Synthetic promoters to induce immune-effectors into the tumor microenvironment

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Harnessing the immune-system to eradicate cancer is becoming a reality in recent years. Engineered immune cells, such as chimeric antigen receptor (CAR) T cells, are facing the danger of an overt life-threatening immune response due to the ON-target OFF-tumor cytotoxicity and Cytokine Release Syndrome. We therefore developed synthetic promoters for regulation of gene expression under the control of inflammation and Hypoxia-induced signals that are associated with the tumor microenvironment (TME). We termed this methodology as chimeric-antigen-receptor-tumor-induced-vector (CARTIV). For proof of concept, we studied synthetic promoters based on promoter-responsive elements (PREs) of IFNγ, TNFα and hypoxia; triple PRE-based CARTIV promoter manifested a synergistic activity in cell-lines and potent activation in human primary T-cells. CARTIV platform can improve safety of CAR T-cells or other engineered immune-cells, providing TME-focused activity and opening a therapeutic window for many tumor-associated antigens that are also expressed by non-tumor healthy tissues.
Cancer treatment is a major challenge facing modern medicine. Cancer cells originate from the patient's own healthy cells, thus any treatment targeting the cancer cells might also harm healthy tissues. Thus, there is an ongoing pursuit for finding new treatments that will target exclusively cancer cells manifesting minimal to zero toxicity to healthy tissues. Precision oncology, developed to increase efficacy and reduce toxicity, usually involves the molecular profiling of tumors identifying targets that discriminate tumor from healthy tissues; while immunotherapy aims to manipulate the immune system in to targets that discriminate tumor from healthy tissues mediating the on-target-off-tumor activity has been proposed by Juillerat et al. A different approach is based on inhibition: an antigen recognition domain fused to an inhibitory receptor. Thus, the CAR T cells can distinguish the tumor and non-tumor cells and restrict their activity to the tumor domain. Another strategy for reduced toxicity is based on splitting the activation signal of the CAR. In this method the full activation signal is divided to two CARs, each recognizing a different antigen, and each is carrying only part of the TCR signaling/stimulation domain. The other is carrying the CD3 and the other is carrying the CD28. Hence, full activation is mediated by the presence of both antigens that are tumor specific are recognized the full activation potential of the T cell will be realized. Another intriguing approach to reduce ON target OFF tumor activity has been proposed by Juillerat et al. where they fused an oxygen sensitive subdomain of HIF1α to a CAR scaffold to generate self-decision making CAR T cell sensitive to the TME hypoxic niche. This approach is of great interest but it relies on a single factor to induce the CAR.

We approached the on-target-off-tumor toxicity problem by restricting spatiotemporal CAR expression through coupling the CAR gene to a synthetic inducible promoter that is sensitive to combined stimuli portraying the tumor microenvironment (TME). TME is the environment around a tumor, including the surrounding blood vessels, immune cells, fibroblasts, signaling molecules and the extracellular matrix. The TME is often hypoxic, demonstrating a characteristic protumorigenic inflammation mediated by both tumor and non-tumor cells of the TME. Hence, we designed synthetic promoters that are (i) combined from promoter-responsive elements (PREs) responding to various TME-associated cytokine stimuli and hypoxic conditions, and (ii) constructed to optimally respond to the combination of stimuli activating the promoter PRE's building blocks. We define this technology as CARTIV: chimeric antigen receptor tumor-induced vector. Specifically, we show that PRE promoter combined from PREs responding to IFNγ, TNFα, and hypoxic stimuli can be employed to restrict CAR expression to the tumor site.

Results

CARTIV design of promoter and reporter construct. Based on consensus Promoter-Response-Elements (PREs) sequences for IFNγ, TNFα or hypoxia, we first designed three CARTIV-adapted PREs (CPREs): GCPRE, KCPRE, and HCPRE for IFNγ, TNFα or hypoxia, respectively (Fig. 1a). To generate CARTIV promoters, the different CPREs, separated by linkers, were then homo- and hetero-combined in a "mix and match" manner, upstream to a minimal herpes virus thymidine kinase (mini TK). Complete sequences of these CPREs and the tested CARTIV promoters are summarized in Supplementary Fig. 1. Figure 1b shows a scheme of 2-CPRE- and 3-CPRE-combined CARTIV synthetic promoters.

To test the activity of the CARTIV synthetic promoters, we cloned them upstream to a fluorescent protein reporter RFP670 in a 3rd generation lentiviral vector. Our vector also harbored another independent constitutive promoter driving a distinct ZsGreen fluorescent reporter (Fig. 1c), allowing to easily identify the infected cells using flow cytometry and to assess the CARTIV promoter activity.

2-CPRE CARTIV promoters are responsive to IFNγ and TNFα. To functionally test CARTIV promoter’s activities in cells, we needed a model that will be responsive to IFNγ, TNFα, and hypoxia. HEK293T cells express IFNγ and TNFα receptors and are responsive to human IFNγ and TNFα in a wide range of concentrations (Supplementary Fig. 2A–C), and are responsive to hypoxia. To validate the elements functionality, we examined the single GCPRE and KCPRE elements as individual promoters (Supplementary Fig. 3) as well as CARTIV synthetic promoter combined from GCPRE and KCPRE; we tested the following promoters: one KCPRE (K1); two, four, and six GCPRE (G2, G4, G6, respectively); three GCPRE and three KCPRE (G3K3); two GCPRE upstream to two KCPRE (G2K2); one GCPRE upstream to one KCPRE (G1K1), and one GCPRE upstream to a short version of the KCPRE sequence (G1K0.6).

