ORIGINAL RESEARCH

Fine-tuning the CAR spacer improves T-cell potency

Norihiro Watanabe, Pradip Bajgain, Sujita Sukumaran, Salma Ansari, Helen E. Heslop, Cliona M. Rooney, Malcolm K. Brenner, Ann M. Leen, and Juan F. Vera

Center for Cell and Gene Therapy, Baylor College of Medicine, Texas Children’s Hospital, and Houston Methodist Hospital, Houston, Texas, USA

ABSTRACT
The adoptive transfer of genetically engineered T cells expressing chimeric antigen receptors (CARs) has emerged as a transformative therapy with curative potential. Structurally, CARs can be divided into four segments: (i) the single chain variable fragment (scFv), which confers antigen specificity, (ii) the spacer, which links the scFv and transmembrane (TM) domain, (iii) the anchoring TM domain, and (iv) endodomain(s) that provide signals for T-cell activation/proliferation/cytolytic effects. Thus, after binding cognate antigen these domains act in concert to induce tumor lysis.3-5
Though much of the field has focused on identifying tumor-specific scFvs and intracellular endodomain combinations (e.g., 41BB and CD28) that promote potent, specific, and sustained antitumor effects in vivo, our study dissect the role of the spacer, traditionally thought to act simply as a stalk facilitating access of the scFv to its target tumor epitope.6,7 Here, we demonstrate the central role that this region plays in modulating transgenic cell phenotype, activation status, migratory capacity, and tumor recognition and we reveal how minor modifications to this single domain can profoundly influence in vivo CAR T-cell potency.

Results
CAR-PSCA T cells exhibit potent in vitro antitumor effects but fail to exert in vivo activity in a xenograft tumor model
To target the tumor-associated antigen (TAA) PSCA, which is overexpressed in a variety of solid tumors including prostate, pancreas, and colon cancer, we constructed a retroviral vector encoding a humanized, codon-optimized, second generation CAR with an IgG1-derived hinge-CH2CH3 spacer, a CD28 transmembrane and signaling domain, and the CD3ζ chain, which we entitled our prototype CAR [P1.CAR] (Fig. 1A). This transgenic molecule was efficiently and stably expressed on the surface of activated T cells (95.9 ± 0.6%, mean ± SE, n = 8; Fig. 1B), conferring cells with the ability to specifically kill PSCA-expressing target cells (K562-PSCA; 73.1 ± 5.9% and Capan-1; 72.0 ± 11.1% specific lysis, mean ± SE, n = 5, 40:1 E:T ratio) but not PSCA-negative targets such as K562 and 293T cells (19.0 ± 2.6% and 8.4 ± 2.0%, respectively). Non-transduced (NT) T cells produced only background levels of lysis (K562; 11.1 ± 4.1%, K562-PSCA; 27.9 ± 7.0%, 293T cells; 6.5 ± 2.1% and Capan-1; 26.9 ± 8.9% specific lysis, mean ± SE, n = 5, 40:1 E:T ratio) (Fig. 1C). To evaluate the in vivo antitumor potential of these CAR T cells, we engrafted 6-week-old NSG mice with 5×106 Capan-1 cells subcutaneously (s.c. - right flank) and after 28 days, when the tumor had reached a volume of > 80 mm3, mice were treated with 10×106 P1.CAR T cells labeled with GFP/firefly luciferase (FFLuc). However, despite CAR T-cell treatment, the tumor continued to increase in size at a rate similar to that observed in control (PBS) mice (Fig. 1D).
To assess whether deficient CAR T-cell trafficking was responsible for this phenomenon, we evaluated T-cell migration by performing sequential luminescence imaging of animals treated with either control (GFP/FFLuc) or P1.CAR T cells. As shown in Fig. 1E control T cells rapidly (within 24 h) localized to secondary lymphoid tissues such as the spleen and lymph nodes. In contrast, P1.CAR T cells failed to migrate to either the tumor or secondary lymphoid tissue. Instead the T cells
were trapped in the lungs, where the luminescence signal progressively increased. To investigate the mechanism behind this “non-specific” expansion, we examined whether interactions between the IgG1-CH2CH3 spacer region of our P1.CAR and Fcγ receptor-expressing cells could be responsible for this phenomenon.8-11 Thus, we cultured NT and P1.CAR T cells at a 1:1 ratio with human monocytes, macrophages and NK cells, all of which express different types of FcγRs (CD64—FcγRI, CD32—FcγRII, and CD16—FcγRIII) at varying intensities (Fig. 1F). As shown in Fig. 1G co-culture with monocytes and macrophages induced P1.CAR T-cell expansion and resulted in the elimination of monocytes and macrophages. However, this phenomenon was not observed in co-cultures with human NK cells, suggesting that this recognition was mediated through interaction with the FcγRs I and II and not CD16 (Fig. 1G).

Modification of the CH2CH3 spacer improves tumor localization

To abrogate FcγR recognition, we made two new CARs - M1.CAR and M2.CAR. In M1.CAR, we mutated the amino acids ELLG (aa233–236) and N (aa297) in the IgG1 CH2 region to PVA and Q, respectively.9,12 To generate the M2.CAR, we substituted the hinge-CH2CH3 IgG1 framework for that of IgG2 (reported to have the lowest potential for interaction with
both human\textsuperscript{13,14} and murine\textsuperscript{11} FcγR-expressing cells) and we additionally mutated aa297 (N) to Q (Fig. 2A). Subsequently, we investigated whether these modifications were sufficient to restore the migratory capacity of our CAR T cells. As shown in Fig. 2B, both the M1 and M2.CARs could be expressed at high levels on CD3/28-activated T cells (95.3 ± 0.8% and 91.3 ± 1.3%, respectively, mean ± SE, n = 8), enabling cells to specifically kill PSCA\textsuperscript{+} targets (72.8 ± 12.9% and 61.5 ± 5.4% specific lysis for M1.CAR and 75.8 ± 5.5% and 63.2 ± 6.1% for M2.CAR against K562-PSCA and Capan-1, respectively, mean ± SE, n = 5, 40:1 E:T ratio), with only background levels of killing against the control PSCA\textsuperscript{-} targets (K562 and 293T) (Fig. 2C).

