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Supplementary Materials for

Combined tumor-directed recruitment and protection from immune suppression enable CAR T cell efficacy in solid tumors

Bruno L. Cadilha*, Mohamed-Reda Benmebarek, Klara Dorman, Arman Oner, Theo Lorenzini, Hannah Obeck, Mira Vänttinen, Mauro Di Pilato, Jasper N. Pruessmann, Stefan Stoiber, Duc Huynh, Florian Märkl, Matthias Seifert, Katrin Manske, Javier Suarez-Gosalvez, Yi Zeng, Stefanie Lesch, Clara H. Karches, Constanze Heise, Adrian Gottschlich, Moritz Thomas, Carsten Marr, Jin Zhang, Dharmendra Pandey, Tobias Feuchtinger, Marion Subklewe, Thorsten R. Mempel, Stefan Endres, Sebastian Kobold*

*Corresponding author. Email: bcadilha@gmail.com (B.L.C.); sebastian.kobold@med.uni-muenchen.de (S.K.)

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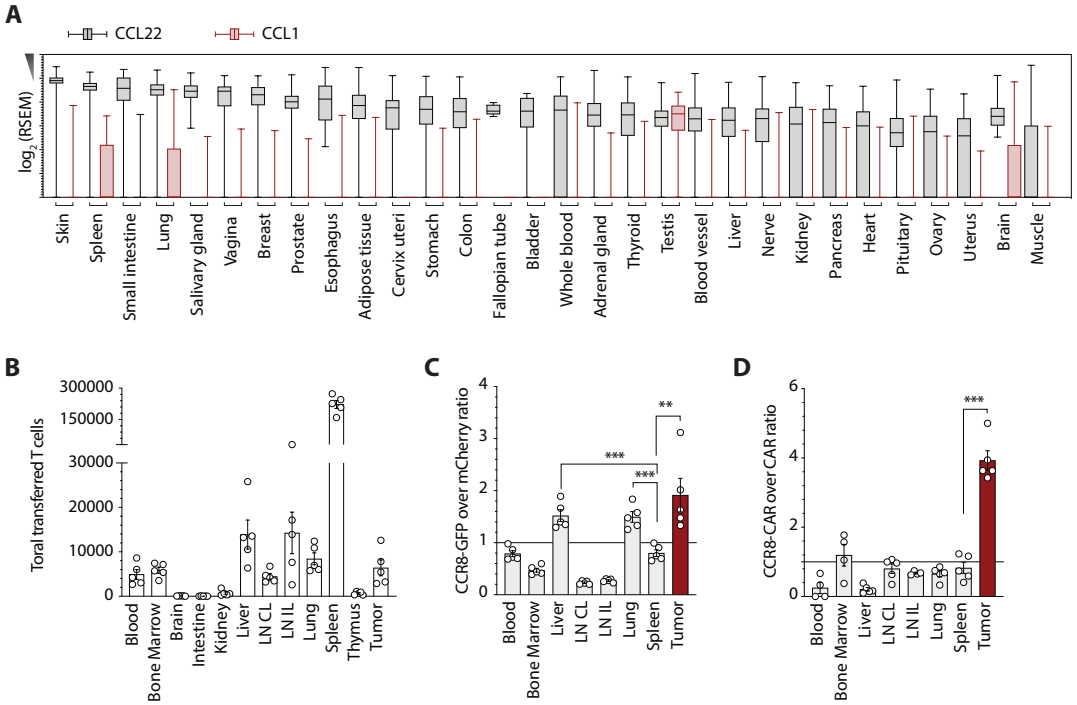
I Supplementary Figures

Supplementary figure S1: CCL22 and CCL1 expression in several healthy human tissues and CCR8-transduced T cell homing pattern in several murine tissues.

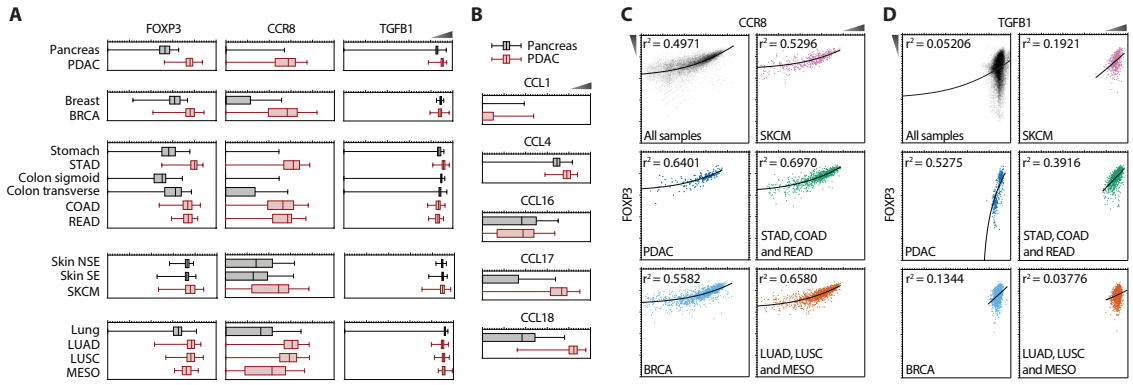


Supplementary figure S1: CCL22 and CCL1 expression in several healthy tissues and CCR8-transduced T cell homing pattern in several murine tissues.

