Glioblastoma is considered one of the most aggressive malignancies in adult and pediatric patients. Despite decades of research no curative treatment is available and it thus remains associated with a very dismal prognosis. Although recent pre-clinical and clinical studies have demonstrated the feasibility of chimeric antigen receptors (CAR) T cell immunotherapeutic approach in glioblastoma, tumor heterogeneity and antigen loss remain among one of the most important challenges to be addressed. In this study, we identify p32/gC1qR/HABP/C1qBP to be specifically expressed on the surface of glioma cells, making it a suitable tumor associated antigen for redirected CAR T cell therapy. We generate p32 CAR T cells and find them to recognize and specifically eliminate p32 expressing glioma cells and tumor derived endothelial cells in vitro and to control tumor growth in orthotopic syngeneic and xenograft mouse models. Thus, p32 CAR T cells may serve as a therapeutic option for glioblastoma patients.
Malignant gliomas are the most common primary brain tumors in the central nervous system, presenting highly infiltrative characteristics and dismal outcomes. Glioblastoma (GBM) is the most aggressive and lethal form among malignant gliomas with an average survival time of 12–18 months. The current standard of care for newly diagnosed GBM patients consists of maximal surgical resection, radiotherapy, and concomitant chemotherapy (temozolomide). Unfortunately, this line of treatment has limited efficacy and the disease progresses or relapses. There is no effective treatment to offer to recurrent GBM patients.

Tumor immunotherapy has become the center of attention in the past decade, with adoptive cell transfer and checkpoint blockade having striking success in the clinics. Chimeric antigen receptors (CARs) combine both antibody-like recognition and T cell activating function, endowing the engineered T cells with the capacity to recognize and kill cancer cells. CAR T cell therapy targeting the CD19 receptor in patients with hematological malignancies (“liquid” cancer) has shown remarkable success, but initial attempts to use the same approach in treating “solid” tumors have encountered several challenges. One of the limitations in solid tumors is the lack of sufficient and specific targets, which can lead to CAR T cells with potential and dangerous “off tumor on target” toxicity. Three published clinical trials have been reported using CAR T therapy in GBM. The targeted antigens in these studies include epidermal growth factor receptor variant III (EGFRvIII), human epidermal growth factor receptor 2 (HER2), and interleukin receptor 13Ra2 (IL-13Ra2). Although all three studies reported evidence for the specific killing of tumor cells expressing these antigens, limited antitumor response has been observed in these clinical trials and patients eventually succumb to the disease.

We have previously reported the successful treatment of preclinical GBM models with a nanosystem targeted to tumor vasculature. This system consists of nanoparticles coated with a tumor-homing peptide, CGKRK, that specifically delivers its payload to tumor cells and tumor endothelial cells in GBM. We identified p32/C1qR/HABP/C1qBP to be the receptor for the CGKRK peptide, expressed in high levels on the surface of tumor cells and tumor-associated endothelial cells. P32, also known as complement component 1, Q subcomponent-binding protein (C1QBP), is predominantly localized in the mitochondrial matrix, where it exerts its function in maintaining oxidative phosphorylation and regulating the synthesis of mitochondrial-DNA-encoded genes.

In this study, we validate the expression of p32 in malignant gliomas and confirm its expression on the cell surface of tumor cells. We then focus our efforts to design a CAR targeting p32 positive glioma cells and provide proof-of-principle evidence that p32-CAR T cells are able to recognize and eliminate not only glioma cells but also tumor endothelial cells. We show that treatment with p32-CAR T cells reduces tumor vascularization and extends the overall survival of mice bearing gliomas. Our data suggest that p32 is a tumor-associated antigen (TAA) in gliomas and that CAR T cell immunotherapy against this target may be employed to achieve both antitumor and anti-angiogenic positive outcomes.

**Results**

**p32 is expressed in murine and human glioma.** Others and we have identified p32 as the receptor for three tumor-homing peptides targeted nanoparticles: Lyp-1, CGKRK, and LinTT. p32 expression is significantly up-regulated in human cancers compared to their corresponding normal tissue, and although it is primarily expressed in the mitochondria, several studies reported the expression of p32 on the surface of malignant cells. We sought to determine the expression profile of p32 in murine and human gliomas. Using Rembrandt datasets we first confirmed upregulation of p32 mRNA in low and high-grade gliomas compared to non-tumor tissue and in all three molecular subtypes of GBM (Supplementary Fig. 1a, b). We exploited a cohort of paired primary and recurrent GBM samples and found higher p32 mRNA levels in recurrent GBM (Supplementary Fig. 1c). Kaplan–Meier survival plot showed that increased expression of p32 in malignant gliomas is associated with worst prognosis with decreased overall survival rates (Supplementary Fig. 1d). Next, human glioma specimens were stained with p32 Ab showing significant enhanced expression with tumor grade and compared to normal brain tissue (Fig. 1a). Similar results were previously observed when p32 expression was assessed using a brain tumor tissue array, showing significant upregulation of p32 in higher grade gliomas compared to normal brain tissue.

