

# TCR-independent Activation in Presence of a Src-family Kinase Inhibitor Improves CAR-T Cell Product Attributes

Gauri Lamture, Alan Baer, Joseph W. Fischer, Winston Colon-Moran, and Nirjal Bhattarai

**Summary:** Chimeric antigen receptor expressing T cells (CAR-T cells) have shown remarkable efficacy against some blood cancers and have potential to treat many other human diseases. During CAR-T cell manufacturing, T cells are activated via engagement of the T-cell receptor (TCR); however, persistent TCR engagement can induce unchecked activation, differentiation, and exhaustion, which can negatively affect CAR-T cell product quality and in vivo potency. In addition, T cells may not uniformly respond to TCR-dependent activation (TCR<sub>D</sub>) contributing to lot-to-lot variability, poor expansion, and manufacturing failures. TCR<sub>D</sub> also presents challenges during manufacturing of allogeneic CAR-T cells when endogenous TCR is deleted to prevent graft-versus-host disease. Thus, novel strategies to activate T cells may help improve CAR-T cell product attributes and reduce manufacturing failures. In this study, we compared the effect of TCR<sub>D</sub> and TCR-independent activation (TCR<sub>I</sub>) on CAR-T cell product attributes. We found that TCR<sub>I</sub> in presence of a Src-kinase inhibitor significantly improved CAR-T cell expansion and yield without affecting viability and CD4/CD8 ratio. Markers of T-cell activation, exhaustion and differentiation were also reduced in these CAR-T cells compared with CAR-T cells manufactured by TCR<sub>D</sub>. TCR<sub>I</sub> did not affect CAR-T cell in vitro potency; however, following co-culture with target cells, CAR-T cells manufactured by TCR<sub>I</sub> released significantly less inflammatory cytokines compared with CAR-T cells manufactured by TCR<sub>D</sub>. Together, these data suggest that manufacturing CAR-T cells by TCR<sub>I</sub> activation in the presence of a Src-kinase inhibitor improves product quality attributes and may help reduce manufacturing failures and improve CAR-T cell safety and efficacy in vivo.

**Key Words:** CAR-T cells, immunotherapy, cell therapy, T cell activation, TCR signaling

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Chimeric antigen receptor (CAR) T cells are novel cellular immunotherapies in which T cells are engineered to express a chimeric receptor to selectively kill desired target cells. Currently there are 5 US FDA-approved CAR-T cell therapies for treating certain leukemia and lymphomas.<sup>1</sup> Due to early success and high response rates observed with these novel cellular drugs, many CAR-T cell-based therapies are in development for treating wide variety of human diseases.<sup>2–6</sup>

CAR-T cell manufacturing is a complex, multistep process, which typically takes about 2–3 weeks. During manufacturing, leukapheresis is either collected from patients for autologous products or from healthy donors for allogeneic products. T cells are then isolated, activated, and modified to express a CAR. Modified T cells expressing CAR are expanded for 2–3 weeks to achieve the desired number of CAR-T cells that meets the target cell dose, after which cells are harvested, washed, and formulated for infusion.<sup>7</sup>

T-cell activation is an important step during CAR-T cell manufacturing as this allows for efficient expression of the CAR with retroviral vectors that are commonly used for CAR gene transfer, and the subsequent expansion of CAR-T cells. Currently, antibodies that engage and cross-link the T-cell receptor (TCR) complex (eg, anti-CD3) and co-receptor (eg, anti-CD28) are commonly used to activate T cells.<sup>7</sup> Following TCR engagement, the Src-family kinases such as Lck and Fyn are activated resulting in activation of proximal TCR signaling.<sup>8</sup> TCR-dependent activation (TCR<sub>D</sub>) poses several challenges during CAR-T cell manufacturing. For example, TCR<sub>D</sub> using antibodies can induce unchecked T cell activation, differentiation, exhaustion and apoptosis,<sup>9,10</sup> which may negatively affect CAR-T cell product quality, yield and reduce potency in vivo. Previous studies have also found a correlation between higher numbers of naive and memory T cells with improved CAR-T persistence and function in vivo.<sup>11,12</sup> Expression of exhaustion markers, such as PD-1, LAG-3, and TIM-3 on CAR-T cells has also been associated with poor therapeutic response in patients.<sup>2</sup> Autologous T cells derived from aged patients or patients with chronic disease can also have impaired proximal TCR signaling, which may contribute to lower activation resulting in reduced CAR expression and/or cellular expansion leading to manufacturing failures. In fact, up to 14% failures have been reported during CAR-T cell manufacturing and inadequate cell expansion is the primary reason for these failures.<sup>13–17</sup> Furthermore, during manufacturing of allogeneic CAR-T cells, endogenous TCRs are often deleted to prevent graft-versus-host disease; however, deletion of

TCR renders these cells unresponsive to TCR<sub>D</sub> activation and requires alternative strategies for T-cell activation and cellular expansion.<sup>18</sup>

To address some of these challenges, previous studies have evaluated various strategies to improve CAR-T cell product attributes during manufacturing. For example, inhibition of nuclear receptor transcription factor (NR4A) improved CAR-T cell function in mice in part by reducing T-cell exhaustion.<sup>19</sup> In another study, transient inhibition of lactate dehydrogenase enhanced the generation of memory cells capable of triggering robust antitumor response.<sup>20</sup> Inhibition of the PI3K/Akt signaling pathway by AKT inhibitor during CAR-T cell manufacturing increased expansion, memory cell phenotype, and anti-tumor activity of CAR-T cells by maintaining MAPK activation and promoting FOXO-1 intranuclear localization.<sup>21</sup> Sun et al<sup>22</sup> recently reported that Src-kinase inhibitor (SKI) PP2, reduced basal CD3 phosphorylation in CAR-T cells and regulated chronic CAR-T cell activation by inhibiting Lck function. This study also highlighted an important role of Lck in controlling CAR-T cell activation and function by modulating proximal TCR signaling pathways.

In our prior study, we found that in addition to proximal TCR signaling, Src-kinases also play an important role in regulating distal T-cell signaling, as it was demonstrated that TCR-independent (TCR<sub>I</sub>) activation of human T cells in the presence of a SKI (PP2) enhanced NFAT1 nuclear translocation and NFAT1-mediated distal T cell signaling.<sup>23</sup> As NFAT1 is a critical transcription factor required for T-cell activation, proliferation, and effector function, we hypothesized that TCR<sub>I</sub> activation of T cells in the presence

of a SKI may help address some of the challenges associated with TCR<sub>D</sub> and improve CAR-T cell product attributes.

