A novel nanobody-based target module for retargeting of T lymphocytes to EGFR-expressing cancer cells via the modular UniCAR platform

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ABSTRACT
Recent treatments of leukemias with chimeric antigen receptor (CAR) expressing T cells underline their impressive therapeutic potential. However, once adoptively transferred into patients, there is little scope left to shut them down after elimination of tumor cells or in case adverse side effects occur. This becomes of special relevance if they are directed against commonly expressed tumor associated antigens (TAAs) such as receptors of the ErbB family. To overcome this limitation, we recently established a modular CAR platform technology termed UniCAR. UniCARs are not directed against TAAs but instead against a unique peptide epitope on engineered recombinant targeting modules (TMs), which guide them to the target. In the absence of a TM UniCAR T cells are inactive. Thus an interruption of any UniCAR activity requires an elimination of unbound TM and the TM complexed with UniCAR T cells. Elimination of the latter one requires a disassembly of the UniCAR-TM complexes. Here, we describe a first nanobody (nb)-based TM directed against EGFR. The novel TM efficiently retargets UniCAR T cells to EGFR positive tumors and mediates highly efficient target-specific and target-dependent tumor cell lysis both in vitro and in vivo. After radiolabeling of the novel TM with $^{64}$Cu and $^{68}$Ga, we analyzed its biodistribution and clearance as well as the stability of the UniCAR-TM complexes. As expected unbound TM is rapidly eliminated while the elimination of the TM complexed with UniCAR T cells is delayed. Nonetheless, we show that UniCAR-TM complexes dissociate in vitro and in vivo in a concentration-dependent manner in line with the concept of a repeated stop and go retargeting of tumor cells via the UniCAR technology.

Introduction
The epidermal growth factor receptor (EGFR) is a member of four related receptor tyrosine kinases (RTKs) named ErbB1 to 4 (also known as Her1 to 4). The ErbB network is an evolutionary conserved multifunctional signaling system involved in the regulation of cell proliferation, differentiation, apoptosis, and survival. The ErbB network is dysregulated in many solid tumors most notably in cancers of the breast, lung, colon, head and neck, and brain. For EGFR targeting tumor therapies, both small molecules working as inhibitors of the intrinsic RTK activity (TKI), or antibodies blocking the generation of intracellular signals e.g. by interacting with the binding of ligands to the extracellular domain of the EGFR were established. Unfortunately, these therapeutic approaches are frequently compromised by the development of refractory disease, e.g., by increased signaling via a non-targeted member of the ErbB network, receptor transactivation or increased production/release of ligands. One idea to overcome such a therapeutic resistance is to go for a direct cytolytic approach e.g., T or NK cells genetically modified with chimeric antigen receptors (CARs) directed against EGFR that can destroy EGFR-overexpressing tumor cells.

In principle, CARs are synthetic receptors consisting of three portions: (i) an extracellular binding moiety, (ii) a transmembrane domain, and (iii) intracellular signaling domain(s). Until today, the extracellular antigen-binding moiety is a recombinant antibody fragment obtained by fusion of the variable heavy and light chain sequences from a monoclonal antibody (mAb). The transmembrane domain is commonly taken from the CD8$^+$ or CD28 receptor. The intracellular signaling domains contain ITAMs of activating immune receptors. Over time, CARs with differing signaling domains were developed and termed as first, second, and third generation CARs. The additional signaling domains in second and third generation
CARs help to improve their survival in patients. The tremendous clinical success of CD19-specific CAR T cells underlines the high potential of the CAR technology. Unfortunately, severe adverse effects, including on-target, off-tumor reactions against healthy tissue or excessive on-target, on-tumor reactions due to heavy tumor loads, can occur which can become even life-threatening. As CD19 expression is not limited to leukemic cells but also found on healthy B cells, patients treated with CD19-specific CARs suffer from lifelong-lasting B cell aplasia. While the lack of B cells is manageable by intravenous immunoglobulin administration, in case of other targets including EGFR such on-target, off-tumor effects are not acceptable. From α-EGFR-based tumor treatments one can expect that retargeting of CAR T cells to this receptor may cause severe cutaneous and cardiac side effects. Indeed, infusion of ErbB2-directed CAR T cells into a patient resulted in lethal side effects. Most likely due to recognition of low level of target antigen in pulmonary endothelial, this patient died from respiratory distress syndrome, multiple organ failure, and cytokine release syndrome.

These considerations emphasize the challenge of an effective but safe tumor treatment by retargeting of immune effector T cells armed with CARs especially against a member of the ErbB receptor family. The obvious major drawback of conventional CAR-armed T cells is their lack of regulation once they are adoptively transferred into a patient. Furthermore, when activated in a patient, CAR T cells will further expand increasing the risk of a tumor lysis and/or cytokine release syndrome in an almost unpredictable manner. Although one can introduce a suicide gene into the CAR T cells or a backdoor to remove the CAR gene via the CRISPR/Cas system or the CAR T cells via an antibody domain, however, all of these approaches lead to a loss of the CAR T cells and thereby to an abrogation of the respective T cell therapy.

To overcome this limitation of conventional CARs, we recently introduced a novel modular platform technology termed UniCAR. To redirect UniCAR T cells to target cells TMs are required. On the one hand, TMs bind to the surface of the tumor cell, on the other hand, they form an immune complex with the antibody domain of the UniCAR via a peptide epitope (E5B9), which is physiologically not accessible on the surface of intact living cells. Consequently, an adoptive transfer of UniCAR-equipped T cells into a patient should cause neither therapeutic nor side effects as also supported from our animal studies. The inert UniCAR-expressing lymphocytes can be redirected via target modules (TM). For this purpose, the peptide epitope recognized by the UniCAR has to be fused to the respective TM. Via the peptide epitope UniCAR T cells will interact with the TM and then attack target-positive cells as schematically summarized in Fig. 1. After elimination of all tumor cells, theoretically UniCAR T cells can be shut down by stopping the infusion of the TM. The same may be possible in case severe side effects occur. Therapy can be continued if the patient relapses or after recovery of the patient at a later time point. Side effects should disappear after elimination of the TM as the UniCAR will be switched off in the absence of a TM. Thus, the activity but also potential adverse effects of UniCAR T cells may be tunable by dosing of the TM. The obvious time limiting parameters for shutting down the UniCAR system is the durability of existing UniCAR-TM complexes and the elimination time of the free TM.

Here, we present a novel TM directed to EGFR which is based on a nb. The novel α-EGFR TM efficiently retargets UniCAR T cells to EGFR-positive cancer cells both in vitro and in a mouse tumor xenograft model. In agreement with our UniCAR concept free TMs are rapidly eliminated. Moreover, we show that TMs can be released from UniCAR-TM complexes.

