofessional/allpages; 2007.

4. Tallman MS, Gilliland DG, Rowe JM. Drug therapy for acute myeloid leukemia. Blood 2005;106:1154–63.

5. Feuring-Buske M, Frankel AE, Alexander RL, Gerhard B, Hogge DE. A diphtheria toxin-interleukin 3 fusion protein is cytotoxic to primitive acute myeloid leukemia progenitors but spares normal progenitors. Cancer Res 2002;62:1730–6.

6. Frankel AE, Powell BL, Hall PD, Case LD, Kreitman RJ. Phase I trial of a novel diphtheria toxin/granulocyte macrophage colony-stimulating factor fusion protein (DT388GMCSF) for refractory or relapsed acute myeloid leukemia. Clin Cancer Res 2002;8:1004–13.

7. Perentesis JP, Waddick KG, Bendel AE, et al. Induction of apoptosis in multidrug-resistant and radiation-resistant acute myeloid leukemia cells by a recombinant fusion toxin directed against the human granulocyte macrophage colony-stimulating factor receptor. Clin Cancer Res 1997;3:347–55.

8. Bross PF, Beitz J, Chen G, et al. Approval summary: gemtuzumab ozogamicin in relapsed acute myeloid leukemia. Clin Cancer Res 2001;7:1490–6.

9. Rajvanshi P, Shulman HM, Sievers EL, McDonald GB. Hepatic sinusoidal obstruction after gemtuzumab ozogamicin (Mylotarg) therapy. Blood 2002;99:2310–4.

10. van Der Velden VH, te Marvelde JG, Hoogeveen PG, et al. Targeting of the CD33-calicheamicin immunoconjugate Mylotarg (CMA-676) in acute myeloid leukemia: *in vivo* and *in vitro* saturation and internalization by leukemic and normal myeloid cells. Blood 2001;97:3197–204.

11. Westcott MM, Abi-Habib RJ, Cohen KA, et al. Diphtheria toxin-murine granulocyte-macrophage colony-stimulating factor-induced hepatotoxicity is mediated by Kupffer cells. Mol Cancer Ther 2004;3:1681–9.

12. Ball ED, McDermott J, Griffin JD, Davey FR, Davis R, Bloomfield CD. Expression of the three myeloid cell-associated immunoglobulin G Fc receptors defined by murine monoclonal antibodies on normal bone marrow and acute leukemia cells. Blood 1989;73:1951–6.

13. Anderson CL. Isolation of the receptor for IgG from a human monocyte cell line (U937) and from human peripheral blood monocytes. J Exp Med 1982;156:1794–806.

14. Fanger MW, Shen L, Graziano RF, Guyre PM. Cytotoxicity mediated by human Fc receptors for IgG. Immunol Today 1989;10:92–9.

15. van de Winkel JG, Anderson CL. Biology of human immunoglobulin G Fc receptors. J Leukoc Biol 1991;49:511–24.

16. Wijngaarden S, van Roon JA, Bijlsma JW, van de Winkel JG, Lafeber FP. Fcγ receptor expression levels on monocytes are elevated in rheumatoid arthritis patients with high erythrocyte sedimentation rate who do not use anti-rheumatic drugs. Rheumatology (Oxford) 2003;42:681–8.

17. Viksman MY, Bochner BS, Peebles RS, Schleimer RP, Liu MC. Expression of activation markers on alveolar macrophages in allergic asthmatics after endobronchial or whole-lung allergen challenge. Clin Immunol 2002;104:77–85.

18. Kiekens RC, Thepen T, Bihari IC, Knol EF, Van De Winkel JG, Bruijnzeel-Koomen CA. Expression of Fc receptors for IgG during acute and chronic cutaneous inflammation in atopic dermatitis. Br J Dermatol 2000;142:1106–13.

19. Thepen T, van Vuuren AJ, Kiekens RC, Damen CA, Vooijs WC, van De Winkel JG. Resolution of cutaneous inflammation after local elimination of macrophages. Nat Biotechnol 2000;18:48–51.

Downloaded from http://aacrjournals.org/mct/article-pdf/7/9/2924/1881828/2924.pdf by guest on 28 September 2022

**20.** van Vuuren AJ, van Roon JA, Walraven V, et al. CD64-directed immunotoxin inhibits arthritis in a novel CD64 transgenic rat model. J Immunol 2006;176:5833 – 8.

**21.** Tur MK, Huhn M, Thepen T, et al. Recombinant CD64-specific single chain immunotoxin exhibits specific cytotoxicity against acute myeloid leukemia cells. Cancer Res 2003;63:8414 – 9.

**22.** Zhong RK, van de Winkel JG, Thepen T, Schultz LD, Ball ED. Cytotoxicity of anti-CD64-ricin a chain immunotoxin against human acute myeloid leukemia cells *in vitro* and in SCID mice. J Hematother Stem Cell Res 2001;10:95 – 105.

**23.** Clement MV, Haddad P, Soulie A, et al. Involvement of granzyme B and perforin gene expression in the lytic potential of human natural killer cells. Res Immunol 1990;141:477 – 89.

**24.** Krahenbuhl O, Rey C, Jenne D, et al. Characterization of granzymes A and B isolated from granules of cloned human cytotoxic T lymphocytes. J Immunol 1988;141:3471 – 7.

**25.** Pipkin ME, Lieberman J. Delivering the kiss of death: progress on understanding how perforin works. Curr Opin Immunol 2007;19:301 – 8.

**26.** Metkar SS, Wang B, Ebbs ML, et al. Granzyme B activates procaspase-3 which signals a mitochondrial amplification loop for maximal apoptosis. J Cell Biol 2003;160:875 – 85.

