Hypoxia-Sensing CAR T-Cells Provide Safety and Efficacy in Treating Solid Tumors

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SUMMARY

Utilizing T-cells expressing chimeric antigen receptors (CARs) to identify and attack solid tumors has proven challenging, in large part, due to the lack of tumor-specific targets to direct CAR binding. Tumor selectivity is crucial as on-target off-tumor activation of CAR T-cells can result in potentially lethal toxicities. This study presents a stringent hypoxia-sensing CAR T-cell system which achieves selective expression of a pan-ErbB-targeted CAR within a solid tumor, a microenvironment characterized by an inadequate oxygen supply. Using murine xenograft models, we demonstrate that despite widespread expression of ErbB receptors in healthy organs, the approach provides anti-tumor efficacy without off-tumor toxicity. This dynamic on/off oxygen-sensing safety switch has the potential to facilitate the unlimited expansion of the CAR T-cell target repertoire for treating solid malignancies.

KEY WORDS

Chimeric antigen receptor, T-cell, hypoxia, HIF1α, cytokine release syndrome, toxicity, cancer, immunotherapy.
INTRODUCTION

There has been significant interest in the prospects of chimeric antigen receptor (CAR) T-cell therapy for solid malignancies and multiple clinical trials are in progress \(^1\). However, the scope of these trials has been restricted by the lack of availability of tumor-specific targets. Upon antigen binding, CARs initiate robust T-cell activation and subsequent cytolytic killing of the target cell through an intracellular signaling domain which most commonly contains both CD3\(\zeta\) and co-stimulatory elements \(^2\). However, the selectivity of CAR-mediated killing of the tumor cells is currently dictated solely by the biodistribution of the CAR antigen. Tumor specificity is vital to the success and safe use of CAR therapy. Intravenously (i.v.) infused anti-ErbB2 CAR T-cells resulted in lethal toxicities in a patient with metastatic colon cancer due to uncontrolled cytokine release syndrome (‘cytokine storm’) following CAR T-cell activation in the lungs \(^3\). The ErbB family of receptors is widely expressed on normal epithelial cells. Nonetheless, ErbB receptors remain attractive tumor-associated targets since they are expressed, or over-expressed, in a wide range of cancers \(^4,5\). Identifying approaches to circumvent off-tumor toxicity has the potential to unlock an entirely new repertoire of CAR antigen targets for carcinomas, which are currently limited. In this study we investigate the opportunities for harnessing tumor hypoxia, a characteristic common to most solid tumors, as a physical cue for licencing CAR T-cell activation specifically within the tumor microenvironment (TME). We find that expressing a CAR under the control of a stringent hypoxia-sensing safety switch avoids on-target off-tumor activation of CAR T-cells whilst delivering efficient anti-tumor killing. This study provides an approach to overcome a major hurdle for the utilization of CAR T-cell therapy against solid malignancies.
RESULTS

Intravenous administration of pan-anti-ErbB CAR T-cells results in lethal toxicity in mice

To investigate the issue of on-target off-tumor CAR T-cell activation we utilized a 2nd generation pan-anti-ErbB CAR T1E28z, named T4-CAR, which has specificity towards 8/9 of the possible ErbB receptor homo- and hetero-dimers and crosses the species barrier, binding both human and mouse receptors equivalently. T4-CAR co-expresses a chimeric cytokine receptor (4αβ) which delivers an intracellular IL-2/IL-15 signal upon binding of IL-4 to the extracellular domain, providing a means to selectively enrich CAR T-cells during ex vivo expansion (Figure 1A,B). T4-CAR has proven safe for intra-tumoral (i.t.) delivery in patients with head and neck squamous cell carcinoma (HNSCC), but i.v. infusion is required to treat patients with metastatic disease. However preclinically, i.v. infusion of human T4-CAR T-cells into NSG mice bearing HN3 tumors (Figure 1C,D and S1A) which express ErbB1-4 (Figure S1B,C) resulted in a lethal toxicity, evident by a rapid loss of weight in these animals (Figure 1D). As observed clinically, analysis of the blood of these mice revealed evidence of cytokine storm (Figure 1E). Using a sub-lethal dose of CAR T-cells concurrently expressing the reporter luciferase (Luc), we evaluated the biodistribution of these cells acutely post i.v. infusion (Figure 1C,F). Imaging revealed that the majority of the infused CAR T-cells accumulated in the lungs and liver, while only a minority reached the tumor despite the expression of ErbB1-4 on the tumor cells (Figure 1G and S1B,C). Profiling of ErbB1-4 mRNA expression confirmed that all four receptors were expressed across all vital organs, including the lungs and liver (Figure S1D-G). Hematoxylin and eosin (H&E) stained tissue sections of the liver and lungs of T4-CAR T-cell infused mice revealed the presence of myeloid cell infiltrates, indicative of CAR-mediated inflammation in these tissues (Figure 1H,I). These data indicate that the liver and lungs represent the two key organs for off-tumor CAR T-cell activation.
A dual oxygen-sensing system provides stringent hypoxia-regulated expression of a CAR

Hypoxia is a characteristic of most solid tumors, where proliferative and high metabolic demands of the tumor cells, alongside inefficient tumor vasculature, result in a state of inadequate oxygen supply (<2% O_2) compared to that of healthy organs/tissues (5-10% O_2). Clinically, hypoxia has been associated with poor prognosis, and resistance to both chemotherapy and radiotherapy. As hypoxia differentiates the TME from that of healthy, normoxic tissue, it represents a desirable marker for the induction of CAR T-cell expression (Figure 1J,K). Although hypoxia has been linked to immune suppression, the killing capacity of T4-CAR T-cells was not negatively impacted by even extreme levels of hypoxia (0.1% O_2) (Figure S2A). Cells have evolved an elegant biological machinery to both detect and rapidly respond to hypoxia through the constitutively expressed transcription factor, hypoxia inducible factor alpha (HIF1α). A previous study has investigated CARs fused with an Oxygen-Dependent Degradation Domain (ODD) of HIF1α. Under conditions of normoxia the ODD becomes ubiquitinated, targeting the protein/CAR for proteasomal degradation. Although a CAR-ODD endowed CAR T-cells with an improved ability to kill tumor cells under hypoxic conditions, the authors observed residual tumor cell killing under normoxic conditions. In an attempt to create a stringent hypoxia-regulated CAR expression system, we developed a dual-oxygen sensing approach for the T4-CAR (Figure 2A). This was achieved by appending a C-terminal 203 amino acid ODD onto the CAR while concurrently modifying the CAR's promoter in the long terminal repeat (LTR) enhancer region of the vector to contain a series of 9 consecutive hypoxia responsive elements (HREs), which permitted HIF1α-mediated transcription of the CAR. This CAR, named ‘HypoxiCAR’, in vitro demonstrated stringent hypoxia-specific presentation of the CAR molecules on the cell surface of human T-cells (Figure 2B). HypoxiCAR T-cells expressed equivalent CAR molecules per cell to the constitutive T4-CAR T-cells when activated in hypoxic (0.1% O_2) conditions (Figure S2B). The dual-hypoxia sensing system incorporated
into HypoxiCAR proved superior to either single-hypoxia sensing modules of the 9xHRE cassette or ODD, which both displayed leakiness in CAR expression (Figure S2C,D) and tumor cell killing (Figure S2E) under conditions of normoxia. HypoxiCAR’s expression of the CAR, utilizing the dual-hypoxia sensing system, was both stringently restricted to hypoxic environments and was also highly dynamic, representing a switch that could be turned both ‘on’ and ‘off’ in an O2-dependent manner (Figure 2B,E and S2D). As an ODD was not appended to the 4αβ receptor of T4-CAR, the leaky expression of the 9xHRE promoter under conditions of normoxia (Figure S2D) was sufficient to allow IL-4 mediated in vitro expansion of HypoxiCAR T-cells under culture conditions of normoxia (Figure 2A). In further in vitro characterization, the exquisite O2 sensitivity of HypoxiCAR was confirmed where CAR expression was absent under O2 concentrations consistent with healthy organs (≥5%) but became detectable on the cell surface at O2 concentrations equivalent to those found in the TME (≤1%) (Figure 2F).

