In Vivo Fluorescence Imaging of the Activity of CEA TCB, a Novel T-Cell Bispecific Antibody, Reveals Highly Specific Tumor Targeting and Fast Induction of T-Cell-Mediated Tumor Killing

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Abstract

Purpose: CEA TCB (RG7802, RO6958688) is a novel T-cell bispecific antibody, engaging CD3ε upon binding to carcinomaembryonic antigen (CEA) on tumor cells. Containing an engineered Fc region, conferring an extended blood half-life while preventing side effects due to activation of innate effector cells, CEA TCB potently induces tumor lysis in mouse tumors. Here we aimed to characterize the pharmacokinetic profile, the biodistribution, and the mode of action of CEA TCB by combining in vitro and in vivo fluorescence imaging readouts.

Experimental Design: CEA-expressing tumor cells (LS174T) and human peripheral blood mononuclear cells (PBMC) were cocultured in vitro or coengrafted into immunocompromised mice. Fluorescence reflectance imaging and intravital 2-photon (2P) microscopy were employed to analyze in vivo tumor targeting while in vitro confocal and intravital time-lapse imaging were used to assess the mode of action of CEA TCB.

Results: Fluorescence reflectance imaging revealed increased ratios of extravascular to vascular fluorescence signals in tumors after treatment with CEA TCB compared with control antibody, suggesting specific targeting, which was confirmed by intravital microscopy. Confocal and intravital 2P microscopy showed CEA TCB to accelerate T-cell–dependent tumor cell lysis by inducing a local increase of effector to tumor cell ratios and stable cross-linking of multiple T cells to individual tumor cells.

Conclusions: Using optical imaging, we demonstrate specific tumor targeting and characterize the mode of CEA TCB–mediated target cell lysis in a mouse tumor model, which supports further clinical evaluation of CEA TCB.

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connected through a short peptide linker and hence lack the IgG Fc domain, which results in short serum half-lives (ca. 2 hours in humans; refs. 3, 7). The necessity to administer blinatumomab via continuous intravenous infusion to maintain therapeutic serum levels over several weeks limits its application in cancer patients. To overcome such fast blood clearance rates, we have recently developed a novel TCB antibody format based on an engineered Fc region, which confers an extended serum half-life due to interaction with the neonatal Fc receptor (FcRn) while abrogating binding to complement components or Fc gamma receptors (FcγRs) through a novel P329G LALA mutation. The latter prevents side-effects resulting from TCB-mediated activation of immune effectors other than T cells (8). CEA TCB (RG7802, RO6958688), the most advanced representative of this novel class of TCbs, currently in phase I clinical trials (NCT02324257) for the treatment of carcinoembryonic antigen (CEA)-expressing solid tumors, mediates cross-linking of human carcinoembryonic antigen (CEA) on tumor cells, CEA TCB on in vivo tumor beds, resulting in the conversion of tumors to highly inflamed, PD-L1–expressing tissues (8). However, the pharmacokinetic as well as biodistribution profile of this molecule in mice and its in vivo mode of action remain largely undefined.

In vitro and in vivo fluorescence imaging approaches have become indispensable tools in pharmaceutical research for the establishment and characterization of novel therapeutic molecules. Allowing for longitudinal and quantitative monitoring of drug biodistribution by macroscopic imaging and therapeutic effects at cellular and subcellular resolution by microscopic readouts, optical in vivo imaging can provide information on the pharmacokinetic drug profile and insights into its mode of action in a target’s physiologic surrounding (10, 11).

In the work presented here, we employed fluorescence-based in vitro and in vivo imaging readouts to assess the pharmacokinetic profile and the mode of action of the novel CEA TCB.

### Materials and Methods

#### Cell culture and preparation of cells for in vitro and in vivo imaging

LS174T human colon carcinoma cells (ATCC CL 188), parental, modified to stably express red fluorescent protein (RFP) or firefly luciferase (FLuc), were cultured in DMEM, high glucose (Gibco/Life Technologies) supplemented with 10% FCS, sodium pyruvate (1 mmol/L; Gibco/Life Technologies), under standard cell culture conditions (37°C, 5% CO₂). In case of the fluorescent reporter cells, puromycin was added to the cell culture medium (3 μg/ml; Gibco/Life Technologies). A detailed description of the generation of the stable LS174T RFP and FLuc cells is found in Supplementary Methods.