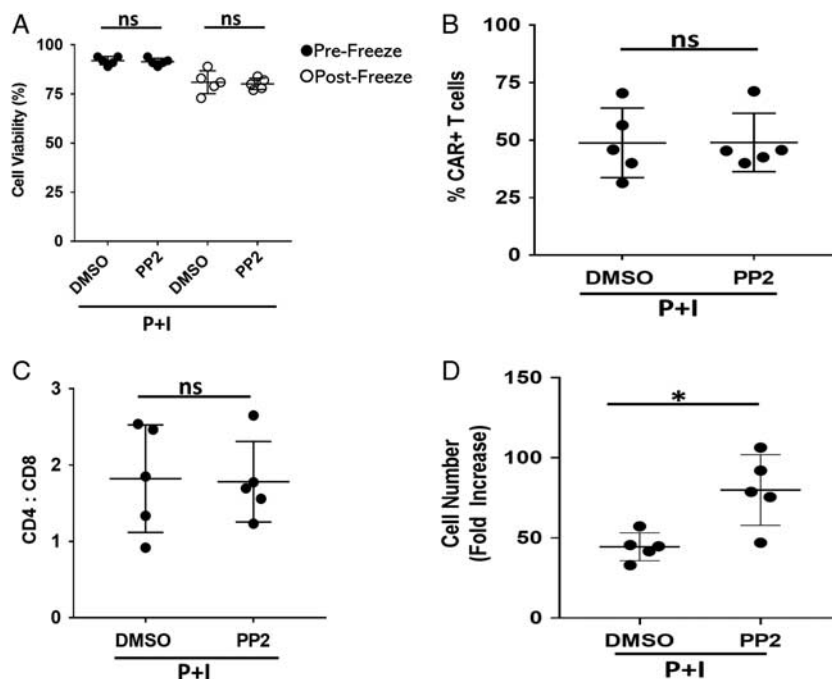
In this study, we assessed the effect of TCR<sub>I</sub> activation in the presence of PP2 on CAR-T cell product attributes and compared the results with CAR-T cells manufactured by traditional TCR<sub>D</sub>.

## RESULTS

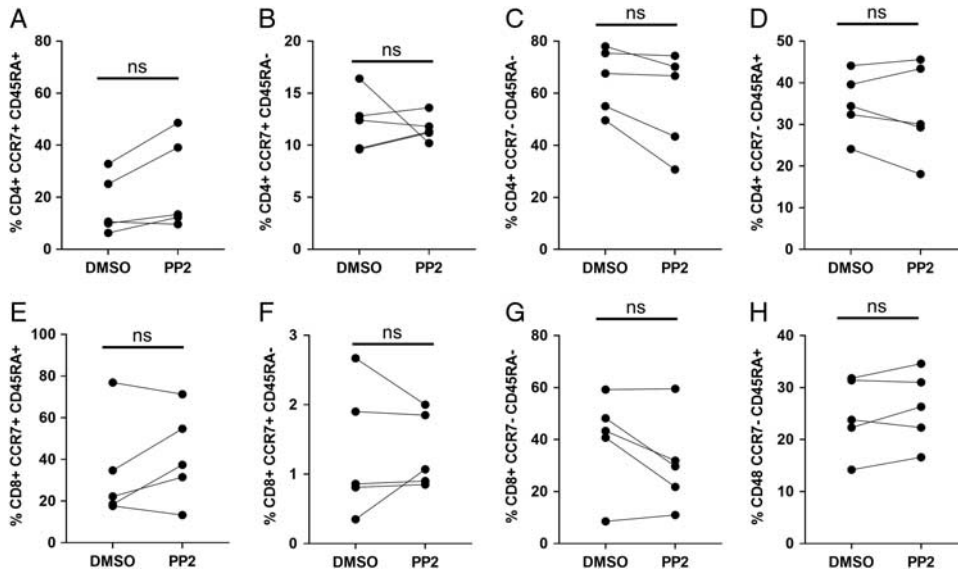
### SKI Does Not Affect CAR-T Cell Product Attributes

We previously used SKI PP2, and found that TCR<sub>I</sub> activation of human T cells in the presence of PP2 enhanced distal T cell signaling mediated by NFAT1.<sup>23</sup> Before performing studies comparing the effect of TCR<sub>I</sub> to TCR<sub>D</sub> on CAR-T cell product attributes, we assessed the effect of PP2 on CAR-T cells to ensure that it does not adversely affect CAR-T cells.

To this end, primary human T cells were treated with PP2, and TCR<sub>I</sub> activation was induced by using PMA/Ionomycin (P+I). Activation with PMA and ionomycin bypasses the TCR and activates distal T-cell signaling pathways in a TCR<sub>I</sub> manner through activation of protein kinase C (PKC) and calcineurin.<sup>23</sup> As a control, T cells from the same donors were also treated with DMSO and activated with P+I. Activated T cells were then transduced with a lentiviral vector to express anti-CD19 CAR. Following transduction, cells were washed to remove PP2 and P+I and expanded for 14 days, after which CAR-T cells were evaluated. Our results show indicate that PP2 treatment did not affect CAR-T cell viability at either harvest (prefreeze) or



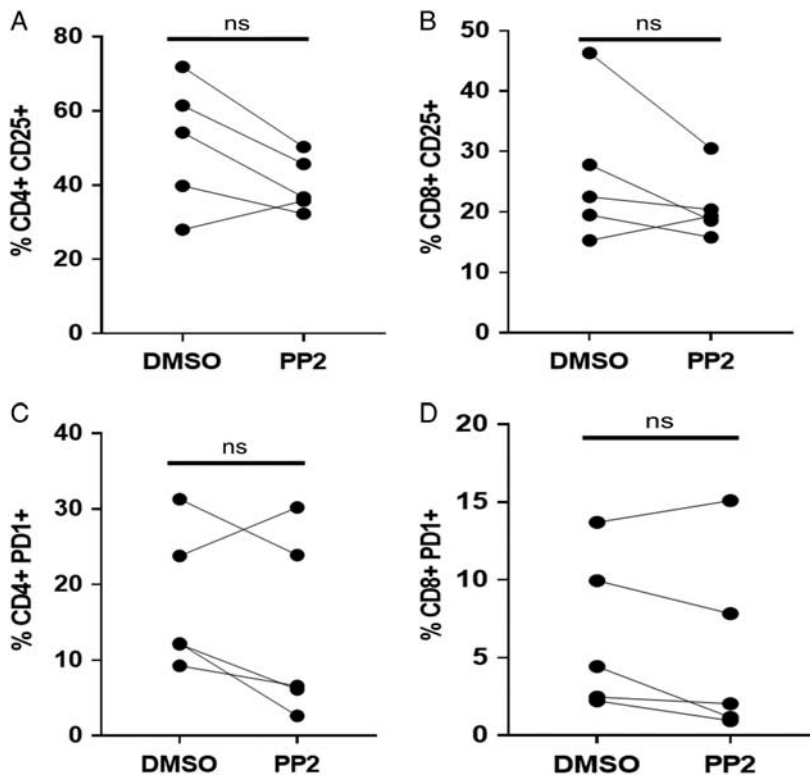
**FIGURE 1.** Effect of Src-kinase inhibitor (PP2) on chimeric antigen receptor expressing T cell (CAR-T cell) product attributes. Primary human T cells were treated with DMSO or PP2 and activated in a TCR-independent manner using PMA and Ionomycin (P+I). Following activation, T cells were transduced with a lentiviral vector to express CD19 CAR. Following 14 days expansion, CAR-T cells were harvested and analyzed for (A) viability, (B) %CAR expression, (C) CD4/CD8 ratio, and (D) cell number. Data represent the average of 5 donors, and the sSD is shown. \* $P < 0.05$ . ns indicates not significant.



