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Novel Humanized and Highly Efficient Bispecific Antibodies Mediate Killing of Prostate Stem Cell Antigen-Expressing Tumor Cells by CD8+ and CD4+ T Cells

Anja Feldmann,*1 Claudia Arndt,*1 Katrin Töpfer,† Slava Stamova,* Franziska Krone,* Marc Cartellieri,* Stefanie Koristka,* Irene Michalk,* Dirk Lindemann,‡§ Marc Schmitz,*§ Achim Temme,† Martin Bornhäuser,§‡ Gerhard Ehninger,§ and Michael Bachmann*§

Prostate cancer is the most common noncutaneous malignancy in men. The prostate stem cell Ag (PSCA) is a promising target for immunotherapy of advanced disease. Based on a novel mAb directed to PSCA, we established and compared a series of murine and humanized anti-CD3-anti-PSCA single-chain bispecific Abs. Their capability to redirect T cells for killing of tumor cells was analyzed. During these studies, we identified a novel bispecific humanized Ab that efficiently retargets T cells to tumor cells in a strictly Ag-dependent manner and at femtomolar concentrations. T cell activation, cytokine release, and lysis of target cells depend on a cross-linkage of redirected T cells with tumor cells, whereas binding of the anti-CD3 domain alone does not lead to an activation or cytokine release. Interestingly, both CD8+ and CD4+ T cells are activated in parallel and can efficiently mediate the lysis of tumor cells. However, the onset of killing via CD4+ T cells is delayed. Furthermore, redirecting T cells via the novel humanized bispecific Abs results in a delay of tumor growth in xenografted nude mice. The Journal of Immunology, 2012, 189: 3249–3259.

Prostate cancer (PCa) is the most frequently diagnosed noncutaneous cancer among American men. In 2010, >200,000 new cases and 32,000 deaths of PCa patients were estimated in the United States (1). Whereas patients with localized PCa are often successfully treated with radical prostatectomy and radiotherapy, effective therapeutic options for patients with metastatic hormone-refractory PCa are limited (2–5). Chemotherapy of patients with hormone-refractory PCa has only palliative effects (6). Thus, new therapeutic options are urgently needed, especially for the treatment of patients with advanced PCa.

Immunotherapy is an attractive approach for the treatment of minimal residual disease and micrometastases. For immunotherapy of human PCa, the prostate stem cell Ag (PSCA) is a promising target (7). PSCA is a GPI-anchored cell-surface protein that is overexpressed in human prostate tumors, including metastatic and hormone-refractory PCa (8). PSCA expression is detected in ~90% of primary PCa, as well as in bone, lymph node, and liver metastases (9, 10). Furthermore, the PSCA expression level positively correlates with advanced clinical stage of PCa (9, 11). In addition to PCa, PSCA is also upregulated in bladder and pancreatic cancers (12, 13).

Over the past decade, a series of different recombinant Ab formats was described (e.g., see Ref. 14). The smallest recombinant Ag-binding unit is known as single-chain fragment variable (scFv). It consists of the V domains of the H (VH) and L (VL) chain of a mAb that are usually connected via a glycine-serine peptide linker sequence. Fusion of two scFvs with different specificities results in bispecific Abs. For retargeting of T cells to tumor cells, recombinant single-chain bispecific Abs were developed that usually target with one arm the activating CD3 complex on T cells and with the other arm a tumor-associated Ag (TAA) on tumor cells. The cross-linking of effector and target cells via such bispecific Abs leads to a polyclonal T cell activation resulting in a serial killing of tumor cells (15). T cell activation and bispecific Ab-mediated killing is independent of the TCR specificity, as well as the MHC class I–peptide complex, and it does not require any costimulatory signal (16, 17). Until today, the impressive capability of redirected T cells to kill tumor cells has been shown in vitro assays, animal models, preclinical trials, and even in first clinical trials (18–25).

Both T cell activation and cytotoxicity have to occur in a strictly target-dependent manner and only after cross-linkage of effector and tumor cell to minimize the risk for a cytokine storm in a clinical setting. Therefore, binding of the anti-CD3 arm of a bispecific Ab to the CD3 complex of T cells in the absence of a target cell must not lead to an activation and a systemic release of cytokines.

The clinical application of murine Abs commonly leads to a human anti-mouse Ab response. Such human anti-mouse Abs can mediate the elimination of the administered Abs and cause un-
wished adverse effects in patients (26, 27). Murine Abs can be humanized to overcome this side effect, for example, by grafting of the murine CDRs into the best fitting human framework regions (FWRs) (28, 29).

In this article, we describe, to our knowledge, the first humanized single-chain bispecific anti-PSCA–anti-CD3 Ab: The Ab was selected from a series of novel murine and humanized single-chain bispecific Abs that all derived from the novel murine monoclonal anti-PSCA Ab MB1. The selected novel humanized bispecific Ab is functional even at femtomolar concentrations, and thus at an almost 1000-fold lower concentration than previously described murine bispecific anti-CD3–anti-PSCA Abs based on the PSCA mAb 7F5 (30–33). We show that the novel single-chain bispecific anti-CD3–anti-PSCA Ab is capable of redirecting both CD8+ and CD4+ T cells to PSCA+ tumor cells in vitro and in experimental mice. Both redirected CD8+ and CD4+ T cells efficiently mediate the killing of target cells. However, the onset of killing via CD4+ T cells is delayed, although both CD8+ and CD4+ T cells are activated simultaneously.

Materials and Methods

Isolation of human PBMCs and T cell subpopulations

Human PBMCs were isolated either from buffy coats supplied by the German Red Cross (Dresden, Germany) or from fresh blood of healthy donors after obtaining oral and written consent by gradient centrifugation over Biocoll (Biochrom, Berlin, Germany). Using the CD8+ or CD4+ T Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany), we isolated CD8+ or CD4+ T cells, respectively, from human PBMCs. The Pan T Cell Isolation Kit (Miltenyi Biotec) was used to isolate untouched T cells from PBMCs. Untouched CD8+, CD4+, or pan (1 × 10^6/ml) T cells were cultured overnight in complete RPMI 1640 medium (30) supplemented with 50 U/ml IL-2 (ImmunoTools, Friesoythe, Germany). Preactivated T cells, which mainly consist of CD25-expressing CD8+ T cells, were generated by 50 U/ml IL-2 (ImmunoTools, Friesoythe, Germany). Preactivated T cells, which permanently express PSCA after transduction (30), were cultured in complete RPMI 1640 containing 100 U/ml IL-2 and 50 ng/ml anti-CD3 mAb OKT3 (eBioscience, San Diego, CA) for 5 d. Afterward, the cells were kept in fresh complete RPMI 1640 medium supplemented with 50 U/ml IL-2 for an additional 3 d.