We validated p32 protein expression by western blot analysis in murine GBM cells, patient-derived GBM83 glioma stem cell (GSC) and human established glioma cells (Supplementary Fig. 1e) and by confocal microscopy in a syngeneic and PDX GBM mouse model, confirming specific expression in tumors but not in normal brain tissue (Fig. 1b). Finally, we examined the expression of p32 on the surface of several murine gliomas derived cells established from our lentiviral-induced adult and pediatric glioma mouse model (005, AFFR53, and O1), as well as on human established cell lines (U87, U118, U178, and U251), and patient-derived glioma stem cells (PD-GSCs) by flow cytometry analysis (Fig. 1b and Supplementary Fig. 2a, b). Among these PD-GSCs we have representatives of both proneural (GBM1079 and GBM1051) and mesenchymal (GBM83, GBM1005, GBM1027) GBM molecular subtypes (Fig. 1b and Supplementary Fig. 2a). All glioma cells stained positive for p32 expression on the cell surface using the same anti-p32 mAb (see the “Methods” section), while human primary cells evaluated were negative (Fig. 1c, d and Supplementary Fig. 3). Besides, we examined the intracytoplasmic and surface expression of p32 in our murine glioma-derived cells and human glioma cells in comparison with primary cortical astrocytes and fibroblasts, and further confirmed that surface expression is restricted to tumor cells (Fig. 1d). Altogether these findings suggest p32 may serve as a TAA in low- and high-grade gliomas.

**Generation and characterization of murine and human p32-specific CAR T cells.** To explore whether the expression of p32 on the surface of glioma cells could serve as an alternative TAA for adoptive cell immunotherapy of brain tumors, a p32-specific 2nd generation CAR construct was designed (Fig. 2a). To generate p32-CAR we cloned a single-chain variable fragment (scFv), clone 2.15, which is directed against the same C1q binding domain of p32 as mAb clone 60.11 used in all our staining experiments described in the previous section. P32 is a highly conserved protein and both the antibody and the scFv clone 2.15 recognize human and murine p32. The scFv is followed by transmembrane and cytoplasmic CD28 and intracellular FcyR domains capable of activating both murine and human T cells. We added a FLAG-tag at the N-term of the scFv for easy validation of expression of the CAR construct on the surface of the transduced T cells and a mcherry fluorescent reporter (separated from the CAR cassette by a P2A skip sequence) to assess transduction efficiency (Fig. 2a). The transduction efficiency of both murine and human activated T cells using the same CAR construct was on average 30–40% (Fig. 2b, c). The phenotypic analysis of CD4 and CD8 subpopulations showed no major changes in their percentages following transduction with the CAR expressing retroviral particles and both CD4 and CD8 subpopulations expressed the p32 CAR (Fig. 2d, e). T cell activation levels were indistinguishable between controls and CAR+
T cells (Fig. 2f, g), and no differences were observed in the expression of representative exhaustion markers (Fig. 2h, i), suggesting that the introduction of the CARs into T cells did not result in tonic signaling. Phenotypic analysis of human lymphocytes showed that p32 CAR⁺ T cells contained central-memory, effector-memory, and T stem cell memory, without significant difference between untransduced and CAR⁺ expressing T cells (Fig. 2j).

Functional evaluation of p32 CAR T cells in vitro. Next, we evaluated the in vitro anti-tumor effect of murine and human p32 CAR T cells. To evaluate the functionality of p32 murine CAR T cells we used two different p32⁺ tumor-derived cell lines, one maintained in a differentiated state, AFFR53, and another line, 005 that we have previously characterized as GSC and that forms typical neurospheres (also termed “tumorspheres”) (Supplementary
CAR T cells with GFP demonstrate that both murine and human p32 CAR T cells T26 or p32 mCAR T cells at different effector T cell to tumor cell (E: tumor) ratios. As shown in Fig. 3a, redirected p32 mCAR T cells effectively and specifically killed p32+ expressing glioma cells, while no effect was observed when co-cultured with p32KD cells. Control irrelevant SP6 mCAR T showed no cytotoxic activity when co-culture with all glioma cells. Specific cytotoxic activity and CAR+ T cell expansion were further confirmed by co-culture of SP6 and p32 mCAR T cells with GFP+ glioma cells and analyzed by flow cytometry (Supplementary Fig. 5a). T cell proliferation in response to p32+ glioma target cells was assessed by CellTracker™ Violet dilution assay (Fig. 3b). Next, we determined the tumor-specific recognition of p32-expressing glioma cells by both intracytoplasmic IFN-γ production (FACS analysis, Fig. 3c) and secretion to the culture media (Fig. 3d).

