Heparanase promotes tumor infiltration and antitumor activity of CAR-redirected T lymphocytes

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Adoptive transfer of chimeric antigen receptor (CAR)-redirected T lymphocytes (CAR-T cells) has had less striking therapeutic effects in solid tumors1–3 than in lymphoid malignancies4,5. Although active tumor-mediated immunosuppression may have a role in limiting the efficacy of CAR-T cells6, functional changes in T lymphocytes after their ex vivo manipulation may also account for the reduced ability of cultured CAR-T cells to penetrate stroma-rich solid tumors compared with lymphoid tissues. We therefore studied the capacity of human in vitro–cultured CAR-T cells to degrade components of the extracellular matrix (ECM). In contrast to freshly isolated T lymphocytes, we found that in vitro–cultured T lymphocytes lack expression of the enzyme heparanase (HPSE), which degrades heparan sulfate proteoglycans, the main components of ECM. We found that HPSE mRNA is downregulated in in vitro–expanded T cells, which may be a consequence of p53 (officially known as TP53, encoding tumor protein 53) binding to the HPSE gene promoter. We therefore engineered CAR-T cells to express HPSE and showed their improved capacity to degrade the ECM, which promoted tumor T cell infiltration and antitumor activity. The use of this strategy may enhance the activity of CAR-T cells in individuals with stroma-rich solid tumors.

CAR-T cells in in vitro cultures consist mostly of memory and effector lymphocytes characterized by potent cytotoxic function1,4,7. However, to exploit their effector functions in vivo, CAR-T cells must traffic to and accumulate in tumor sites. These processes involve a complex sequence of events, beginning with the adhesion of T cells to endothelial cells and followed by chemokine-chemokine receptor interactions, that modulates their extravasation into antigen-rich tissues8–10. During this process, T lymphocytes actively degrade the main components of the subendothelial basement membrane and the ECM, including the heparan sulfate proteoglycans (HSPGs)11. The ECM is an integral component of the stroma, and therefore T cells attacking stroma-rich solid tumors must be able to degrade HSPGs in order to access tumor cells and exert antitumor effects.

The release of specific enzymes by T cells is fundamental to the degradation of ECM. One of these enzymes is HPSE, which is the only known mammalian β-D-endoglycosidase capable of cleaving the heparan sulfate chains of HSPGs12–14. HPSE is first synthesized as a latent precursor protein of ~65 kDa and then cleaved into two subunits of ~8 and ~50 kDa that heterodimerize to form the enzymatically active protein13. HPSE is produced in large amounts by activated CD4+ T lymphocytes, neutrophils, monocytes and B lymphocytes15–17. However, the exact contribution of HPSE in mediating the tumor infiltration of in vitro–cultured, tumor-specific T cells remains unexplored.

We first assessed whether ex vivo–expanded human T cells are defective in their capacity to degrade the ECM. Using a Matrigel-based cell invasion assay, we compared the invasion capacity of freshly isolated resting T cells (FI-T cells), briefly activated T cells (BA-T cells; 24 h of activation with CD3-specific (OKT3) and CD28-specific antibodies) and long-term ex vivo–expanded T cells (LTE-T cells; activation with OKT3 and CD28-specific antibodies and ex vivo culture for 12–14 d). Consistent with previously reported data in rodents12, BA-T cells showed superior invasion of the ECM compared to FI-T cells (34% ± 8% vs. 23% ± 8%, respectively; P = 0.05). Conversely, LTE-T cells had a significantly reduced ability to degrade ECM (8% ± 6%) compared to both BA-T (P = 0.01) and FI-T (P = 0.022) cells (Fig. 1a). To dissect the mechanisms responsible for this observation, we evaluated the expression and function of HPSE in each cell population. In accordance with the cell invasion assay, both CD4+ and CD8+ T cells from FI-T and BA-T cells retained the active form of HPSE (50 kDa), whereas the enzyme was not detectable by western blotting and immunofluorescence in LTE-T cells (Fig. 1b,c). The loss of HPSE expression in LTE-T cells was not determined by the culture medium or cytokines used for T cell growth, as we observed similar results using either human type AB serum or fetal bovine serum, and interleukin (IL)-2, IL-7 or IL-15 as T cell growth factors (Supplementary Fig. 1). We also found that the loss of HPSE expression in response to stimulation with OKT3 and anti-CD28 antibodies and cytokines is observed in naïve (CD45RA+) central memory T lymphocytes characterized by potent cytotoxic function1,4,7.