**HypoxiCAR T-cells provide hypoxia-restricted tumor cell killing in vitro**

Having validated HypoxiCAR’s ability to sense hypoxia, we sought to investigate its ability to elicit hypoxia-dependent killing of tumor target cells. SKOV3 ovarian cancer cells were seeded onto culture plates and co-incubated with T4-CAR or HypoxiCAR T-cells under normoxic and hypoxic (0.1% O2) conditions. Despite equivalent transduction efficiencies and CD4+:CD8+ T-cells ratios (Figure 2C,D), HypoxiCAR T-cells displayed efficient hypoxia-dependent killing of the SKOV3 cells, almost equivalent to T4-CAR T-cells, with no significant killing observed under normoxic conditions (Figure 2G). Target-cell destruction was strictly CAR-dependent as when the intracellular tail of HypoxiCAR was truncated, to prevent CD3ζ signaling, killing was abrogated (Figure 2G). In addition, HypoxiCAR T-cells exhibited stringent hypoxia-restricted secretion of both IL-2 (Figure 2H) and IFN-γ (Figure 2I), two cytokines which play an important role in the T-cell response.22,23
HypoxiCAR T-cells express CAR selectively in the TME in vivo

To evaluate whether hypoxia could restrict HypoxiCAR expression to a TME while concurrently remaining switched ‘off’ in healthy organs in vivo, human HypoxiCAR T-cells were injected concurrently i.v. and i.t. in NSG mice bearing hypoxic HN3 tumors with an approximate volume of 500mm³ (Figure 3A and 1J,K). Three days after HypoxiCAR T-cell infusion, tissues resident T-cells were assessed for CAR expression ex vivo using flow cytometry. As predicted by the in vitro analyses (Figure 2), HypoxiCAR T-cells had no detectable surface CAR molecules when recovered from the blood, lungs, or liver of the mice, but did express surface CAR molecules within the hypoxic TME (Figure 3B,C and S2F). A similar observation was made in NSG mice bearing SKOV3 tumors (Figure 3D,E). To establish if the ‘Hypoxi’ construct elements would remain active at different stages of tumor growth, a Hypoxi-Luc reporter was developed in which the HRE promoter was used to drive expression of a Luc-ODD. This reporter was stably transduced into the SKOV3 and HN3 cell lines (Figure S3A,B). Luc-ODD, despite not being detectable in tumor cells under normoxic conditions (Figure S3C), was detected in vivo at all stages of tumor growth, even prior to the tumor becoming palpable, in both SKOV3 (Figure S3D) and HN3 tumors (Figure S3E). This highlights the potential for HypoxiCAR T-cells to target tumors from an early stage of development.

HypoxiCAR T-cells circumvent treatment-limiting toxicities and provide anti-tumor efficacy in vivo

To test the anti-tumor efficacy of HypoxiCAR, high-dose T4-CAR, HypoxiCAR or non-transduced human T-cells were infused into mice at day 16 post injection of HN3 tumor cells, just prior to tumors becoming palpable (Figure 4A and S3E). In keeping with the absence of CAR expression on the T-cells in normoxic tissues (Figure 3), HypoxiCAR circumvented the
treatment-limiting toxicity seen following i.v. infusion of high-dose T4-CAR T-cells. Mice infused with HypoxiCAR T-cells displayed no acute drop in weight post-infusion (Figure 4B), no evidence of cytokine storm in the systemic circulation (Figure 4C), nor signs of tissue damage in the lungs or liver (Figure 4D,E). Importantly, while mice infused i.v. with human T4-CAR T-cells all reached their humane endpoints at 28 h (Figure 4B), those infused with HypoxiCAR T-cells displayed no signs of toxicity while tumor growth was effectively prevented (Figure 4F). To directly confirm that HypoxiCAR T-cells accumulated at the site of disease when tumor control was observed, HypoxiCAR T-cells were co-transduced to express a constitutively expressed Renilla (r)Luc and injected into mice bearing established SKOV3 tumors (Figure 4G). HypoxiCAR T-cells suppressed tumor growth in mice bearing palpable SKOV3 tumors (Figure 4H) without obvious toxicity (Figure S4A). Tracking the biodistribution of reporter HypoxiCAR T-cells in vivo confirmed their infiltration into the TME and persistence in these animals as well as presence in the tumor, for at least 26 days post infusion (Figure 4I,J and S4B). Although, there was an observable drop in the prevalence of HypoxiCAR T-cells 11 days post infusion which preceded the loss of tumor control (Figure S4B). As such, HypoxiCAR overcomes a major hurdle that currently precludes the systemic administration of CAR T-cells targeting antigens that are expressed in normal tissues throughout the body.

**T-cells efficiently infiltrate and stabilize HIF1α in hypoxic tumor islands in human cancer**

Hypoxia has been extensively studied in HNSCC. To assess which patients might be most appropriate for HypoxiCAR T-cell immunotherapy, we firstly generated an HRE-regulated gene signature using patient tumor sample transcriptomic data which included; PGK1, SLC2A1, CA9, ALDOA and VEGFA (Figure S5A). Expression of this 5-gene signature, although expressed across all tumors, did increase with tumor size (suggesting
larger tumors were more hypoxic; Figure S5B), but did not differentiate between HNSCC subtypes (Figure S5C) and was prognostic of poorer survival in stage 3 and 4 cancers (Figure S5D). Other groups have also demonstrated hypoxia-gene signatures to be predictive of adverse prognosis in HNSCC 26-28, and such signatures could be utilized to guide patient selection for HypoxiCAR therapy.

Immunohistochemistry staining of HNSCC tumor sections for stabilized HIF1α, the master transcription factor for HypoxiCAR expression, revealed large regions of the tumors where HIF1α had become stabilized (Figure 4K). Although several factors can stabilize HIF1α, hypoxia represents the most probable explanation for this observation 29. Heterogeneity in both HIF1α stabilization and intra-tumoral T-cell infiltration was seen between patients, however those tumors with the highest prevalence and/or intensity of HIF1α stabilization did not exclude T-cells from entering the inter-epithelial space (Figure 4L), nor from entering HIF1α stabilized regions of the tumor (Figure 4M). Using immunofluorescence, we also confirmed that CD3⁺ T-cells infiltrating HIF1α stabilized tumor regions also stabilized HIF1α themselves (Figure 4N and SSE), suggesting that in these environments HypoxiCAR T-cells would become activated. These observations suggest that HypoxiCAR could find clinical application in hypoxic tumor types such as HNSCC, where gene expression (Figure S5), staining of biopsy samples for HIF1α/CD3 (Figure 4K) and imaging techniques such as PET/CT using a hypoxia-radiotracer such as ⁶⁴Cu-ATSM ²⁵ might provide biomarkers to confirm the presence of a hypoxic TME and guide patient selection ³⁰.

**DISCUSSION**

Approaches to improve tumor-specificity of CAR T-cells have been developed, such as T-cell receptor-mimetic CARs with specificity for HLA-presented antigens ³¹, combined targeting of tumor antigens ³²-³⁴, or tuning of CAR affinity to preferentially target high density antigens ³⁵. This study demonstrates an alternative approach to achieve cancer-selective
immunotherapy, exploiting one of the most innate characteristics of the TME. The dual ‘hypoxia-sensing’ system described here achieves compelling anti-tumor efficacy while abrogating off-tumor toxicity of a CAR that recognizes multiple targets in normal tissues.

