T cells engineered to express chimeric antigen receptors (CAR-T cells) have shown impressive clinical efficacy in the treatment of B cell malignancies. However, the development of CAR-T cell therapies for solid tumors is hampered by the lack of truly tumor-specific antigens and poor control over T cell activity. Here we present an avidity-controlled CAR (AvidCAR) platform with inducible and logic control functions. The key is the combination of (i) an improved CAR design which enables controlled CAR dimerization and (ii) a significant reduction of antigen-binding affinities to introduce dependence on bivalent interaction, i.e. avidity. The potential and versatility of the AvidCAR platform is exemplified by designing ON-switch CARs, which can be regulated with a clinically applied drug, and AND-gate CARs specifically recognizing combinations of two antigens. Thus, we expect that AvidCARs will be a highly valuable platform for the development of controllable CAR therapies with improved tumor specificity.
CARs are synthetic proteins consisting of antigen-binding domains linked to T-cell-activating signaling domains (Fig. 1a). T cells genetically engineered to express CARs (CAR-T cells) efficiently eliminate antigen-expressing target cells, which is reflected by the impressive efficacy of CD19-directed CAR-T cell therapies in patients with B cell malignancies. A major challenge in translating this success to other malignancies is the fact that CAR antigens are almost exclusively tumor-associated antigens (TAAs), which are not truly tumor specific. Since most TAAs are also expressed in vital healthy tissues, efficient TAA targeting is expected to result in unacceptable on-target/off-tumor toxicity, which has indeed been observed in several clinical studies. Ultimately, this could preclude increasing the efficacy of CAR-T cell therapies to levels required for sustained remission of solid tumors. The problem of on-target/off-tumor toxicity is further aggravated by the fact that the activity of CAR-T cells is difficult to control in a reversible manner.

To address the lack of control and tumor specificity associated with CAR-T cells, several promising strategies have been developed including CARs for combinatorial antigen recognition, affinity-tuned CARs to minimize killing of healthy cells expressing low levels of target antigen and CARs whose function can be regulated by administration of small molecules or soluble proteins. Despite these important advances, there is still a need for improvement. For example, currently available systems for the control of CAR-T cells are either based on small molecules which are suboptimal for clinical application or on suicide

![Diagram of CARs and their applications](https://example.com/diagram.png)

**Fig. 1 Principle and applications of AvidCARs.** a Schematic of a conventional CAR (high-affinity CAR) typically containing a high-affinity antigen-binding domain (usually a single-chain variable fragment, scFv) fused via a dimerizing hinge region (derived from, e.g., CD8α) to the cytoplasmic domains of a costimulatory receptor (mostly 4-1BB or CD28) and CD3ζ. Due to high-affinity binding, monovalent interaction with the target antigen is sufficient for CAR activation. b Schematic of a low-affinity CAR in which a low-affinity antigen-binding domain is fused to the same conventional CAR backbone shown in (a). We hypothesized that such low-affinity CARs require bivalent interaction with the antigen for efficient activation. We further hypothesized that this avidity-based concept (i.e., the dependency on bivalent antigen recognition) can be exploited for various applications: c for generating ON-switch AvidCARs whose function is regulated by a dimerizing small molecule; d for constructing AND-gate AvidCARs which are dimerized by a soluble antigen B, leading to avidity-based recognition of antigen A; e, f and for the design of heterodimeric AND-gate AvidCARs specifically recognizing a combination of two surface bound antigens with two different recognition domains, respectively. This AND-gate concept can be realized in a nonswitchable format by constitutive CAR heterodimerization (e) or in a switchable version by inducing heterodimerization with a small molecule (f).
switches which irreversibly destroy the CAR-T cells. Furthermore, previously described AND-gate CARs suffer from low specificity or their inability to differentiate between a double-positive cell and two single-positive cells, which express the antigens A and B complementarily and are located in close proximity to each other. That is, in this SynNotch strategy, expression of the CAR can be induced by healthy cells expressing antigen A, enabling recognition of nearby healthy cells expressing antigen B. Furthermore, the SynNotch system is based on xenogeneic and thus potentially immunogenic proteins.

One critical parameter defining the potency and function of CARs is the affinity of their antigen-binding domains. Importantly, the interaction with a target antigen not only depends on the affinity of an individual antigen-binding domain, but also on the valency of the interaction. That is, a multivalent interaction amplifies the individual affinities through a phenomenon known as avidity. Many types of natural immune reactions are based on low affinities, which are amplified by avidity effects, such as antigen binding by IgM during early humoral immune responses. However, the influence of avidity effects on CAR function has mostly been neglected in the CAR field so far, even though most currently used CARs are based on naturally dimeric components.

Here, we demonstrate that this aspect can be exploited for the integration of inducible and logic control functions into CAR molecules. We show that it is possible to generate avidity-controlled CARs (AvidCARs) which are highly potent and dependent on bivalent antigen engagement (Fig. 1). These AvidCARs are based on two main design principles: controlled CAR dimerization and low-affinity antigen binding. We exemplify the potential of the AvidCAR platform (1) by generating a controllable ON-switch CAR that can be regulated by CAR homodimerization with a clinically applied small molecule (Fig. 1c); (2) by constructing an AND-gate CAR that is triggered only in the presence of both a surface molecule on tumor cells and a soluble protein found in tumor stroma (Fig. 1d); and (3) by generating a heterodimeric AND-gate CAR which enables the combinatorial recognition of two different antigens exclusively when co-presented on the surface of the same cell. This latter AND-gate AvidCAR is designed either in a constitutive (Fig. 1e) or in a switchable version regulated by a dimerizing small molecule (Fig. 1f). Finally, we also investigate the suitability of different antigen-binding modules and various co-stimulatory domains for the design of efficient AvidCARs. Together, our study illustrates the potential of exploiting avidity effects and demonstrates that the AvidCAR platform is a versatile concept for inducible and combinatorial CAR control.

**Results**

**Construction of avidity-controlled CARs.** Since a critical design principle of AvidCARs is the control over CAR dimerization, the usage of CAR components potentially causing uncontrolled dimerization or oligomerization needs to be avoided. Given the recent report on scFv-mediated CAR tonic signaling (probably caused by scFv-mediated clustering) we looked for an antigen-binding domain based on a single protein domain, which prevents intermolecular dimerization as was observed with several scFvs. Therefore, we chose the human epidermal growth factor receptor (hEGFR)-specific binder E11.4.1, which was previously engineered based on the single-domain protein rCsso7d. As described above, we hypothesized that the construction of an AvidCAR also requires the incorporation of low-affinity binding domains (Fig. 1b). For this purpose, we reduced the affinity of the high-affinity binder E11.4.1 by performing an alanine scan, resulting in eight different E11.4.1 mutants. To determine the affinities of those mutants, they were expressed as monomeric proteins (Supplementary Fig. 1a) and titrated on Jurkat cells expressing truncated hEGFR (hEGFRt, Supplementary Fig. 1c). These titration experiments yielded an affinity of 13 nM for the high-affinity binder E11.4.1, which is in close agreement with the previously published value (17 nM). Among the mutants we identified two (E11.4.1-G25A and E11.4.1-G32A) with substantially decreased, yet measurable affinities of 125 and 877 nM, respectively (Fig. 2a). Complementary affinity determination by surface plasmon resonance (SPR) analysis with the entire extra-cellular domain of hEGFR yielded highly comparable $K_d$ values (Supplementary Fig. 1b).