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Figure 1 LTE-T cells show reduced invasion of the ECM and loss of the enzyme HPSE. (a) ECM invasion assay of CD14+ monocytes (M), FI-T cells, BA-T cells and LTE-T cells. Monocytes freshly isolated from peripheral blood showed the highest capacity to degrade the ECM (63% ± 23%). BA-T cells showed superior invasion of ECM compared to FI-T cells (**P = 0.05) and LTE-T cells (**P = 0.01), while LTE-T cells also showed inferior invasion compared to FI-T cells (***P = 0.022); analysis of variance (ANOVA) followed by a log-rank (Mantel–Cox) test for multiple comparisons. Data summarize means ± s.d. of five donors. We compared all four cell subsets for each donor. (b) Western blot showing the expression of HPSE in monocytes (M), CD4+ and CD8+ T cells at different culture time points. Data are representative of four donors. Positive controls are HPSE-transfected 293T cells. (c) Immunofluorescence staining for HPSE in MCF-7 cells, M, FI-T cells and LTE-T cells. Nuclei are stained with DAPI and shown in blue, whereas HPSE is stained with red fluorescent dye (Alexa Fluor 555). Magnification is 20×; scale bars, 50 μm. (d) qRT-PCR of HPSE in CD4+ and CD8+ T cells at different culture time points. Data summarize means ± s.d. of four donors. (e) HPSE enzymatic activity assessed in supernatants collected from CD4+ and CD8+ T cells at different culture time points. Monocytes and tumor cell lines CHLA-255, A549 and DU-145 are positive controls. Data summarize means ± s.d. of five donors. We compared all four cell subsets for each donor. (f) Western blot showing the expression of HPSE and full-length p53 protein in T cells before (t = 0) and after activation with immobilized OKT3 and CD28-specific antibodies for 18 and 72 h. Shown are results from one representative of three donors. (g,h) p53 ChIP in LTE-T cells cultured for 14 d (g), and in CD45RA+ cells before (t = 0) and after stimulation with OKT3 and CD28-specific monoclonal antibodies (t = 72 h) (h). Input is DNA that has been sonicated but not immunoprecipitated; IgG and p53 are DNA immunoprecipitated by the isotype and p53-specific antibody that recognizes the full-length protein. Relative quantification was performed comparing the intensities of PCR bands of IgG and p53 to input PCR band. For this representative sample, relative quantifications are: IgG, 20% and p53, 90% for LTE-T cells (g); IgG, 2% and p53, 4% at t = 0 and IgG, 53% and p53, 100% at t = 72 h for CD45RA+ cells (h). Shown is one representative of three donors.

(4CD4RO*CD62L+) and effector memory (CD45RO*CD62L−) T cells isolated from the peripheral blood, suggesting that this is a general phenomenon and that it is not T cell subset specific (Supplementary Fig. 2). The absence of HPSE protein in LTE-T cells was associated with the downregulation of the HPSE mRNA. As shown in Figure 1d, HPSE mRNA decreased immediately after activation in both CD4+ and CD8+ T cells compared to CD14+ monocytes (P < 0.005 and P < 0.031, respectively) and remained low over the subsequent 14 d of culture. Re-stimulation of LTE-T cells with OKT3 and anti-CD28 antibodies on day 14 of culture did not induce re-expression of either the HPSE mRNA or protein (Fig. 1d). The lack of cellular HPSE in LTE-T cells was also confirmed by the absence of enzymatic activity in the culture supernatant. As shown in Figure 1e, HPSE enzymatic activity was detected in supernatants collected within the first 72 h after activation of FI-T cells. This detection can be attributed to enzyme accumulation in the culture medium. However, the enzymatic activity returned to background levels 72 h later (from 0.34 ± 0.2 U ml and 0.45 ± 0.27 U ml to 0.22 ± 0.06 U ml) for both CD4+ and CD8+ T cells (Fig. 1e). This observation is in line with previous studies reporting that preformed HPSE protein is stored in an intracellular compartment and released as an early event in response to T cell activation18. We found that HPSE is also absent in Epstein–Barr virus–specific cytotoxic T cells that are stimulated in vitro by antigen-presenting cells, suggesting that HPSE loss in LTE-T cells is not caused by a supra-physiological activation of these cells mediated by OKT3 antibody19 (Supplementary Fig. 2). Previous studies showed that mutated p53 with loss of function in tumor cells is associated with overexpression of HPSE20. Because there is an accumulation of full-length p53 in LTE-T cells20,21, we suggest that the lack of HPSE mRNA expression in LTE-T cells may be due to the accumulation of full-length p53 in LTE-T cells, which binds to the HPSE gene promoter (Fig. 1f–h and Supplementary Fig. 3). The immediate transnational implication of these findings is that T cells that are engineered in vitro and cultured for adoptive immunotherapy lack HPSE expression when infused into subjects, and they are thus impaired in their capacity to degrade components of the ECM of the stroma. It is also important to note that the cleavage of HPSG chains by HPSE releases preformed stored chemokines into the stroma22,23. Because chemokines also guide the migration of T cells toward their target cells within the tumor microenvironment, the lack of HPSE may further indirectly compromise the antitumor effects of T cells by reducing their migration.

We thus hypothesized that engineering LTE-T cells to express HPSE through retroviral gene transfer would improve their invasion capability. LTE-T cells transduced with the HPSE(I)GFP retroviral vector expressed GFP (Fig. 2a) and HPSE (Fig. 2b). HPSE expression
LTE-T cells modified to express HPSE show enhanced degradation of the ECM.

We transduced LTE-T cells with retroviral vectors encoding HPSE and GFP (HPSE(I)GFP) or both the GD2-specific CAR and HPSE (CAR(I)HPSE). (a) GFP expression by both CD4+ and CD8+ LTE-T cells at days 12–14 of culture. (b) Western blot showing the expression of HPSE in control and transduced LTE-T cells at days 12–14 of culture. (c) qRT-PCR of HPSE in control and CAR(I)HPSE+ LTE-T cells cultured in IL-2 deficient medium for 3, 7 and 10 d. Data are representative of two donors. (d) HPSE(I)GFP+ LTE-T cells were sorted based on GFP expression. After sorting we used the ECM invasion assay to compare control, nonsorted HPSE(I)GFP+ LTE-T cells and GFP-sorted HPSE(I)GFP+ LTE-T cells. Data summarize mean ± s.d. of five donors, **P < 0.001. (e) ECM invasion assay of HPSE-transduced LTE-T cells in the presence or absence of the inhibitor heparin H1. Data summarize mean ± s.d. of four donors, ***P < 0.01; Student’s t-test.