Similar line of experiments was conducted with transduced p32 human CAR T cells co-cultured with either human U87 glioma cells (maintained in the presence of serum; differentiated state) or GBM83 patient-derived GSCs.30 Untransduced (UT) T cells were used as controls in all the experiments with human glioma cells.31 While p32 hCAR T cells were able to exert their cytotoxic effect (Fig. 3e and Supplementary Fig. 5b), proliferate (Fig. 3f) and secrete IFN-γ (Fig. 3g) when co-culture with glioma cells, UT T cells showed little to no response. These results demonstrate that both murine and human p32 CAR T cells recognize p32+ glioma target cells specifically and are able to kill only p32-expressing glioma cells.

P32 CAR T cells show antitumor activity in both syngeneic and xenograft models. To evaluate the antitumor effect of p32 mCAR T cells in vivo in a syngeneic immunocompetent model, we transplanted orthotopically in NUDE mice. The latency of GBM83 tumors is on average 20 days so three days after injection of the tumors is just the right time to assess tumor growth using in vivo bioluminescence imaging, and then transplanted orthotopically in NUDE mice. The latency of GBM83 tumors is on average 20 days so three days after injection of the tumors (the presence of a lesion was confirmed by luciferase positive signal) mice received one dose (1 × 107 cells) of UT or p32 hCAR T cells or UT T cells were infused intratumorally. Kaplan–Meier survival curve shows significantly improved overall survival results for mice treated with p32 hCAR T cells compared to the control group (Fig. 4b). For the next set of experiments we decided to use a more aggressive model of GBM, this time with patient-derived glioma stem cells (GBM83), derived from a mesenchymal aggressive tumor.32,33 First, GBM83 cells were transduced with firefly luciferase, allowing us to track tumor growth using in vivo bioluminescence imaging, and then transplanted orthotopically in NUDE mice. The latency of GBM83 tumors is on average 20 days so three days after injection of the tumors (the presence of a lesion was confirmed by luciferase positive signal) mice received one dose (1 × 107 cells) of UT or p32 hCAR T cells (containing ~4 × 106 hCAR+ T cells) intratumorally and another dose intraventricularly (2.5 × 106 total T cells, ~1 × 106 CAR+) (Fig. 4c). The latest route of injection was selected based on the positive and improved results obtained in pre-clinical and clinical trials with CAR T cells in GBM.1 While UT control mice showed continuous tumor growth and rapidly succumb to the disease, mice treated with p32 hCAR T cells showed prolonged survival (Fig. 4c). No signs of toxicity or adverse effects were observed in the treated mice (Supplementary Fig. 6d). Comparison of bioluminescence imaging data revealed a significant difference between days 6 and 17 of treatment between UT and p32 hCAR T treated group (Fig. 4d, e). One indication of on-target effect is the decrease of antigen expression in the remaining tumor after treatment. To that end, we analyzed at endpoint the resulting tumors after treatment with p32 hCAR T and UT cells, and compared their p32 expression by immunofluorescent confocal microscopy (Fig. 4f) and flow cytometry analysis (Fig. 4g). We found that p32 expression levels decreased in p32 hCAR T treated mice compared to UT control mice. Next, we sought to analyze the expression of PD−1 on gated CD3+ infiltrating T cells from both UT and p32 hCAR T treated tumors, and found that in all treated...
samples PD−1 was highly expressed (Fig. 4h). Based on these results and previously published work evaluating patients treated with CAR T9 we can only speculate that T cells in the tumors become exhausted and are probably affected by the glioma immunosuppressive microenvironment.

**Antiangiogenic effect of p32 CAR T cells.** When we first identified p32 as the receptor of the CGKRK homing-peptide nanosystem, we not only found it expressed on glioma cells, like the 005 GSCs, but also in tumor-derived endothelial cells (TDEC) and tumor vasculature12. Under hypoxic conditions, 005 GSCs

