Published OnlineFirst January 19, 2015; DOI: 10.1158/2326-6066.CIR-14-0186

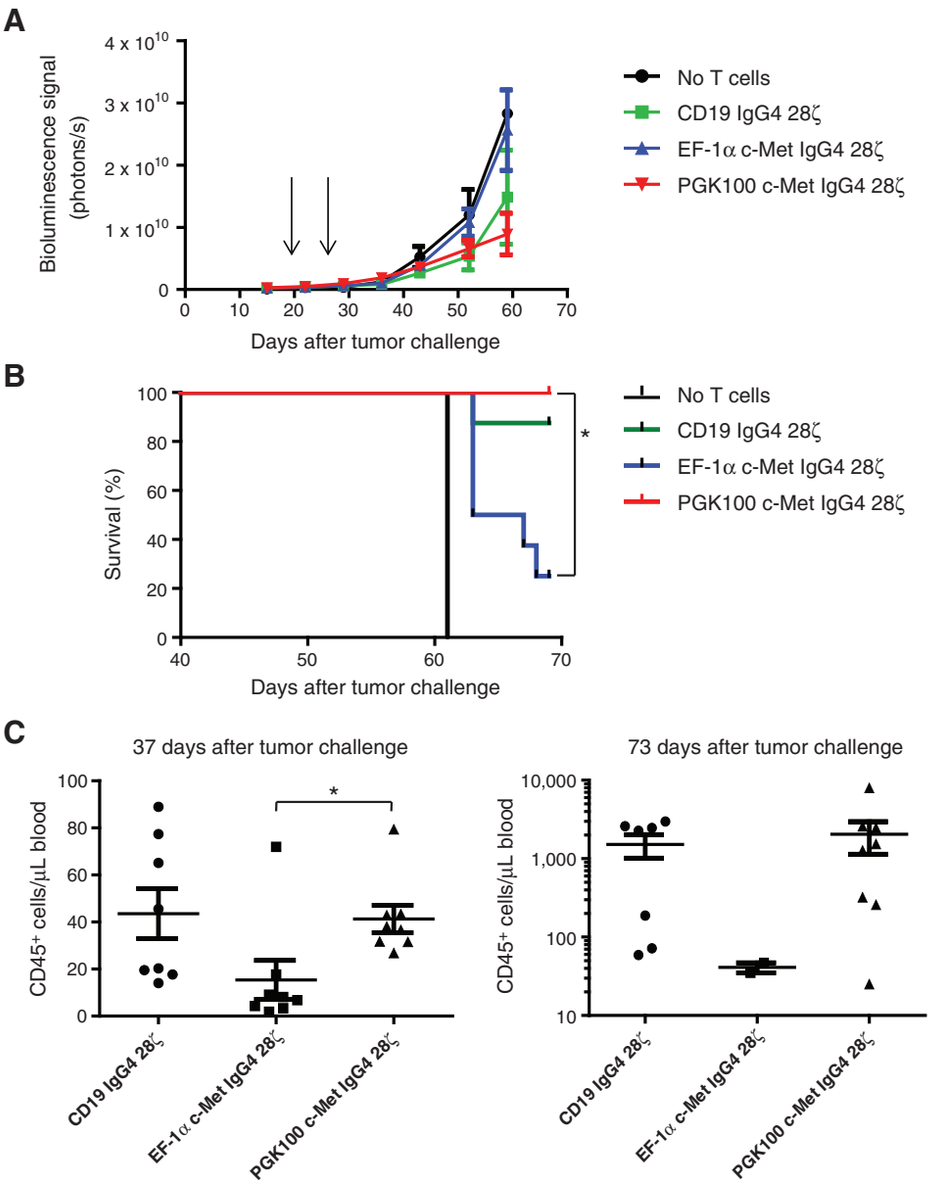

Figure 7.
A–C, *in vivo* efficacy of c-Met IgG4 28ζ CAR T cells. $CD4^+$ and $CD8^+$ T cells transduced to express CD19 IgG4 28ζ or c-Met IgG4 28ζ CAR under the influence of either EF-1α promoter or PGK100 promoter were infused (two administrations, 16×10^6 cells in total) into mice (for no T cells $n = 2$; for the rest $n = 8$ per group) bearing intraperitoneal SKOV3 tumors preestablished for 16 days. A, bioluminescence signal was acquired every week as a surrogate for tumor growth. $P < 0.01$ EF-1α versus PGK100 group. B, the Kaplan–Meier analysis. *, $P < 0.05$, EF-1α versus PGK100, log-rank (Mantel–Cox) test was used for statistical analysis. C, the absolute number of human $CD45^+$ T cells was determined in the blood on days 37 (left) and 73 (right), respectively. Only 2 mice survived in the EF-1α c-Met IgG4 28ζ CAR group on d73. *, $P < 0.05$. A two-tailed Student *t* test was used for statistical analysis. The design of the CAR constructs is shown in Supplementary Fig. S1.

growth phenotype. However, high-level expression is not sufficient to induce constitutive growth, as this phenomenon is only observed when the CAR encodes the CD28 transmembrane and cytosolic domain.

As far as we are aware, this is the first report of constitutive expression of the endogenous IL2 gene in primary nontransformed T cells. Previous studies have shown that constitutive expression of IL2 and CD25 occurs under conditions that lead to transformation of T cells, most prominently in HTLV-1 infection (41). It is likely that sustained signaling of the CD28 cytosolic domain encoded by the CAR is responsible for the constitutive secretion of IL2 and numerous other cytokines. It is interesting that both HTLV-1–mediated expression of IL2 by tax and IL2 secretion driven by the endogenous CD28 pathway have been reported to be resistant to cyclosporine (42, 43), an immunosuppressant that inhibits the calcineurin phosphatase. Consistent with the above, we have not observed constitutive proliferation of CAR T cells encoding ICOS, a signaling molecule that is closely related to CD28 (44).