**FIGURE 2.** Src-kinase inhibitor (PP2) does not affect T cell subsets. T cells subsets were measured in CAR-T cells manufactured following DMSO or PP2 treatment and TCR-independent activation. Naive, CCR7<sup>+</sup>CD45RA<sup>+</sup> (A, E); central memory, CCR7<sup>+</sup>CD45RA<sup>-</sup> (B, F); effector memory, CCR7<sup>-</sup>CD45RA<sup>-</sup> (C, G); and terminally differentiated effector memory cells (T<sub>EMRA</sub>), CCR7<sup>-</sup>CD45RA<sup>+</sup> were measured in CD4 and CD8 T cells (D, H). Data obtained from 5 donors is shown. ns indicates not significant.

following cryopreservation and thaw (postfreeze) compared with T cells treated with DMSO control (Fig. 1A). Furthermore, CAR expression as measured by %CAR<sup>+</sup> T cells and the CD4/CD8 ratio did not change following PP2

treatment (Figs. 1B, C). It is interesting to note that PP2 treatment significantly improved cell expansion resulting in higher cellular yields after 14 days of expansion compared with DMSO treatment (Fig. 1D).



**FIGURE 3.** Src-kinase inhibitor (PP2) does not affect T cell activation and exhaustion. Effect of PP2 on T-cell activation and exhaustion was assessed by measuring surface expression of (A, B) CD25 (activation) and (C, D) PD-1 (exhaustion) on CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Data obtained from 5 donors is shown. ns indicates not significant.

To ensure that increased cellular expansion in PP2 treatment condition did not affect T-cell differentiation, T-cell subsets were also assessed as previously described.<sup>24</sup> Naive ( $CCR7^+CD45RA^+$ ), central memory ( $CCR7^+/CD45RA^-$ ), effector memory ( $CCR7^-/CD45RA^-$ ) and terminally differentiated effector memory cells ( $T_{EMRA}$ ,  $CCR7^-/CD45RA^+$ ) were measured in PP2 treated CAR-T cells and compared with DMSO treated CAR-T cells. PP2 treatment did not significantly change T-cell subsets compared with the DMSO control in both CD4 (Figs. 2A–D) and CD8 (Figs. 2E–H) T cells. This suggests that PP2 treatment does not alter T-cell subsets during ex vivo manufacturing of CAR-T cells compared with a DMSO control.

Next, we assessed if PP2 treatment affects T-cell activation or exhaustion. Surface expression of CD25 (activation marker) and PD-1 (exhaustion marker) were assessed by flow cytometry. We found that neither CD25 (Figs. 3A, B) nor PD-1 expression (Figs. 3C, D) significantly changed in CAR-T cells manufactured following PP2 treatment compared with CAR-T cells manufactured following DMSO treatment.

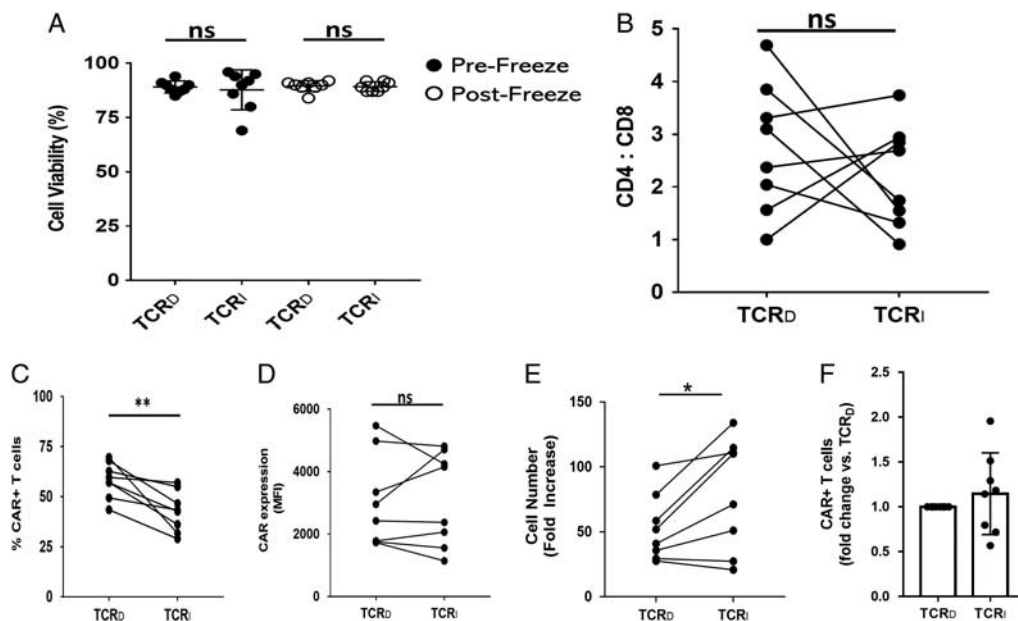
Together, these data suggest that PP2 treatment does not affect CAR-T cell viability, CAR expression, CD4/CD8 ratio, activation, differentiation, and exhaustion compared with DMSO treatment. Furthermore, these data also suggest that PP2 treatment may promote cellular expansion and increase yield during the manufacture of CAR-T cells.