Results

Development of a novel nanobody-based TM for retargeting of T cells to EGFR-positive cancer cells

As mentioned in the introduction section and schematically summarized in Fig. 1, we recently described a modular CAR platform termed UniCAR. To redirect UniCAR T cells to target cells TMs are required. On the one hand, TMs bind to the surface of the tumor cell, on the other hand, they form an immune complex with the antibody domain of the UniCAR via a peptide epitope (E5B9) recognized by the UniCAR (Fig. 1).
So far, all of our TMs were based on scFvs delineated from IgG type murine or humanized mAbs (Fig. 1). The first aim of this study was to learn whether the molecular structure of a TM is limited to scFvs or other antibody derivatives may also work for redirection of UniCAR T cells. We decided to construct a TM based on a single-domain camelide-derived nb. The underlying camelide ab is directed against EGFR.41 The structure of such a nb–based UniCAR-TM immune complex is schematically summarized in Fig. 1.

After cloning and sequencing the novel TM had to be expressed and purified. In previous studies, we found that TMs based on scFvs derived from murine mAbs are not efficiently expressed in *Escherichia coli*. For that reason, we express them using eukaryotic cell lines transduced with the open reading frame of the respective TM. In contrast to scFvs, it was reported that nbs can be expressed with high efficacy in *E. coli*.42 We therefore, compared the expression of the novel nb-based TM in *E. coli* and Chinese Hamster Ovarian (CHO) cells.

The schematic structure of the prokaryotic and eukaryotic nb-based TM is shown in Fig. 2(AI and AII). Expression in CHO cells requires an N-terminal signal peptide sequence (Fig. 2AI and SP), which is absent in the prokaryotic construct (Fig. 2AII). To facilitate the interaction of UniCAR T cells with the E5B9 epitope the epitope sequence was N- and C-terminally flanked by a glycine serine linker each consisting of four glycine residues and one serine (Fig. 2, G4S). For purification of the nb from total extracts a His6-tag was added to the nb-based TMs. To avoid C-terminally truncated, prematurely terminated inactive contaminations, the His6-tag was fused to the C-terminus. The respective recombinant nb was purified from either total *E. coli* extract or cell culture supernatant of CHO cells by performing Ni-NTA affinity chromatography (see Materials and methods). The α-EGFR TM expressed and isolated from CHO cells was termed as α-EGFR TM (eu), while the α-EGFR TM expressed and isolated from *E. coli* extracts was termed as α-EGFR TM (pro). Both purified α-EGFR TMs were analyzed by SDS-PAGE (Fig. 2BI) and immunoblotting (Fig. 2BII). Histagged proteins were detected using an anti-His Ab (Fig. 2BII).

From SDS-PAGE analysis (Fig. 2BI, lane 1) but also from HPLC size exclusion chromatography (Fig. 2C, (eu)), it is obvious that the purified eukaryotic TM contains additional high molecular weight (HMW) contaminations, which appear to be mostly absent in the prokaryotic material (Fig. 2BI, lane 2 and Fig. 2C, (pro)). As these HMW species (i) are resistant to SDS

Figure 2. Development of the novel nb-based α-EGFR TM. (A) Two α-EGFR TM constructs (AI, α-EGFR TM (eu); AII, α-EGFR TM (pro)) were cloned for expression either in CHO cells (α-EGFR TM (eu)) or in E. coli (α-EGFR TM (pro)). As schematically shown, both nb-based α-EGFR TM constructs consist of the open reading frame encoding the EGFR-specific nb. For binding to the UniCAR the E5B9-tag is fused to the C-terminus. Furthermore, both TMs are tagged with 6xhis residues at the C-terminus for protein purification and detection. To enable eukaryotic expression, the α-EGFR TM (eu) construct additionally contains an N-terminal signal peptide (SP). (B) The elution fraction of the purified α-EGFR TM (eu) (lane 1) and α-EGFR TM (pro) (lane 2) was separated via SDS-PAGE and subsequently stained with Coomassie brilliant blue G-250 (BI) or transferred onto a nitrocellulose membrane for detection of the purified α-EGFR TM (eu) (lane 1) and α-EGFR TM (pro) (lane 2) via its C-terminal his-tag (BII). M, molecular weight marker. (C) Both TMs were further analyzed by size exclusion HPLC using either 15 μL of the α-EGFR TM (eu) (10 μg) or 7.5 μL of the α-EGFR TM (pro) (15 μg).
Estimation of the binding capability of the novel nanobody-based α-EGFR TM

First functional analysis included the estimation of the binding capability of the novel nb-based TMs. For that purpose, the α-EGFR TM (eu) and α-EGFR TM (pro) were analyzed by FACS analysis on naturally EGFR-expressing cell lines, including A431 and FaDu cells (Fig. 3). As positive control, cells were stained with a mAb against EGFR (Fig. 3, upper panels). A431 and FaDu cells were selected as FaDu cells have an intermediate expression of EGFR on their surface, while A431 express EGFR at high density as also supported by the estimated MFI values. Detection of the bound TMs was performed via the C-terminal E5B9-tag (Fig. 3, lower panels). Both the α-EGFR TM (eu) (Fig. 3, lower panels, light gray) and the α-EGFR TM (pro) (Fig. 3, lower panels, dark gray) are able to bind to both A431 and FaDu cells. The measured MFI values are lower for the α-EGFR TM (eu). The lower MFI values for the α-EGFR TM (eu) are in line with the $K_d$ values estimated for α-EGFR TM (eu) with 80 nM and 13 nM for α-EGFR TM (pro) (Fig. 4).

Most importantly, these binding studies underline that after binding to its target antigen EGFR the E5B9-tag of the TM is still accessible for interaction with an α-ESB9-tag mAb, which is a prerequisite for the interaction with α-ESB9-directed UniCAR-expressing T cells.

Lysis of EGFR-positive tumor cells by retargeted UniCAR T cells occurs in a TM-dependent and target-specific manner

For further functional analysis, human T cells from healthy donors were transduced with lentiviral vectors encoding the UniCAR sequence containing a dual CD28/CD3ζ signaling domain (UniCAR 28/ζ). Transduction was performed as described under Materials and methods. As negative controls, T cells were transduced with lentiviral vectors encoding the UniCAR sequence lacking the signaling domain (UniCAR Stop) or encoding only the EGFP marker protein (vector control). To compensate different transduction rates of UniCAR-positive T cells, the transduction efficacy was estimated by FACS analysis and transduced cells were sorted to > 90% purity to allow comparison between different human donors. Transduction and sorting was performed as described previously (see also Materials and methods). For analysis of tumor cell lysis, chromium release assays were performed. Release of radiolabeled chromium was measured after 48 h of incubation (Fig. 5, Fig. 6).