To test our hypothesis that only low-affinity, but not high-affinity CARs are dependent on bivalent interaction, we incorporated the high-affinity (E11.4.1; $K_d$ of 13 nM), intermediate-affinity (E11.4.1-G25A; $K_d$ of 125 nM), or low-affinity (E11.4.1-G32A; $K_d$ of 877 nM) hEGFR binders into CARs comprising the hinge and transmembrane region of CD8a, followed by the signaling domains of 4-1BB and CD3ζ (termed BBz-CAR, Fig. 2b). In addition, each of these CARs was also created in a second version in which the two extracellular cysteine residues in the CD8a hinge region, which are known to cause CD8a dimerization, were mutated to serine residues, resulting in a CAR backbone termed Ser-BBz. All CAR variants were expressed at similar levels in primary human T cells (Supplementary Fig. 2a). In co-culture with hEGFRt-expressing target cells (Supplementary Fig. 2b) both the high- and intermediate-affinity CARs triggered target cell lysis and IFN-γ secretion both in the BBz-, as well as in the Ser-BBz-format, albeit at slightly higher efficiency in the dimeric BBz version (Fig. 2c and Supplementary Fig. 2c). In contrast, the low-affinity CAR only mediated lysis and IFN-γ production when expressed with dimerization-promoting cysteine residues in its CD8a hinge (Fig. 2c and Supplementary Fig. 2c), thus supporting our initial hypothesis that low-affinity CARs are dependent on bivalent interactions.

Next, we used a mathematical model to investigate whether the observed avidity-driven activation of AvidCARs is mediated by an increase in the effective lifetime of the CAR–antigen interaction (by allowing for increased rebinding) and/or by increased binding (i.e., more CAR occupancy by antigen). A longer lifetime is thought crucial for pMHC binding to the T-cell receptor (TCR) as a result of kinetic proofreading and this has recently been shown to be the case also for CARs. Thus, to investigate how avidity alters CAR occupancy and lifetime, we set up a mathematical model of antigen binding to a bivalent CAR with kinetic proofreading (e.g., representing CAR phosphorylation) where a tunable parameter (σ) was used to modulate avidity (Supplementary Fig. 3a). The model shows that increasing avidity has only a modest effect on CAR occupancy but a dramatic effect on the amount of phosphorylated CAR, highlighting that increased avidity strongly prolongs the duration of individual CAR–antigen interactions, thereby enhancing the signaling capacity.

In summary, this mathematical model predicts that increased avidity leads to improved antigen sensitivity (Supplementary Fig. 3b). To investigate this dependence of AvidCAR sensitivity on avidity experimentally, we switched to another assay system based on small molecule-mediated CAR dimerization. For that purpose, the Fk506-binding protein (FKBP12) and a mutant FKBP12-rapamycin binding domain (FRB) were integrated into the Ser-BBz-CARs for small molecule (AP21967)-induced CAR heterodimerization (Fig. 2d). To additionally investigate the impact of antigen dimerization on avidity, we integrated the FKBP12-F36V domain into hEGFRt for small molecule (AP20187)-mediated antigen homodimerization (Fig. 2d). Primary human T cells expressing either high-affinity CARs or the corresponding low-
affinity AvidCARs were then co-cultured ± AP21967 (for CAR dimerization) and ± AP20187 (for antigen dimerization) with Jurkat target cells expressing the antigen hEGFR at different densities ranging from hundreds to several hundred thousand molecules per cell (Supplementary Fig. 4a). Target cell lysis mediated by the high-affinity CAR was largely independent of the dimerization state of both the CAR and the antigen, strongly suggesting that the high-affinity CAR was not dependent on bivalent interaction (Fig. 2e, left diagram). In contrast, the low-affinity AvidCAR efficiently triggered activation only in a dimeric form (Fig. 2e, right diagram, black and red solid lines), again demonstrating its dependency on bivalent interaction, i.e., avidity. Furthermore, the antigen sensitivity of this dimeric AvidCAR was strongly increased if also the antigen was dimerized (red solid line vs.

Fig. 2 Generation of AvidCARs. a Flow cytometric determination of the affinities of hEGFR-specific binder mutants. Soluble binder proteins (sfGFP fused to E11.4.1 or mutants thereof) were titrated on hEGFR Jurkat cells. Shown are the median fluorescence intensities (MFI) of bound E11.4.1-variants (three independent experiments; mean values ± SD). Colored lines indicate the high-, intermediate-, and low-affinity binders used in the following experiments. The insert in the diagram shows the structure of the original Sso7d binder scaffold with its mutated binding surface in pink (PDB 1SSO). b, c Dependence of CAR function on binder affinity and CAR dimerization. b Schematic of the tested CARs. Selected rcSso7d-based binders were fused to a CAR backbone in which the CD8α hinge contained either two cysteine- or two serine residues instead (see SEQ-IDs 1 and 2 in Supplementary Data 1, respectively). c The figure shows the cytolytic activity of mock-T cells (no CAR) and T cells expressing the monovalent (Ser-BBz) and bivalent (BBz) CARs with different affinities against hEGFR Jurkat target cells. (Luciferase-based assay; four independent experiments with four different donors; **p < 0.01, calculated by two-tailed paired t-test). d, e Influence of CAR and antigen dimerization on the activity of AvidCARs and their high-affinity versions. d Schematic of the experimental system for controlled dimerization of CARs (containing domains for conditional heterodimerization by the compound AP21967; see SEQ-IDs 3 and 4 in Supplementary Data 1) and for separately regulated dimerization of the antigen hEGFR (containing a domain for conditional homodimerization by AP20187; SEQ-ID 21). e T cells, which expressed CARs with either high- or low-affinity binders (i.e., Kd of 13 nM or 877 nM, respectively), were co-cultured with Jurkat cells expressing different levels of hEGFR. The effect of CAR and antigen dimerization was tested by separately regulating these two dimerization events with AP21967 and AP20187, respectively. The figure shows the lysis of hEGFR-expressing Jurkat cells as determined by a flow cytometry-based cytotoxicity assay (two independent experiments with three different donors; mean values ± SD). Exact p values are given in Supplementary Data 2. Source data are provided as a Source data file.
black solid line, Fig. 2e, right diagram). Thus, enhancing the avidity effect of the dimeric AvidCAR—mediated through additional antigen dimerization in this experiment—resulted in increased antigen sensitivity, as predicted by the mathematical model described above. Similar activation patterns were observed when measuring IFN-γ release (Supplementary Fig. 4b). High-affinity CAR and AvidCAR constructs were expressed at similar levels, hence excluding expression-based effects (Supplementary Fig. 4c).