Cell lines

The PSCA+ PCa cell line PC3 wild type (wt) and the PC3-PSCA cells, which permanently express PSCA after transduction (30), were cultured in complete RPMI 1640 medium. The bladder cancer cell lines RT4 and HT-1376 that naturally express PSCA were grown in complete RPMI 1640.

Construction and analysis of single-chain bispecific Abs

The construction of the murine single-chain bispecific Abs scBsDb CD3xPSCA(7F5) and scBsTaFv CD3-PSCA(7F5) derived from the anti-CD3 mAb MT-301 and anti-PSCA mAb 7F5 were described previously (30). The novel anti-PSCA mAb MB1 was established from hyperimmunized mice using the hybridoma technique (30, 33). The mice were immunized with murine cells that were transduced with the human PSCA reading frame. For amplification of the H and L chain sequences of the anti-PSCA mAb MB1, cDNA was prepared from the hybridoma line as described previously (30). Based on the novel anti-PSCA mAb MB1, the anti-CD3 mAb MT-301 (30–32), and the anti-La mAb 5B9 (M. Bachmann, unpublished observations), we cloned a series of bispecific Abs of which we selected the murine scBsTaFv PSCA (MB1)-CD3, humanized scBsTaFv CD3-PSCA(7F5), and humanized scBsTaFv CD3-PSCA(7F5) for this report. The latter one serves as negative control. The structure of these single-chain bispecific Abs is shown schematically in Supplemental Fig. 1A.

These constructs were cloned as follows: The Vh and Vl domains of the anti-PSCA mAb MB1 were amplified from the cDNA using the primer pair VlF1 (5′-TGTATTCTGCCGAGAGTCTGGTCAACCATGCA-3′) and VlG1Rev (5′-GGGACGCGCCCGCGCCGCGCCGCGG-3′) or the primer pair VlF2 (5′-TTTTTTTGCGCGCTCCGCGCGCCGCGGCGG-3′) and VlG2Rev (5′-GGGGGCGCGCGCGCGCGCGCGCGGCGGCGG-3′). All PCRs were performed with the Advantage HD 2 PCR Kit (Clontech, Mountain View, CA). For construction of the murine scFv PSCA(MB1), the Vl domain was amplified by using the primer pair #1 (5′-GGGCC-AGGCCGCCAGGCCGCCAGGCCGCCAGGCCGCCAGGAGTCGAC-3′) and #2 (5′-GGGCC-AGGCCGCCAGGCCGCCAGGCCGCCAGGCCGCCAGGAGTCGAC-3′) and the Vh domain with the primer pair #3 (5′-GGGCC-AGGCCGCCAGGCCGCCAGGCCGCCAGGCCGCCAGGAGTCGAC-3′) and #4. The scFv PSCA(MB1) was fused by PCR in the order VhG3S-Vl, and cloned into the vector pSecTaFv2B via the HindIII restriction enzyme site following the primers #1 and #4. The scFv CD3 was amplified by the primer pair #5 (5′-GGGCC-AGGCCGCCAGGCCGCCAGGCCGCCAGGCCGCCAGGAGTCGAC-3′) and #6 (5′-GGGCC-AGGCCGCCAGGCCGCCAGGCCGCCAGGCCGCCAGGAGTCGAC-3′) and afterward cloned via Apal into the vector pSecTag2B as scFv PSCA(MB1) to construct the murine scBsTaFv PSCA(MB1)-CD3.

The humanization of the Abs was achieved by CDR grafting as schematically shown in Supplemental Fig. 1B. Furthermore, the Ab sequences were optimized for protein expression. The in silico developed sequences were realized by fusion of overlapping oligonucleotides by PCR. Alternatively, the whole sequence was synthesized. Overlapping oligonucleotides or whole gene sequences were obtained by Eurofins MWG Operon (Ebersberg, Germany). The sequences encoding the humanized scFv CD3, PSCA(MB1), or 5B9 were synthesized. Afterward, scFv CD3 was cloned via HindIII into the vector pSecTag2B, and secondly, scFv PSCA(MB1) or scFv 5B9 was cloned via Xhol/Apal into the same vector. Resulting vectors encode the humanized scBsTaFv CD3-PSCA(MB1) or the humanized control scBsTaFv CD3-5B9.

For permanent production of murine scBsTaFv PSCA(MB1)-CD3, humanized scBsTaFv CD3-PSCA(MB1), or humanized scBsTaFv CD3-3B9, 3T3 cells were transduced using a lentiviral packaging system. Therefore, the sequence of the recombinant Ab including the N-terminal IgG leader and the C-terminal myc- and histidine (his)-tag were cut out of pSecTag2B with Nhel and Mis1 and cloned into the lentiviral vector p6NST50, which was digested with Xbal and KspAI. All the restriction enzymes were purchased from Fermentas (St. Leon-Rot, Germany).

The purification and analysis of the isolated his-tagged recombinant Abs from cell culture supernatants were performed as described previously (30). Protein analysis was performed by SDS-PAGE and immunoblotting. After transfer of proteins to nitrocellulose membrane, isolated Abs were detected by a mouse anti-penta-his mAb (Qiagen, Hilden, Germany) and an anti-mouse IgG conjugated with alkaline phosphatase (Dianova, Hamburg, Germany) using BCIP/NBT (Roche Diagnostics, Mannheim, Germany) as substrate. To analyze the Ab binding properties, we stained cells with the recombinant Abs and detected them using anti-myc/FITC mAb (Miltenyi Biotec) in a FACScalibur flow cytometer (BD Biosciences, Heidelberg, Germany) as described previously (30). Kd values were determined as described previously (30).

Size exclusion fast protein liquid chromatography

Size exclusion fast protein liquid chromatography was performed at a flow rate of 0.5 ml/min by using Superose 200 (Sigma-Aldrich Chemie, Steinheim, Germany) as gel filtration medium. The column was connected to a BioLogic DuoFlow Chromatography System (Bio-Rad Laboratories, München, Germany) as described previously (30).