Primary human T cells were isolated from peripheral blood mononuclear cells (PBMC), obtained from buffy coats (Blutspende Zurich) or from whole blood from healthy donors, by negative magnetic isolation using the Pan T-cell isolation kit II (Miltenyi Biotech) and stained with carboxyfluorescein succinimidyl ester (CFSE; 1, 5, or 10 μmol/L; Sigma Aldrich) in PBS at 37°C for 10 minutes. After the staining, cells were washed in complete cell medium twice before being mixed with LS174T RFP tumor cells at an effector-to-target (E:T) ratio as stated (in vitro confocal microscopy), respectively 5:1 (in vivo 2P imaging). For in vitro live cell imaging of T-cell mediated apoptosis, pSIVA (Abcam) was added to the culture medium according to the manufacturer’s instructions. For 2P intravital imaging studies, cells were re-suspended in RPMI (Gibco/Life Technologies) to a final concentration of 1 × 10⁶ tumor cells, 5 × 10⁶ CFSE-labeled T cells, and 5 × 10⁶ PBMCs (without T cells) per 30-μL injection volume.

#### Confocal imaging and data analysis

Confocal laser scanning microscopy was performed on a Zeiss LSM700 microscope. Images were acquired by sequential 3–4 channel scanning (2 fluorescence channels and transmission signal) using 405, 488, 553, and 639 nm lasers for excitation. Z-Stacks of 4- or 5-μm interslice distance were generated. For live cell imaging, different positions were assigned and sequentially imaged over 5, 24, or 48 hours. During imaging, cells were maintained under standard cell culture (37°C, 5% CO₂). Tumor cells undergoing apoptosis in the presence of CEA TCB or control therapy as indicated by pSIVA were counted using the Cell Counter plugin available from ImageJ and normalized to total tumor cell area. The time from initial T-cell/tumor cell contact to the induction of apoptosis was determined manually by observing the engagement of a T-cell to a tumor cell and measuring the time...
to tumor cell death on time lapse series. Apoptosis was recognized either by pSIVA or morphologic changes (rounding up of cells, cell fragmentation, increased cellular fluorescence intensities). Ten movies per condition were analyzed. The frequency of T cells bound per tumor cell 0 and 24 hours after therapy was determined manually using the Cell Counter plugin available from ImageJ.

**In vivo tumor models**

All animal protocols were approved by the Cantonal Veterinary Office in Zurich (license Roche). To establish LS174T/T cell cognoids for fluorescence reflectance imaging, 1 × 10^6 LS174T-FLuc cells were mixed with freshly isolated PBMCs at E:T ratios of 3:1 and subcutaneously injected into the flank of NOD mice (NOD/Shi-scid/IL-2Rγnull mice, 8 weeks, female, Taconic). Tumor sizes were monitored by caliper measurements and CFSE-labeled T cells, and 5 × 10^6 FLuc cells were mixed with freshly isolated PBMCs at E:T ratios of 3:1 and subcutaneously injected into the skinfold chamber (30-μL injection volume) in anesthetized animals, 24 hours after surgery. Animals were maintained under specific pathogen-free (SPF) conditions for the duration of the experiment.

**In vivo fluorescence reflectance imaging**

For *in vivo* fluorescence imaging, animals were gas-anesthetized using isoflurane (3% during anesthesia induction, 1% during imaging; Provet AG) and an oxygen–air mixture as carrier gas. Spectral imaging was performed in a fluorescence reflectance imaging system (IVIS Spectrum; Perkin Elmer) using the following excitation/emission filter pairs (605 nm/660 nm; 640 nm/680 nm; 640 nm/700 nm; 640 nm/720 nm; 640 nm/740 nm; 640 nm/760 nm; 640 nm/780 nm). After acquisition of a precontrast, baseline image, each animal was administered an intranasal injection of 50 μg of Alexa 647–labeled CEA TCB or Alexa 647–labeled control TCB. Images were acquired at 30 minutes, 1, 3, 5, 8, 24, 48, 72, and 96 hours after antibody injection. Mice were euthanized at the last imaging time point and tumors extracted for histologic analysis. Spectral unmixing was performed using the Living Image Software (Perkin Elmer) based on a spectral library including the Alexa 647 spectrum (obtained by imaging a solution of Alexa 647-labeled TCB dissolved in PBS (250 μg/mL)) and the tissue background spectrum [determined by measuring the fluorescence spectrum in a baseline animal in a region of interest (ROI) drawn on the back]. For analysis, ROIs were drawn in the tumor region, defined on bioluminescence images, acquired at the end of every imaging day by injection of n-luciferin (30 mg/kg; Perkin Elmer) and in the eye as indication of the blood serum level. Fluorescence intensities were further normalized to intensities measured in image background ROIs. Animals per experimental group were analyzed. For further analysis of extravascular versus vascular TCB concentrations in the tumor and eye, respectively, a mathematical modeling approach was employed as further described in Supplementary Methods.