remained high in transduced LTE-T cells starved of cytokines in culture for more than 10 d, suggesting stable transgene expression (Fig. 2c). In functional assays, HPSE(I)GFP+ LTE-T cells better degraded ECM (48% ± 19%) than did control untransduced LTE-T cells (29% ± 18%; P = 0.025) (Fig. 2d). The addition of the HPSE inhibitor heparin H1 (ref. 24) confirmed that the invasion of HPSE(I)GFP+...
LTE-T cells is HPSE-specific, as invasion was reduced from 74% ± 14% in the absence of inhibitor to 29% ± 9% (P < 0.01) in the presence of heparin H1 (Fig. 2e). We then assessed whether HPSE expression leading to improved cell invasion could be coupled with antitumor specificity. We used neuroblastoma (NB) as a cancer model because this tumor type has been targeted in a clinical trial with a CAR specific to the NB-associated antigen GD2 with some clinical responses. On day 14 of culture, CAR expression was 71% ± 14% and 56% ± 6% in LTE-T cells transduced with CAR and CAR(I)HPSE vectors, respectively (Fig. 3a). HPSE was detected only in LTE-T cells transduced with the CAR(I)HPSE vector (Fig. 3b). Both CAR+ and CAR(I)HPSE+ LTE-T cells lysed the GD2+ human NB cell line LAN-1 (71% ± 22% and 41% ± 16%, respectively, at a 20:1 effector-to-target cell (E:T) ratio in a 51Cr-release assay (P = nonsignificant (ns)), and the GD2+ human NB cell line CHLA-255 (76% ± 7% and 55% ± 13%, respectively (P = ns), whereas both CAR+ and CAR(I)HPSE+ LTE-T cells showed negligible activity against the GD2+ Raji cell line (<8%) (Fig. 3c). Control LTE-T cells lacking the CAR lysed none of these targets. The cytolytic activity was associated with a preserved T171 cytokine profile, as CAR+ and CAR(I)HPSE+ LTE-T released similar amounts of interferon (IFN)-γ (927 ± 328 and 527 ± 320 pg/ml × 10⁶ cells, respectively; P = ns) and IL-2 (83 ± 6 and 61 ± 27 pg/ml × 10⁶ cells, respectively, P = ns) in response to stimulation with GD2+ tumor cells (Fig. 3d). In sharp contrast to their comparable cytolytic function, only CAR(I)HPSE+ LTE-T cells degraded the ECM (66% ± 1%) compared to CAR+ or control LTE-T cells (13% ± 9% and 16% ± 10%, respectively (P = 0.004 and P < 0.001) (Fig. 3e). To prove ex vivo that LTE-T cells co-expressing HPSE and CAR have increased antitumor activity in the presence of ECM, we plated LTE-T and tumor cells in a Matrigel cell invasion assay, in which LTE-T cells must degrade the ECM to reach and eliminate the tumor targets. After 3 d of culture, both CAR+ and CAR(I)HPSE+ LTE-T cells eliminated LAN-1 tumor cells equally well in the absence of the ECM (<3% GFP+ tumor cells) compared to control LTE-T cells (31% ± 6% GFP+ tumor cells) (Fig. 3f,g). By contrast, in the presence of ECM, CAR(I)HPSE+ LTE-T cells eliminated all but 16% ± 8% of LAN-1 cells, compared to 37% ± 12% in the presence of CAR+ LTE-T cells (P = 0.001) (Fig. 3f,g). Control LTE-T cells did not show antitumor activity (45% ± 9% GFP+ tumor cells). We obtained identical results with the CHLA-255 human NB cell line (Supplementary Fig. 4). Thus, only LTE-T cells co-expressing HPSE and CAR show robust antitumor activity in the presence of the ECM. The improved antitumor activity of CAR(I)HPSE+ LTE-T cells was achieved without causing detectable detrimental effects on T lymphocytes. Indeed, CAR(I)HPSE+ LTE-T cells expanded in vitro, retained the same phenotype of CAR+ LTE-T cells and did not show increased activation-induced cell death in response to either OKT3-specific antibody, which binds CD3, or 1A7 anti-idiotypic antibody, which causes cross-linking of the GD2-specific CAR (Supplementary Fig. 5).

