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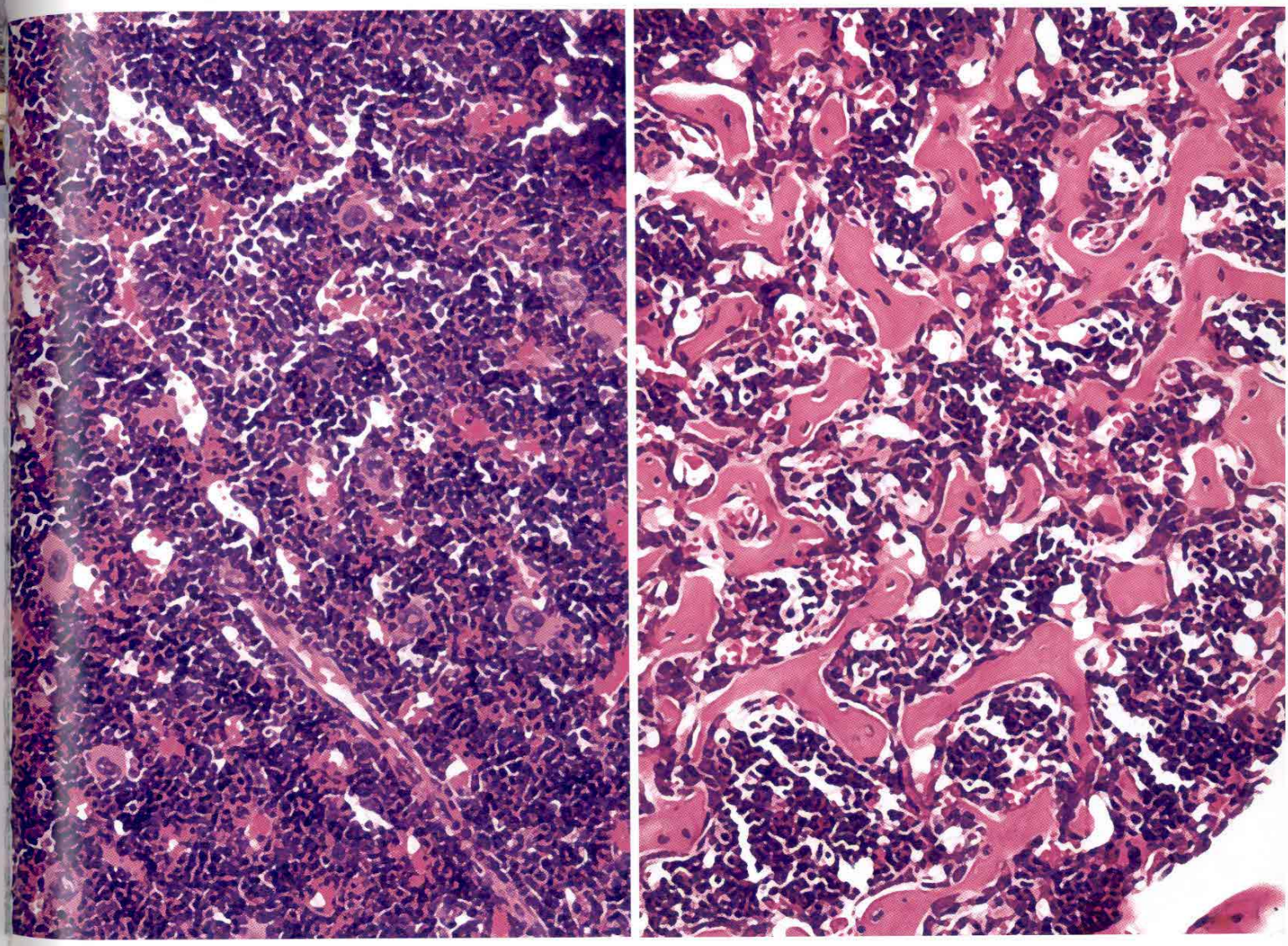

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Chimeric Receptors Containing CD137 Signal Transduction Domains Mediate Enhanced Survival of T Cells and Increased Antileukemic Efficacy *In Vivo*

Michael C. Milone[1,2], Jonathan D. Fish[3,4], Carmine Carpenito[1], Richard G. Carroll[1], Gwendolyn K. Binder[1], David Teachey[3,4], Minu Samanta[2], Mehdi Lakhal[1], Brian Gloss[1], Gwenn Danet-Desnoyers[5], Dario Campana[6,7], James L. Riley[1,2], Stephan A. Grupp[3,4] and Carl H. June[1,2]

[1]*Abramson Family Cancer Research Institute, University of Pennsylvania, Philadelphia, Pennsylvania USA;* [2]*Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania USA;* [3]*Department of Pediatrics, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania USA;* [4]*Division of Oncology, Children's Hospital of Philadelphia, Philadelphia, Pennsylvania, USA;* [5]*Department of Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, USA;* [6]*Department of Oncology, St Jude Children's Research Hospital, Memphis, Tennessee, USA;* [7]*Department of Pathology, St Jude Children's Research Hospital, Memphis, Tennessee, USA*