**Intravital 2P imaging**

2P microscopy was performed on a Zeiss 710 combined confocal and multiphoton system using a 20× objective (Plan Apochromat, Zeiss, N/A = 1). To this end, mice were gas-anesthetized by isoflurane, placed on a heating pad and fixed onto the microscopic stage. During the time of an experiment, the body temperature of the experimental animal was monitored and kept constant at 37°C using a rectal temperature probe. Simultaneous excitation of CFSE, second harmonics, and Alexa 647 was achieved at an excitation wavelength of 880 nm while dsRed was excited at 760 nm. Sequential 3D stacks were acquired at step sizes of 5 μm. For the visualization of blood vessels, mice received an intravenous injection of A645-angioSense (Perkin Elmer; 2 nmol/L per animal) immediately before imaging. A detailed description of the image analysis is found in Supplementary Methods.

**Skinfold chamber surgery**

The skinfold chamber surgery was performed according to the procedures described by Koehl and colleagues (12). Briefly, NOG mice were anesthetized using isoflurane in combination with an air–oxygen mixture as carrier gas and placed on a heating pad. During the entire surgery, their body temperature was monitored through a rectal probe and kept constant at 37°C. After formation of a dorsal skinfold on the back of the mice undergoing surgery, a round piece of skin of approximately 10 mm diameter was removed from the first layer in the fold, leaving a facial plane with associated vasculature on the opposing side. The titanium frame of the skinfold chamber (AP Trading) was subsequently sutured onto the skin, tightening the dorsal window at the upper edge. Screws were then used to pierce the skinfold and fix the skinfold chamber onto the animal. After mounting screws and nuts, the superficial layer of fat was removed under the stereomicroscope using fine forceps. The exposed skin layer was rinsed with saline and finally a coverslip was fixed onto the chamber by a spanning ring thereby closing the window. Mice were allowed to wake under a warming infrared lamp.

**In vitro antibody binding**

Adherent tumor target cells were trypsinized (0.05% trypsin/EDTA; Gibco #25300096) and washed with PBS. Binding was assessed using 0.1 Mio cells/well of a 96-well round-bottom plate, and incubation with the indicated antibody concentrations for 30 minutes at 4°C. Cells were washed twice with cold FACS buffer (PBS, 0.5% BSA) and incubated with diluted goat anti-human IgG Fc–specific secondary antibody (Jackson Immuno Research, #109-096-098) for further 30 minutes at 4°C. Cells were washed twice with cold FACS buffer, resuspended in 150 μL cold FACS buffer/well, and analyzed using a BD FACS Fortessa equipped with FACS Diva. Data were analyzed using Flowjo software (Tree Star Inc.).

More information regarding experimental methods has been included in the Supplementary Data.

**Results**

CEA TCB induces tumor cell lysis within 24 hours and significantly reduces the time of T-cell–mediated cytotoxicity in *vitro*