Fig. 1 The promoter response elements used to construct the CARTIV promoters and the vector used. a CARTIV promoter response elements (CPREs) used in CARTIV promoters. b Two of the tested promoters. Yellow: IFNγ PRE (GCPRE), turquoise: NFκB PRE (KCPRE), green: hypoxia PRE (HCPRE); purple: minimal herpes simplex virus thymidine kinase (Mini TK). c The vector used in the CARTIV system. Direct activation of the CARTIV vector, including an independent constitutive reporter.
Fig. 2 CARTIV promoters show a robust and additive effect with IFNγ and TNFα stimulation in HEK293 cells. a Representative FACS plots of HEK293T cells infected using lentiviral vectors with RFP670 under the control of the indicated CARTIV promoter and ZsGreen controlled by the ef1α core promoter. At 72 h following infection, the cells were incubated for 48 h with the indicated cytokines (250 U/ml for each cytokine), harvested and analyzed by flow cytometry. Data shown are ZsGreen-positive, single-discriminated, and DAPI-negative results. b Geometric mean of RFP670 in ZsGreen-positive cells normalized to uninfected cells, showing average of triplicates, error bars indicate standard deviation. A two-way ANOVA was performed. c Synergism level calculated by dividing the geometric mean of Zs+ cells stimulated with IFN and TNF by the geometric mean of cells stimulated with IFN or TNF. A one-way ANOVA was performed, error bars indicate standard deviation. Results are from one representative experiment of three full experiments performed (including the G2K2); the G1K0.6, G1K1, and G3K3 were compared in more than eight independent experiments.

assessed by gating on ZsGreen+ cells (the transduced cells) and testing the geoMEAN of the RFP670 reporter (Supplementary Fig. 4). The K1 promoter manifested a strong activity as a single element (73.38 fold increases compared to non-stimulated) when compared to the GCPR that showed no substantial difference in expression (2.87, 6.38, and 6.43 fold increases for G2, G4, and G6 accordingly when compared to non-stimulated) between 4 and 6 GCPR promoters (Supplementary Fig. 3). Following stimulation with IFNγ alone, the GCPR manifested low activity for G1K0.6 and G1K1 and higher activity for the G2K2 and the G3K3 (1.35, 1.57, 3.16, and 3.47 fold increases for G1K0.6, G1K1, G2K2, and G3K3 accordingly when compared to non-stimulated) as seen in Fig. 2a. Following stimulation with TNFα alone, the HCPRE manifested a pronounced activity that maximized in the G3K3 promoter (2.9, 5.01, 13.88, and 32.82 fold increases for G1K0.6, G1K1, G2K2, and G3K3 accordingly when compared to non-stimulated) as seen in Fig. 2a. As expected, a single-factor reactivity was increasing with the number of repeats; nevertheless, the most synergistic activation following a combined stimulation was best observed for the G1K0.6 promoter induction by IFNγ and TNFα (Fig. 2c). The CARTIV promoters having more repeats showed pronounced activation (16.13, 15.75, 46.61, and 84.33 fold increases for G1K0.6, G1K1, G2K2, and G3K3 accordingly when compared to non-stimulated), but their synergistic response was relatively lower, likely due to the strong induction by TNFα alone (Fig. 2c).

The hypoxia element better location is immediately upstream to the mini TK. Gaining good activation by one inflammatory factors and even better with synergistic two factors, we next set out to find a third signal to combine in our CARTIV promoters. In solid tumors it is common to find hypoxia due to insufficient vascularization and blood supply23–26. Therefore, we designed CARTIV promoters combined from GCPR, KCPRE and also HCPRE for hypoxia-response (Fig. 3a). We first aimed to find if there might be a preferential location of the HCPRE within the CARTIV promoter. A series of three promoters were constructed to consist of G, K, and H elements that differ only in the location of the hypoxia element relative to the minimal TK (Fig. 3a). CARTIV+ HEK293T subjected to hypoxic conditions for 18 h and ZsGreen+ cells were assessed for the expression of the RFP670 reporter. Interestingly, all three promoters responded to the hypoxia stimuli, a 1.69, 2.07, and 3.91 fold increase compared to the normoxic conditions for the H2G2K2, G2H2K2, and the G2K2H2 accordingly; however, the response was most profound when the HCPRE was located downstream to the other CPREs and adjacent to the minimal TK (Fig. 3b, c). This finding was unanticipated since the HRE consensus sequence is usually found between 100 and 1000 bases upstream to the ATG start codon29 and may appear also downstream of the TSS33. Hence, our data demonstrate critical importance for the order of PRE elements within a CARTIV promoter that is not predictable and require experimental examination.