Figure 2. Modification of the IgG derived-hinge-CH2CH3 spacer results in improved tumor localization (A) Representation of modified 2G.CAR.PSCA constructs (M1.CAR and M2.CAR)—vector map and schematics. (B) M1.CAR and M2.CAR expression on primary T cells shown for representative donor. (C) In vitro cytolytic activity of M1 and M2.CAR T cells as assessed in a 4 h \textsuperscript{51}Cr-release assay using PSCA\textsuperscript{+} (K562-PSCA and Capan-1) and PSCA\textsuperscript{-} targets (K562 and 293T cells). Data represents mean ± SE (n = 5). Significance was determined by two-way ANOVA. \( p \textless 0.05 \) compared with NT cells. (D) Representative dot plots and summary FACS data (n = 6 independent co-culture experiments) quantifying T cells (CD3) and FcγR-expressing cells on day 3 of co-culture using counting beads. Significance was determined by an unpaired two-tailed t-test and \( p \textless 0.05 \) when test conditions were compared with control (NT) cells. (E) In vivo T cell distribution of GFP/FFluc (control) and GFP/FFluc.CAR T cells as measured by bioluminescence imaging. (F) Tumor volume in NSG mice engrafted s.c. with Capan-1 and treated with PBS (open), P1.CAR (black), M1.CAR (blue) and M2.CAR T cells (red). Significance was determined by two-way ANOVA. \( p \textless 0.05 \).
To investigate whether our modifications mitigated FcγR recognition we co-cultured NT, P1, M1, and M2.CAR T cells with monocytes or macrophages (Fig. 2D) and after 3 d quantified residual cells by flow cytometry. As before, co-culture with P1.CAR T cells resulted in the elimination of macrophages/monocytes, while our M1 or M2.CAR and NT co-cultures all showed limited T-cell expansion and retention of macrophages/monocytes, suggesting that our modifications had successfully minimized FcγR recognition (Fig. 2D). However, while M1 and M2.CARs behaved similarly in vitro, our in vivo studies revealed that M2.CAR T cells were more efficiently able to mobilize from the lungs and localize at the tumor (Fig. 2E). Nevertheless, their antitumor activity was overwhelming (Fig. 2F) — for reasons unrelated to target antigen expression (Fig. S5B), highlighting the need for further CAR optimization.

**CAR T-cell senescence**

To determine whether CAR expression adversely impacted T-cell persistence, we initiated a series of in vitro studies to assess long-term T-cell expansion. Thus, we cultured P1.CAR T cells in media supplemented with IL2 (50 IU/mL) (without antigen or FcγR stimulation) and media and cytokines were replenished every 2–3 d for a total of 30 d. The gene expression profile, phenotype, and function of cells were assessed on days 10, 20, and 30 post-transduction. As shown in Fig. 3A, prolonged in vitro expansion did not impact cytolytic capacity, as measured in a short-term (4 h) chromium release assay with DU145 cells (57.0 ± 3.8%; day 10, 56.0 ± 7.0%; day 20, 54.7 ± 7.9%; day 30, n = 3, 40:1). However, when we performed a long-term (6 d) co-culture with DU145 cells (1:2—E:T) we observed an inverse correlation between antitumor activity and cell age (tumor cell fold expansion: 8.8 ± 1.5; day 10, 19.8 ± 1.5; day 20, 32.0 ± 7.2; day 30—mean ± SE, n = 4), which could be ascribed, at least in part, to decreased T-cell proliferation (T-cell fold expansion: 18.1 ± 2.4; day 10, 10.0 ± 1.1; day 20, 4.8 ± 0.6; day 30—mean ± SE, n = 4) (Fig. 3B). To explore this phenomenon further we compared the gene expression profile of P1.CAR T cells cultured for 20 or 30 d with that of cells maintained in culture for 10 d and found that genes associated with maintaining a naive/central memory phenotype (e.g., CCR7, SELL, CD27, and CD28) were progressively downregulated, whereas those associated with a differentiated T-cell profile (e.g., EOMES, FAS ligand (FASL), and Granzyme B (GZMB)) were upregulated (Fig. 3C). Indeed, Fig. 3D shows all the genes that were significantly upregulated or downregulated, clearly illustrating that prolonged culture induced cellular differentiation.

To confirm our microarray analyses and determine if our M1 and M2.CAR T cells exhibited an aging profile similar to that of our P1.CAR T cells, we performed phenotypic analyses to examine their memory profile (Fig. 3E, left panel and Fig. S1A). Although NT cells retained a naive profile over time, there was a progressive increase in effector memory T cells in all three CAR populations (P1, M1, and M2). Furthermore, although NT cells retained expression of both CD27 and CD28 over time, there was a progressive decline in expression of these molecules in all three CAR T-cell products (Fig. 3E, right panel and Fig. S1B), suggesting that CAR expression resulted in accelerated T-cell aging/differentiation.

**Tonic signaling is responsible for accelerated CAR T-cell aging**

To investigate whether these phenotypic and functional changes could be related to spontaneous CAR signaling, we generated a truncated version of the P1.CAR lacking the intra-cellular signaling domains (∆CAR; Fig. 4A), which we expressed on T cells (mean 89.2 ± 1.6% transduction, n = 7) (Fig. 4B). Next, we measured CD3ζ phosphorylation in the absence of cognate antigen stimulation and, as shown in Fig. 4C, all except the ∆CAR T cells exhibited evidence of CD3 signaling, which resulted in a chronic activation state characterized by persistently elevated CD25 levels both on CD8+ (Fig. 4D) and CD4+ (Fig. S2) T cell. Fig. 4D left panel shows CD25 expression on CD8+ T cells from a representative donor and right panel shows summary data (mean ± SE, n = 6). As expected, this tonic signal promoted cell cycle progression, characterized by transition from resting (G0) state to G1, S, and G2/M (Fig. 4E). As a consequence, P1-, M1-, and M2-modified cells exponentially expanded in the absence of the antigenic stimulation (Fig. 4F) and non-specifically produced effector cytokines (Fig. 4G), implicating tonic signaling as the mechanism underlying the accelerated aging we had detected.