To assess CEA TCB binding to the human CEA–expressing colon cancer cell line LS174T, the model utilized throughout this study (13), fluorescently (Alexa 647) labeled CEA TCB and nonbinding control TCB (comprising the same CD3 binder fused to two nontargeting Fv regions) (DP47 TCB) were added to LS174T cells at different antibody concentrations. Fluorescence intensities were measured by flow cytometry, revealing specific binding of CEA TCB from concentrations as low as 5 nmol/L with a mean effective concentration of binding (ECso) of 23.4 nmol/L. (Fig. 1A). No signal was detected for the nonbinding control TCB. In *vitro,
Figure 1.
CEA TCB increases the fraction of apoptotic tumor cells within 24 hours by accelerating tumor cell lysis and simultaneous engaging of multiple T cells by individual tumor cells. A, binding of fluorescently labeled (Alexa 647) CEA TCB or control TCB to tumor cells. CEA TCB EC_{50} = 23.4 nmol/L. Values, means ± SD. B, tumor cell lysis (LS174T) at 24, 48, and 72 hours of CEA TCB or control TCB treatment (different antibody concentrations were used) in the presence of pan-T cells (E:T ratio, 15:1) measured via the release of LDH. The following EC_{50} values were calculated for CEA TCB: EC_{50(24 h)} = 6.28 nmol/L, EC_{50(48 h)} = 1.41 nmol/L, EC_{50(72 h)} = 0.24 nmol/L. Values, means ± SD from triplicates. Nonlinear dose–response curve fits are shown. C, confocal imaging of tumor cell killing. CFSE-labeled T cells and LS174T RFP cells were plated at an E:T ratio of 3:1. While LS174T cells rapidly proliferated to confluence in the presence of control TCB, tumor cells treated with CEA TCB showed increased apoptosis. Scale bar, 50 μm. D, image series illustrating the dynamics of T-cell (unlabeled) mediated LS174T RFP tumor cell killing by CEA TCB (E:T = 1:1). pSIVA, an Annexin-based green fluorescent cell death probe, was used to detect tumor cell apoptosis [dual color cells (red/green)]. Scale bar, 20 μm. (Continued on the following page.)
LS174T tumor cell binding translated to efficient, time-dependent tumor cell killing in the presence CD3+ T cells (E:T = 15:1) as shown in cell killing assays (LDH release) performed at 24, 48, and 72 hours of T-cell and tumor cell coinoculation in the presence of CEA TCB or nonbinding TCB, respectively (Fig. 1B). This finding is not restricted to LS174T cells, but has also been shown for other tumor cells (ref. 8; Supplementary Fig. S1). Live cell confocal imaging of cocultures of LS174T cells, stably expressing the red fluorescent protein (RFP), and human pan T cells, freshly isolated from PBMCs and labeled with carboxyfluorescein succinimidyl ester (CFSE), at an E:T of 3:1, further confirmed a decrease in tumor cell number as well as CEA TCB–induced apoptosis (visualized through a caspase-3/7 imaging probe) as early as 16 hours after CEA TCB addition. In contrast, tumor cells cocultured with pan T cells in the presence of nonbinding control TCB rapidly grew to confluency (Fig. 1C; Supplementary Videos S1 and S2). In CEA TCB–treated cocultures, T-cell binding was observed to lead to tumor cell death within only a few hours as illustrated by the image series shown in Fig. 1D. In line with a CEA TCB–mediated T-cell cytotoxic activity dependent on tumor cell/T-cell cross-linking, the lymphocyte activation markers CD69 and CD25 were upregulated on T cells 48 hours after CEA TCB, but not upon nonbinding control TCB addition on CD4+ and CD8+ T cells in the presence of tumor cells (Fig. 1E and Supplementary Figs. S1C, S1D, and S2). When comparing the efficiency by which different T-cell populations (CD3+, CD8+, and CD4+) mediated tumor cell (MKN45) lysis in the presence of either CEA TCB or nonbinding control TCB, we found a significant contribution of both the CD4+ Th and CD8+ T killer cells, almost 40% CD8+ cells were faster in mediating the cytotoxic activity (Supplementary Fig. S3). The highest cytotoxic activity of CEA TCB towards MKN45 cells was however, observed in the presence of pan CD3+ T cells (including both CD8+ and CD4+ T-cell subsets). Quantification of tumor apoptosis by means of pSIVA, an Annexin-binding dye, which fluoresces only when bound to apoptotic cells showed a significantly increased fraction of apoptotic tumor cells 24 and 48 hours after CEA TCB addition (Fig. 1F) at an E:T ratio of 1:1. Further analysis of the lag time from initial T-cell to tumor cell contact to apoptosis (Fig. 1G) demonstrated CEA TCB to decrease the lag time to apoptosis by almost 50%, suggesting that TCBS shorten the T-cell to tumor cell interaction time required for target cell death induction (Fig. 1G). Moreover, when analyzing the number of T cells associated with an individual tumor cell at 0 and 24 hours after antibody addition, we observed an increased frequency of tumor cells bound to 2 or 3 T cells at later time points after CEA TCB, but not control TCB treatment (Fig. 1H). In conclusion, these data show that CEA TCB binds specifically to CEA-positive LS174T tumor cells, triggers the activation of T cells, mediates cross-linking of multiple T cells to an individual tumor cell, and decreases the time from initial T-cell contact to tumor cell apoptosis, which translates into accelerated T-cell–dependent tumor cell lysis.