Cytotoxicity assay

The assay was performed as described previously (30). Statistical significance of differences in specific tumor cell lysis was analyzed by one-way ANOVA and Bonferroni multiple-comparison test using GraphPad Prism software. The p values <0.05 were considered significant.

Degranulation assay

In one well of a round-bottom 96-well plate, 1 × 10^5 PC3-PSCA tumor cells were cultured with 1 × 10^4 freshly isolated CD4+ or CD8+ T cells in the presence or absence of 30 pmol/ml recombinant Abs. In addition, 5 μl anti-CD107α/IFN-γ mAb (BD Biosciences) and complete RPMI 1640 were pipetted to each well (final volume of 200 μl). After 1 h of incubation at 37°C in 1 μl of 2 mM monensin (Sigma-Aldrich Chemie; diluted in ethanol) was added. After an additional incubation of 4 and 19 h, respectively, the degranulation marker CD107α present on the cell surface of T cells was measured using a MACSQuant Analyzer (Miltenyi Biotec). Beforehand, the cells were stained with anti-CD4/VioBlue (Miltenyi Biotec), anti-CD8/PE-Cy5, and anti-CD3/FITC mAbs (BD Biosciences).

T cell activation assay and cytokine ELISA

After a 5- and/or 20-h incubation of 5 × 10^4 tumor cells with 5 × 10^4 freshly isolated CD4+, CD8+, or pan T cells in the presence or absence of

NOVEL HUMANIZED ANTI-CD3–ANTI-PSCA BISPECIFIC AB
Mice were sacrificed by CO2 gas overdose when tumor size exceeded 18 mm² measured continuously with a caliper in two perpendicular dimensions. Vein of each mouse of groups 2 and 3, respectively. The tumor growth was monitored. Humanized scBsTaFv CD3-PSCA(MB1) were injected into the tail vein of each mouse of the third group. A mix of PC3-PSCA tumor cells (3×10⁶) in PBS was injected s.c. at the right flank of each mouse of the other groups. In addition, each mouse of the second group received 10⁶ humanized scBsTaFv CD3-PSCA(MB1) (day 0). At days 1, 2, and 3, 10⁶ murine T cells of each mouse of the third group received 10⁶ PSCA+ mor cells, whereas the control humanized scBsTaFv CD3-5B9, whereas each mouse of the second group received 10 μg humanized scBsTaFv CD3-PSCA(MB1) (day 0). At days 1, 2, and 3, 10 μg humanized scBsTaFv CD3-5B9 and humanized scBsTaFv CD3-PSA(MB1) were injected into the tail vein of each mouse of groups 2 and 3, respectively. The tumor growth was measured continuously with a caliper in two perpendicular dimensions. Mice were sacrificed by CO2 gas overdose when tumor size exceeded 18 mm² in one direction or when the animals showed pathological symptoms. The tumor area was calculated according to the formula, A = b × h × π / 4. Statistical analysis of all groups was performed by one-way ANOVA and a post hoc Tukey’s multiple-comparison test (significance p < 0.05).

Results

Development and functional comparison of murine and humanized anti-CD3–anti-PSCA Ab fragments based on the novel anti-PSCA mAb MB1

Based on the novel anti-PSCA mAb MB1, a series of murine and humanized versions of single-chain bispecific anti-CD3–anti-PSCA Abs were established and characterized. These various bispecific Abs differed with respect to the order of their Ab domains, the order of the respective VH or VL chains within the Ab domains, the linker sequences, and size (data not shown). The structure of the murine and humanized single-chain bispecific anti-CD3–anti-PSCA Abs, which we selected and present in more detail later, is schematically summarized in Supplemental Fig. 1A. They were selected because of their improved expression, stability, and/or functionality. The chosen murine and humanized anti-CD3–anti-PSCA(MB1) Abs were both constructed in the scBsTaFv format, but differ with regard to orientation of CD3 and PSCA domains, VH-VL arrangement, and linker length in the PSCA domain. In addition, a humanized single-chain bispecific control Ab was established in the scBsTaFv format that targets the intracellular protein La/SS-B (34). This control Ab is based on the anti-La mAb 5B9 (M. Bachmann, unpublished observations). Humanization was achieved by CDR grafting as schematically summarized in Supplemental Fig. 1B. Sequence alignments revealed that the newly generated V L and V H domains have a 100% homology in the FWRs to the most similar human V genes in germline configuration at amino acid level (Table I). Regarding the CDRs, the grade of homology ranged between 35 and 60% (Table I). The humanized anti-CD3 V H, anti-CD3 V L, anti-PSCA(MB1) V H, and anti-PSCA(MB1) V L domains have an 80, 74, 84, and 81% identity to the corresponding murine counterparts at amino acid level, respectively. The novel single-chain bispecific Abs were termed as murine scBsTaFv PSCA(MB1)-CD3, humanized scBsTaFv CD3-PSCA(MB1), and humanized scBsTaFv CD3-5B9.

After cloning, permanent cell lines were established for expression of the Abs. Affinity-purified Abs were analyzed by SDS-PAGE and staining with Coomassie brilliant blue (Supplemental Fig. 2AI) or immunoblotting (Supplemental Fig. 2AII). All three scBsTaFv Abs were expressed and purified as full-length proteins with the expected molecular mass of ~60 kDa (Supplemental Fig. 2AII). As expected, all novel single-chain bispecific Abs bound to CD3 on T cells (Supplemental Fig. 2B, upper panel). Furthermore, the murine scBsTaFv PSCA(MB1)-CD3 and the humanized scBsTaFv CD3-PSCA(MB1) also bound to PSCA on PSCA+ tumor cells, whereas the control humanized scBsTaFv CD3-5B9 failed to bind to PSCA-expressing tumor cells (Supplemental Fig. 2B, lower panel).

The K D values for the CD3 domain of the murine scBsTaFv PSCA(MB1)-CD3 and the humanized scBsTaFv CD3-PSCA (MB1) were estimated with 4×10⁻⁷ mol/l and 5×10⁻⁷ mol/l, respectively. For the PSCA domain, we estimated K D values of 6×10⁻⁷ mol/l and 9×10⁻⁷ mol/l for the murine scBsTaFv PSCA(MB1)-CD3 and the humanized scBsTaFv CD3-PSCA (MB1), respectively.

