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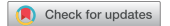
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ORIGINAL RESEARCH



A bispecific nanobody approach to leverage the potent and widely applicable tumor cytolytic capacity of V γ 9V δ 2-T cells

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ABSTRACT

Though V γ 9V δ 2-T cells constitute only a small fraction of the total T cell population in human peripheral blood, they play a vital role in tumor defense and are therefore of major interest to explore for cancer immunotherapy. V γ 9V δ 2-T cell-based cancer immunotherapeutic approaches developed so far have been generally well tolerated and were able to induce significant clinical responses. However, overall results were inconsistent, possibly due to the fact that these strategies induced systemic activation of V γ 9V δ 2-T cells without preferential accumulation and targeted activation in the tumor. Here we show that a novel bispecific nanobody-based construct targeting both V γ 9V δ 2-T cells and EGFR induced potent V γ 9V δ 2-T cell activation and subsequent tumor cell lysis both *in vitro* and in an *in vivo* mouse xenograft model. Tumor cell lysis was independent of *KRAS* and *BRAF* tumor mutation status and common V γ 9V δ 2-T cell receptor sequence variations. In combination with the conserved monomorphic nature of the V γ 9V δ 2-TCR and the facile replacement of the tumor-specific nanobody, this immunotherapeutic approach can be applied to a large group of cancer patients.

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Introduction

Although the majority of human T cells expresses an $\alpha\beta$ -T cell receptor (TCR), a smaller proportion of T cells expresses a $\gamma\delta$ -TCR. The most predominant $\gamma\delta$ -T cell subset in human peripheral blood consists of V γ 9V δ 2-T cells that account for approximately 1–5% of all T cells. V γ 9V δ 2-T cells are able to induce apoptosis in a broad spectrum of cancer cells and their reduced frequency and/or impaired functionality in the peripheral blood is a commonly observed phenomenon in cancer patients.^{1–8} In melanoma patients, reduced V γ 9V δ 2-T cell levels in the tumor microenvironment are related to more advanced clinical stages and reduced V γ 9V δ 2-T cell levels in the peripheral blood were recently shown to be a negative predictor for response upon treatment with ipilimumab.^{9,10} These observations clearly point to a vital role for V γ 9V δ 2-T cells in natural and induced immunity to cancer. In contrast to conventional T cells, ligand recognition by V γ 9V δ 2-T cells is independent of MHC-molecule presentation, tumor neo-epitope burden and classical immuno-editing.^{11,12} This underscores their great potential as anti-tumor effector cells, a potential that has been hitherto largely untapped.

V γ 9V δ 2-T cells become activated by the recognition of non-peptidic phosphoantigens (pAg).^{13–15} These are upregulated by stressed cells, including malignant cells, as a consequence of an enhanced activity of the mevalonate pathway¹⁶ or through the non-mevalonate pathway upon bacterial infection.^{14,17,18} Furthermore, therapeutic agents such as aminobisphosphonates (NBP) can inhibit the mevalonate pathway and thus lead to intracellular pAg accumulation. Upon elevated intracellular levels of pAg in target cells, the GTPase RhoB translocates from the nucleus to the cytoplasm where it presumably binds to the membrane protein butyrophilin 3A1 (BTN3A1, also known as CD277). This binding might induce a conformational change of BTN3A1 that in turn is sensed by the V γ 9V δ 2-TCR and results in V γ 9V δ 2-T cell activation.^{19–23} Activation of V γ 9V δ 2-T cells can be enhanced by interactions between the NKG2D receptor expressed on most V γ 9V δ 2-T cells and by stress-related MICA, MICB and ULBP molecules that are upregulated in infected or transformed cells.^{3,24} This, in combination with enhanced pAg levels, allows V γ 9V δ 2-T cells to distinguish “normal” cells from “altered-self” or tumor cells.²⁵ Activated

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V γ 9V δ 2-T cells produce pro-inflammatory cytokines (e.g. IFN- γ , TNF- α , TRAIL and the chemokines MIP-1 and RANTES) in addition to cytolytic mediators (perforin, granzyme B) to induce the specific lysis of target cells, which is regulated through the perforin pathway or through Fas- and TRAIL-induced apoptosis.^{25,26}

Their monomorphic recognition of activating ligands, their effective induction of tumor cell lysis and their rapid effector response provide V γ 9V δ 2-T cells with a unique combination of features that make them of major interest for cancer immunotherapeutic approaches. As a result, several clinical trials have been initiated to evaluate the use of V γ 9V δ 2-T cells in the treatment of both hematological and solid malignancies. Clinically explored approaches have included adoptive transfer of *ex vivo* expanded V γ 9V δ 2-T cells and the *in vivo* activation of V γ 9V δ 2-T cells through the administration of NBPs or synthetic pAg, alone or in combination with low-dose IL-2 treatment.^{27,28} These V γ 9V δ 2-T cell-based therapeutic approaches were well tolerated and capable of inducing clinically relevant anti-tumor responses in several cases. However, the overall results were inconsistent and are possibly related to the fact that these approaches induced a systemic V γ 9V δ 2-T cell activation without necessarily affecting their preferential accumulation and activation in the tumor microenvironment, where these cells should exert their anti-tumor effects.

To date, various bispecific T cell engagers (BiTEs) targeting both CD3 and a tumor antigen through the coupling of single-chain variable fragments (scFv) have been developed and were shown to induce clinical responses.²⁹ However, as CD3 is expressed by all T cells, including immunosuppressive regulatory T cells (Tregs) that actually predominate in the tumor microenvironment and are related to poor prognosis³⁰, antibody-based constructs designed to exclusively trigger immune cells with a pro-inflammatory function, such as V γ 9V δ 2-T cells, might well constitute a more effective approach.³¹ Recently, we have reported on the generation of a set of V γ 9V δ 2-TCR specific nanobodies with activating properties that could form the basis for a novel therapeutic approach aimed at tumor-specific V γ 9V δ 2-T cell accumulation and activation.³² Nanobodies (or VHHs) are defined by the variable antigen binding regions derived from heavy chain only antibodies, naturally occurring in camelids (i.e. llamas, camels and dromedaries).^{33,34} Single-domain VHH have several advantages over full-length antibodies or scFv when used for the generation of multivalent and/or multispecific molecules. Due to the absence of light chain domains, pairing issues do not apply, VHHs refold easily and they are provided with increased solubility. Moreover, VHHs can easily be produced by bacteria or yeast allowing time and cost reduction during manufacturing.^{35,36} Furthermore, VHH domains are low immunogenic because of their high homology with human VH genes and the absence of the Fc-region.^{29,36} VHHs are ten times smaller than conventional antibodies allowing them to reach clefts in antigen structures and granting them with enhanced tissue penetration as compared with conventional antibodies.^{37,38}

Here, we describe the generation and evaluation of a bispecific VHH-based construct that combines inhibition of the epidermal growth factor receptor (EGFR)-signaling pathway via an antagonistic anti-EGFR VHH with the target-dependent

activation of effector V γ 9V δ 2-T cells via an anti-V γ 9V δ 2-TCR VHH. V γ 9V δ 2-T cells activated in this manner produced pro-inflammatory cytokines such as IFN- γ and TNF- α and efficiently lysed EGFR-expressing tumor cell lines both *in vitro* and *in vivo*. This therapeutic effect was independent of *KRAS* or *BRAF* mutations, which are normally associated with resistance to anti-EGFR monoclonal antibody (mAb) therapy.^{39,40} Moreover, variations in V γ 9V δ 2-TCR δ 2-CDR3 sequence that are known to be associated with reduced V γ 9V δ 2-T cell responses¹ to pAg stimulation did not affect cell killing efficacy. This novel bispecific VHH-based immunotherapeutic approach can be applied to many tumor types by simply replacing the tumor-specific VHH and does not require further individualization due to the conserved monomorphic nature of the V γ 9V δ 2-TCR.

Results

Selection of a human V γ 9V δ 2-TCR specific and -activating VHH

V γ 9V δ 2-TCR specific VHHs were generated by immunizing two *Lama glamas* multiple times with human V γ 9V δ 2-T cells pooled from different healthy donors. Through phage display and after screening for V γ 9V δ 2-TCR specific fragments, 20 different V γ 9V δ 2-TCR specific VHHs were identified, either directed to the V δ 2- or to the V γ 9-chain, and either with activating or with non-activating potential as determined using a V γ 9V δ 2-TCR transduced JurMa luciferase reporter cell line. The specificity of these V γ 9V δ 2-TCR specific VHHs and their applicability for flow cytometry, immunocytochemistry, and magnetic activated cell sorting was previously reported.³² The VHHs with activating potential identified in this screen were then tested for their capability to induce activation of human healthy donor-derived V γ 9V δ 2-T cells via cross-linking. For this purpose, V γ 9V δ 2-T cells were cultured with plate-bound VHHs for 24 hrs. Activation of V γ 9V δ 2-T cells was determined by assessing up-regulation of the activation marker CD25, induction of CD107a expression reflecting the release of cytotoxic granules, and the intracellular production of IFN- γ as determined by flow cytometry. As a positive control we used NBP-pretreated HeLa cells in which the endogenous pAg isopentenyl pyrophosphate (IPP) accumulates as a result of NBP's inhibitory effect on farnesyl pyrophosphate synthase. These screens led to the identification of the anti-V δ 2 VHH 6H4 and the anti-V γ 9 VHH 6H1 as the most consistently activating VHHs, inducing V γ 9V δ 2-T cell activation in all three assays across multiple donors (Fig. 1A-C). Their ability to activate V γ 9V δ 2-T cells was further confirmed by studying the activation of V γ 9V δ 2-T cells from PBMC directly *ex-vivo* (Supplementary Fig. 1).

