

# A bispecific diabody directed against prostate-specific membrane antigen and CD3 induces T-cell mediated lysis of prostate cancer cells

P. Bühler · P. Wolf · D. Gierschner · I. Schaber · A. Katzenwadel ·  
W. Schultze-Seemann · U. Wetterauer · M. Tacke · M. Swamy ·  
W. W. A. Schamel · U. Elsässer-Beile

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## Abstract

**Background** Although cancer of the prostate is one of the most commonly diagnosed cancers in men, no curative treatment currently exists after its progression beyond resectable boundaries. Therefore, new agents for targeted treatment strategies are needed. Cross-linking of tumor antigens with T-cell associated antigens by bispecific monoclonal antibodies have been shown to increase antigen-specific cytotoxicity in T-cells. Since the prostate-specific membrane antigen (PSMA) represents an excellent tumor target, immunotherapy with bispecific diabodies could be a promising novel treatment option for prostate cancer.

**Methods** A heterodimeric diabody specific for human PSMA and the T-cell antigen CD3 was constructed from

the DNA of anti-CD3 and anti-PSMA single chain Fv fragments (scFv). It was expressed in *E. coli* using a vector containing a bicistronic operon for co-secretion of the hybrid scFv V<sub>H</sub>CD3-V<sub>L</sub>PSMA and V<sub>H</sub>PSMA-V<sub>L</sub>CD3. The resulting PSMAxCD3 diabody was purified from the periplasmic extract by immobilized metal affinity chromatography (IMAC). The binding properties were tested on PSMA-expressing prostate cancer cells and PSMA-negative cell lines as well as on Jurkat cells by flow cytometry. For in vitro functional analysis, a cell viability test (WST) was used. For in vivo evaluation the diabody was applied together with human peripheral blood lymphocytes (PBL) in a C4-2 xenograft-SCID mouse model.

**Results** By Blue Native gel electrophoresis, it could be shown that the PSMAxCD3 diabody is mainly a tetramer. Specific binding both to CD3-expressing Jurkat cells and PSMA-expressing C4-2 cells was shown by flow cytometry. In vitro, the diabody proved to be a potent agent for retargeting PBL to lyse C4-2 prostate cancer cells. Treatment of SCID mice inoculated with C4-2 tumor xenografts with the diabody and PBL efficiently inhibited tumor growth.

**Conclusions** The PSMAxCD3 diabody bears the potential for facilitating immunotherapy of prostate cancer and for the elimination of minimal residual disease.

P. Bühler and P. Wolf equally contributed to the work.

P. Bühler · P. Wolf · D. Gierschner · I. Schaber · A. Katzenwadel ·  
W. Schultze-Seemann · U. Wetterauer · U. Elsässer-Beile (✉)  
Department of Urology, Experimental Urology,  
University of Freiburg, Breisacher Str. 117,  
79106 Freiburg, Germany  
e-mail: ursula.elsaesser@uniklinik-freiburg.de

P. Bühler  
Faculty of Biology, University of Freiburg,  
Schänzle Str. 1, 79104 Freiburg, Germany

M. Tacke  
Institute of Molecular Medicine and Cell Research,  
University of Freiburg, Stefan-Meier-Strasse 17,  
79106 Freiburg, Germany

M. Swamy · W. W. A. Schamel  
Department of Molecular Immunology,  
Max-Planck-Institute for Immunobiology and  
University of Freiburg, Biologie III,  
Stübeweg 51, 79108 Freiburg, Germany

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## Introduction

Currently, there is no effective therapy for advanced prostate cancer, which necessitates the development of new and targeted therapies [13]. Among the different identified prostate antigens, the prostate-specific membrane antigen

(PSMA) has been shown to represent an excellent target for immunotherapy because it is (1) selectively expressed in the prostate, (2) abundantly expressed as protein at all stages and even upregulated in androgen-insensitive metastatic disease, (3) presented at the cell surface without being released into the circulation, and (4) associated with enzymatic or signaling activity [10].

In the field of cancer immunotherapy research, the retargeting of T-cells to tumor cells by bispecific antibodies (BsAbs) is an appealing therapeutic concept because cytotoxic T-cells range among the most potent effector cells of the immune system [31, 32]. The promise of BsAbs is an antibody target-dependent, polyclonal T-cell response. This does not depend on a specific T-cell receptor and MHC class I/peptide complex, and can support serial killing by cytotoxic T cells. Various strategies have been utilized for the creation of BsAbs. In a first generation, they have been produced mainly by using quadroma technology [1]. The most effective approach for tumor rejection appeared to be targeting via the CD3 component of the T-cell receptor complex molecule. The feasibility and effectiveness of this immunotherapeutic concept have been studied extensively in pre-clinical models and in some cases also in clinical trials [2, 3]. However, the immunogenicity of these BsAbs derived from rodent monoclonal antibodies was a major drawback for their clinical use. Additionally, their production and purification in large quantities proved to be difficult.

Recent advances in genetic engineering enabled the preparation of recombinant BsAbs as dimeric antibody fragments [12]. Diabodies are the smallest available BsAbs, and the distance between the two antigen-binding sites is sufficient to link two cells [21]. This compact size contributes to a low immunogenicity and a high tumor penetration [34].

Kipriyanov et al. constructed a bispecific diabody with dual specificity for both the human B-cell antigen CD19 and the  $\epsilon$ -chain of the CD3/T-cell receptor complex, which is able to retarget human lymphocytes to malignant B-cells [16]. This construct was used in the present study as a model for the generation of a diabody against PSMA and the CD3 complex.

## Material and methods

### Cell lines

The human prostate carcinoma LNCaP subline C4-2 was used as PSMA-positive target. As PSMA-negative control the prostate cancer cell lines DU 145 and PC-3 were used. The Jurkat cell line served as positive target for CD3 and CD28.

Cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in RPMI 1640 medium supplemented with penicillin (100 U/ml), streptomycin (100 mg/l) and 10% fetal calf serum (Biochrom, Berlin, Germany) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>.

### Monoclonal antibodies and scFv

The anti-PSMA scFv, called A5, directed against native cell-adherent PSMA was obtained in our group from the monoclonal antibody (mAb) 3/A12 as described previously. This scFv could be shown to be highly specific for PSMA-expressing prostate cancer cells [8, 33]. The anti-CD3 scFv was obtained from Dr. Kipriyanov, DKFZ, Heidelberg, Germany [14]. Anti-CD28 mAb (15E8) was obtained from Dr. Miedema, Netherlands Red Cross Blood Transfusion Service.

### Expression vectors for diabody construction

The *E. coli* K12 strain XL1-blue (Stratagene, La Jolla, CA, USA) was used as the cloning and expression host. The plasmid pHOG-3-19, containing the recombinant DNA for V<sub>H</sub>CD3 and V<sub>L</sub>CD19, and the plasmid pHOG-19-3, containing the recombinant DNA for V<sub>H</sub>CD19 and V<sub>L</sub>CD3, were obtained from Dr. Kipriyanov, DKFZ, Heidelberg, Germany [16].

### scFv and diabody expression and purification

*E. coli* XL1-blue cells (La Jolla, CA, USA) transformed with the plasmid for diabody or scFv were grown overnight in 2xYT-AG-medium, then diluted 1:20 and grown as 600 ml cultures at 37°C. When cultures reached OD 0.8, bacteria were pelleted by centrifugation at 1,500g for 10 min and resuspended in the same volume of fresh 2xYT-medium containing 50 µg/ml ampicillin, 0.4 M sucrose and 1 mM IPTG. Then the bacteria were incubated at room temperature for 18–20 h. Cells were harvested by centrifugation at 5,000g for 10 min and 4°C. To isolate soluble periplasmic proteins, the pelleted bacteria were resuspended in 30 ml of ice-cold 50 mM Tris-HCl, 20% sucrose, 1 mM EDTA (pH 8.0). After incubation for 1 h on ice, the spheroblasts were centrifuged at 20,000g for 30 min at 4°C yielding soluble periplasmic extract in the supernatant, which was dialyzed against 50 mM Tris-HCl, 1 M NaCl, (pH 7.0).

Purification was achieved by immobilized metal affinity chromatography (IMAC). This was performed using a 1 ml column of chelating Sepharose (Amersham Biosciences, Freiburg, Germany) charged with Ni<sup>2+</sup> and equilibrated with a buffer containing 50 mM Tris-HCl and 1 M NaCl

(pH 7.0). The periplasmic extract was loaded on the column, washed with 20 column volumes of equilibration buffer containing 30 mM imidazole and then eluted with the same buffer containing 250 mM imidazole. The eluted material was dialyzed against PBS. Determination of the protein content was performed with the Micro BCA Protein Reagent Kit (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions.

#### Western blot analysis

SDS-PAGE was performed by standard procedures according to the manufacturer's instructions (Invitrogen, Karlsruhe, Germany). For Western blotting, the diabody and scFvs were subjected to SDS-PAGE and transferred to nitrocellulose membranes. The blots were blocked overnight in PBS containing 5% non-fat milk and incubated with a peroxidase-coupled anti-human-c-myc antibody (Roche Diagnostics, Mannheim, Germany) for 1 h, and then developed by using 3,3'-diaminobenzidine as substrate. For Blue Native (BN)-PAGE [22, 23] analysis, the diabody and scFvs were diluted to 0.2 µg in 10 µl BN buffer (500 mM 6-aminohexanoic acid, 20 mM NaCl, 10% glycerol, 2 mM EDTA, 20 mM bis-Tris, pH 7 and protease inhibitors) and resolved on 4–18% BN-PAGE as described [29]. The proteins were transferred semi-dry to a PVDF membrane, and Western blotting was performed as above, and developed with the ECL chemiluminescence system. The high molecular weight calibration kit for native electrophoresis (GE Healthcare) was also loaded and stained with Coomassie brilliant blue for molecular weight standards.

#### Flow cytometry

The target cells were freshly harvested from tissue culture flasks and a single cell suspension was prepared in PBS with 3% FCS and 0.1% NaN<sub>3</sub>. Approximately, 10<sup>5</sup> cells were incubated with 50 µl of scFv or diabody for 1 h on ice. After three rounds of washing with PBS, 25 µl anti-human-c-myc mAb (Roche Diagnostics, Mannheim, Germany) was added at 10 µg/ml and incubated for 40 min on ice. After three washings with PBS, the cells were incubated with 25 µl of goat anti-mouse Ig-RPE (Becton Dickinson, Mountain View, CA, USA) for 40 min on ice. For inhibition experiments with mouse anti-PSMA mAb, a rat anti-c-myc mAb (Serotec LTD, Oxford, UK) and goat anti-rat IgG (H + L)-RPE (Catlag Lab Burlingame, CA, USA) were taken. The cells were then washed repeatedly and resuspended in 200 µl PBS containing 1 µg/ml propidium iodide, 3% FCS and 0.1% NaN<sub>3</sub>. The relative fluorescence of stained cells was measured using a FACScan<sup>®</sup> flow cytometer and the CellQuest<sup>®</sup> software (Becton Dickinson). Mean fluores-

cence intensity values (MFI) were considered after subtraction of the background staining with secondary antibody and RPE-labeled goat anti-mouse Ig alone.