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**Fig. 2 CAR design and human and murine T Cell transduction.** a Schematic representation of the retroviral vector expressing the p32 CAR (F = FLAG, L = Linker, LTR = long terminal repeat) and its transduction efficiency in mouse b and human c T cells. The transduction efficiency was measured by mcherry positive cells and by FLAG cell surface expression. Untransduced T cells were used as control (mUT = mouse and hUT = human). Histograms are representative of three independent experiments. d, e Frequency of CD4+ / CD8+ T cells 3 days post transduction showing no significant (ns) variation after CAR transduction of mouse (d) and human (e) T cells. Graphs on the right panel represent the quantification of N = 4 mice and N = 4 human donors, respectively. f, g Activation marker expression 2 days after transduction of mouse (f) and human (g) lymphocytes. Graphs on the right panel represent the quantification of N = 4 mice and N = 4 human donors, respectively. h, i Quantification of representative exhaustion marker expression 4 days (h, mouse, N = 3) and 10 days (i, human, N = 3 donors) after initial activation. j Phenotypic analysis of human UT (hUT) and CAR+(hp32) T cells at 10 days post transduction showing the frequency of central memory T cells (Tem, CD45RA−CCR7−), stem central memory T cells (Tscm, CD45RA−CCR7+), effector T cells (Teff, CD45RA+CCR7−) and effector memory T cells (Tem, CD45RA−CCR7−) in CD8+ and CD4+ T cells; N = 4 donors. Each dot in the graphs represents a donor’s average of three independent experiments and final data is presented as mean ± SEM. Statistical significance was determined using an unpaired, two-sided t test when comparing between two groups (d, e, g, i, j), and multiple comparisons ANOVA test when comparing more than two groups (f, h). Source data are provided as a Source Data file.
Fig. 3 Murine and human p32 CAR T cells specifically target p32 expressing glioma cells. a Specific cytotoxicity towards p32-positive and the corresponding p32 KD cells (knockdown; transduced with lentivirus expressing shRNA targeting p32) was measured by LDH activity in culture media. Target cells were co-cultured with either a non-relevant (mSP6) or p32 murine CAR T (mp32) cells for 6 h at the indicated E:T ratios. Data represent mean ± SEM. N = 3. One-way ANOVA, Tukey’s multiple comparisons test. P value shown in graph corresponds to the comparison of the mp32 vs mSP6. P < 0.004 for the remaining comparisons. b CellTrace™ Violet dilution assay of labeled p32 CAR T murine lymphocytes co-cultured with the indicated glioma cells for 3 days (E:T = 3:1). Labeled unstimulated p32 CAR T cells alone were used as control. Representative histogram is shown out of four independent experiments. c, d Transduced murine lymphocytes were incubated with the target cells (E:T = 2:1) for 24 h, and IFN-γ production was measured by FACS intracellular staining (c) and by ELISA (d). N = 3, representative histogram is shown. Dots in graph (d) show average for each independent experiment. Data are shown as mean ± SEM. Statistical significance was determined using one-way ANOVA test with multiple comparisons adjustment. e Human target glioma cells were co-cultured with control untransduced T cells (hUT) or CAR-p32 T cells (hp32) at the indicated E:T ratios. Eighteen hours later, the cytotoxic action of the human CAR T cells was measured by quantifying luciferase activity in tumor target cells. Data represent mean ± SEM. N = 5 for GBM and N = 4 for U87 independent experiments, two different human donors were used. Unpaired t test with Welch’s correction was used for statistical analysis. Two-tailed P value is shown. f IFN-γ secretion (ELISA; N = 3 independent experiments with two different donors) was also assessed using hUT and hp32 T cells following co-culture with the indicated human glioma cells. Data are shown as mean ± SEM. Unpaired t test was used. Two-tailed P value is shown. All source data are provided as a Source Data file.
have the capacity to transdifferentiate and give rise to TDEC both in vitro (Fig. 5a) and in vivo. Confocal microscopy (Fig. 5a) and FACS analysis confirmed the expression of p32 in 005 cells cultured in EGM2 endothelial media supplemented with deferroxamine (DFO), an iron chelator that mimics hypoxic conditions by blocking proline hydroxylase. Next, we co-culture p32 or SP6 mCAR T cells with 005 TDEC at different effector to target ratios and as shown in Fig. 5b p32 mCAR T cells effectively killed p32 expressing TDEC. The cytolytic activity of p32 mCAR T cells was corroborated by IFN-γ release in the culture supernatant. Interestingly, when we analyzed tumor sections from our previous in vivo experiments by confocal microscopy, we observed that while tumors in control groups infused with UT or control SP6 CAR T cells were highly vascularized and stained...
positive for the endothelial marker vWF, the p32 CAR T cell treated mice showed significantly less blood vessels in the tumors (Fig. 5e and quantification by ImageJ in Fig. 5e). The same trend was observed when GBM83 tumors infused with either UT or p32 CAR T cells were dissociated at endpoint and analyzed by flow cytometry for CD31 staining (Supplementary Fig. 7). To further confirm that this anti-angiogenic effect is related to the expression of p32 on TDEC and tumor vasculature, we engineered GBM83 cells to overexpress ErbB2 (Supplementary Fig. 8a). Next we transduce T cells with an ErbB-2-specific CAR (hN29) (Supplementary Fig. 8b) and evaluated the in vitro functionality of hN29 CAR T on ErbB-2-GBM83 cells (Supplementary Fig. 8c–e). Finally, we infused either UT or hN29 CAR T (same dose and location as with hp32 CAR T experiments) to ErbB-2-GBM83 injected mice and followed tumor growth and survival (Supplementary Fig. 8f–h). While we observed an effect on tumor growth and survival with the hN29 CAR T cell treatment, no difference in tumor vasculature was obtained (Supplementary Fig. 8i–k). Altogether, our results suggest that p32 CAR T cells are able not only to recognize tumor cells but also tumor-associated endothelial cells, suggesting a possible antiangiogenic effect of p32 CAR T cells by targeting the tumor vasculature.