It has been described that bispecific Abs tend to form dimers. Therefore, size exclusion chromatographies were performed (Suppl-

### Table 1. Sequence identity at the amino acid level of the scFvs after humanization

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<th>FWR1</th>
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The murine scFvs were aligned to the NCBI human V genes database using IgBlast (http://www.ncbi.nlm.nih.gov/igblast). The best fitting human V gene was identified and the FWR of the human V gene grafted onto the murine scFvs. Homology (%) between the resulting humanized scFvs and the best fitting human V genes in germline configuration for the different regions is given. In the last column, the homology (%) at amino acid level between the humanized and the original murine scFv is indicated.
To estimate the amount of dimers present in purified Ab fractions of the humanized scBsTaFv CD3-PSCA(MB1), we did not detect protein dimers (data not shown). An Ab sample that was stored for 6 mo at 4°C contained a small fraction (~7%) with an apparent m.w. of a dimer of the humanized scBsTaFv CD3-PSCA(MB1).

The capability of the novel murine and humanized anti-CD3–anti-PSCA bispecific Abs to lyse PSCA+ tumor cells was compared with previously described murine anti-CD3–anti-PSCA (7F5) bispecific Abs, which were cloned from the anti-PSCA mAb 7F5 (30). For this purpose, preactivated T cells were cocultured with chromium-labeled PSCA+ PC3-PSCA tumor cells (E:T ratio of 20:1) in the presence of humanized scBsTaFv CD3-PSCA(MB1), murine scBsTaFv PSCA(MB1)-CD3, murine scBsDb CD3xPSCA(7F5), or murine scBsTaFv CD3-PSCA(7F5) in different concentrations. The average of specific lysis and the SEM are shown (x, data not determined). Data represent one donor out of three.

According to Fig. 2A, the humanized scBsTaFv CD3-PSCA (MB1) mediated a significant specific tumor cell lysis at E:T ratios of 1:1, 2:1, 5:1, 10:1, and 20:1. In addition, prestimulated T cells and naturally PSCA+ bladder cancer cells (RT4, HT-1376) or PSCA+ PC3 wt cells (E:T ratio of 20:1) were cultured with or without 30 pmol/ml humanized scBsTaFv CD3-PSCA(MB1) or the control humanized scBsTaFv CD3-5B9. (B) In addition, prestimulated T cells and naturally PSCA+ bladder cancer cells (RT4, HT-1376) or PSCA+ PC3 wt cells (E:T ratio of 20:1) were cultured with or without 30 pmol/ml humanized scBsTaFv CD3-PSCA(MB1) or the control humanized scBsTaFv CD3-5B9. After 20 h of incubation, the chromium release was measured and the specific tumor cell lysis was calculated. Data are presented as mean ± SD of three (A) or five (B) individual donors, respectively (**p < 0.001 with respect to no Ab and humanized control scBsTaFv CD3-5B9, one-way ANOVA with Bonferroni multiple-comparison test).
tios between 1:1 and 20:1. Tumor cell lysis is strictly dependent on T cell cross-linkage to the target cell as: 1) the humanized scBsTaFv CD3-5B9 was not able to redirect preactivated T cells to PC3-PSCA tumor cells (Fig. 2A), and 2) the humanized scBsTaFv CD3-PSCA(MB1) was not able to mediate the killing of PSCA− PC3wt cells (Fig. 2B). These data show that the expression of the target Ag is necessary for the induction of target cell lysis.

**FIGURE 3.** Activation of CD4+ and CD8+ T cells dependent upon their cross-linkage with PSCA+ tumor cells via humanized scBsTaFv CD3-PSCA (MB1). Unstimulated CD4+ or CD8+ T cells were cocultured with PSCA+ PC3-PSCA tumor cells (PSCA+) without any Ab (transparent graph with dashed line), in the presence of the negative control humanized scBsTaFv CD3-5B9 (transparent graph with gray line) or the humanized scBsTaFv CD3-PSCA (MB1) (transparent graph with black line). In addition, PSCA− PC3 wt cells were incubated in the presence of T cells with or without humanized scBsTaFv CD3-PSCA(MB1) as control. After 5 and 20 h of incubation, the cells were stained with anti-CD25/PE, anti-CD69/PE-Cy5, and anti-CD4/FITC, or anti-CD8/FITC mAbs. The expression of the activation markers CD69 (A) and CD25 (B) on CD4+ or CD8+ T cells was analyzed by flow cytometry. IgG isotype controls were used to define gates. In case of CD4+ T cells, marker M1 was set to CD25high cells. The mean fluorescence intensity and the percentage of the CD4+ or CD8+ T cells stained positive for CD69 or CD25 on their cell surface are shown in the M1 region in the histograms. Data shown were obtained from one donor and are representative of data from four donors.
Furthermore, we could demonstrate that humanized scBsTaFv CD3-PSCA(MB1) can specifically recruit T cells for efficient killing of various bladder carcinoma cell lines that naturally express PSCA on their cell surface (Fig. 2B).

Taken together, we developed novel murine and humanized bispecific anti-PSCA–anti-CD3 Abs. Both the murine scBsTaFv PSCA(MB1)-CD3 and the humanized scBsTaFv CD3-PSCA (MB1) efficiently redirect T cells to kill PSCA-expressing tumor cells in an Ag-specific manner. Interestingly, the humanized version of the murine scBsTaFv PSCA(MB1)-CD3 had slightly improved killing capabilities. The humanized scBsTaFv CD3-PSCA (MB1) efficiently redirected T cells to tumor cells even at a fem-
tomolar concentration and, thus, at an almost thousand-fold lower concentration than the previously described bispecific Abs based on the anti-PSCA mAb 7F5.