(**A**) Boxplots comparing CCL22 and CCL1 gene expression levels in several healthy tissues. Samples analyzed for skin n = 557, spleen n = 101, small intestine n = 93, lung n = 289, salivary gland n = 56, vagina n = 86, breast n = 180, prostate n = 101, esophagus n = 557, adipose tissue n = 516, cervix uteri n = 11, stomach n = 175, colon n = 309, phallopian tube n = 6, bladder n = 10, whole blood n = 338, adrenal gland n = 129, thyroid n = 280, testis n = 166, blood vessel n = 557, liver n = 111, nerve n = 279, kidney n = 29, pancreas n = 168, heart n = 378, pituitary n = 108, ovary n = 89, uterus n = 79, brain n = 557 and muscle n = 397. Scales are depicted in a $\log_2$ scale (minimum 0.5 and maximum 16, major tick interval

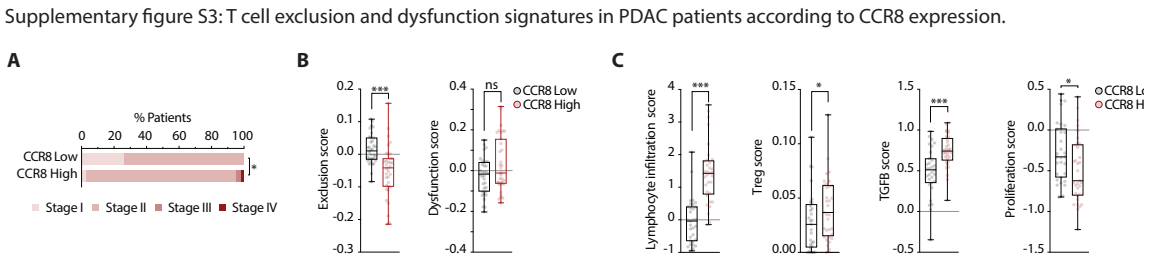
of 1 power of 2) and mRNA normalization was estimated by the TCGA using the RSEM (RNA-seq by expectation maximization) method. (**B** to **D**) Flow cytometry ACT tracking experiments with a similar layout to the one described in Figure 2I. n = 5 mice. (**B** and **C**) Panc02-tumor bearing mice treated with a mixed ratio of CCR8-GFP or mCherry-transduced T cells. n = 5 mice. (**D**) Panc02-EpCAM-tumor bearing mice treated with a mixed ratio of CCR8-CAR- or CAR -ransduced T cells. n = 5 mice.

Supplementary figure S2: FOXP3, CCR8 and TGFB1 genes are upregulated in pancreatic adenocarcinomas and other cancer types.



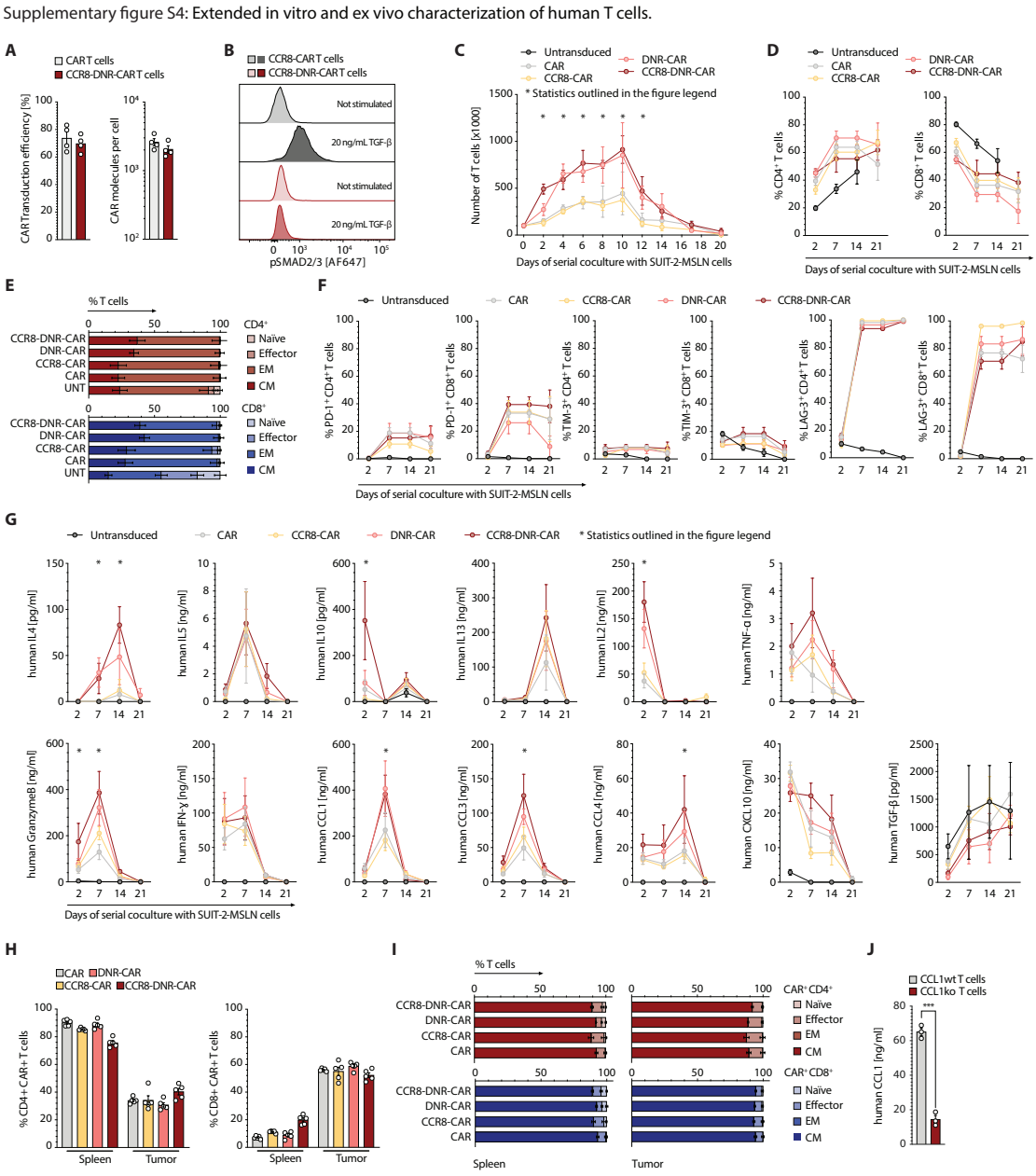
Supplementary figure S2: FOXP3, CCR8 and TGFB1 genes are upregulated in pancreatic adenocarcinomas and other cancer types.