**27.** Edwards KM, Davis JE, Browne KA, Sutton VR, Trapani JA. Anti-viral strategies of cytotoxic T lymphocytes are manifested through a variety of granule-bound pathways of apoptosis induction. Immunol Cell Biol 1999; 77:76 – 89.

**28.** Zhao J, Zhang LH, Jia LT, et al. Secreted antibody/granzyme B fusion protein stimulates selective killing of HER2-overexpressing tumor cells. J Biol Chem 2004;279:21343 – 8.

**29.** de Kruif J, Tijmensen M, Goldsein J, Logtenberg T. Recombinant lipid-tagged antibody fragments as functional cell-surface receptors. Nat Med 2000;6:223 – 7.

**30.** Graziano RF, Tempest PR, White P, et al. Construction and characterization of a humanized anti-γ-Ig receptor type I (FcγRI) monoclonal antibody. J Immunol 1995;155:4996 – 5002.

**31.** Sambrook J, Russell DW. Molecular cloning: a laboratory manual. 3rd ed. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory; 2001.

**32.** Barth S, Engert A, Stöcker M. Apoptotic agents. Patent Application 2001:WO/2001/080880.

**33.** Stocker M, Tur MK, Sasse S, Krussmann A, Barth S, Engert A. Secretion of functional anti-CD30-angiogenin immunotoxins into the supernatant of transfected 293T-cells. Protein Expr Purif 2003;28: 211 – 9.

**34.** Stocker M, Pardo A, Hetzel C, Reutelingsperger C, Fischer R, Barth S. Eukaryotic expression and secretion of EGFP-labeled Annexin A5. Protein Expr Purif 2008;58:325 – 31.

**35.** Krasinskas AM, Wasik MA, Kamoun M, Schretzenmair R, Moore J, Salhany KE. The usefulness of CD64, other monocyte-associated antigens, and CD45 gating in the subclassification of acute myeloid leukemias with monocytic differentiation. Am J Clin Pathol 1998;110: 797 – 805.

**36.** Guyre CA, Barreda ME, Swink SL, Fanger MW. Colocalization of FcγRI-targeted antigen with class I MHC: implications for antigen processing. J Immunol 2001;166:2469 – 78.

**37.** van Roon JA, van Vuuren AJ, Wijngaarden S, et al. Selective elimination of synovial inflammatory macrophages in rheumatoid arthritis by an Fcγ receptor I-directed immunotoxin. Arthritis Rheum 2003;48: 1229 – 38.

**38.** Anderson CL, Guyre PM, Whitin JC, Ryan DH, Looney RJ, Fanger MW. Monoclonal antibodies to Fc receptors for IgG on human mononuclear phagocytes. Antibody characterization and induction of superoxide production in a monocyte cell line. J Biol Chem 1986;261:12856 – 64.

**39.** Borchmann P, Schnell R, Fuss I, et al. Phase 1 trial of the novel bispecific molecule H22xKi-4 in patients with refractory Hodgkin lymphoma. Blood 2002;100:3101 – 7.

**40.** Curnow RT. Clinical experience with CD64-directed immunotherapy. An overview. Cancer Immunol Immunother 1997;45:210 – 5.

**41.** Sundarapandiyan K, Keler T, Behnke D, et al. Bispecific antibody-mediated destruction of Hodgkin's lymphoma cells. J Immunol Methods 2001;248:113 – 23.

**42.** van Roon JA, Bijlsma JW, van de Winkel JG, Lafeber FP. Depletion of synovial macrophages in rheumatoid arthritis by an anti-FcγRI-calicheamicin immunoconjugate. Ann Rheum Dis 2005;64:865 – 70.

**43.** Dalken B, Giesubel U, Knauer SK, Wels WS. Targeted induction of apoptosis by chimeric granzyme B fusion proteins carrying antibody and growth factor domains for cell recognition. Cell Death Differ 2006;13: 576 – 85.

**44.** Kurschus FC, Kleinschmidt M, Fellows E, et al. Killing of target cells by redirected granzyme B in the absence of perforin. FEBS Lett 2004;562: 87 – 92.

**45.** Liu Y, Cheung LH, Hittelman WN, Rosenblum MG. Targeted delivery of human pro-apoptotic enzymes to tumor cells: *in vitro* studies describing a novel class of recombinant highly cytotoxic agents. Mol Cancer Ther 2003;2:1341 – 50.

**46.** Liu Y, Cheung LH, Thorpe P, Rosenblum MG. Mechanistic studies of a novel human fusion toxin composed of vascular endothelial growth factor (VEGF)121 and the serine protease granzyme B: directed apoptotic events in vascular endothelial cells. Mol Cancer Ther 2003;2:949 – 59.

**47.** Bots M, Medema JP. Granzymes at a glance. J Cell Sci 2006;119: 5011 – 4.

**48.** Froelich CJ, Orth K, Turbov J, et al. New paradigm for lymphocyte granule-mediated cytotoxicity. Target cells bind and internalize granzyme B, but an endosomolytic agent is necessary for cytosolic delivery and subsequent apoptosis. J Biol Chem 1996;271:29073 – 9.

**49.** Fuchs H, Bachran C, Li T, Heisler I, Durkop H, Sutherland M. A cleavable molecular adapter reduces side effects and concomitantly enhances efficacy in tumor treatment by targeted toxins in mice. J Control Release 2007;117:342 – 50.

**50.** Barth S, Huhn M, Matthey B, et al. Ki-4(scFv)-ETA′, a new recombinant anti-CD30 immunotoxin with highly specific cytotoxic activity against disseminated Hodgkin tumors in SCID mice. Blood 2000; 95:3909 – 14.

Downloaded from http://aacrjournals.org/mct/article-pdf/7/9/2924/1881828/2924.pdf by guest on 28 September 2022