Together, these experiments demonstrate that it is possible to engineer AvidCARs which depend on bivalent interaction and whose function can thus be regulated by drug-mediated dimerization. Moreover, the comparable efficacy of the monomeric and dimeric high-affinity CAR (Fig. 2e) is in line with a previous report showing that a single CD3ζ domain is sufficient for efficient signaling40.

**scFVs may preclude controlled CAR dimerization.** Global efforts in the development and clinical testing of novel CAR-T cell constructs almost exclusively rely on scFv-based binders. To test the suitability of scFVs for generating AvidCARs, we incorporated the hHER2-specific low-affinity scFv 4D5-5 (Kd of 1.1 μM)15 into both the BBz- and the Ser-BBz-CAR (Fig. 3a). In contrast to the rcSso7d-based CARs described above, in the scFv-based CAR the Cys→Ser-mutation had little effect on target cell lysis and IFN-γ secretion by CAR-T cells co-cultured with hHER2tpos target cells (Fig. 3b), despite even lower expression levels of the CAR containing the Ser-mutation (Supplementary Fig. 5a). Given those functional differences compared to the rcSso7d-based CARs despite similarly low affinities, we hypothesized that scFv-mediated oligomerization might prevent the formation of strictly monomeric CARs. Dimerization of scFvs has previously been reported for soluble scFvs, resulting in the formation of so-called diabodies, in which the VH of one scFv molecule pairs with the VL of another scFv molecule35–37 (Fig. 3c).

To investigate whether intermolecular dimerization of VH and VL between scFvs can also trigger CAR oligomerization, we expressed this scFv as an Fv, i.e., without the linker connecting VL and VH, thus preventing such intermolecular dimerization (Fig. 3d and Supplementary Fig. 5b). In this Fv-based CAR, the VH was fused to the Ser-BBz-backbone, whereas the VL was anchored via a separate CD8α(Ser) transmembrane domain without an intracellular signaling region. Importantly, compared to the scFv-based CAR, significantly reduced cytotoxicity was observed with the Fv-based CAR (Fig. 3e), strongly suggesting that removing the linker from the scFv prevents CAR dimerization and thereby avidity-based activation of low-affinity CARs. Of note, CAR activity could be restored by AP20187-induced homodimerization of two Fv-based CAR molecules (Fig. 3e). This demonstrates (1) that separate VH- and VL-containing constructs assemble into a CAR molecule with a functional Fv unit and (2) that in the absence of scFv-mediated oligomerization, avidity can be controlled by inducing dimerization. As expected, in the absence of the VL, construct the dimerization of the VH-construct had no effect, confirming that antigen binding requires an assembled Fv comprising VH and VL (Fig. 3e). Furthermore, all constructs were expressed at similar levels, thus excluding expression-mediated effects (Supplementary Fig. 5c).

Together, these data demonstrate that intermolecular VH/VL dimerization of at least certain scFvs can cause CAR oligomerization. As a consequence, such clustering scFvs are undesired in our context not only because of tonic CAR signaling34,41, but also because of preventing the avidity-based control of AvidCARs.

**Influence of different co-stimulatory domains.** Next, we investigated whether the dependence of low-affinity CARs on dimerization is also observed with other co-stimulatory domains by exchanging the 4-1BB domain in the Ser-BBz-CAR backbone for the endodomain of CD28, ICOS, OX40, or CD2 in our hEGFR-specific rcSso7d-based AvidCAR as described above. All CARs additionally contained an FKBPI2-F36DV domain for conditional homodimerization by AP20187 (Fig. 3f). In a cytotoxicity experiment with primary human T cells expressing those CARs, significant dimerization dependence was observed with 4-1BB-, ICOS- and CD2-mediated co-stimulation, but not if CD28 or OX40 were incorporated (Fig. 3g). IFN-γ secretion was significantly induced by dimerization of 4-1BB- and CD2-based CARs (Supplementary Fig. 5e). Albeit not being significant, a trend was also observed with the endodomain of OX40. In summary, these data suggest that among the co-stimulatory domains tested, the ones derived from 4-1BB or CD2 are best suited for construction of efficient AvidCARs—at least with the binding affinities and antigen densities tested in these experiments.

**AvidCARs with AND-gate function.** Current bispecific CARs are characterized by high-affinity binding domains and, hence, do not possess AND-gate function but OR-gate function, i.e., activation in the presence of either antigen A or B or both42–44. Moreover, the homodimeric/oligomeric nature of current CAR designs facilitates multivalent engagement of a single antigen by several identical binding domains, thus additionally precluding efficient AND-gate function. Here, we tested whether our AvidCAR platform, in which undesired homodimerization can be prevented, allows for the generation of bispecific CARs with robust AND-gate function. To this end, we generated an additional low-affinity binder recognizing a different model antigen. As a starting point, we chose the hHER2-specific single-domain binder zHER2-2AK, which is based on the previously engineered affibody ZHER22,45. High-affinity binding of zHER2-2AK to hHER2 was confirmed by SPR, yielding a Kd of 17 nM (Supplementary Fig. 6b). Again, to reduce its affinity, the affibody was subjected to an alanine scan. The resulting 12 mutants were incorporated into the Ser-BBz-CAR backbone comprising the FKBPI2-F36DV domain for functional screening by controlled homodimerization. In a cytotoxicity assay, the CAR containing the binder mutant zHER2-2AK-R10A triggered lysis of hHER2tpos cells in a dimerization-dependent manner (Supplementary Fig. 6a), indicating that the reduced affinity of this mutant necessitates bivalent antigen engagement. Indeed, subsequent SPR analysis confirmed that this affibody-mutant binds to hHER2 with low affinity (Kd of 865 nM), which is comparable to that of the hEGFR-specific rcSso7d-based binder E11.4.1-G32A described above.