Although the vast majority of $\gamma\delta$ -T cells in the human peripheral blood consist of V γ 9V δ 2-T cells, $\gamma\delta$ -T cells expressing either the V γ 9-chain (i.e. V γ 9⁺V δ 2⁻ $\gamma\delta$ -T cells) or the V δ 2-chain (i.e. V γ 9⁻V δ 2⁺ $\gamma\delta$ -T cells) exist; these, however, do not respond to pAg stimulation. As the relative frequency of V γ 9⁻V δ 2⁺ $\gamma\delta$ -T cells is very low and substantially lower than the level of V γ 9⁺V δ 2⁻ $\gamma\delta$ -T cells^{17,41}, we reasoned that a V δ 2-TCR chain specific VHH would more selectively target

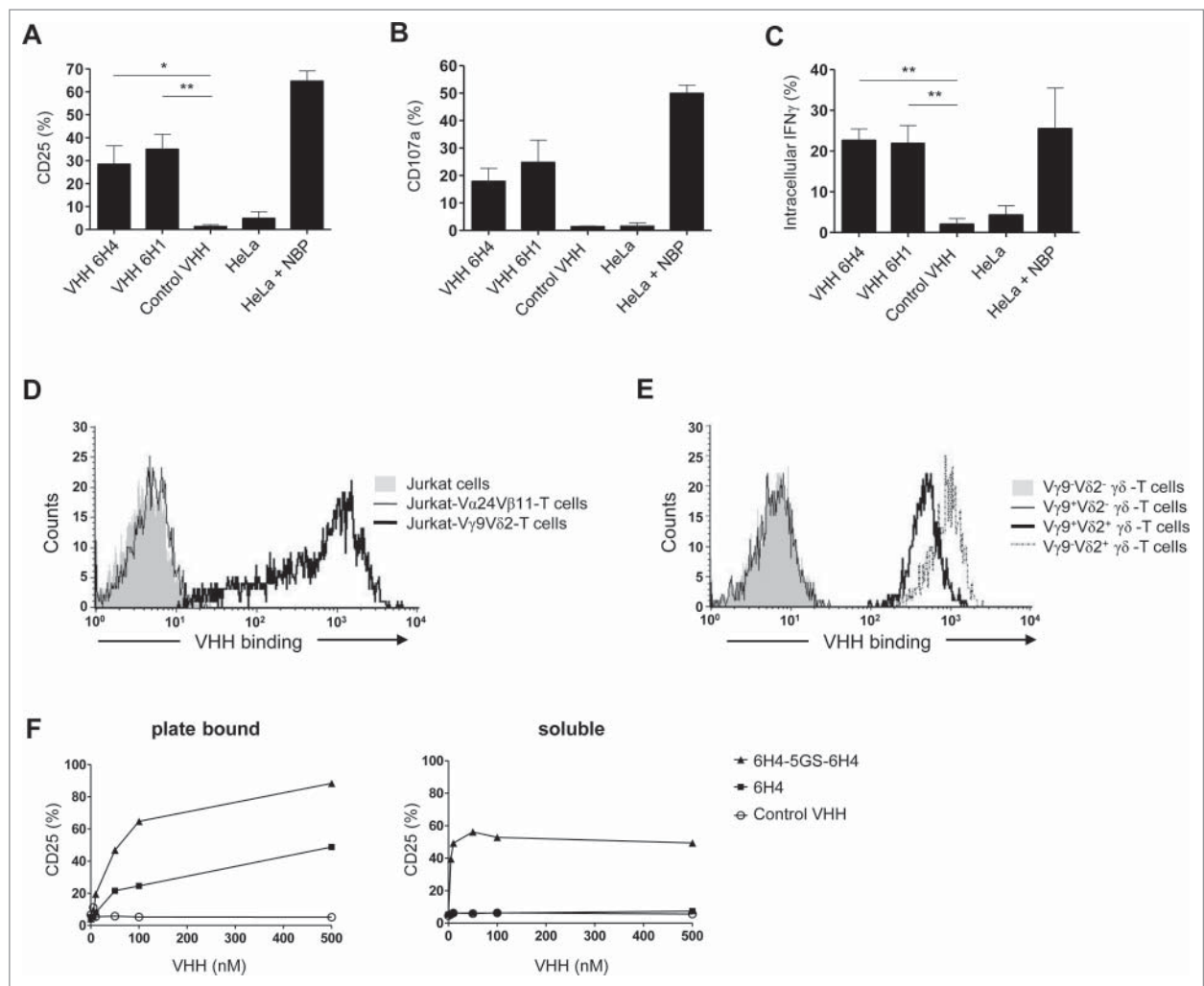


Figure 1. Characteristics of $V\gamma 9V\delta 2$ -T cell activating VHH. (A-C) $V\gamma 9V\delta 2$ -T cells were cultured with individual plate bound (wells coated with 500 nM) anti- $V\gamma 9V\delta 2$ -TCR VHH, control VHH R2, HeLa cells or NBP-pretreated HeLa cells in a 1:1 ratio. After 24 hrs, $V\gamma 9V\delta 2$ -T cell activation was determined by assessing the percentage of $V\gamma 9V\delta 2$ -T cells positive for (A) CD25, (B) CD107a, or (C) intracellular IFN- γ using flow cytometry. Shown are means subtracted by background levels \pm SEM of $n = 3$ -5 experiments. p -Values were calculated with a one-way ANOVA and Bonferroni's post-hoc test. (* indicates $p < 0.05$ and ** indicates $p < 0.01$). (D) The anti- $V\delta 2$ VHH 6H4 (40 nM) binds to Jurkat- $V\gamma 9V\delta 2$ -TCR cells (thick line), but not to Jurkat cells without TCR expression (filled grey) or Jurkat- $V\alpha 24V\beta 11$ -TCR cells (dotted line). (E) The anti- $V\delta 2$ VHH 6H4 (350 nM) binds to healthy donor-derived $V\gamma 9^+V\delta 2^+$ (thick line) and $V\gamma 9^+V\delta 2^+ \gamma\delta$ -T cells but not to $V\gamma 9^-V\delta 2^-$ (filled grey) or $V\gamma 9^+V\delta 2^- \gamma\delta$ -T cells (dotted line). (F) $V\gamma 9V\delta 2$ -T cells were cultured with plate bound or soluble monovalent VHH (filled squares), bivalent VHH (filled triangles) or control VHH R2 (open circles) at the indicated concentrations for 24 hrs. Expression of CD25 was assessed using flow cytometry. Representative figures of $n = 3$ experiments are shown. Abbreviations: aminobisphosphonates (NBP); Gly $_4$ Ser (GS).

$V\gamma 9V\delta 2$ -T cells and would therefore be the preferred VHH to be used in a bispecific VHH construct aimed at the specific targeting of $V\gamma 9V\delta 2$ -T cells. For this reason, the $V\delta 2$ -TCR specific VHH 6H4 (Fig. 1D and 1E) was selected for further experiments.

To determine whether coupling of two anti- $V\gamma 9V\delta 2$ -TCR VHHs into one bivalent VHH construct could result in an even stronger activation of $V\gamma 9V\delta 2$ -T cells, two 6H4 VHH genes were engineered into one construct and separated by a flexible Gly $_4$ Ser-linker (GS) of varying length (5-30 amino acids). First, bivalent VHH 6H4-5GS-6H4 was compared to the monovalent VHH 6H4 with respect to its ability to activate $V\gamma 9V\delta 2$ -T cells in a plate-bound assay. At all tested concentrations, stimulation with the bivalent VHH resulted in a stronger $V\gamma 9V\delta 2$ -T cell activation as compared to the monovalent VHH (Fig. 1F). However, to be optimally effective in a tumor targeting construct it is desirable that the VHH does not induce $V\gamma 9V\delta 2$ -T cell activation either on its own or in the absence of tumor cells.

We observed that when $V\gamma 9V\delta 2$ -T cells were cultured with the bivalent VHH 6H4-5GS-6H4 added in solution, a strong $V\gamma 9V\delta 2$ -T cell activation was induced, even at very low concentrations (Fig. 1F). This was independent of the linker length between both 6H4 VHHs (data not shown). In contrast, when monovalent VHH 6H4 was added in solution to $V\gamma 9V\delta 2$ -T cell cultures, $V\gamma 9V\delta 2$ -T cells did not become activated (Fig. 1F). As $V\gamma 9V\delta 2$ -TCR cross-linking by the bivalent VHH constructs would likely result in non-specific systemic activation as opposed to target-specific activation of $V\gamma 9V\delta 2$ -T cells, the monovalent anti- $V\gamma 9V\delta 2$ TCR VHH 6H4 was selected for incorporation into a bispecific tumor-targeting VHH construct.

Generation and functional evaluation of a bispecific anti-EGFR-anti- $V\gamma 9V\delta 2$ -TCR VHH construct

To generate a bispecific VHH construct, the anti- $V\gamma 9V\delta 2$ -TCR VHH 6H4 was joined to the previously generated and

characterized high-affinity anti-EGFR VHH 7D12. This VHH is able to compete with EGF for EGFR binding and inhibits both EGFR phosphorylation and EGFR⁺ tumor cell proliferation *in vitro* and *in vivo*.^{42,43} To determine the optimal format with respect to binding and functionality, multiple bispecific VHH constructs were created with variations in orientation and spacing between the individual VHHS. First, bispecific 7D12-6H4 and 6H4-7D12 VHH constructs were generated with a flexible Gly₄Ser-linker of 10 amino acids to determine whether target binding and affinity of the individual VHHS was maintained in the bispecific format and whether this was dependent on their orientation. Whereas the affinity of 6H4 to V γ 9V δ 2-T cells did not differ by its relative (i.e. N-terminal or C-terminal) position in the bispecific VHH, the anti-EGFR 7D12 VHH clearly bound to EGFR⁺ A431 tumor cells more efficiently when it was positioned at the N-terminus (Fig. 2A). Therefore, the 7D12-6H4 format was considered optimal and variations in the linker length were made to assess whether this would influence binding efficiency and functionality. As shown in Fig. 2B, linker length variations did not influence the binding of the construct to target cells. Neither did these influence the efficacy of V γ 9V δ 2-T cell activation, degranulation or tumor cell lysis induced by the bispecific VHH construct upon co-culture of V γ 9V δ 2-T cells with EGFR-expressing tumor cells (Fig. 2C). This was also observed when the linker length was

replaced to the smallest Gly₄Ser-linker consisting of 5 amino acids (data not shown). As a small linker is least prone to proteolysis *in vivo*, we used the 5 amino acid (5GS) linker for all subsequent experiments.