G1K06H1 promoter is responsive to triple-stimuli in lower cytokine concentrations. Following the above mentioned findings for double GCPR-KCPRE promoter (Fig. 2) and the location of the HPRE (Fig. 3), we designed the G1K06H1 synthetic promoter with one copy of GCPR, 60% of the KCPRE, and one HCPRE. CARTIV+ HEK293 cells were incubated with 500 U/ml IFNγ and 500 U/ml TNFα for 24 h then placed under hypoxic or normoxic conditions for an additional 18 h; in this experimental setup the hypoxia stimuli did not add to the activation induced by IFNγ and TNFα; Initial experiments with 500 U/ml of each cytokine found little effect by the addition of hypoxia (Fig. 4a). We further investigated this promoter in lower levels of cytokine stimuli that better correlates with the physiological conditions in the TME17,34–36. Cells were incubated for 24 h with all possible combinations of 500, 125, 32, 8, 2, and 0 U/ml of IFNγ and TNFα, and then placed under hypoxic or normoxic conditions for an additional 18 h (Fig. 4b, c). Data clearly indicates that in the lower concentration of IFNγ and TNFα, the added effect of the hypoxia...
stimulus on the RFP670 reporter expression is more profound. Figure 4d shows the synergistic induction by fold-expression following the hypoxia stimulus; when both IFN\(\gamma\) and TNF\(\alpha\) stimuli were lower than 32 U/ml the fold induction due to addition of the hypoxia stimulus was the highest. The "turn on" and "turn off" kinetics of the G1K06H1 promoter were tested by adding 500 U/mL of IFN\(\gamma\) and TNF\(\alpha\) and tracking the increase or decrease in florescence over 48 h. The promoter showed a t\(_{1/2}\) of 9.78 h on rate and a t\(_{1/2}\) of 12.37 h off rate (Supplementary Fig. 5).

G1K06H1 promoter in NK92 cells and primary human T cells is responsive to combined TNF\(\alpha\) and hypoxia stimuli. Following the experiments with the HEK293T cell line, we aimed to investigate the activity of our CARITV promoter in immune effector cells that are clinically relevant to CAR-based treatment. We used primary human T cells as well as the NK92 natural killer cell line \(^7\). Following stable transduction of the G1K0.6H1 CARTIV, cells, primary human T cells or the NK-92 cell line, were stimulated with 250 U/ml of IFN\(\gamma\), TNF\(\alpha\), and Hypoxia. Both NK92 and primary human T cells manifested a substantial response to the combined stimuli of hypoxia and TNF\(\alpha\) as compared to controls (a 5.206 and 3.84 fold increase compared to the non-stimulated for the NK92 and human primary T cells accordingly), and to single-factor stimulation (Fig. 5a, b). Interestingly, in this set of experiments IFN\(\gamma\) stimulus did not show substantial nor significant effect with either T- or NK-cells (Fig. 5b). This relative low impact of IFN\(\gamma\) was probably due to the levels of endogenous IFN\(\gamma\) secreted by these cultured T or NK cells. Direct measure of IFN\(\gamma\) from these cells cultures indeed
Fig. 4 The G1K06H1 promotor shows an additive response to hypoxia and cytokines with physiological cytokine concentrations. 

a) Representative plots of HEK293T cells infected using lentiviral vectors with RFP670 under the control of the G1K06H1 promotor and ZsGreen controlled by the eif4a core promotor. At 72 h following infection, the cells were treated for 48 h with 500 U/ml indicated cytokines and for 18 h under hypoxic or normoxic conditions, harvested and analyzed by flow cytometry. Data shown are ZsGreen-positive, single-discriminated and DAPI-negative results.

b) From the top panel to the bottom—cells were treated for 48 h with 500, 125, 32, 8, 2 or 0 U/mL of TNF respectively and veering IFN concentrations and placed under hypoxic or normoxic conditions for 18 h, cells were analyzed by flow cytometry as described above. Showing average of duplicates, error bars indicate standard deviation.

c) From the top panel to the bottom—cells were treated for 48 h with 500, 125, 32, 8, 2 or 0 U/mL of IFN respectively and veering TNF concentrations and placed under hypoxic or normoxic conditions for 18 h, cells were analyzed by flow cytometry as described above. Showing average of duplicates, error bars indicate standard deviation.

d) Ratios of reporter expression with different cytokine concentrations under hypoxic or normoxic conditions. Results are from one representative experiment of four performed.
revealed endogenous secretion (Fig. 5c, showed for primary human T cells), suggesting for possible positive auto-regulation in such culture conditions.

