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transduced or transduced with a continuous CAR and cultured under conditions that lead to long-term growth expressed very low but detectable transcripts specific for c-Met, while mesothelin transcripts remained undetectable. Given that both c-Met and mesothelin-specific CARs displayed the continuous growth phenotype, the low level of c-Met expression in activated T cells is unlikely to be necessary for the sustained growth of T cells. In addition, the absence of fratricide in the cultures is consistent with ligand-independent continuous growth. Finally the results described above were replicated in T cells obtained from at least 10 different healthy donors.

Signaling CD28 and CD3ζ domains is required for constitutive CAR T-cell proliferation

To determine the contribution of signaling to the observed phenotype, we constructed a series of CARs that were identical except that the endodomain was replaced with ICOS, 4-1BB, CD28 and zeta only (**Supplementary Fig S1**). When T cells were transduced with lentiviral vectors encoding these CARs, only the c-Met IgG4 28ζ CAR T cells exhibited continuous proliferation (**Fig 1D**). Given that the scFv was held constant in these experiments, signaling from the CD28 transmembrane and cytosolic domain is required for the phenotype.

Constitutive expression of IL2 and a diverse array of cytokines and chemokines

To our knowledge, the observation that CARs can mediate long-term constitutive proliferation of primary T cells has not been reported previously. To begin to understand the mechanism leading to constitutive proliferation, we first determined the levels of various cytokines and other immune-related factors in the supernatants from the cultures that might be sustaining their unusual longevity. Analysis at the protein level revealed that the culture supernatants from continuous CARs contained high levels of cytokines characteristic of both Th1 and Th2 $CD4^+$ T cells (**Fig 2**). In contrast, the supernatants of non-continuous CAR T cells had low levels of cytokines that decreased with time of culture. The differences were large in magnitude, as the cytokine concentrations in the supernatants of continuous CARs were 100 to >1000-fold higher than those in the non-continuous CAR cultures. The cytokines likely contributed to the

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proliferation because transfer of day 56-conditioned medium from continuous CAR T cell cultures induced activation of unstimulated naïve $CD4^+$ T cells (**Supplementary Fig S4**). These results were confirmed at the transcriptional level, with prominent expression of transcripts for IFNγ, TNFα, IL2, IL4, IL13, IL3 and GM-CSF in cells isolated from the constitutively proliferating CAR T cells compared to those from non-continuous CAR T cells (**Fig 3**). Consistent with this finding, we observed that continuous CAR T cells outgrew normal T cells in cultures that were initially composed of mixtures of CAR T cells and T cells that did not express CARs (**Fig 4A**). In addition to the sustained transcription and secretion of cytokines and chemokines, continuous CAR $CD4^+$ T cells had elevated levels of granzyme B and perforin (**Fig 3**), consistent with the potent cytotoxic effector function that was observed (**Supplementary Fig S2**) and reported(30). The growth was not driven by fetal growth factors because the continuous CAR phenotype occurred in culture medium supplemented with human serum as well as with fetal bovine serum (**Supplementary Fig S5**).

Molecular signature of constitutive CAR T-cell proliferation

We performed gene array analysis to investigate the mechanism leading to long-term CAR T-cell proliferation. The molecular signature of key transcription factors and genes involved in T-cell polarization, growth and survival is shown in **Supplementary Fig S6**. The master transcription factors T-bet (TBX21), Eomes, and GATA-3 were induced and maintained at high levels in the continuous CAR $CD4^+$ T cells. In contrast, FoxP3 and RORC were expressed at comparable levels in continuous CAR T cells, untransduced activated T cells, and in CAR T cells with the non-continuous proliferative phenotype. As early as day 11, Bcl-xL was highly expressed in continuous CAR T cells compared to the non-continuous CAR and other control T-cell populations ($p < 0.001$), suggesting that resistance to apoptosis as well as enhanced proliferation contributes to the long-term proliferation of CAR T cells. The day 11 microarray samples were derived from cells that were >90% CAR-positive. Consistent with their substantial proliferative capacity, continuous CAR T cells maintained low level expression of KLRG1, a gene often expressed in terminally differentiated and senescent $CD4^+$ T cells (34).