V γ 9V δ 2-T cell activation and subsequent tumor cell lysis was formally demonstrated to depend on simultaneous binding of the bispecific VHH construct to V γ 9V δ 2-T cells and EGFR-expressing tumor cells by the use of control constructs incorporating the irrelevant VHH R2⁴⁴ (Fig. 3A). Of note, V γ 9V δ 2-T cell activation and degranulation levels induced by the 7D12-5GS-6H4 bispecific VHH construct were equivalent to those observed when V γ 9V δ 2-T cells were co-cultured with NBP-pretreated EGFR⁺ tumor cells. The maximum level of tumor cell lysis induced by V γ 9V δ 2-T cells was observed at concentrations as low as 10 nM of the 7D12-5GS-6H4 bispecific construct. Importantly, this was at least as effective as when tumor cells were pretreated with 100 μ M NBP. Furthermore, when titrating down the 7D12-5GS-6H4 concentration, efficient lysis of EGFR⁺ tumor cells was observed at concentrations as low as 10 pM in a 1:1 effector:target cell ratio. Importantly, this was not observed when the immortalized human B-cell line JY, lacking EGFR expression, was used as target (Fig. 3B) and here-with demonstrated the specificity of this targeting approach.

As treatment with anti-EGFR mAbs such as cetuximab or panitumumab is often accompanied by skin toxicity,^{45,46} we

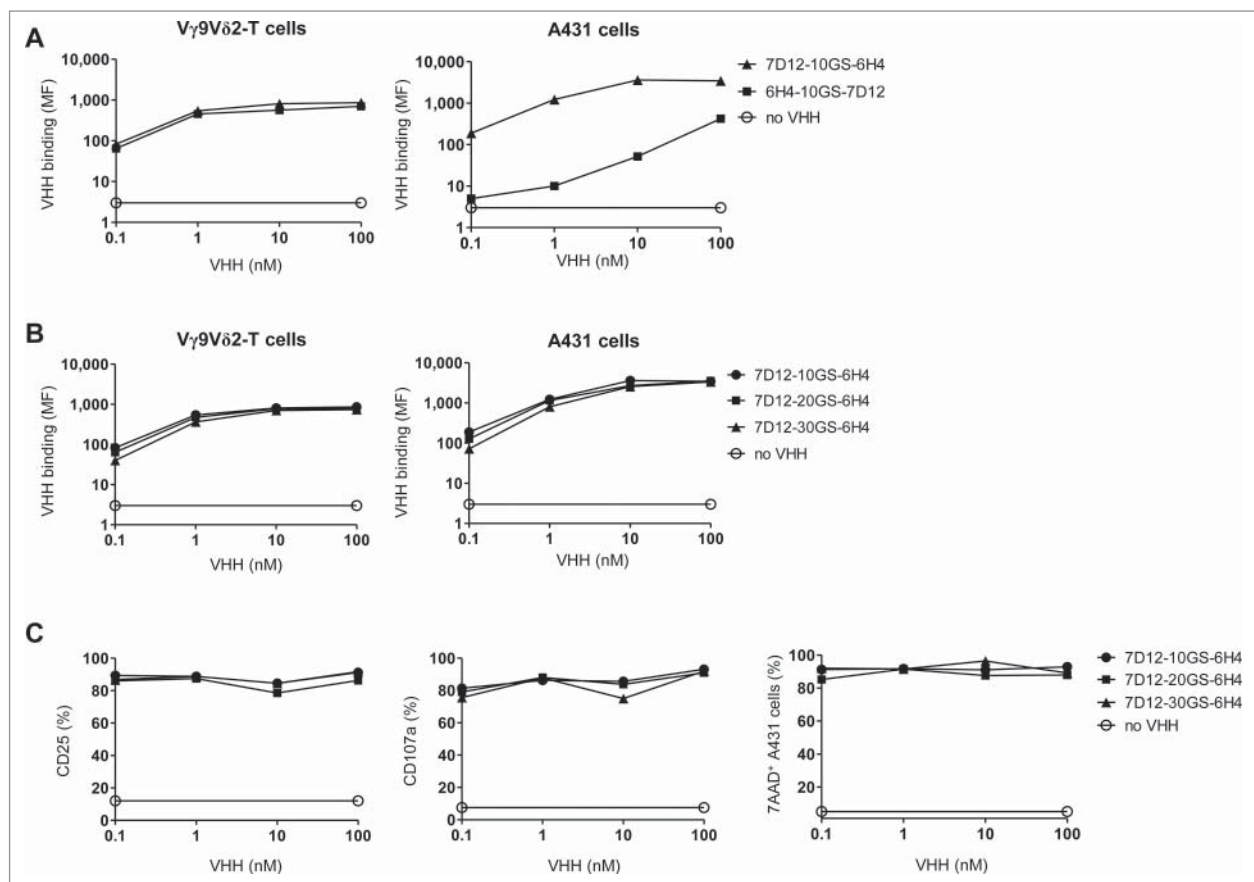


Figure 2. The effect of orientation and linker length in the bispecific VHH. (A and B) V γ 9V δ 2-T cells (left) or EGFR-expressing A431 cells (right) were incubated in the presence or absence of the indicated VHHS and bound VHH was assessed by flow cytometry. Mean fluorescence intensity (MF) of bound VHH to the cells is depicted. (C) V γ 9V δ 2-T cells and A431 cells were co-cultured in a 1:1 ratio for 24 hrs in the presence or absence of the indicated bispecific VHH. Both CD25 (left) and CD107a (middle) expression on V γ 9V δ 2-T cells were assessed by flow cytometry. The percentage of lysed A431 cells (right) was determined using 7-AAD staining and flow cytometry. Representative figures of $n = 3$ experiments are shown. Abbreviations: Gly₄Ser (GS).

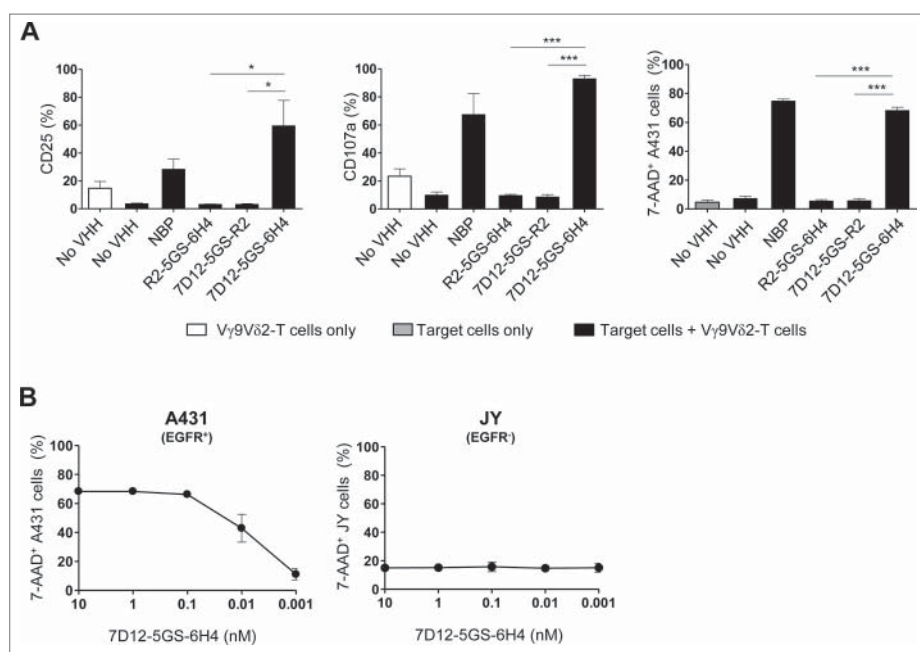


Figure 3. The 7D12-5GS-6H4 bispecific VHH induces $V\gamma 9V\delta 2$ -T cell activation and lysis of EGFR⁺ A431 tumor cells (A-B) or EGFR⁻ JY cells (B) in a 1:1 ratio in the presence of the 7D12-5GS-6H4 bispecific VHH or a bispecific control VHH. VHH concentrations: (A) 10 nM; (B) as indicated. For control situations, $V\gamma 9V\delta 2$ -T cells were co-cultured with target cells in the absence of VHH (no VHH; negative control) or with NBP-pretreated target cells (positive control). After 24 hrs, $V\gamma 9V\delta 2$ -T cell activation and degranulation was determined by assessing the percentage of CD25 or CD107a expression, respectively by flow cytometry. The percentage of lysed target cells was determined using 7-AAD staining and flow cytometry. A) *White bars* represent $V\gamma 9V\delta 2$ -T cell mono-cultures in the absence of VHH, *grey bars* represent target cell mono-cultures in the absence of VHH and *black bars* represent $V\gamma 9V\delta 2$ -T cell co-cultures with target cells and indicated VHH. B) Co-cultures of target cells with $V\gamma 9V\delta 2$ -T cells and indicated amount of 7D12-5GS-6H4 bispecific VHH. Shown are mean \pm SEM of $n = 3-4$ experiments. p -Values were calculated with a one-way ANOVA and Bonferroni's post-hoc test (* indicates $p < 0.05$ and *** indicates $p < 0.001$). Abbreviations: aminobisphosphonates (NBP); Gly₄Ser (GS).

explored whether primary skin-derived (EGFR⁺) keratinocytes were lysed by $V\gamma 9V\delta 2$ -T cells in the presence of the 7D12-5GS-6H4 construct. EGFR expression was confirmed by flow cytometry demonstrating that the mean fluorescence index (MFI) of EGFR expression on keratinocytes was 3.2 ± 1.5 ($n = 3$, mean \pm SD) (as a reference, the MFI of EGFR on A431 was 9.4, and the MFI of EGFR on JY was 0.8). Even at high concentrations of the bispecific targeting construct, only minor activation and cytolytic activity of $V\gamma 9V\delta 2$ -T cells was observed in the presence of primary keratinocytes (Supplementary fig. 2).