**Discussion**

Despite ongoing success in CD19+ B cell hematologic malignancies with CAR T cell therapy, progress in the solid tumor arena still has to face many obstacles. Among the known list of challenges is the identification of a suitable neoantigen or TAA to re-direct the engineered CAR T cells. GBM shares a high level of biological complexity with other solid tumors, with an emphasis on molecular and cellular heterogeneity, the latest reflected by a spectrum of dynamic cell states. Mono-specific CAR T cell therapy demonstrated feasibility and safety when tested in GBM, but eventually resulted in the outgrowth of tumors lacking the expression of the single antigen targeted. Therefore, the identification of an array of glioma-associated antigens is critical for the strategic success of CAR T therapy in highly heterogeneous solid tumors like GBM.

We found in previous work that p32 is the binding partner of CGKRR and LinTTL tumor-homing peptides-coated nanoparticles in GBM. In this study, we confirmed and validated the upregulation and surface expression of p32 in glioma cells and associated endothelial cells. Most importantly, we could not detect p32 expression in normal brain tissue or healthy tissue from other organs.

Higher expression of p32 has been reported in several types of tumors including melanoma, colon, ovarian, endometrial, prostate, and breast, suggesting a potential role in tumorigenesis. Indeed, genetic knockdown of p32 shifted the metabolism of tumor cells from oxidative phosphorylation to glycolysis and resulted in reduced tumor formation in vivo. In gliomas, p32 expression was found to be significantly correlated with the expression of c-myc, and to be involved in the reprogramming of glutamine metabolism in these tumors. Silencing p32 resulted in impaired cell proliferation in vitro and had an anti-tumor effect in vivo. In line of this evidence, mitochondrial localized p32 has been recently evaluated as a therapeutic target in gliomas using a small molecule inhibitor. While mitochondrial p32 functional role has been studied, its translocation to the cell surface is not completely understood. p32 is also known as hyaluronic acid binding protein (HABP). Hyaluronic acid (HA) is an important component of the brain extracellular matrix (ECM) and is one of the major components of GBM-ECM. In addition to anatomical and physical aspects, specific ECM components in GBM, such as Tenascin C, fibronectin, and hyaluronan were shown to contribute to different aspects of tumor biology (e.g., invasiveness, proliferation, angiogenesis). Based on a recently reported study, we speculate that increased levels of HA may disrupt the normal localization of p32 in the mitochondria and induce its translocation to the cell surface. P32 expressed on the cell surface of tumor cells can interact with HA and this interaction may contribute to GBM highly invasive and proliferative capacity.

In the present study, we took advantage of the selective expression of p32 on the surface of glioma cells and describe the design and characterization of a p32-specific CAR T that efficiently kills glioma cells both in differentiated and stem-like states. Cancer cell plasticity has been proposed to be an alternative mechanism to promote cancer cell diversity and to contribute to intra-tumor heterogeneity. Plasticity in solid tumors has been linked to the epithelial-to-mesenchymal transition, and in GBM, it confers the ability to shift from a differentiated state to an undifferentiated or stem-like state. We also reported another interesting scenario in cancer cell plasticity: the transdifferentiation of glioma cells to TDEC, demonstrating the complexity of this phenomenon in brain tumors. We found p32 to be expressed on the surface of differentiated cells, GSCs, and TDEC, and our in vitro studies demonstrated the ability of p32 CAR T cells to specifically recognize and efficiently kill all these populations of glioma cells representing different phenotypic/differentiation states. GSCs have been reported to be more resistant to conventional therapy such as chemotherapy and radiotherapy, and some even blame their resistance to be the source of recurrence in GBM patients. Besides, we have previously shown that GSC differentiation into TDEC is independent of VEGF and FGF, suggesting that TDECs involvement in tumor angiogenesis might be one of the resistance mechanisms against...
anti-VEGF therapies. The ability of p32 CAR T cells to recognize and eliminate glioma cells in different phenotypic/differentiated states provides an advantage to this strategy over the existing conventional therapies and the possibility of combining these therapeutic approaches for the treatment of GBM.