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Hierarchical clustering analysis of the microarray data set indicates that the CAR T cells with constitutive T-cell proliferation have a unique molecular signature (**Supplementary Fig S7**). It is notable that by day 11, cMet IgG4 28ζ CAR T cells with the long-term growth phenotype closely cluster in the dendrogram. In contrast, naïve T cells were most closely related to untransduced T cells and non-continuous CARs on day 11 of culture (**Supplementary Fig S7**). Similarly, fully activated day 6 T cells from all groups cluster together, while T cells expressing the continuous CAR constructs diverge by day 11 to display a unique RNA signature differs from that in untransduced or non-continuous CAR T cells on day 6 (**Supplementary Fig S8**). The differentially expressed genes in the continuous CAR (c-Met IgG4) and non-continuous CAR (CD19 CD8α) T cells were plotted as a heat map to depict the relationship of the two populations (**Fig 5**). When analyzed using a stringent 5-fold cutoff on day 11 of culture, 183 genes were upregulated and 36 genes were down regulated in continuous CARs compared to the non-continuous CAR T cells. A list of the differentially expressed genes is presented in **Supplemental Tables 2 and 3**. Most notably the continuous CAR T cells are enriched for genes related to control of the cell cycle and a diverse group of cytokines.

Constitutive signal transduction by continuous CARs

To further investigate the mechanisms of the continuous CAR-dependent and ligand-independent T-cell growth, we interrogated the canonical signal transduction pathways that are implicated in T-cell activation and growth (**Fig 4B**). T cells expressing non-continuous or continuous CARs had similar levels of phosphorylation on Akt, ERK1/2, NF-κB p65 (RelA) and S6 on day 6 of culture. In contrast, only the continuous CAR T cells had sustained activation of Akt pS473, ERK1/2 pT202 and pY204, and RelA pS529 at days 10 and 25 of culture. However, the expression of continuous CARs in cells had only a minor effect on S6 pS240 phosphorylation, indicating that the expression of CARs do not lead to universal activation of T-cell signaling pathways. Constitutive signal transduction together with sustained cytokine secretion indicate that both cell intrinsic and extrinsic effects of CARs can lead to the long-term expansion of primary human T cells.

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In the experiments described above, primary human T cells were subjected to a single round of activation with anti-CD3 and anti-CD28 beads, and then followed in culture without the addition of exogenous cytokines. This method of culture was chosen because it has been used in clinical trials, and the initial activation is necessary to mediate high-efficiency transduction of CARs. To determine if the initial activation of T cells by anti-CD3 and anti-CD28 signaling is required for the subsequent constitutive signaling by CARs, we expressed various CARs in a Jurkat T-cell line that stably expresses GFP under the control of the NFAT promoter (**Supplementary Fig S9**). The cells were analyzed 3 days after transduction; only the continuous CARs as classified by the growth phenotype in primary T cells, led to constitutive NFAT activation in Jurkat cells. This effect was cell intrinsic as only the Jurkat cells that expressed CARs on the surface had GFP expression. In contrast, expression of non-continuous CARs (SS1 CAR with a truncated cytosolic domain and the CD19 CARs) did not lead to constitutive NFAT activation in Jurkat cells.

Level of surface expression contributes to continuous CAR T-cell phenotype

In previous studies we showed that CARs expressed under the control of different eukaryotic promoters in primary T cells had widely varying levels of surface expression (29). To determine if the level of surface expression contributes to the continuous CAR phenotype, the initial experiments were conducted using the EF-1α or CMV promoters to express the CARs, resulting in a higher or lower expression (**Fig 6C**). By day 9 of culture, there was a 5-fold reduction in surface expression of the CAR driven by the CMV promoter. As in previous experiments, the c-Met CAR displayed a continuous phenotype when under the control of EF-1α. In contrast, the same CAR reverted to a non-continuous CAR phenotype when expressed under the control of the CMV promoter (**Fig 6A and B**).