#### Preparation of effector cells

Human peripheral blood mononuclear cells (PBMC) were isolated from fresh buffy coats of random donors by Ficoll (Pharmacia, Freiburg, Germany) density gradient centrifugation. The cells of the interphase were washed twice, counted and tested for viability with trypan blue dye. The PBMC were incubated overnight in 10 ml RPMI 1640 medium supplemented with penicillin (100 U/l), streptomycin (100 mg/l) fetal calf serum (10%) and low-dose IL-2 (10 U/ml) at 37°C, 5% CO<sub>2</sub> leading to the adherence of monocytes. The non-adherent PBL were used as effector cells.

#### Measurement of in vitro cytotoxicity

The metabolism of the red tetrazolium salt WST to a water-soluble formazan dye was determined according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). Target cells were seeded at 1.5 × 10<sup>4</sup> cells/well of a 96-well plate and grown for 24 h until a confluent cell layer was formed. Dilutions of the diabody in aliquots of 50 µl/well were added to the plates together with the effector cells at a target:effector ratio of 1:10 and incubated for 48 h at 37°C and 5% CO<sub>2</sub>. After this time, the cultures were pulsed with 15 µl/well WST reagent and incubated for 90 min at 37°C and 5% CO<sub>2</sub>. Then the spectrophotometrical absorbance of the samples was measured at 450 nm (reference 690 nm). As control for the PBL background in each WST experiment, PBL were incubated at the same lymphocyte concentration without tumor cells for 48 h and developed with WST. The value of these wells was then subtracted from the values of the test wells.

#### SCID mouse Xenograft model

Six-week-old SCID mice were purchased from Charles River Laboratories (Sulzfeld, Germany). The animals were kept under sterile and standardized environmental conditions (20 ± 1°C room temperature, 50 ± 10% relative humidity, 12 h light–dark rhythm) and received autoclaved food, water and bedding. All experiments were performed according to the German animal protection law with permission from the responsible local authorities. For tumor inoculation, 2 × 10<sup>6</sup> C4-2 cells in 100 µl PBS were mixed with 100 µl Matrigel (Collaborative Biomedical Products, Chicago, USA) at 4°C and administered s.c. at the right flank of each animal. Growing tumors were measured daily using a caliper in two perpendicular dimensions. Between day 6 and 12 after tumor inoculation, the animals were

treated i.v. either with peripheral blood lymphocytes (PBL) plus diabody, or with PBL alone, or with the vehicle. When the tumors of the control group reached a diameter of 12 mm, the animals were killed by CO<sub>2</sub> gas overdose. From the tumor diameters, the mean radius ( $r$ ) and hence the tumor volumes were calculated using the formula  $V = 4/3\pi r^3$ . Statistical analysis of the mean tumor volume of the corresponding treatment groups versus the vehicle control group was calculated performing a Student's  $t$  test.

## Results

### Construction of the diabody plasmid

In the plasmid pHOG-3-19, the sequence for V<sub>L</sub>CD19 was exchanged against V<sub>L</sub>PSMA by a PCR and the restriction sites Hind III and Not I, thus generating the plasmid pHOGCD-3-PSMA (Fig. 1a). In the plasmid pHOG-19-3, the sequence of V<sub>H</sub>CD19 was exchanged against V<sub>H</sub>PSMA by using the restriction sites NcoI and Hind III for the generation of pHOG-PSMACD-3 (Fig. 1b). The expression plasmid of the PSMAxCD3 diabody for co-secretion of the two hybrid scFvs was constructed by ligation of the Bgl II/XbaI restriction fragment from pHOGCD-3-PSMA comprising the vector backbone and the Bgl II/Xba I fragment from pHOG-PSMACD-3 (Fig. 1c). Both hybrid scFv operons are followed by sequences coding for a human c-myc tag for immunodetection and a hexahistidine tag for purification.

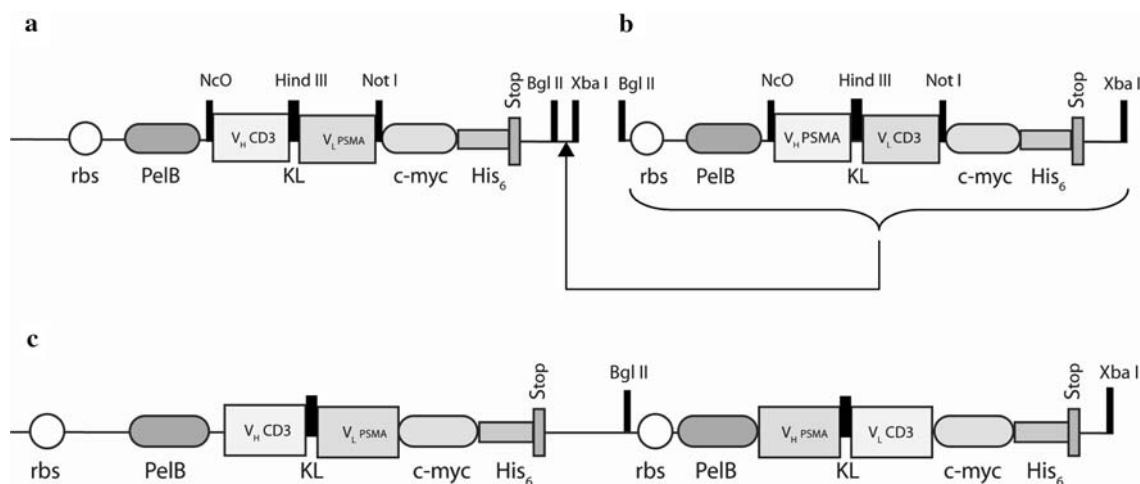
### Diabody expression and purification

Upon induction with IPTG, the PSMAxCD3 diabody was expressed by co-secretion of the two hybrid scFvs

V<sub>H</sub>PSMA-V<sub>L</sub>CD3 and V<sub>H</sub>CD3-V<sub>L</sub>PSMA from *E.coli* XL1-blue cells as periplasmic native protein and was also released into the culture medium. The periplasmic extract was purified by IMAC. Yields were estimated by the protein content according to BCA-testing and the 32 KD band on SDS-gels compared to a BSA standard. Between 100 and 300 μg diabody per liter of culture were obtained from the periplasmic extract. Purity of the diabody was determined by SDS-PAGE and Coomassie brilliant blue staining (Fig. 2a). The diabody was kept under sterile conditions and stored at -80°C.