**Activation and cytokine release of CD4+ and CD8+ T cells dependent upon their cross-linkage with PSCA+ tumor cells by humanized scBsTaFv CD3-PSCA(MB1)**

In addition, the ability of humanized scBsTaFv CD3-PSCA(MB1) to activate CD4+ or CD8+ T cells in a target-dependent manner was analyzed in a T cell activation assay. Unstimulated CD4+ or CD8+ T cells from healthy donors were cocultured with PSCA-expressing PC3-PSCA tumor cells either in the absence of any Ab or in the presence of the humanized scBsTaFv CD3-PSCA(MB1). As a negative control, the humanized scBsTaFv CD3-5B9 was used. In addition, PC3 wt cells that do not express PSCA on the cell surface were incubated with or without the humanized scBsTaFv CD3-PSCA(MB1) in the presence of CD4+ or CD8+ T cells. After 5 and 20 h of incubation, the surface expression of the activation markers CD69 and CD25 was measured by FACS analysis. A significant portion of CD4+ T cells isolated from human donors was found to have a low-to-intermediate expression of CD25 per se. For analysis of the CD25 upregulation on CD4+ T cells, we therefore defined only the CD4+CD25high T cell population as CD25+ T cells. CD4+CD25mid T cells were considered as CD25− cells. According to the results shown in Fig. 3, the humanized scBsTaFv CD3-PSCA(MB1) induced an upregulation of both activation markers on CD4+ as well as on CD8+ T cells after 20 h. The expression of the early activation marker CD69, but not of the late activation marker CD25, was already increased after 5 h (Fig. 3A, 5 h). In contrast, CD69 and CD25 were not upregulated on T cells in the presence of the humanized control scBsTaFv CD3-5B9 or in case PSCA+ PC3 tumor cells were used as target cells (Fig. 3). The representative data for one of four donors are shown in Fig. 3.

To analyze whether the cross-linkage of CD4+ or CD8+ T cells with PSCA+ tumor cells via the humanized scBsTaFv CD3-PSCA(MB1) leads to the production of proinflammatory cytokines, the concentration of TNF and IFN-γ was determined in the supernatants of the T cell activation assay by ELISA. In general, the humanized scBsTaFv CD3-PSCA(MB1) mediated the release of TNF and IFN-γ from CD4+ and CD8+ T cells in the presence of PC3-PSCA tumor cells, but the estimated amounts of released TNF or IFN-γ substantially differed between different donors (Fig. 4A, donors 1–3). Furthermore, it is shown (Fig. 4A) that CD8+ T cells from donors 1 and 2 released more TNF and IFN-γ than the corresponding CD4+ T cells, whereas CD4+ T cells dominated the cytokine release in donor 3.

To analyze whether secreted TNF originates from T cells or tumor cells, we additionally measured intracellular TNF production (Fig. 4B). As shown by intracellular cytokine staining, TNF was only detectable in CD4+ and CD8+ T cells after cultivation with PC3-PSCA cells in the presence of humanized scBsTaFv CD3-PSCA(MB1) (Fig. 4B). Moreover, we could demonstrate that Ab-mediated induction of TNF expression is less pronounced compared with TNF production after unspecific stimulation of T cells with aCD3/aCD28 beads. In any case, there was no cytokine production in the presence of the control humanized scBsTaFv CD3-5B9 (Fig. 4A, donors 1–3; Fig. 4BI, PC3-PSCA) or PSCA− tumor cells representatively shown for one donor (Fig. 4A, donor 3, wt; Fig. 4BII, PC3wt).

According to these results, the humanized scBsTaFv CD3-PSCA (MB1) induced the activation of CD4+ and CD8+ T cells in the presence of PSCA-expressing tumor cells as shown by the up-regulation of CD69 and CD25, and the release of TNF and IFN-γ. As indicated by intracellular TNF staining, secreted TNF originates from Ab-activated T cells (Fig. 4B). T cell activation and cytokine release strictly depend on the presence of PSCA on the surface of the target cells and cross-linkage of effector and tumor

**FIGURE 5.** Redirection of CD4+ or CD8+ T cells to kill PSCA+ tumor cells by humanized scBsTaFv CD3-PSCA(MB1). In a chromium release assay, unstimulated CD4+ or CD8+ T cells were cultured with PSCA+ PC3-PSCA tumor cells in the presence or absence of the control humanized scBsTaFv CD3-5B9 or the humanized scBsTaFv CD3-PSCA(MB1) at an E:T ratio of 10:1 for 5 (A) and 20 h (B). In addition, PSCA− PC3 wt cells were incubated with or without the humanized scBsTaFv CD3-PSCA(MB1) in the presence of T cells as a control. The average of specific tumor cell lysis and SD of three different donors are shown (***p < 0.001, no Ab and humanized control scBsTaFv CD3-5B9; ##p < 0.01, Ab-mediated specific lysis induced by CD4+ compared with CD8+ T cells, one-way ANOVA with Bonferroni multiple-comparison test).
cells via the humanized scBsTaFv CD3-PSCA(MB1). Thus, an Ag-unspecific T cell activation or cytokine release was not observed in the in vitro assays.

**Ag-specific killing of PSCA$^+$ tumor cells by CD4$^+$ and CD8$^+$ T cells via humanized scBsTaFv CD3-PSCA(MB1)**

By performing a chromium release assay, we investigated whether the humanized scBsTaFv CD3-PSCA(MB1) can also trigger unstimulated CD4$^+$ or CD8$^+$ T cells to lyse PSCA$^+$ tumor cells. The average of specific tumor cell lysis of three different donors is shown in Fig. 5. According to the obtained data, CD8$^+$ and CD4$^+$ T cells were able to kill PC3-PSCA tumor cells via humanized scBsTaFv CD3-PSCA(MB1) with similar efficiency. After 20 h of incubation at an E:T ratio of 10:1, both CD4$^+$ and CD8$^+$ T cells mediated a significant tumor cell lysis of 67 and 65%, respectively (Fig. 5B), but obviously CD8$^+$ T cells reacted earlier than CD4$^+$ T cells (Fig. 5A). After 5 h of incubation, killing effects of CD4$^+$ and CD8$^+$ T cells differed significantly in presence of humanized scBsTaFv CD3-PSCA(MB1). CD8$^+$ T cells already killed, on average, 19% of the tumor cells, whereas almost no specific lysis was determined in the presence of CD4$^+$ T cells (Fig. 5A). Upon incubation of T cells with PC3-PSCA tumor cells in the presence of the humanized scBsTaFv CD3-5B9, no specific tumor cell lysis was observed. Furthermore, PSCA$^-$ PC3 wt cells were not eliminated by both T cell subpopulations in the presence of the humanized scBsTaFv CD3-PSCA(MB1).