To generate the hEGFR/hHER2-bispecific AND-gate AvidCAR, those low-affinity binders were incorporated into two separate Ser-BBz-CAR constructs with complementary heterodimerization domains (Fig. 4a). As we had hypothesized, primary human T cells expressing this AND-gate AvidCAR efficiently triggered lysis of target cells expressing both hEGFRt and hHER2t, while lysis of cells expressing only hEGFRt or hHER2t was comparable to background lysis by CARneg T cells (Fig. 4b and Supplementary Fig. 6c). This experiment clearly demonstrates the dependency of this heterodimeric AvidCAR on combinatorial antigen recognition. Moreover, cytotoxicity was only observed in the presence of the heterodimerizing agent, demonstrating that this AND-gate AvidCAR can be additionally controlled by administration of a small molecule. Of note, the incorporation of the high-affinity versions of both binders (Kd values of 13 and 17 nM for E11.4.1 and zHER2-2AK, respectively) abrogated the AND-gate character and the dependence on...
dimerization (Fig. 4b), thus confirming the necessity of low-affinity antigen binding. Both the high-affinity and the low-affinity heterodimeric CARs were expressed at similar levels, excluding any major expression-based bias (Supplementary Fig. 6d). Fusion of the two low-affinity binders in tandem into a single, monomeric CAR molecule (TanCAR) (Fig. 4a), also yielded specificity for double-positive target cells (Fig. 4b), albeit the possibility to conditionally control CAR function with a small molecule.

Together, these data demonstrate that bispecific AvidCARs can be engineered for robust combinatorial antigen recognition, which ultimately allows for specific elimination of target cells co-

**Fig. 3** Influence of extra- and intracellular CAR domains. a, b Clustering of a hHER2-specific scFv. scFv-containing CARs were either based on a BBz and Ser-BBz-backbone, as shown in (a) (SEQ-IDs 5 and 6, respectively). b The figure shows the cytolytic and secretory activity of mock-T cells (no CAR) and T cells expressing the BBz- or Ser-BBz-CAR in co-cultures with hHER2ppp Jurkat cells [flow cytometry-based assay; three independent experiments with different donors; *p < 0.05, calculated by two-tailed paired t-test (left panel) or by two-tailed ratio-paired t-test (right panel)]. c Schematic of diabody formation of two scFvs by intermolecular heterodimerization of VHs and VLs. d, e Prevention of diabody formation enables defined CAR dimerization. d Schematic of a CAR with VH and VL on separate polypeptide chains for preventing diabody formation (SEQ-IDs 7 and 8). Both constructs are shown in the heterodimerized state together forming a hHER2-specific Fv-based CAR. e Cytopotic activity of mock-T cells (no CAR) or T cells expressing either the hHER2-specific scFv-CAR [shown in (a)] or the VH and VL constructs [shown in (d)] in co-cultures with hHER2ppp Jurkat cells. AP20187 was added for homodimerization of the VH-construct as indicated (filled red boxes). [flow cytometry-based assay; two independent experiments with three different donors; *p < 0.05, calculated by two-tailed paired t-test and by two-tailed unpaired t-test (scFv Ser-BBz vs. VH + VL chain)]. f, g Influence of co-stimulatory domains. f Schematic of hEGFR-specific rcsS07d-based low-affinity CARs which contain different co-stimulatory domains. The 4-1BB domain of the AvidCAR was substituted by the endodomain of CD28, ICOS, OX40, or CD2 (SEQ-IDs 9, 10, 11, 12, and 13, respectively). The FKBP12-F36V domain was integrated for conditional homodimerization. g rcsS07d-based low-affinity CARs with different co-stimulatory domains were expressed in T cells and co-cultured with hEGFRppp Jurkat cells. The figure shows the cytolytic activity of the T cells and its dependence on AP20187-mediated CAR dimerization (flow cytometry-based assay; two independent experiments with three different donors; *p < 0.05, calculated by two-tailed paired t-test). Exact p values are given in Supplementary Data 2. Source data are provided as a Source data file.
expressing two antigens on their surface, but not their single-positive counterparts.

**AND-gate with a surface and a soluble antigen.** Based on the data described above, we hypothesized that the AvidCAR concept also enables the construction of CARs that are specifically activated by combinatorial recognition of a surface antigen and a tumor-resident soluble factor, which in this case acts as a CAR-dimerizing agent. For that purpose, we designed a hEGFR-specific AvidCAR additionally containing the heterodimeric Fc antigen binding (Fcab) mutant JanusCT6, which recognizes vascular endothelial growth factor (VEGF) and expressed this AvidCAR construct in primary human T cells (Supplementary Fig. 6e). As VEGF is naturally present as a dimer, we hypothesized that it could homodimerize the AvidCAR molecules and thereby facilitate bivalent recognition of hEGFR (Fig. 4c). Indeed, VEGF triggered lysis of hEGFRt-expressing Jurkat cells, thus confirming our hypothesis (Fig. 4d). Of note, VEGF had no effect on target cell viability in the presence of T cells expressing either no CAR or a VEGF-independent high-affinity CAR, thus excluding any
direct toxic effects caused by VEGF. Summing up, we designed an AvidCAR that recognizes the combination of a cell surface and a soluble antigen, thus further broadening the application range of the AvidCAR platform.

Regulation and specificity of AvidCARs in vivo. As shown above, the activity of ON-switch AvidCARs can be regulated by conditional dimerization with small molecules. To test this principle in vivo, we lentivirally transduced primary human T cells with a hEGFR-specific ON-switch AvidCAR, whose antigen-binding domain specifically recognizes human, but not murine EGFR (mEGFR) (Supplementary Fig. 7a). This CAR additionally harbors the FKBPI2-F36V domain for conditional homodimerization by AP20187. CD19pos Nalm-6 target cells were stably transduced with hEGFRt (Supplementary Fig. 7b), enabling the direct comparison of the hEGFR-directed AvidCAR with two control CARs: (1) a high-affinity version of this AvidCAR and (2) a CD19-specific reference BBz-CAR based on the scFv FMC63 (aCD19-BBz; Supplementary Data 1). Initial in vitro experiments confirmed that (1) both control CARs (aCD19-BBz-CAR and high-affinity ahEGFR-Ser-BBz-CAR) efficiently triggered lysis of CD19pos/hEGFRtpos Nalm-6 cells, (2) that the activity of the hEGFR-specific ON-switch AvidCAR was dependent on the presence of the dimerizer AP20187 (Fig. 5a) and that (3) the parental hEGFRt pos Nalm-6 cells were not recognized by hEGFR-directed CARs (Fig. 5b). Similar effects were observed irrespective of the effector:target ratio used (Fig. 5a and b). In vivo, both control CARs efficiently delayed tumor outgrowth. Remarkably, the switchable ahEGFR-AvidCAR mediated comparable tumor control, but only upon administration of the dimerizing small molecule (on-state; Fig. 5c, Supplementary Fig. 7c). Moreover, even if the switchable ahEGFR-AvidCAR-T cells were initially administered without the dimerizing small molecule (off-state, leading to tumor outgrowth), the data indicate that antitumor activity could still be triggered by a single administration of the small molecule 11 days after CAR-T cell administration (Fig. 5c and Supplementary Figs. 7c and 8a). To investigate the effects of these CAR-T cells over a longer time span, we generated a Kaplan–Meier curve (Fig. 5d). Again, while the ahEGFR-AvidCAR-T cells had no effect in the absence of the dimerizer, long-term administration of the dimerizing small molecule resulted in significant prolongation of survival—similar to that observed upon treatment with either the high-affinity version of the ahEGFR-CAR or the standard aCD19-BBz-CAR. Together, these results confirm that CAR function can be controlled in vivo by regulating CAR avidity. Importantly, this AvidCAR represents a clinically applicable ON-switch CAR, as the domain FKBPI2-F36V (used for conditional homodimerization by AP20187 in this experiment) can also be homodimerized by the closely related investigational drug AP1903 (rimiducid).