To explore the contribution of CAR expression to continuous phenotype, we constructed a panel of promoters driving surface expression that varied by 10- to 20-fold (**Supplementary Fig S10 and Fig S1D**) using a series of PGK truncation mutants. The growth characteristics of c–Met IgG4 28ζ CAR T cells were compared to the same CAR expressed with the EF-1a promoter (**Suppl Fig S10**). In both $CD4^+$

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and $CD8^+$ T cells the c–Met IgG4 28ζ CAR was continuous when driven by EF-1α and non-continuous when driven by the PGK100 truncation mutant (**Suppl Fig S10A and B**). However, bright surface expression is necessary but not sufficient for the continuous CAR phenotype because as was shown in figure 1, when the CD19 CARs are expressed at similar levels using the EF-1α promoter they display the non-continuous CAR phenotype. This result suggests that structural characteristics of the particular scFv, in addition to constitutive signaling through CD28 contribute to the continuous CAR phenotype.

Continuous CARs induce T-cell differentiation and proliferation without transformation

Polychromatic flow cytometry was used to characterize CAR T cells with constitutive proliferation. The expression of T-cell molecules associated with activation and differentiation was examined in cultures of cells expressing or not expressing the CAR (**Supplementary Fig S11**). Additionally, untransduced T cells were followed over time after a single round of stimulation with anti-CD3 and anti-CD28 beads (**Supplementary Fig S12**). The results show that a progressive enrichment for CAR T cells, so that by day 23 of culture, essentially all cells expressed the CAR. This was associated with bright expression of CD25 at all times on the CAR T cells, whereas CD25 became undetectable by day 14 in the non-transduced companion control culture (**Supplementary Fig S12**). Similarly, CD70 was expressed at progressively higher frequencies in CAR T-cell culture, a feature not observed in the control culture. In contrast, CD27, the ligand for CD70, was expressed in the control cultures, while CD27 progressively decreased in the CAR T-cell cultures. CD28, CD62L and CCR7 expression was maintained in the control cultures while many of the continuous CAR T cells became dim or negative for these molecules. In contrast, PD-1 was transiently expressed in the control cultures at day 6, while the CAR T cells had a prominent subpopulation of cells that retained expression of PD-1. Finally, Crtam, a molecule associated with cell polarity regulation (35), was induced in the continuous CAR T cell cultures and expression of Crtam was notably restricted to the T cells expressing CARs at the surface.

The potential for the CAR T cells to transform was assessed by long-term cultures *in vitro* and by transfer of CAR T cells to immunodeficient mice. The long-term cultured CAR T cells do not have constitutive

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expression of telomerase, as assessed by hTERT expression (**Supplementary Fig S6B**), and telomere length decreases with time in cultures of continuous CAR T cells (**Supplementary Fig S13**). In contrast, transformed human T cells have been reported to have constitutive telomerase activity (36). To date, in more than 20 experiments, transformation has not been observed in T cells transduced with continuous CARs. The continuous cytokine-independent polyclonal T-cell proliferation mediated by the CD28:CD3ζ CARs was independent of the specificity of the endogenous TCR, and was not the result of clonal outgrowth because the T-cell populations remained diverse during culture (**Supplementary Fig S14**). As a potentially more sensitive assay to detect cellular transformation, NSG (NOD-SCID-$\gamma c^{-/-}$) mice were used, as previous studies have shown that adoptively transferred transformed and malignant T cells can form tumors in immunodeficient mice (37). Groups of mice were infused with fully activated T cells or with continuous CAR T cells and proliferation assessed by quantification of T cells in the mice and effector function assessed by the induction of xenogeneic graft versus host disease in the mice. By day 60, xeno-reactivity (grade 1-3 xGVHD) was observed in 5/10 mice in the untransduced group compared to 3/10 in the c-Met IgG4 CAR group. Tumor formation was not observed at necropsy, and the levels of T-cell engraftment were similar ($p= 0.39$) in mice engrafted with continuous CAR T cells or untransduced primary T cells that were stimulated with anti-CD3 and anti-CD28 (**Supplementary Fig S15**).