**CARTIV G1K06H1 can induce the expression of a functional CAR.** Following the good induction of reporter-genes, we next aimed to find if CARTIV promoter could also induce CAR receptors to the cell's surface. Therefore, we constructed a Herceptin-based 3rd generation CAR (Supplementary Fig. 1) Open Reading Frame (ORF) just following the G1K06H1 promoter to study its direct induction by stimuli. The CAR employed was composed of a Herceptin based scFv, a CD28 and 4-1BB costimulatory motifs and a CD3ζ chain (Fig. 6a), suggesting for activation of effector cells when encountering target cells that express high levels of the cognate ERBB2. To assess for surface-expression, cells were stained using an ERBB2-Fc chimeric protein, the CAR cognate ligand. ZsGreen+ positive T cells manifested clear expression of the CAR following the stimulation, as compared to non-stimulated ZsGreen− positive T cells, and to the ZsGreen− T cells in the same well (Fig. 6b). We then tested the functionality of this CAR by employing target cells that manifest high, dull or no expression of the ERBB2 target protein on their cell membrane (Supplementary Fig. 6a, b). Activity of the T cells was measured using the CD107a-based degranulation assay. The percentage of degranulating cells was calculated as the fraction of CD107a positive cells derived from the ZsGreen positive population. Following stimulation, the T cells incubated with ERBB2dull and ERBB2high target cells showed substantial levels of...
degranulation when compared to HER2 negative Jurkat cells, with different basal-levels differences among target-cells probably due to secreted cytokines (Fig. 6c). The degranulation observed for the T cells induced to express the CAR and incubated with HER2 positive cells was substantially higher (a 3.1, 4.82, 3.02, 4.14, and 2.58 fold change for MCF7, JIMT1, SKOV3, HeLa, and HEK293T target cells accordingly when compared to the non-stimulated T-cells) than the degranulation observed for HER2 negative target Jurkat cells. Therefore, the CAR transcribed following the stimuli of the CARTIV promoter and expressed on the cell’s surface is also functional specifically against its cognate target. Figure 6d show experimental raw data for MCF7 as target cells. CAR-positive T cells (ZsGreen+ cells) are substantially activated upon stimuli, when incubated with HER2-positive target cells (Fig. 6d, upper panels), while CAR-negative T cells (ZsGreen−) are not activated upon stimuli on HER2-positive target cells (Fig. 6d, bottom panels). These data support the possible improvement of CAR-T activity against tumor, but not against normal tissues.

GIK06H1 promoter in NK92 cells is responsive to a tumor microenvironment when compared to a non-tumor site in a CDX model. Next, we investigated whether CARTIV promoter could be induced in vivo within the TME as compared to a non-TME site. A cell line derived xenograft (CDX) model was established by injecting the HER2-positive JIMT-1 tumor cells into immunocompromised NSG mice. When JIMT-1 tumors reached ~150 mm3, NK-92 cells harboring RFP670-encoded by the G1K06H1 promoter were injected intra-tumorally and in parallel (into the same mouse) subcutaneously in Matrigel (Fig. 7a). Forty-eight hours after inoculation, mice were sacrificed and tumor and matrigel were extracted and dissociated. NK-92 cells, residing within the tumor, Matrigel were gated based on GFP expression (Supplementary Fig. 7A), and their expression of the RFP670 reporter was measured by flow cytometry. When we compared the RFP670 reporter expression in NK-92 from the tumor site to NK-92 from the Matrigel, a clear enhancement of expression was observed in the tumor site (Fig. 7b, representative). The increase of expression at the tumor site was statistically significant (Fig. 7c). In additional set of experiments on mice bearing JIMT-1 CDXs, NK-92 cells harboring RFP670-encoded by the G1K06H1 promoter were injected intra-tumorally and in parallel (into the same mouse) intravenously. Forty-eight hours after inoculation, we analyzed RFP670 expression in GFP+ cells derived from the TME or from the blood. Again, a clear significant enhancement of RFP670 expression, a 1.57 fold increase, was observed in cells derived from the tumor site as compared to cells from a non-TME source, i.e. the blood (Supplementary Fig. 7B). These results suggest that the G1K06H1 CARTIV...
promoter is induced in vivo in response to the tumor microenvironment when compared to a non-tumor site.

**Discussion**

CAR-T gained clinical success, with approved treatments against uncured cancers, and hundreds of current clinical trials. However, the risk of harming normal tissues is preventing its application to many patients. Focusing effector immune cell's activities onto tumors, while sparing healthy tissues can work together with current CAR-T approach, as well as with any other adoptive-transferred engineered immune cells. The CARTIV approach presented hereby is suggesting the combination of multiple promoter-elements that may reside thousands and even million-base, we speculate that natural promoters are spread over multiple PREs. This is perhaps not surprising since the actual binding-sites of transcription factors are usually less than dozen bases. While endogenous promoters are usually surprisingly short. While endogenous promoters are usually proximal promoters that are around hundred bases before and after the transcription Start Site (TSS), and enhancer elements that may reside thousands and even million-base, we find sufficient abilities of short 200 base pair promoters to include features by CAR-T cells, but it is still specific for the single tumor-antigen. CARTIV approach includes multiple PREs having specificity to TME rather than a tumor-antigen, in order to reduce risk of ON-target OFF-tumor activities.

Turning CAR-T ON/OFF throughout the body may allow to reduce cytotoxic damage, but also alleviate the anti-cancer activities. The CARTIV approach brings the ability to focus CAR-T, or any engineered immune-cell, onto spatiotemporal zone within the body. This may allow to reduce or even completely avoid the ON-target OFF-tumor activity while sustaining high effector function on the TME.