T-cells that have infiltrated the TME can egress \(^3\), highlighting a potential safety concern if hypoxia-experienced HypoxiCAR T-cells expressing CAR were to re-enter healthy normoxic tissue. However, CD8\(^+\) T-cell migration has been demonstrated to cease within regions where it encounters tumor cells expressing its cognate antigen \(^3\), suggesting that once HypoxiCAR T-cells have expressed their CAR and engaged with tumor cells expressing ErbB receptors, their ability to egress could be limited. Furthermore, based on our \textit{in vitro} observations (Figure 2E), CAR expression is rapidly reduced upon potential exit from the tumor, substantially limiting the risk for unwanted off-tumor activation of the infused CAR T-cells.

The hypoxic TME is not conducive to efficient immune reactions \(^1\), where hypoxia can activate immune-suppressive programs in stromal cells such as macrophages \(^3\), regulate the expression of immune checkpoint molecules \(^3\) and promote a more aggressive tumor cell phenotype \(^4\). T-cell activation/killing can also result in increased hypoxia in the TME \(^4\). Encouragingly however, and in agreement with the findings by others \(^4\), we found that hypoxia did not negatively affect T-cell effector function \textit{in vitro} (Figure 2G-I and S2A).

HypoxiCAR T-cells also were able to prevent the growth of hypoxic tumors (Figure 4F,H) suggesting that, in the models tested, the TME was not an absolute barrier to HypoxiCAR’s effector function. Although the HypoxiCAR T-cell dose used in this study did not entirely eradicate SKOV3 tumors (Figure 4H), the loss of tumor control coincided with an observable drop in the number of HypoxiCAR T-cells resident in the mice (Figures S4B), which could account for this observation. Also, SKOV3 tumors induced lower overall expression of CAR compared to HN3 tumors (Figure 3), which agrees with SKOV3 tumors being relatively less hypoxic (Figure S3D,E). These observations highlight important considerations for translation and appropriately selecting the dose and patient for receiving HypoxiCAR T-cells.
to achieve efficient anti-tumor control. Importantly, as we also demonstrated that T-cells are not excluded from HIF1α stabilized regions of human tumors (Figure 4K-N), it is likely that HypoxiCAR T-cells should be able to access the appropriate TMEs to activate CAR expression. Although we did not observe evidence of treatment-limiting toxicity in mice infused with high therapeutic doses of HypoxiCAR T-cells (Figure 4B-E), there are microenvironments in healthy tissues such as the intestinal mucosa where ‘physiologic hypoxia’ has been observed 43. Such tissues might represent sites where off-tumor activation of HypoxiCAR T-cells could take place. However, an additional suicide switch 44 could be incorporated into HypoxiCAR to provide an additional level of safety for clinical testing.

In summary, ‘HypoxiCAR’ provides a stringent and broadly applicable strategy to overcome the paucity of safe targets available for the treatment of solid malignancies.

LIMITATIONS OF STUDY

The current study utilizes a single CAR in xenograft tumor models with immunocompromised mice. Utilizing the HypoxiCAR approach within further CARs and tumor models, potentially including syngeneic mouse CAR T-cells in immunocompetent preclinical models of cancer, would provide further insight into the effectiveness and utility of the approach.

The current study also does not establish a maximal tolerated dose of HypoxiCAR T-cells compared to constitutive CAR T-cells, and also the effectiveness of multiple dose regimens, which would both provide important further insight for translation. Given that HypoxiCAR T-cells do not express CARs on their surface during their in vitro expansion phase, it would also be interesting to further study if, in the absence of ‘tonic’ signaling, HypoxiCAR T-cells have a superior phenotype for adoptive cell therapy.
Although the ‘HypoxiCAR’ approach does represent a strategy to circumvent off-tumor activation, there are further hurdles which are acknowledged to hinder the effectiveness of CAR immunotherapy for treating solid malignancies. These include, a lack of cell trafficking and entry to the tumor and the immune suppressive TME, which may require further consideration to achieve maximal efficacy of the approach for translation. However, some of these hurdles may be addressed through evaluating HypoxiCAR T-cells within combination therapy strategies, such as alongside immune checkpoint blockade or even radiotherapy, where the latter could potentially preferentially target more oxygenated regions of the tumor. The long-term effect of HypoxiCAR T-cells on healthy organs where regions of HIF-1α stabilization have been described and also pathophysiological conditions such as ischemia, which would represent environments where off-tumor activation may be possible, also require careful investigation for translation.

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AUTHOR CONTRIBUTIONS

P.K., J.M., J.N.A. conceived the project, designed the approach, interpreted the data and wrote the manuscript. P.K., J.W.O., K.I.L., R.H., C.L.S., M.O., M.Y.M.T, T.M., D.L.Y. performed experiments and interpreted the data. D.M.D., N.W., C.G., S.T. provided key expertise and interpretation.

Declarations of Interest

Disclosures: J.M. is co-founder and chief scientific officer, T.M. is an employee, and D.M.D., D.L.Y. are consultants to Leucid Bio, which is a spinout company focused on development of cellular therapeutic agents. J.N.A., J.M. and P.K. are named inventors on a patent submitted in relation to this work. All other authors have declared that there are no competing financial interests or conflicts of interest in relation to this study.

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FIGURE LEGENDS

**Figure 1. Intravenously infused T4-CAR T-cells cause inflammation in healthy organs.**

- **(A)** Diagram depicting T4-CAR. **(B)** Example histograms of surface CAR expression on live (7AAD-) CD3+ T4-CAR or non-transduced human T-cells assessed using flow cytometry before and after expansion in IL-4. **(C-E)** Day 13 post subcutaneous HN3 tumor cell inoculation, mice were infused i.v. with vehicle or 10x10^6 non-transduced or T4-CAR T-cells.
(n=5). (C) Schematic diagram depicting the experiment. (D) Weight change of the mice. Arrow denotes T-cell infusion and cross denotes humane endpoint. (E) Serum cytokines 24h post-infusion. (F) Low-dose human ErbB-CAR/Luc T-cells (4.5x10^6) were infused i.v. into SKOV3 tumor bearing NSG mice and 4 days later, bioluminescence imaging was performed on the whole body and dissected organs. (G) Quantification of the photons/s/unit area as percent of all organs (n=6), LN- inguinal lymph node, SI-small intestine. (H,I) H&E stained sections (left) and quantitation of myeloid infiltration (right) in the lung (H) and liver (I) 5 days post i.v. infusion of low-dose 4.5x10^6 T4-CAR, non-transduced T-cells or vehicle. Arrows indicate myeloid infiltrates. (J,K) IHC staining of tissue sections for reductively-activated pimonidazole in tumor bearing NSG mice (J) and quantitation of the staining, scoring between 0-3, from no staining (0) to intense staining (3) as a % area of the tissue (K). All experiments are representative of a biological repeat. In line charts the dots mark mean and error bars s.e.m. Bar charts show mean and points individual mice. * P<0.05, ** P<0.01.