Finally, we also tested the AND-gate function of an AvidCAR in vivo. For this purpose, we inserted domains for constitutive heterodimerization into the hEGFR/hHER2-specific AvidCAR described above, since the persistent administration of sufficient amounts of the currently available heterodimerizer AP21967 in mice is not feasible. That is, the domains for conditional heterodimerization were replaced with drug-independent heterodimerizing leucine zipper domains (EE and RR, respectively; Fig. 6a; Supplementary Data 1). The two polypeptide chains of this switchable AvidCAR were expressed at high levels (Supplementary Fig. 8b, c) and formed a functional CAR with potent AND-gate function, as demonstrated by specific lysis of hEGFRt pos/hHER2t pos target cells, but no or only weak lysis of single-positive target cells (Fig. 6b). Subsequently, this ahEGFR/ahHER2-AvidCAR was introduced lentivirally into primary human T cells and tested for its function in an in vivo model, where four different Nalm-6 populations (hEGFRt/hHER2t double-positive, both types of single-positive controls and double-negative version) were mixed 1:1:1:1 and subsequently administered intravenously (i.v.) to NSG mice. Ten days after CAR-T cell inoculation, the four different Nalm-6 populations were analyzed in the bone marrow, liver, blood, and spleen (Fig. 6c, d, and Supplementary Figs. 9–11). Strikingly, the ahEGFR/ahHER2-AvidCAR-T cells specifically killed double-positive, while ignoring single-positive Nalm-6 cells, even though they were present in the same tissue of the same animal (Fig. 6c, d and Supplementary Fig. 10). Notably, this high specificity was obtained despite even higher antigen expression levels in the single-positive controls (Fig. 6c and Supplementary Fig. 8d). In addition to its high specificity, the efficacy of the ahEGFR/ahHER2-AvidCAR-T cells was comparable to that of high-affinity ahEGFR-CAR-T cells (equally efficient in the liver; only slightly less efficient in the bone marrow; Fig. 6d and Supplementary Fig. 10). No Nalm-6 cells were detected in the blood and spleen of diseased mice (Supplementary Fig. 11). As expected, the high-affinity ahEGFR-CAR mediated killing of both hEGFRt/hHER2t double-positive and hEGFRt single-positive Nalm-6 cells (Fig. 6c, d and Supplementary Fig. 10). Together, these in vivo data demonstrate that the ahEGFR/ahHER2-AvidCAR is both efficacious and highly specific, even if double-positive target cells and single-positive controls are co-localized in the same tissues. To the best of our knowledge, this is the first example of an AND-gate CAR that can selectively target double-positive cells without requiring a safe distance between single-positive healthy bystander cells.
In this study we present the concept of AvidCARs. The key design principles were (1) the development of strictly monomeric CAR backbones by removing cysteines in the hinge region to prevent uncontrolled disulfide-mediated CAR dimerization; (2) the incorporation of antigen-binding domains whose affinities are substantially reduced down to levels comparable to those of TCRs ($K_d > 0.5 \mu M$); and (3) the use of single-domain antigen-binding domains, which seems to be preferable to exclude potential oligomerization that has been observed for some scFvs.

As described above, AvidCARs were successfully engineered using an EGFR-targeting rcSso7d-based binder and an affibody directed against HER2. Of note, similar dimerization-independent AvidCAR activation was also observed when using low-affinity nanobodies targeting green fluorescent protein (GFP) and HER2, respectively (Supplementary Fig. 12). Thus, in total we have successfully generated AvidCARs with four different targeting domains based on three different protein scaffolds, demonstrating the generalizability of the AvidCAR concept. An important feature, that all of these targeting domains (rcSso7d, affibodies and nanobodies) have in common, is their single-domain architecture. In contrast, scFvs are composed of two domains, which are known to cause mispairing and thus oligomerization to various extent depending on the scFv mutant, linker length, and environmental conditions. This has long been known for soluble scFvs and recently such scFv clustering has also been associated with tonic signaling in CAR-T cells. In our study, we provide direct biochemical evidence that scFv clustering indeed takes...
Fig. 6 Specificity of an AND-gate AvidCAR in vivo. **a** Schematic of a constitutively heterodimeric leucine zipper based AND-gate AvidCAR (αhEGFR/αhHER2-AvidCAR; SEQ-IDs 25 and 26). **b** Lysis of single- and double-antigen positive target cells by T cells expressing either no CAR or the αhEGFR/αhHER2-AvidCAR (flow cytometry-based assay; three independent experiments with four different donors; ***p < 0.001, **p < 0.01, calculated by a mixed model, with CAR group, antigen, and their interaction as fixed effects and the donor as a random effect to take into account the correlated structure, unadjusted two-sided p values are given). T cells were co-cultured with mRNA-electroporated Jurkat target cells expressing either hEGFRt or hHER2t or both antigens. **c, d** Specificity and efficacy of AND-gated AvidCAR-T cells in vivo. NSG mice were injected via tail vein with 0.5 × 10⁶ cells of a 1:1:1:1 mixture of Nalm-6, hEGFRt-Nalm-6, hHER2t-Nalm-6, and hHER2t-hEGFRt-Nalm-6 cells (Supplementary Fig. 9a) and 3 days later with 10 × 10⁶ CAR-T cells or mock-T cells, as indicated. As a positive control we used the high-affinity αhEGFR-CAR, i.e., the Ser-BBz backbone (with integrated FKBP12-F36V domain) fused to the high-affinity EGFR-binder, from which the low-affinity version for the AvidCAR was derived. Mice were sacrificed 13 days after tumor injection. Bone marrow and liver was analyzed for tumor cell infiltration. **c** Representative flow cytometric analysis of the ratio of the four different Nalm-6 cell populations in bone marrow. See Supplementary Fig. 10 for the flow cytometric analyses of the organs of all mice and Supplementary Fig. 9b for the used gating strategy. **d** Total number of Nalm-6 cells and T cells in the bone marrow and liver of NSG mice (n = 7, every data point represents one animal; *p < 0.05, **p < 0.01, ***p < 0.001, calculated by a two-way ANOVA analysis of log-transformed values). Exact p values are given in Supplementary Data 2. Source data are provided as a Source data file.
place on the CAR-T cell surface. Moreover, our experiments also demonstrated that scFvs that tend to cluster do not allow for the generation of efficient AvidCARs. However, we do not exclude that other scFvs may be suitable for the construction of AvidCARs. We would also like to note that we do not propose the separate expression of VH and VL as a suitable solution in case of oligomerizing scFvs. This design was only used as a model system to study the mechanism of scFv clustering (Fig. 3d, e). Instead, we strongly favor the use of single-domain binding scaffolds, especially in the context of AvidCARs. In fact, various different single-domain binding scaffolds have already been used in CARs and due to their favorable properties might be attractive for CARs in general. Regarding the potential immunogenicity it should be noted that while the single-domain binding scaffolds used here are based on nonhuman proteins, there are also several alternatives derived from human protein domains, such as monobodies.