**The CH2CH3 spacer is responsible for tonic T-cell signaling**

To determine if removing the CH2CH3 region would abrogate tonic signaling, we deleted this region entirely (X2.CAR, Fig. 5A), modified activated T cells (87.1 ± 2.0% transduction, mean ± SE, n = 8) (Fig. 5B), and confirmed that these cells were unable to interact with monocytes or macrophages (Fig. S3A). We next monitored the activation status of ∆CAR (negative control), M2.CAR (positive control), and X2.CAR T cells using CD25 expression as a readout. As shown in Figs. 5C and S3B, X2.CAR T cells exhibited a profile similar to that of ∆CAR T cells with minimal CD25 expression over 30 d of culture. This quiescent state was confirmed by cell cycle analysis showing that the majority of X2.CAR T cells were in G0 (mean 69.2 ± 8.4%, n = 3 - Fig. 5D). Consequently, non-specific expansion and cytokine production was low (Fig. 5E and G) and cells maintained an undifferentiated phenotype (Figs. 5F and S3C). Finally, we assessed the ability of X2.CAR T cells to kill PSCA+ targets. In a 4-h 51Cr release assay (Fig. 5H) transgenic cells exhibited potent antitumor activity against targets expressing high levels of PSCA (K562-PSCA and Capan-1; Fig. S3D). However, when cultured with target cells that expressed low PSCA levels (Fig. S3D), X2.CAR T cells demonstrated little/no tumor cell recognition (DU145—mean 20.7 ± 5.8% vs 57.5 ± 4.3%; X2 vs M2, CFPAC-1—mean 9.9 ± 1.5% vs 28.3 ± 4.0%; X2 vs M2, n = 5, 40:1) (Fig. 5H). Taken together this data suggests that although tonic signaling can be mitigated by removing the CH2CH3 region, deletion of this region can adversely affect antigen recognition and subsequent target lysis.

**Inclusion of CH3 as a spacer restores cytolytic abilities without accelerating cell aging**

To generate an effective CAR that retained its cytolytic capacity, we generated an additional vector with an intermediate
length spacer comprising only the IgG2 hinge-CH3 domain (X2.CAR—mean 86.4 ± 2.2% transduction, n = 8; Fig. 6A and B). To first determine whether transgenic expression of this construct enabled T-cell recognition of targets expressing both high (K562-PSCA and Capan-1) and low (DU145 and CFPAC-1) PSCA levels, we performed a chromium release assay. As shown in Fig. 6C, inclusion of the CH3 region facilitated recognition of all PSCA-expressing target cells.

Figure 3. Accelerated cell senescence in CAR-modified T cells. (A) Cytolytic activity (as measured in a 4-h 51Cr-release assay; ET = 40:1) of P1.CAR T cells in culture for 10, 20, or 30 d after transduction when cultured with 293T (PSCA−) and DU145 cells (PSCA+). The bar graph represents mean ± SE (n = 3). Significance was determined by one-way ANOVA for DU145; n.s: not significant. (B) Summary of FACS result of 3 independent co-culture experiments quantifying T cells and DU145 cells on day 6 of co-culture using counting beads. Significance was determined by one-way ANOVA with Bonferroni’s multiple comparisons test and p < 0.05 when the test conditions were compared with day 10 T-cell co-cultures. (C) Volcano plot of microarray analysis performed on P1.CAR T cells cultured for 10 or 20 d with differentially expressed genes displayed (n = 3 donors). (D) Fold change of gene expression in P1.CAR T cells maintained in culture for either 20 or 30 d and compared with the gene profile of the same cells maintained in culture for 10 d. All listed genes were significantly upregulated or downregulated as determined by FDR-corrected ANOVA analysis (p < 0.05). (E) Surface phenotypes of CD8+ T cells were analyzed on days 10, 20, and 30 after transduction. The top panel shows representative data –CCR7/CD45RO (left) and CD27/CD28 (right), whereas the pie charts show summary data (mean ± SE, n = 6) on day 30 of culture. Significance was determined by an unpaired two tailed t-test. p<0.05 compared with NT cells. Tnaive: naive, Tcm: central memory, Tem: effector memory, Temra: terminally differentiated.
(K562-PSCA; mean 74.8 ± 2.8%, Capan-1; 68.8 ± 6.6%, DU145; 48.4 ± 5.2%, CFPAC-1; 19.6 ± 3.5%, n = 5, 40:1) with no non-specific target recognition. Furthermore, based on assessment of phenotype (Fig. 6D and E), cell cycle status (Fig. 6F), expansion (Fig. 6G), and cytokine production (Fig. 6H) this enhanced antitumor activity was not gained at the expense of non-specific T-cell activation. Finally, since this construct lacked the CH2 region, as expected there was no evidence of Fc–FcγR interaction (Fig. S4).

**In vivo CAR antitumor activity**

Based on our previous *in vitro* data (presented in Figs. 1, 2, 5, and 6), we predicted that *in vivo*: (i) P1.CAR T cells would be
Figure 5. The CH2CH3 spacer is responsible for tonic T-cell signaling. (A) Representation of X2.CAR construct—vector map and schematic. (B) X2.CAR expression on primary T cells from a representative donor. (C) The representative histogram of CD25 expression on CD8 T cells (left panel) and summarized for 6 donors (right panel, mean ± SE). (D) Representative FACS plot showing cell cycle analysis. The pie chart represents mean ± SE (n = 3). Significance was determined by an unpaired two-tailed t-test. *p < 0.05 compared with M2.CAR. (E) Fold-expansion of in vitro cultured cells (gray: ΔCAR, red: M2.CAR, green: X2.CAR). (F) The phenotype of CD8 T cells was analyzed on days 10, 20, and 30 after transduction. Top panel shows representative data—CCR7/CD45RO (left) and CD27/CD28 (right), while the pie charts show summary data (mean ± SE; n = 6) on day 30 of culture. Significance was determined by an unpaired two-tailed t-test. *p < 0.05 compared with M2.CAR. (G) Cytokine production (GM-CSF, IFNγ, and TNFα) measured in cell supernatant from unstimulated CAR T cells using a Luminex assay (n = 3). Significance was determined by an unpaired two-tailed t-test. *p < 0.05 compared with M2.CAR. (H) The cytolytic activity of CAR T cells as measured in a 4-h 51Cr-release assay against PSCAbright (K562-PSCA and Capan-1), PSCAdim (DU145 and CFPAC-1), and PSCAbright (K562 and 293T cells) targets (n = 5; mean ± SE). Significance was determined by two-way ANOVA. *p < 0.05 compared with NT cells.
trapped in the lungs and rapidly eliminated, (ii) M1 and M2. CAR T cells would traffic to the tumor (and secondary lymph nodes) but would not control tumors due to cell senescence as a consequence of tonic signaling, (iii) X2.CAR T cells would effectively traffic to the tumor, persist in vivo and kill “high” PSCA-expressing tumor cells but fail to eliminate those expressing low levels of target antigen, whereas (iv) X2.CAR T cells would effectively traffic to the tumor, persist in vivo and control tumor growth, resulting in a survival benefit. To assess if this was indeed the case, NSG mice were engrafted s. c. with Capan-1 cells and when the tumor had reached a volume of 80 mm³, animals were administered i.v. with $10^6$ FFluc-CAR T cells (P1, M1, M2, X2, or X2). In vivo T-cell migration and proliferation was monitored by luminescence imaging, while tumor volume was measured by calipers. As shown in Figs. 7A and S5A, only M1, M2, X2, and X2.CAR T cells were able to escape the lungs, and persist at the tumor and secondary lymphoid organs (Fig. 7B and C), which resulted in delayed tumor growth. However, the antitumor activity mediated by X2.CAR T cells was significantly stronger than that exhibited by the other three constructs (Fig. 7D), resulting in a survival benefit in this cohort of animals (Fig. 7E). Finally, although all tumors eventually relapsed, in the X2.CAR T cell-treated group, the recurrent tumors were PSCA negative (Fig. S5B), illustrating that tumor immune escape was due to antigen modulation rather than CAR deficiency.