As shown in Fig. 5A, UniCAR 28/ζ T cells incubated with an α-EGFR TM were able to eliminate A431 tumor cells. As expected, the cytotoxic capability depends on the effector to target cell (et) ratio. In addition, also the FaDu cell line, expressing the target antigen EGFR at lower levels, could be eradicated by UniCAR T cells (Fig. 5B). Importantly, the killing of both tumor cell lines was strictly dependent on the presence of the respective TM (50 nM), either α-EGFR TM (eu) (Fig. 5, AI and BI) or α-EGFR TM (pro) (Fig. 5, AII and BII). No killing was observed with UniCAR 28/ζ T cells in the absence (Fig. 5, Fig. 6A and B, w/o Ab) of any TM. Moreover, T cells modified with the UniCAR Stop or the EGFP-encoding vector control did not attack the target cells (Fig. 5).

Figure 3. Binding of the novel TMs to EGFR-expressing tumor cells. To analyze binding properties of the α-EGFR TMs, A431 and FaDu cells were stained with the respective TM (20 ng/μL). The specific binding was detected via the E5B9-tag using an α-ESB9 mAb and a PE-conjugated α-mouse-IgG mAb. As positive control, cells were labeled with mAb α-EGFR/PE-Cy7. The histograms show cells stained with either the control Ab (dark gray graphs, upper panel) or the α-EGFR TM (eu) (light gray graphs, lower panel) or α-EGFR TM (pro) (dark gray graphs, lower panel) and their respective controls (transparent graphs). The numbers represent the percentage of antigen-positive cells and mean fluorescence intensity (MFI) of stained cells.

Figure 4. Estimation of the $K_d$ values of the novel α-EGFR TMs. Increasing amounts of the α-EGFR TM (eu) (left panel) or α-EGFR TM (pro) (right panel) were used for staining of A431 cells. Binding was detected via the E5B9-tag using an α-ESB9 mAb and a PE-conjugated α-mouse-IgG mAb. The respective $K_d$ value was calculated from the resulting binding curve (see also Materials and methods).
As expected, the killing correlates with the concentration of the respective TM (Fig. 6). Increasing the concentration of the \(\alpha\)-EGFR TM (eu) (Fig. 6A) shows that a plateau is reached at a concentration of about 5 nM. Interestingly, this plateau can already be reached with the \(\alpha\)-EGFR TM (pro) (Fig. 6B) at a concentration of 0.5 nM. Thus, the killing efficacy of UniCAR T cells mediated by the \(\alpha\)-EGFR TM (pro) appears to be superior to the \(\alpha\)-EGFR TM (eu). This interpretation is supported by the estimated EC_{50} values of 0.1 nM for the \(\alpha\)-EGFR TM (pro) and 1.7 nM for the \(\alpha\)-EGFR TM (eu) (Fig. 6C).

**Release of cytokines by retargeted UniCAR T cells occurs in a TM-dependent manner**

For analysis of cytokine release, UniCAR 28/\(\zeta\) T cells were incubated with A431 target cells either in the presence (50 nM) (Fig. 7, UniCAR CD28/\(\zeta\) + A431 + \(\alpha\)-EGFR TM (eu) or + \(\alpha\)-EGFR TM (pro)) or absence (Fig. 7, UniCAR CD28/\(\zeta\) + A431) of the respective TM. The concentration of cytokines secreted into the supernatants after 48 h of co-cultivation was measured for three individual donors.

In a first step, cytokine concentrations were estimated for three donors using a multiplex assay (the MACSplex Cytokine 12 Kit, see Materials and methods) (Fig. 7A). This bead-based assay allowed us to detect and quantify the cytokines IFN-\(\gamma\), IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12, IL-17A, and TNF-\(\alpha\) in parallel. With the exceptions of GM-CSF, IFN-\(\gamma\), IL-2, IL-4, IL-9, and TNF-\(\alpha\) (Fig. 7A) no other cytokines could be detected at a significant concentration including IL-6 (Data not shown). Cytokines were only detected in samples from UniCAR expressing T cells in the presence of (i) target cells and (ii) either the \(\alpha\)-EGFR TM (eu) (Fig. 7A, gray bars) or the \(\alpha\)-EGFR TM (pro) (Fig. 7A, black bars). Cytokines could not be detected in supernatants of negative controls (Fig. 7A, UniCAR T cells in the absence of a TM, white bars).

In a second step, the concentrations of the selected cytokines IFN-\(\gamma\), IL-2, and TNF were estimated by enzyme-linked immunosorbent assay (ELISA) (Fig. 7B) (see Materials and methods) for the same three donors. As shown in Fig. 7, the analyzed cytokines strongly varied from donor to donor. In some cases, cytokines could not even be detected. Nonetheless, cytokine release was strictly dependent on the combination of UniCAR T cells, TM, and target cells. In comparison, no cytokine release was detectable in the control samples. Cytokine levels were usually lower for the \(\alpha\)-EGFR TM (eu) which may be related to its lower efficacy.

**The UniCAR system triggers antitumor effects in experimental mice in dependence on the presence of the EGFR-TM**

To show a TM-dependent in vivo antitumor activity of UniCAR-expressing T cells against EGFR-positive cells, a mouse tumor xenograft model was established. For this purpose, A431 cells were transduced to express firefly luciferase (see Materials and methods). To reduce the number of experimental mice, we limited all in vivo analyses to the \(\alpha\)-EGFR TM (pro). For analysis of in vivo retargeting, per mouse, 1.5 \(\times\) 10^6 A431-Luc cells
more luciferase activity could be detected in all the treated animals (Fig. 8, group B) while luciferase activity could easily be detected in the control mice (Fig. 8, group A). These data indicate that UniCAR T cells armed with TMs specific for EGFR can also eliminate EGFR-positive tumor cells in vivo.

**Biodistribution of the EGFR-TM in tumor-bearing mice**

For analysis of the biodistribution especially the elimination time of the free TM, we decided to radiolabel the α-EGFR TM (pro) as it contains less contaminations compared with the α-EGFR TM (eu) (see also Fig. 2C). For this purpose, the α-EGFR TM (pro) was conjugated with the chelator NODAGA (see Materials and methods). As NODAGA binds randomly to lysine residues, the average number of chelator molecules per molecule of antibody varies. According to MALDI-TOF analysis (Fig. 9A), in average 1.2 molecules of NODAGA were conjugated to one molecule of the α-EGFR TM (pro). The resulting TM was termed as α-EGFR TM (pro) (NODAGA)1.2. NODAGA can be used for chelating of both ⁶⁴Cu and ⁶⁸Ga. For small animal PET, the ⁶⁴Cu-labeled TM should reveal a higher resolution, while the ⁶⁸Ga-labeled TM will be the preferred radiopharmaceutical compound for human imaging. Therefore, we wanted to look for obvious differences with respect to the biodistribution of the ⁶⁴Cu-labeled- versus the ⁶⁸Ga-labeled TM in experimental mice. Both radiolabeled compounds had specific activities larger than 24 GBq/μmol and were of high radichemical purity (>95%). The stability test at room temperature showed that after 24 h the radiochemical purity of the [⁶⁴Cu]Cu-α-EGFR TM (pro) (NODAGA)1.2 had decreased to just 88% in the injection solution. After SDS-PAGE and autoradiography of [⁶⁴Cu]Cu-α-EGFR TM (pro) (NODAGA)1.2 the molecular mass of the major labeled protein fraction was estimated with approximately 18 kDa (Fig. 9B), which is in a good agreement with the 17486.367 (m/z) as estimated by MALDI-TOF MS (Fig. 9A). In agreement with the HPLC analysis (Fig. 2C) only minor contaminations were detectable even by autoradiography (Fig. 9B).