To validate our findings in vivo, we first established xenograft models of NB by implanting NSG-strain mice intraperitoneally (i.p.) with either CHLA-255 or LAN-1 NB cell lines in the presence of Matrigel to allow the formation of complex and structured tumors. We used the i.p. route to minimize confounding variables related to T cell homing to the tumor, a known issue in NB xenograft models when the tumor is inoculated subcutaneously. After 10 d, mice received
either control, CAR+ or CAR(I)HPSE+ LTE-T cells i.p. As shown in Figure 4a, mice implanted with CHLA-255 tumor cells and treated with CAR(I)HPSE+ LTE-T cells had significantly improved survival by day 40 compared to mice treated with control LTE-T (P < 0.001) or CAR+ LTE-T (P < 0.007) cells. Among treated mice, we found that 6 of 22 infused with CAR+ LTE-T cells, and 18 of 26 infused with CAR(I)HPSE+ LTE-T cells were macroscopically tumor free when we ended the observation at day 40 (P = 0.008). We obtained similar results in mice engrafted with LAN-1 tumor cells. Mice infused with CAR(I)HPSE+ LTE-T cells had significantly improved survival compared to mice treated with control (P < 0.0001) or CAR+ LTE-T cells at day 50 (Fig. 4b and Supplementary Fig. 6). In another set of experiments, we euthanized mice on day 12–14 after T cell infusion to measure tumor T cell infiltration. Tumors collected from mice infused with CAR(I)HPSE+ LTE-T cells had greater T cell infiltration (4.6% ± 2.4%) compared to those treated with control (0.6% ± 0.5; P = 0.029) or CAR+ LTE-T cells (0.1% ± 0.1; P = 0.043) (Fig. 4c). Tumors collected from euthanized mice also showed a significant reduction in weight in recipients infused with CAR(I)HPSE+ LTE-T cells compared to control (0.8 g ± 0.6 g vs. 3.3 g ± 2.4 g) (P = 0.039), and when compared to mice infused with CAR+ LTE-T cells (0.8 g ± 0.6 g vs. 2.5 g ± 2 g), although this difference was not statistically significant (P = 0.093) (Fig. 4d). Because NB cell lines require Matrigel to form complex and structured tumors when infused i.p., we also implemented a third NB model in which CHLA-255 tumor cells labeled with firefly luciferase were implanted in the kidney of Nod scid gamma (NSG) mice without using Matrigel26, and 255 tumor cells labeled with firefly luciferase were implanted in the kidney of Nod scid gamma (NSG) mice without using Matrigel26, and infused them via tail injection. T cell biodistribution evaluated under physiological conditions, HPSE expression by T cells preferential accumulation in lung or liver of CAR+ LTE-T and CAR(I)HPSE+ LTE-T cells were infused intravenously (i.v.). Tumor sections from mice infused i.v. with CAR(I)HPSE+ LTE-T cells showed enhanced infiltration of CD3+ T cells compared to CAR+ LTE-T cells (357 ± 72 and 173 ± 32 cells, respectively; P = 0.028) (Fig. 4e–g). Long-term observation of infused mice also showed improved survival of recipients treated with CAR(I)HPSE+ LTE-T cells by day 50 (P < 0.005) (Fig. 4h and Supplementary Fig. 6).

Finally, we extended our observation to CAR+ LTE-T cells targeting the solid tumor-associated antigen chondroitin sulfate proteoglycan 4 (CSPG4)27 in an aggressive melanoma model, suggesting that the positive effect of HPSE in CAR-T cells can be extrapolated to other targeted antigens and solid tumors (Supplementary Fig. 7). In contrast, the co-expression of HPSE in LTE-T cells redirected with a CD19-specific CAR did not seem to have a role in B-lymphoid malignancies, which are generally stroma-poor compared to solid tumors (Supplementary Fig. 8).

Under physiological conditions, HPSE expression by T cells is regulated to avoid tissue damage from T cell extravasation into nonpathologic tissues16,17,28,29. To rule out concerns about non-specific infiltration of normal tissues, such as lung or liver, by HPSE-engineered LTE-T cells, we evaluated in vivo T cell biodistribution. For these experiments, we labeled CAR(I)HPSE+ and CAR+ LTE-T cells with the vector encoding GFP and firefly luciferase and infused them via tail injection. T cell biodistribution evaluated by in vivo imaging at different time points after T cell inoculation and immunohistochemical analysis at early and late passages did not show differences between the two groups of mice, suggesting no preferential accumulation in lung or liver of HPSE-engineered LTE-T cells (Supplementary Fig. 9a,b).

In conclusion, HPSE deficiency in in vitro-engineered and cultured tumor-specific LTE-T cells may limit their antitumor activity in stroma-rich solid tumors. Other enzymes such as matrix metalloproteases (MMPs) are also involved in modifications of ECM components and may compensate for HPSE deficiency40. However, we found that some MMPs are also downregulated upon TCR activation and cytokine exposure (Supplementary Fig. 10a,b). We thus suggest that inducing expression of HPSE in LET-T cells co-expressing a tumor-specific CAR improves their capacity to degrade the ECM without compromising their viability, expansion or effector function, and it promotes increased antitumor activity. The proposed strategy may enhance the antitumor activity of CAR-redirected T cells in subjects with stroma-rich solid tumors.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

G.D., I.C. and B.S. designed experiments; I.C., V.H., G.W. and E.S.K. performed the experiments; I.C., B.S. and G.D. analyzed the data; I.C. and G.D. wrote the manuscript; H.L. performed the statistical analysis; M.M.I. performed the pathology; D.M. provided his expertise in the heparanase field and provided crucial reagents; all the authors reviewed and approved the final version of the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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14. Yurchenco, P.D. & Schittny, J.C. Molecular architecture of basement membranes. *FASEB J.* 4, 1577–1590 (1990).

15. Fridman, R. et al. Soluble antigen induces T lymphocytes to secrete an endoglycosidase that degrades the heparan sulfate moiety of subendothelial extracellular matrix. *J. Cell. Physiol.* 130, 85–92 (1987).

16. Naparstek, Y., Cohen, I.R., Fuxs, Z. & Vlodavsky, I. Activated T lymphocytes produce a matrix-degrading heparan sulphate endoglycosidase. *Nature* 310, 241–244 (1984).

17. Vlodavsky, I. et al. Expression of heparanase by platelets and circulating cells of the immune system: possible involvement in diapedesis and extravasation. *Invasion Metastasis* 12, 112–127 (1992).