Figure 2. HypoxiCAR T-cell CAR surface expression and effector function is stringently restricted to hypoxic environments. (A) Diagram depicting HypoxiCAR in normoxia and hypoxia. (B) Example histograms of surface CAR expression on live (7AAD−) CD3+ T4-CAR, HypoxiCAR and non-transduced human T-cells in normoxic or 18h hypoxic (0.1% O_2) conditions assessed using flow cytometry. (C) Genomic DNA from T4-CAR, HypoxiCAR and non-transduced T-cell preparations subjected to qPCR for T2A copy number relative to that of Tbp in the genomic DNA. (D) The relative prevalence of CD4+/CD8+ T-cells among CD3+ T-cells, assessed using flow cytometry in the T-cells, T4-CAR and HypoxiCAR preparations (n=6). (E) Surface CAR expression on HypoxiCAR T-cells at the indicated times under hypoxia (0.1% O_2) and re-exposure to normoxia assessed normalized to 18h hypoxia (n=6). (F) Surface CAR expression on HypoxiCAR T-cells after 18h exposure to 20, 5, 1 and 0.1 % O_2 (n=6), values normalized to 0.1% O_2. (G-I) In vitro SKOV3 tumor cell killing by T4-CAR, HypoxiCAR, CD3ζ-truncated HypoxiCAR (CD3ζ−; to
prevent intracellular signaling) and non-transduced T-cells (effector to target cell ratio 1:1) in normoxic and 0.1% O₂ hypoxic conditions. (H) Quantification of IL-2 and (I) IFN-γ released into the media from the respective T-cells after 24h and 48h exposure to SKOV3 cells, respectively, under normoxic and 0.1% O₂ hypoxic conditions. All experiments are representative of a biological repeat. Bar on charts shows mean and points an individual healthy donor. In line charts, the dots mark mean and error bars s.e.m. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001.

**Figure 3.** HypoxiCAR T-cells express CAR on their surface specifically in tumors. (A) Schematic diagram depicting the experiment. (B-E) Subcutaneous tumor-bearing NSG mice were concurrently injected both i.v. and i.t. with human HypoxiCAR T-cells (2.5x10⁵ i.t. and 7.5x10⁵ i.v.) 72h prior to sacrifice. Representative histograms showing surface CAR expression on live nucleated (7AAD⁻, Ter119⁻) CD45⁺ CD3⁺ HypoxiCAR T-cells in the indicated enzyme-dispersed tissues and blood (B) and frequency of CAR expression (C) in HN3 tumor bearing mice (n=9). Where panels (D) and (E) represent the same respective analysis in SKOV3 tumor-bearing NSG mice (n=8). All experiments are representative of a biological repeat. Bar charts shows the mean and each point an individual mouse. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001.

**Figure 4.** HypoxiCAR T-cells provide anti-tumor efficacy without systemic toxicity. (A-C) Sixteen days post subcutaneous HN3 tumor cell inoculation, mice were infused i.v. with either vehicle or 10x10⁶ T4-CAR, HypoxiCAR or non-transduced human T-cells (control) (n=4 mice). (A) Schematic diagram depicting the experiment. (B) Weight change of the mice. (C) Serum cytokines 24h post-infusion. (D, E) Low dose (4.5x10⁶) T4-CAR or HypoxiCAR T-cells were infused i.v. into NSG mice and five days later the indicated tissues were excised, and myeloid infiltration was scored in the lung (D) and liver (E) (n=5-6). (F)
HN3 tumor growth curves from (A-C), arrow marking the point of CAR T-cell infusion. (G-J) Schematic diagram depicting the experiment in which HypoxiCAR T-cells or T-cells were transduced to express a constitutive rLuc/eGFP ‘reporter’ to allow in vivo tracking (G). Mice bearing established SKOV3 tumors were infused i.v. with either vehicle (n=6) or 10x10^6 reporter HypoxiCAR (n=7) or reporter T-cells (n=5) (H). Bioluminescence imaging was performed on the whole body of mice to track the biodistribution of the infused HypoxiCAR T-cells at day 26 post infusion. Red box marks the SKOV3 tumor (I). Quantification of the % photon flux (photons/s/unit area) signal detected specifically in the tumor out of total photon flux across the whole body (J). (K-M) Example IHC stained human HNSCC section for HIF1α (red) and CD3 (brown) (K). The abundance of inter-epithelial T-cells (IETs) (L) (example marked by black arrow (K)), low/absent n=40 and high n=52 assessed against the HIF1α stabilization score of the tumor (L). For the tumors where IETs were ‘high’, TILs directly infiltrating HIF-1α stabilized regions of the tumor (H-TILs, examples marked by white arrows (K)) were scored as absent (n=6 of 52 tumors) or present (n=46 of 52 tumors) and plotted against the HIF1α stabilization score of the tumor (M). (N) Immunofluorescence images from a human oral tongue carcinoma stained with DAPI (nuclei; blue) and antibodies against CD3 (green) and HIF1α (red); white denotes CD3 and HIF1α co-localization. All experiments are representative of a biological repeat. Bar charts shows the mean and each point an individual mouse. In line charts, the dots mark the mean and error bars s.e.m. Box plots show median and upper/lower quartiles, whiskers show highest and lowest value.* P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact Dr James Arnold (james.n.arnold@kcl.ac.uk).
**Material Availability**

Constructs and other reagents generated in this study will be made available from the Lead Contact for academic/non-commercial research purposes on request without restriction under a Material Transfer Agreement. Commercial use of the constructs generated or derivatives would be subject to a licensing agreement as intellectual property rights are in place.

**Data and Code Availability**

The datasets supporting the current study have not been deposited in a public repository but are available from the corresponding author upon request.

**Experimental Model and Subject Details**

**Mice**

NSG (NOD-scid IL2Rgamma<sup>null</sup>) mice were purchased from Charles River. Male NSG mice were used for studies involving HN3 (a human male cell line) and female NSG mice were used for studies involving SKOV3 (a human female cell line). All mice used for ectopic tumor studies were adults of 6-8 weeks of age and approximately 22 g in weight. Mice were maintained in individually ventilated cages in the King’s College London Biological Services Unit. The use of animals for this study was approved by the Ethical Review Committee at King’s College London and the Home Office, UK. Experiments using animals were performed under Home Office Licence P95C5B41D.
Cell lines

SKOV3 human (female) ovarian adenocarcinoma cells were originally purchased from ATCC and were re-authenticated for this study by ATCC. HN3 human (male) head and neck adenocarcinoma cells were acquired from Ludwig Institute for Cancer Research, London and grown in D10 medium, Dulbecco’s modified Eagle’s medium (DMEM; Gibco) supplemented with 10% Fetal calf serum (FCS; Thermo Fisher Scientific) and GlutaMAX (Thermo Fisher Scientific). Cell lines were confirmed to be free of mycoplasma for this study using the MycoAlert® Mycoplasma Detection Kit (Lonza).

Primary human cells

Primary T cells were isolated from peripheral blood mononuclear cells (PBMCs) from fresh blood obtained as described below from healthy volunteers and cultured in RPMI 1640 supplemented with 5% human serum (Sigma-Aldrich) and 1X penicillin/streptomycin and the indicated cytokines. Donors were between 20-40 years of age with an equal ratio of male:female donors used. No age/sex/gender-specific differences were observed. Blood was obtained from healthy volunteers under approval of the Guy's and St Thomas’ Research Ethics Committee (REC reference 09/H0804/92).

Human tumor tissue

Human FFPE HNSCC tumor tissue was obtained with informed consent under ethical approval from the King's Health Partners Head and Neck Cancer Biobank (REC reference 12/EE/0493) and stained for IHC and immunofluorescence analyses as described below. The patient demographic of HNSCC tissue included in this study was; total n=92 / sex: female (F) n=34, male (M) n=58 / ages (yrs): 66 +/-11 (s.d.) / T-stage (T): T1 n=18, T2 n=16, T3 n=17, T4 n=41). Which was composed of the following patient demographic for the
indicated tumor subtypes; hypopharynx (total n=3 / sex: F n=2, M n=1 / ages (yrs): 59, 74, 78 / T4 n=3), larynx (total n=20 / sex: F n=0, M n=20 / average age (yrs): 63 +/-10 (s.d.) / T1 n=1, T3 n=5, T4 n=14), oral cavity (total n=67 / sex: F n=32, M n=35 / average age (yrs): 67+/-11 (s.d.) / T1 n=16, T2 n=15, T3=12, T=24), tonsil (total n=2 / sex: F n=1, M n=1 / ages (yrs): 65,39 / T1 n=1, T2 n=1). No subtype-specific differences were observed.