Figure 10 summarizes the biodistribution of the [⁶⁴Cu]Cu-α-EGFR TM (pro) (NODAGA)1.2 (Fig. 10A,C) and the [⁶⁸Ga]Ga-α-EGFR TM (pro) (NODAGA)1.2 (Fig. 10B,D) as either percentage of the total activity of the injected dose (%ID) or activity concentration (SUV) for a total of four A431-Luc tumor-bearing mice. As summarized in Fig. 10, both the [⁶⁴Cu]Cu-α-EGFR TM (pro) (NODAGA)1.2 and the [⁶⁸Ga]Ga-α-EGFR TM (pro) (NODAGA)1.2 show a similar biodistribution pattern 2 h after intravenous injection. Both compounds were mainly eliminated by glomerular filtration into the urine while hepatobiliary elimination was slow. Obviously, the elimination of the ⁶⁸Ga-activity (63±4%ID) was higher compared with the ⁶⁴Cu-activity (28±1%ID) while the ⁶⁴Cu-labeled TM appeared to be more efficiently trapped in the kidneys (40.9±2.0%ID vs. 17.3±2.2%ID). Tumor to blood ratios and tumor to muscle ratios were comparable and estimated with 2.2±0.6 vs. 2.9±1.0, and 12.8±2.3 vs. 7.9±1.1, respectively (Fig. 10E).

Finally, small animal PET/CT was performed using the [⁶⁴Cu]Cu-α-EGFR TM (pro) (NODAGA)1.2. For this purpose, A431-Luc tumors were established either in the right hind flank (data not shown) or the right shoulder (e.g., Fig. 11) of experimental mice (see Materials and methods). The dynamic PET studies with the [⁶⁴Cu]Cu-α-EGFR TM (pro) (NODAGA)1.2 showed even after 2 h...
a clear delineation of the respective tumors in the mice shoulder (e.g., Fig. 11) and leg (Data not shown). The maximal tumor activity concentration (2.7±0.2 SUV) was reached at 22 min p.i. and decreased after 48 h to 0.9±0.1 SUV. The tumor to blood (4.5±0.5) and to muscle (7.2±0.7) ratios reached the maximum at 2 h after injection.

To analyze the pharmacokinetic behavior of the nb-based α-EGFR TM in more detail, time-activity curves (TAC) of regions of interest (ROI) derived from the PET studies of the four analyzed A431-Luc tumor-bearing mice were estimated. The data points were collected over a time range of 20–35 h p.i. of the [64Cu]Cu-α-EGFR TM (pro) (NODAGA)1,2 (Fig. 12). The TAC of the ROI over the heart represents primarily the blood activity concentration (SUVmean) (Fig. 12A), indicating a rapid elimination of the TM with half-lives of 4.2 min and 23.2 min of the fast and slow distribution and elimination phase, respectively. According to the TAC of the tumor, the TM activity reaches its maximum in the tumor 22 min p.i. with a clearance half-life of 3.7 h (Fig. 12B). Based on the TAC of the total activity (%ID), we estimated a maximum of 3.2%ID in the tumor (Fig. 12C). The TACs of the tumor ratios to muscle and blood, respectively, show an increasing image contrast up to 2 h p.i. (Fig. 12D,E). Two hours p.i. the accumulation of the 64Cu-activity in the kidneys reaches its maximum of 46.4%ID.

![Figure 7](image_url). Cytokine release from EGFR-redirected UniCAR T cells as estimated by either (A) a multiplex assay or (B) ELISA. (A and B) A431 cells were incubated in the presence of genetically engineered UniCAR 28/z T cells either in the absence (UniCAR CD28/z + A431, white bars) or presence of 50 nM of the α-EGFR TM (eu) (gray bars) or α-EGFR TM (pro) (black bars) at an et ratio of 5:1 for 48 h. Cytokines secreted into cell culture supernatants were estimated for three individual donors. As controls UniCAR T cells were co-cultivated with A431 cells in the absence of 50 nM TM as indicated in (A). As additional controls T cells were engrafted with the vector control or the UniCAR Stop construct and incubated in the presence of 50 nM TM as indicated in (B). (A) Using the MACSPlex Cytokine 12 Kit, variable amounts of the cytokines GM-CSF, IFNγ, IL-2, IL-4, IL-9, and TNF-α were detected (x, not detectable, n.d. not defined) but not the other cytokines IFN-α, IL-5, IL-6, IL-9, IL-10, IL-17A (Data not shown). (B) The concentration of selected cytokines including IFNγ (left), IL-2 (middle) and TNF (right) in co-culture supernatants was also estimated by ELISA. Mean cytokine concentrations and SD of triplicates for three independent donors are shown (x, not detectable).
Dissociation of existing UniCAR-TM complexes in vitro

As mentioned in the introduction section, one prerequisite for switching UniCAR activity on and off is a reliable disassembly of active UniCAR-TM complexes. To learn about the stability of UniCAR-TM complexes, T cells transduced with UniCAR 28/β were pre-incubated with TMs (50 nM of α-EGFR TM (eu) (Fig. 13A) or α-EGFR TM (pro) (Fig. 13B) for 30 min and washed several times (see Materials and methods) and indicated in Fig. 13. Thereafter, the capability of such pre-incubated and washed UniCAR-TM complexes to lyse chromium-labeled A431 tumor cells was estimated by chromium release assays (et ratio 5:1). For comparison non-pre-incubated UniCAR 28/β T cells were cultivated with A431 cells in the presence or absence of the respective TM. After 48 h, chromium release was measured. As negative controls served T cells transduced with the vector control or the UniCAR Stop construct. As shown in Fig. 13, washing of UniCAR-TM complexes results in a significant reduction of UniCAR T cell activity even down to almost background (Fig. 13A). The activity of the UniCAR-α-EGFR TM (eu) complexes (Fig. 13A) can be easier switched off by washing than the α-EGFR TM (pro) ones (Fig. 13B). This is in agreement with the titration experiments shown in Fig. 6C. According to these titration experiments the combination of UniCARs with the α-EGFR TM (eu) requires higher concentrations of TM for maximum activity while already lower concentrations of the α-EGFR TM (pro) are sufficient for maximum activity of UniCARs.