To analyze whether the killing mechanism correlates with the degranulation of T cells, we measured the exposure of the granular membrane protein CD107a on the surface of CD4$^+$ and CD8$^+$ T cells by flow cytometry. After 20-h incubation of T cells and PC3-PSCA tumor cells with humanized scBsTaFv CD3-PSCA(MB1), an increase of CD107a$^+$ T cells was detected in comparison with incubation in the absence of any Ab or the presence of the humanized scBsTaFv CD3-5B9 control Ab (Fig. 6B). Under these conditions, no striking differences between CD4$^+$ and CD8$^+$ T cells were observed (Fig. 6B, scBsTaFv CD3-PSCA(MB1)). At an earlier time point (5-h cocultivation), however, more CD8$^+$ T cells presented the degranulation marker on their cell surface than CD4$^+$ T cells (Fig. 6A, scBsTaFv CD3-PSCA(MB1)). In contrast, upon the incubation of T cells and PC3-PSCA tumor cells with the humanized scBsTaFv CD3-5B9 control Ab, the CD107a signal did not increase in comparison with the control without any Ab (Fig. 6).

Thus, both redirected CD8$^+$ and CD4$^+$ T cells are activated by cross-linkage with target cells via the bispecific Ab, and both types of redirected T cells can mediate the lysis of the tumor cells.

**FIGURE 6.** Degranulation of CD4$^+$ and CD8$^+$ T cells after their cross-linkage with PSCA$^+$ tumor cells via humanized scBsTaFv CD3-PSCA(MB1). Unstimulated CD4$^+$ or CD8$^+$ T cells were cultured with PSCA$^+$ PC3-PSCA tumor cells in the presence or absence of the control humanized scBsTaFv CD3-5B9 or the humanized scBsTaFv CD3-PSCA(MB1). After 5 (A) or 20 h (B) of incubation, the degranulation marker CD107a was analyzed on the cell surface of T cells. All samples contained anti-CD107a/PE mAbs. Before measurement of the cells in the MACSQuant Analyzer, the cells were stained with anti-CD4/VioBlue, anti-CD8/PE-Cy5, and anti-CD3/FITC mAbs. The dot plots and the percentage of CD4$^+$ or CD8$^+$ T cells expressing CD107a on their cell surface are shown. One representative donor out of three is shown.
However, the onset of killing mediated by CD4+ T cells is significantly delayed. For both CD8+ and CD4+ T cells, the killing capability correlates with the occurrence of the degranulation marker CD107a on their cell surface as shown for one representative donor (Fig. 6).

**Antitumor effect of humanized scBsTaFv CD3-PSCA(MB1)** in vivo

The antitumor effect of humanized scBsTaFv CD3-PSCA(MB1) in vivo was analyzed in a xenotransplantation mouse model. Therefore, a mix of $3 \times 10^6$ PC3-PSCA tumor cells, $6 \times 10^6$ human PBMCs, and $10 \mu g$ humanized scBsTaFv CD3-PSCA(MB1) was injected s.c. at the right flank of eight mice (group 3 at day 0). Afterward (at days 1, 2, and 3), $10 \mu g$ humanized scBsTaFv CD3-PSCA(MB1) was injected into the tail vein of each mouse. As one negative control, an additional eight mice of group 2 were treated in the same way using the humanized scBsTaFv CD3-5B9 control Ab instead of the humanized scBsTaFv CD3-PSCA(MB1). Furthermore, at day 0, only $3 \times 10^6$ PC3-PSCA tumor cells were injected s.c. at the right flank of each mouse in group 1 to observe the tumor cell growth per se. The tumor growth was constantly measured and the tumor area was calculated. As shown in Fig. 7, the mice of group 3 that received PC3-PSCA cells, PBMCs, and humanized scBsTaFv CD3-PSCA(MB1) showed a significant reduction of tumor growth. In contrast, the tumors of the control mice treated with PBMCs and the humanized scBsTaFv CD3-5B9 control Ab (group 2) grew as well as the untreated ones (group 1).

**Discussion**

Although some patients with primary local prostate tumor can be successfully treated, half of the patients experience recurrence after local treatment. Especially for those 10–20% of patients with primarily advanced metastatic disease, no effective therapy options are available (35, 36). Therefore, novel strategies for PCa therapy are needed. One promising approach is the retargeting of T cells to PCa cells by bispecific Ab fragments.

For retargeting of T cells to PCa+ PCa, we established novel bispecific anti-CD3–anti-PSCA Abs that were derived from the novel mouse anti-human PSCA mAb MB1 and the previously described mouse anti-human CD3 mAb MT-301. With regard to clinical application, the murine anti-CD3(MT-301) and the novel murine anti-PSCA(MB1) domain were successfully humanized. According to size exclusion, chromatography-purified Ab fractions mainly comprise the monomeric form of the humanized scBsTaFv CD3-PSCA(MB1). Even after storage of 6 mo at 4°C, only a small portion of the bispecific Ab had aggregated. Interestingly, the humanized scBsTaFv CD3-PSCA(MB1) is even slightly superior over the murine scBsTaFv PSCA(MB1)-CD3 counterpart and able to efficiently redirect T cells for killing of PSCA-expressing tumor cells at femtomolar concentrations. It remains currently open whether this improvement of efficacy is a result of the humanized sequence or caused by the minor differences in the Ab format of the humanized and murine versions.

T cell activation and redirected tumor cell lysis is strictly Ag dependent: the cross-linkage of T cells and PSCA+ tumor cells by humanized scBsTaFv CD3-PSCA(MB1) is a prerequisite for T cell activation as shown by the upregulation of CD69 and CD25, the release of TNF and IFN-γ, and the killing of tumor cells. As shown previously, PSCA expression varies and is downregulated in cultured PCa cell lines. To have cells with a standardized level of PSCA expression, we, therefore, used in some of our experiments PSCA-overexpressing cell lines. However, we could also demonstrate that T cells redirected by the humanized scBsTaFv CD3-PSCA(MB1) were capable of killing naturally PSCA-expressing tumor cells.

In contrast with our previous data (30), in this study, we observed that not only CD8+ T cells but also CD4+ T cells were redirected by bispecific Abs. It is, however, important to mention that the onset of killing mediated by CD4+ T cells is delayed. After a 5-h incubation with Ag-expressing target cells, redirected CD8+ T cells showed expression of the degranulation marker CD107a on the surface and substantial cytotoxic activity, whereas no CD107a was detected on redirected CD4+ T cells nor any killing observed after this time period. After a longer incubation time (20 h), however, both redirected T cell subpopulations efficiently lysed PSCA+ target cells. According to our data, there is no obvious correlation between the killing efficiency of redirected T cells and the amount of cytokines released after their cross-linkage with tumor cells: 1) after an activation period of 5 h, the expression level of the activation marker CD69 is even higher on CD4+ than on CD8+ T cells. In contrast, their contribution to redirected tumor cell lysis is marginal. 2) Furthermore, the amounts of cytokines released differed dramatically between different donors; however, T cells from all donors efficiently lysed the target cells. 3) In addition, the utilization process slightly improved the killing efficiency of the bispecific Ab, whereas the amounts of cytokines released tend to decrease in comparison with its murine counterpart.