### TCR<sub>I</sub> Activation Significantly Improves Cellular Expansion of CAR-T Cells

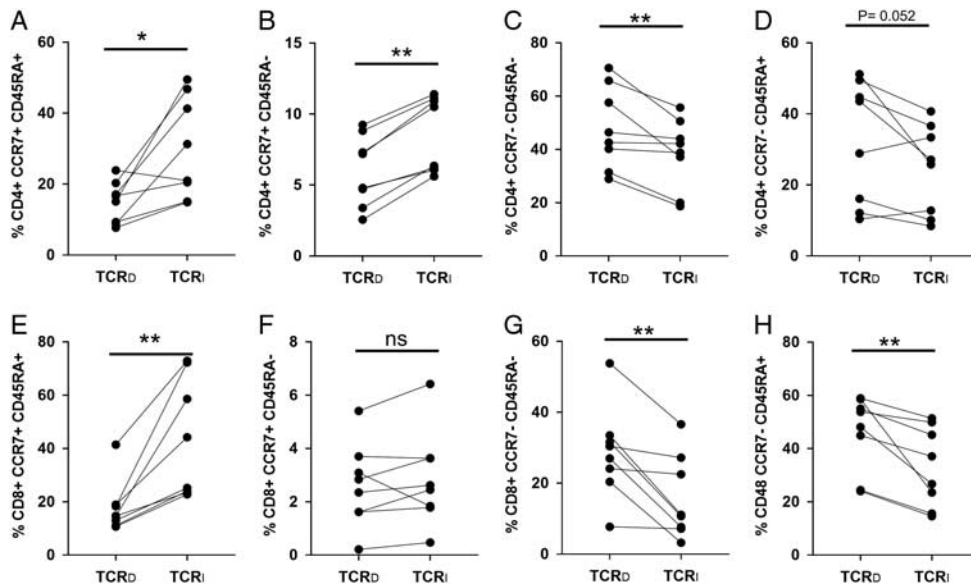
As we found that PP2 treatment does not negatively affect CAR-T cell attributes, we next compared CAR-T cells manufactured following TCR<sub>I</sub> activation in the presence of PP2 against CAR-T cells manufactured following traditional TCR<sub>D</sub> activation. The current standard manufacturing process for CAR-T cells use antibodies such as

anti-CD3 and anti-CD28 that engage with the TCR and the co-receptor for T-cell activation.<sup>7,18,25,26</sup> This method activates the proximal TCR signaling pathways, of which Src-family kinases play an essential role.<sup>14,23,27,28</sup> It has been well established that autologous CAR-T cell products manufactured from patient's T cells are prone to lot-to-lot variation, heterogeneous TCR expression, and may not have optimal proximal TCR signaling.<sup>14,29</sup> During CAR-T cell manufacturing, these factors can contribute to sub-optimum cellular expansion resulting in failure to meet established quality requirements. Inadequate cellular expansion and failure to meet the required clinical dose has been one of the major reasons for lot failures during CAR-T cell manufacturing.<sup>17,29,30</sup> As noted previously, we found that following TCR<sub>I</sub> and PP2-treatment, CAR-T cell expansion was significantly improved compared with the DMSO treatment, to this end, we next compared the effect of TCR<sub>I</sub> (PI) in the presence of PP2, with TCR<sub>D</sub> (anti-CD3/CD28) on CAR-T cell product attributes.

Primary human T cells were activated with either anti-CD3/CD28 for TCR<sub>D</sub> or PMA/Ionomycin in the presence of PP2 for TCR<sub>I</sub>. Activated cells were then transduced with a lentiviral vector to express anti-CD19 CAR, and expanded for 14 days, after which both CAR-T cell manufacturing schemes were compared and evaluated. We found that cell viability was not significantly different between CAR-T cells manufactured via TCR<sub>D</sub> or TCR<sub>I</sub> at harvest (prefreeze) or following cryopreservation (postfreeze) (Fig. 4A). Prior studies have found a correlation between higher CD4/CD8 ratios in the starting leukapheresis and improved clinical outcomes during CAR-T cell therapy.<sup>31,32</sup> Thus, to ensure that TCR<sub>I</sub> does not change the CD4/CD8 ratio in the final CAR-T cell product compared to TCR<sub>D</sub>, CD4/CD8 ratios were assessed. While there was considerable variation between donors with respect to CD4/CD8 ratios; it did not



**FIGURE 4.** Effect of T-cell receptor (TCR)-dependent and TCR-independent activation on CAR-T cell product attributes. Primary human T cells were activated with either anti-CD3/CD28 (TCR-dependent, TCR<sub>D</sub>) or PMA/Ionomycin in presence of PP2 (TCR-independent, TCR<sub>I</sub>). Following activation, T cells were transduced with a lentiviral vector to express CD19 CAR. Following 14 days expansion, CAR-T cells were harvested and analyzed for (A) viability, (B) CD4/CD8 ratio, (C) %CAR expression, (D) CAR MFI (E) total cell number, and (F) CAR+ T cell number. Data represent the average of 8 donors, and the SD is shown. \* $P < 0.05$ , \*\* $P < 0.01$ . ns indicates not significant.



**FIGURE 5.** T-cell receptor (TCR)-independent activation increases naive and central memory T-cell subsets and reduces effector memory subsets compared to TCR-dependent activation. T-cell subsets were measured in CAR-T cells manufactured following TCR-dependent (TCR<sub>D</sub>) or TCR-independent (TCR<sub>I</sub>) activation. Naive (A, E), CCR7<sup>+</sup>CD45RA<sup>+</sup>; central memory, CCR7<sup>+</sup>CD45RA<sup>-</sup> (B, F); effector memory, CCR7<sup>-</sup>CD45RA<sup>-</sup> (C, G); and terminally differentiated effector memory cells (T<sub>EMRA</sub>), CCR7<sup>-</sup>CD45RA<sup>+</sup> were measured in CD4 and CD8 T cells (D, H). Data obtained from 8 donors is shown. \**P*<0.05, \*\**P*<0.01. ns indicates not significant.

change significantly between CAR-T cells manufactured following TCR<sub>I</sub> compared to TCR<sub>D</sub> (Fig. 4B). It is interesting to note that while the frequency of CAR<sup>+</sup> cell was significantly lower in CAR-T cells manufactured by TCR<sub>I</sub> (Fig. 4C), CAR expression as measured by mean fluorescence intensity (MFI) was not different (Fig. 4D). Finally, consistent with previous observation (Fig. 1D), we found that TCR<sub>I</sub> significantly improved cellular expansion and yield compared to TCR<sub>D</sub> (Fig. 4E), as 5 of 8 donors had significantly increased CAR<sup>+</sup> T-cell numbers (Fig. 4F). Since equal number of cells were plated in both conditions at the start of expansion, and all other factors except the method of T-cell activation was identical in both conditions, the observed difference in cellular yield is likely due to the proposed TCR<sub>I</sub> of T cells.

Together, these data suggest that TCR<sub>I</sub> in the presence of PP2 significantly improves CAR-T cell expansion and yield without significantly affecting viability, CD4/CD8 ratios and CAR expression.