18. Bartlett, M.R., Underwood, P.A. & Parish, C.R. Comparative analysis of the ability of leucocytes, endothelial cells and platelets to degrade the subendothelial basement membrane: evidence for cytokine dependence and detection of a novel sulfatase. *Immunol. Cell Biol.* 73, 113–124 (1995).

19. Smith, C.A. et al. Production of genetically modified Epstein-Barr virus-specific cytotoxic T cells for adoptive transfer to patients at high risk of EBV-associated lymphoproliferative disease. *J. Hematother.* 4, 73–79 (1995).

20. Baraz, L., Haupt, Y., Elkin, M., Peretz, T. & Vlodavsky, I. Tumor suppressor p53 regulates heparanase gene expression. *Oncogene* 25, 3939–3947 (2006).

21. Mondal, A.M. et al. p53 isoforms regulate aging- and tumor-associated replicative senescence in T lymphocytes. *J. Clin. Invest.* 123, 5247–5257 (2013).

22. Gallagher, J.T. Heparan sulfate: growth control with a restricted sequence menu. *J. Clin. Invest.* 108, 357–361 (2001).

23. Iozzo, R.V. Matrix proteoglycans: from molecular design to cellular function. *Annu. Rev. Biochem.* 67, 609–652 (1998).

24. Nakajima, M., Irimura, T., Di, F.N. & Nicolson, G.L. Metastatic melanoma cell heparanase. Characterization of heparan sulfate degradation fragments produced by B16 melanoma endoglucuronidase. *J. Biol. Chem.* 259, 2283–2290 (1984).

25. Craddock, J.A. et al. Enhanced tumor trafficking of GD2 chimeric antigen receptor T cells by expression of the chemokine receptor CCR2b. *J. Immunother.* 33, 780–788 (2010).

26. Patterson, D.M., Shohet, J.M. & Kim, E.S. Preclinical models of pediatric solid tumors (neuroblastoma) and their use in drug discovery. *Curr. Protoc. Pharmacol.* 52, 14.17.1–14.17.18 (2011).

27. Geldres, C. et al. T lymphocytes redirected against the chondroitin sulfate proteoglycan-4 control the growth of multiple solid tumors both *in vitro* and *in vivo*. *Clin. Cancer Res.* 20, 962–971 (2014).

28. Arvatz, G., Barash, U., Nativ, O., Ilan, N. & Vlodavsky, I. Post-transcriptional regulation of heparanase gene expression by a 3′ AU-rich element. *FASEB J.* 24, 4969–4976 (2010).

29. Lu, W.C., Liu, Y.N., Kang, B.B. & Chen, J.H. Trans-activation of heparanase promoter by ETS transcription factors. *Oncogene* 22, 919–923 (2003).

30. Wilson, T.J. & Singh, R.K. Proteases as modulators of tumor-stromal interaction: primary tumors to bone metastases. *Biochim. Biophys. Acta* 1785, 85–95 (2008).
**ONLINE METHODS**

**Cell lines.** 293T, DU-145 and CHLA-255 cell lines were cultured in IMDM ( Gibco, Invitrogen, Carlsbad, CA) supplemented with 10% FBS (FBS, HyClone, Thermo Scientific, Pittsburgh, PA) and 2 mM GlutaMax (Invitrogen, Carlsbad, CA). MCF-7, Raji, K562, LAN-1, Daudi and SENMA cells were cultured in RPMI1640 (HyClone) supplemented with 10% FBS and 2 mM GlutaMax. A549 cells were cultured in DMEM (GIBCO) supplemented with 10% FBS and 2 mM GlutaMax. Cells were maintained in a humidified atmosphere containing 5% CO₂ at 37 °C. Tumor cell lines MCF-7, CHLA-255, A549 and DU-145 produced HPSE. All cell lines were routinely tested for mycoplasma and for surface expression of target antigens. Furthermore, all cell lines were authenticated except for CHLA-255, which was established from a neuroblastoma subject and SENMA, which was established in our laboratory from a melanoma subject. However, we routinely verified that this line retained the surface expression of the target antigens.

**Isolation and culture of primary human T lymphocytes.** Peripheral blood mononuclear cells (PBMCs) were isolated from samples obtained from healthy volunteers from the protocol entitled ‘Humoral and cellular immune responses to tumor-associated antigens (TAA)—healthy blood and skin donors’ which is being conducted after approval by the Institutional Review Board of Baylor College of Medicine, or anonymous buffy coats (purchased as discarded material from the blood bank) of healthy donors (Gulf Coast Regional Blood Center, Houston, TX) using Lymphoprep density separation (Fresenius Kabi Norge, Oslo, Norway). Monocytes were obtained from PBMCs by positive magnetic separation with CD14 microbeads (Miltenyi Biotec, Auburn, CA). CD8⁺ and CD4⁺ T cells were also obtained from PBMCs by negative magnetic selection (Miltenyi Biotec). In selected experiments, naive (CD45RA⁺), central-memory (CD45RO⁺CD62L⁺) and effector-memory (CD45RO⁺CD62L⁻) T cells were also separated from PBMCs by negative magnetic selection (Miltenyi Biotec). T lymphocytes were activated with immobilized OKT3 (1 mg/ml) and anti-CD28 (no. 555725, Becton Dickinson Biosciences, Franklin Lakes, NJ) (1 μg/ml) antibodies and then expanded in complete medium containing 45% RPMI 1640 and 45% Glick’s medium (Irvine Scientific, Santa Ana, CA, USA) supplemented with 10% FBS and 2 mM GlutaMax. Cells were fed twice a week with recombinant interleukin-2 (IL-2) (50 U/ml) (Chiron Therapeutics, Emeryville, CA). We defined as FI-T cells: freshly isolated resting T cells from peripheral blood that comprise naive, effector-memory and central-memory T cells; BA-T cells: briefly activated T cells that result from incubation of FI-T with OKT3 and CD28-specific antibodies for 24 h; LTE-T cells: long-term ex vivo expanded T cells that result from BA-T cultured ex vivo for 12–14 d and consist mostly of central-memory and effector-memory T cells. At day 14 of culture, LTE-T cells were reactivated with OKT3 and CD28-specific antibodies and cultured for additional 24 h. For the experiments in which we compared the invasion capacity of FI-T versus BA-T versus LTE-T cells side by side, we obtained three separated blood draws at different time points from the donors to make LTE-T, BA-T and FI-T cells to be able to run all the samples in parallel in the invasion assay.