7D12-5GS-6H4 induces $V\gamma 9V\delta 2$ -T cell-mediated lysis of EGFR⁺ tumor cells irrespective of KRAS or BRAF mutation status

Of note, the 7D12 VHH retained its capacity to inhibit EGFR signaling in a dose-dependent manner upon incorporation into the bispecific VHH construct. As demonstrated by the analysis of EGFR phosphorylation in EGFR expressing tumor cells upon their exposure to EGF, this inhibitory activity was equivalent to that of the monovalent 7D12 VHH (Fig. 4A). To test if the 7D12-5GS-6H4 bispecific VHH exerted antitumor activity even in the presence of activating mutations in the EGFR signaling pathway, $V\gamma 9V\delta 2$ -T cells were co-cultured with EGFR⁺ human colon cancer cell lines carrying either a mutation in *KRAS* (i.e. SW480 cells, expressing one of the most common and oncogenic *KRAS* mutations G12V) or *BRAF* (i.e. HT-29 cells, expressing the most common *BRAF* mutation V600E)^{47,48} in the presence or absence of 7D12-5GS-6H4. As shown in Fig. 4B-I, 7D12-5GS-6H4 induced potent $V\gamma 9V\delta 2$ -T cell activation, degranulation and tumor cell

lysis of EGFR⁺ colon tumor cells, irrespective of their *KRAS* or *BRAF* mutation status. Next, the antitumor activity of the 7D12-5GS-6H4 bispecific VHH was assessed using primary tumor material from colon cancer patients. For this purpose, $V\gamma 9V\delta 2$ -T cells were co-cultured for 4 hrs at a 1:1 effector:target ratio with tumor cells of three different colon carcinoma patients (1 patient with a *RAS*^{wt} *BRAF*^{wt} tumor, 1 patient with a *KRAS*^{mt} tumor, and 1 patient with a *BRAF*^{mt} tumor). In the presence of the 7D12-5GS-6H4 bispecific VHH efficient lysis of primary colon cancer cells was observed (Fig. 4J). Again, no apparent differences between the wild-type tumor and *KRAS* or *BRAF* mutant primary tumors could be noted. EGFR expression was confirmed on all primary colon cancer samples using flow cytometry (Fig. 4K).

7D12-5GS-6H4 activates $V\gamma 9V\delta 2$ -TCR-G115 with various $\delta 2$ -CDR3 sequence variations

The sequence and length of the $\delta 2$ -CDR3 region of the $V\gamma 9V\delta 2$ -TCR varies between individuals and in part determines the TCR affinity and cytolytic capacity upon binding of pAg expressing target cells.¹ Since VHH 6H4 specifically binds to the V $\delta 2$ -chain of the $V\gamma 9V\delta 2$ -TCR, we determined if common V $\delta 2$ -CDR3 variations would influence the binding of the VHH 6H4 to the $V\gamma 9V\delta 2$ -TCR and whether this affected its $V\gamma 9V\delta 2$ -T cell activating capacity. To this end, JurMa cells were transduced to express the wildtype $V\gamma 9V\delta 2$ -TCR-G115 or the $V\gamma 9V\delta 2$ -TCR-G115 with $\delta 2$ -CDR3 variations in the 110–112 region (98–103 by Kabat numbering). This region was either replaced by i) a single alanine, creating a “short length” mutant ($\delta 2$ -G115_{LM1}) with complete abolishment of

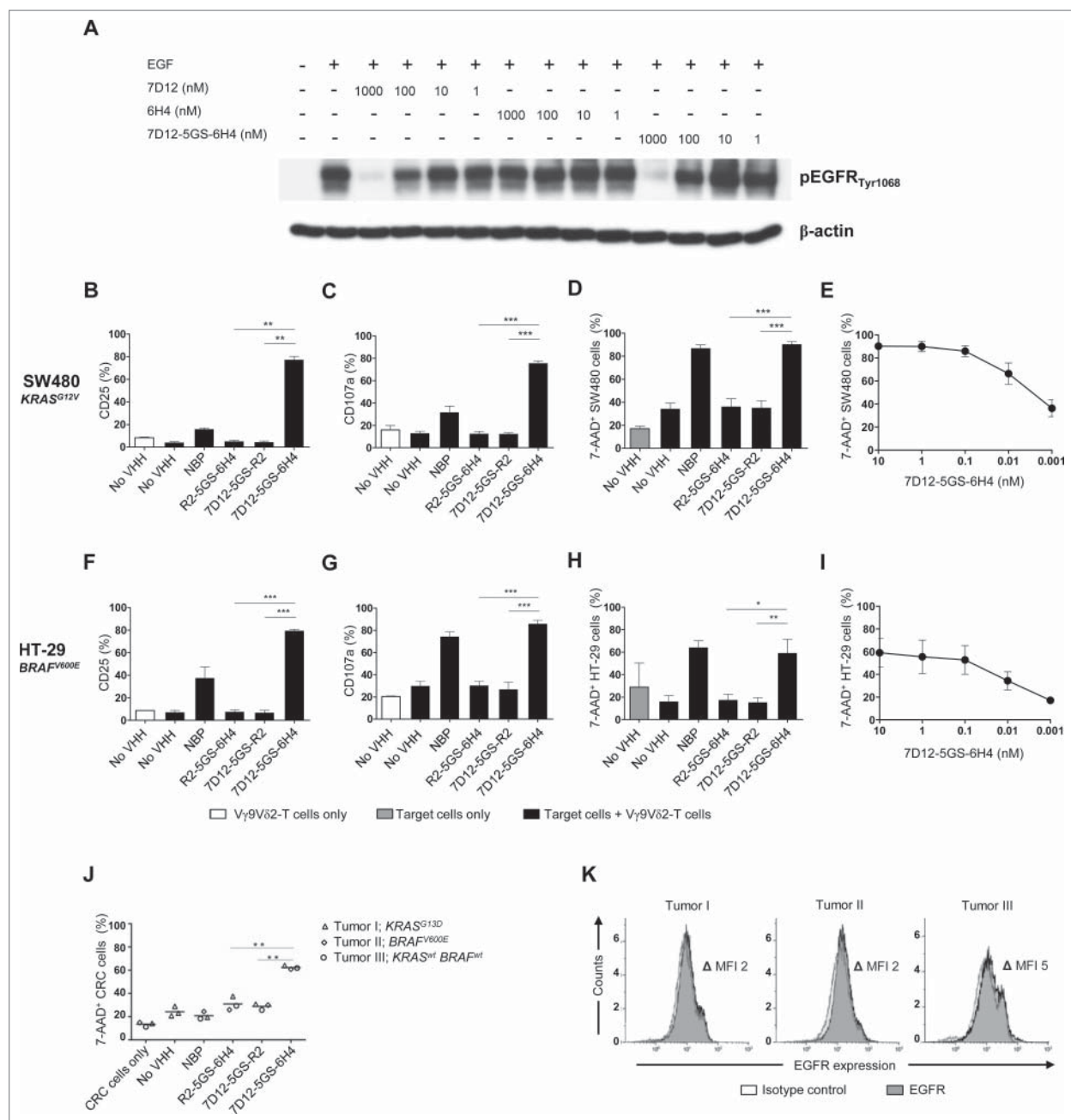


Figure 4. The 7D12-5GS-6H4 bispecific VHH inhibits EGFR signaling but does not depend on this to induce tumor cell lysis. (A) The anti-EGFR 7D12 VHH retains its capacity to inhibit phosphorylation of EGFR when incorporated in a bispecific 7D12-5GS-6H4 VHH format. Her14 cells were incubated with a mixture of 8 nM human EGF and the indicated VHH. Cell lysates were run on SDS-PAGE gel and western blotted for phosphorylated EGFR_{Tyr1068} and β -actin as a loading control. (B-E) $V\gamma 9V\delta 2$ -T cells were cultured with or without the EGFR⁺ colon tumor cells SW480 *KRAS^{G12V}* (B-E) or HT29 *BRAF^{V600E}* (F-I) for 24 hours or primary colon cancer cells for 4 hours (J) in a 1:1 ratio in the presence of the 7D12-5GS-6H4 bispecific VHH or a bispecific control VHH. VHH concentrations: B-D, F-H and J) 10 nM; E and I) as indicated. For control conditions, $V\gamma 9V\delta 2$ -T cells were co-cultured with target cells in the absence of VHH (no VHH; negative control) or with NBP-pretreated target cells (positive control). $V\gamma 9V\delta 2$ -T cell activation and degranulation was determined by assessing the percentage of CD25 (B) and (F) or CD107a (C and G) expression, respectively by flow cytometry. The percentage of lysed target cells was determined using 7-AAD staining and flow cytometry (D-E and H-J). B-D and (F-H) *White bars* represent $V\gamma 9V\delta 2$ -T cell mono-cultures in the absence of VHH, *grey bars* represent target cell mono-cultures in the absence of VHH and *black bars* represent $V\gamma 9V\delta 2$ -T cell co-cultures with target cells and indicated VHH. E and (I) Co-cultures of target cells with $V\gamma 9V\delta 2$ -T cells and the indicated amount of 7D12-5GS-6H4 bispecific VHH. J) Lysis of patient derived primary colon cancer cells; tumor 1: mutation in *KRAS* exon 2, c.38G>A, p.G13D; tumor 2: mutation in *BRAF* exon 15, c.1799 T>A, p.V600E; tumor 3: RAS and BRAF wild-type. (K) All three colon cancer samples expressed EGFR as determined by flow cytometry. Shown are means \pm SEM of $n = 3$ experiments. p -Values were calculated with a one-way ANOVA and Bonferroni's post-hoc test (* indicates $p < 0.05$, ** indicates $p < 0.01$ and *** indicates $p < 0.001$). Abbreviations: aminobisphosphonate (NBP); Gly₄Ser (GS); mean fluorescence index (MFI).