**Microbes**

One Shot Stbl3™ chemically competent *E. coli* (Thermo Fisher Scientific) were grown in Luria Bertani (LB) or LB Agar (Sigma-Aldrich) plates containing 100 µg/ml ampicillin (Santa Cruz Biotechnology) in a 37ºC incubator.

**METHOD DETAILS**

**CAR/Reporter construct cloning**

Human T1E28z CAR (pan-ErbB-targeting CAR)-containing SFG retroviral vector was modified to generate the constructs utilized in this study. The full-length ODD cDNA encoding for amino acids 401-603 from human HIF1α was synthesized as a gBlock® (Integrated DNA Technologies) and was appended onto the C-terminus of CD3ζ within the T1E28z CAR through overlap PCR using Platinum Pfx DNA polymerase (Thermo Fisher Scientific) according to the manufacturer’s instructions. The forward primer 5’-

GCCTACCAAGAACAACTGGAC-3’ and reverse primer 5’-

TCCAGCGGCTGGGGCGCGAGGGGGCAGGGCC-3’ were used to amplify 4αβ-T2A-T1E28z CAR while introducing ODD-compatible sticky ends. The ODD was amplified using forward primer 5’-GGCCCTGCCCCCTCGCGCCCCAGCCGCTGGA-3’ and reverse primer 5’-GACTAATCCGGATCCTCGAGTGGCTGTTACTGGAATACTGTAACTGTGCTTTGAGG-3’ which also introduced a CD3ζ-compatible sticky end. The PCR fragments were then fused
using the forward primer 5’-GCCTACCAAGAACAACCTGGAC-3’ and the reverse primer 5’-
GACTAATCCGATCCTCGAGTGGCTTTACTGGAATACTGTAACGTGTCTTGGAGG-3’.

PCR products were run on 1.2% Agarose (Sigma-Aldrich) gels and product size was 
estimated against a 1kb Plus DNA ladder (Thermo Fisher Scientific). Fragments of the 
expected size were excised and purified using the QIAquick® Gel Extraction kit. The 4αβ T2A
T1E28z CAR-ODD sequence which contained flanking Agel and Xhol cleavage sites was 
cloned into the SFG vector to replace the wild type T1E28z CAR with the T1E28z ODD
CAR. Agel and Xhol restriction endonucleases (New England Biolabs) were used to cleave
Agel and Xhol restriction enzyme cleavage sites in the SFG plasmid to remove the existing
4αβ T2A CAR from the vector backbone. Agel and Xhol restriction endonucleases were also 
used to cleave the Agel and Xhol restriction enzyme sites which were built to flank the 4αβ
T2A T1E28z CAR-ODD cDNA. Vector and constructs that had been restriction
endonuclease-digested were agarose gel-purified using the QIAquick® Gel Extraction kit
(Qiagen) and ligated using T4 ligase (Thermo Fisher Scientific). The final ODD modified 4αβ
T2A T1E28z CAR-ODD construct was 3212bp in length (including the 609bp ODD) from the
start to the stop codon. Plasmids were transformed into One Shot Stbl3™ chemically 
competent E. coli (Thermo Fisher Scientific). Transformed E. coli were selected using
ampicillin (100µg/ml; Santa Cruz Biotechnology) containing Luria Bertani (LB) Agar (Sigma-
Aldrich) plates. Transformed colonies were then grown up in LB broth (Sigma-Aldrich) with
100 µg/ml ampicillin and purified using either Qiagen Plasmid Midi or Maxi kits. Final
constructs were sequence verified (Source BioScience). The constitutively expressed
reporter construct has previously been described 47 and contained a Click Beetle Luciferase
(Luc) and eGFP separated by a viral P2A sequence 48. The reporter construct was PCR
amplified using Platinum Pfx DNA polymerase (Thermo Fisher Scientific) according to the
manufacturer’s protocol with the forward primer 5’- CCATGGTGAGCGTGAGAAAAATG-3’
and the reverse primer 5’- CTCGAGTTTACTTGACGCTCGCCATGC-3’. The amplified
product was digested with NcoI and Xhol restriction endonuclease (New England Biolabs)
and cloned into the SFG vector using the Ncol and Xhol restriction sites and T4 DNA ligase
(Thermo Fisher Scientific). A full length ODD (as described above) was also appended onto the C-terminus of Luc from the reporter construct by overlap PCR using the primers: forward 5'-GAGAAGGCCGGCTGCCCCAGCCGCTGGA-3' and reverse 5'-
CCTCAAAGCAGTTACAGTATTCCAGGAACGGAGCTACTAACTTCAG-3' to amplify the ODD flanked with complementary overhangs. Subsequently, overlapping fusion PCR using primers: forward 5'-CCATGGTGAAGCGTGAGAAAAATG-3' and reverse 5'-
CTCGAGTTACTTGTACGCTCGTCATGC-3' was performed to generate a fragment encoding Luciferase-ODD-P2A-eGFP flanked by Ncol and Xhol restriction sites, which were used to insert Luciferase-ODD-P2A-eGFP into the SFG vector. The HRE modification was targeted in the 3’ LTR of the SFG retroviral vector, as the 3’ LTR region is copied to the 5’ LTR upon integration 49. DNA containing 9 tandem 5’
GGCCCTACGTGCTCGTCTCACACACGCTGTCTGAC-3’ HRE motifs (total length 306bp) derived from the human EPO gene, containing both HIF-binding and ancillary sites, was synthesized as a gBlock® (Integrated DNA Technologies) and sub-cloned into the 3’ LTR of the SFG vector, to replace an equivalent sized fragment within the enhancer region, between the Nhel and Xbal restriction endonuclease sites upstream of the native murine leukemia virus promoter. As Nhel and Xbal were not unique restriction sites in the vector, to achieve specific modification of the 3’ LTR, we generated using overlapping fusion PCR a larger fragment flanked by Xhol/EcoRI unique restriction endonuclease sites. This fragment was identical to the vector DNA Xhol/EcoRI region except that the Nhel/Xbal region was replaced by the 9 HRE gBlock®. The T1E28z CAR ODD CD3’ truncated control construct (for CD3-truncated HypoxiCAR) was synthesized as gBlock® (Integrated DNA Technologies) with flanking SbfI and Xhol restriction sites and sub-cloned into the HRE-modified SFG vector using SbfI and Xhol restriction endonucleases (New England Biolabs). To generate the bicistronic Luciferase-T2A-CAR construct for in vivo tracking CAR T-cells, a gBlock® (Integrated DNA Technologies), which was designed to include Luciferase-T2A-T1E peptide binder flanked with AgeI and NotI restriction sites, was inserted into the T1E28z CAR construct.
**Human T-cell isolation**

Blood obtained from healthy volunteers, under approval of the Guy's and St Thomas' Research Ethics Committee (REC reference 09/H0804/92), was collected into Falcon tubes containing anti-coagulant (10% Citrate), mixed at 1:1 with RPMI 1640 and layered over Ficoll-Paque Plus (GE Healthcare). Samples were centrifuged at 750 g for 30 mins at 20ºC (acceleration and brake set to 0) to separate the PBMC cell fraction. The interface between the plasma and the Ficoll layer, which contained the PBMCs, was harvested using a sterile Pasteur pipette and washed in RPMI 1640. T-cells were purified from the PBMC fraction using human Pan T-cell isolation kit (Miltenyi Biotec) and isolated using a MidiMACs™ separator and LS columns (Miltenyi Biotec) according to the manufacturer's protocol. Purified human T-cells were activated using CD3/CD28 Human T-Activator Dynabeads (Gibco) at a 1:1 cell to bead ratio and seeded in tissue culture plates at 3x10⁶/ml in RPMI 1640 supplemented with 5% human serum (Sigma-Aldrich) and 1X penicillin/streptomycin. The following day, 100 IU/ml recombinant human IL-2 (PROLEUKIN) was added to the cultures.