It is known that also tumor cells are able to produce and secrete soluble TNF. However, it is highly unlikely that TNF measured in supernatants after culturing tumor and T cells in the presence of humanized scBsTaFv CD3-PSCA(MB1) originates from PC3-PSCA cells. First, the intracellular staining of CD4+ and CD8+ T cells revealed that only the Ab-mediated cross-linkage with PSCA+ tumor cells specifically induces the TNF production in both T cell subpopulations. Second, no TNF was detectable in culture supernatants after incubation of T cells and PC3-PSCA cells without any Ab or with the humanized control Ab. Finally, the cross-linkage of T cells and tumor cells via a bispecific Ab that simultaneously targets PSCA and another cell surface marker on T cells (instead of CD3) did not result in any cytokine production (data not shown).

According to our FACS data, the cytotoxic activity of both types of redirected T cells correlates with their degranulation. For these reasons, we assume that the underlying killing mechanism
is related to the perforin-granzyme pathway. A delayed onset of tumor cell lysis by redirected CD4+ T cells and a similar killing mechanism was also proposed for the single-chain EpCAM/CD3-bispecific Ab construct MT110 and the CD19xCD3 bispecific Ab. It was shown that tumor cell lysis is mediated by the perforin-dependent, granule-exocytosis pathway but not by death ligands FasL, TRAIL, or TNF-α. In addition, we and others could visualize that cross-linkage of T cells and TAA-expressing tumor cells via anti-CD3–anti-TAA bispecific Abs results in the formation of cytolytic synapses before tumor cell lysis occurs (16, 42, 43). Taken together, our data indicate that both redirected CD8+ and CD4+ T cells are able to kill target cells most likely by the perforin/granzyme pathway. Although CD8+, but not CD4+, T cells contain preformed perforin/granzyme granules, CD4+ T cells have to produce them after activation and cross-linkage, which could explain their delay in killing.

One critical prerequisite for a clinical application of a bispecific Ab is that binding of the anti-CD3 domain to the CD3 complex of T cells alone does not lead to the activation of T cells and the release of cytokines. According to our data, the binding of the humanized anti-CD3 domain used in our bispecific Abs does not lead to an activation of T cells or a significant release of cytokines. This finding holds also true for experiments performed in the presence of high Ab concentrations of 5000 ng/ml (Supplemental Fig. 4). Significant amounts of cytokines were only released after cross-linkage of T cells to Ag-presenting target cells. Thus, in a clinical setting, cytokines should only be released locally and may not lead to a life-threatening systemic cytokine storm, but rather help to overcome the immunosuppressive environment in a tumor.

The high potential of the novel humanized anti-CD3–anti-PSCA (MB1) bispecific Ab as a novel immunotherapeutic drug for PCa therapy was also underlined by our in vivo data in a first mouse model, although these results have to be confirmed by future studies using improved orthotopic autologous animal models.

Mainly regulatory T cells (Tregs) promote the tumor progression by suppressing the antitumor immune response (44, 45). In fact, the accumulation of Tregs in tumor-bearing patients correlates with poor survival prognosis (46–48). According to our recently published data, anti-CD3–anti-TAA bispecific Abs are also able to activate Tregs in a target-dependent manner resulting in the suppression of proliferation and cytokine production of autologous CD4+ T effector cells (49). Thus, a systemic application of a bispecific Ab could lead to an activation of Tregs that could limit its antitumor effect. However, an antitumor effect of bispecific Abs mediated by whole WBC preparations was not only shown in animal models (including in our study) but was also impressively demonstrated after a systemic application of bispecific Abs in first clinical trials (23, 24). Thus, an activation of Tregs in tumor tissues must not necessarily be critical for a systemic application of bispecific Abs. As also supported by our data, CD8+ T cells are faster activated than CD4+ T cells. Consequently, Ab-redirected CD8+ T cells have a time slot to eliminate tumor cells before Tregs are activated and can suppress the antitumor immune response. Nonetheless, the impact of Tregs in the context of an immunotherapy based on T cells redirected via bispecific Abs needs further investigation, for example, whether an adoptive transfer of effector T cells that are preloaded with bispecific Abs ex vivo is superior over a systemic application of the bispecific Abs.

In summary, in this article, we provide experimental evidence including a xenograft mouse model that the humanized scBsTaFv CD3-PSCA(MB1) is capable of redirecting T cells to PSCA+ tumor cells in a strictly Ag-dependent manner. Both CD8+ and CD4+ T cells contribute to the tumor eradication most likely via the perforin/granzyme pathway. The onset of killing mediated by CD4+ T cells is delayed compared with CD8+ T cells. Because of its high efficacy, its target specificity, and its little, if any, risk for inducing systemic cytokine storms, the humanized scBsTaFv CD3-PSCA(MB1) is a promising candidate for the treatment of PCa or other PSCA-expressing tumors.

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Disclosures

M.B. and G.E. hold patents related to the bispecific Ab. The other authors have no financial conflicts of interest.

References

Supplementary Figure Legends

**FIGURE S1.** Development of novel anti-CD3-anti-PSCA bispecific antibodies for retargeting of T cells to PSCA-positive tumor cells, design of a control ab redirected to an irrelevant intracellular antigen, and humanization of murine variable ab domains. (A) Schematic gene structure of murine (mu) scBsTaFv PSCA(MB1)-CD3, humanized (hu) scBsTaFv CD3-PSCA(MB1), and hu scBsTaFv CD3-5B9. To allow secretion of recombinant proteins the Igκ leader sequence was fused to the N-terminus as signal peptide (SP). Recombinant abs were detected and purified via their myc-tag and 6xhistidine (his)-tag. The mu scBsTaFv PSCA(MB1)-CD3 consists of the variable domain of the heavy (V\textsubscript{H}) and light (V\textsubscript{L}) chain of the mouse anti-CD3 mab MT-301 and the mouse anti-PSCA mab MB1. These murine anti-CD3 and anti-PSCA(MB1) V\textsubscript{H} and V\textsubscript{L} sequences were humanized and used to clone the hu scBsTaFv CD3-PSCA(MB1). The control ab hu scBsTaFv CD3-5B9 contains the same humanized anti-CD3 domain and the humanized mouse anti-5B9 V\textsubscript{H} and V\textsubscript{L} sequences. (B) Schematic construction of humanized V\textsubscript{H} or V\textsubscript{L} sequences.