pAg/BTN3A1-reactivity; ii) 9 alanine amino acids, creating an "enlongated length" mutant (in $\delta 2$ -G115_{LM9}) with approximately 40% reduced pAg/BTN3A1-reactivity compared to wild-type $V\gamma 9V\delta 2$ -TCR-G115; iii) the $\delta 2$ -CDR3 sequence of the naturally weakly pAg/BTN3A1-reactive c13 clone ($\delta 2$ -G115_{c13}); or iii) the $\delta 2$ -CDR3 sequence of the naturally highly

pAg/BTN3A1-reactive c15 clone ($\delta 2$ -G115_{c15}) (Supplementary table 1).¹ The $\delta 2$ -G115 length mutants and $\delta 2$ -G115_{c13} showed a slightly reduced binding of VHH 6H4 compared to $\delta 2$ -G115_{WT} and $\delta 2$ -G115_{c15} (Fig. 5A). However, and more importantly, no significant difference was observed in the ability of VHH 6H4 to trigger activation as determined by CD69

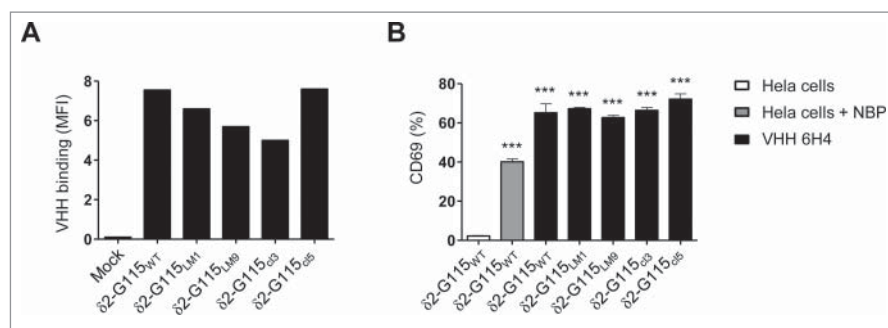


Figure 5. The anti- $V\gamma 9V\delta 2$ -TCR specific VHH 6H4 efficiently activates $V\gamma 9V\delta 2$ -T cells harbouring $\delta 2$ -CDR variations. (A) Indicated JurMa transductants were incubated with 500 nM VHH 6H4 and bound VHH was assessed by flow cytometry. Mean fluorescence intensity (MFI) of VHH bound to the cells is depicted. A representative figure of $n = 3$ experiments is shown. (B) Indicated JurMa transductants were cultured with HeLa cells (negative control, white), NBP-pretreated HeLa cells (positive control, grey) or plate bound (wells coated with 500 nM) VHH 6H4 (black). After 24 hrs, the activation status of the cells was determined by assessing CD69 expression on the cells by flow cytometry. Indicated significant differences are relative to values of $\delta 2$ -G115_{WT} cells stimulated with HeLa cells. A representative figure of triplicate samples (mean \pm SEM) of $n = 3$ experiments is shown. p -Values were calculated with a one-way ANOVA and Bonferroni's post-hoc test (***) indicates $p < 0.001$). Abbreviations: aminobisphosphonates (NBP).

expression on the JurMa cells expressing the modified $V\gamma 9V\delta 2$ -TCRs as compared to the JurMa cells expressing the $V\gamma 9V\delta 2$ -TCR-G115_{WT} (Fig. 5B). Thus, donor sequence variations in $\delta 2$ -CDR3 that are known to impact pAg recognition, do not substantially affect the capacity of the 7D12-5GS-6H4 bispecific VHH construct to trigger $V\gamma 9V\delta 2$ -T cell activation and function.

7D12-5GS-6H4 enhances the $V\gamma 9V\delta 2$ -T cell-mediated inhibition of tumor outgrowth *in vivo*

We next assessed the effect of the 7D12-5GS-6H4 VHH on the outgrowth of human EGFR-overexpressing tumors *in vivo*. Immunodeficient BRGS mice were engrafted with SW480^{Gluc} tumor cells carrying a KRAS^{G12V}-mutation and transduced to stably express Gaussia luciferase (Gluc). Expression of Gluc allowed real-time monitoring of viable primary and metastatic tumor cell load and response to treatment using bioluminescence imaging (BLI).⁴⁹ At days 1, 4, and 7, mice were treated with either PBS, cetuximab, $V\gamma 9V\delta 2$ -T cells, or $V\gamma 9V\delta 2$ -T cells in combination with 7D12-5GS-6H4 (Fig. 6A). At day 35, BLI clearly demonstrated that mice treated with the combination of $V\gamma 9V\delta 2$ -T cells and 7D12-5GS-6H4 had an overall lower tumor burden compared to the mice treated with PBS, cetuximab or $V\gamma 9V\delta 2$ -T cells alone (Fig. 6B and C). Most importantly, and in accordance with the BLI data, mice treated with the combination of $V\gamma 9V\delta 2$ -T cells and 7D12-5GS-6H4 had a significantly improved overall survival compared with mice treated with either PBS, cetuximab or $V\gamma 9V\delta 2$ -T cells alone (Fig. 6D). These results confirm the expected treatment failure with cetuximab of KRAS mutated colorectal cancer cells and demonstrate that EGFR-mediated tumor targeting by 7D12-5GS-6H4 enhances the $V\gamma 9V\delta 2$ -T cell-mediated inhibition of tumor outgrowth *in vivo*, even for cetuximab resistant (i.e. KRAS mutated) tumors.

Discussion

$V\gamma 9V\delta 2$ -T cells have a unique combination of features that make them highly promising for use in cancer immunotherapy, i.e. the recognition of ligands exclusively exposed by stressed or

altered cells in an MHC-independent manner, a rapid innate-like response, the ability to induce efficient target cell lysis via multiple routes (Fas/FasL and perforin pathway) against a wide variety of tumor targets, the induction of dendritic cell maturation, and even efficient antigen presentation to $\alpha\beta$ -T cells.^{22,25,26} Several attempts have been made to clinically exploit $V\gamma 9V\delta 2$ -T cell activation in cancer patients but results thus far lack consistency.^{27,28} This is likely related to the absence of a specific trigger for the activated $V\gamma 9V\delta 2$ -T cells to home to and infiltrate tumor sites.

Here, we explored whether the antitumor activity of $V\gamma 9V\delta 2$ -T cells could be enhanced and directed to the tumor by using a bispecific VHH construct that would allow $V\gamma 9V\delta 2$ -T cell accumulation and activation specifically at the tumor site. As a model tumor antigen we selected EGFR, which is a key factor in epithelial malignancies as its activity enhances tumor growth, invasion, and metastasis.^{50,51} Agents aimed at EGFR inhibition, such as anti-EGFR mAbs competing for ligand binding (e.g. cetuximab and panitumumab) and EGFR-specific tyrosine kinase inhibitors (TKI; e.g. erlotinib or gefitinib), are currently registered treatments for various advanced-stage epithelial cancers, including non-small-cell lung cancer, colorectal cancer, pancreatic cancer, and head and neck squamous cell carcinoma.⁵² Treatment with these agents is related to improved progression free and overall survival, though the overall efficacy is generally limited and frequently restricted to certain patient subsets, leaving ample room for improvement.⁵²

From a set of 20 $V\gamma 9V\delta 2$ -TCR specific VHHs generated from immunized llamas and selected by phage display, we selected the $V\delta 2$ -specific VHH 6H4 on the grounds that it consistently induced $V\gamma 9V\delta 2$ -T cell activation and since targeting the $V\delta 2$ -chain would be more specific for $V\gamma 9V\delta 2$ -T cells than targeting the $V\gamma 9$ -chain, as in general $V\gamma 9^+V\delta 2^- \gamma\delta$ -T cells are more abundant than $V\gamma 9^-V\delta 2^+ \gamma\delta$ -T cells in the human peripheral blood.^{17,41} As bivalent formats of the $V\delta 2$ -specific VHH 6H4 already induced striking activation of $V\gamma 9V\delta 2$ -T cells in the absence of target cells, probably due to the crosslinking of the TCRs, and this was expected to result in systemic activation of $V\gamma 9V\delta 2$ -T cells when applied therapeutically, we decided to use the monovalent VHH 6H4 for incorporation in the bispecific VHH in order to minimize the chances of non-