(**A** and **B**) Boxplots comparing FOXP3, CCR8 (and its ligands) and TGFB1 gene expression levels in various cancers to corresponding healthy tissue in various cancers. (**C** and **D**) Correlation of CCR8 and FOXP3 and correlation of TGFB1 and FOXP3, respectively, in different tissues. r^2 was used to evaluate the fit estimated by the Pearson's squared method. PDAC pancreatic adenocarcinoma, BRCA breast invasive carcinoma, STAC stomach adenocarcinoma, COAD colon adenocarcinoma, READ rectum adenocarcinoma, NSE not-sun exposed, SE sun exposed, SKCM skin cutaneous melanoma, LUAD lung adenocarcinoma, LUSC lung squamous cell carcinoma, MESO mesothelioma. Samples analyzed for pancreas tissue n = 167, PDAC n = 183, breast mammary tissue n = 179, BRCA n = 1212, stomach n = 175, STAC n = 450, colon sigmoid n = 141, colon transverse n = 167, COAD n = 331, READ n = 103, skin NSE n = 233, skin SE n = 324, SKCM n = 470, lung n = 288, LUAD n = 574, LUSC n = 548 and MESO n = 87. All scales are depicted in a $\log_2$ scale (minimum 0.5 and maximum 16, major tick interval of 1 power of 2) and mRNA normalization was estimated by the TCGA using the RSEM (RNA-seq by expectation maximization) method.



Supplementary figure S3: T cell exclusion and dysfunction signatures in PDAC patients according to CCR8 expression.

(**A**) PDAC staging correlation with CCR8-expression. (**B**) TIDE analysis of CCR8 expression in T cell exclusion and dysfunction signatures in PDAC patients. (**C**) Lymphocyte infiltration, T_{reg}, TGFB and Proliferation scores according to CCR8-expression in PDAC patients. PDAC patients from the TCGA database were included according to their CCR8 expression in a CCR8 High group (top 20th percentile) (n = 26) or in a CCR8 Low group (bottom 20th percentile) (n = 26). Statistical analysis for (**A**) was performed using a Chi-squared test, and two-tailored Mann-Whitney test for (**B** and **C**).



Supplementary figure S4: Extended *in vitro* and *ex vivo* characterization of human T cells.

(**A**) Transduction efficiency and surface expression of CAR in CAR- and CCR8-DNR-CAR-transduced T cells, as determined by flow cytometry and QIFIKIT (Agilent Technologies), respectively. (**B**) Phospho-specific flow cytometry of SMAD2/3. **(C** to **G)** Long-term coculture assay was carried out as described: 10^5 CAR-, CCR8-CAR-, DNR-CAR- or CCR8-