In addition to the diversity within the tumor (intra-tumor heterogeneity), genetic profiling of GBM samples from different patients revealed inter-tumor heterogeneity, suggesting that at least three molecular subtypes of GBM exist: proneural (PN), classical (CL), and mesenchymal (MES)33,46. These molecular subtypes can also co-exist within the same tumor (spatial heterogeneity) or change over the course of tumor progression or as a result of therapy (temporal heterogeneity)46–48. Both our TCGA analysis and flow cytometry experiments using MES and PN GSCs showed that p32 expression is observed in all subtypes of GBM. Altogether, expression of p32 was found in both low and high-grade gliomas, different cell states, and different GBM subtypes, making it a glioma-relevant target for immunotherapy.

Fig. 5 P32 CARTs exert an antiangiogenic action both in vitro and in vivo. a 005 cells differentiated into tumor-derived endothelial cells (TDEC) in EGM medium with deferoxamine (DFO; to mimic hypoxia) were analyzed for Von Willebrand factor (vWF, endothelial marker) (upper panels) and p32 (lower panels) expression by confocal microscopy. Representative image of three independent experiments. b Cytotoxic action of mSP6 and mp32 (murine CAR) T cells against TDEC assessed by LDH cytotoxicity assay. Data represent mean ± SEM. N = 3 independent experiments. Unpaired t test with Welch’s correction was used for statistical analysis. Two-tailed P value is shown. c Recognition of TDEC by CAR T cells (measured by IFN-γ release) Data are shown as mean ± SEM, each dot is the average for an individual experiment (N = 3). d, e Tumor tissue stained for von Willebrand factor (vWF) and analyzed by confocal microscopy shows a clear decrease of blood vessels in the p32CAR treated animals. e Quantification of vWF staining. Data are shown as mean ± SEM. Each dot shown in the graph represents average of three measurements done per slide. A total of 9 (mSP6, hp32 for both models), 10 (mp32) or 11 (hUT) slides originated from 3 different mice were stained per group. For c, e unpaired t test was used and two-tailed P value is shown. Source data are provided as a Source Data file.
observed in our pre-clinical models, mice bearing GBM tumors eventually succumb to the disease. In all the in vivo experimental models, p32-CAR T cells induced the regression of GBM growth, and it is important to highlight that this effect was assessed on endogenous levels of p32 expression, no overexpression or ex vivo modifications were performed to the transplanted glioma cells. A positive indication of on-target effect is the decrease of antigen expression in the tumors treated with p32-CAR T cells compared to UT treated group, and no toxicity was observed following local and systemic injection of p32-specific CAR T cells. We believe that in solid tumors, due to the significant tumor heterogeneity, more than one tumor antigen should be targeted, using for example technologies such as the CART-BiTE recently reported in GBM25, or the convertible CARs where multiple antigens can be simultaneously or sequentially targeted26. Here the benefit of p32 as a surface-expressed antigen in the tumor and not in healthy tissue adds one more option to the limited pool of CAR targetable antigens in GBM. As nicely summarized in a recent review, one of the main challenges CAR T therapies for GBM faces is the intra/intratumor heterogeneity and antigen loss30. The degree of expression of each antigen tested in the aforementioned CAR T GBM clinical trials presented regional, temporally and inter-patient heterogeneity. And while successful eradication of TAA positive cells was observed in each different study, it was also accompanied by the progression of glioma cells not expressing the relevant antigen. Increasing the pool of TAAs to target and the engineered CAR T repertoire in GBM will help overcome in part this particular obstacle. Furthermore, although we observed a short window of CAR specific activity (tumor growth by bioluminescence imaging) a multifactorial in situ immuno-suppressive response drives the expression of exhaustion markers such us PD-1 on infiltrating T cells, leading to tumor re-growth. These observations suggest the possibility of combining CAR T adoptive transfer with inhibition of checkpoint players such as the PD1/PD-L1 axis.

In conclusion, our study provided evidence of specific surface expression of p32 in gliomas, and the potent antitumor and antiangiogenic activity of p32-specific CAR T cells against glioma and tumor-associated and derived endothelial cells, indicating it may be of relevance for the treatment of glioma patients. **Methods**