**Retroviral transduction**

To produce retrovirus with tropism for human cells, RD114 pseudotyped retroviral particles were generated by triple transfection, using Peq-Pam plasmid (Moloney GagPol; a gift from Dr Martin Pule, UCL), RDF plasmid (RD114 envelope; a gift from Prof. Mary Collins, UCL) and the SFG plasmid of interest, using FuGENE HD transfection reagent (Promega), of HEK 293T cells as previously described ⁴¹. For the in vivo experiments evaluating the therapeutic efficacy of CAR T-cells in which the SKOV3 tumor cells expressed Click Beetle luciferase, T-cells were tracked by co-transduction with a construct containing Renilla luciferase. Co-transduction was conducted using 1:1 ratios of virus containing a red-shifted Renilla reniformis luciferase 8.6-535 variant (rluc; Genscript) ⁵⁰, which contained the Renilla
luciferase separated from eGFP by a furin-T2A sequence (rluc/eGFP) \(^{51}\), alongside the respective retroviral particles containing the indicated CAR constructs. Supernatants containing viral particles were harvested and incubated with the cells of interest for at least 48 h to allow their transduction. T-cells were transduced in non-tissue culture treated plates that were pre-coated with 4 \(\mu g/cm^2\) RetroNectin (Takara Bio) overnight at 4 \(^\circ\)C. Prior to the retroviral transduction of human T-cells, CD3/CD28 Human T-Activator Dynabeads (Gibco) were removed and fresh IL-2 was added as stated in the T-cell isolation section. In the case of T-cell transduction with the bicistronic 4\(\alpha\beta\)-T2A-CAR construct, following T-cell transduction, human IL-4 (Peprotech) at 30 ng/ml final concentration was added to the culture every 2-3 days to selectively enrich the transduced T-cell population. Adherent cell lines, including SKOV3 and HN3, were transduced with retrovirus, produced as indicated before, in media solution containing Polybrene (Santa Cruz Biotechnology Inc) at 4\(\mu g/ml\) final concentration to increase infection efficiency. Cells modified to express Luc/eGFP were purified by cell sorting using BD FACSAria III (BD Biosciences) based on their eGFP fluorescence.

**Quantitative PCR**

Genomic DNA was extracted from cells using a DNeasy Blood & Tissue Kit (Qiagen) according to manufacturer’s protocol and quantitative PCR was performed using KiCqStart SYBR Green qPCR ReadyMix with ROX (Sigma-Aldrich) according to the manufacturer’s protocol using custom designed primers to generate amplicons from \(Tbp\), \(Luc\) or \(T2A\) sequences in the genome. The primers used were: human \(TBP\) 5’-

\[\text{TTTGGTGTGGCTTCAGTCGC-3'}\] and \(5'\)-\(\text{ATACCTAGAAACAGGAGTTGCTCA-3'}\), \(T2A\) 5’-

\[\text{CGGAGAAAGCGCAGC-3'}\] and \(5'\)-\(\text{GGGTCCGGGGTTCTCTT-3'}\). Amplification of the genes of interest was detected on an ABI 7900HT Fast Real Time PCR instrument (Thermo Fisher Scientific).
Quantitative reverse transcriptase PCR

mRNA was extracted using TRIzol (Thermo Fisher Scientific) method and quantitative reverse transcription (qRT) PCR was performed as previously described using the EXPRESS one-step Superscript RT PCR kit and the following primers/probes purchased from Thermo Fisher Scientific: Erbb1 Mm01187858_m1, Erbb2 Mm00658541_m1, Erbb3 Mm01159999_m1, Erbb4 Mm01256793_m1 and Tbp Mm01277045_m1. Expression of all genes is represented relative to the house-keeping gene Tata-binding protein for both human and murine experiments. Assays were performed using an ABI 7900HT Fast Real Time PCR instrument (Thermo Fisher Scientific).

In vitro studies

In vitro hypoxia was achieved using a hypoxia incubator chamber (Stemcell Technologies) purged at 25L/min for 4 mins with gas containing either; 0.1, 1 or 5% O₂, 5% CO₂ and nitrogen as a balance (BOC), after which the chamber was sealed. This process was repeated again after 1 h. Hypoxia-mediated HIF1α stabilization was, in some cases, mimicked by using the chemical CoCl₂ (Sigma-Aldrich), which inhibits HIF1α hydroxylation, at 100µM final concentration, unless otherwise stated. In in vitro cytotoxicity assays 1x10⁴ Luc/eGFP-expressing SKOV3 cells were seeded in 96-well tissue culture plates and transduced or non-transduced T-cells were added in the well at the indicated effector to target ratios. Co-cultures were incubated for 24, 48 and 72 h time points in normoxia or experimental hypoxia as indicated and target cell viability was determined by luciferase quantification following the addition of 1µl of 15mg/ml XenoLight D-luciferin (PerkinElmer) in PBS per 100µl of media. Luminescence was quantified using a FLUOStar Omega plate reader (BMG Labtech). At the 24 and 48 h co-culture time points a sample of media was taken from the co-culture and subsequently used for IL-2 and IFN-γ quantification, respectively. IL-2 was quantified using Human IL-2 ELISA Ready-SET-Go! Kit, 2nd Generation (eBioscience) as per manufacturer’s protocol. IFN-γ was quantified using Human
IFN-γ DuoSet ELISA kit (Bio-Techne) as per manufacturer’s protocol. In both ELISAs cytokine concentration was determined by absorbance measurements at 450 nm on a Fusion alpha-FP spectrophotometer (Perkin-Elmer).

**In vivo studies**

Tumor cell lines (2.5x10^5 cells in PBS) were inoculated by subcutaneous (s.c.) injection into female (for SKOV3) and male (for HN3) mice that were six to eight weeks of age. Once tumors were palpable, digital caliper measurements of the long (L) and short (S) dimensions of the tumor were performed every 2 or 3 days. Tumor volume was established using the following equation: Volume = (S^2 x L)/2. The indicated doses of CAR T-cells were injected in 200µl PBS through the tail vein using a 26 G needle. Where i.t. injection was used, cells were injected directly into the tumor in 50µl PBS. Blood samples were taken from mice in EDTA-coated Microvette™ tubes (Sarstedt) and plasma was extracted by centrifugation of these samples at 2,000 g for 5 mins. Cytokine concentrations were blindly measured externally by Abcam using the FirePlex™ Human Th1/Th2/Th17 and FirePlex™ Mouse Inflammation Immunoassay panels. After mice had been humanely sacrificed at the end of a study period, where tissues were excised they were immersed in excess formalin solution (10%) neutral buffered (Sigma-Aldrich), paraffin embedded, sectioned and stained with H&E using standard protocols. Blinded analysis of histopathology was performed by a FRCPath-qualified specialist in veterinary pathology. Scores were assigned for evidence of inflammation using a non-linear semi-quantitative grading system from 0 to 5 where 0 = no significant change and 5 = whole organ or tissue affected for each observation. Tumor tissue, and other organs, for flow cytometry analyses were enzyme-digested to release single cells as previously described. In brief, tissues were minced using scalpels, and then single cells were liberated by incubation for 60 mins at 37°C with 1 mg/ml Collagenase I from *Clostridium Histolyticum* (Sigma-Aldrich) and 0.1 mg/ml Deoxyribonuclease I (AppliChem) in RPMI (Gibco). Released cells were then passed through a 70 µm cell
strainer prior to staining for flow cytometry analyses. Viable cells were numerated using a hemocytometer with trypan blue (Sigma-Aldrich) exclusion.