CEA TCB specifically targets CEA-expressing tumor cells in vivo

In a next step, we assessed CEA TCB binding to LS174T tumors in vivo. To this end, a single dose of 2 mg/kg Alexa 647–labeled CEA or nonbinding control TCB was intravenously applied to experimental animals, carrying a subcutaneous LS174T/huPBMC cograft in their flank. Fluorescence reflectance images of animals lying on the side were acquired before and at different time points after antibody injection up to 96 hours postinjection (Fig. 2A). Signal intensities were quantified by spectral unmixing in regions of interest (ROI) placed in the eye, reflecting the antibody serum concentration as described before (14), and encompassing the tumors (Fig. 2B). To separate extravascular and vascular components in the tumor region, we employed a mathematical modeling approach (described in detail in the Materials and Methods section). Deconvolution of the extravascular and vascular components in the tumor region revealed higher ratios of extravascular to vascular signals in tumors of mice treated with the targeted CEA TCB (Aint/Aext = 9.07) than with the nonbinding control TCB (Aint/Aext = 3.38) construct suggesting the former to specifically bind to tumor cells (Fig. 2B). The SE of the estimate for these two ratios was calculated smaller than 20%. Plasma elimination half-lives of the two constructs were determined to be 30.1 and 62.4 hours for the targeted CEA TCB and the nonbinding control TCB, respectively. Likely, this difference can be attributed to target-mediated drug disposition, thus providing further evidence for specific targeting of CEA TCB. The half-life for vascular–extravascular exchange was found to be similar for the two constructs, of the order of 15 hours.

Specific tumor targeting was further confirmed by intravital 2P microscopy in LS174T RFP/huPBMC cografts subcutaneously injected into dorsal skinfold windows sutured onto NOG mice and ex vivo by confocal/epifluorescence microscopy on tumor tissue sections from LS174T RFP/huPBMC (labeled T cells) cografts. Intravenous injection of Alexa 647–labeled CEA TCB and nonbinding control TCB antibodies showed a specific, tumor cell–associated fluorescent A647 signal 24 hours after antibody injection only in case of CEA TCB, but not after control TCB injection (Fig. 2C–E). Only a minor A647 fluorescent signal was detected after labeled nonbinding control TCB injection (Fig. 2C and Supplementary Fig. S4). The labeled CEA TCB antibody signal was mostly detected on the cell surface of tumor cells, but to a certain degree also intracellularly, indicating some internalization within 24 hours in vivo (Fig. 2C–E). Higher magnification confocal images as well as z-stacks and 3D projections thereof, further revealed cross-linking of CFSE-positive T cells to CEA TCB–labeled tumor cells (Fig. 2D and E; Supplementary Videos S3 and S4). Thus, compared to the non-binding control TCB, CEA TCB specifically binds to CEA-expressing tumor cells and shows engagement of T cells in vivo.

Continued)