Inducing expression of effector-genes to a limited spatiotemporal resolution is an inherent feature of the immune system. Both innate and adaptive cells change gene expression following activation. The CARTIV promoter includes response elements for some of the main inflammatory cytokines that define in vivo the zone of effector cells destructive activities. Although, IFNγ is usually referred to as an antitumor cytokine it can also mediate pro tumorigenic transformation and progression. Similarly, TNFα is secreted by inflammatory cells and is related to inflammation-associated carcinogenesis. It has been previously shown that the presence of IFNγ in the TME could be directly associated with tumor virulence. Our data is presenting the potent ability to combine promoter elements of various response factors and gain an induced activation by the combination of external stimulants. The length of the CPRE we used in the CARTIV approach is surprisingly short. While endogenous promoters are usually including proximal promoters that are around hundred bases before and after the transcription Start Site (TSS), and enhancer elements that may reside thousands and even million-base, we find sufficient abilities of short 200 base pair promoters to include multiple PREs. This is perhaps not surprising since the actual binding-sites of transcription factors are usually less than dozen bases. We may speculate that natural promoters are spread over larger length simply since there is no evolutionary reason for...
them to squeeze. Using rational-design and prior knowledge is suggested for the opportunity to create synthetic promoters with complex response to multiple factors within relatively short sequences of DNA.

Special note should be given to the non-trivial combination of the CPRE location that we observed in the CARTIV approach. Catabolic response of CARTIV promoters is designed to allow robust focus of activity within TME, and little or even no activity in normal tissues. Considering safety, having single-factor regulation will possibly improve the constitutive-expression which is currently used, but will inevitably have limited abilities to restrict activity within TME only. Synergistic induction is therefore desired to gain specificity into the tumor microenvironment. Surprisingly, the combination of multiple CPRES into a concise CARTIV promoter found non-trivial significance for the exact sequences and for the order by which the different CPRES are located (Fig. 1). Especially surprising was the dramatic effect of the hypoxia CPRE localization (Fig. 3).

In the CARTIV design we aimed to achieve synergistic induction by multiple factors. The factors used in this study may have a substantial range that in turn may affect the response elements activities. TNFα is a major inflammatory cytokine, having systemic and local activities; IFNγ is having a more local-microenvironment function that was reported specifically to affect cytotoxic T cells via local paracrine and even autocrine fashion53; hypoxia is considered a hallmark of cancer, but levels may range from pO2 of 80 mmHg down to zero, with substantial heterogeneity within tumor microenvironments54. Interestingly, IFNγ is also secreted from T cells and NK cells, possibly making a positive feed-forward activation, but also possibly limiting usage in case one require the lowest background expression. The later may require further modification of our technology, for example, by replacing the IFNγ CPRE.

As mentioned above, the range of biological-activity in vivo is known to differ substantially, as TNFα has the long-range diffusion and IFNα is local, and hypoxia is simply doing as the pO2 for each cell. Our data indicate that the synergistic effect on CARTIV promoters is largely dependent on the concentrations of the relevant factors (Fig. 4). Strikingly, the synergism of TNFα + IFNγ + hypoxia was most prominent at the low-range of cytokine concentration tested, which correspond with physiological levels34,35. This is most likely a feature of the specific CARTIV promoter in these experiments, as other vectors having multiple binding-sites to the same factor showed higher response to same cytokine levels (Fig. 2). Therefore, we may suggest that synthetic promoters can be designed and optimized to the relevant concentrations of factors; the synergism of multiple factors is most beneficial when each is contributing some mediocre induction. Future studies may use these principles to further design promoters for additional factors and gaining sensitivities to their relevant physiological levels. It is probable that by combining different or additional CPRES, employing various ratios between them and testing alternative relative CPRES locations, one could achieve a more robust promoter activation and CAR expression that will then be assessed in vivo for successful tumor regression followed by employing this approach in a clinical setting.

To summarize, CARTIV promoters are an approach to focus engineered immune cells activities into tumor microenvironment. With growing interest in CAR-T, and their safety limitations, we have major interest in applying CARTIV for CAR-T. Further use of such CARTIV promoter may provide immune-focus for any engineered adoptive-transfer approach of effector cells. We anticipate multiple utilization for the synthetic promoters combining multiple response elements.
CARTIV promoter activity assay. HEK293T cells plated at 1 x 10^5 per well in a 96 well flat bottom; NK92 or human primary T cells at 2 x 10^4 in a 96 well U-shape. Cytokines IFNγ, TNFα (PeproTech) added to final concentrations of 32–500 U/mL as indicated per experiment. In experiments involving Hypoxia, it was induced for the last 16–20 h of the experiment. Cells were harvested, washed once in PBS 2% FCS, suspended with DAPI 1 µg/mL and FACS measured using Beckman Coulter® Gallios® flow cytometer. Data were analyzed using Kaluza®.

CARTIV promoter kinetics assay. HEK293T cells plated at 5 x 10^5 per well in a 24-well plate; cells were either stimulated using IFNy and TNFα 500 U/mL for 48 h and then washed twice in complete DMEM or supplemented with IFNy and TNFα 500 U/mL before starting the measurement. Cells were imaged in a Lionheart™ FX Automated Microscope every hour for 48 h. Data were filtered by gating on GFP positive cells and then was plotted using a python script utilizing the matplot and seaborn libraries. Growth and decay curves were fitted to data using the curve_fit function in the scipy library.