### TCR<sub>I</sub> Activation Enriches Naive and Memory T-Cell Subsets in CAR-T Cell Product

Previous studies have assessed the role of various T-cell subsets on the safety and efficacy of CAR-T cells. CAR-T cells containing higher numbers of central memory or stem-like memory cells have been associated with improved expansion, persistence and efficacy *in vivo*.<sup>33,34</sup> Furthermore, single-cell analysis of various CAR-T cell subsets have revealed that different populations of cells contribute to effector function,<sup>35</sup> with one study demonstrating that increased frequency of CD8<sup>+</sup>CD45RA<sup>+</sup>CCR7<sup>+</sup> (naive T cells) correlated with significantly improved CAR-T cell expansion in lymphoma patients.<sup>36</sup> Together, these studies suggest that CAR-T cell products containing higher numbers of naive or memory T-cell subsets may have beneficial effects *in vivo*. To assess the effect of TCR<sub>I</sub> on T-cell subsets, CAR-T cells manufactured by either TCR<sub>I</sub> or TCR<sub>D</sub> were analyzed for naive (CCR7<sup>+</sup>CD45RA<sup>+</sup>),

central memory (CD45RA<sup>-</sup>CCR7<sup>+</sup>), effector memory (CD45RA<sup>-</sup>CCR7<sup>-</sup>) and TEMRA (CD45RA<sup>+</sup>CCR7<sup>-</sup>) by flow cytometry.

We found that CAR-T cells manufactured via TCR<sub>I</sub> had a significantly higher frequency of naive and central memory T cells among the CD4 compartment, when compared with CAR-T cells manufactured via TCR<sub>D</sub> (Figs. 5A, B). Similarly, a significantly higher frequency of naive cells was also found in the CD8 compartment; however, central memory cells were not significantly different (Figs. 5E, F). Consistent with these results, the frequency of effector memory cells in both CD4 and CD8 compartments and TEMRA cells in the CD8 compartment were significantly reduced in CAR-T cells manufactured by TCR<sub>I</sub> compared with CAR-T cells manufactured by TCR<sub>D</sub> (Figs. 5C, G, H). Although the difference in frequency of CD4<sub>TEMRA</sub> did not reach statistical significance (*P*=0.052), a consistent trend of lower frequencies of CD4<sub>TEMRA</sub> cells were observed in the majority of CAR-T cell manufactured by TCR<sub>I</sub> (Fig. 5D).

Together, these data suggest that TCR<sub>I</sub> in the presence of PP2 results in higher frequency of naive and central memory cells in the CAR-T cell product compared with TCR<sub>D</sub>.

### CAR-T Cells Manufactured Via TCR<sub>I</sub> Process Have Reduced Expression of Activation and Exhaustion Markers

It has been well documented that chronic T-cell activation and exhaustion can impair T-cell effector function,<sup>37</sup> and that persistent TCR engagement with anti-CD3 can result in chronic activation and exhaustion of CAR-T cells. In clinical and nonclinical studies, T-cell exhaustion has been increasingly associated with CAR-T cell dysfunction, and various strategies to reduce T-cell exhaustion are in development.<sup>33,38-40</sup> Furthermore, higher frequencies of CD8<sup>+</sup>PD1<sup>-</sup> cells in the CAR-T cell product has been associated with an improved clinical response in leukemia

patients.<sup>33</sup> To assess if CAR-T cells manufactured via TCR<sub>I</sub> in the presence of PP2 reduces unwanted activation and exhaustion of CAR-T cells, surface expression of CD25 (activation marker) and PD-1 (exhaustion maker) were assessed by flow cytometry. We found that the frequency of activated CD8<sup>+</sup>CD25<sup>+</sup> cells was significantly reduced in CAR-T cells manufactured via TCR<sub>I</sub> compared with the TCR<sub>D</sub> process (Fig. 6A). Although, the difference between the CD4<sup>+</sup>CD25<sup>+</sup> T-cell percentage did not reach statistical significance between CAR-T cells manufactured via the 2 different processes, 5 of 8 lots manufactured by the TCR<sub>I</sub> process had lower frequencies of CD4<sup>+</sup>CD25<sup>+</sup> cells compared with TCR<sub>D</sub> (Fig. 6A). T-cell exhaustion as measured by CD4<sup>+</sup>PD1<sup>+</sup> and CD8<sup>+</sup>PD1<sup>+</sup> cells was also significantly reduced in CAR-T cell products manufactured via TCR<sub>I</sub> compared with the TCR<sub>D</sub> (Fig. 6B).

In clinical studies, a specific population of T cells (CD8<sup>+</sup>CD27<sup>+</sup>PD1<sup>-</sup>) in the CAR-T cell product has been significantly associated with better clinical outcomes in

chronic lymphocytic leukemia patients.<sup>33</sup> A higher percentage of CD8<sup>+</sup>CD27<sup>+</sup> cells has been also associated with improved efficacy in melanoma patients.<sup>41</sup> Since, we observed a reduction in PD1<sup>+</sup> cells in CAR-T cells manufactured via TCR<sub>I</sub>, we next assessed the frequency of CD8<sup>+</sup>CD27<sup>+</sup>PD1<sup>-</sup> cells. We found that CAR-T cells manufactured following TCR<sub>I</sub> had a significantly higher percentage of CD8<sup>+</sup>CD27<sup>+</sup>PD1<sup>-</sup> cells compared with CAR-T cells manufactured following TCR<sub>D</sub> (Fig. 6C).

Together, these data suggest that TCR<sub>I</sub> results in CAR-T cells with lower activation and exhaustion profiles and may also increase the frequency of a specific and favorable populations of CD8<sup>+</sup>CD27<sup>+</sup>PD1<sup>-</sup> cells that have been associated with improved clinical outcomes.

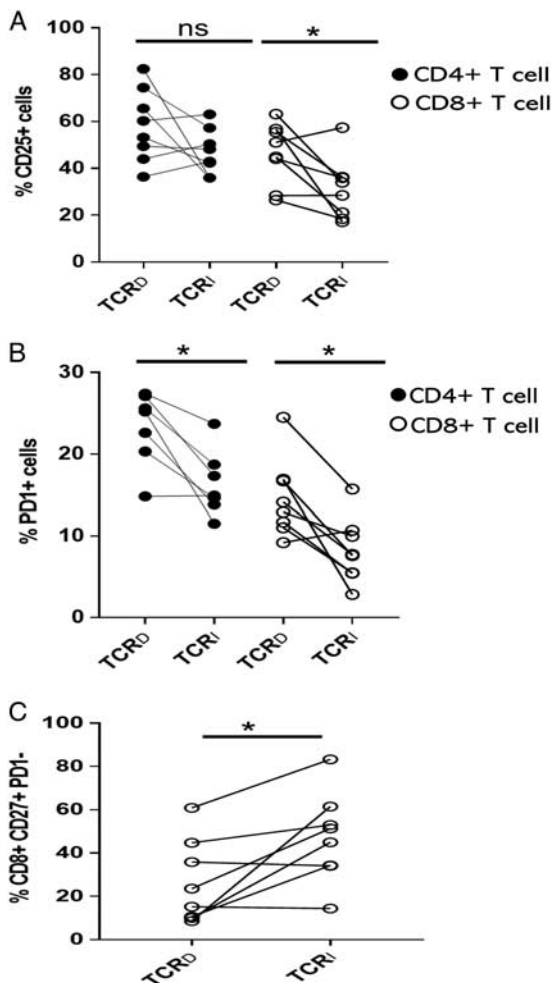
### CAR-T Cells Manufactured Via TCR<sub>I</sub> Process Secrete Significantly Lower Levels of Inflammatory Cytokines