**Discussion**

The recent clinical success of CAR T cells in treating hematological malignancies has energized the field of immunotherapy and precipitated a wave of associated research. To date, many of these studies have focused on evaluating the impact of incorporating intracellular domains, while research into the role of the spacer has been limited. However, our work highlights the central role that this domain plays in various biological processes including T-cell migration, “tonic signaling,” and antigen recognition. Indeed, this is the first study to clearly delineate the influence of a single CAR component on multiple downstream effects, and demonstrates how apparently minor engineering modifications can have major therapeutic consequences.

Our current study originated from observations made using our second generation CAR targeting PSCA with an IgG1-derived hinge-CH2CH3 spacer domain. In our published work, we demonstrated both the in vitro and in vivo antitumor activity of T cells was modified with this CAR when delivered intraperitoneally to SCID mice engrafted with Capan-1, a PSCA⁺ pancreatic cancer cell line. However, our subsequent efforts to reproduce these findings in NSG mice engrafted with the same tumor but treated systemically with CAR T cells failed to produce tumor regression. Indeed, imaging studies clearly showed limited T-cell trafficking as our cells became trapped in the lungs.

In seeking an explanation for this finding, we came across studies reporting on the capacity of human IgG to interact with both murine and human Fcγ-R-expressing cells. For example, using a CD19 tumor model, Hudecek et al. showed that interactions between the IgG4-derived spacer of their CD19-CAR T cells and FcγR-expressing murine Ly6C⁺ cells led to their sequestration in the lungs.

However, in our study, the problem was easily resolved by simply mutating the FcγR binding site (within the CH2 region) as follows: (i) aa233 was deleted and aa234–236 (FLG) were substituted for PVA; and (ii) N (aa297) was substituted for Q. However, similar modifications to our construct only diminished but did not abrogate these Fc–FcγR interactions (Figs. 2E and S5A), thus highlighting the need for additional investigation.

Fcγ receptors can be sub-divided into three classes (FcγRI, FcγRII, and FcγRIII), which are differentially expressed by immune cells and display different IgG binding affinities. FcγRI (CD64) and FcγRII (CD32) are mainly expressed by monocytes, macrophages, dendritic cells, and neutrophils and bind with highest affinity to IgG1 and lowest to IgG2. In contrast, FcγRIII (CD16) is expressed by NK cells and binds with highest affinity to IgG3 and lowest to IgG2. Hence, to abrogate Fc–FcγR interactions, we substituted the IgG1 framework of our CAR for that of IgG2 and, as illustrated in Figs. 2E and S5A, this modification did indeed allow T cells to egress from the lungs, which improved tumor localization.

Although T-cell migration and tumor localization are necessary pre-requisites for antitumor responses, they are not sufficient. Indeed, at the tumor site CAR T cells must proliferate, and persist in a functional state to provide long-term tumor control. Recent work from a number of groups has highlighted the relationship between cellular phenotype and in vivo T-cell expansion/persistence. Indeed, Sommermeyer et al. reported on the superior antitumor effects achieved when tumor-bearing mice were treated with a mix of central memory-derived CD8⁺ and naive-derived CD4⁺ CAR.CD19 T cells. These findings were subsequently clinically validated by Turtle et al. who reported achieving bone marrow remissions in 27 of 29 B-ALL patients who received lymphodepleting chemotherapy followed by infusions of CD4⁺ and CD8⁺ CAR.CD19 mixed at a defined ratio. Cieri et al. also explored the activity of different T-cell subsets in vitro and in vivo and demonstrated that the culture of naive-derived CD3/28-activated T cells in IL7+15 enriched for cells with a stem-like profile (CD45RA⁻CD62L⁺CCR7⁺CD95⁻) that showed an enhanced capacity for proliferation, differentiation, and self-renewal upon antigen encounter.

Not surprisingly, therefore, the quest to prevent T-cell differentiation with the addition of homeostatic cytokines and/or AKT inhibitors during the in vitro expansion phase remains an area of active research. However, our findings additionally highlight the importance of selecting a CAR whose configuration does not induce tonic signaling, with its consequent adverse impact on cellular phenotype.

To date two CAR domains have been associated with tonic signaling: our PSCA spacer region and the CAR-GD2 scFv framework identified by Long et al. In the NCI study tonic signaling was suspected with the observation that CAR-GD2 appeared to be chronically activated based on (i) increased cell size, (ii) upregulation of CD25, and (iii) the appearance of an exhausted phenotype (expression of PD1, TIM3, and LAG3). Our findings differed somewhat since our CAR-PSCA-modified T cells appeared to be of an effector memory profile...
(based on expression of CCR7/CD45RO and loss of CD27 and CD28) but not exhausted. Indeed, none of our CARs (ΔCAR, P1.CAR, M1.CAR, M2.CAR, X2.CAR, and X32.CAR) induced upregulation of PD1, unlike their CAR-GD2-modified counterpart (which also had an IgG1-derived CH2CH3 domain) (Fig. S6). This not only highlights the difference between the two studies but also supports our assertion that different levels of tonic signaling can affect T cells in diverse ways and that reversing these effects may require customized strategies. Indeed, for Long et al., substitution of the original CD28 endodomain for that of 41BB resolved this issue, whereas we achieved the same result by reducing the spacer length.