**Materials**

DMEM, RPMI, fetal bovine serum (FBS), L-glutamine, penicillin, streptomycin, and mycoplasma detection kit were purchased from Biological Industries Ltd. (Kibbutz Beit HaEmek, Israel). DMEM/F-12 (1:1)w/v glut w/o HEPESS (Cat# 1500-050) 500 ml (Cat. No. 11320074), N–2 Supplement (100X), Liquid 5 ml (Cat No. 17920408), B–27 Supplement W/O Vit A (50X/10 ml) (Cat. No. 12587010), B–27 Supplement (50X) (Cat. No. 17504044), Glutamax (Cat. No. 10565-018), were purchased from Gibco. Recombinant Human FGFR-basic (Cat No. 100-188) was purchased from Peprotech. Recombinant Human EGF (Cat No. G5021) was purchased from Promega. RetroNectin (Cat No. T202) was purchased from Takara (Japan). Ficol-Paque PLUS (Pharmacia Biotech, Uppsala, Sweden), GentleMACS C tubes for cell separation (Cat. No. 103-093-334), CD45 MicroBeads for cell isolation (Cat. No. 130-052-301) were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany), IFN-γ mouse IFN-γ DuoSet ELISA (Cat No. DY485) and IFN-γ Human IFN-γ DuoSet ELISA (Cat No. DY285B) were purchased from R&D systems. Cell tracer violet (Cat No. C34557A) was purchase from Invitrogen. Percoll medium (Cat. No. p937) and all other chemical reagents, including salts and solvents, were purchased from Sigma-Aldrich (Rehovot, Israel).

**Cell culture**

Human embryonic kidney 293T cells (HEK 293T), U87, U118, U178 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). U251 human GBM cell line was obtained from the European Collection of Authenticated Cell Cultures (ECACC) (Porton Down, Salisbury, UK). Cell lines from ATCC have been authenticated; ATCC uses morphology, karyotyping, and PCR-based approaches to confirm the identity of human cell lines. The ICLAC identifies U118 as misidentified line, a derivative of U138, possibly sharing a common donor. We only used this cell line once in this study to check the expression of p32 on surface (FACS analysis). U251 cell line was authenticated by the European Collection of Authenticated Cell Cultures (ECACC) using morphology, karyotyping, PCR-based techniques, and Cytochrome oxidase I assay, following manufacturer validated procedures. GBM 3, GBM1005, GBM1027, GBM1051 were obtained from Prof. Ichiro Nakano. GBM25 was obtained from Prof. SM Pollard/Peter Dirks. BL line was obtained from Prof. Santosh Kesari. NCH421K line was obtained from Prof. C. Herold-Mende and received from Prof. Tantem Teeszlu. GBM3 is a patient-tumor-derived GSC (newly diagnosed female, 72 years old, 30–35% Ki67 index35). Human primary cells H–6067, H–6034, H–6013, H–6044 were purchased from cell biology, and N7805–100 was purchased from Gibco. 005 is a murine GSC line generated from a lentiviral HRasV12 induced tumor in a p53−/− knockout mouse29 and AFFR35 are transformed primary astrocytes with HRas-shp35 lentivirus29. O1 cells were derived from FGFR1mut-shp35 GBM1027. Cell lines 005, AFFR53, and O1 and patient-derived cell lines were not authenticated by the authors. For a complete list and summary of all the cell lines please refer to Supplementary Table 1.

**Gene knockdown and luciferase/GFP expression**

For lentiviral HRasV12 induced tumor in a p53−/− knockout mouse29, and it is important to highlight that this effect was assessed on endogenous levels of p32 expression, no overexpression or ex vivo modifications were performed to the transplanted glioma cells. A positive indication of on-target effect is the decrease of antigen expression in the tumors treated with p32-CAR T cells compared to UT treated group, and no toxicity was observed following local and systemic injection of p32-specific CAR T cells. We believe that in solid tumors, due to the significant tumor heterogeneity, more than one tumor antigen should be targeted, using for example technologies such as the CART-BiTE recently reported in GBM25, or the convertible CARs where multiple antigens can be simultaneously or sequentially targeted26. Here the benefit of p32 as a surface-expressed antigen in the tumor and not in healthy tissue adds one more option to the limited pool of CAR targetable antigens in GBM. As nicely summarized in a recent review, one of the main challenges CAR T therapies for GBM faces is the intra/intratumor heterogeneity and antigen loss30. The degree of expression of each antigen tested in the aforementioned CAR T GBM clinical trials presented regional, temporally and inter-patient heterogeneity. And while successful eradication of TAA positive cells was observed in each different study, it was also accompanied by the progression of glioma cells not expressing the relevant antigen. Increasing the pool of TAAs to target and the engineered CAR T repertoire in GBM will help overcome in part this particular obstacle. Furthermore, although we observed a short window of CAR specific activity (tumor growth by bioluminescence imaging) a multifactorial in situ immuno-suppressive response drives the expression of exhaustion markers such us PD-1 on infiltrating T cells, leading to tumor re-growth. These observations suggest the possibility of combining CAR T adoptive transfer with inhibition of checkpoint players such as the PD1/PD-L1 axis.