**Cell invasion assay.** The capacity of each cell subset to degrade ECM was examined in vitro using the BioCoat Matrigel Invasion assay (Becton Dickinson Biosciences) according to the manufacturer’s instructions. Five percent FBS was used as a chemoattractant in the low chamber. All experiments were performed in duplicate. Data are expressed as the percentage of invasion through the Matrigel and the membrane relative to the migration through the control membrane (8 μm polyethylene terephthalate membrane pores). The percentage of invasion was calculated as follows: (mean of cells invading through the Matrigel chamber membrane/mean of cells migrating through the control insert membrane) × 100. In specific experiments, we simultaneously evaluated the invasion and antimigratory activity of LTE-T cells. Briefly, we used the BioCoat Matrigel Invasion assay and plated LAN-1-GFP⁺ or CHLA-255-GFP⁺ cells (1.4 × 10⁵) in the bottom of a 24-well plate and LTE-T cells (2.5 × 10⁵ cells) in the upper chamber/insert. Chambers and inserts were removed 24 h later. After 3 d of culture cells were then collected from the lower chamber and quantified by flow cytometry to identify tumor cells and T cells, respectively.

**Western blotting.** 20 μg of proteins were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). The antibodies and dilutions used in these experiments were as follows: mouse anti-human HPAl–HPSE (1:100 and 1:6500 dilution, clone nos. HP130 and HP3-17, respectively) (InSight Biopharmaceuticals Ltd., Rehovot, Israel) that recognizes both the 65-kDa precursor and the 50-kDa active form of HPSE-1, rabbit anti-human HPAl polyclonal (1:4000 dilution) (no. CLANT155, Cedarlane, Burlington, NC), mouse anti-human p53 (1:200 dilution, clone DO-1) (Santa Cruz Biotechnology, Santa Cruz, CA) that recognizes the full-length p53 protein, mouse anti-human β-actin (1:10000 dilution, clone C4) (Santa Cruz Biotechnology) and horseradish peroxidase–conjugated secondary antibodies (1:5000, goat anti-mouse no. sc-2005 and goat anti-rabbit no. sc-2004) (Santa Cruz Biotechnology). Blots were then incubated with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific).

**Immunofluorescence.** Cells were fixed with 4% paraformaldehyde. After permeabilization with 0.1% Triton X-100, cells were incubated with 5% goat serum (Cell Signaling Technology, Danvers, MA) and 1% BSA to block non-specific binding. Cells were then stained with the primary antibody against human HPSE1 (HPAl, clone HP130) (InSight Biopharmaceuticals Ltd.) (1:100 dilution at 25 °C for 2 h). Cells were then probed with Alexa Flour 550 goat anti-mouse secondary antibody (no. 44095, 1:500 dilution at 25 °C for 2 h) (Cell Signaling Technology). Fluorescent signals were detected using a fluorescence microscope (Olympus IX70, Leeds Instruments Inc., Irving, TX). DAPI (BiLegand, San Diego, CA) was used for nuclear staining.

**Cell invasion assay.** The capacity of each cell subset to degrade ECM was examined in vitro using the BioCoat Matrigel Invasion assay (Becton Dickinson Biosciences) according to the manufacturer’s instructions. Five percent FBS was used as a chemoattractant in the low chamber. All experiments were performed in duplicate. Data are expressed as the percentage of invasion through the Matrigel and the membrane relative to the migration through the control membrane (8 μm polyethylene terephthalate membrane pores). The percentage of invasion was calculated as follows: (mean of cells invading through the Matrigel chamber membrane/mean of cells migrating through the control insert membrane) × 100. In specific experiments, we simultaneously evaluated the invasion and antimigratory activity of LTE-T cells. Briefly, we used the BioCoat Matrigel Invasion assay and plated LAN-1-GFP⁺ or CHLA-255-GFP⁺ cells (1.4 × 10⁵) in the bottom of a 24-well plate and LTE-T cells (2.5 × 10⁵ cells) in the upper chamber/insert. Chambers and inserts were removed 24 h later. After 3 d of culture cells were then collected from the lower chamber and quantified by flow cytometry to identify tumor cells and T cells, respectively.

**Enzyme-linked immunosorbent assay (ELISA).** Cytokine release by LTE-T cells in response to stimulation with GD2⁺ LAN-1 cells was analyzed using IFN-γ and IL-2–specific ELISAs (R&D Systems, Minneapolis, MN). HPSE activity was measured using a heparan sulfate (HS) degrading enzyme assay (Cruz Biotechnology, Santa Cruz, CA) that recognizes the full-length β-actin (1:10000 dilution, clone C4) (Santa Cruz Biotechnology) and horseradish peroxidase–conjugated secondary antibodies (1:5000, goat anti-mouse no. sc-2005 and goat anti-rabbit no. sc-2004) (Santa Cruz Biotechnology). Blots were then incubated with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific).