E, analysis of CD69, an early lymphocyte activation marker, on CD4+ and CD8+ T-cell populations by FACS, 48 hours after incubation of T cells with LS174T in the presence of CEA TCB or control TCB, respectively. E:T ratio = 15:1. Values, means ± SD from triplicates. Nonlinear dose–response curve fits are shown. F, quantification of apoptotic tumor cells. CFSE-labeled T cells and LS174T RFP cells were plated at an E:T ratio of 1:1, treated with the apoptotic marker pSIVA and imaged at 24 and 48 hours after CEA TCB treatment. Values shown as means ± SEM. Statistical analysis was done by Mann–Whitney test. Ten movies from four independent experiments were analyzed for each treatment condition. G, analysis of the time from initial T-cell engagement to tumor cell killing after CEA or control TCB treatment of LS174T RFP cells in the presence of T cells (E:T ratio = 3:1). Statistical analysis was done by Mann–Whitney test. Ten movies from four independent experiments were analyzed for each treatment condition. H, frequency distribution of tumor cells showing simultaneous binding of 1, 2, 3, or 4 T cells at 0 or 24 hours after CEA or control TCB addition. E:T ratio = 3:1.
Figure 2.
CEA TCB shows specific tumor cell targeting in vivo. A, fluorescence reflectance images acquired at baseline and 8, 48, and 96 hours after intravenous administration of fluorescently labeled (Alexa 647) CEA TCB or nonbinding control TCB using a LS174T/huPBMC cografting tumor model. Three animals per group were analyzed. B, quantification of fluorescence signal intensities and simulations thereof for region of interests in the eye, representing serum blood levels and tumor. Simulation of the extravascular-vascular ratio within the tumor region (right y-axis on the last graph) reveals specific accumulation of CEA TCB but not of the nonbinding control TCB in the tumor region. BG, background. C, confirmation of CEA TCB binding to tumor cells by intravital multiphoton microscopy after intravenous injection of fluorescently labeled (Alexa 647) nonbinding control- or CEA TCB in mice-bearing LS174T/huPBMC cografts subcutaneously injected into the dorsal skinfold chamber. Scale bar, 100 μm (overviews) and 20 μm (high magnification insets). Arrowheads mark tumor cells showing membrane-bound CEA TCB. Maximum intensity projections are shown. D and E, analysis of CEA TCB binding by ex vivo confocal imaging on tumor sections obtained from LS174T RFP/huPBMC (CFSE-labeled T cells) bearing mice, 24 hours after fluorescently labeled CEA TCB injection. Higher magnification images show CEA TCB-mediated cross-linking of tumor and T cells. Scale bars, 10 μm. Maximum intensity projections are shown.
CEA TCB leads to a drastic increase in tumor-associated T cells and induces tumor cell death within 24 hours in vivo.

For the assessment of the CEA TCB activity in vivo, LS174T RFP cells and human PBMCs containing CFSE-labeled T cells (E:T 5:1) were implanted subcutaneously into the dorsal skinfold chamber as outlined in Fig. 3A. 2P microscopic imaging was started 4 days after tumor crograft inoculation, before intravenous injection of a single dose of 2 mg/kg CEA or nonbinding control TCB, respectively (Fig. 3B; Supplementary Video S5, baseline). Mosaic images of tumor cells and T cells acquired at baseline showed no significant difference between mice. However, 24 hours after antibody application, an increased density of T cells within and surrounding the tumor of mice treated with CEA TCB, but not the non-binding control TCB was observed (Fig. 3B, 24 hours). Moreover, CEA TCB treatment led to an increased number of fragmented, round, high intensity cells detected after 24 hours, suggestive of tumor cell apoptosis.

Figure 3.
CEA TCB significantly increases the number of tumor-infiltrated T cells and tumor cell apoptosis in vivo. A, outline of the experimental procedure for the 2P microscopic imaging studies in the skinfold chamber mouse model. B, representative 2P microscopic images acquired in skinfold chamber-bearing NOG mice after subcutaneous injection of huPBMC (containing CFSE-labeled T cells)/LS174T RFP crografts (T-cell to tumor cell ratio, 5:1). The same animals were analyzed at baseline and 24 hours after CEA TCB or nonbinding control TCB treatment. Scale bar, 100 μm (top) and 50 μm (bottom). C, quantification of the T-cell to tumor cell ratio in the microscopic field of views, the tumor T-cell infiltration, and the number of apoptotic events before and after TCB treatment. Values are shown as means ± SEM. Statistical analysis was done by paired t test. Images from the following number of animals were analyzed: baseline conditions, 3 animals control TCB, 4 animals CEA TCB; 24 hours after treatment, 2 animals control TCB, 4 animals CEA TCB.
increased tumor cell apoptosis (Fig. 3B; Supplementary Video SS). Quantitative analysis of the T-cell to tumor cell ratio and the T-cell–positive area fraction confirmed increased tumor-associated infiltration of T cells 24 hours after CEA TCB administration (Fig. 3C). The increased number of tumor-associated T cells translated to an increased frequency of apoptotic events within the tumor mass as determined by morphologic changes associated with tumor cell apoptosis (Fig. 3B and C). In conclusion, CEA TCB treatment triggered a strong increase in T-cell number present within the tumor mass 24 hours after antibody injection which correlated with a higher frequency of tumor cell apoptosis.