Importantly, our observations were not restricted to CAR-PSCA or to a second generation CAR as evaluation of a first-generation CAR-MUC1 expressing different spacer regions...
(M2, X2, and X2) resulted in the progressive increase of an undifferentiated phenotype (Fig. S7A). Similarly, shortening the CAR-CD19 spacer (IgG1-derived hinge) resulted in a similar phenomenon (Fig. S7B) confirming the broad implications of our observations.

Finally, to produce tumor elimination, the scFv must engage with antigen and Moritz and colleagues were the first to demonstrate a direct correlation between the spacer length and the capacity of CAR ErbB-2 to engage with Her2.6 However, these results were not reproduced by Hombach et al. using a CD30-targeted CAR7 implying that target epitope location must also be considered. Indeed, subsequent studies using CARs targeting a range of antigens (CEA, NCAM, ST4, CD1928, MUC128, CD2229, ROR130, and CD17131) have borne out this assertion. Our results suggest that our PSCA scFv binds to a PSCA epitope located proximal to the cell membrane as the use of a long CAR spacer resulted in the greatest target recognition.

The field of CAR engineering is rapidly growing with efforts to enhance potency by the incorporation of genes to (a)
enhance in vivo persistence (cytokines, co-stimulatory endodomains), (b) ensure safety (suicide genes, iCARs, co-stimulatory CARs), and (c) protect cells from the inhibitory tumor microenvironment (switch receptors). However, our study serves as a cautionary tale that CARs, in their most basic form, are complex molecules and additional work is required to harness their full potential. Thus, before adding complexity, we must first understand the platform.

Materials and methods

Donors and cell lines

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy volunteers after informed consent on protocols approved by the Baylor College of Medicine Institutional Review Board. K562 (chronic erythroid leukemia cell line), 293T (human embryonic kidney cell line), Capan-1 (pancreatic cancer cell line), DU145 (prostate cancer cell line), and CFPAC-1 (pancreatic cancer cell line) were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in IMDM containing 20% heat-inactivated fetal bovine serum (FBS) (Hyclone, Waltham, MA) with 2 mM L-GlutaMAX. DU145 and CFPAC-1 cells were maintained in Iscove’s Modified Dulbecco’s Medium (IMDM; Gibco BRL Life Technologies, Inc., Gaithersburg, MD). Capan-1 cells were grown in IMDM containing 20% heat-inactivated fetal bovine serum (FBS) (Hyclone, Waltham, MA) with 2 mM L-GlutaMAX (Gibco BRL Life Technologies, Inc.), whereas other cell lines were grown in their specific media containing 10% FBS with 2 mM L-GlutaMAX.

Generation of retroviral constructs and retroviral transfection

Our original second generation CAR-PSCA has an IgG1-derived hinge-CH2CH3 spacer and CD28 and CD3ζ intracellular domains (P1.CAR). To generate our modified CARs, we synthesized the following DNA segments (Invitrogen, Grand Island, NY): (i) IgG1-hinge-CH2CH3 spacer region with mutations of ELLG (aa233–236) and N (aa297) to PVA and Q, respectively—M1.CAR; (ii) IgG2-hinge-CH2-CH3 with N297Q mutation—M2.CAR; (iii) IgG2-hinge—X2.CAR; and IgG2-hinge-CH3—X2.CAR. To generate the novel CAR constructs, the spacer sequences in the original P1.CAR construct were removed by enzymatic digestion (BamH1 and PstMI) and replaced with the new spacers. The γ-retroviral vectors encoding the fusion protein (GFP/FFluc) and the retroviral supernatant was plated in a non-tissue culture-treated 24-well plate (1 ml/well) that had been pre-coated with a recombinant fibronectin fragment (FN CH-296; Retronectin; TAKARA BIO INC, Otsu, Japan), and centrifuged at 2,000g for 90 min. After removal of supernatant, OKT3/CD28-activated PBMCs (0.1×10⁶/ml) were resuspended in complete media supplemented with IL2 (100U/ml) and 2ml was added to each well of a 24-well plate, which was subsequently spun at 400g for 5 min, and then transferred to a 37 °C, 5% CO₂ incubator. Subsequently, cells were split and fed every 2–3 d with fresh media plus IL2 (50 U/ml). To track T-cell numbers over time, viable cells were counted using trypsin blue. To co-express CAR and GFP-firefly luciferase (GFP/FFluc) (for in vivo bioluminescence imaging), T cells were first modified to express the CAR, as previously described, and were transduced after 24 h to co-express GFP/FFluc using the same protocol.

Generation of K562 modified to express PSCA

We synthesized PSCA (Invitrogen, Grand Island, NY), which was incorporated into the pVITRO1-blasti-mcs vector (Invivogen, San Diego, CA) by enzymatic digestion (Agel and NheI) and transfected into K562 using the GeneJuice® Transfection Reagent (EMD Millipore, Darmstadt, Germany). Transfected cells were selected and maintained in the presence of 10 ng/ml of Blasticidin (Invivogen, San Diego, CA).

FcγR-expressing cell preparation

Monocytes were isolated from PBMCs using human CD14 microbeads (MACS system; Miltenyi Biotec Inc., San Diego, CA). Macrophages were generated by culturing monocytes with 100 ng/ml GM-CSF for 7 d. NK cells were expanded by stimulating 5×10⁶ PBMCs with 5×10⁶ irradiated K562-mbL15-41BBL35,36 in 500 U/ml IL2 in G-Rex10 devices (Wilson Wolf Manufacturing, Minneapolis, MN) followed by depletion of residual CD3⁺ cells using CD3 microbeads (MACs system; Miltenyi Biotec, Inc.).