Our collective results suggest that overexpression of the CD28 transmembrane and cytosolic domains in the context of some CARs can lead to constitutive signaling. Thus, it is likely that the regulation of endogenous CD28 gene expression is a critical determinant of T-cell homeostasis, consistent with studies showing that overexpression of CD28 ligands leads to T-cell hyperplasia in mice (45).

It is not well understood why human T cells progressively downregulate CD28 expression with age and cell division (46). The constitutive CAR T cells maintained CAR expression at bright levels and had far more rapid downregulation of the endogenous CD28 molecule than noncontinuous CARs or nontransduced T cells. A dileucine motif in CD28 contributes to limiting expression of CARs on mouse T cells, and mutating this sequence leads to increased expression of the CAR (47). The constitutive CAR T cells

Published OnlineFirst January 19, 2015; DOI: 10.1158/2326-6066.CIR-14-0186

that we have tested used the wild-type dileucine motif in the CD28 endodomain.

One of the limitations of our results is that we do not yet have a complete mechanistic understanding of the properties of CAR design that result in noncontinuous CAR T-cell growth that is ligand-dependent or continuous CARs that are ligand-independent. Our data indicate that given a permissive scFv, a 5- to 10-fold change in the level of expression can lead to the continuous CAR phenotype. This may explain why other laboratories have not detected this phenomenon using other expression systems. In addition, we have not examined the role of the hinge region in these studies. Hudecek and colleagues (48) have recently compared the influence of a CH2–CH3 hinge [229 amino acids (AA)], CH3 hinge (119 AA), and short hinge (12AA) on the effector function of T cells expressing ROR1-specific CARs and concluded that T cells expressing "short hinge" CARs had superior antitumor activity when ROR1 is targeted.