CEA TCB promotes T-cell/tumor cell interactions and leads to long-term engagement of multiple T cells by individual tumor cells in vivo

To further explore the in vivo mode of action of CEA TCB and to validate the in vitro finding that CEA TCB treatment leads to increased numbers of T cells cross-linked to a single tumor cell, we analyzed time-lapse videos for the maximal number of T cells simultaneously bound to an individual tumor cell. In line with the in vitro results, we found a substantial fraction of tumor cells, showing parallel interactions with 2 or more T cells simultaneously after CEA TCB treatment. In contrast, after nonbinding control TCB application, a majority of tumor cells (60%) showed interaction with only one T cell. When assessing the number of T cells, long-term engaged (>15 minutes) by a single tumor cell, there was a higher frequency of tumor cells stably binding more than one T cells 24 hours after CEA TCB application compared with baseline conditions or after treatment with nonbinding control TCB (Fig. 4A–C). When analyzing the average T-cell movement by automated analysis, we observed an increased speed after CEA TCB treatment (Fig. 4D). Particularly, CEA TCB increased the motile fraction of cells, showing speeds higher than 5 μm/minute. Consistently, T cells of CEA TCB–treated mice also showed an increased persistence index, that is, better maintained the directionality of their motion for the duration of the trajectories analyzed compared with T cells in tumors of control TCB–treated mice (Supplementary Fig. S5). Further suggesting a shift towards longer and more numerous T-cell interactions, analysis of the total fraction of time T cells spent interacting with cancer cells showed a significant increase 24 hours after CEA TCB treatment compared with the control TCB condition (Fig. 4E). Hence, CEA TCB–mediated tumor cell lysis is associated with an overall increase in cancer/T-cell interactions, an increased maximal number of T cells simultaneously engaged by an individual tumor cell, as well a shift towards long-term engagement of more than one T cell by single tumor cells, which, in a combined manner, sensitizes tumor cells to T-cell–mediated killing.

Discussion

Fluorescence imaging is widely used for preclinical characterization and validation of newly developed anticancer therapies. Whole-body fluorescence reflectance imaging allows noninvasive and longitudinal assessment of drug biodistribution as well as pharmacokinetic profiles with high sensitivity and temporal resolution in a cost-efficient manner (15–17). Complementing macroscopic imaging, intravital multiphoton microscopy has to-date become an important tool for the study

the in vivo mode of action of therapeutic molecules such as antibodies at cellular and subcellular resolution within the tissue physiologic context (18). In the current study, we have combined macroscopic and microscopic fluorescence imaging readouts for further characterization of the biodistribution and pharmacokinetic properties as well as the mode of action of CEA TCB, a novel T-cell bispecific antibody, which engages T cells via binding to CD3, in a polyclonal fashion independent of antigen specificity of the recruited T cells.

Fluorescence reflectance imaging performed at time points from 0 to 96 hours after intravenous injection of fluorescently labeled CEA- or nonbinding control TCB into mice-bearing tumor cell/PBMC co grafts combined with mathematical modeling for analysis of the specific extravascular signal contribution within the tumor area, revealed highly specific targeting of CEA TCB to CEA-expressing tumor cells, but no targeting by the nonbinding control TCB within 24 hours. In addition, CEA TCB was shown to cross-link T cells to tumor cells in vivo. As expected from the inclusion of an Fc region, the half-life of the TCB described here was determined to be significantly longer than the one estimated for blinatumomab in mice (30 hours vs. estimated 2 hours; ref. 19), confirming one of the major advantages of this novel TCB construct, currently in clinical trials. Although the nonbinding control antibody showed higher blood serum levels and a decreased blood clearance rate compared with CEA TCB [most likely due to CEA-dependent target-mediated drug disposition (TMDD) of CEA TCB] the former did not target the tumor specifically as determined by the extravascular tumor vascular concentration ratio within tumor areas and by 2P acquisitions of tumor cells. This suggests CEA TCB accumulates in the tumor mainly through CEA targeting with only minor contributions from CD3 binding in the cografting model employed throughout our studies due to its low monovalent affinity for CD3ε.