One of the hallmark challenges with CAR-T cell therapy is the development of systemic inflammatory toxicities known as cytokine release syndrome (CRS), neurotoxicity and in some patients development of macrophage activation syndrome/hemophagocytic lymphohistiocytosis, following CAR-T cell infusion.<sup>42,43</sup> Although the mechanisms contributing these inflammatory toxicities are not completely understood, previous studies have suggested that inflammatory cytokines produced by CAR-T cells contribute to these systemic inflammatory toxicities in part by activating bystander myeloid cells.<sup>42,44-47</sup> T-cell-derived proinflammatory cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and IFN- $\gamma$  have been suggested to play a role in activating these bystander myeloid cells,<sup>44,48</sup> thus, various strategies to modulate expression of these cytokines have been proposed to improve the safety of CAR-T cells.<sup>44,45,49</sup>

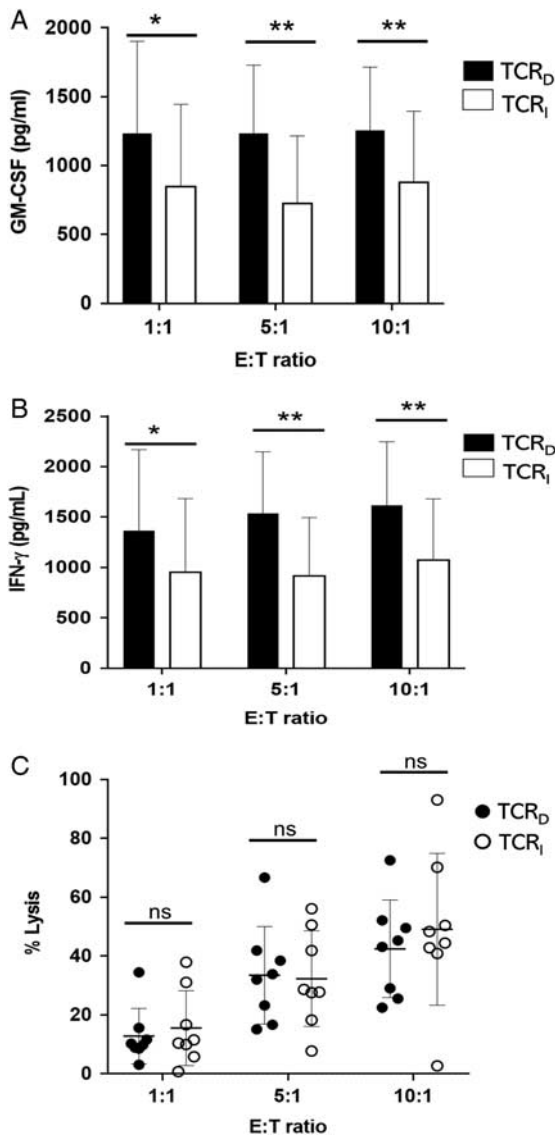
To assess if TCR<sub>I</sub> has any effect on inflammatory cytokine production by CAR-T cells, CD19 CAR-T cells were manufactured following TCR<sub>I</sub> or TCR<sub>D</sub>. CAR-T cells (effector) were co-cultured with CD19<sup>+</sup> Nalm6 cells (target) at various effector to target ratio. Following 24-hour co-culture, cell-free supernatant was assessed for GM-CSF and IFN- $\gamma$  by ELISA. We found that both GM-CSF (Fig. 7A) and IFN- $\gamma$  (Fig. 7B) levels were significantly lower in co-culture supernatant obtained from CAR-T cells manufactured via TCR<sub>I</sub> compared with co-culture supernatant obtained from CAR-T cells manufactured via TCR<sub>D</sub> at all effector to target (E: T) ratios.

To ensure that TCR<sub>I</sub> does not negatively affect CAR-T cell potency, we next assessed CAR-T cell-mediated cytotoxicity. CD19 CAR-T cells (effector) that were either manufactured following TCR<sub>I</sub> or TCR<sub>D</sub> were co-cultured with CD19<sup>+</sup> Nalm6 cells (target) expressing luciferase at various effector to target ratio. Following 24 hours of co-culture, CAR-T cell-mediated cytotoxicity was measured by assessing lysis of target cells as described in the methods section. We found that cytotoxicity (in vitro potency), as measured by the lysis of Nalm6 cells, was not significantly different between CAR-T cells manufactured following TCR<sub>I</sub> compared with TCR<sub>D</sub> at all effector to target ratios (Fig. 7C).

Together, these data suggest that the manufacture of CAR-T cells via TCR<sub>I</sub> reduces the production of inflammatory cytokines but does not affect potency compared with CAR-T cells manufactured via traditional TCR<sub>D</sub>.



**FIGURE 6.** T-cell receptor (TCR)-independent activation reduces expression of activation and exhaustion markers in CAR-T cells compared TCR-dependent activation. Surface expression of markers for (A) T cell activation (CD25) and (B) exhaustion (PD-1) on CD4 and CD8 T cells and (C) percentage of CD8<sup>+</sup>CD27<sup>+</sup>PD1<sup>-</sup> cells were assessed in CAR-T cells manufactured either by TCR-dependent (TCR<sub>D</sub>) or TCR-independent (TCR<sub>I</sub>) process. \**P* < 0.05. ns indicates not significant.



**FIGURE 7.** Chimeric antigen receptor expressing T cells (CAR-T cells) manufactured via T-cell receptor (TCR)-independent process secrete significantly lower levels of inflammatory cytokines. Following co-culture with CD19<sup>+</sup> Nalm6 (target cells, T), inflammatory cytokines (A) GM-CSF and (B) IFN<sub>γ</sub> released by CAR-T cells (effector cells, E) and (C) cytotoxicity (in vitro potency) of CAR-T cells manufactured either by TCR-dependent (TCR<sub>D</sub>) or TCR-independent (TCR<sub>I</sub>) process were measured at various effector to target (E: T) ratios. For cytokines, data represent the average of 8 donors, and the SD is shown. Cytotoxicity of CAR-T cells from all 8 donors is shown. \**P* < 0.05, \*\**P* < 0.01. GM-CSF indicates granulocyte-macrophage colony-stimulating factor; ns, not significant.