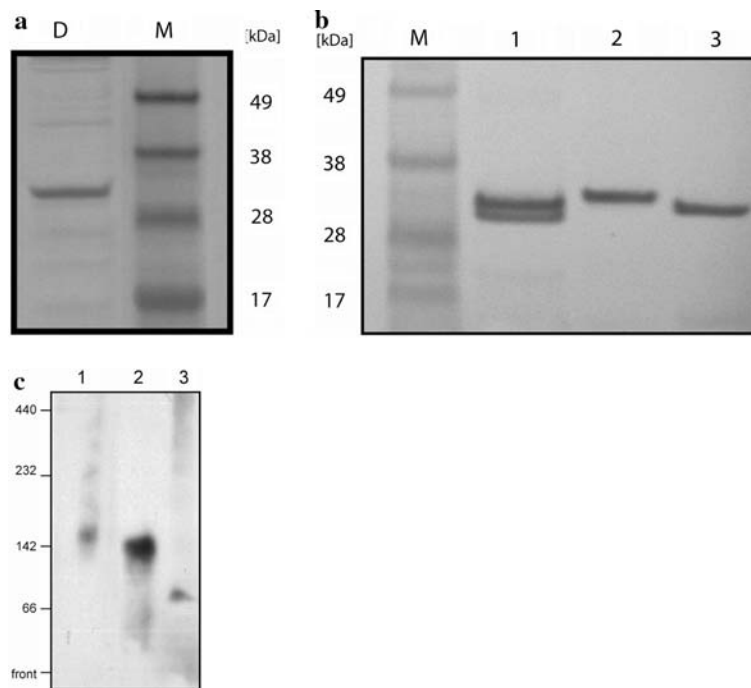
### Analysis of the PSMAxCD3 diabody

To analyze the quaternary structure of the diabody, it was resolved by gel-electrophoresis in either reducing denaturing conditions (SDS-PAGE) or native conditions (BN-PAGE), followed by Western blotting. Under denaturing, reducing conditions the PSMAxCD3 diabody was detected as protein double band at 32 kD (Fig. 2b, lane 1), corresponding to the two hybrid scFvs, V<sub>H</sub>PSMA-V<sub>L</sub>CD3 and V<sub>H</sub>CD3-V<sub>L</sub>PSMA. Separation of the diabody into a double band was only seen by Western blotting and not in the Coomassie-stained SDS gel, as smaller amounts were loaded for Western blotting, which allows higher resolution. When separated under native conditions by BN-PAGE [28, 29], the diabody is mainly present as a band whose molecular weight (~150 kD) corresponds to a tetramer (Fig. 2c, lane 1). Additional bands of higher MW are visible, possibly representing complexes of a larger order. The anti-PSMA scFv alone is also a tetramer (lane 2), whereas the anti-CD3 scFv is a dimer at ~70 kD (Fig. 2c, lane 3). Together these findings indicate that the variable regions of the anti-PSMA scFv are responsible for tetramerization.



**Fig. 1** Construction scheme for the PSMAxCD3 diabody plasmid (c) from the hetero scFv V<sub>H</sub>CD3-V<sub>L</sub>PSMA (a) and V<sub>H</sub>PSMA-V<sub>L</sub>CD3 (b). The positions of the sequences of the variable regions of the light and

heavy chains of the anti-PSMA-scFv and the anti-CD3-scFv, the ribosome binding sites (*rbs*), pelB leader sequences (*pelB*), c-myc epitopes (*c-myc*), hexa-histidine tags (*His<sub>6</sub>*) and stop codons (*stop*) are indicated



**Fig. 2** **a** SDS-PAGE of the PSMAxCD3 diabody (*lane D*). The gel was stained with Coomassie brilliant blue. *Lane M* contains a molecular weight marker. **b** Gel electrophoresis under denaturing, reducing conditions of PSMAxCD3 diabody (*lane 1*), anti-PSMA scFv (*lane 2*) and anti-CD3 scFv (*lane 3*). Proteins were subjected to reducing SDS-PAGE and transferred to a Western blot membrane. The blot was developed with peroxidase-coupled anti-c-myc-mAb, and 3,3'-diamino-

benzidine. *Lane M* contains a prestained molecular weight marker. **c** Gel electrophoresis under native conditions of PSMAxCD3 diabody (*lane 1*), anti-PSMA scFv (*lane 2*) and anti-CD3 scFv (*lane 3*). Proteins were separated by BN-PAGE and Western blotted with the peroxidase-coupled anti-c-myc-mAb and the ECL detection system. The molecular weights of the native protein standards are indicated on the *left* in kilo Daltons