CAR expression assay. Following activation, human primary T cells were washed once, stained using an ERBB2-Fc chimeric protein (R&D, USA, MN) 4 µg/mL for 1 h on ice, washed twice in PBS 2% FCS and stained with a Goat anti human APC IgG™ (The Jackson Laboratories, USA, MD). FACS measured using Beckman Coulter® Gallios® flow cytometer. Data were analyzed using Kaluza®.

T cell functional assay. Primary human T cells or NK92 cells were plated at 5 x 10^4 per well in 96 plates and supplemented with 250 U/IFNγ, TNFα or both, after 24 h cells were placed at hypoxic conditions for 16–18 h. Supernatants were collected cyto
tubes quantified using standard ELISA as described elsewhere.

In vivo CARTIV activation assay. NOD.Cg-Ptkdc Il2rg/Slo (NSG) mice were purchased from The Jackson Laboratory. For IMM-139 cell line-derived xenograft, 6-week-old female mice were injected s.c in the neck. Two days later, tumors were dissociated for a single cell suspension. 

Statistics and reproducibility. For FACS data shown are means ± s.d. of geometric mean of the indicated gate from three independent experiments unless otherwise noted. A two-tailed t test was performed to compare between two groups. Unless otherwise noted, all graphs were generated using GraphPad as well as the statistical analysis.

Data availability. Supplementary Data 1 gives all data on all experimental replicates presented in the main work. Additional Source data regarding figures and Supplemental Figures will be available on request from the corresponding author.

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References

1. Drake, C. G., Lipson, E. J. & Brahmer, J. R. Breathing new life into immunotherapy: review of melanoma, lung and kidney cancer. Nat. Rev. Clin. Oncol. https://doi.org/10.1038/nrclinonc.2013.208 (2014).

2. Morgan, R. A. et al. Case report of a serious adverse event following the administration of t cells transduced with a chimeric antigen receptor recognizing ERBB2. Mol. Ther. https://doi.org/10.1038/mt.2010.24 (2010).

3. Lamers, C. H. J. et al. Treatment of metastatic renal cell carcinoma with CAIX CAR-engineered T cells: Clinical evaluation and management of on-target toxicity. Mol. Ther. https://doi.org/10.1038/mt.2013.17 (2015).

4. Lamers, C. H. J. et al. Treatment of metastatic renal cell carcinoma with autologous T-lymphocytes genetically retargeted against carbonic anhydrase IX: first clinical experience. J. Clin. Oncol. https://doi.org/10.1200/JCO.2006.55.9964 (2006).

5. Kochenderfer, J. N. et al. B-cell depletion and remissions of malignancy along with cytokine-associated toxicity in a clinical trial of anti-CD19 chimeric antigen-receptor-transduced T cells. Blood https://doi.org/10.1182/blood-2011-10-384388 (2012).

6. Brentjens, R. J. et al. CD19-targeted T cells rapidly induce molecular remissions in adults with chemotherapy-refractory acute lymphoblastic leukemia. Sci. Transl. Med. https://doi.org/10.1126/scitranslmed.3005930 (2013).

7. Gargett, T. & Brown, M. P. The inducible caspase-9 suicide gene system as a ‘safety switch’ to limit on-target, off-tumor toxicities of chimeric antigen receptor T-cells. Front. Pharmacol. https://doi.org/10.3389/fphar.2014.00235 (2014).

8. Diacou, I. et al. Inducible caspase-9 selectively modulates the toxicities of CD19-specific chimeric antigen receptor-modified T cells. Mol. Ther. https://doi.org/10.1038/mlmt.2017.01.011 (2017).

9. Fedorov, V. D., Themeli, M. & Sadelain, M. PD-1- and CTLA-4-based immunotherapy responses. Sci. Transl. Med. https://doi.org/10.1126/scitranslmed.3006597 (2011).

10. Wilke, S. et al. Dual targeting of ErbB2 and MUC1 in breast cancer using chimeric antigen receptors engineered to provide complementary signaling. J. Clin. Immunol. https://doi.org/10.1007/s10875-012-9689-9 (2012).

11. Lanitis, E. et al. Chimeric antigen receptor T Cells with dissociated signaling domains exhibit focused antitumor activity with reduced potential for toxicity in vivo. Cancer Immunol. Res. https://doi.org/10.1158/2326-6066.CIR-13-0008 (2013).

12. Juillerat, A. et al. An oxygen sensitive self-decision making engineered CAR T-cell. Sci. Rep. https://doi.org/10.1038/srep39833 (2017).

13. Tesh, O., Porgador, A. & Rubin, E. Extracting tumor immune status from expression profiles: correlating renal cancer prognosis with tumor-associated immune. Oncoarget https://doi.org/10.18632/oncoarget.5052 (2015).

14. Bhat, M. Y. et al. Comprehensive network map of interferon gamma signaling. J. Cell Commun. Signal. https://doi.org/10.1186/s12979-018-0486-y (2018).