DNR-CAR-transduced T cells were cocultured with 2 x 10^4 SUIT-MSLN tumor cells. T cells were serially transferred to new tumor cells every 2 days. (**C**) An equal volume of T cells was collected at each T cell transfer to monitor T cell proliferation. Day 2 CCR8-DNR-CAR vs CCR8-CAR p = 0.028, CCR8-DNR-CAR vs CAR p = 0.045. Day 4 CCR8-DNR-CAR vs CCR8-CAR p = 0.047, DNR-CAR vs CCR8-CAR p = 0.045. Day 6 CCR8-DNR-CAR vs CCR8-CAR p = 0.011, CCR8-DNR-CAR vs CAR p = 0.007. Day 8 CCR8-DNR-CAR vs CCR8-CAR p = 0.004, DNR-CAR vs CCR8-CAR p = 0.024, CCR8-DNR-CAR vs CAR p = 0.012, DNR-CAR vs CAR p = 0.045. Day 10 CCR8-DNR-CAR vs CCR8-CAR p < 0.001, DNR-CAR vs CCR8-CAR p = 0.010, CCR8-DNR-CAR vs CAR p = 0.002, DNR-CAR vs CAR p = 0.042. Day 12 CCR8-DNR-CAR vs CCR8-CAR p = 0.036. n = 6 healthy donors. (**D**) Changes in the frequency of $CD4^+$ and $CD8^+$ T cells assessed by flow cytometry after 2, 7, 14 and 21 days of coculture. (**E**) Phenotypic characterization of T cell products using CD45RO and CCR7 expression, allowing for the differentiation of effector memory (EM), central memory (CM), effector or naïve T cells in after 2 days of coculture. (**F**) Frequency of expression of PD-1, TIM-3 and LAG-3 in $CD4^+$ and $CD8^+$ T cell populations after 2, 7, 14 and 21 days of coculture. (**G**) Secreted IL-4, IL-5, 1L-10, 1L-13, IL-2, TNF-α, granzyme B, IFN-ɣ, CCL1, CCL3, CCL4, CXCL10 and TGF-β cytokine concentrations determined by ELISA across different coculture conditions after 2, 7, 14 and 21 days of coculture. CCL1 on day 7 for CCR8-DNR-CAR vs CCR8-CAR p = 0.013, CCR8-DNR-CAR vs CAR p = 0.043, DNR-CAR vs CCR8-CAR p < 0.001, DNR-CAR vs CAR p = 0.007. CCL3 on day 7 for CCR8-DNR-CAR vs CCR8-CAR p < 0.001, CCR8-DNR-CAR vs CAR p < 0.001, DNR-CAR vs CAR p = 0.035. CCL4 on day 14 for CCR8-DNR-CAR vs CCR8-CAR p = 0.009, CCR8-DNR-CAR vs CAR p = 0.029. Granzyme B on day 7 CCR8-DNR-CAR vs CCR8-CAR p = 0.005, CCR8-DNR-CAR vs CAR p < 0.001, DNR-CAR vs CAR p = 0.006. IL-2 on day 2 CCR8-DNR-CAR vs DNR-CAR p = 0.028, CCR8-DNR-CAR vs CCR8-CAR p <

0.001, CCR8-DNR-CAR vs CAR $p < 0.001$, DNR-CAR vs CCR8-CAR $p < 0.001$, DNR-CAR vs CAR $p < 0.001$. IL-4 on day 14 CCR8-DNR-CAR vs CCR8-CAR $p < 0.001$, CCR8-DNR-CAR vs CAR $p < 0.001$, DNR-CAR vs CCR8-CAR $p = 0.026$, DNR-CAR vs CAR $p = 0.009$. IL-10 on day 2 CCR8-DNR-CAR vs DNR-CAR $p < 0.001$, CCR8-DNR-CAR vs CCR8-CAR $p < 0.001$, CCR8-DNR-CAR vs CAR $p < 0.001$. TGF-β concentrations was significantly increased over time for all conditions, $p = 0.001$. (**H** and **I**) Data for tumor and spleen infiltrating T cells after 19 days *in vivo,* from the experiment in Figure 5M. n = 5 mice per group. (**H**) Frequency of expression of $CD45^+CD3^+$ SUIT-2-Mesothelin-CCL1 infiltrating $CD4^+$ and $CD8^+$ T cells. (**I**) Phenotypic characterization using CD45RO and CCR7 expression, allowing for the differentiation of effector memory (EM), central memory (CM), effector or naïve T cells. (**J**) Technical validation of CCL1 knock-out from primary human T cells through CCL1 ELISA on supernatant of T cells activated for 24 hours with anti-CD3 and anti-CD28 antibodies. n = 3 healthy donors.

Supplementary movie 1: Multiphoton intra-vital microscopy tracking of tumor infiltrating CCR8-GFP T cells versus mCherry T cells.

ACT tracking experiment in a mouse implanted with a tumor within a dorsal skin-fold chamber to enable multi-photon intra-vital imaging.

II Methods for supplementary Figures

Data mining

We utilized the bioinformatics tool UCSC Xena to retrieve data from TGCA (The Cancer Genome Atlas). TCGA RNA sequencing datasets were analysed in comparison to GTex (Genotype-tissue Expression) healthy tissue reference datasets concerning the expression of multiple genes. Correlations were assessed through r^2, goodness of fit estimated by the Pearson's squared. mRNA normalization was estimated by the TCGA using the RSEM

(RNA-seq by expectation maximization) method. For Tumor Immune Dysfunction and Exclusion (TIDE) analysis on PDAC patients we used the TIDE tool with CCR8 as the query gene (http://tide.dfci.harvard.edu/query/) (*54*). PDAC datasets were stratified by bulk tumor expression levels of CCR8 in groups that included the 20% higher and 20% lower expression levels of CCR8. These high and low groups were then evaluated for evidence of T cell dysfunction and exclusion. Immune tumor microenvironment was analyzed using the iAtlas deconvolution tool (https://www.cri-iatlas.org/) (*55*) and datasets stratified as for TIDE analysis.

Long-term coculture assay

T cell and tumor cell cocultures were set-up at indicated effector to target ratios. T cells expressing either CAR, CCR8-CAR, DNR-CAR or CCR8-DNR-CAR were coincubated with SUIT-MSLN tumor cells, and serially transferred to newly plated SUIT-MSLN cells every two to three days. To assess the impact of regular antigen restimulation on transduced T cells, collected supernatant to assess the cytokine profile of the cells after 2, 7, 14 and 21 days of coculture. DuoSet ELISA kits were purchased from R&D to assess the cytokine concentrations of human TNF-α (DY210), CCL3 (DY270), CCL4 (DY271), CXCL10 (DY266), IL-2 (DY202), IL-4 (DY204), IL-5 (DY205), IL-10 (DY217B), IL-13(DY213), granzyme B (DY2906-05), and TGF-β (DY240). At the same time points, we used flow cytometry to assess T cell proliferation and phenotype. An equal volume of cells was stained using antibodies from Biolegend against human CD3 (HIT3a), CD4 (A161A1), CD8 (SK1), PD-1 (EH12.2H7), LAG-3 (11C3C65), TIM-3(F38-2E2), as well as the Miltenyi antibody against c-myc (SH1-26E7.1.3) to assess CAR expression. CountBright counting beads were used to assess and normalize T cell proliferation across conditions and over time.