Our experiments demonstrate that AvidCARs with low-affinity antigen-binding domains require bivalent interaction to be efficiently triggered. Such behavior can be explained by the fact that CAR signaling, i.e., the sufficient phosphorylation of signaling proteins, requires a certain duration of interaction with its target antigens as has been shown for the TCR and other immunoreceptors. For low-affinity binding domains, this duration is too short in a monovalent interaction and must be prolonged by interaction via a second binding domain, as supported by both our mathematical model and experimental data, in particular those presented in Fig. 2e. We demonstrate that this mechanism can be exploited to regulate CAR function by conditional dimerization and/or for combinatorial recognition of two different antigens. Such AND-gate function mediated by bivalent interaction of a bispecific CAR would not be possible with current homodimeric CAR formats, which may explain why bispecific CARs have so far only been used as OR gates. Finally, due to the dependence on bivalent interaction, we could also use our AvidCAR platform as a functional reporter system for identifying clustering tendencies of different CAR components such as a human retinol binding protein 4, an engineered protein scaffold based on leucine zippers to be able to study this AND-gate AvidCAR in vivo (Fig. 6). Of note, recently we introduced a small molecule-regulated heterodimerization module with a constitutive version localized proteins may exist that are not known yet. Finally, due to antigen downregulation. It is expected that avidity effects and thereby CAR sensitivity can be further increased by optimizing the design of AvidCARs (e.g., length and flexibility of spacers), which was not investigated in the examples presented (Fig. 4). However, it is also possible to enhance the avidity effect and thereby AvidCAR sensitivity. Therefore, co-localized antigens are particularly attractive candidates. Of note, many TAAs naturally exist in a dimeric/oligomeric state and are clustered into nanodomains or interact directly with other antigens. Prominent examples of co-localization include heterodimers of EGFR family members either among themselves or with other growth factor receptors, or the co-localization of receptors and gangliosides. The architecture of the mammalian cell membrane is still incompletely understood and many more co-localized proteins may exist that are not known yet.

Another consequence of the avidity-based mechanism of AvidCARs is the strong capability to sense antigen density. Antigen density determines the probability that a second binding domain can catch an antigen before the first one dissociates from its antigen. That is, antigen density strongly determines the lifetime of the interaction between a bivalent low-affinity CAR and its antigens—and thus CAR signaling. This mechanism is exploited in affinity tuning of current CARs to limit CAR-T cell cytotoxicity to tumor cells expressing elevated target antigen levels. Due to the same mechanism, AND-gate AvidCARs integrate the dependence on two different antigens (i.e., AND-gate function) with the ability to sense antigen density, which can further increase tumor specificity.

Nevertheless, for other applications, it is desirable to enhance the antigen sensitivity of the AvidCARs, e.g., for antigens expressed at low levels and/or to reduce the risk of tumor escape due to antigen downregulation. It is expected that avidity effects and thereby CAR sensitivity can be further increased by optimizing the design of AvidCARs (e.g., length and flexibility of spacers), which was not investigated in the examples presented (Fig. 4). However, it is also possible to enhance the avidity effect and thereby AvidCAR sensitivity. Therefore, co-localized antigens are particularly attractive candidates. Of note, many TAAs naturally exist in a dimeric/oligomeric state and are clustered into nanodomains or interact directly with other antigens. Prominent examples of co-localization include heterodimers of EGFR family members either among themselves or with other growth factor receptors, or the co-localization of receptors and gangliosides. The architecture of the mammalian cell membrane is still incompletely understood and many more co-localized proteins may exist that are not known yet.

Together, our study highlights and explores a previously neglected aspect in the CAR field, which is the bivalent antigen engagement due to the dimeric nature of virtually all currently applied CAR molecules. Therefore, the presented data will contribute to an improved mechanistic understanding of CAR function and biology. Moreover, we present a strategy for specifically exploiting this bivalent antigen recognition in a controlled manner, ultimately opening up a range of applications in which an AvidCAR integrates multiple input signals, e.g., (1) a cell surface antigen and a clinically applied small molecule, (2) two different cell surface antigens and a small molecule or (3) a cell surface antigen and a soluble factor enriched in the tumor microenvironment. Thus, we expect that AvidCARs will be a highly valuable platform for the development of controllable CAR therapies with improved tumor specificity.

**Methods**

**Cell culture.** Buffy coats from de-identified healthy donor’s blood were purchased from the Austrian Red Cross, Vienna, Austria. Primary human T cells were isolated by negative selection using the RosetteSep Human T-Cell Enrichment Cocktail (STEMCELL Technologies) and cryopreserved in RPMI-1640 GlutaMAX medium (Thermo Fisher Scientific) supplemented with 20% FCS (Sigma-Aldrich) and 10% DMSO (Sigma-Aldrich) until further use. After thawing, primary human T cells were immediately activated with Dynabeads Human T-Activator αCD3/αCD28 beads (Thermo Scientific) at a 1:1 ratio according to the manufacturer’s instructions. T cells were expanded before the experiments for at least 14 days in T-cell medium, consisting of RPMI-1640 GlutaMAX supplemented with 10% FCS, 1% penicillin–streptomycin (Thermo Scientific) and 200 U/mL recombinant human IL-2 (Peprotech). Half of the medium was exchanged every other day and cell densities were kept between 0.4 and 2 × 10⁶ cells/mL. Nalm-6 cells and Jurkat cells (gifts from Dr. Sabine Streit and Dr. Michael Dvorzák, respectively; CCRI, Vienna, Austria) were maintained in RPMI-1640 GlutaMAX supplemented with 10% FCS, 1% penicillin–streptomycin, and 200 U/mL recombinant human IL-2 (Peprotech). A stable clone of Nalm-6 cells expressing both transgenes was established by limiting dilution cloning and subsequently was co-transduced with a lentiviral vector encoding firefly luciferase (fluc), puromycin N-acetyl transferase (thermo Scientific) and/or hHER2t. A stable clone of Nalm-6 cells expressing both transgenes was established by limiting dilution cloning and subsequently was co-transduced with a lentiviral vector encoding fluc and enhanced GFP (eGFP) to
ensure high expression of the luciferase reporter gene. Cell lines were regularly tested for mycoplasma contamination using the MycoAlert PLUS Mycoplasma Detection kit (LONZA) and for squelent monkey retroviral contamination by PCR (performed by Labdia Labordiagnostik GmbH, Austria). Cell line authentication was performed by SNP-profiling at Multiplexion GmbH, Germany.