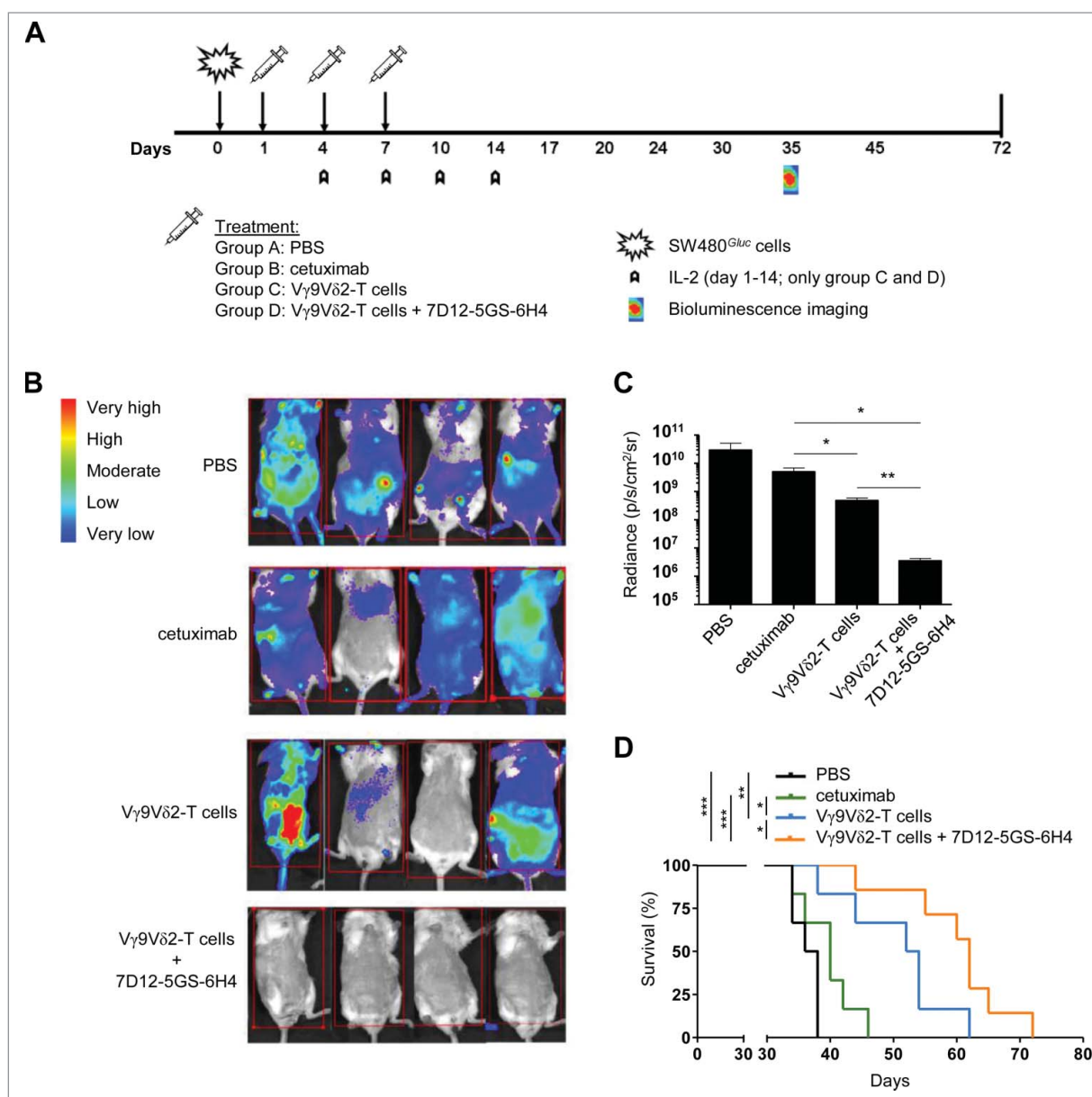


Figure 6. The 7D12-5GS-6H4 bispecific VHH inhibits tumor growth *in vivo*. Immunodeficient BRGS mice grafted with SW480^{Gluc} cells were treated with PBS (control group; (A), cetuximab (500 μ g i.p.; group (B), V γ 9V δ 2-T cells (1×10^7 i.v.; group (C) or V γ 9V δ 2-T cells and 7D12-5GS-6H4 VHH (1×10^7 and 1 μ g, respectively, both i.v.; group (D) at days 1, 4 and 7. IL-2 (10,000 U, i.p.) was administered on days 1, 4, 7, 10, and 14 to the groups receiving V γ 9V δ 2-T cells. A) A schematic overview of the treatment schedule. B and C) Bioluminescence imaging at day 35 of 4 mice per treatment group. B) Heat map indicating the sites and relative level of tumor cell activity in individual mice. Red squares indicate the image field used for quantification analysis. C) Quantified bioluminescence signal measured per mouse expressed as the measured radiance normalized to the number of pixels, time and angle of imaging. Shown are means \pm SEM of $n = 4$ mice per group. p -Values were calculated with a unpaired T-test (* indicates $p < 0.05$). D) Kaplan-Meier analyses of mouse survival, $n = 6$ mice per group. p -Values were calculated with a Mantel-Cox test (* indicates $p < 0.05$, ** indicates $p < 0.01$ and *** indicates $p < 0.001$). Abbreviations: Gly₄Ser (GS).

specific and tumor unrelated activation upon systemic administration. By joining an anti-EGFR VHH (7D12) to the 6H4 VHH, we created a bispecific VHH construct targeting both EGFR and the V γ 9V δ 2-TCR. The generated 7D12-5GS-6H4 bispecific VHH induced strong activation and degranulation of V γ 9V δ 2-T cells resulting in potent lysis of EGFR expressing tumors at picomolar concentrations in an EGFR and V γ 9V δ 2-TCR dependent fashion. Previously, we determined that VHH 7D12 inhibited EGFR phosphorylation and pathway activation by binding to EGFR domain III, thus preventing its conformational change to an active state.^{42,43} Importantly, integration of anti-EGFR VHH 7D12 into the bispecific format did not alter its ability to inhibit EGF-induced signaling in EGFR

overexpressing cancer cells. Moreover, and in contrast to the currently available anti-EGFR mAb therapies which are mainly effective through the inhibition of EGFR signaling⁵², the 7D12-5GS-6H4 bispecific VHH construct described here also induced efficient V γ 9V δ 2-T cell-mediated lysis of both colon cancer cell lines and primary colon cancer cells carrying common *KRAS* or *BRAF* mutations. Mutations such as these in the proto-oncogenes of the RAS family (e.g. *KRAS*, *NRAS*, *HRAS*) and *BRAF* frequently occur in e.g. colorectal, pancreatic and lung cancers, which together account for a major proportion of cancer cases. This often makes tumors resistant to the currently available anti-EGFR therapies (e.g. mAbs and TKI)^{39,40} and leads to poor prognosis^{53,54}. Our data demonstrate that 7D12-5GS-6H4

has a dual mechanism of action by combining the inhibition of EGF-induced signaling (involved in tumor survival, growth and metastasis) with the direct induction of tumor cell lysis. The effective and superior anti-tumor effect of the bispecific VHH construct was confirmed using mice xenograft experiments. Mice grafted with human EGFR-overexpressing *KRAS*-mutated tumor cells that were treated with the bispecific VHH in combination with $V\gamma 9V\delta 2$ -T cells showed significant reduction of tumor outgrowth and improved overall survival. These are promising results that suggest that the bispecific 7D12-5GS-6H4 VHH construct may also inhibit growth of EGFR⁺ tumors in patients independent of the *RAS* or *BRAF* mutation status of the tumor and might thereby allow a more widespread applicability of EGFR-targeted treatments by bypassing the need for *RAS* and *BRAF* mutation analyses.⁵⁵ Moreover, the data presented here provide a proof of concept for $V\gamma 9V\delta 2$ -T cell targeted therapy for a broad range of tumor types, which may be facilitated by simply exchanging the anti-EGFR VHH for VHHs targeting various other tumor antigens.

Interestingly, the ability to overcome the therapeutic barrier posed by *RAS* mutations was also noted for bispecific T cell engagers (BiTEs) wherein the scFv variable domains of cetuximab or panitumumab were fused to a scFv against CD3 expressed by T cells.⁵⁶ However, though some BiTEs have induced clinical responses, limitations of this approach include the requirement for continuous infusions of the drug due to its short serum half-life time and the fact that it targets CD3 which is expressed by all T cells including immunosuppressive T cells such as Tregs.²⁹ Tregs predominate in the tumor microenvironment, actively suppress the activation and proliferation of effector T cells and are related to unfavorable prognosis.³⁰ For this reason, antibody-based constructs designed to exclusively trigger immune cells with a pro-inflammatory function, such as $V\gamma 9V\delta 2$ -T cells, may be preferable over the targeting of CD3.³¹

In a recently published preclinical study the antitumor efficacy of $V\gamma 9V\delta 2$ -T cells was explored through the use of a tribody targeting the $\gamma 9$ -chain of the $V\gamma 9V\delta 2$ -TCR and the tumor antigen Her2/neu. This (Her2)₂ $V\gamma 9$ tribody efficiently lysed Her2/neu overexpressing pancreatic cells *in vitro* and in mouse xenografts.⁵⁷ Although this nearly full-sized (~100kD) antibody approach underscores the potential of tumor-targeted $V\gamma 9V\delta 2$ -T cell-based immunotherapies, more specific targeting of the $V\gamma 9V\delta 2$ -T cell population can be achieved using an antibody (fragment) directed to $V\delta 2$ compared to $V\gamma 9$, as in our bispecific VHH. Furthermore, the development and use of whole mouse mAbs has several limitations including the mispairing of heavy and light chains and the risk of developing human-anti-mouse antibodies (HAMA) in patients which leads to antibody neutralization and adverse events in the form of a cytokine release syndrome.^{29,36} These limitations can be overcome by the use of VHHs. VHH are low immunogenic because they share high homology with human *VH* genes and are devoid of an Fc-region.^{29,33,36} Because of the single domain nature of VHHs, pairing issues do not apply. This advantage in combination with their small size and the fact that they do not require post-translational modifications, allow VHHs to be easily produced in bacteria or yeast, which remain the most cost- and time-efficient production systems to date.^{36,58,59} VHHs are known for their high stability at elevated temperature and pH,

providing them with enhanced solubility and making them less prone to aggregation.^{36,60} The small size of VHHs (~30 kDa for a bispecific VHH) also facilitates deep tumor tissue penetration compared to larger sized antibody constructs, but like other small antibody fragments (such as BiTEs) this is also associated with a short serum half-life time due to fast renal clearance. This can be circumvented by fusion of the VHH to a serum albumin binding VHH.^{29,38,42,61}

As anti-EGFR mAb therapy can be complicated by (generally well manageable) skin toxicity as a result of mAb binding to EGFR expressed on keratinocytes, it was encouraging to see only minor keratinocyte lysis when keratinocytes were cultured with $V\gamma 9V\delta 2$ -T cells in the presence of the 7D12-5GS-6H4 bispecific VHH. Although preclinical tests such as this do not necessarily predict safety in patients, these results are encouraging with regards to the future clinical exploration of this particular bispecific VHH construct.