Flow cytometry

Cell surface staining

The following antibodies were used in this study: CD3-PerCP (clone SK7/Cat# 347344), CD27-PE (L128/340425), CD28-APC (CD28.2/559770), CD25-PE (M-A251/555432), CD64-APC (10.1/561189), CD32-APC (FLI8.26/559769), CD45RO-APC (UCHL1/340384), CCR7-FITC (150503/561272), CD33-PE (P67.6/347787), PD1-PE (MIH4/557946), rat anti-mouse IgG1-APC (X56/550874) (BD Biosciences, San Jose, CA), CD4-APC (13B8.2/IM2468U), CD3-Krome Orange (13B8.2/A96417), CD8-Pacific Blue (B9.11/A82791), CD8-PC7 (SFC121Thy2D3/6607102), CD16-APC-AlexaFluor750 (3GB/A66330), CD3-APC-AlexaFluor750 (UCHT1/A66329; Beckman Coulter, Inc.), anti-PSCA (7FS/sc-80654), and mouse IgG1 (Mountain View, CA). Cells were cultured in complete media (RPMI-1640 containing 45% Clicks medium (Irvine Scientific, Inc., Santa Ana, CA), 10% FBS, and 2 mM L-GlutaMAX), which was supplemented with recombinant human IL2 (50 U/mL, NIH, Bethesda, VA) on day 1. On day 3, retroviral supernatant was plated in a non-tissue culture-treated 24-well plate (1 ml/well) that had been pre-coated with a recombinant fibronectin fragment (FN CH-296; Retronectin; TAKARA BIO INC, Otsu, Japan), and centrifuged at 2,000g for 90 min. After removal of supernatant, OKT3/CD28-activated PBMCs (0.1×10⁶/ml) were resuspended in complete media supplemented with IL2 (100U/ml) and 2ml was added to each well of a 24-well plate, which was subsequently spun at 400g for 5 min, and then transferred to a 37 °C, 5% CO₂ incubator. Subsequently, cells were split and fed every 2–3 d with fresh media plus IL2 (50 U/ml). To track T-cell numbers over time, viable cells were counted using trypsin blue. To co-express CAR and GFP-firefly luciferase (GFP/FFluc) (for in vivo bioluminescence imaging), T cells were first modified to express the CAR, as previously described, and were transduced after 24 h to co-express GFP/FFluc using the same protocol.
(sc-3877; Santa Cruz Biotechnology, Inc., Dallas, TX). CAR molecules were detected using Goat anti-human F(ab’)2 antibody conjugated with AlexaFluor647 (109-606-097; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Cells were stained with antibody for 20 min at 4°C. All samples were acquired on a Gallios™ Flow Cytometer (Beckman Coulter, Inc., Brea, CA), and the data were analyzed using Kaluza® Flow Analysis Software (Beckman Coulter, Inc.).

**Intracellular staining**

T cells were fixed with formaldehyde solution (1.5%) (F1635, Sigma-Aldrich, St. Louis, MO), washed, permeabilized with pre-chilled 100% methanol (Fisher Scientific, Pittsburgh, PA) for 15 min on ice, and then washed thrice. For phospho-FACs, cells were stained with anti-CD247 (pY142)-AlexaFluor647 antibody (K25–407.369/558489) (BD Biosciences) for 60 min at room temperature in the dark. For cell cycle analysis, cells were stained with anti-kI67-AlexaFluor647 (Ki-67/350510) (BioLegend, San Diego, CA) and 7-AAD (BD Biosciences) for 30 min in the dark at room temperature.

**51Chromium-release assay**

The cytotoxicity and specificity of engineered T cells were evaluated in a standard 4–6-h 51Cr-release assay, as previously described.15

**Co-culture experiments**

For co-culture experiments with FcγR-expressing cells, T cells and target cells were co-cultured at a 1:1 ratio in 2 mL of complete media in a 24-well plate for 3 d. Subsequently, cells were harvested and stained with CD3, CD4, and CD8 (T cells) and CD33 (monocyte/macrophage) or CD16 (NK cells). For co-culture experiments with tumor cells, 5×106 T cells were co-cultured with 1×105 GFP/FFlu-T cells (Capan-1 cells) in 4 mL of complete media in a 6-well plate for 6 d. To quantify cells by flow, CountBright™ Absolute Counting Beads (C36950; Invitrogen, Eugene, OR) were added (50 μL) and 7-AAD was added to exclude dead cells. Acquisition was halted at 5,000 beads.

**Cytokine detection**

To measure cytokine production 1×106 T cells, which were maintained in culture for 10 d after transduction, were plated in a single well of a 24-well tissue culture plate with 2 mL of complete media and cultured for 24 h. Subsequently, supernatants were collected and stored at –80°C. Cytokine levels were analyzed using MILLIPLEX MAP High Sensitivity Human Cytokine 13 Plex (Merck Millipore, Billerica, MA) according to manufacturer’s instructions.

**Microarray analysis**

Total RNA was extracted from T cells cultured for different culture period using the RNAeasy Mini kit (QIAGEN, Valencia, CA) and quantified using the NanoDrop 2000 (Thermo Fisher Scientific, Inc., Waltham, MA). RNA expression profiling was performed using the GeneChip PrimeView Human Gene Expression Array (Affymetrix, Inc., Santa Clara, CA) by Genome Exploration, USA (Memphis, TN).

**In vivo study**

NOD.Cg-Prkdcsid Il2rgtm1Wjl/SzJ mice (NSG mice, 6–8 weeks old, The Jackson Laboratory) were engrafted s.c. (right flank) with Capan-1 cells (5×106/animal) and once the tumors were > 80 mm3 (~day 28) the animals were treated with 10×106 engineered GFP/FFlu-T cells i.v. Tumor size was measured by calipers and tumor volume was calculated formulas follows: tumor volume (mm3) = length × width × width/2. T-cell migration and distribution were evaluated by bioluminescence imaging recorded two times per week using a Lumina IVIS imaging system (Caliper Life Sciences, Inc., Hopkinton, MA), and analyzed by Living image software (Caliper Life Sciences, Inc.). To assess PSCA expression, mice were sacrificed, tumors were dissected, and single cell suspensions were prepared, as previously published.38 Briefly, extracted tumors were minced and dissociated by incubating the cells with 200 U/mL collagenase IV (Gibco BRL Life Technologies, Inc.) at 37°C in a water bath for 2 h. Every 20 mins, cells were vortexed for 1 min and at the end of the incubation period tissue debris and dead cells were removed by density centrifugation using Lymphoprep (Axis-Shield, Oslo, Norway).

**Statistics**

Statistical analysis was performed using Graphpad Prism 6 software (GraphPad Software, Inc., La Jolla, CA). Two-way ANOVA was used for 51Cr-release assay and in vivo tumor growth. One-way ANOVA was used in comparing the cytolytic function of T cells cultured for 10, 20, or 30 d. To analyze microarray results for different T cells of different ages generated from three independent donors, we used FDR-corrected ANOVA. All other experiments were analyzed using an unpaired two-tailed t-test.