15. Gilkes, D. M., Semenza, G. L. & Wirtz, D. Hypoxia and the extracellular matrix: drivers of tumour metastasis. Nat. Rev. Cancer https://doi.org/10.1038/nrc3726 (2014).

16. Bhandari, V. et al. Molecular landmarks of tumor hypoxia across cancer types. Nat. Genet. https://doi.org/10.1038/s41588-018-0318-2 (2019).

17. Ma, Y. et al. IL-6, IL-8 and TNF-a levels correlate with disease stage in breast cancer patients. Adv. Clin. Exp. Med. https://doi.org/10.17219/acem/62120 (2017).

18. Binnewies, M. et al. Understanding the tumor immune microenvironment (TIME) for effective therapy. Nat. Med. https://doi.org/10.1038/s41591-018-0014-x (2018).

19. Alkasalias, T., Moyano-Galceran, L., Arsenian-Henriksson, M. & Leith, K. Fibroblasts in the tumor microenvironment: Shield or spear? Int. J. Mol. Sci. https://doi.org/10.3390/ijms19051272 (2018).

20. Viallard, C. & Larrivée, B. Tumor angiogenesis and vascular normalization: alternative therapeutic targets. Angiogenesis https://doi.org/10.1007/s10451-017-9562-9 (2017).

21. Landskron, G., De La Fuente, M., Thuwajit, P., Thuwajit, C. & Hermoso, M. A. Chronic inflammation and cytokines in the tumor microenvironment. J. Immunol. Res. https://doi.org/10.1155/2014/149185 (2014).

22. Erdogan, B. & Webb, D. J. Cancer-associated fibroblasts modulate growth factor signaling and extracellular matrix remodeling to regulate tumor metastasis. Biochem.Soc. Trans. https://doi.org/10.1042/BST20160387 (2017).

23. Huang, Y., Lin, D. & Taniguchi, C. M. Hypoxia inducible factor (HIF) in the tumor microenvironment: friend or foe? Sci. China Life Sci. https://doi.org/10.1007/s11427-017-9178-y (2017).

24. Mayer, A. & Vaupel, P. Multiparametric analysis of the tumor microenvironment: Hypoxia markers and beyond. Adv. Exp. Med. Biol. https://doi.org/10.1007/978-3-319-53525-8_14 (2017).

25. Ackerman, D. & Simon, M. C. Hypoxia, lipids, and cancer: Surviving the harsh tumor microenvironment. Trends Cell Biol. https://doi.org/10.1016/j.tcb.2014.06.001 (2014).

26. Semenza, G. L. The hypoxic tumor microenvironment: a driving force for breast cancer progression. Biochem. Biophys. Acta. https://doi.org/10.1016/j.bbamcr.2015.05.036 (2016).