In conclusion, we here describe the development of a bispecific VHH construct with a dual mechanism of action, combining ligand deprivation crucial for tumor cell proliferation and survival with the efficient and exclusive lysis of EGFR expressing tumor cells by conserved immune effector $V\gamma 9V\delta 2$ -T cells. Since EGFR is a widely expressed and clinically validated tumor antigen, a large patient group could benefit from this therapy. This group can be even broadened by the fact that, in contrast to currently available anti-EGFR therapies, the effectiveness of this therapy will not be influenced by downstream mutations in e.g. *RAS* or *BRAF* and, due to the monomorphic nature of the $V\gamma 9V\delta 2$ -TCR this immunotherapeutic approach requires no further individualization. Furthermore, as recently VHHs directed to various other tumor antigens have been developed⁶² and continue to be developed, these can easily be exchanged for the anti-EGFR VHH enabling future $V\gamma 9V\delta 2$ -T cell targeted therapy for a broad range of tumor types.

Materials & methods

Cell lines

HeLa, A431, HT29, Colo320 and SW480 cell lines were obtained from ATCC and, as well as Her14⁶³ cultured in DMEM+, i.e. Dulbecco's Modified Eagle's Medium (Lonza, catalog #BE12-614 F) supplemented with 10% (v/v) fetal calf serum (FCS) (HyClone GE Healthcare, catalog #SV30160.03), 100 IU/mL sodium penicillin, 100 μ g/mL streptomycin sulfate and 2.0 mM L-glutamine (Life Technologies, catalog #10378-016).

The SW480 cell line was stably transduced with lentivirus carrying the *Gussia Luciferase (Gluc)* and *Cerulean Fluorescent Protein (CFP)* genes (LV-CFP-Gluc)⁶⁴, kindly provided by Tom Würdinger (VU University medical center (VUmc), Amsterdam, NL), to generate the SW480^{Gluc} cell line. CFP positive SW480^{Gluc} cells were sorted and used for tumor injection in mice when CFP expression was >95% as determined by flow cytometry.

JY cells and Jurkat transductants³² were cultured in IMDM+, i.e. Iscove's modified Dulbecco's medium (Lonza, catalog #BE12-722 F) supplemented with 10% (v/v) FCS, 0.05 mM β -mercaptoethanol, 100 IU/mL sodium penicillin, 100 μ g/mL streptomycin sulfate and 2.0 mM L-glutamine. JurMa cell lines transduced with $V\gamma 9V\delta 2$ -TCR-G115_{WT} and

δ 2-CDR3 variants, were generated as previously described¹ and cultured in IMDM+.

Primary cells

Keratinocytes were isolated from human adult skin as described previously.⁶⁵ In brief, epidermal sheets were separated from dermis by incubation with Dispase II (Roche, catalog #04942078001) overnight at 4 °C. Subsequently, keratinocytes were isolated from the epidermis by a 10 minute 0.125% trypsin incubation (HyClone GE Healthcare, catalog # SH3004201) and seeded per 3×10^6 cells in keratinocyte culture medium on 9-cm-diameter tissue culture dishes coated with 0.5 μ g/cm² human placental collagen IV (Sigma-Aldrich, catalog #C5533). Keratinocyte culture medium consisted of Dulbecco's Modified Eagle's Medium and Ham's F12 (Invitrogen, catalog #21765-029) in a 3:1 ratio, supplemented with 10% (v/v) FCS, 100 IU/mL sodium penicillin, 100 μ g/mL streptomycin sulfate, 2.0 mM L-glutamine, 1 μ mol/L hydrocortisone (Sigma-Aldrich, catalog #H0888), 1 μ mol/L isoproterenol hydrochloride (Sigma-Aldrich, catalog #I6504), 0.09 μ mol/L insulin (Sigma-Aldrich, catalog #I5500), and 2 ng/ml human keratinocyte growth factor (Sigma-Aldrich, catalog #K1757). All cell lines were maintained at 37 °C with 5% CO₂ in a humidified atmosphere and tested mycoplasma negative.

Colon cancer tissues from patients participating in trials conducted at the VU University medical center were collected and processed after written informed consent as described previously⁶⁶. In brief, tumor material was washed, cut into small fragments and digested mechanically using collagenase A (Roche Diagnostics, The Netherlands) based growth medium. Single cell suspensions were obtained by passing the digested material through a 45 μ m sterile filter after which the samples were frozen under controlled conditions in liquid nitrogen. Before use, the colon cancer samples were thawed, dead cells were removed by a ficoll based density gradient separation and resuspended in DMEM+. The mutational status of KRAS exon 2/3/4, NRAS exon 2/3/4 and BRAF exon 15 was assessed by a high resolution melting (HRM) assay followed by Sanger sequencing of HRM-PCR products with an aberrant melt curve, essentially as described previously.^{67,68}

Flow cytometry and monoclonal antibodies

FITC-labeled anti-TCR V δ 2 (catalog #555738), FITC-labeled anti-IFN- γ (catalog #554700), FITC-labeled anti-CD69 (catalog #347823), PE-labeled anti-CD107a (catalog #555801), PE-labeled anti-CD25 (catalog #55542), PE-labeled pan $\gamma\delta$ -TCR (catalog #333141), APC-labeled anti-CD25 (catalog #340907), and 7-AAD (catalog #559925) were obtained from BD Biosciences. PerCP-labeled anti-TCR V δ 2 (catalog #331410), PE-labeled anti-TCR V γ 9 (catalog #331308) and APC-labeled anti-TCR V γ 9 (catalog #331310) were from Biolegend. RPE-labeled goat-anti-mouse F(ab')₂ fragment (catalog #R0480) was obtained from Dako. Anti-Myc tag mAb clone 4A6 (catalog #05-724) was obtained from Merck Millipore and anti-Myc tag mAb clone 9E10 was produced in-house. Alexa488-labeled cetuximab was a kind gift of Rens Braster and Yvette van Kooyk (VUmc, Amsterdam, NL). All stainings for flow

cytometry were performed in PBS supplemented with 0.1% BSA and 0.02% sodium-azide. Intracellular IFN- γ production was determined by adding GolgiPlug to the cell culture for the final 4 hrs of the experiment. Cells were fixed and permeabilized with the Fixation/Permeabilization Solution Kit from BD Biosciences (catalog #555028) and stained with anti-IFN- γ mAb.

Stained cells were measured with a FACS Calibur or LSRFortessa (BD Biosciences) and analyzed with CellQuest (BD Biosciences) or Kaluza software (Beckman-Coulter).

Generation of donor-derived V γ 9V δ 2-T and pan $\gamma\delta$ -T cell lines

Healthy donor-derived V γ 9V δ 2-T cells were isolated, expanded and cultured as described previously.³² In brief, V γ 9V δ 2-T cells were isolated by magnetic-activated cell sorting from PBMC using FITC-labeled anti-TCR V δ 2 or PE-labeled anti-TCR V γ 9 mAb in combination with anti-mouse IgG MicroBeads (Miltenyi Biotec, catalog #130-048-401). Purified V γ 9V δ 2-T cells were stimulated weekly with irradiated and NBP-pretreated (100 μ M Pamidronate for 2–3 hrs, Teva Pharmachemie, catalog #12J08RD) human mature monocyte derived dendritic cells or an irradiated feeder mix (PBMC of 2 healthy human donors and Epstein Barr Virus transformed B cells with addition of 50 ng/ml PHA). V γ 9V δ 2-T cells were used for experiments when V γ 9⁺V δ 2⁺-TCR expression was >90% and CD25 expression was <40% as determined by flow cytometry.

V γ 9V δ 2-T cell lines were cultured in Ysells+, i.e. Ysells medium⁶⁹ supplemented with 1% human AB serum (Collect, MP Biomedicals, catalog #2931949), 50 U/ml rhIL-2 (Proleukin, Novartis), 0.05 mM β -ME, 100 IU/mL sodium penicillin, 100 μ g/mL streptomycin sulfate and 2.0 mM L-glutamine. V γ 9V δ 2-T cell lines and tumor target cell lines were cultured in IMDM+ medium during experiments. The V γ 9V δ 2-T cell lines were maintained at 37 °C with 5% CO₂ in a humidified atmosphere and tested mycoplasma negative.

V γ 9⁺V δ 2⁺, V γ 9⁺V δ 2⁻, V γ 9⁻V δ 2⁺ and V γ 9⁻V δ 2⁻ $\gamma\delta$ -T cell lines were obtained from human PBMC as follows. PBMC were stained with PE-labeled pan $\gamma\delta$ -TCR antibody and anti-mouse IgG MicroBeads by MACS isolation. This pan $\gamma\delta$ -T cell line was expanded with a feeder mix and subsequently stained with FITC-labeled anti-TCR V δ 2 and PE-labeled anti-TCR V γ 9 antibodies to allow flow cytometric cell sorting of 4 separate populations (i.e. V γ 9⁻V δ 2⁺, V γ 9⁺V δ 2⁻, V γ 9⁺V δ 2⁺ and V γ 9⁻V δ 2⁻ $\gamma\delta$ -T cells).

Generation, production and purification of bivalent and bispecific VHHs

To generate bivalent or bispecific VHHs, genes of VHH 6H4³², VHH 7D12⁴² or VHH R2⁴⁴ were PCR-amplified using Phusion High-Fidelity DNA Polymerase (New England Biolabs, catalog #M0530) and appropriate primers encoding the N- or C-terminal end of the VHH gene, a restriction endonuclease site, and a linker sequence (composed of Gly₄Ser repeats). PCR products were purified by gel extraction using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, catalog #740609), digested with restriction endonucleases to allow cloning into appropriate plasmids.

For *in vitro* experiments, PCR products were cloned to plasmid pMek219, verified by sequencing and produced in TG1 bacteria as described previously³². Produced VHH were derived from the bacterial periplasm by a PBS freeze-thawing step and purified by immobilized metal ion affinity chromatography (IMAC) using Talon resin (Clontech, catalog # 635504). VHH were eluted with 150 mM imidazole and dialyzed twice against PBS.