**RNA isolation and quantitative real-time PCR (qRT-PCR).** For the qRT-PCR, 100 ng of total RNA was used to prepare cDNA (TaqMan One Step PCR Master Mix Reagents Kit) (Applied Biosystems, Carlsbad, CA). Specific primers and probes that were designed, tested, and standardized by Applied Biosystems for HPSE and p53 were used (HPSE: Hs00935036_m1; p53: Hs01034249_m1). The difference in cycle threshold values (ΔCT) of HPSE was normalized to the ΔCT of GAPDH (glyceraldehyde-3-phosphate dehydrogenase, Hs99999905_m1), and the fold change in expression was expressed relative to CD14⁺ monocytes, considered as a positive control while mesenchymal stem cells were used as a negative control. For p53 mRNA quantification the difference in cycle threshold values (ΔCt) of p53 was normalized to the ΔCt of GAPDH (glyceraldehyde-3-phosphate dehydrogenase, Hs99999905_m1), and the fold change in expression was expressed relative to FI-T cells.

**Multiplex for matrix metalloproteases (MMPs).** Analysis of MMPs was performed using Milliplex Map kit panel 1 and 2 (Millipore) according to the manufacturer’s instructions. In particular, FI-T cells were activated with OKT3 and anti-CD28 mAbs in absence of IL-2 (50 U/ml or 2000 U/ml) or IL-7 and IL-15 (10 ng/ml and 5 ng/ml, respectively). T cells were fed twice a week. At days 3, 7 and 10 of culture, supernatants and T cells were collected and 60 μg of sample were tested per well.

**p53 chromatin immunoprecipitation (ChiP) assay.** LTE-T cells, CD45RA⁺ and CD45RO⁺ T cells were collected and fixed with formaldehyde (Merck, Darmstadt, Germany) to a final concentration of 1%. Fixation proceeded at room temperature for 10 min and was stopped by the addition of glycine to a final concentration of 0.125 mol/l. The cells were then washed twice...
with cold PBS. Pellets were resuspended in 350 µl of lysis buffer and protease inhibitors mixture (Active Motif, Carlsbad, CA), washed and resuspended in 350 µl of optimized ChIP lysis buffer and protease inhibitors mixture (0.3% SDS, 10mM EDTA, 0.5 mM EGTA and 50 mM Tris-HCl pH 8) and sonicated into chromatin fragments of an average length of 500 bp, as determined empirically by agarose gel electrophoresis of fragmented chromatin samples. Chromatin was kept at ~80 °C. The chromatin solution was incubated with a pS3-specific antibody that recognizes the full-length human protein (no. FL393, Santa Cruz) at 4 °C overnight with rotation. The immunoprecipitation was performed using Chip-IT Express kit (Active Motif, Carlsbad, CA) according to the manufacturer’s instructions. Amplifications (37 cycles) were performed using specific primers (Supplementary Fig. 11), yielding PCR products ~200 bp in length (location of primers relatively to the origin of the promoter is indicated in parentheses after each primer pair). PCR products were separated by 1.5% agarose electrophoresis in Tris-borate-EDTA buffer and stained with ethidium bromide.

Retroviral constructs, transient transfection and transduction of T lymphocytes. HPSE cDNA (accession number NM_000666) was cloned into the SFG retroviral backbone that also encodes the GFP (SFG.HPSE[1GFp] (Supplementary Fig. 12). The construct for the GD2-specific CAR containing the CD28, OX40 and ζ endodomains was previously described (SFG.CAR)34. We then generated a bicistronic vector to co-express the HPSE and CAR-GD2 using an IRES (SFG.CAR(H)HPSE) (Supplementary Fig. 12). The retroviral vector encoding the fusion protein GFP–firefly luciferase (GFP.FFluc) for in vivo imaging of T cells and CD19-specific and CSPG4-specific CARs were previously described.35 Transient retroviral supernatant was produced as previously described. A specific inhibitor of HPSE, Ronestarpat (ST00001) (a chemically modified heparin 10KNa-Ro, H, property of Sigma-tau Research Switzerland S.A.) (3 µg ml⁻¹)36,37, was added to the media during the virus preparation to increase its titer. Activated T lymphocytes were then transduced with retroviral supernatants using retroenkein-coated plates (Takara Bio Inc., Shiga, Japan). After removal from the retroenkein plates, T cell lines were maintained in complete T cell medium in a humidified atmosphere containing 5% CO₂ at 37 °C in the presence of IL-2 (50 U ml⁻¹) for 2 weeks.

Flow cytometry. We performed flow cytometry analysis using the following antibodies: antibodies specific to CD45, CD56, CD8, CD4, CD3, CD45RA, CD45RO, CD62L, 7AAD and annexin V (all from Becton Dickinson, San Jose, CA) and CCR7 (from E&D) conjugated with FITC, PE, PerCP or APC fluorochromes. Expression of GD2 and CSPG4 antigens on tumor cell lines was assessed with anti-GD2 (clone 14.g2a, BD) and anti-CSPG4 (clone no. 1E6.4, Miltenyi-Biotec), respectively. The expression of GD2-specific CAR was detected using a specific anti-idiotype antibody (1A7). Samples were analyzed with a BD FACScalibur system equipped with a filter set for quadruple fluorescence signals and the CellQuest software (BD Biosciences). For each sample we analyzed a minimum of 10,000 events.