Phospho-Flow of SMAD2/3

The antibody from BD against pSMAD2/3 (O72-670) was used to assess downstream signaling of TGF-β according to the manufacturers protocol.

Cell surface molecule quantification through quantitative analysis kit

Surface antigen density of constructs was evaluated through flow cytometry with QIFIKIT (Agilent) using a Biolegend antibody against c-Myc (9E10).

REFERENCES AND NOTES

1. M. Kalos, B. L. Levine, D. L. Porter, S. Katz, S. A. Grupp, A. Bagg, C. H. June, T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leukemia. *Sci. Transl. Med.* **3**, 95ra73 (2011).

2. S. L. Topalian, F. S. Hodi, J. R. Brahmer, S. N. Gettinger, D. C. Smith, D. F. McDermott, J. D. Powderly, R. D. Carvajal, J. A. Sosman, M. B. Atkins, P. D. Leming, D. R. Spigel, S. J. Antonia, L. Horn, C. G. Drake, D. M. Pardoll, L. Chen, W. H. Sharfman, R. A. Anders, J. M. Taube, T. L. McMiller, H. Xu, A. J. Korman, M. Jure-Kunkel, S. Agrawal, D. McDonald, G. D. Kollia, A. Gupta, J. M. Wigginton, M. Sznol, Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N. Engl. J. Med.* **366**, 2443–2454 (2012).

3. S. A. Roseberg, N. P. Restifo, Adoptive cell transfer as personalized immunotherapy for human cancer. *Science* **348**, 62–68 (2015).

4. M.-R. Benmebarek, H. C. Karches, L. B. Cadilha, S. Lesch, S. Endres, S. Kobold, Killing mechanisms of chimeric antigen receptor (CAR) T cells. *Int. J. Mol. Sci.* **20**, 1283 (2019).

5. J. H. Park, I. Rivière, M. Gonen, X. Wang, B. Sénéchal, K. J. Curran, C. Sauter, Y. Wang, B. Santomasso, E. Mead, M. Roshal, P. Maslak, M. Davila, R. J. Brentjens, M. Sadelain, Long-term follow-up of CD19 CAR therapy in acute lymphoblastic leukemia. *N. Engl. J. Med.* **378**, 449–459 (2018).

6. S. S. Neelapu, F. L. Locke, N. L. Bartlett, L. J. Lekakis, D. B. Miklos, C. A. Jacobson, I. Braunschweig, O. O. Oluwole, T. Siddiqi, Y. Lin, J. M. Timmerman, P. J. Stiff, J. W. Friedberg, I. W. Flinn, A. Goy, B. T. Hill, M. R. Smith, A. Deol, U. Farooq, P. McSweeney, J. Munoz, I. Avivi, J. E. Castro, J. R. Westin, J. C. Chavez, A. Ghobadi, K. V. Komanduri, R. Levy, E. D. Jacobsen, T. E. Witzig, P. Reagan, A. Bot, J. Rossi, L. Navale, Y. Jiang, J. Aycock, M. Elias, D. Chang, J. Wiezorek, W. Y. Go, Axicabtagene ciloleucel CAR T-cell therapy in refractory large B-cell lymphoma. *N. Engl. J. Med.* **377**, 2531–2544 (2017).

7. N. N. Shah, T. J. Fry, Mechanisms of resistance to CAR T cell therapy. *Nat. Rev. Clin. Oncol.* **16**, 372–385 (2019).

8. S. Stoiber, L. B. Cadilha, M.-R. Benmebarek, S. Lesch, S. Endres, S. Kobold, Limitations in the design of chimeric antigen receptors for cancer therapy. *Cell* **8**, 472 (2019).

9. P. M. Sullivan, L. Arguedas-Jimenez, A. Johnson, J. Yokoyama, Y. Yuzefpolskiy, M. Jensen, V. Kalia, S. Sarkar, A novel preclinical immunocompetent CAR T cell mouse model for solid tumors. *J. Immunol.* **202**, 71.16 (2019).

10. L. Zitvogel, J. M. Pitt, R. Daillère, M. J. Smyth, G. Kroemer, Mouse models in oncoimmunology. *Nat. Rev. Cancer* **16**, 759–773 (2016).

11. C. H. Karches, M.-R. Benmebarek, M. L. Schmidbauer, M. Kurzay, R. Klaus, M. Geiger, F. Rataj, B. L. Cadilha, S. Lesch, C. Heise, R. Murr, J. vom Berg, M. Jastroch, D. Lamp, J. Ding, P. Duewell, G. Niederfellner, C. Sustmann, S. Endres, C. Klein, S. Kobold, Bispecific antibodies enable synthetic agonistic receptor-transduced T cells for tumor immunotherapy. *Clin. Cancer Res.* **25**, 5890–5900 (2019).