The role, if any, of CAR T cells with continuous proliferation in potential clinical applications remains to be determined. We recently reported safety and clinical benefit with CD19 CARs that use the 4-1BB signaling domain (7, 8). T cells expressing this CAR have enhanced ligand-independent proliferation (29) but do not have the long-term continuous growth phenotype that we describe in this report. CARs containing CD28 signaling domains have now been tested with safety in several clinical trials (5, 49–52). However, it is important to note that those trials expressed the CARs after manufacturing with a different cell culture system and with a retroviral vector rather than the lentiviral vector that we have used in the present work. Whether continuous CARs, such as those that we report here, would be useful and safe can only be established in future clinical trials. Overall, our present data suggest that strategies to identify CARs with a noncontinuous growth phenotype should be used to optimize antitumor efficacy and CAR persistence.

Disclosure of Potential Conflicts of Interest

A.D. Posey Jr and M.C. Milone report receiving commercial research grants from Novartis. L.J.N. Cooper has received speakers bureau honoraria from Miltenyi Biotec; has ownership interest (including patents) in Targazyme; and is a consultant/advisory board member for Ferring Pharmaceuticals, Janssen Pharmaceuticals, and Cellectis. M.J. Frigault, Y. Zhao, M.C. Basil, M. Kalos, and C.H. June have ownership interest in pending patents related to CAR technology licensed by the University of Pennsylvania to Novartis. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: M.J. Frigault, J. Lee, J. Scholler, S. Ang, L.J.N. Cooper, C.M. Paulos, Y. Zhao, M. Kalos, M.C. Milone, C.H. June

Development of methodology: M.J. Frigault, J. Lee, C. Carpenito, J. Scholler, O.U. Kawalekar, S. Guedan, A.D. Posey Jr, S. Ang, L.J.N. Cooper, C.M. Paulos, Y. Zhao, M.C. Milone

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M.J. Frigault, J. Lee, M.C. Basil, S. Motohashi, O.U. Kawalekar, S.E. McGettigan, L.J.N. Cooper, J.M. Platt, F.B. Johnson

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.J. Frigault, J. Lee, J. Scholler, L.J.N. Cooper, J.M. Platt, F.B. Johnson, C.M. Paulos, M. Kalos, M.C. Milone, C.H. June

Writing, review, and/or revision of the manuscript: M.J. Frigault, J. Lee, M.C. Basil, S. Guedan, L.J.N. Cooper, F.B. Johnson, C.M. Paulos, M. Kalos, M.C. Milone, C.H. June

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M.J. Frigault, S.E. McGettigan

Study supervision: M.J. Frigault, M. Kalos

Acknowledgments

The authors thank John Tobias and the Microarray Core, Brian Keith for advice, Spencer Small for experimental help, and the Human Immunology Core for healthy donor lymphocytes. Data and materials are available from the authors under a material transfer agreement.

Grant Support

This work was supported by funding from NIH grants R01CA120409, R01CA105216, 2PN2EY016586, 2P30CA016520, P01CA066726, 3T32-GM007170-36S1, the Abramson Family Cancer Institute, and Novartis.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received October 1, 2014; revised December 23, 2014; accepted December 26, 2014; published OnlineFirst January 19, 2015.