**DISCUSSION**

Current ex vivo manufacturing processes for CAR-T cells are complex, consisting of numerous steps spanning multiple days and is technically challenging.<sup>7</sup> T-cell activation is a critical and essential process during CAR-T cell manufacturing, as it allows for efficient cellular expansion to achieve the desired target dose. Furthermore, it allows for efficient transduction, as retroviral vectors are often used for CAR gene transfer. Current manufacturing processes use

antibodies that cross-link the TCR and a co-receptor such as anti-CD3 and anti-CD28 for T-cell activation. This method activates the proximal/upstream TCR signaling pathways mediated by Src-family of kinases such as Lck and Fyn. However, T-cell activation via TCR engagement presents many challenges as persistent stimulation via antibodies can induce unchecked T-cell activation, differentiation, exhaustion and apoptosis,<sup>9,10</sup> which may negatively affect CAR-T cell product quality, yield and reduce potency in vivo. Furthermore, T cells obtained from aged patients or patients with chronic disease for autologous CAR-T cell manufacture may have impaired proximal TCR signaling, resulting in manufacturing failures.<sup>13–16</sup> In addition, TCR<sub>D</sub> activation may not be feasible for certain allogeneic CAR-T cell products where endogenous TCRs are deleted to prevent graft-versus-host disease. These challenges need to be addressed to improve CAR-T cell product quality and help promote its widespread use.

In this study, we found that T cell activation in a TCR<sub>I</sub> manner, in the presence of an SKI (PP2), significantly improved CAR-T cell expansion and reduced activation, differentiation and exhaustion compared to CAR-T cells manufactured by activating T cells in a TCR<sub>D</sub> manner. Previously, we found that in addition to proximal TCR signaling pathways, Src-family kinases also regulate distal T-cell signaling, and that inhibition of Src-kinases significantly improved distal T-cell signaling mediated by NFAT1.<sup>23</sup> Since NFAT1 is an essential transcription factor that modulates activation, differentiation and effector function of T cells,<sup>23,50</sup> the improvement of product attributes observed in CAR-T cells manufactured following TCR<sub>I</sub> activation in the presence of PP2 may be due to modulation of NFAT1 activity. These results are significant to the field, as clinical manufacturing failures for autologous CAR-T cells in leukemia and lymphoma patients ranging from 1% to 14%.<sup>17</sup> In most cases, these failures are due to poor CAR-T cell expansion resulting in failure to meet target dose.<sup>17</sup> Remanufacturing may not be always feasible due to patient health condition or other ongoing treatments and is costly. Thus, alternative strategies such as TCR<sub>I</sub> activation during CAR-T cell manufacturing, which significantly improved cellular expansion and yield, may help reduce manufacturing failures.

CAR-T cells with a higher proportion of naive and memory cells and lower proportions of differentiated effector cells have also been associated with better persistence and activity in vivo.<sup>33,34,36,51</sup> We found that TCR<sub>I</sub> activation resulted in higher levels of naive and central memory cells and lower levels of effector cells in the CAR-T cell product. This suggests that CAR-T cells manufactured following TCR<sub>I</sub> activation may have better persistence and improved function in vivo.

Similarly, chronic T-cell activation and exhaustion have been suggested to negatively affect CAR-T cell persistence and function.<sup>52</sup> Many preclinical studies have demonstrated that T-cell exhaustion significantly limits CAR-T cell function in vivo.<sup>38–40</sup> Other studies have found correlations between increased levels of CD8<sup>+</sup>CD27<sup>+</sup>PD1<sup>-</sup> cells in CAR-T cell product with higher rates of response,<sup>33</sup> and the CD8<sup>+</sup>CD27<sup>+</sup>PD1<sup>-</sup> population was shown to increase STAT3 activation, which resulted in persistence and long-term remission.<sup>33</sup> A loss in tumor control in NSG mice was also observed when the CD8<sup>+</sup>CD27<sup>+</sup>PD1<sup>-</sup> population was removed.<sup>33</sup> In our study, we found that TCR<sub>I</sub> activation reduced T-cell activation and exhaustion and increased the frequency of CD8<sup>+</sup>CD27<sup>+</sup>PD1<sup>-</sup> cells in the CAR-T cell product. CD27 is an important surface marker

associated with memory cells and its expression is dependent on calcium ionophores such as ionomycin,<sup>53</sup> thus, we speculate that the use of ionomycin during TCR<sub>1</sub> activation may have facilitated CD27 expression and reduced unwanted chronic T-cell activation and exhaustion.

CAR-T therapy is also associated with systemic inflammatory toxicities, known as CRS, along with neurotoxicity.<sup>48,54,55</sup> During these inflammatory toxicities, cytokines such as GM-CSF and IFN- $\gamma$  are released at high levels.<sup>48,56</sup> These inflammatory cytokines, released by activated CAR-T cells, in turn activate bystander immune cells such as monocytes and macrophages, which then produce IL-6, IL-1 $\beta$ , etc., and contributes to a cascading response of CRS and neurotoxicity.<sup>46,47,54</sup> It is interesting to note that we found that CAR-T cells manufactured following TCR<sub>1</sub> activation produced significantly less GM-CSF and IFN- $\gamma$  following co-culture with target cells compared with CAR-T cells manufactured via TCR<sub>D</sub> activation. Although the mechanism for lower inflammatory cytokine expression by CAR-T cells manufactured following TCR<sub>1</sub> activation is not clear, current studies are under way to gain mechanistic insights into this observation. Importantly, TCR<sub>1</sub> activation did not affect CAR-T cell cytotoxicity in vitro, suggesting that TCR<sub>1</sub> activation may improve CAR-T cell product safety by reducing release of inflammatory cytokines, without affecting its potency.

In our study, we used CAR-T cells manufactured from healthy donor T cells to assess the effects of TCR<sub>1</sub> activation on CAR-T cell product quality attributes. While this has direct application to allogenic CAR-T cell products (typically relying on healthy donors), in light of the autologous nature of most current CAR-T cell products, it would have been helpful to also assess the effect of TCR<sub>1</sub> activation on CAR-T cells using diverse patient T cells; however, access to patient cells presents a limiting factor. Nevertheless, prior studies have demonstrated that healthy donor T cells are an appropriate surrogate of patient T cells for CAR-T cell product development, and they are routinely used during preclinical assessments.<sup>7,57</sup> Thus, we believe that these studies performed using healthy donor T cells are informative and applicable for CAR-T cell product development.