Chromium-release assay. The cytotoxic activity of T cells was evaluated using a standard 6-h ⁵¹Cr-release assay.

Xenogenic mouse models. We used the NSG mouse model to assess the in vivo antitumor effect of control and transduced T cells. All mouse experiments were approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine. 8–10-week-old male and female NSG mice (Jackson Laboratory, Bar Harbor, Maine) were injected i.p. with either CHLA-255 or LAN-1 neuroblastoma cells (GD2⁺) (2.5 × 10⁶) or SENMA melanoma cells (CSPG4⁺) (5 × 10⁴) resuspended in Matrigel (BD Biosciences). These tumor cell lines were labeled with Firefly luciferase. 10–12 d after neuroblastoma inoculation and 2 d after melanoma inoculation, LTE-T cells were injected i.p. (2 × 10⁶ cells per mouse). No randomization was used. Investigators were not blinded, but mice were matched based on the signal of tumor cells before assignment to control or treatment groups. Mice were euthanized when signs of discomfort were detected by the investigator or as recommended by the veterinarian who monitored the mice three times a week. When valuable, tumor growth was also monitored by bioluminescence. For the in vivo biodistribution of T cells, 5 × 10⁶ LTE-T cells per mouse labeled with the GFP, FFluc vector were infused via tail injection. For in vivo imaging, we used the Xenogen-IVIS Imaging System as previously described.31 In the orthotopic model32, an inoculum of 10⁸ CHLA-255 luciferase-transduced tumor cells suspended in 0.1 ml of PBS was surgically implanted under the renal capsule of 5–7-week-old female mice using a 27-gauge needle. CAR⁺ and CAR(H)HPSE⁻ LTE-T cells were infused i.v. (1.5 × 10⁸ per mouse) 7 d later, and tumor regression was measured by bioluminescence imaging. By day 10, some mice were sacrificed and tumors collected, fixed and stained with anti-human CD3 mAb (no. A0452, Dako North America Inc., Carpinteria, CA). Mice were euthanized when signs of discomfort were detected by the investigator or as recommended by the veterinarian who monitored the mice three times a week.

Tissue processing and immunohistochemistry. Tissue samples were fixed, processed and stained according to standard procedures. We performed H&E staining and labeling of human T cells using polyclonal rabbit anti-human CD3 mAb (A0452, Dako North America Inc., Carpinteria, CA) and for detection we used Dako LSAB + System-HRP (K0679, Dako). Tumors were scored without knowledge of the treatment used by the pathologist (M. Ittmann) by counting human infiltrating T lymphocytes in ten high-power fields at the edge of the tumor.

Statistical analyses. Unless otherwise noted, data are summarized as mean ± s.d. Student’s t-test (two-sided) was used to determine statistically significant differences between samples, with P < 0.05 indicating a significant difference. When multiple comparison analyses were required, statistical significance was evaluated by a repeated-measures ANOVA followed by a log-rank (Mantel–Cox) test for multiple comparisons. The mouse survival data were analyzed using the Kaplan–Meier survival curve, and Fisher’s exact test was used to measure statistically significant differences. Animals were excluded only in the event of their death after tumor implant but before T cell infusion. Neither randomization nor blinding was done during the in vivo study. However, mice were matched based on the tumor signal for control and treatment groups before infusion of control or gene modified T cells. When bioluminescence signal intensity was used to monitor tumor growth, values were log transformed and then compared using a two-sample t-test. In contrast, the analysis of the pathologist M. Ittmann, aimed at quantifying tumor infiltration by human T cells, was performed in a blinded fashion. We estimated the sample size considering the variation and mean of the samples. We tried to reach a conclusion using as small a sample size as possible. We estimated the sample size to detect a difference in means of 2 s.d. at the 0.05 level of significance with 80% power. Graph generation and statistical analyses were performed using Prism version 5.0d software (GraphPad, La Jolla, CA).

31. Lyon, E. et al. Immunotherapy of metastatic melanoma using genetically engineered GD2-specific T cells. Clin. Cancer Res. 15, 5882–5886 (2009).
32. Roy, M. et al. Antisense-mediated suppression of Heparanase gene inhibits melanoma cell invasion. Neoplasia 7, 253–262 (2005).
33. Zhang, L., Sullivan, P., Suyama, J. & Marchetti, D. Epidermal growth factor-induced heparanase nuclear localization augments DNA topoisomerase I activity in brain metastatic breast cancer. Mol. Cancer Res. 8, 278–290 (2010).
34. Püle, M.A. et al. A chimeric T cell antigen receptor that augments cytokine release and supports clonal expansion of primary human T cells. Mol. Ther. 12, 933–941 (2005).
35. Vera, J. et al. T lymphocytes redirected against the kappa light chain of human immunoglobulin efficiently kill mature B lymphocyte-derived malignant cells. Blood 108, 3890–3897 (2006).
36. Naggii, A. et al. Modulation of the heparanase-inhibiting activity of heparin through selective desulfation, graded N-acetylation, and glycol splitting. J. Biol. Chem. 280, 12103–12113 (2005).
37. Zhang, L., Ngo, J.A., Wetzel, M.D. & Marchetti, D. Heparanase mediates a novel mechanism in laptatin-resistant brain metastatic breast cancer. Neoplasia 17, 101–113 (2015).