27. Becker, T., Kovarik, P. & Meinke, A. GAS elements: a few nucleotides with a major impact on cytokine-induced gene expression. J. Interf. Cytokine Res. https://doi.org/10.1089/jir.1997.17.121 (1997).
28. Scharer, C. D., Barwick, B. G., Youngblood, B. A., Ahmed, R. & Boss, J. M. Global DNA methylation remodeling accompanies CD8 T cell effector function. J. Immunol. https://doi.org/10.4049/jimmunol.1301395 (2013).
29. Gao, S., Zhou, J., Zhao, Y., Toselli, P. & Li, W. Hypoxia-response element (hre)-directed transcriptional regulation of the rat hsf1 oxidase gene in response to cobalt and cadmium. Toxicol. Sci. https://doi.org/10.1093/toxsci/ki5327 (2013).
30. Aron, S., Flemington, E. K. & Deininger, P. L. The human thymidine kinase gene promoter. Deletion analysis and specific protein binding. J. Biol. Chem. 264, 2343–2349 (1989).
31. Hu, C.-J., Wang, L.-Y., Chodosh, L. A., Keith, B. & Simon, M. C. Differential roles of hypoxia-inducible factor 1 (HIF-1) and HIF-2 in hypoxic gene regulation. Mol. Cell. Biol. https://doi.org/10.1128/mcb.23.24.9361-9374.2003.
32. Land, S. C. & Tee, A. R. Hypoxia-inducible factor 1a is regulated by the mammalian target of rapamycin (mTOR) via an mTOR signaling motif. J. Biol. Chem. https://doi.org/10.1074/jbc.M611782200 (2007).
33. McGarry, E. C., Rondon, I. J. & Beckman, B. S. Post-transcriptional regulation of erythropoietin mRNA stability by erythropoietin mRNA-binding protein. J. Biol. Chem. https://doi.org/10.1074/jbc.C727.138628 (1997).
34. Kim, Y. W. et al. Association of serum and intratumoral cytokine profiles with tumor stage and neutrophil lymphocyte ratio in colorectal cancer. Antioxidant Res. 34, 3481–3487 (2014).
35. Kawaguchi, K. et al. Alteration of specific cytokine expression patterns in patients with breast cancer. Sci. Rep. 9, 2924 (2019).
36. Wang, S. U. Y., Wei, Y. F., Du, H. L., Ren, L. L. & Li, S. H. Clinical efficacy and T-lymphocyte subset, serum interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin-2 (IL-2) levels on treatment of chronic aplastic anemia patients by Shenfu injection combined with stanozol and cyclosporin A. Zhongguo Zhongxi Zaichi 30, 383–385 (2005).
37. Tang, X. et al. First-in-man clinical trial of CAR NK-92 cells: safety test of CD33-CAR NK-92 cells in patients with relapsed and refractory acute myeloid leukemia. Am. J. Cancer Res. 8, 1083–1089 (2018).
38. Zhao, Y. et al. A Hecreptin-based chimeric antigen receptor with modified signaling domains leads to enhanced survival of transduced T lymphocytes and antitumor activity. J. Immunol. https://doi.org/10.4049/jimmunol.0990447 (2009).
39. Tanner, M. et al. Characterization of a novel cell line established from a patient with Herceptin-resistant breast cancer. Mol. Cancer Ther. 3, 1585–1592 (2004).
40. Gross, G. & Esthar, Z. Therapeutic potential of T cell chimeric antigen receptors (CARs) in cancer treatment: countering off-tumor toxicities for safe CAR T cell therapy. Annu. Rev. Pharmacol. Toxicol. https://doi.org/10.1146/annurev-pharmaco-010814-124844 (2014).
41. Xin, Y. J., Hubbard-Lucey, V. M. & Tang, J. The global pipeline of cell therapies for cancer. Nat. Rev. Drug Discov. https://doi.org/10.1038/d41573-019-00090-x (2019).
42. D’Azzo, E., Schob, K., Nauerth, M. & Busch, D. H. T cell engineering for adoptive T cell therapy: safety and receptor avidity. Cancer Immunol. Immunother. https://doi.org/10.1007/s00262-019-03995-9 (2019).
43. Gross, G., Waks, T. & Esthar, Z. Expression of immunoglobulin-V-T-cell receptor chimeric molecules as functional receptors with antibody-type specificity. Proc. Natl Acad. Sci. USA https://doi.org/10.1073/pnas.86.24.10024 (1989).
44. Hwu, P. et al. Lysis of ovarian cancer cells by human lymphocytes redirected with a chimeric gene composed of an antibody variable region and the Fc receptor γ chain. J. Exp. Med. https://doi.org/10.1084/jem.178.1.361 (1993).
45. Lee, D. W. et al. Current concepts in the diagnosis and management of cytokine release syndrome. Blood https://doi.org/10.1182/blood-2014-05-552729 (2014).
46. Di Stasi, A. et al. Inducible apoptosis as a safety switch for adoptive cell therapy. N. Engl. J. Med. https://doi.org/10.1056/NEJMoa1106152 (2011).
47. Roybal, K. T. et al. Precision tumor recognition by T cells with combinatorial antigen-sensing circuits. Cell https://doi.org/10.1016/j.cell.2016.01.011 (2016).
48. Wu, C. Y., Roybal, K. T., Puchner, E. M., Onuffer, J. & Lim, W. A. Remote control of therapeutic T cells through a small molecule-gated chimeric receptor. Science https://doi.org/10.1126/science.aab4077 (2015).
49. Cartellieri, M. et al. Switching CAR T cells on and off: a novel modular platform for retargeting of T cells to AML blasts. Blood Cancer J. https://doi.org/10.1038/bjcc.2016.61 (2016).
50. Mestermann, K. et al. The tyrosine kinase inhibitor dasatinib acts as a pharmacologic on/off switch for CAR T cells. Sci. Transl. Med. https://doi.org/10.1126/scitranslmed.aaz5907 (2019).
51. Kulemzin, S. V. et al. Design and analysis of stably integrated reporters for inducible expression in human T cells and CAR NK-cell lines. BMC Med. Genomics https://doi.org/10.1186/s12920-019-0489-4 (2019).
52. Wu, C. Y., Roybal, K. T., Puchner, E. M., Onuffer, J. & Lim, W. A. Remote control of therapeutic T cells through a small molecule-gated chimeric receptor. Science https://doi.org/10.1126/science.aab4077 (2015).
53. Bhat, P., Leggatt, G., Waterhouse, N. & Frazer, I. H. Interferon-γ derived from cytokytic lymphocytes directly enhances their motility and cytotoxicity. Cell Death Dis. https://doi.org/10.1038/cddis.2017.67 (2017).
54. Epe, B. & Halpern, J. A. In vivo ϒ2O imaging of tumors: oxygenmetry with very low-frequency electron paramagnetic resonance. Methods Enzymol. 564, 501–527 (2015).
55. Edri, A. et al. The Ebola-glycoprotein modulates the function of natural killer cells. Front. Immunol. https://doi.org/10.3389/fimmu.2018.01428 (2018).

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Author contributions
Y.G. performed a large portion of the experimental work, analysis, and contributed substantially to the design and data interpretation. O.S. performed substantial experimental work. A.O. contributed to data analysis in the time laps experiments and performed graphical editing to the figures. A.C. performed experimental work in our proof of concept stage. K.K. and K.Y. established and maintained our animal model under the supervision and guidance of M.E. R.G. contributed to the study design, supervision, and data interpretation. A.P. led the study, supervised the experimental work and data analysis, and let the writing and styling of the study.

Competing interests
The authors declare no competing interests.

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