#### Antigen-binding specificity of the PSMAxCD3 diabody and the parental scFvs

Flow cytometric experiments demonstrated a specific binding of the PSMAxCD3 diabody to PSMA-positive C4-2 cells (Fig. 3b) and to CD3-expressing Jurkat cells (Fig. 3f). The mean fluorescence intensities (MFI) at saturating conditions were about 30 on C4-2 cells and about 15 on Jurkat cells and therefore comparable to those obtained using the parental monospecific anti-PSMA scFv (Fig. 3a) and anti-CD3 scFv (Fig. 3e). In contrast to the PSMAxCD3 diabody, the separately induced hybrid scFv  $V_H$ PSMA- $V_L$ CD3 and scFv  $V_H$ CD3- $V_L$ PSMA did not show any binding to either C4-2 cells (Fig. 3c, d) or Jurkat cells (Fig. 3g, h). This indicates that both variable regions ( $V_H$  and  $V_L$ ) are necessary to reconstitute the binding site of an antibody. The saturation curves of the PSMAxCD3 diabody and the parental anti-PSMA scFv at concentrations between 0.5 and 500 nM on C4-2 cells is shown in Fig. 4a. From this graph, a half-maximal saturation was calculated to be 100 nM for the diabody and 80 nM for the scFv. The corresponding curves of the diabody and the anti-CD3 scFv on Jurkat cells are shown in Fig. 4b. Half-maximal saturation was calculated to be 30 nM for the diabody and the scFv.

Binding of the PSMAxCD3 diabody to C4-2 cells was inhibited by parental monoclonal antibody 3/A12 (Fig. 5a) and binding to Jurkat cells was inhibited by mAb OKT3 (Fig. 5b).

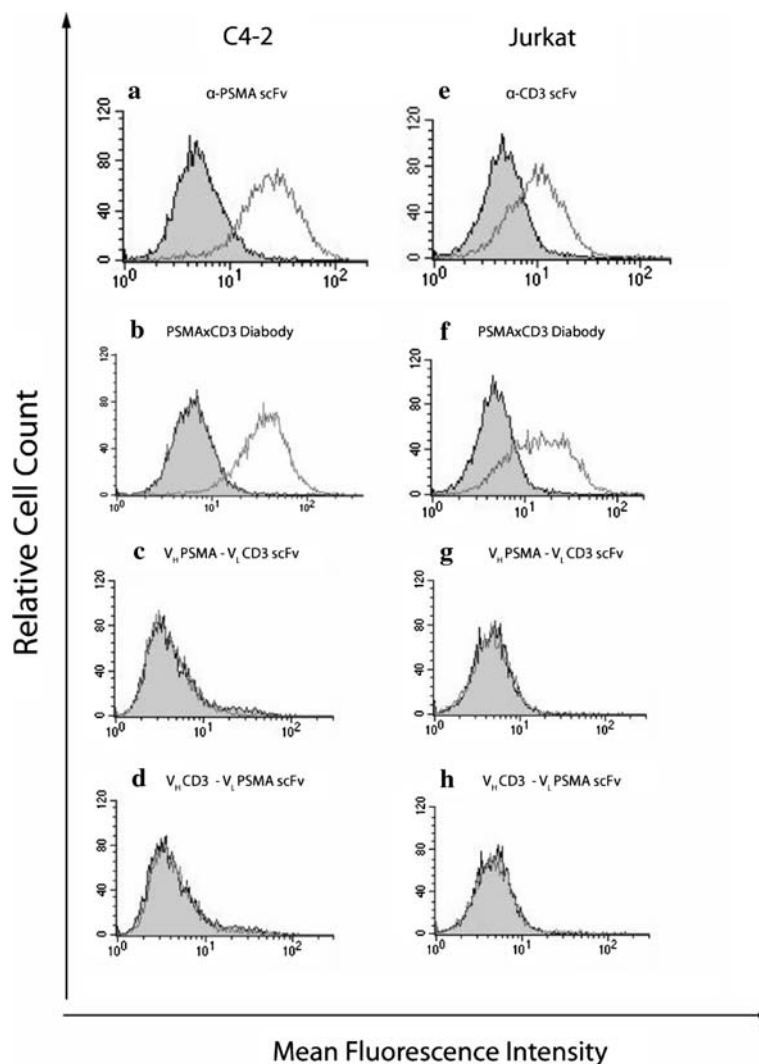
No binding of the diabody was found on PSMA- and CD3-negative DU145 and PC-3 cells. This is in accordance with the parental anti-PSMA scFv, which was shown to be highly specific for PSMA-expressing cells and did not bind to the PSMA-negative target cell lines DU 145, PC-3, HeLa, MCF7, HCT15, MB453, K562 and SW20 [33]. The specificity of the parental anti-CD3 scFv has also been shown previously [14].

The *in vitro* stability of the diabody was tested by binding experiments before and after incubation at 37°C for 24 h. Under these conditions, the binding capacity was reduced by 25%. Storage of the diabody at 4°C or frozen at -20°C for several weeks brought about no alteration in the binding capacity.

#### Induction of specific cytotoxicity by the PSMAxCD3 diabody *in vitro*

The ability of the bispecific diabody to induce tumor cell lysis by redirecting T-cell mediated cytotoxicity was inves-

**Fig. 3** Binding of anti-PSMA scFv (a), PSMAxCD3 diabody (b), hybrid scFv  $V_H$ PSMA- $V_L$ CD3 (c) and hybrid scFv  $V_H$ CD3- $V_L$ PSMA (d) to PSMA-positive C4-2 cells. Binding of anti-CD3 scFv (e), PSMAxCD3 diabody (f), hybrid scFv  $V_H$ PSMA- $V_L$ CD3 (g) and hybrid scFv  $V_H$ CD3- $V_L$ PSMA (h) to CD3-positive Jurkat cells. Cells were stained with diabody or scFv at 300 nM, mouse-anti-c-myc mAb and goat anti-mouse Ig-RPE. Histograms represent logarithms of PE-fluorescence on flow cytometer. Negative control (gray histograms) with mouse-anti-c-myc mAb, and goat anti-mouse Ig-RPE



tigated with PBL from healthy donors, which were monocyte depleted. PSMA-expressing C4-2 prostate cancer cells served as target cells and PSMA-negative DU-145 cells as controls.