References

1. Jena B, Dotti G, Cooper L. Redirecting T-cell specificity by introducing a tumor-specific chimeric antigen receptor. Blood 2010;116:1035–44.
2. Bonini C, Brenner MK, Heslop HE, Morgan RA. Genetic modification of T cells. Biol Blood Marrow Transplant 2011;17:S15–20.
3. Ertl HC, Zaia J, Rosenberg SA, June CH, Dotti G, Kahn J, et al. Considerations for the clinical application of chimeric antigen receptor (CAR) T cells: observations from a recombinant DNA advisory committee (RAC) symposium June 15, 2010. Cancer Res 2011;71:3175–81.
4. Kohn DB, Dotti G, Brentjens R, Savoldo B, Jensen MC, Cooper LJ, et al. CARS on track in the clinic: report of a meeting organized by the blood and marrow transplant clinical trials network (BMT CTN) sub-committee on cell and gene therapy. Washington D.C., May 18, 2010. Mol Ther 2011; 19:432–8.
5. Savoldo B, Ramos CA, Liu E, Mims MP, Keating MJ, Carrum G, et al. CD28 costimulation improves expansion and persistence of chimeric antigen receptor—modified T cells in lymphoma patients. J Clin Invest 2011;121: 1822–5.
6. Restifo NP, Dudley ME, Rosenberg SA. Adoptive immunotherapy for cancer: harnessing the T cell response. Nat Rev Immunol 2012;12:269–81.
7. Porter DL, Levine BL, Kalos M, Bagg A, June CH. Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. N Engl J Med 2011;365: 725–33.
8. Kalos M, Levine BL, Porter DL, Katz S, Grupp SA, Bagg A, et al. T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leukemia. Sci Transl Med 2011;3: 95ra73.
9. Finney HM, Lawson ADG, Bebbington CR, Weir ANC. Chimeric receptors providing both primary and costimulatory signaling in T cells from a single gene product. J Immunol 1998;161:2791–7.
10. Alvarez-Vallina L, Hawkins RE. Antigen-specific targeting of CD28-mediated T cell co-stimulation using chimeric single-chain antibody variable fragment-CD28 receptors. Eur J Immunol 1996;26: 2304–9.
11. Feldhaus AL, Evans L, Sutherland RA, Jones LA. A CD2/CD28 chimeric receptor triggers the CD28 signaling pathway in CTLL.2 cells. Gene Ther 1997;4:833–8.
12. Geiger TL, Nguyen P, Leitenberg D, Flavell RA. Integrated src kinase and costimulatory activity enhances signal transduction through single-chain chimeric receptors in T lymphocytes. Blood 2001;98:2364–71.
13. Arakawa F, Shibaguchi H, Xu ZW, Kuroki M. Targeting of T cells to CEA-expressing tumor cells by chimeric immune receptors with a highly specific single-chain anti-CEA activity. Anticancer Res 2002:4285–9.
14. Haynes NM, Trapani JA, Teng MWL, Jackson JT, Cerruti L, Jane SM, et al. Rejection of syngeneic colon carcinoma by CTLs expressing single-chain