12. M. P. Avanzi, O. Yeku, X. Li, D. P. Wijewarnasuriya, D. G. van Leeuwen, K. Cheung, H. Park, T. J. Purdon, A. F. Daniyan, M. H. Spitzer, R. J. Brentjens, Engineered tumor-targeted T cells mediate enhanced anti-tumor efficacy both directly and through activation of the endogenous immune system. *Cell Rep.* **23**, 2130–2141 (2018).

13. S. Viaud, J. S. Y. Ma, I. R. Hardy, E. N. Hampton, B. Benish, L. Sherwood, V. Nunez, C. J. Ackerman, E. Khialeeva, M. Weglarz, S. C. Lee, A. K. Woods, T. S. Young, Switchable control over in vivo CAR T expansion, B cell depletion, and induction of memory. *Proc. Natl. Acad. Sci. U.S.A.* **115**, E10898–E10906 (2018).

14. W. A. Lim, C. H. June, The principles of engineering immune cells to treat cancer. *Cell* **168**, 724–740 (2017).

15. G. Plitas, C. Konopacki, K. Wu, P. D. Bos, M. Morrow, E. V. Putintseva, D. M. Chudakov, A. Y. Rudensky, Regulatory T cells exhibit distinct features in human breast cancer. *Immunity* **45**, 1122–1134 (2016).

16. Y. Barsheshet, G. Wildbaum, E. Levy, A. Vitenshtein, C. Akinseye, J. Griggs, S. A. Lira, N. Karin, $CCR8^{+}FOXp3^{+}$ Treg cells as master drivers of immune regulation. *Proc. Natl. Acad. Sci. U.S.A.* **114**, 6086–6091 (2017).

17. R. Wieser, L. Attisano, J. L. Wrana, J. Massagué, Signaling activity of transforming growth factor beta type II receptors lacking specific domains in the cytoplasmic region. *Mol. Cell. Biol.* **13**, 7239–7247 (1993).

18. C. C. Kloss, J. Lee, A. Zhang, F. Chen, J. J. Melenhorst, S. F. Lacey, M. V. Maus, J. A. Fraietta, Y. Zhao, C. H. June, Dominant-negative TGF-β receptor enhances PSMA-targeted human CAR T cell proliferation and augments prostate cancer eradication. *Mol. Ther.* **26**, 1855–1866 (2018).

19. M. D. Miller, S. Hata, R. De Waal Malefyt, M. S. Krangel, A novel polypeptide secreted by activated human T lymphocytes. *J. Immunol.* **143**, 2907–2916 (1989).

20. M. D. Miller, M. S. Krangel, The human cytokine I-309 is a monocyte chemoattractant. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 2950–2954 (1992).

21. T. J. Curiel, G. Coukos, L. Zou, X. Alvarez, P. Cheng, P. Mottram, M. Evdemon-Hogan, J. R. Conejo-Garcia, L. Zhang, M. Burow, Y. Zhu, S. Wei, I. Kryczek, B. Daniel, A. Gordon, L. Myers, A. Lackner, M. L. Disis, K. L. Knutson, L. Chen, W. Zou, Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat. Med.* **10**, 942–949 (2004).

22. T. Brand, W. R. MacLellan, M. D. Schneider, A dominant-negative receptor for type beta transforming growth factors created by deletion of the kinase domain. *J. Biol. Chem.* **268**, 11500–11503 (1993).

23. C. M. Bollard, T. Tripic, C. R. Cruz, G. Dotti, S. Gottschalk, V. Torrano, O. Dakhova, G. Carrum, C. A. Ramos, H. Liu, M. F. Wu, A. N. Marcogliese, C. Barese, Y. Zu, D. Y. Lee, O. O'Connor, A. P. Gee, M. K. Brenner, H. E. Heslop, C. M. Rooney, Tumor-specific T-cells engineered to overcome tumor immune evasion induce clinical responses in patients with relapsed hodgkin lymphoma. *J. Clin. Oncol.* **36**, 1128–1139 (2018).

24. P. S. Adusumilli, L. Cherkassky, J. Villena-Vargas, C. Colovos, E. Servais, J. Plotkin, D. R. Jones, M. Sadelain, Regional delivery of mesothelin-targeted CAR T cell therapy generates potent and long-lasting CD4-dependent tumor immunity. *Sci. Transl. Med.* **6**, 261ra151 (2014).

25. R. J. Brentjens, M. L. Davila, I. Riviere, J. Park, X. Wang, L. G. Cowell, S. Bartido, J. Stefanski, C. Taylor, M. Olszewska, O. Borquez-Ojeda, J. Qu, T. Wasielewska, Q. He, Y. Bernal, I. V. Rijo, C. Hedvat, R. Kobos, K. Curran, P. Steinherz, J. Jurcic, T. Rosenblat, P. Maslak, M. Frattini, M. Sadelain, CD19-targeted T cells rapidly induce molecular remissions in adults with chemotherapy-refractory acute lymphoblastic leukemia. *Sci. Transl. Med.* **5**, 177ra38 (2013).

26. A. Di Stasi, B. De Angelis, C. M. Rooney, L. Zhang, A. Mahendravada, A. E. Foster, H. E. Heslop, M. K. Brenner, G. Dotti, B. Savoldo, T lymphocytes coexpressing CCR4 and a chimeric antigen receptor targeting CD30 have improved homing and antitumor activity in a Hodgkin tumor model. *Blood* **113**, 6392–6402 (2009).