**Study approval**

For human cells, healthy volunteers gave written informed consent according to protocols approved by the Baylor College of Medicine Institutional Review Board and performed in accordance with the guidelines established by the Declaration of Helsinki. All animal studies were performed under a protocol approved by the Animal Research Committee of Baylor College of Medicine.

**Disclosure of potential conflicts of interest**

Watanabe N, Brenner MK, and JF Vera have filed I.P. and plan to submit a related patent.

**Funding**

This research was supported in part by the NIH-NCI (P01 CA094237, P50 CA126752, and P50 CA186784) as well as the Adrienne Helis Malvin Medical Research Foundation through its direct engagement in the continuous active conduct of medical research in conjunction with Baylor College of Medicine. In addition, the Elsa U. Pardee Foundation and the National Science Foundation through its direct engagement in the continuous active conduct of medical research in conjunction with Baylor College of Medicine.
Pancreas Foundation provided support to N.W. J.F.V. is supported by a Mentored Research Scholars Grant in Applied and Clinical Research, MRSG-14-197-01—LIB from the American Cancer Society. During the execution of the work, the Center for Cell and Gene Therapy had a research collaboration with Celgene and Bluebird bio. We would like to thank Dr. Maksim Mamonkin from the Baylor College of Medicine for the CD19-CAR construct. We thank Texas Children’s Hospital for the use of the Small Animal Imaging Facility and we also appreciate the support of the Flow Cytometry and Cell and Vector Production shared resources in the Dan L. Duncan Comprehensive Cancer Center support grant P30 CA125123. H.E.H. is supported by a Dan L. Duncan Chair and M.K.B. by a Fayez Sarofim Chair.

Author contributions
NW, AML, and JFV designed the experiments and interpreted the data. NW, PB, SS, and SA performed the experiments and analyzed the data. NW, AML, and JFV designed the experiments and interpreted the data.

References
1. Jensen MC, Riddell SR. Design and implementation of adoptive therapy with chimeric antigen receptor-modified T cells. Immunol Rev 2014; 257(1):127-44; PMID:24329794; http://dx.doi.org/10.1111/imr.12139
2. Dotti G, Gottschalk S, Savoldeo B, Brenner MK. Design and development of therapies using chimeric antigen receptor-expressing T cells. Immunol Rev 2014; 257(1):107-26; PMID:24329793; http://dx.doi.org/10.1111/imr.12131
3. Maude SL, Frey N, Shaw PA, Aplenc R, Barrett DM, Bunin NJ, Chew A, Gonzalez VE, Zheng Z, Lacey SF et al. Chimeric antigen receptor T cells for sustained remissions in leukemia. N Engl J Med 2014; 371(16):1507-17; PMID:25317870; http://dx.doi.org/10.1056/NEJMoa1407222
4. Berentjens RJ, Riviere I, Park JH, Davila ML, Wang X, Stefanis J, Taylor C, Yeh R, Bartido S, Borquez-Ojeda O et al. Safety and persistence of adoptively transferred allogeneic CD19-targeted T cells in patients with relapsed or chemotherapy refractory B-cell leukemias. Blood 2011; 118(18):4817-28; PMID:21849486; http://dx.doi.org/10.1182/blood-2011-04-348540
5. Eshhar Z. The T-body approach: redirecting T cells with antibody binding and improve T cell persistence and antitumor efficacy. Mol Ther 2015; 23(4):757-68; PMID:25366031; http://dx.doi.org/10.1038/mt.2014.208
6. Overdijk MB, Verploegen S, Ortiz Buijsse A, Vink T, Leusen JH, Bleeker WK, Parren PW. Crosstalk between human IgG isotypes and murine effector cells. J Immunol 2012; 189(7):3430-8; PMID:22956577; http://dx.doi.org/10.4049/jimmunol.1200356
7. Hombach A, Hombach AA, Abken H. Adoptive immunotherapy with genetically engineered T cells: modification of the IgG1 Fc ‘space’ domain in the extracellular moiety of chimeric antigen receptors avoids ‘off-target’ activation and unintended initiation of an innate immune response. Gene Ther 2010; 17(10):1206-13; PMID:20555360; http://dx.doi.org/10.1038/gt.2010.91
8. Bruhns P, Iannascoli B, England P, Mancardi DA, Fernandez N, Jorieux S, Daeron M. Specificity and affinity of human Fc gamma receptors and their polymorphic variants for human IgG subclasses. Blood 2009; 113(16):3716-25; PMID:19018092; http://dx.doi.org/10.1182/blood-2008-09-179754
9. Jonsson F, Daeron M. Mast cells and company. Front Immunol 2012; 3:16; PMID:22566901; http://dx.doi.org/10.3389/fimmu.2012.00016
10. Anurathapalan U, Chan RC, Hindi HF, Muchalra R, Baigain P, Hayes BC, Fisher WS, Heslop HE, Rooney CM, Brenner MK et al. Kinetics of tumor destruction by chimeric antigen receptor-modified T cells. Mol Ther 2014; 22(3):623-33; PMID:24213558; http://dx.doi.org/10.1038/mt.2013.262
11. Smith KG, Clatworthy MR. FcgammaRIIB in autoimmunity and infection: evolutionary and therapeutic implications. Nat Rev Immunol 2010; 10(5):328-43; PMID:20142406; http://dx.doi.org/10.1038/nri2762
12. Gattinoni L, Lugli E, Ji Y, Pos Z, Paulos CM, Quigley MF, Almeida JR, Gostick E, Yu Z, Carpenito C et al. A human memory T cell subset with stem cell-like properties. Nat Med 2011; 17(10):1290-7; PMID:21926977; http://dx.doi.org/10.1038/nm.2446
13. Wang X, Berger C, Wong CW, Forman SJ, Riddell SR, Jensen MC. Engraftment of human central memory-derived effector CD8+ T cells in immunodeficient mice. Blood 2011; 117(6):1888-98; PMID:21123821; http://dx.doi.org/10.1182/blood-2010-10-310599
14. Berger C, Jensen MC, Lansdorp PM, Gough M, Elliott C, Riddell SR. Adoptive transfer of effector CD8+ T cells derived from central memory cells establishes persistent T cell memory in primates. J Clin Invest 2008; 118(1):294-305; PMID:18060041; http://dx.doi.org/10.1172/JCI32103
15. Xie Y, Akpinarli A, Maris C, Hipkiss EL, Lane M, Kwon EK, Muranski P, Restifo NP, Antony PA. Naive tumor-specific CD4 (+) T cells differentiated in vivo eradicate established melanoma. J Exp Med 2010; 207(3):651-67; PMID:20156973; http://dx.doi.org/10.1084/jem.20091921
16. Xu Y, Zhang M, Ramos CA, Durett A, Liu E, Dakhova O, Liu H, Creighton CJ, Gee AP, Heslop HE et al. Closely related T-memory stem cells correlate with in vivo expansion of CAR-CD19-T cells and are preserved by IL-7 and IL-15. Blood 2014; 123(24):3750-9; PMID:24782509; http://dx.doi.org/10.1182/jem.2014-0515274
17. Zeng R, Spolski R, Finkelstein SE, Oh S, Kovanen PE, Hinrichs CS, Pise-Manski LA, Berger C, Gooley TA, Cherian S, Hudecek M, Kossashkull K, Maloney DG, Turtle CJ, Riddell SR. Chimeric antigen receptor-modified T cells derived from defined CD8+ and CD4+ subsets confer superior antitumor activity. J Exp Med 2015; 208(12):2679-89; PMID:26369987; http://dx.doi.org/10.1083/jem.20150513
18. Sommermeyer D, Hudecek M, Kossashkull K, Maloney DG, Turtle CJ, Riddell SR. Chimeric antigen receptor-modified T cells derived from defined CD8+ and CD4+ subsets confer superior antitumor activity in vivo. Leukemia 2016; 30(2):492-500; PMID:26369987; http://dx.doi.org/10.1038/leu.2015.247
19. Turtle CJ, Hanaﬁ LA, Berger C, Goley TA, Cherian S, Hudecek M, Sommermeyer D, Melville K, Pender B, Budiarto TM et al. CD19 CAR-T cells of defined CD4+-CD8+ composition in adult B cell ALL patients. J Clin Invest 2016; 126(6):2123-38; PMID:27111235; http://dx.doi.org/10.1172/JCI85309
20. Cieri N, Camisa B, Cocchiarella F, Forcato M, Oliveira G, Provasi E, Bondanza A, Bordignon C, Peccatori J, Cieri F et al. IL-7 and IL-15 instruct the generation of human memory stem T cells from naive
precursors. Blood 2013; 121(4):573-84; PMID:23160470; http://dx.doi. org/10.1182/blood-2012-05-431718