Figure 8. Retargeting of EGFR-positive tumor cells in experimental mice. A431 cells were transduced to express firefly luciferase resulting in A431-Luc cells. Per mouse, $1.5 \times 10^6$ A431-Luc cells were mixed with $1.5 \times 10^6$ UniCAR 28/β T cells and 100 μg of the α-EGFR TM (pro). As “untreated” control served $1.5 \times 10^6$ A431-Luc cells mixed with $1.5 \times 10^6$ UniCAR 28/β T cells without any TM. The respective mixture (100 μL) was injected subcutaneously into SCID/beige mice resulting in two groups of animals representing untreated (group A) or treated (group B) mice. Luminescence imaging of anesthetized mice was performed 10 min after i.p. injection of 200 μL of D-luciferin potassium salt (15 mg/mL) starting at day one (D1), and followed at day 2 (D2), and day 8 (D8).

Figure 9. MALDI-TOF MS analysis (A) and SDS-PAGE followed by autoradiography (B) of 64Cu-radiolabeled α-EGFR TM (pro). (A) A mixture of non-modified and NODAGA modified TMs were analyzed with MALDI-TOF MS. The molecular mass of the unmodified α-EGFR TM (pro) was determined with 16676.611 (m/z). The mean of the molecular mass of the NODAGA conjugated TMs was estimated with 17486.367 (m/z), which yields an average of 1.2 NODAGA chelator groups per molecule of the α-EGFR TM (pro) (NODAGA)1.2. (B) After SDS-PAGE, the autoradiogram of the [64Cu]Cu-α-EGFR TM (pro) (NODAGA)1.2 shows a main fraction with a molecular mass of approximately 18 kDa.
Figure 10. Biodistribution of $^{64}$Cu- and $^{68}$Ga-radiolabeled α-EGFR TM (pro). After conjugation of the α-EGFR TM (pro) with NODAGA the resulting α-EGFR TM (pro) (NODAGA)$_{1.2}$ was radiolabeled with either $^{64}$Cu or $^{68}$Ga. The biodistribution of the $^{64}$Cu-α-EGFR TM (pro) (NODAGA)$_{1.2}$ complex is shown in (A and C). The biodistribution of the $^{68}$Ga-α-EGFR TM (pro) (NODAGA)$_{1.2}$ complex is shown in (B and D). The biodistribution is given as percentage of the total activity of the injected dose (%ID) and the activity concentration (SUV) based on four A431-Luc tumor-bearing mice. (E) Target to background ratios including tumor to muscle, and tumor to blood ratios.

Figure 11. Small animal PET/CT. Orthogonal sections (left) scaled to either maximum (upper) or visualize the tumor (lower) or maximum intensity projections (right) of a selected A431-Luc tumor-bearing mouse at 2 h after single intravenous injection (ki, kidneys, tu, tumor, li, liver, bl, bladder).
As already shown by PET analysis (Fig. 11) i.v. injected TMs are rapidly eliminated via the kidneys with half-lives below 30 min. To avoid first pass effects of complexes between tumor cells, TMs, and UniCAR T cells in the lung, we decided to estimate the elimination of either free TMs or TMs complexed with either tumor cells or UniCAR T cells or both after s.c. injection (Fig. 14). Before the in vivo application of the A431-Luc and UniCAR T cells they were incubated with the [64Cu]Cu-α-EGFR (TM) (pro) (NODAGA)1.2 and washed with medium. After that only 1.5±0.7% of the activity in the incubation medium was bound to the cells. These preparations were s.c. injected in the mice and the activity time curves over the

**Figure 12. Time-activity curves (TAC) of regions of interest (ROI).** The TAC curves are derived from PET studies of four A431-Luc tumor-bearing mice. The data points were collected over 20 h to 35 h after injection of the [64Cu]Cu-α-EGFR (TM) (pro) (NODAGA)1.2. (A) TAC of the ROI over the heart representing primarily the blood activity concentration (SUV mean) supporting the fast elimination of the TM with half-lives of 4.2 min and 23.2 min of the fast and slow distribution and elimination phase, respectively. (B) TAC of the tumor activity concentration with a maximum at 22 min p.i. and the clearance with half-life of 3.7 h. (C) TAC of the total activity (%ID) in the tumor with 3.2% ID at the maximum. (D), (E) TAC of the tumor ratios to muscle and blood, respectively, showing the increasing image contrast up to 2 h. (F) accumulation of the 64Cu-activity in the kidneys with a maximum of 46.4% ID after 2 h.

**Dissociation of existing UniCAR-TM complexes in vivo**

As already shown by PET analysis (Fig. 11) i.v. injected TMs are rapidly eliminated via the kidneys with half-lives below 30 min. To avoid first pass effects of complexes between tumor cells, TMs, and UniCAR T cells in the lung, we decided to estimate the elimination of either free TMs or TMs complexed with either tumor cells or UniCAR T cells or both after s.c. injection (Fig. 14). Before the in vivo application of the A431-Luc and UniCAR T cells they were incubated with the [64Cu]Cu-α-EGFR (TM) (pro) (NODAGA)1.2 and washed with medium. After that only 1.5±0.7% of the activity in the incubation medium was bound to the cells. These preparations were s.c. injected in the mice and the activity time curves over the
injection site and organs of interest were acquired. As shown in Fig. 14 (A and B (TM)), free TMs injected s.c. are still rapidly eliminated. After 90 min the TM can be found in the kidneys (Fig. 14A, 90 min). Based on the dynamic PET data a half-life of 1.7 h was estimated for the free TM (Fig. 14C, TM). The prolonged half-life compared with i.v. application is not unexpected as the TM has first to move from the tissue into blood vessels. Also not unexpected, when complexes between TMs and tumor cells were s.c. injected the occurrence of the TM in the kidneys is further delayed (Fig. 14A, TM\textsuperscript{C}A431-Luc, 90 min). Based on the dynamic PET data (Fig. 14B, TM\textsuperscript{C}A431-Luc), a half-life of 19.4 h was estimated for the TM bound to tumor cells (Fig. 14C, TM\textsuperscript{C}A431-Luc). Interestingly, the elimination curve of the TM complexed with UniCAR expressing T cells (Fig. 14B, T\textsuperscript{C}UniCAR) lays between the curve of the free TM (Fig. 14B, TM) and the TM tightly bound to the A431-Luc cells (Fig. 14B, TM\textsuperscript{C}A431-Luc). These data also indicate that TMs are released from UniCAR-TM complexes. Based on the dynamic PET data (Fig. 14B, TM\textsuperscript{C}UniCAR), a half-life life of 7 h was estimated for the TM bound to T cells. The complex between TM, UniCAR T cell and A431-Luc cells follows closely the kinetic between A431-Luc cells+TM, which points again to a tight binding between the TM and the tumor cells.