antibody receptors codelivering CD28 costimulation. J Immunol 2002: 5780–6.
15. Maher J, Brentjens RJ, Gunset G, Riviere I, Sadelain M. Human T-lymphocyte cytotoxicity and proliferation directed by a single chimeric TCR zeta/CD28 receptor. Nat Biotechnol 2002;20:70–5.
16. Finney HM, Akbar AN, Lawson ADG. Activation of resting human primary T cells with chimeric receptors: costimulation from CD28, inducible costimulator, CD134, and CD137 in series with signals from the TCR zeta chain. J Immunol 2004;172:104–13.
17. Gyobu H, Tsuji T, Suzuki Y, Ohkuri T, Chamoto K, Kuroki M, et al. Generation and targeting of human tumor-specific Tc1 and Th1 cells transduced with a lentivirus containing a chimeric immunoglobulin T-cell receptor. Cancer Res 2004;64:1490–5.
18. Moeller M, Haynes NM, Trapani JA, Teng MW, Jackson JT, Tanner JE, et al. A functional role for CD28 costimulation in tumor recognition by single-chain receptor-modified T cells. Cancer Gene Ther 2004;11:371–9.
19. Teng MW, Kershaw MH, Moeller M, Smyth MJ, Darcy PK. Immunotherapy of cancer using systemically delivered gene-modified human T lymphocytes. Hum Gene Ther 2004;15:699–708.
20. Friedmann-Morvinski D, Bendavid A, Waks T, Schindler D, Eshhar Z. Redirected primary T cells harboring a chimeric receptor require costimulation for their antigen-specific activation. Blood 2005;105:3087–93.
21. Pule MA, Straathof KC, Dotti G, Heslop HE, Rooney CM, Brenner MK. A chimeric T cell antigen receptor that augments cytokine release and supports clonal expansion of primary human T cells. Mol Ther 2005;12: 933–41.
22. Westwood JA, Smyth MJ, Teng MW, Moeller M, Trapani JA, Scott AM, et al. Adoptive transfer of T cells modified with a humanized chimeric receptor gene inhibits growth of Lewis-Y-expressing tumors in mice. Proc Natl Acad Sci U S A 2005;102:19051–6.
23. Willemsen RA, Ronteltap C, Chames P, Debets R, Bolhuis RLH. T cell retargeting with MHC class I-restricted antibodies: the CD28 costimulatory domain enhances antigen-specific cytotoxicity and cytokine production. J Immunol 2005;174:7853–8.
24. Kowolik CM, Topp MS, Gonzalez S, Pfeiffer T, Olivares S, Gonzalez N, et al. CD28 costimulation provided through a CD19-specific chimeric antigen receptor enhances *in vivo* persistence and antitumor efficacy of adoptively transferred T cells. Cancer Res 2006;66:10995–1004.
25. Loskog A, Giandomenico V, Rossig C, Pule M, Dotti G, Brenner MK. Addition of the CD28 signaling domain to chimeric T-cell receptors enhances chimeric T-cell resistance to T regulatory cells. Leukemia 2006;20: 1819–28.
26. Shibaguchi H, Luo NX, Kuroki M, Zhao J, Huang J, Hachimine K, et al. A fully human chimeric immune receptor for retargeting T-cells to CEA-expressing tumor cells. Anticancer Res 2006;26:4067–72.
27. Teng MWL, Kershaw MH, Jackson JT, Smyth MJ, Darcy PK. Adoptive transfer of chimeric Fc(epsilon)RI gene-modified human T cells for cancer immunotherapy. Human Gene Therapy 2006;17:1134–43.
28. Brentjens RJ, Santos E, Nikhamin Y, Yeh R, Matsushita M, La Perle K, et al. Genetically targeted T cells eradicate systemic acute lymphoblastic leukemia xenografts. Clin Cancer Res 2007;13:5426–35.
29. Milone MC, Fish JD, Carpenito C, Carroll RG, Binder GK, Teachey D, et al. Chimeric receptors containing CD137 signal transduction domains mediate enhanced survival of T cells and increased antileukemic efficacy *in vivo*. Mol Ther 2009;17:1453–64.
30. Carpenito C, Milone MC, Hassan R, Simonet JC, Lakhal M, Suhoski MM, et al. Control of large, established tumor xenografts with genetically retargeted human T cells containing CD28 and CD137 domains. Proc Natl Acad Sci U S A 2009;106:3360–5.
31. Lukens JN, VanDeerlin V, Clark CM, Xie SX, Johnson FB. Comparisons of telomere lengths in peripheral blood and cerebellum in Alzheimer's disease. Alzheimer's Dementia 2009;5:463–9.
32. Hombach A, Wieczarkowiecz A, Marquardt T, Heuser C, Usai L, Pohl C, et al. Tumor-specific T cell activation by recombinant immunoreceptors: CD3 zeta signaling and CD28 costimulation are simultaneously required for efficient IL-2 secretion and can ge integrated into one combined CD28/CD3 zeta signaling receptor molecule. J Immunol 2001;167:6123–31.