26. Crompton JG, Sukumar M, Roychoudhuri R, Clever D, Gros A, Eil RL, Tran E, Hanada K, Yu Z, Palmer DC et al. Akt inhibition enhances expansion of potent tumor-specific lymphocytes with memory cell characteristics. Cancer Res 2015; 75(2):290-305; PMID:25432172; http://dx.doi.org/10.1158/0008-5472.CAN-14-2277

27. Guest RD, Hawkins RE, Kirillova N, Cheadle EJ, Arnold J, Neill A, Irlam J, Chester KA, Kemshead JT, Shaw DM et al. The role of extracellular spacer regions in the optimal design of chimeric immune receptors: evaluation of four different scFvs and antigens. J Immunother 2005; 28(3):203-11; PMID:15838376

28. Wilkie S, Picco G, Foster J, Davies DM, Julien S, Cooper L, Arif S, Mather SJ, Taylor-Papadimitriou J, Barrett DM et al. Anti-CD22-chimeric antigen receptors targeting B-cell precursor acute lymphoblastic leukemia. Blood 2013; 121(7):1165-74; PMID:23243285; http://dx.doi.org/10.1182/blood-2012-06-438002

29. Hudecek M, Lupo-Stanghellini MT, Kosasih PL, Sommermeyer D, Jensen MC, Rader C, Riddell SR. Receptor affinity and extracellular domain modifications affect tumor recognition by ROR1-specific chimeric antigen receptor T cells. Clin Cancer Res 2013; 19(12):3153-64; PMID:23620405; http://dx.doi.org/10.1158/1078-0432.CCR-13-0330

30. Kunkele A, Johnson AJ, Rolczynski LS, Chang CA, Hoglund V, Kelly-Spratt KS, Jensen MC. Functional tuning of CARs reveals signaling threshold above which CD8+ CTL antitumor potency is attenuated due to cell Fas-FasL-dependent AICD. Cancer Immunol Res 2015; 3 (4):368-79; PMID:25576337; http://dx.doi.org/10.1158/2326-6066. CIR-14-0200

31. Veras JF, Hoyos V, Savoldo B, Quintarelli C, Giordano Attianese GM, Leen AM, Liu H, Foster AE, Heslop HE, Rooney CM et al. Genetic manipulation of tumor-specific cytotoxic T lymphocytes to restore responsiveness to IL-7. Mol Ther 2009; 17(5):880-8; PMID:19259067; http://dx.doi.org/10.1038/mt.2009.34

32. Leen AM, Sukumaran S, Watabe T, Thomas G, Szigeti K, Davis E, Wahl M, Nisitani S, Yamashiro J, Le Beau MM et al. Prostate stem cell antigen: a cell surface marker overexpressed in prostate cancer. Proc Natl Acad Sci USA 1998; 95(4):1735-40; PMID:9465086

33. Mair C, Iwamoto S, Campana D. Genetic modification of primary natural killer cells overcomes inhibitory signals and induces specific killing of leukemic cells. Blood 2005; 106(1):376-83; PMID:15755898; http://dx.doi.org/10.1182/blood-2004-12-4797

34. Fujisaki H, Kakuda H, Shimasaki N, Imai C, Ma J, Lockey T, Eldridge P, Leung WH, Campana D. Expansion of highly cytotoxic human natural killer cells for cancer cell therapy. Cancer Res 2009; 69(9):4010-7; PMID:19383914; http://dx.doi.org/10.1158/0008-5472.CAN-08-3712

35. Lapteva N, Szmania SM, van Rhee F, Rooney CM. Clinical grade purification and expansion of natural killer cells. Crit Rev Oncog 2014; 19 (1-2):121-32; PMID:24941378

36. Rasheed Z, Wang Q, Matsui W. Isolation of stem cells from human pancreatic cancer xenografts. J Vis Exp 2010 (43); PMID:20972397; http://dx.doi.org/10.3791/2169