Persistence of T cells engineered with chimeric antigen receptors (CARs) has been a major barrier to use of these cells for molecularly targeted adoptive immunotherapy. To address this issue, we created a series of CARs that contain the T cell receptor-ζ (TCR-ζ) signal transduction domain with the CD28 and/or CD137 (4-1BB) intracellular domains in tandem. After short-term expansion, primary human T cells were subjected to lentiviral gene transfer, resulting in large numbers of cells with >85% CAR expression. In an immunodeficient mouse xenograft model of primary human pre-B-cell acute lymphoblastic leukemia, human T cells expressing anti-CD19 CARs containing CD137 exhibited the greatest antileukemic efficacy and prolonged (>6 months) survival *in vivo*, and were significantly more effective than cells expressing CARs containing TCR-ζ alone or CD28-ζ signaling receptors. We uncovered a previously unrecognized, antigen-independent effect of CARs expressing the CD137 cytoplasmic domain that likely contributes to the enhanced antileukemic efficacy and survival in tumor bearing mice. Furthermore, our studies revealed significant discrepancies between *in vitro* and *in vivo* surrogate measures of CAR efficacy. Together these results suggest that incorporation of the CD137 signaling domain in CARs should improve the persistence of CARs in the hematologic malignancies and hence maximize their antitumor activity.

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INTRODUCTION

With the advent of efficient gene transfer technologies, such as murine retroviral and HIV-derived lentiviral vectors, it has become feasible to confer novel antigenic specificity to T cells by transfer of chimeric antigen receptors (CARs) with stable, long-term expression. This technology has been used to generate T cells specific for HIV and several human tumor antigens, and some of these engineered T cells have been tested in Phase I/II studies in humans demonstrating the feasibility and relative safety of this approach.[1–3] One study has demonstrated antitumor activity in patients with neuroblastoma given a single CAR infusion.[4]

CARs combine the antigen recognition domain of antibody with the intracellular domain of the T cell receptor-ζ (TCR-ζ) chain or FcγRI protein into a single chimeric protein that are capable of triggering T-cell activation in a manner very similar to that of the endogenous TCR.[5,6] Several studies demonstrate that the addition of costimulatory domains, particularly the intracellular domain of CD28 can significantly augment the ability of these receptors to stimulate cytokine secretion and enhance antitumor efficacy in preclinical animal models using both solid tumors and leukemia that lack the expression of the CD28 receptor ligands CD80 and CD86.[7–9] Inclusion of domains from receptors such as the tumor necrosis factor receptor family members, CD134 (OX-40) and CD137 (4-1BB) into CARs has also been shown to augment CAR-mediated T-cell responses.[10,11] Gene transfer approaches using these engineered CARs may therefore provide significant improvements over current adoptive immunotherapy strategies that must rely on the endogenous TCR specificities, for which significant issues of TCR repertoire limitation and impaired tumor major histocompatibility complex class I expression may exist.

Correspondence: *Michael C. Milone, Department of Pathology and Laboratory Medicine, Abramson Family Cancer Research Institute, University of Pennsylvania, BRB 2/3, 421 Curie Boulevard, Philadelphia, Pennsylvania 19104-5160, USA. E-mail: milone@mail.med.upenn.edu or Carl H. June, Department of Pathology and Laboratory Medicine, Abramson Family Cancer Research Institute, University of Pennsylvania, BRB 2/3, 421 Curie Boulevard, Philadelphia, Pennsylvania 19104-5160, USA. E-mail: cjune@exchange.upenn.edu*