In conclusion, our study provided evidence of specific surface expression of p32 in gliomas, and the potent antitumor and antiangiogenic activity of p32-specific CAR T cells against glioma and tumor-associated and derived endothelial cells, indicating it may be of relevance for the treatment of glioma patients.
Flow cytometry antibodies. Anti-mouse CD3ε-APC (Biologent, Cat. No. 100311, clone 145-2C11, lot B291091, dilution 1:100). Anti-mouse CD8α-alexa 488 (Biologent, Cat. No. 100723, clone 53-6-7, lot B295426, dilution 1:200). Anti-mouse CD4 BV 785 (Biologent, Cat. No. 100453, clone GK1.5, lot B236697, dilution 1:100). Anti-mouse CD25-PE (Biologent, Cat. No. 101904, clone 3C7, lot B243773, dilution 1:50). Anti-mouse LAG3-PerCP-Cy5.5 (Biologent, Cat. No. 125211, clone C9B7W, lot B261544, dilution 1:200). Anti-mouse PD-1-PerCP-Cy5.5 (Biologent, Cat. No. 125211, clone C9B7W, lot B261544, dilution 1:200). Anti-human CD3ε-APC (Biologent, Cat. No. 300439, clone UCHT1, lot B286610, 5 μl per test). Anti-human CD4-FITC (Biologent, Cat. No. 317408, clone OKT4, lot B2349453, 5 μl per test). Anti-human CD8α e450 (Biologent, Cat. No. 40888842, clone RPA-TB, lot N98809, 5 μl per test). Anti-human CD25-FITC (Biologent, Cat. No. 302604, clone BC-96, lot B253407, 5 μl per test). Anti-human LAG3-APC (Biologent, Cat. No. 360212, clone 7H2C5, lot B294090, 5 μl per test). Anti-human CD38-APC (Biologent, Cat. No. 304112, clone HI100, lot B276650, dilution 1:200). Anti-human CCR7-FITC APC (Biologent, Cat. No. 353216, clone G043H7, lot B949440, dilution 1:200). Anti-human PD-1-FITC (Biologent, Cat. No. 621611, clone A17188B, dilution 1:200). Rabbit anti-FLAG (Cell Signalling, Cat. No. 14793, clone 145-2C11, lot B291091, 5 μl per test). Anti-mouse HRP (General Biological). Cell Signalling (Cat. No. 2147H, Dilution 1:500, sample concentration as a corresponding primary antibody). Anti-mouse/human C1QBP-PE (Santa Cruz, Cat. No. sc-23884, clone 60.11, Lot L0403, Dilution: 20 μl per test).

Flow cytometry and immunofluorescence staining. Efficiency of T cell transduction was assessed using anti-FLAG antibody along with mcherry fluorescent reporter. Flow cytometry analysis 100,000 cells were stained with the appropriate antibody according to the antibodies manufacture instructions. Briefly, the cells were incubated with TruStain FcX™-Fc blocker CD16/32 (Biologent, Cat. No. 101520, clone 93, Lot no. B275367, dilution 1:100) for 15 min to reduce non-specific staining, followed by staining with primary antibodies listed above for 30 min on ice. Cells were fixed in 4% paraformaldehyde for 15 min. Then cells were permeabilized using permeabilization buffer (BD Bioscience, Cat. No. 554714) according to the manufacturer’s protocol. Fluorescence intensity was assessed using an Attune NxT Flow Cytometry and analysis was performed using Kaluza 2.1 software (Beckman Coulter, USA).

Negative controls included iso-type antibodies and where appropriate, untransduced T cells stained using the test antibodies. Gating strategy for all flow cytometry experiments is included in Supplementary Fig. 9.

Flow cytometry analysis 100,000 cells were stained with the appropriate antibodies according to the antibodies manufacture instructions. Brie
for MAC. Data represent mean ± SEM, n values are listed in figure legends. Log-rank (Mantel-Cox test) analysis was used to determine the statistical significance of Kaplan–Meier survival plots. P < 0.05 was considered statistically significant.

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

**Data availability**

The glioma patient sequencing data analyzed in this study are available and were downloaded from [http://gliovis.bioinfo.cnio.es](http://gliovis.bioinfo.cnio.es) (Rembrandt adult dataset was selected for tumors from different histological types and GBM molecular subtypes, and CGGA adult dataset to compare primary and recurrent tumors). The remaining data generated or analyzed during this study are available within the article and its supplementary information files. Source data are provided with this paper.

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Author contributions
D.F.M. designed and supervised the project. L.R.N., I.M., and S.T. performed most of the experiments. T.W., A.G.L., and Z.E. provided valuable reagents, helped with human T cell transduction, and reviewed the data. L.A.V. provided the p32 scFv and reviewed the data. M.H. and T.T. performed the p32 staining of human GBM samples. L.R.N., I.M., and D.F.M. wrote or edited the manuscript. All authors have seen, corrected, and approved the final manuscript.

Competing interests
The authors declare no competing interests.

Additional information
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