The homo- and heterodimerization of proteins in vitro was induced by the addition of APD2187 or AP21967 (both TakaRa) to the cell suspensions at a final concentration of 100 nM. The respective transgene expression was monitored using Western blotting and the manufacturer’s instructions. Briefly, the cells were stained with a saturating concentration of a PE-labeled aHGF-R antibody (clone AY13; BioLegend). Subsequently, the geometric mean of the fluorescence intensity was determined, subject to background subtraction using unstained or isotype-labeled cells and then used to normalize the number of respective cell ABC. ABC values were corrected for the PE-conjugation efficiency of the respective antibody (i.e., PE dye-to-antibody ratio) yielding effective surface densities.

Generation of mutant binders by alanine scanning. Site-directed mutagenesis of the hGF-R and hHER2-specific binder scaffolds was performed using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Genomics), according to the manufacturer’s instructions. Mutagenic primers were designed using the QuikChange Primer Design Software (Agilent Genomics) and synthesized by Biomers, Germany. Primer sequences can be found in Supplementary Data 3. In the case of the hHER2-specific binder, the aptamer 45HER2-ART was generated from the parental aptamer zHER2-AK, which binds to hHER2 with an affinity of 50 nM, the 45 sequence by introducing mutations N23A and S33K for preventing N-glycosylation.

Quantitation of target antigen density. The surface density of target antigen molecules on Jurkat cells was quantified using the QuantIBRITE Phycoerythrin (PE) Fluorescence Quantiitation Kit (Becton Dickinson) according to the manufacturer’s instructions. Jurkat cells were stained using the hEGFR- and hHER2-specific binder scaffolds and the manufacturer’s instructions. Briefly, the cells were stained with a saturating concentration of a PE-labeled aHGF-R antibody (clone AY13; BioLegend). Subsequently, the geometric mean of the fluorescence intensity was determined, subject to background subtraction using unstained or isotype-labeled cells and then used to normalize the number of respective cell ABC. ABC values were corrected for the PE-conjugation efficiency of the respective antibody (i.e., PE dye-to-antibody ratio) yielding effective surface densities.
ultrafiltration with a Vira-Cell 375 ultrasonic processor (Sonics & Materials, Inc.). After two washing steps using the same buffer, the pellet was resuspended in 20 µL. Subsequently, 20 µm TRIS-HCl, 5 µm EDTA, 7.5 µK, loading buffer (dithiothreitol, pH 7.5), stirred for 2 h at room temperature followed by centrifugation (39,000 x g, 25 min, 4 °C). The supernatant containing the unfolded protein was diluted tenfold into refolding buffer (20 µm TRIS-HCl, 7.5 µm CuCl2, pH 8.4) and stirred overnight at room temperature. Following a dialysis step against 20 mM TRIS-HCl (pH 8.0), the protein was purified using a 6 µL Resource Q column, a 1 mL Hitrap Phenyl FF (Low Sub) column and a HiLoad 16/600 Superdex 200 pg (all from GE Healthcare) according to the manufacturer's instructions. Pure VEGF samples were stored at ~80 °C.

Surface plasmon resonance. SPR analysis was performed with a Biacore T200 instrument (GE Healthcare). All experiments were performed in degassed and filtered PBS, pH 7.4, supplemented with 0.1% BSA (Sigma-Aldrich) and 0.05% Tween-20 (Merck Millipore) at 25 °C. Immobilization of hEGFR-Fc and hHER2-Fc (both in 1 mg/mL) was performed on a Protein A sensor chip (GE Healthcare) at a flow rate of 10 µL/min for 60 s at a concentration of 6.7 and 4 µg/mL, respectively, yielding a density of ~400 response units. Five different concentrations of the respective binder scaffold (depending on the expected Kd value) were injected at a flow rate of 30 µL/min for 15 s (for binders E11.4.1, E11.4.1-G25A, E11.4.1-G32A, and zHER2-AK-R10A) or 60 s (for zHER2-AK) in the single-cycle kinetic mode. Subsequently, a dissociation step was performed for 30 s (all E11.4.1-based binders), 60 s (zHER2-AK-R10A), or 180 s (zHER2-AK). Protein A sensor chips were regenerated by applying 10 µM Glycine-HCl, pH 1.5, at a flow rate of 30 µL/min for 30 s. The Kd value was either obtained by steady state binding analysis or by fitting the sensorgram to a 1:1 Langmuir model in the kinetic mode using the Biacore T200 Evaluation Software (GE Healthcare).

Flow cytometry-based cytotoxicity assay: the assay principle is based on the determination of the ratio of antigenpos and antigenneg target cells that expressed either a green or red fluorescent protein, respectively, and were mixed 1:1 before co-culture with T cells. For this purpose, Jurkat cells were electroporated on the day before the assay (1) with mRNA encoding eGFP and mRNA encoding the respective target antigen, and alternatively (2) with mRNA encoding mCherry. Those cells were co-cultured for 4 h at 37 °C in round-bottom 96-well plates with 100 µL PBS (100 µL PBS were injected i.v. into the tail veins of NSG mice (male and female, 8–12 weeks of age). Three or 5 days later (as indicated), when tumor burden became detectable by bioluminescence imaging (BLI), CAR-T cells (10 x 106 in 100 µL PBS) were injected i.v. into the tail veins of the NSG mice. Dimerization of the ON-switch AvidCAR was induced by intraperitoneal (i.p.) administrations of 2 mg/kg AP20187 (Takara). AP20187 was prepared by dissolution in ethanol yielding a concentration of 12.5 mg/mL and further dilution to the final working concentration of 0.5 mg/mL in vehicle solution [4% ethanol, 10% PEG-400 and 1.7% Tween-80 (both Sigma-Aldrich) in water]. Those working stocks of AP20187 were sterile filtered and used for injection within 30 min. Injection of only the vehicle solution served as control. The mice were monitored daily and sacrificed by cervical dislocation at the first sign of disease (weight loss, rough fur, beginning paralysis).