In these experiments with an effector–target ratio of 10:1, the PSMAxCD3 diabody appeared to be potent in retargeting PBL to lyse the PSMA-positive C4-2 cells, whereas PSMA-negative DU145 cells were not lysed at the same conditions (Fig. 6a). PBL from different donors varied as seen by the error bars. The PBL alone (at ET ratio 10:1) and the diabody alone at 15 nM were not toxic to both cell lines. Cytotoxicity could be blocked by addition of an excess of parental mAb 3/A12 (Fig. 6b). The addition of anti-PSMA scFv or anti-CD3 scFv together with PBL showed only minimal cytotoxic activity compared to the PSMAxCD3 diabody (Fig. 7). Addition of anti-CD28 mAb for co-stimulation at concentrations between 100  $\mu$ g and 5 mg/ml brought about no enhancement of the diabody effects on C4-2 cells (data not shown).

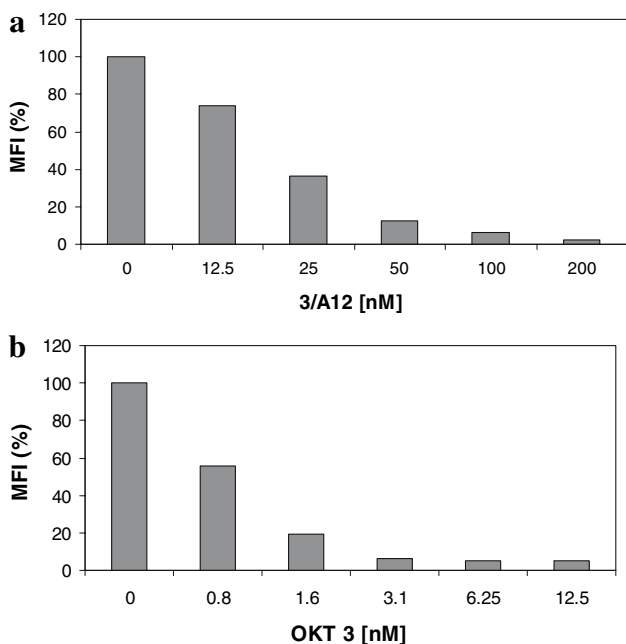
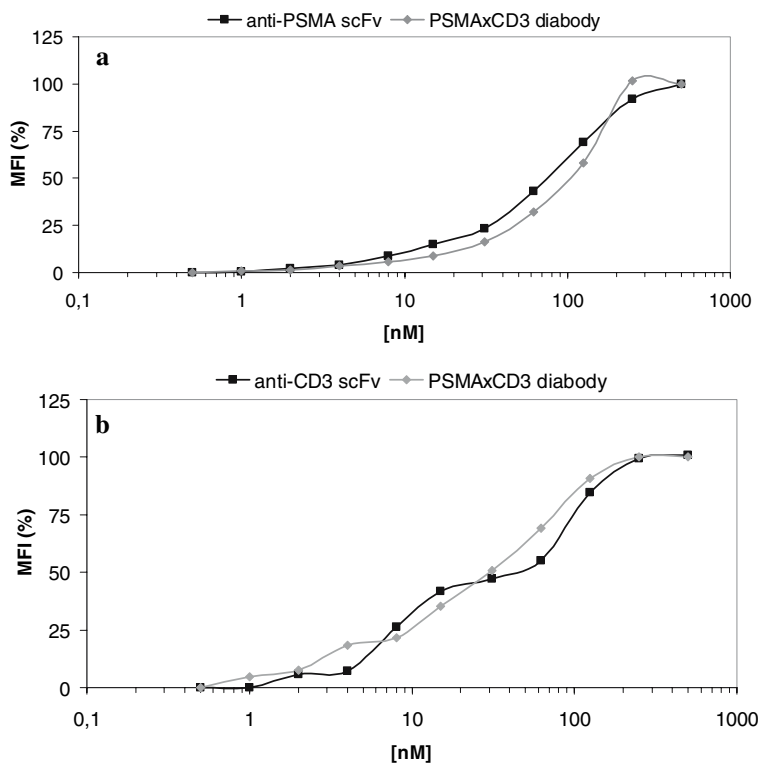
Effect of the PSMAxCD3 diabody on xenotransplanted C4-2 tumors in SCID mice

The in vivo effects of the PSMAxCD3 diabody were tested in SCID mice xenotransplanted with the human LNCaP subline C4-2. Mice were engrafted with  $2 \times 10^6$  C4-2 cells s.c., which was shown to generate palpable tumors in more than 95% of the animals.

In a first series of four animals, toxicity of the PSMAxCD3 diabody was tested at doses of 5, 10, 15 and 20  $\mu$ g per injection. Doses up to 15  $\mu$ g were well tolerated.

For therapy, a series of 21 animals were used. On day 6 after tumor inoculation, the animals were randomized into three groups of seven animals. Group a was treated with  $5 \times 10^6$  PBL as effector cells on day 6 and additionally with 10  $\mu$ g PSMAxCD3 diabody on days 6, 7, 10, 11, and 12. Group b was treated with  $5 \times 10^6$  human PBL on day 6. The animals of group c were treated with saline vehicle. All animals were killed on day 20 after tumor inoculation,

**Fig. 4** Saturation curves of the PSMAxCD3 diabody and the parental anti-PSMA scFv to PSMA-positive C4-2 cells (a) and of PSMAxCD3 diabody and parental anti-CD3 scFv to CD3-positive Jurkat cells (b) at concentrations between 0.5 and 500 nM



**Fig. 5** Inhibition of the binding of PSMAxCD3 diabody to C4-2 cells by parental mAb 3/A12 (a) and to Jurkat cells by parental mAb OKT3 (b). Cells were preincubated with the mAb at different concentrations (5–200 nM) and then stained with PSMAxCD3 diabody at 200 nM, rat anti-c-myc mAb and goat anti-rat IgG(H + L)-RPE

when the tumors of the control animals had reached 12 mm diameter and/or begun to ulcerate.