33. Skibinski G, Skibinska A, James K. The role of hepatocyte growth factor and its receptor c-met in interactions between lymphocytes and stromal cells in secondary human lymphoid organs. Immunology 2001;102:506–14.
34. Voehringer D, Koschella M, Pircher H. Lack of proliferative capacity of human effector and memory T cells expressing killer cell lectinlike receptor G1 (KLRG1). Blood 2002;100:3698–702.
35. Yeh JH, Sidhu SS, Chan AC. Regulation of a late phase of T cell polarity and effector functions by Crtam. Cell 2008;132:846–59.
36. Hsu C, Jones SA, Cohen CJ, Zheng Z, Kerstann K, Zhou J, et al. Cytokine-independent growth and clonal expansion of a primary human $CD8^+$ T-cell clone following retroviral transduction with the IL-15 gene. Blood 2007;109:5168–77.
37. Newrzela S, Cornils K, Heinrich T, Schlager J, Yi JH, Lysenko O, et al. Retroviral insertional mutagenesis can contribute to immortalization of mature T lymphocytes. Mol Med 2011;17:1223–32.
38. Zhao Y, Moon E, Carpenito C, Paulos CM, Liu X, Brennan A, et al. Multiple injections of electroporated autologous T cells expressing a chimeric antigen receptor mediate regression of human disseminated tumor. Cancer Res 2010;70:9062–72.
39. Huang X, Wilber AC, Bao L, Tuong D, Tolar J, Orchard PJ, et al. Stable gene transfer and expression in human primary T-cells by the Sleeping Beauty transposon system. Blood 2006;107:483–91.
40. Singh H, Manuri PR, Olivares S, Dara N, Dawson MJ, Huls H, et al. Redirecting specificity of T-cell populations for CD19 using the sleeping beauty system. Cancer Res 2008;68:2961–71.
41. McGuire KL, Curtiss VE, Larson EL, Haseltine WA. Influence of human T-cell leukemia virus type I tax and rex on interleukin-2 gene expression. J Virol 1993;67:1590–9.
42. Good L, Maggirwar SB, Harhaj EW, Sun SC. Constitutive dephosphorylation and activation of a member of the nuclear factor of activated T cells, NF-AT1, in Tax-expressing and type I human T-cell leukemia virus-infected human T cells. J Biol Chem 1997;272:1425–8.
43. June CH, Ledbetter JA, Gillespie MM, Lindsten T, Thompson CB. T-cell proliferation involving the CD28 pathway is associated with cyclosporine-resistant interleukin 2 gene expression. Mol Cell Biol 1987;7:4472–81.
44. Guedan S, Chen X, Madar A, Carpenito C, McGettigan SE, Frigault MJ, et al. ICOS-based chimeric antigen receptors program bipolar TH17/TH1 cells. Blood 2014;124:1070–80.
45. Yu X, Fournier S, Allison JP, Sharpe AH, Hodes RJ. The role of B7 costimulation in CD4/CD8 T cell homeostasis. J Immunol 2000;164: 3543–53.
46. Goronzy JJ, Li G, Yu M, Weyand CM. Signaling pathways in aged T cells—a reflection of T cell differentiation, cell senescence and host environment. Semin Immunol 2012;24:365–72.
47. Nguyen P, Moisini I, Geiger T. Identification of a murine CD28 dileucine motif that suppresses single-chain chimeric T-cell receptor expression and function. Blood 2003;102:4320.
48. Hudecek M, Sommermeyer D, Kosasih PL, Silva-Benedict A, Liu L, Rader C, et al. The nonsignaling extracellular spacer domain of chimeric antigen receptors is decisive for *in vivo* antitumor activity. Cancer Immunol Res 2015;3:125–35.
49. Brentjens RJ, Riviere I, Park JH, Davila ML, Wang X, Stefanski J, et al. Safety and persistence of adoptively transferred autologous CD19-targeted T cells in patients with relapsed or chemotherapy refractory B-cell leukemias. Blood 2011;118:4817–28.
50. Kochenderfer J, Wilson W, Janik J, Dudley M, Stetler-Stevenson M, Feldman S, et al. Eradication of B-lineage cells and regression of lymphoma in a patient treated with autologous T cells genetically-engineered to recognize CD19. Blood 2010;116:4099–102.
51. Till BG, Jensen MC, Wang J, Qian X, Gopal AK, Maloney DG, et al. CD20-specific adoptive immunotherapy for lymphoma using a chimeric antigen receptor with both CD28 and 4-1BB domains: pilot clinical trial results. Blood 2012;119:3940–50.
52. Kochenderfer JN, Dudley ME, Feldman SA, Wilson WH, Spaner DE, Maric I, et al. B-cell depletion and remissions of malignancy along with cytokine-associated toxicity in a clinical trial of anti-CD19 chimeric-antigen-receptor-transduced T cells. Blood 2012;119:2709–20.