In vivo bioluminescence imaging. BLI was performed at the PIL (Medical University of Vienna) using an IVIS Spectrum In Vivo Imaging System (Perkin Elmer). D-luciferin (Perkin Elmer) was freshly dissolved in PBS to a final concentration of 15 mg/mL and sterile filtered. Mice were anesthetized with isoflurane (Abbott) and received i.p. injections of the luciferin solution (doseage of 150 mg/kg body weight). After 15–20 min, mice were transferred to the IVIS Imaging System and bioluminescence was measured immediately in medium binning mode with an automatic acquisition time (ranging from 1 s to 2 min) to obtain unsaturated images. The image acquisition was performed within the region of interest which encompassed the entire body of the mouse. Tumor growth was monitored every 3–4 days. Living Image Software (Caliper) was used to analyse the data.
In vivo model for the AND-gate AvidCAR. NSG mice were injected via tail vein with 0.5 × 10⁶ cells of a 1:1:1 mixture of Nalm-6, hEGFR–Nalm-6, and HER2–Nalm-6 and 14H2RM–EGFR–Nalm-6 cells and 3 days later with 10 × 10⁶ CAR-T cells or mock-T cells, as indicated. Mice were sacrificed 13 days after tumor injection. Bone marrow single-cell suspensions were obtained by flushing one femur with ~10 mL sterile PBS and passing through a 70 µm cell strainer (Corning). After disruption of the liver through a 70 µm cell strainer and several washing steps, lymphocytes were separated from hepatocytes using a 33.75% Percoll gradient (GE Healthcare). Spleen single-cell suspensions were obtained by two consecutive passages through 70 µm cell strainers. Blood was drawn retro-orbitally from isoflurane-anesthetized mice and collected in EDTA-containing tubes (Greiner). Fifty µL blood was used for the staining with fluorescent antibodies, followed by red blood cell lysis using an RBC lysis/solution (Biolegend).

Mathematical modeling. The multivalent interaction between a bivalent antigen and a bivalent CAR that can undergo kinetic proofreading leads to a large number of distinct chemical states. Therefore, it is not practical to manually enumerate them. To overcome this, we used the rule-based framework of BioNetGen to define five elementary reaction: intermolecular binding (kab), intramolecular binding (kab), intramolecular phosphorylation (kap), intermolecular phosphorylation (kap), and the kinetic proofreading phosphorylation rate constant (k). We have assumed that (1) the intramolecular unbinding rate (i.e., the unbinding rate between a single CAR and antigen when both CARs are bound to antigen) is equal to the intermolecular unbinding (i.e., unbinding rate of a single CAR and antigen when only a single CAR in the dimer is bound to antigen) and (2) the kinetic proofreading phosphorylation rate constant can only take place when at least one CAR in the dimer is bound to antigen. When a single CAR in the dimer is bound, the binding rate of the second CAR in the dimer to the second antigen in the dimer is expected to be dependent on the intermolecular on-rate (since the same binding interface is formed) multiplied by a factor that accounts for the local concentration (and potentially other factors, such as structural alignment) that we define to be σ with units of concentration. This parameter controls the avidity of the CAR with σ = 0 producing a monovalent CAR and increasing values of σ lead to increasing avidity. Collectively, these elementary reactions lead BioNetGen to 72 chemical states that were evaluated as a system of 72 coupled nonlinear coupled ordinary differential equations. The initial conditions were σ = 0 for all chemical species except the concentration of the CAR (1000/µm²) and antigen (varied as indicated). The parameter values used were k_a = 5 × 10⁶ µm⁻¹ s⁻¹, k_d = 10⁻⁴, and k_p = 0.1/s. These equations were solved numerically in Matlab (Mathworks, MA) to produce the panels in Supplementary Fig. 3b showing the bound receptor and phosphorylated receptor. To calculate the cellular response in Supplementary Fig. 4b we have assumed a saturating Hill function connecting phosphorylated receptor to the cellular response using a Hill function with a Hill coefficient of 5. To calculate the cellular response in Supplementary Fig. 3b showing the bound receptor and phosphorylated receptor. To calculate the cellular response we have assumed a saturating Hill function connecting phosphorylated receptor to the cellular response using a Hill function with a Hill coefficient of 5. The cellular response is given by:

\[
\text{Cellular response} = \frac{\text{Bound receptor} \times \text{Phosphorylated receptor}}{1 + \text{Bound receptor}}
\]

Statistical analysis. Statistical analysis was performed using GraphPad Prism 7 software for Windows (GraphPad Software Inc.) and SAS software (Version 9.4, SAS Institute). Graphs were generated using GraphPad Prism 7 software. Data are presented as individual data points or as means ± SD. Statistical analysis was performed using two-tailed paired t-tests, two-tailed ratio-paired t-test, or two-tailed unpaired t-test, as indicated. Statistical Analysis of Figs. 4b and 6b was done with a mixed model to compare fixed-effect means taking into account that the data are correlated. Statistical analysis of Figs. 5d and 6d was done by log-rank analysis or two-way ANOVA, respectively.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The authors declare that all data supporting the findings of this study are available within the article and its Supplementary information files or from the corresponding authors upon reasonable request. The source data for the Figs. 2a, c, e, 3b, e, g, 4b, d, 5a–c, and 6b, d, as well as Supplementary Figs. 2c, 4a, b, d, 5a, 6a, b, and 12c, e are provided as a Source data file. Source data are provided with this paper.

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Author contributions

B.S., C.M.S., C.U.Z., T.P., and M.A.S. performed the experiments and analyzed data. B.S., M.E.P., J.B.H., M.W.T., and M.L. designed the experiments and critically reviewed the data. B.K., M.C.B., and E.M.P. assisted in animal models. B.S., E.M.P., and E.M.P. contributed reagents and helped with experimental design. E.M.P. assisted in mathematical modeling. B.S., O.D., C.O., W.H., M.W.T., and M.L. wrote the paper. All authors edited and approved the paper.

Competing interests

B.S., C.U.Z., C.O., M.W.T., and M.L. have filed three patent applications that relate to the technology described in this paper: patent applicant: St. Anna Kinderkrebsforschung (Zimmermannplatz 10, 1090 Vienna, Austria), Universität für Bodenkultur Wien (Gregor Mendel-Strasse 33, 1180 Vienna, Austria); name of inventor(s): B.S., M.W.T., and M.L. designed the experiments and critically reviewed the data. B.K., M.C.B., and E.M.P. assisted in animal models. E.L. contributed valuable reagents and helped with experimental design. O.D. assisted in mathematical modeling. B.S., O.D., C.O., W.H., M.W.T., and M.L. wrote the paper. All authors edited and approved the paper.