**Discussion**

As summarized in the introduction section, one major drawback of conventional CAR T cells is their risk of on-target, off-tumor side effects, which can lead to life-threatening adverse reactions. In case of a cross-reaction of the extracellular CAR domain with healthy tissues, reactivity of CAR T cells has to stop latest after sufficient tumor cell elimination. Moreover, in case of unexpected side effects caused by the CAR system, it should be possible to turn it off as fast as possible. However, there is little play for steering the function of conventional CAR T cells especially without losing them. After an adoptive transfer and the activation of CAR T cells, they will even start to proliferate, which may further enhance the risk of and/or exacerbate occurring side effects. Although CAR-modified T cells might first destroy tumor cells overexpressing the target antigen, once transferred into a patient, they remain active until
all cells expressing the antigen or any cross-reactive epitope will be eliminated, including at low levels in healthy tissues. Thus, the application of the CAR technology to tumor targets that are not limited to tumor cells, including for example the ErbB receptor family appears to be rather risky.

Recently, we described a novel modular CAR strategy (Uni-CARs) for retargeting of tumor cells. In contrast to conventional CARs, UniCAR T cells are “blind.” In the presence of a TM, UniCAR T cells form an immune complex with a TM. The specificity of the TM tells such armed UniCAR T cells which cells to attack. Theoretically, the modular UniCAR format has the advantage of an internal self-limiting switch as this label would be used in humans. Although, the $^{64}$Cu-labeled TM appeared to be more efficiently trapped in the kidney, otherwise the biodistribution of both radiolabeled TMs did not dramatically differ. Consequently, the differences of the charges of the $^{64}$Cu$^{2+}$ NODAGA complex, which should result in a negatively charged complex, and the $^{68}$Ga$^{3+}$ NODAGA complex, which should be neutral, did not significantly affect the biodistribution pattern and the target to background ratios. Both the biodistribution analysis and the immuno PET data point to a rapid elimination of the TM via the kidneys with a half-life of about 4–20 min. In real life the situation may be even more complex as a portion of the TM is bound and complexed with UniCAR T cells and/or tumor cells. Consequently, switching UniCAR T cells off does not only require the elimination of unbound TM but the TMs bound by UniCAR T cells have first to be released from this complex and then eliminated. Both our in vitro and in vivo experiments support our concept that TMs can be released from UniCAR T cell-TM complexes and, thus, the capability of a self-limiting switch. The short half-life of TMs will require a continuous infusion. Especially, at the start of a UniCAR therapy, only a portion of the UniCAR T cells will be complexed with TMs and the elimination even of the bound TMs will thus be much faster than the half-life estimated under our “saturated” conditions. Therefore, even a rapid safety shut off appears possible. If not one could try to further accelerate the dissociation of the complex between the UniCAR T cells and TMs by competing the interaction via a peptide representing the E5B9 tag. One still open question is what happens with the TMs if too tightly bound to tumor cells. It appears likely that these TMs may be uptaken by the target cells and thereby eliminated.

In summary, our data show that TMs for re-targeting of UniCAR T cells are not limited to molecules based on scFvs but that even smaller molecules, such as nbs can be used for the generation of TMs. The nb-based TMs cannot only be used for re-targeting of UniCAR T cells, but also as theranostic tools for PET-imaging of tumors. Moreover, their rapid elimination may provide a sufficient safety window for switching UniCAR T cells on and off.

**Materials and methods**

**Cell lines**

The CHO (ATCC CCL—61) cells were used for production of the recombinant $\alpha$-EGFR TM. They were cultured in complete RPMI 1,640 medium. Human Embryonic Kidney cells
HEK293T (ATCC CRL-11268) and the pharynx squamous cell carcinoma cell line FaDu (ACC 784) were kept in complete DMEM medium. For cultivation of the epidermoid carcinoma cell line A431 (ATCC CRL-1555) and A431-Luc+ cells (see below) complete DMEM medium supplemented with 1 mM sodium pyruvate (Biochrom GmbH, Berlin, Germany) was used. To express the enzyme luciferase (Photinus pyralis) A431 cells were transduced with the open reading frame encoding the firefly luciferase gene as described previously. The resulting cells were termed A431-Luc+ cells. All cells were kept at 37°C in a humidified atmosphere (5% CO2).

**Isolation and cultivation of human peripheral blood mononuclear cells (PBMCs) and Pan T cells**

Isolation of primary human T cells from peripheral blood mononuclear cells (PBMCs) out of buffy coats (supplied by German Red Cross, Dresden, Germany) or from fresh blood of healthy donors was performed as described previously. The study including the consent form was approved by the local ethics committee of the university hospital of the medical faculty of “Carl Gustav Carus” TU-Dresden (EK27022006). Isolated T cells were cultured in RPMI 1,640 complete medium supplemented with 50 U/mL IL-2 (ImmunoTools, Friesoythe, Germany) at densities of 1–2 × 10^6 cells/mL.

**Lentiviral transduction of T cells**

Production of lentiviral particles and transduction of primary human T cells was performed as described previously. Briefly, T cells were activated with anti-CD3/CD28 coated polyclonal T cell activator beads (Thermo Fisher Scientific, Waltham, MA, USA) at a bead to cell ratio of 1:4. After 24 h concentrated lentivector viral supernatant was added 5 times for the next 48 h before beads were removed. During genetic modification and expansion, T cells were maintained in RPMI supplemented with 200 U/mL IL-2 (Proleukin®, Novartis Pharmaceuticals, Horsham, UK), 5 ng/mL IL-7 and 5 ng/mL IL-15 (ImmunoTools). Three to 4 d later, cells were sorted using a FACSAria II (BD Biosciences). Isolated T cells were rested in RPMI supplemented with cytokines for additional 5–6 d. Media was substituted for complete RPMI lacking any recombinant cytokines 24 h before experiments started.

**Construction of the expression vectors encoding the novel α-EGFR TM**

The α-EGFR TM was constructed based on the cameldile α-EGFR Ab (clone 7C12). Synthesis of the gene NotI-α-EGFR nb-XhoI was performed by and purchased from the company Eurofins Genomics (Ebersberg, Germany). To enable eukaryotic expression of the novel α-EGFR TM, the α-EGFR nb sequence was cloned upstream of the ESB9 peptide epitope into the expression vector pSecTag2B-ESB9-his using NotI/XhoI (Thermo Fisher Scientific) restriction enzyme sites. The ESB9 sequence is taken from the sequence of the natural autoantigen La(SS)-B. The pSecTag2B-ESB9-his vector is a derivative of the original vector pSecTag2B (Invitrogen GmbH, Karlsruhe, Germany), which contains the ESB9 tag instead of the c-myc tag downstream of the multiple cloning site.