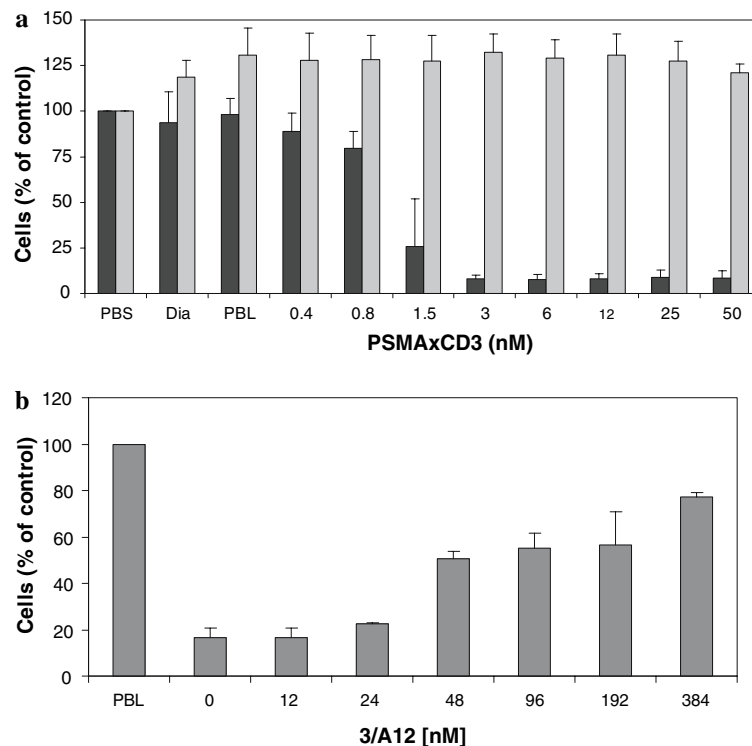
In the saline-treated control group, the animals developed fast growing tumors between day 10 and 15 after

tumor inoculation. In contrast, animals receiving human PBL showed reduced tumor growth. Animals receiving human PBL and additionally PSMAxCD3 diabody showed a delayed starting point of tumor growth and, finally, clearly reduced tumor growth (Fig. 8). The differences in the mean tumor volume of the animals of group a (diabody + PBL) and group c (vehicle) proved to be significant ( $P = 0.001$ ), as well as the differences in the mean tumor volume of the animals of group a and group b (PBL alone) were significant ( $P = 0.04$ ).

**Discussion**

The limited options in the treatment of prostate cancer have encouraged the search for new immunotherapeutic strategies. The retargeting of cellular effector systems by bispecific antibodies is a promising approach. Such recombinant diabodies have been generated against a number of tumor-associated antigens, but not yet against prostate cancer. By virtue of its abundant and restricted surface expression on prostate epithelial cells, PSMA represents an attractive antigen for a targeted therapy. However, in contrast to other tumor-associated antigens [19], there are only very few approaches that use PSMA. The reason for this may be that only few mAbs and scFvs binding to cell-adherent PSMA have yet been published [5, 11, 17, 20, 30].

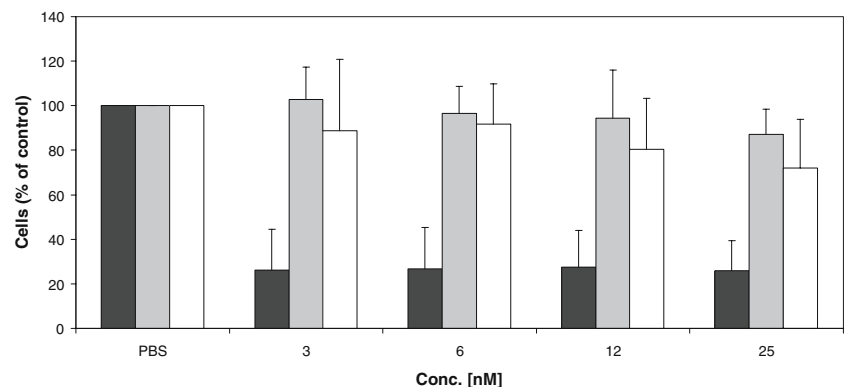
For the construction of the PSMAxCD3 diabody described in the present paper, we used the recombinant



**Fig. 6 a** Effects of treatment of PSMA-positive C4-2 cells (*black*) and PSMA-negative DU 145 cells (*gray*) with PSMAxCD3 diabody and PBL effector cells (effector:target ratio 10:1). Cell viability was measured in WST assays after 48 h incubation as described. The results are expressed as the percentage of viable cells compared to the control (= cells incubated with PBS). Additionally, the data of treatment with diabody alone at 15 nM without PBL (Dia) and the data of treatment with PBL alone without diabody (PBL) are given. The results were corrected for the background of the effector cells. Data represent mean

values of determinations with three different blood donors each in triplicate. Standard deviations (SD) are indicated by the *error bars*. **b** Blocking of the cytotoxic activity of the PSMAxCD3 diabody by an excess of parental anti-PSMA mAb. C4-2 cells were incubated with PBL and diabody at 12 nM together with parental mAb 3/A12 at different concentrations. Cell viability was measured in WST assays after 48 h incubation. Determination was with three different donors. SD are indicated by the *error bars*