To gain insight into the mode of action of CEA TCB, we performed in vitro cell assays and live cell confocal microscopy of cocultures of LS174T tumor cells and T cells and in vivo multiphoton microscopy of LS174T/huPBMC co grafts in mice. In vitro killing assays showed CEA TCB–dependent T-cell activation and T-cell–mediated tumor cell lysis 24 hours onward, which is in line with studies performed with T-cell engagers of the BiTE class targeting solid tumor antigens (20). In line with earlier studies showing increased T-cell velocities upon activation (21), our in vivo results show CEA TCB to significantly increase the average speed of T cells. Although both CD4+ and CD8+ T cells were found to be involved in the CEA TCB–mediated cytotoxicity, CD8+ proved to be more efficacious than CD4+ T cells in killing of tumor cells. However, the highest killing rates were observed in the presence of pan T-cells composed of both CD8+ and CD4+ T-cell subsets. Interestingly, we observed a higher frequency of tumor cells bound to multiple T cells 24 hours after CEA TCB treatment compared with control conditions, indicating this antibody to accelerate tumor cell lysis by the induction of simultaneous binding of multiple T cells to one specific target cell. Indeed, analysis of long-term (>15 minutes) T-cell engagement by tumor cells in vivo by means of multiphoton microscopy, confirmed this shift to a higher fraction of tumor cells showing stable interactions with multiple T cells. In line with this finding, the maximum number of T cells found to simultaneously interact with a single tumor cell was also increased after...
CEA TCB treatment in vivo. Binding of multiple T cells by one individual target cell has been described before and linked to faster target killing rates (22–24). The observed CEA TCB induced shortening of the time from initial T-cell engagement to tumor cell apoptosis, may, in addition, also result from very efficient stimulation of CD3/TCR downstream signaling after TCB-mediated cross-linking of tumor and T cells described before (8).

Hoffmann and colleagues report the BiTE molecule blinatumomab to exert so-called serial killing, that is, to induce T cells to sequentially kill multiple target cells (25). At the E:T ratios employed in our confocal live cell studies in vitro, and within the relatively short time-spans of in vivo imaging, it was not possible to analyze serial tumor cell killing. Our data rather suggest that under CEA TCB treatment conditions, several T cells can contribute to tumor cell death. However, this does not exclude that serial killing also occurs after CEA TCB treatment at low T-cell to target cell ratios.

In vivo analysis of T-cell recruitment further revealed increased densities of tumor associated T cells 24 hours after CEA TCB treatment. However, this does not exclude that serial killing also occurs after CEA TCB treatment at low T-cell to target cell ratios.
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TCB–mediated activation. The imaging data presented in this study are fully in line with previous findings obtained by histologic analysis of CEA TCB–treated tumor samples, showing that CEA TCB–mediated tumor regression is accompanied by increased numbers of tumor-infiltrating leukocytes (TIL; ref. 8). Typically, increased E:T ratios are associated with more efficient target cell lysis (22, 26, 27).

Overall, our data suggest CEA TCB to accelerate T-cell–dependent tumor cell death by (i) locally increasing E:T ratios, (ii) cross-linking of multiple, stably interacting T cells to individual tumor cells, and (iii) decreasing the lag time from initial T-cell engagement to the induction of tumor cell apoptosis.

Conclusions

Our results show that CEA TCB, a novel half-life extended T-cell bispecific antibody with monovalent CD3 and bivalent CEA binding, specifically targets CEA-expressing tumor cells in a cografting tumor model in mice in vivo, and demonstrate its cytolytic activity in the preclinical setting in vitro as well as in vivo. Using microscopic imaging, we have gained additional insights into the mode of action of T-cell bispecific antibodies: through increasing the density of tumor-associated T cells and by promoting binding of multiple T cells to one individual tumor cell these molecules significantly accelerate T-cell–mediated tumor cell elimination. Our results strongly encourage further preclinical and clinical evaluation of T-cell bispecific antibodies for the treatment of solid tumors.

Disclosure of Potential Conflicts of Interest

C. Klein holds ownership interest (including patents) in Roche. No potential conflicts of interest were disclosed by the other authors.

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