Subsequently, the open reading frame of the α-EGFR TM including the N-terminal murine Igκ leader sequence as well as the C-terminal polyhistidine (His6)-tag was cut out with Nhel/MssI restriction enzymes (Thermo Fisher Scientific) and cloned into the XbaI/HpaI (Thermo Fisher Scientific) digested lentiviral vector p6NST50, resulting in the transduction vector p6NST50-α-EGFR TM. To establish the prokaryotic expression vector, the sequence of the α-EGFR TM was cloned into the expression vector pET-28a (Novagen, Darmstadt, Germany). For this purpose, the gene sequence of the α-EGFR TM was amplified via PCR with the Advantage® HF2 PCR Kit (Clontech Laboratories, Inc., Mountain View, CA, USA) by using the primer #1 (5'-GGTCTAGAGGATATACCATGCGGTTCACTGCG-3') and primer #2 (5'-CGGGCTAGCTTAAACTCAATGATGATG-3'). The resulting gene product Nhel-α-EGFR TM-NcoI was subcloned into the pGEM®-T Easy vector (Promega GmbH, Mannheim, Germany) and subsequently inserted into the vector pET-28a via Nhel/NcoI (Thermo Fisher Scientific) restriction enzyme sites, resulting in the expression vector pET-28a-α-EGFR TM.

**Expression and purification of recombinant antibodies**

To obtain a permanent eukaryotic cell line for production of the α-EGFR TM (eu) the vector p6NST50-α-EGFR TM was stably transduced into CHO wt cells as described previously. Purification of the his-tagged recombinant proteins from cell culture supernatant was performed by affinity chromatography on Ni-NTA columns (Qiagen, Hilden, Germany) according to previously published protocols.

For prokaryotic expression of the α-EGFR TM the E. coli strain SHuffle® T7 (New England BioLabs GmbH, Frankfurt am Main, Germany) was transformed with the vector pET-28a-α-EGFR TM using standard heat shock method. One single clone of genetically modified bacteria was grown over night at 37°C in 10 mL SOC medium containing 2% [w/v] BactoTryptone (BD Biosciences), 0.5% [w/v] BactoTrypton (BD Biosciences), 10 mM NaCl (VWR International GmbH, Darmstadt, Germany), 2.5 mM KCl (Fluka Chemie AG, Neu-Ulm, Germany), 10 mM MgCl2 (VWR International GmbH), 10 mM MgSO4 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), 20 mM glucose (Carl Roth GmbH & Co. KG, Karlsruhe, Deutschland), and 10 µg/mL kanamycin (Carl Roth GmbH & Co. KG). The next day, 400 mL SOC medium were inoculated with 2 mL overnight culture and incubated at 30°C until optical density at 600 nm reached values ranging between 0.5 and 0.8. Subsequently, TM expression was induced by adding isopropyl-β-D-1-thiogalactopyranoside (Gerbu Biotechnik GmbH, Würzburg, Germany) at a final concentration of 1 mM. After cultivation at 37°C for additional 18 h TM producing cells were harvested via centrifugation (10 min, 4°C, 10,000g), resuspended in 10 mM imidazole buffer (10 mM imidazole, 150 mM NaCl, 1xPBS) supplemented with 1% [v/v] phenylmethylsulfonyl fluoride (Merck KGaA, Darmstadt, Germany) and 0.1% lysozyme (Sigma-Aldrich Chemie GmbH) and sonicated. After centrifugation (15 min, 4°C, 10,000g), the EGFR-specific TM was purified by Ni-NTA affinity chromatography. Imidazole-containing elution fractions were dialyzed overnight against 1xPBS (Biochrom GmbH).
Purified TMs were analyzed via SDS-PAGE and immunoblotting as described previously. Recombinant Abs were detected by a mouse mAb directed to the his-tag (Qiagen) and a polyclonal rabbit α-mouse IgG conjugated with alkaline phosphatase (Dianova GmbH, Hamburg, Germany).

**High-performance liquid chromatography**

For size exclusion high-performance liquid chromatography (SE-HPLC) analysis 10-15 μL samples containing the respective α-EGFR TM were applied to a size exclusion column (Agilent Bio SEC-3 (3 μm, 150 A, 7.8 × 300 mm²) from Agilent Technologies, Böblingen, Germany). HPLC chromatography was performed using a Chromaster HPLC-System 600 (VWR-Hitachi, Darmstadt, Germany). As running buffer we used 100 mM Na₂HPO₄ adjusted to pH 7.0 with HCl. Proteins were detected by UV at 280 nm.

**Flow cytometric analysis**

Detection of the extracellular EGFR domain on tumor cells was performed by using a murine mAb against human EGFR (clone A13) purchased from BioLegend (Fell, Germany). The binding was performed by using a murine mAb against human EGFR (clone Krefeld, Germany) as detection Ab. Stained cells were analyzed using a MACSQuant Analyzer and the MACSQuantify software (Miltenyi Biotec GmbH). Relative mean fluorescence intensities (MFI) of the stained cells were analyzed with GraphPad Prism 6 software (GraphPad Software Inc., La Jolla, CA, USA). The resulting binding curve was used to calculate the respective Kₐ value of the one-site specific binding.

**Cytotoxicity assay**

To analyze T-cell mediated tumor cell killing standard chromium release assays were performed. Genetically modified effector T cells were co-cultivated with 5 × 10⁵ [⁵¹Cr]-labeled target cells (A431 or FaDu cells) in the presence or absence of recombinant Ab constructs at different e:t ratios as indicated. Additional assays were performed with 0.25 × 10⁵ T cells pre-incubated with TM (50 nM) in 2 mL RPMI 1,640 complete medium for 30 min at 37°C. Subsequently T cells were washed several times with 2 mL of RPMI 1,640 complete medium, counted and co-cultivated with labeled target cells (e:t ratio 5:1). After 48 h specific lysis of tumor cells was determined as described previously. Quantification was performed via the MACSplex assay using the MACSplex Cytokine 12 Kit (Miltenyi Biotec GmbH), the MACSQuant® Analyzer (Miltenyi Biotec GmbH) and the MACSQuantify® software (Miltenyi Biotec GmbH) according to the manufacturer’s instructions. In parallel enzyme-linked immunosorbent assay was conducted as described previously.