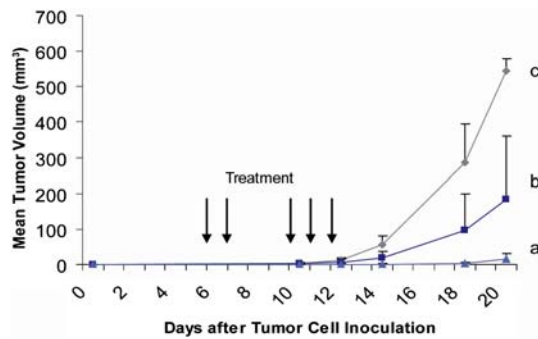
**Fig. 7** Effects of treatment of PSMA-positive C4-2 cells with PSMAxCD3 diabody (*black*) or anti-PSMA scFv (*gray*) or anti-CD3 scFv (*white*) and PBL. (WST assay, E:T ratio 10:1, incubation time 48 h). The results are expressed as the percentage of viable cells compared to the control (= cells treated with PBS). Data represent mean values of three determinations with different donors. SD are indicated by the *error bars*



DNA of an anti-CD3 scFv, which had been modified to improve its stability and folding in bacteria [14]. The second scFv was highly specific for cell-adherent PSMA [33]. The molecular design of the diabody and its bicistronic secretion was in accordance with the CD19 × CD3 diabody published by Kipriyanov et al. [16]. This construct has been shown to be a dimer in native conditions with two antigen-binding sites at opposite ends of the molecule, separated by

65 Å. This size has been suggested as being optimal because it provides rapid tissue penetration, high target retention and rapid blood clearance [12]. Interestingly, our PSMAxCD3 diabody proved to be a tetramer as analyzed by BN-PAGE. This indicates the presence of two binding sites each for CD3 and PSMA. Kipriyanov et al. have shown that a tetravalent CD3 × CD19 tandem diabody was superior to the corresponding heterodimeric diabody [15].





**Fig. 8** Treatment of C4-2 xenografted SCID mice with PSMAxCD3 diabody. Mice were inoculated with  $2 \times 10^6$  C4-2 cells on day 0 and treated as follows: group a:  $5 \times 10^6$  human PBL on day 6 after tumor inoculation and additionally  $10 \mu\text{g}$  PSMAxCD3 diabody on day 6, 7, 10, 11 and 12; group b:  $5 \times 10^6$  human PBL on day 6; group c: PBS vehicle. The mean tumor volume is shown for each group. SD are indicated by the error bars

Currently it is not known whether our diabody forms a dimer of dimers or whether it is a bispecific tetrabody [12]. Flow cytometry measurements have shown that it binds to CD3-positive Jurkat cells and to PSMA-positive C4-2 cells with an affinity similar to that of the parental scFvs. In vitro testing showed the diabody's ability to induce an efficient lysis of PSMA-expressing target cells by human PBL. Furthermore, the diabody has proven to be clearly more effective than either of the parental scFv alone. However, in this in vitro test the antitumor activity of the diabody was not enhanced in the presence of anti-CD28 mAb, which induces a well-known co-stimulatory signal [4]. This may be partly explained by the use of preactivated PBL and is in accordance with other in vitro and in vivo studies [9, 16, 18, 25].

The ultimate goal of any antitumor immunotherapy is the in vivo eradication of tumor cells. In a first animal experiment we tested the potency of the PSMAxCD3 diabody to mediate T-cell dependent tumor lysis in a fairly stringent in vivo model of immunodeficient mice bearing a subcutaneously growing human prostate cancer xenograft. The therapy scheme was chosen according to literature suggestions [24] and aimed to treat small tumor masses. Therefore, we started the application of preactivated PBL and diabody as early as 6 days after tumor inoculation. With this treatment regime we could show that the PSMAxCD3 diabody is effective against prostate cancer cells in vivo in the absence of any T-cell co-stimulatory compounds. The efficacy under these conditions supports that human T-cells did reach the tumor site after injection into the tail vein and could act sufficiently long with a sufficient number of functional T-cells. The limited number and accessibility of human T-cells in our model may underestimate the potential of the PSMAxCD3 diabody in humans where cytotoxic T-cells are present in larger numbers and found widespread

in the organism. This availability of T-cells would make a repeated application of the diabody much more efficient, raising the opportunity to treat larger tumor masses. The current mouse model rather allows the investigation of a minimal residual disease situation. This is in agreement with other authors who showed good antitumor effects in human B-lymphoma xenografts after application of CD19  $\times$  CD3 diabody within a few days after tumor inoculation [6, 7]. A direct comparison of early and late diabody application was shown by Schlereth et al. [24] in a human colon carcinoma xenograft model after application of an EP-CAMxCD3 diabody. These authors also found a much better tumor remission rate in the groups with early application due to a limited half-life of human lymphocytes in the SCID mouse, a smaller workload for the cytotoxic T-cells and an increased penetration in smaller tumors.

In comparison to other models, a shortcoming of our C4-2 xenograft model is the short experimental time. Since the C4-2 tumors are very fast growing and mostly ulcerating, the animals had to be euthanized 20–25 days after tumor inoculation. A slower growing cell line that allows longer observation times may be used in further studies. For further preclinical development, pharmacological properties as well as immunological side effects have to be tested in an immunocompetent individual. However, since the CD19  $\times$  CD3 diabody could be safely applied to chimpanzees [26], and also used in a first clinical trial [12], no severe side effects like a cytokine storm in the Phase I trial of the anti-CD28 monoclonal antibody [27] may be expected with the PSMAxCD3 diabody.

In conclusion, the present study describes a first approach to establish a new immunotherapy with retargeting of effector T cells for prostate cancer. We have demonstrated that the PSMAxCD3 diabody induces highly efficient T-cell mediated killing of prostate cancer cells. Therefore, this diabody deserves further development in preclinical and clinical settings as a promising therapeutic agent.

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