27. B. Molon, S. Ugel, F. Del Pozzo, C. Soldani, S. Zilio, D. Avella, A. De Palma, P. Mauri, A. Monegal, M. Rescigno, B. Savino, P. Colombo, N. Jonjic, S. Pecanic, L. Lazzarato, R. Fruttero, A. Gasco, V. Bronte, A. Viola, Chemokine nitration prevents intratumoral infiltration of antigen-specific T cells. *J. Exp. Med.* **208**, 1949–1962 (2011).

28. G. D'Amico, G. Frascaroli, G. Bianchi, P. Transidico, A. Doni, A. Vecchi, S. Sozzani, P. Allavena, A. Mantovani, Uncoupling of inflammatory chemokine receptors by IL-10: Generation of functional decoys. *Nat. Immunol.* **1**, 387–391 (2000).

29. M. H. Kershaw, G. Wang, J. A. Westwood, R. K. Pachynski, H. L. Tiffany, F. M. Marincola, E. Wang, H. A. Young, P. M. Murphy, P. Hwu, Redirecting migration of T cells to chemokine secreted from tumors by genetic modification with CXCR2. *Hum. Gene Ther.* **13**, 1971–1980 (2002).

30. J. A. Craddock, A. Lu, A. Bear, M. Pule, M. K. Brenner, C. M. Rooney, A. E. Foster, Enhanced tumor trafficking of GD2 chimeric antigen receptor T cells by expression of the chemokine receptor CCR2b. *J. Immunother.* **33**, 780–788 (2010).

31. I. Siddiqui, M. Erreni, M. van Brakel, R. Debets, P. Allavena, Enhanced recruitment of genetically modified CX3CR1-positive human T cells into Fractalkine/CX3CL1 expressing tumors: Importance of the chemokine gradient. *J. Immunother. Cancer* **4**, 21 (2016).

32. D. R. Principe, B. DeCant, E. Mascariñas, E. A. Wayne, A. M. Diaz, N. Diaz, R. Hwang, B. Pasche, D. W. Dawson, D. Fang, D. J. Bentrem, H. G. Munshi, B. Jung, P. J. Grippo, TGFβ signaling in the pancreatic tumor microenvironment promotes fibrosis and immune evasion to facilitate tumorigenesis. *Cancer Res.* **76**, 2525–2539 (2016).

33. L. Gorelik, R. A. Flavell, Transforming growth factor-beta in T-cell biology. *Nat. Rev. Immunol.* **2**, 46–53 (2002).

34. D. A. Thomas, J. Massagué, TGF-β directly targets cytotoxic T cell functions during tumor evasion of immune surveillance. *Cancer Cell* **8**, 369–380 (2005).

35. G. A. Rabinovich, D. Gabrilovich, E. M. Sotomayor, Immunosuppressive strategies that are mediated by tumor cells. *Annu. Rev. Immunol.* **25**, 267–296 (2007).

36. J. Eyquem, J. Mansilla-Soto, T. Giavridis, S. J. van der Stegen, M. Hamieh, K. M. Cunanan, A. Odak, M. Gonen, M. Sadelain, Targeting a CAR to the *TRAC* locus with CRISPR/Cas9 enhances tumour rejection. *Nature* **543**, 113–117 (2017).

37. J. Feucht, J. Sun, J. Eyquem, Y. J. Ho, Z. Zhao, J. Leibold, A. Dobrin, A. Cabriolu, M. Hamieh, M. Sadelain, Calibration of CAR activation potential directs alternative T cell fates and therapeutic potency. *Nat. Med.* **25**, 82–88 (2019).

38. C. S. Hinrichs, N. P. Restifo, Reassessing target antigens for adoptive T-cell therapy. *Nat. Biotechnol.* **31**, 999–1008 (2013).

39. M. Rapp, S. Grassmann, M. Chaloupka, P. Layritz, S. Kruger, S. Ormanns, F. Rataj, K.-P. Janssen, S. Endres, D. Anz, S. Kobold, C-C chemokine receptor type-4 transduction of T cells enhances interaction with dendritic cells, tumor infiltration and therapeutic efficacy of adoptive T cell transfer. *Onco. Targets. Ther.* **5**, e1105428 (2015).

40. E. J. Cheadle, V. Sheard, D. G. Rothwell, J. S. Bridgeman, G. Ashton, V. Hanson, A. W. Mansoor, R. E. Hawkins, D. E. Gilham, Differential role of Th1 and Th2 cytokines in autotoxicity driven by CD19-specific second-generation chimeric antigen receptor T cells in a mouse model. *J. Immunol.* **192**, 3654–3665 (2014).

41. J. N. Brudno, J. N. Kochenderfer, Toxicities of chimeric antigen receptor T cells: Recognition and management. *Blood* **127**, 3321–3330 (2016).

42. L. Gorelik, R. A. Flavell, Abrogation of TGFbeta signaling in T cells leads to spontaneous T cell differentiation and autoimmune disease. *Immunity* **12**, 171–181 (2000).

43. P. J. Lucas, S. J. Kim, S. J. Melby, R. E. Gress, Disruption of T cell homeostasis in mice expressing a T cell-specific dominant negative transforming growth factor β II receptor. *J. Exp. Med.* **191**, 1187–1196 (2000).