**FIGURE S2.** Expression and antigen binding of murine and humanized anti-CD3-anti-PSCA(MB1) bispecific antibodies. (A) The his-tagged ab fragments were permanently expressed by 3T3 cells and purified from cell culture supernatants via Ni-NTA affinity chromatography. After SDS-PAGE the purified proteins were stained with Coomassie-Brilliant-Blue (AI) or transferred onto a Western blot membrane and detected by an anti-his mab (AII). M, protein marker; 1, murine scBsTaFv PSCA(MB1)-CD3; 2, humanized scBsTaFv CD3-PSCA(MB1); 3, humanized scBsTaFv CD3-5B9. (B) For flow cytometry analysis pre-activated T cells (upper panel) or PC3-PSCA cells (lower panel) were stained with murine scBsTaFv
Humanized bispecific abs for treatment of prostate cancer

PSCA(MB1)-CD3, humanized scBsTaFv CD3-PSCA(MB1), or humanized scBsTaFv CD3-5B9 and the bound scBsTaFvs were detected via a FITC-conjugated anti-myc mab (black graphs). As negative control the cells were incubated with the secondary anti-myc/FITC mab alone (transparent graphs). The mean fluorescence intensity (MFI) and percentage of stained cells are shown in the M1 region of the histograms.

**FIGURE S3.** Size exclusion chromatography of the humanized scBsTaFv CD3-PSCA(MB1). To determine the amount of dimers and oligomers in purified ab fractions we performed size exclusion FPLC using a Superose 200 column. (A) Size exclusion profile of an ab fraction of the humanized scBsTaFv CD3-PSCA(MB1) that was stored for 6 months at 4 °C. (B) Calibration of gel filtration column was performed using the gel filtration marker kit for protein molecular weights 12,000-200,000 Da (Sigma-Aldrich Chemie GmbH). V0 (void volume) value was determined by dextran blue and Ve (elution volume) values of standard proteins were used to construct standard curve for estimation of protein sizes in the analyzed ab fraction. P1/2, protein 1/2.

**FIGURE S4.** Target cell dependent activation and cytokine release from T cells using high concentrations of humanized scBsTaFv CD3-PSCA(MB1). Non-stimulated T cells and PC3-PSCA cells were incubated alone (transparent graph with dashed line or white bars, respectively), with the control humanized scBsTaFv CD3-5B9 (transparent graph with grey line or grey bars, respectively) or the humanized scBsTaFv CD3-PSCA(MB1) (transparent graph with black line or black bars, respectively). Additionally, PSCA-negative PC3 wild type (wt) cells were cultured with T cells in the absence or presence of the humanized scBsTaFv CD3-PSCA(MB1). Co-cultivation of effector and target cells with 5000 ng/ml of recombinant antibody
was performed at an e:t ratio of 10:1 over a period of 24 hours. (A) T cells were analyzed by flow cytometry for expression of the activation markers CD25 and CD69. Mean fluorescence intensity (MFI) of total cells and percentage of CD25+ or CD69+ T cells in M1 region are shown. Marker was adjusted to appropriate IgG isotype control (grey graphs). Results of one representative donor of three are shown. (B) Release of pro-inflammatory cytokines by T cells was determined by ELISA of culture supernatants (x, not detectable). Data represent mean ± SD of three independent donors (n.s., not significant; ***p < 0.001 with respect to no ab and humanized control scBsTaFv CD3-5B9, one-way ANOVA with Bonferroni multiple comparison test).
Supplementary Figure S1

A

**mu scBsTaFv PSCA(MB1)-CD3**

\[
\begin{align*}
&V_H MB1 & V_L MB1 & V_H CD3 & V_L CD3 \\
&SP X_{10} & (G,S)_3 & X_{14} & (G,S)_3 & \text{myc his}
\end{align*}
\]

**hu scBsTaFv CD3-PSCA(MB1)**

\[
\begin{align*}
&V_H CD3 & V_L CD3 & V_H MB1 & V_L MB1 \\
&SP X_{5} & (G,S)_3 & X_{5} & (G,S)_3 & \text{myc his}
\end{align*}
\]

**hu scBsTaFv CD3-5B9**

\[
\begin{align*}
&V_H CD3 & V_L CD3 & V_H 5B9 & V_L 5B9 \\
&SP X_{5} & (G,S)_3 & X_{5} & (G,S)_3 & \text{myc his}
\end{align*}
\]

B

**murine \(V_H/V_L\)**

\[
\begin{align*}
&FWR1 & CDR1 & FWR2 & CDR2 & FWR3 & CDR3 \\
&\vdots & \vdots & \vdots & \vdots & \vdots & \vdots
\end{align*}
\]

**human \(V_H/V_L\)**

\[
\begin{align*}
&FWR1 & CDR1 & FWR2 & CDR2 & FWR3 & CDR3 \\
&\vdots & \vdots & \vdots & \vdots & \vdots & \vdots
\end{align*}
\]

**theoretical humanized \(V_H/V_L\)**

**overlapping oligos**

**humanized \(V_H/V_L\)**

\[
\begin{align*}
&FWR1 & CDR1 & FWR2 & CDR2 & FWR3 & CDR3
\end{align*}
\]

- searching for homologue human IgG1
- design of artificial humanized \(V_H/V_L\) sequence
- synthesis and annealing of oligos
- amplification of humanized \(V_H/V_L\)
Supplemental Figure S4

A

wt

PSCA

--- 27.4 (24.2%)

--- 23.6 (22.4%)

--- 34.9 (25.5%)

--- 27.6 (24.3%)

--- 377.5 (67.0%)

--- 4.5 (2.3%)

--- 4.4 (2.4%)

--- 5.0 (3.5%)

--- 4.8 (3.2%)

--- 110.0 (69.2%)

CD25

CD69

B

TNF release

IFNγ release

no ab

scBsTaFv CD3-5B9

scBsTaFv CD3-PSCA(MB1)

PC3-PSCA

PC3wt

PC3-PSCA

PC3wt