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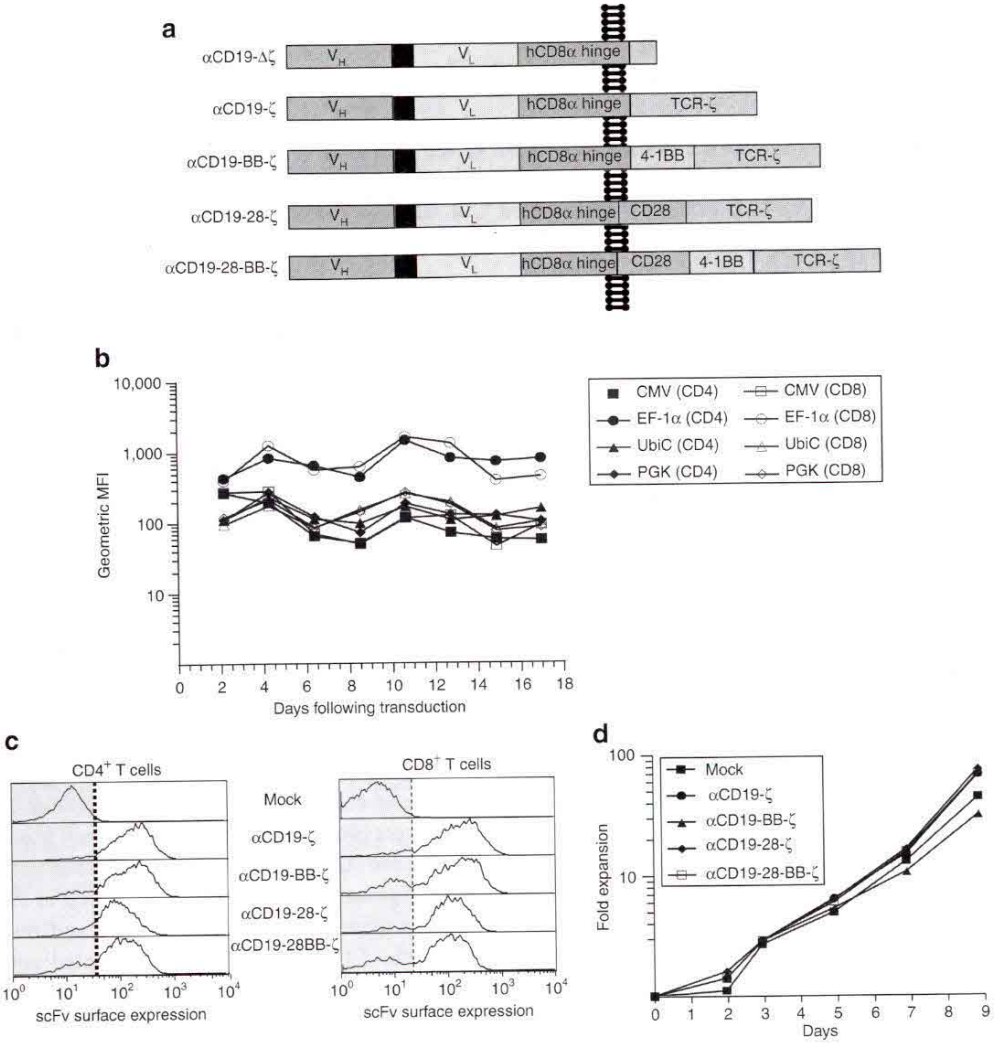

Figure 1 Lentiviral gene transfer combined with αCD3/αCD28 coated magnetic bead activation of T cells permits generation of large numbers of CD19-specific chimeric antigen receptor (CAR$^+$) T cells. (**a**) A schematic diagram showing the CD19-specific CAR used in this study. (**b**) Comparison of green fluorescent protein (GFP) expression under the control of different eukaryotic promoters in primary human CD4$^+$ and CD8$^+$ T cells over time. GFP fluorescence was compared in the indicated T cell subset in cells that were stimulated with αCD3/αCD28 coated beads followed by lentiviral transduction at an multiplicity of infection (MOI) of 0.2 on day 1 with vector expressing enhanced GFP under the control of the promoter indicated. Flow cytometric detection of GFP fluorescence was calibrated using Rainbow Calibration Particles (Spherotech, Lake Forest, IL) to correct for day-to-day variation. (**c**) αCD19-specific CAR surface expression in primary human CD4$^+$ and CD8$^+$ T cells. Expression was examined 6 days following transduction with the indicated CAR-encoding lentiviral vector at a MOI of ~8. (**d**) *In vitro* expansion of CD4$^+$ and CD8$^+$ T cells following activation with αCD3/αCD28 coated magnetic beads and transduction of the indicated CAR on day 1. Data are representative of >3 independent experiments.

In this study, we have addressed the issue of limited *in vivo* persistence of CARs by defining the relative contributions of TCR-ζ, CD137 and CD28 signaling domains in mice engrafted with hematopoietic malignancies. We chose the human CD19 antigen as our initial target for several reasons: (i) CD19 displays a pattern of expression that is highly restricted to B cells and B-cell progenitor cells,[12] (ii) CD19 does not appear to be expressed by hematopoietic stem cells permitting the targeting of the B-cell lineage without affecting other hematopoietic lineages,[13] and (iii) CD19 is widely expressed by malignant cells that are derived from the B-cell lineage including most lymphomas and lymphocytic leukemias.[14] After optimizing the generation of CARs with an efficient T-cell culture process, *in vitro* studies indicate that incorporation of either CD28 or 4-1BB signaling domains enhances activity over TCR–ζ, confirming previous studies. In contrast, compared to CARs that contain CD28, our *in vivo* studies indicate that CARs containing CD137 have superior antileukemic efficacy and improved persistence in a primary human acute lymphoblastic leukemia xenograft model. Furthermore, we also find that CARs expressing CD137 signaling domains can provide significant activity that appears to be antigen independent and may contribute to the efficacy of CARs *in vivo*.

RESULTS

Efficient generation of CAR$^+$ T cells using artificial bead-based antigen-presenting cells and lentiviral gene transfer

Lentiviral vectors can transfer genes into activated CD4$^+$ and CD8$^+$ human T cells with high efficiency but expression of the vector-encoded transgene depends on the internal promoter that drives its transcription. Therefore, successful CAR expression and

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