44. T. Kamada, Y. Togashi, C. Tay, D. Ha, A. Sasaki, Y. Nakamura, E. Sato, S. Fukuoka, Y. Tada, A. Tanaka, H. Morikawa, A. Kawazoe, T. Kinoshita, K. Shitara, S. Sakaguchi, H. Nishikawa, PD-1^+ regulatory T cells amplified by PD-1 blockade promote hyperprogression of cancer. *Proc. Natl. Acad. Sci. U.S.A.* **116**, 9999–10008 (2019).

45. K. Ghani, X. Wang, P. O. de Campos-Lima, M. Olszewska, A. Kamen, I. Riviere, M. Caruso, Efficient human hematopoietic cell transduction using RD114- and GALV-pseudotyped retroviral vectors produced in suspension and serum-free media. *Hum. Gene Ther.* **20**, 966–974 (2009).

46. S. Kobold, S. Grassmann, M. Chaloupka, C. Lampert, S. Wenk, F. Kraus, M. Rapp, P. Duwell, Y. Zeng, J. C. Schmollinger, M. Schnurr, S. Endres, S. Rothenfußer, Impact of a new fusion receptor on PD-1-mediated immunosuppression in adoptive T cell therapy. *J. Natl. Cancer Inst.* **107**, djv146 (2015).

47. M.-R. Benmebarek, B. L. Cadilha, M. Herrmann, S. Lesch, S. Schmitt, S. Stoiber, A. Darwich, C. Augsberger, B. Brauchle, L. Rohrbacher, A. Oner, M. Seifert, M. Schwerdtfeger, A. Gottschlich, F. Rataj, N. C. Fenn, C. Klein, M. Subklewe, S. Endres, K.-P. Hopfner, S. Kobold, A modular and controllable T cell therapy platform for acute myeloid leukemia. *Leukemia*, 1–15 (2021).

48. S. A. Oh, A. Seki, S. Rutz, Ribonucleoprotein transfection for CRISPR/Cas9-mediated gene knockout in primary T cells. *Curr. Protoc. Immunol.* **124**, e69 (2019).

49. K. Labun, T. G. Montague, M. Krause, Y. N. Torres Cleuren, H. Tjeldnes, E. Valen, CHOPCHOP v3: Expanding the CRISPR web toolbox beyond genome editing. *Nucleic Acids Res.* **47**, W171–W174 (2019).

50. K. L. Heckman, L. R. Pease, Gene splicing and mutagenesis by PCR-driven overlap extension. *Nat. Protoc.* **2**, 924–932 (2007).

51. D. Sommermeyer, J. Neudorfer, M. Weinhold, M. Leisegang, B. Engels, E. Noessner, M. H. Heemskerk, J. Charo, D. J. Schendel, T. Blankenstein, H. Bernhard, W. Uckert, Designer T cells by T cell receptor replacement. *Eur. J. Immunol.* **36**, 3052–3059 (2006).

52. S. Das, E. Sarrou, S. Podgrabinska, M. Cassella, S. K. Mungamuri, N. Feirt, R. Gordon, C. S. Nagi, Y. Wang, D. Entenberg, J. Condeelis, M. Skobe, Tumor cell entry into the lymph node is controlled by CCL1 chemokine expressed by lymph node lymphatic sinuses. *J. Exp. Med.* **210**, 1509–1528 (2013).

53. C. A. Bauer, E. Y. Kim, F. Marangoni, E. Carrizosa, N. M. Claudio, T. R. Mempel, Dynamic Treg interactions with intratumoral APCs promote local CTL dysfunction. *J. Clin. Invest.* **124**, 2425–2440 (2014).

54. P. Jiang, S. Gu, D. Pan, J. Fu, A. Sahu, X. Hu, Z. Li, N. Traugh, X. Bu, B. Li, J. Liu, G. J. Freeman, M. A. Brown, K. W. Wucherpfennig, X. S. Liu, Signatures of T cell dysfunction and exclusion predict cancer immunotherapy response. *Nat. Med.* **24**, 1550–1558 (2018).

55. V. Thorsson, D. L. Gibbs, S. D. Brown, D. Wolf, D. S. Bortone, T. H. Ou Yang, E. Porta-Pardo, G. F. Gao, C. L. Plaisier, J. A. Eddy, E. Ziv, A. C. Culhane, E. O. Paull, I. K. A. Sivakumar, A. J. Gentles, R. Malhotra, F. Farshidfar, A. Colaprico, J. S. Parker, L. E. Mose, N. S. Vo, J. Liu, Y. Liu, J. Rader, V. Dhankani, S. M. Reynolds, R. Bowlby, A. Califano, A. D. Cherniack, D. Anastassiou, D. Bedognetti, Y. Mokrab, A. M. Newman, A. Rao, K. Chen, A. Krasnitz, H. Hu, T. M. Malta, H. Noushmehr, C. S. Pedamallu, S. Bullman, A. I. Ojesina, A. Lamb, W. Zhou, H. Shen, T. K.

Choueiri, J. N. Weinstein, J. Guinney, J. Saltz, R. A. Holt, C. S. Rabkin, A. J. Lazar, J. S. Serody, E. G. Demicco, M. L. Disis, B. G. Vincent, I. Shmulevich, The immune landscape of cancer. *Immunity* **48**, 812–830.e14 (2018).