**Antibody conjugation**

After purification of the α-EGFR TM (pro) by Ni-NTA affinity chromatography (see above) the TM was dialyzed against PBS and stored at a concentration of 1 mg/mL. For immuno-PET Imaging studies (see below), we conjugated the α-EGFR TM (pro) with the chelator NODAGA. Prior to the conjugation, PBS buffer was exchanged by 0.1 M borate-buffered saline (pH 8.5) using spin filtration (3 times at 7°C and 4000 rpm; Amicon Ultra-4, 10,000 MWCO). Then 40 equivalents of 2,2′-(1-carboxy-4-((4-isothiocyanatobenzyl)amino)-4-oxobuty1)-1,4,7-triazonane-1,4-diyldiacetic acid (p-SCN-Bz-NODAGA) ester were added to the α-EGFR TM (pro) solution (1 mg/mL). The mixture was left at 4°C for 20 h. Excess chelator was removed by spin filtration with PBS.

**MALDI-TOF MS analysis**

The average number of chelators per α-EGFR TM (pro) molecule was determined using MALDI-TOF MS. A thin layer of a saturated sinapinic acid solution in ethanol was applied onto the target plate (Bruker Daltonik GmbH) and left to dry. On top of this layer, a mixture (50:50 v/v) of the sample and a saturated sinapinic acid solution in 0.1% TFA, 30% acetonitrile in water was added. The samples were left to dry before the analysis was performed (AutoFlexTM, Bruker, Germany). To determine the number of attached chelator molecules the non-conjugated and the purified conjugate were mixed on the target plate and measured simultaneously. We estimated a ratio of 1.2 chelator per molecule of TM. Therefore, the conjugated TM was termed as α-EGFR TM (pro) (NODAGA)1,2.

**Radiolabeling**

The production of ⁶⁴Cu was performed at Cyclone(R) 18/9 (Helmholtz-Zentrum Dresden-Rossendorf) in a ⁶⁴Ni(p, n)⁶⁴Cu nuclear reaction with specific activities of 150–250 GBq/mmol Cu diluted in HCl (10 mM). For radiolabeling of the α-EGFR TM (pro) (NODAGA)₁₂, TM with ⁶⁴Cu, the pH of the ⁶⁴Cu solution was adjusted to pH 5.2 using NH₄OH and 1.6 nmol of the α-EGFR TM (pro) (NODAGA)₁₂ were added. For radiolabeling of the α-EGFR TM (pro) (NODAGA)₁₂ with ⁶⁸Ga, the pH of the ⁶⁸Ga solution was adjusted to pH 4.2 using NH₄OH and 0.9 nmol of the α-EGFR TM (pro) (NODAGA)₁₂ were added. The respective mixtures were shaken at 37°C for 30 min. Then 1 μmol EDTA was added and the radiolabeled TM was separated by spin filtration with PBS. The labeling process was monitored using instant thin-layer chromatography (ITLC). After chelating, the reaction mixture was supplemented with EDTA, and the radiolabeling efficiency was determined using both ITLC and SE-HPLC. SDS-PAGE of the labeled
conjugates, followed by silver staining and autoradiography was performed to further evaluate the TM-specific conjugation.

**Immuno-PET imaging, biodistribution analysis, and optical imaging of tumor xenograft models**

All animal experiments were performed at the Helmholtz-Zentrum Dresden-Rossendorf (HZDR) according to the guidelines of German Regulations for Animal Welfare and have been approved by the Landesdirektion Dresden (24–9165.40–4, 24.9168.21–4/2004–1). General anesthesia was induced with 10% (v/v) and maintained with inhalation of 8% (v/v) desflurane (Suprane, Baxter, Germany) in 30/10% (v/v) oxygen/air.

For immuno-PET imaging and biodistribution analysis, naïve, athymic male nude (NMRI-Foxn1nu/Foxn1nu) mice (Janvier, France), aged 5–8 week, were inoculated subcutaneously in the right hind flank or the right shoulder with 1 × 10⁶ A431-Luc cells in PBS. Six to 8 weeks after cell inoculation animals bearing tumors between 100 and 500 mm³ as measured by a caliper and visual inspection were selected for PET or biodistribution studies.

To evaluate tumor targeting of [64Cu]Cu-α-EGFR TM (pro) (NODAGA)₁.₂ and in vivo EGFR expression levels, immuno-PET imaging was performed in four A431-Luc NMRI nu/nu tumor-bearing mice. [64Cu]Cu-α-EGFR TM (pro) (NODAGA)₁.₂ (approximately 3.7 MBq) were intravenously injected into a lateral tail vein of the mice, and dynamic scans were acquired over 2 h and static scans were done 24, and 48 h after injection using a small animal PET/CT scanner (NanoPET/CT, Mediso). Quantitative data were expressed as SUV, activity concentration normalized to the body weight, that is defined as tissue concentration (MBq/mL)/injected dose (MBq)/body weight (g) in (g/mL), tumor to muscle and tumor to blood ratios. Images were visualized using ROVER software (ABX GmbH). The TAC curves were calculated as average±SEM.

To evaluate the biodistribution and tumor targeting of [64Cu]Cu-α-EGFR TM (pro) (NODAGA)₁.₂ and [68Ga]Ga-α-EGFR TM (pro) (NODAGA)₁.₂ 4 A431-Luc NMRI nu/nu tumor-bearing mice obtained a single intravenous injection of the respective radiotracer (~0.5 MBq). 2 h after injection the mice were killed, blood, selected organs and tissues were taken, dried, weighted and measured in a cross-calibrated γ-counter. Quantitative data were expressed as percentage injected dose (%ID) and SUV.

For optical imaging and pharmacokinetic analyses, 1.5 × 10⁶ A431-Luc cells were cultivated for 2 h at 37°C either alone or with 1.5 × 10⁶ human T cells genetically modified with the signaling construct UniCAR 28/18 in the presence or absence of 100 μg of the α-EGFR TM (pro). Cell mixtures (100 μL) were subcutaneously injected into the right thigh of experimental SCID/beige mice. Luminescence imaging (exposure times 1 sec, 10 sec, and 60 sec) was performed using a dedicated small animal multimodal imaging system (Xtreme, Bruker, Germany) 10 min after i.p. injection of 200 μL of D-luciferin potassium salt (15 mg/mL) (Thermo Fisher, Dreieich, Germany). In parallel, an X-ray photograph was taken from the same animals at the same position.

**Statistics**

To determine statistically significant differences in experiments data analysis was performed with GraphPad Prism 6 software (GraphPad Software Inc.) using one-way ANOVA with post-hoc Bonferroni Multiple Comparison test. p values <0.05 were considered significant.

**Disclosure of potential conflicts of interest**

MB and AE have filed patent applications related to the UniCAR platform.

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**Author contributions**

A.F., R.B., and M.B. designed experiments. S.A., C.A., R.B., D.B., S.K., F.L., P.Z.-W., A.K., and S.G. performed experiments or evaluated data. H.J.P., J.S., and M.B. provided critical materials. M.C., A.E., G.E., and M.S., discussed the data and provided critical suggestions. S.A., C.A., R.B., and M.B. analyzed and interpreted data, M.B. invented the CAR technology and wrote the paper.

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