For *in vivo* experiments, PCR products were cloned to a modified version of the pFastBac I plasmid (Thermo Fisher Scientific) containing a Honeybee Melittin Signal Sequence (HMSS) and a C-terminal his-tag. Bacmid DNA and virus were essentially prepared according to the Bac-to-Bac manual (Thermo Fisher Scientific). Briefly, the pFastBac constructs were transformed into EMBACY cells⁷⁰ and bacmid DNA was isolated. Sf9 insect cells were transfected with the bacmid DNA and the virus was subsequently amplified in a sf9 suspension culture. Collected virus was used to infect sf9 cells for protein expression. Medium containing the secreted proteins was harvested 3 days post infection and dialyzed 2 x against 25 mM HEPES pH 7.5 and 200 mM NaCl. Proteins were purified on a 5 ml HiTrap Ni²⁺ column, eluted with 200 mM imidazole in 25 mM HEPES pH 7.5, 200 mM NaCl. Proteins were further purified by size exclusion chromatography on a S75 16/60 Superdex column (GE Healthcare) equilibrated with PBS buffer. Fractions containing the proteins were pooled, concentrated and passed through a 0.22 μ M filter.

The purity of produced protein was checked on a coomassie blue-stained protein gel before use.

Binding analysis of VHH

To determine the binding of VHH to cells, 5×10^4 V γ 9V δ 2-T cells, A431 cells, Jurkat or JurMa transductants were incubated with VHH at the indicated concentrations for 30 minutes. Bound VHH was detected with anti-Myc-tag antibody clone 4A6 and RPE-labeled goat-anti-mouse F(ab')₂ fragment by flow cytometry.

Functional analysis of monospecific, bivalent and bispecific VHH

To determine the effect of monovalent and bivalent V γ 9V δ 2-TCR specific VHH on V γ 9V δ 2-T cell activation, V γ 9V δ 2-TCR expressing cells were either cultured in the presence of plate bound or soluble VHHs. For plate bound conditions, wells of a 96-well flat-bottom culture plate (Greiner, catalog #655180) were coated with 4 μ g/ml mouse-anti-Myc clone 9E10 in PBS overnight at 4 °C. Wells were washed three times with PBS and incubated for 2 hrs with the indicated concentrations of VHH in PBS. After the wells were again washed three times with PBS, 10^5 V γ 9V δ 2-T cells, 10^5 V γ 9V δ 2-TCR-G115 transduced JurMa cells or 2.5×10^5 PBMC were added per well in a final volume of 200 μ l IMDM+. For soluble VHH conditions, the indicated concentration of VHH in PBS was added to 10^5 V γ 9V δ 2-T cells in a 96-well flat-bottom culture plate. For control conditions, V γ 9V δ 2-T cells were co-cultured with untreated (negative control) or NBP-treated (100 μ M Pamidronate for 2–3 hrs, positive control) HeLa cells in a 1:1 ratio. Cells were cultured in a final volume of 200 μ l IMDM+ for 24 hrs.

To determine the effect of bispecific anti-EGFR-anti-V γ 9V δ 2-TCR VHH on V γ 9V δ 2-T cell activation, degranulation and target cell lysis, 5×10^4 target cells (A431, JY, HT29, Colo320, SW480 or primary colon cancer cells) were labeled with 40 nM CFSE (Sigma-Aldrich, catalog #21888) or 5 μ M PBSE (Thermo Fisher Scientific, catalog #P10163), according to the manufacturer's protocol and allowed to adhere for 4hrs in a 96-well flat bottom culture well. V γ 9V δ 2-T cells were incubated with the indicated concentrations of VHH for 1 hr at 4 °C, washed three times with PBS and added in a 1:1 ratio to the target cells and cultured for 24 hrs in a final volume of 200 μ l IMDM+. In case of keratinocytes, primary keratinocytes were plated 2 days beforehand on collagen IV coated wells to obtain a viable cell pool at the start of the experiment. In case of primary colon cancer cells, these were not adhered to the plate before the addition of V γ 9V δ 2-T cells and cells were co-cultured for 4 hrs instead of 24 hrs. For the NBP-pretreated positive control, target cells were incubated with 100 μ M Pamidronate during cell adherence and washed by a 3x PBS rinse before the addition of V γ 9V δ 2-T cells.

To determine degranulation of V γ 9V δ 2-T cells, anti-CD107a mAb and GolgiStop (BD Bioscience, catalog #554724) were added to the co-culture for the final 4 hrs of the experiment. At the end of the experiment, cells were harvested and stained with anti-V δ 2 and/or anti-V γ 9 mAb or CD3 mAb to identify V γ 9⁺V δ 2⁺-T cells. CD25 and CD69 expression on V γ 9V δ 2-T cells was determined with an anti-CD25 and anti-CD69 mAb, respectively. Cells were stained with 7-AAD according to the manufacturer's protocol to distinguish lysed cells. mAbs bound to the cells and 7-AAD staining were analyzed by flow cytometry.

Inhibition of EGF-induced EGFR phosphorylation

Inhibition of EGF-induced EGFR phosphorylation was performed as described before.⁷¹ 10^5 Her14 cells were seeded per well in a 12-wells plate in DMEM+ and allowed to adhere. After 8 hrs, the medium was replaced by DMEM^{min}, i.e. Dulbecco's Modified Eagle's Medium supplemented with 0.1% (v/v) FCS, 100 IU/mL sodium penicillin, 100 μ g/mL streptomycin sulfate and 2.0 mM L-glutamine. The following day, cells were washed once with PBS, after which a mixture of the indicated VHHs was added to the cells in combination with 8 nM recombinant human EGF (Peprotech, catalog # AF-100-15) in DMEM^{min} for 15 minutes at 37 °C. Subsequently the cells were washed three times with ice-cold PBS and resuspended in 2x Laemmli protein sample buffer. Half of the sample was loaded and run on a SDS-PAGE gel and western blotted. Phosphorylated EGFR was detected with an anti-phosphoEGF Receptor (Tyr1068) polyclonal antibody and an anti-rabbit-HRP mAb (both from Cell Signaling Technology, catalog #2234 and #7074, respectively). Blots were stained with an anti- β -actin (Sigma-Aldrich, catalog #21888) and anti-mouse HRP mAb (Cell Signaling Technology, catalog #7076) to demonstrate that equal amounts of cell lysate were loaded on gel.

In vivo studies

Immunodeficient BRGS mice (BALB/c Rag2^{-/-}Il2rg^{-/-} Sirpa^{NOD})⁷² were housed in isolators under pathogen-free

conditions and randomly divided in 4 treatment groups ($n = 6/\text{group}$). At day 0, mice received an intravenous (i.v.) tail vein injection with 0.5×10^6 SW480^{Gluc} cells. At days 1, 4 and 7 mice were treated with either a) 500 μg cetuximab i.p., b) 1×10^7 V γ 9V δ 2-T cells i.v., c) 1×10^7 V γ 9V δ 2-T cells in combination with 1 μg of the bispecific 7D12-5GS-6H4 VHH i.v., or d) an equal volume of sterile PBS i.v.. Mice that received V γ 9V δ 2-T cells were injected at days 1, 4, 7, 10 and 14 with 10,000 U human recombinant IL-2 i.p. to stimulate the proliferation of activated V γ 9V δ 2-T cells. Bioluminescence imaging (BLI) was performed at day 35 in 4 randomly selected mice from each study group. For this procedure, mice were anesthetized with inhalation anesthetics (isoflurane/oxygen) and injected i.v. retro-orbitally with 4 mg/kg coelenterazine (PJK GmbH, native-CTZ). BLI was recorded using an IVIS imaging system (PerkinElmer) and images were analysed using Living Image 4.0 software. BRGS mouse experiments were approved by the animal ethical committee of the Institut Pasteur (Reference # 2007–006), Paris, France, and validated by the French Ministry of Education and Research (Reference # 02162.01).

Statistical analyses

Statistical analyses were performed in GraphPad Prism version 5 (La Jolla, CA, USA). For *in vitro* analyses, a one-way or two-way ANOVA with a Bonferroni's post-hoc test was used as appropriate. For *in vivo* data analysis of BLI, an unpaired T-test was used. For the survival analysis, a Mantel-Cox test was used. All findings were considered significant when *p*-values were < 0.05 .

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Disclosures

We would like to state that authors Renée de Bruin, Tanja de Gruijl, Henk Verheul and Hans van der Vliet have a patent on V γ 9V δ 2-TCR specific nanobodies. Hans van der Vliet is CSO/CMO of Lava Therapeutics. Author Jürgen Kuball reports grants from Pierre Fabre, Novartis, and Miltenyi Biotech. He is scientific co-founder and CSO of gadeta (www.gadeta.nl) and inventor of multiple patents on $\gamma\delta$ TCR receptors and isolation strategies for engineered immune cells. The other authors have no financial conflicts of interest.

Authorship contributions

RB	performed research, collected data, analyzed and interpreted data, performed statistical analysis, and wrote the manuscript
JV	performed research, analyzed and interpreted data and wrote the manuscript
SL	performed research, analyzed and interpreted data and performed statistical analysis and wrote the manuscript.
FS	performed research, analyzed and interpreted data and wrote the manuscript
SLL	performed research, analyzed and interpreted data and wrote the manuscript
RL	performed research, analyzed and interpreted data and wrote the manuscript
AS	performed research, analyzed and interpreted data and wrote the manuscript
ZS	designed research, performed research and revised the manuscript critically
JK	designed research, interpreted data and revised the manuscript critically
CM	designed research and revised the manuscript critically
EH	designed research and revised the manuscript critically
RR	designed research and revised the manuscript critically
JD	designed research and revised the manuscript critically
PBH	designed research and revised the manuscript critically
HMWV	designed research and revised the manuscript critically
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