**Bioluminescence Imaging**

For assessing Luc bio-distribution in vivo mice were injected intraperitoneally (i.p.) with 200µl (15mg/ml) XenoLight D-luciferin (PerkinElmer) in sterile PBS 10 mins prior to imaging to detect Click Beetle Luciferase. *Renilla reniformis* Luciferase was detected using 100µl (150µg/ml) RediJect Coelenterazine Bioluminescent Substrate (PerkinElmer) immediately prior to imaging. Animals were anesthetized for imaging and emitted light was detected using the *In vivo* Imaging System (IVIS®) Lumina Series III (PerkinElmer) and data analyzed using the Living Image software (PerkinElmer). Light was quantified in photons/second/unit area.

**Immunohistochemistry**

To measure hypoxia within tissues, mice were injected i.p. with 60mg/kg pimonidazole HCl (Hypoxyprobe, HPI, Inc) dissolved in PBS, 2 h before sacrifice. Tissues were transferred into formalin solution (10%) neutral buffered (Sigma-Aldrich) for at least 24 h prior to paraffin embedding. Paraffin-embedded tissues were cut into 5 µm sections and mounted onto glass microscope slides (VWR). Sections were dewaxed in Histo-Clear™ (National Diagnostics) for 6 mins, prior to rehydration through 100%, 95%, 70% ethanol and tap water for 3 min each at room temperature (RT). Antigens were retrieved using Access Revelation (Biocore LLC) at 95°C for 20 mins. Sections were washed three times in 100 mM Tris, 140 mM NaCl, 0.1% Tween 20 pH7.4 (TBST) wash buffer for 10 mins prior to applying a wax circle. Tissue peroxidases were quenched using 0.3% H₂O₂ (Sigma-Aldrich) for 10 mins at RT. Sections were washed again in TBST prior to blocking with 10% goat serum (Sigma-Aldrich) 0.1% Triton X-100 in TBST for 1 h at RT. The stable protein adducts formed with the reductively
activated pimonidazole in hypoxic tissue were detected using rabbit anti-pimonidazole antisera (1:100 Pab2627, Hypoxyprobe, HPI, Inc) O.N. at 4°C. Sections were washed with TBST as above and bound rabbit IgG was detected using Dako EnVision™+ System-HRP (DAB) (K4010, Agilent Technologies) according to the manufacturer’s instructions. Tissue sections were counterstained with hematoxylin and washed clear with tap water prior to dehydration through 70%, 95%, 100% ethanol, and Histo-Clear™ (National Diagnostics) for 3 mins each prior to mounting with a coverslip using DePex (SERVA).

FFPE human HNSCC tumor tissues sections were deparaffinized and dual-antibody stained using a BenchMark ULTRA IHC/ISH system (Roche). Deparaffinized sections were pretreated with Cell Conditioning 1 (CC1) buffer (Roche) for 36 minutes and then incubated with mouse anti-human CD3ε (1:50 F7.2.38 Dako) for 32 mins at 37°C followed by amplification and detection using ultraView Universal DAB Detection Kit (Roche). Subsequently, tissues were incubated with Rabbit anti-human HIF1α (1:1000 EP1215Y, Abcam) for 32 mins at 37°C followed by amplification and detection using ultraView Universal Alkaline Phosphatase Red Detection Kit (Roche). Tissues were counterstained with hematoxylin and bluing reagent (Roche), dehydrated and mounted with a cover slip.

Images were acquired using a NanoZoomer Digital Slide Scanner (Hamamatsu) and IHC staining for stable protein adducts of hypoxyprobe and HIF1α were quantified using an H-score which represented the sum of the respective stain intensities from 0-3 (3 being highest), multiplied by the percent area that each intensity occupied across the tumor. Intra-epithelial T-cells (IETs) were scored as low/absent if CD3+ cells were sparse or absent and ‘high’ if prevalent in the stromal regions surrounding the tumor. Tumor infiltrating lymphocytes (TILs) were scored as ‘present’ if there was >3 areas across the section where CD3+ cells could be found within the tumor tissue.
**Immunofluorescence**

Sections from formalin fixed paraffin embedded (FFPE) human head and neck cancer, principally oral cavity (tongue) and tonsil, were de-paraffinized and antigen retrieved using a Ventana® BenchMark ULTRA (Roche Tissue Diagnostics). Immunofluorescence was performed as previously described \(^\text{47}\). The following antibodies were used at 1:100 dilutions, mouse anti-CD3ε (F7.2.38, Dako) and Rabbit anti-HIF1α (EP12151, Abcam). Primary antibodies were detected using donkey IgG antibodies purchased from Thermo Fisher Scientific at 1:100: AlexaFluor\textsuperscript{®} 488 anti-mouse IgG and AlexaFluor\textsuperscript{®} 568 anti-rabbit IgG. Nuclei were stained using 1.25 μg/ml 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (Thermo Fisher Scientific). Images were acquired using a Nikon Eclipse Ti-E Inverted Spinning Disk confocal microscope system and associated NIS Elements software. Co-localization of staining was evaluated using thresholding on the NIS Elements Software.

**Flow cytometry**

Flow cytometry was performed as previously described \(^\text{53}\). The following antibodies were purchased from suppliers indicated in the Key Resources Table and were used at 1 μg/ml unless stated otherwise: anti-human CD3ε Brilliant Violet 421™ (SK7; Biolegend\textsuperscript{®}), anti-human CD8α Alexa Fluor 488 (RPA-T8), anti-human CD4 PE (RPA-T4), anti-human CD45 Brilliant Violet 510™ (HI30 Biolegend\textsuperscript{®}), anti-mouse CD4 FITC (Clone: RM4-5), anti-mouse CD8α eFluor\textsuperscript{®}450 (Clone: 53-6.7), anti-mouse CD3ε PE (Clone: 145-2C11), neutralizing anti-mouse CD16/CD32 (Clone: 2.4G2). Background fluorescence was established using fluorescence minus one staining. The T1E28z CAR was stained with a biotinylated anti-human EGF antibody (Bio-Techne: BAF236) and detected using Streptavidin APC. Human ErbB family members were detected using anti-ErbB1 (ICR62), anti-ErbB2 (ICR12; both ICR antibodies were gifts of Professor Suzanne Eccles, Institute of Cancer Research, Sutton), anti-ErbB3 PE (BioLegend\textsuperscript{®}, 1B4C3), anti-ErbB4 (NOVUS Biologicals, Clone: H4.77.16). Antibodies that there not conjugated to a fluorochrome were detected using goat anti-Mouse...
IgG (H+L) highly cross-adsorbed secondary conjugated to Alexa Fluor Plus 488 (Thermo Fisher Scientific) or goat anti-rat IgG APC (BioLegend®) as appropriate. eGFP was detected by its native fluorescence. Dead cells and red blood cells were excluded using 1 µg/ml 7-amino actinomycin D (Cayman Chemical Company) alongside anti-Ter-119 PerCP-Cy5.5 (Ter-119; Thermo Fisher Scientific). Data were collected on a BD FACS Canto II (BD Biosciences). Data was analyzed using FlowJo software (Freestar Inc.).