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Comparison of antitumor effects mediated by continuous and non-continuous CAR T cells

To extend the above phenotypic, functional and transcriptional studies, a series of experiments were conducted in NSG mice with advanced vascularized tumor xenografts. The human ovarian cancer cell line SK-OV3 was selected as a representative c-Met-expressing tumor. We compared the antitumor efficacy of c-Met IgG4 28ζ CAR T cells expressed under continuous or non-continuous conditions using the promoter system shown in Supplementary figures S10. Mock transduced and CD19 IgG4 28ζ CAR T cells served as specificity controls. NSG mice bearing day 16 intraperitoneal tumors were injected intravenously with the T-cell preparations and serial bioluminescence imaging was used as a measure of tumor growth. Surprisingly, the non-continuous c-Met CAR cells with the PGK100 promoter had improved antitumor efficacy compared to the EF-1α group as measured by bioluminescence and survival (**Fig 7A and B**). Consistent with the improved antitumor effects, the engraftment and persistence of the non-continuous PGK100 CAR T cells was better than that of the continuous EF-1α CAR T cells (**Fig 7C**). Analysis of tumors from mice with flank tumors showed that there are many more T cells infiltrating the tumors in mice with the CARs using the weaker promoter (**Supplementary Figure S16**). In addition, the numbers of circulating CAR T cells were significantly higher when mice were treated with CARs driven by weaker rather than by stronger promoters. Together these results suggest that efficacy of CAR T cells *in vivo* is a function of the density of CAR expression and that this can have a substantial impact on antitumor efficacy and persistence of CAR T cells both systemically and at the tumor site. Mice treated with the irrelevant CD19 CAR had improved survival compared to mice given no T-cell injection, consistent with an allogeneic effect. However mice treated with the continuous c-Met CAR T cells using the EF-1α promoter were inferior in all experimental endpoints: bioluminescence, survival and *in vivo* CAR T-cell persistence.

DISCUSSION

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To our knowledge this is the first description of "continuous CARs", i.e. primary T cells that exhibit prolonged exponential expansion in culture that is independent of ligand and of addition of exogenous cytokines or feeder cells. The constitutive secretion for several months of large amounts of cytokines by non-transformed T cells was unexpected. The continuous CAR T cells progressively differentiate during culture towards terminal effector cells and transformation has not been observed. The mechanism leading to the growth phenotype includes signal transduction involving canonical TCR and CD28 signal transduction pathways that is independent of cognate antigen. Another mechanism identified is the level of scFv surface expression, as CARs that expressed brightly at the cell surface had sustained proliferation, while CARs that expressed at lower levels did not exhibit sustained proliferation and cytokine secretion. Furthermore, the scFv appears to have important effects on determining the growth phenotype. We have not investigated the role of the hinge domain in these studies.

These results are notable for several reasons. The nature of the scFv has a role in the phenotype, as we have observed continuous CAR phenotype with scFvs that are specific for c-Met and mesothelin but not in the case of FMC63 that is specific for CD19. An implication of this finding is that one cannot assume that the behavior of a signaling domain coupled to a given scFv will be the same when expressed with a distinct scFv. The method of CAR expression also contributes to the growth phenotype. To date we have not observed constitutive growth of T cells when the CARs are expressed by electroporation of mRNA or plasmids encoding *Sleeping Beauty* transposons (38-40). When expressed using lentiviral vectors, we have only observed continuous growth in vectors that employ the EF-1α promoter but not when driven by CMV or truncated PGK promoters. In previous studies comparing several promoters in lentiviral vectors, we found that this promoter resulted in more stable and higher level expression in primary CD4 and CD8 T cells (29). The particular design of the hinge and extracellular domain does not appear to have a major contribution to the continuous growth phenotype as we have observed this phenomenon with CARs that encode either the longer IgG4 hinge or the shorter CD8α scaffold. High level expression of the CAR appears to be necessary for the continuous 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 non-transformed 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 non-continuous CARs or non-transduced 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 that we have tested employed 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 non-continuous 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 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 (48).

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 non-continuous growth phenotype should be used to optimize antitumor efficacy and CAR persistence.

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AUTHOR CONTRIBUTIONS

In order of appearance: M.J.F. designed research, conducted research, analyzed data, and edited the manuscript; J.L designed research, conducted research, analyzed data, and edited the manuscript; M.C.B designed research, conducted research, analyzed data, and edited the manuscript; C.C. conducted