**QUANTIFICATION OF STATISTICAL ANALYSIS**

*Computational analysis of cancer patient data*

RSEM normalized expression datasets from the Cancer Genome Atlas (TCGA) were downloaded from the Broad Institute Firehose resource ([https://gdac.broadinstitute.org/](https://gdac.broadinstitute.org/)). The HRE-regulated gene expression signature was generated by taking the mean normalized log2-transformed expression value of the component signature genes. The HRE-regulated gene signature was comprised of HRE-regulated genes for which a positive correlation was observed between all genes. As *VEGFA* and *ENO1* did not correlate with each other (Figure S5A), we elected to include *VEGFA* over *ENO1* in the signature as, by comparison, *VEGFA* provided a greater sensitivity for tumor T-stage and patient prognosis. This final signature included genes associated with glucose metabolism (*ALDOA, PGK1, SLC2A1*), pH regulation (*CA9*) and angiogenesis (*VEGFA*). All TCGA data was analyzed using R version 3.5.1 ([https://www.r-project.org/](https://www.r-project.org/)). Overall survival analyses were generated by partitioning all HNSCC patients into quartiles based on hypoxia score and taking the top and bottom quartile expression ranked hypoxia score values. Kaplan-Meier survival curves were plotted using GraphPad Prism (GraphPad).
Statistics

Normality and homogeneity of variance were determined using a Shapiro-Wilk normality test and an F-test, respectively. Statistical significance was then determined using a two-sided unpaired Students t test for parametric or Mann-Whitney test for nonparametric data using GraphPad Prism 6 software. When comparing paired data, a paired ratio Students t test was performed. A Welch’s correction was applied when comparing groups with unequal variances. Statistical analysis of in vitro killing curves was performed using repeated measures ANOVA followed by Tukey’s post hoc tests. Homoscedasticity of residual variance and normality assumptions were met. Statistical analysis of tumor growth curves was performed as described $^{54}$. Correlation analyses were performed using Pearson correlation. The pairwise Wilcoxon Rank Sum Test with Benjamini-Hochberg correction was used to measure statistical differences between clinical groups and hypoxia score in cancer patient data from the TCGA. The log-rank (Mantel-Cox) test was used to determine statistical significance for overall survival in cancer patient data from TCGA. No outliers were excluded from any data presented.
Supplemental Information

Figure S1. Flow cytometry gating strategies and ErbB receptor expression in cell lines and healthy tissues. Related to Figures 1-4. (A, B) Example of the flow cytometry gating strategy for live (7AAD−), singlet, CD3+ T-cells (A) and tumor cell lines (B). (C) Live gated tumor cell (SKOV3 and HN3) surface expression of ErbB1-4 (colored histograms) against their respective isotype control staining (grey histograms). (D-G) mRNA expression of Erbb1 (D), Erbb2 (E), Erbb3 (F), Erbb4 (G) genes relative to the housekeeping gene Tbp in the indicated tissues (n = 6). All experiments are representative of a biological repeat. Bar charts show the group mean and each point represents each individual mouse.
Figure S2. HypoxiCAR’s dual oxygen-sensing modules synergize to provide superior stringency in sensing hypoxia. Related to Figures 2 and 3. (A) In vitro SKOV3 tumor cell killing by human T4-CAR T-cells (CAR⁺ effector T-cell to target tumor cell ratio 1:1) in normoxic and hypoxic conditions (0.1% O₂) (n=4). (B) HypoxiCAR and T4-CAR T-cells exposed to 18h hypoxic (0.1% O₂) conditions were assessed for relative surface CAR expression/cell presented as median fluorescence intensity of staining/cell (MFI) using flow cytometry analyses (n=5). (C-E) Schematic diagram depicting the constructs, and their modular arrangements, which were stably transduced into human T-cells (C). Surface CAR expression on T4-CAR, HRE-CAR, CAR-ODD and HypoxiCAR human T-cells at the indicated times under hypoxia (0.1% O₂) or normoxia assessed using flow cytometry analyses, values normalized to 18h hypoxia (n=4) (D). In vitro SKOV3 tumor cell killing by T4-CAR, HRE-CAR, CAR-ODD and HypoxiCAR human T-cells (CAR⁺ effector T-cell to target tumor cell ratio 4:1) in normoxic and hypoxic conditions (0.1% O₂) (E). (F) Representative flow cytometry dot/contour plots for the gating strategy from enzyme-dispersed tumors and healthy tissues alongside the blood from mice which had been injected both i.v. and i.t. with 7.5x10⁵ and 2.5x10⁵ of human HypoxiCAR T-cells, respectively, 72 h prior to sacrifice. Stained for live cells (7AAD, Ter119), CD45⁺ CD3⁺ T-cells and their surface CAR expression. Positive gates were applied based on isotype staining. All experiments are representative of a biological repeat. For line graphs, dots mark the mean and error bars the s.e.m.
Figure S3. Hypoxia is a characteristic of even early tumor microenvironments. Related to Figure 4. (A) Schematic diagram depicting the ‘dual-sensing’ hypoxia reporter construct, and its modular arrangements, which were stably transduced into SKOV3 and HN3 tumor cells. (B) Representative flow cytometry histograms, gating on live (7AAD-) cells, from the hypoxia reporter (Hypoxi-Luc transduced) cell lines. eGFP can be observed under conditions of normoxia in these cells, as in the absence of an ODD, the HRE module alone results in leaky expression (Figure S2C-E). (C) Assessing luciferase activity in Hypoxi-Luc transduced tumor cell lines in the presence or absence of 100µM CoCl2, to mimic hypoxia-mediated HIF1α stabilization. (D, E) Representative bioluminescence images of 3 representative mice at day 14 post inoculation with hypoxia reporter SKOV3 (D) and HN3 (E) cell lines (left of each panel) and the tumor volume (red line, y-axis left) plotted against the bioluminescence signal (blue line, y-axis right) across n=6 mice for each respective tumor cell line (right panel). All experiments are representative of a biological repeat. For line graphs, dots mark the mean and error bars the s.e.m.
Figure S4. HypoxiCAR T-cell toxicity and persistence in mice. Related to Figure 4. Thirty two days post subcutaneous injection of SKOV3 tumor cells, when tumors were palpable, mice were infused i.v. with either vehicle (n=6) or 10x10^6 luciferase reporter HypoxiCAR (n=7) or control reporter T-cells (n=7) (schematic for experiment shown in Figure 4G). (A) Weight change of the mice post infusion. (B). Bioluminescence imaging was performed on the whole body of mice to track the prevalence of the infused HypoxiCAR T-cells on the indicated days. Arrow marks the point of HypoxiCAR T-cell infusion. Data presented as photons/second (p/s). Experiments is representative of a biological repeat. Line charts, the dots mark the mean and error bars s.e.m. * $P<0.05$, ** $P<0.01$. 
Figure S5. HRE-regulated gene signature and HIF1α in HNSCCs to identify patient cohorts more likely to benefit from HypoxiCAR immunotherapy. Related to Figure 4. (A-D) An HRE-regulated gene signature was constructed from known HRE-regulated genes in the HNSCC TCGA dataset (n=528). (A) Correlation plot showing pairwise correlation of HRE-regulated genes in the HNSCC. The size of the dot represents the P value of the correlation where $P > 0.05$ and the color of the dot represents the Pearson correlation coefficient (r) (left) and heatmap displaying the Pearson correlation coefficient for the individual genes in the TCGA HNSCC dataset (right). (B) Signature expression based on T stage (T1 n=48, T2 n=136, T3 n=99, T4 n=174). (C) Expression of the HRE-regulated gene score in HNSCC based on subtype (hypopharynx n = 10, larynx n = 116, oral cavity n = 316, oropharynx n = 79). (D) Survival curve for patients with Stage 3 and 4 HNSCC for high and low expression of the HRE-regulated gene signature (n=87 respectively). (E) Additional representative confocal images from sections from two further HNSCC tumors stained with DAPI (nuclei; blue) and antibodies against CD3 (green) and HIF1α (red), white events denote CD3 and HIF1α co-localizing pixels. Images are representative of multiple tissues and sections. Box plots show median and upper/lower quartiles, whiskers show highest and lowest value. Bar chart shows the group mean and each dot represents an individual mouse and tumor. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$. 