In conclusion, our data suggests that the proposed method of TCR<sub>1</sub> T-cell activation for CAR-T cell manufacturing may improve numerous product quality attributes. In addition, the use of a synthetically derived and inexpensive chemicals (PI and PP2), provides a significant reduction in expense, along with an improved safety profile when compared with traditional animal tissue derived antibodies. While the mechanism for improved CAR-T cell product attributes following TCR<sub>1</sub> activation is not clearly understood, current studies are underway to address this. Our previous study found that TCR<sub>1</sub> activation in presence of SKI increased NFAT1 activity.<sup>23</sup> Since NFAT1 plays an important role in T-cell activation, expansion and function, the observed improvement in CAR-T cell attributes following TCR<sub>1</sub> activation in the presence of an SKI may be due to increased activity of NFAT1 in these cells. Future studies are warranted to better understand mechanisms for improvement of these product quality attributes, which may help develop strategies to improve CAR-T cell manufacturing, safety, efficacy and reduce manufacturing failures.

## METHODS

### Peripheral Blood Mononuclear Cells (PBMCs)

Healthy donor buffy coats were obtained from the National Institutes of Health (NIH) Blood Bank. All the

donors provided written consent for their blood products to be used in research projects and all the samples were de-identified. This study was exempted by the FDA's IRB, Research Involving Human Subject Committee (RIHSC). PBMCs were isolated using Ficoll-Hypaque gradient centrifugation. Isolated PBMCs were washed in PBS and resuspended in complete RPMI 1640 media supplemented with 2 mM L-glutamine, 100 IU/mL penicillin, 10  $\mu$ g/mL streptomycin and 10% heat-inactivated fetal calf serum, and incubated overnight at 37°C, 5% CO<sub>2</sub>. Following day, T cells in suspension were harvested and the adherent cells were discarded.

### Cell Lines

Nalm6 cells were purchased from ATCC (Manassas, VA) and were cultured in complete RPMI 1640 media and were maintained at 37°C, 5% CO<sub>2</sub>. CRISPR/Cas9 was used to generate a B2M knock out (KO) Nalm6 cell line. The gRNA was designed as previously described.<sup>58</sup> The B2M gRNA (GCCGAGATGTCTCGCTCCG) was cloned into plasmid PX459 (pSpCas9(BB)-2A-Puro; Plasmid #62988) (Addgene, Cambridge, MA). The B2M negative cells were bulk sorted using flow cytometry. A stable luciferase expressing Nalm6 cell line was generated by transducing Nalm6 B2M KO cells with a lentiviral vector encoding firefly luciferase and EGFP (pLentiviro3/TO/V5-GW/EGFP-Firefly Luciferase; plasmid #119816) (Addgene).

### Lentiviral Vector Production

Plasmid encoding CD19 CAR [Lenti-anti-CD19- scFv -h (CD28-41BB-CD3z)] was purchased from Creative Biolabs. HEK 293T cells were used for the lentiviral vector production. Briefly, plasmids encoding VSV-G envelope, CD19 scFv and packaging plasmid pCD/NL-BH\*DDD (a gift from Dr Jakob Reiser, FDA) were mixed with PEI in Opti MEM, incubated for 15 minutes after which transfection mix was added to the HEK293T cells. Cells were incubated with transfection mix for a minimum of 5 hours, after which cells were washed and incubated at 37°C, 5% CO<sub>2</sub>. Supernatant containing lentiviral vector was collected at 72 hours and concentrated using 100kDa cutoff Amicon Ultra-15 Centrifugal Filter Units (EMD Millipore). Concentrated vector was aliquoted and stored at -80°C until further use. An aliquot of frozen vector was used to determine infectious titer in Jurkat human T cell line. Titer was determined by incubating 1 million Jurkat cells with serially diluted (10-fold dilutions) vector in presence of polybrene (0.8  $\mu$ g/mL). CAR expression was determined at 72 hours using FITC conjugated Protein L (AcroBio) by flow cytometry. Vector titer was determined by following method,  $T = (P \times N) / (D \times V)$ . Where, T = titer (TU/ml); P = % FITC+ cells; N = number of cells at the time of transduction; D = dilution factor; V = total volume of viral inoculum.

### CAR-T Cells

T cells from each donor were split and CAR-T cells were manufactured either by activating T cells in a TCR<sub>D</sub> or TCR<sub>1</sub> manner. In TCR<sub>D</sub> process, T cells were activated using anti-CD3/CD28 activation reagent (Stemcell Technologies Inc.) for 48 hours. This method activates the proximal TCR signaling pathways. In TCR<sub>1</sub> process, T cells were treated with SKI (PP2) (SelleckChem) at 1  $\mu$ g/mL for 16 hours, followed by activation with phorbol 12-myristate 13-acetate (50 ng/mL) and ionomycin (1  $\mu$ g/mL) for 24 hours. This method bypasses the proximal TCR signaling and activates distal T-cell signaling pathways. Activation of T cells with CD3/CD28 for



48 hours was chosen as it is standard practice in the field to allow sufficient T-cell activation for lentiviral vector transduction. However, we found that in our preliminary studies 24 hours activation with PMA/Ionomycin was sufficient to fully activate T cells,<sup>23</sup> which is likely due to its more potent mode of action. Thus, a 24-hour time point was chosen for PI activation. T cells were activated with anti-CD3/CD28 or PMA/Ionomycin were transduced with a lentiviral vector to express CD19 CAR by spinoculation at multiplicity of infection of 10. Briefly, cells were spun at 1800 rpm for 1 hour at 25°C in a 6-well non-tissue culture treated plate coated with RetroNectin (Takara Bio). After spinoculation, cells were incubated at 37°C, 5% CO<sub>2</sub> for at least 5 hours followed by change in culture media. Cells were monitored daily, and cell density was maintained at 0.5×10<sup>6</sup> cells/mL in Immunocult-XF T cell expansion medium (Stemcell Technologies Inc.) supplemented with 5% human AB serum (Stemcell Technologies Inc.), IL-7 (1 ng/mL; PeproTech), and IL-2 (50 ng/mL; PeproTech). Following 14 days of cellular expansion, CAR-T cells were harvested, washed twice in sterile PBS and cryopreserved in cell freezing media (70% FBS, 20% RPMI, 10% DMSO). To assess the effect of PP2 on CAR-T cell product attributes, healthy donor T cells were split, and were treated with either DMSO or PP2 for 16 hours, followed by activation with PMA/Ionomycin for 24 hours. Following activation, CAR-T cells were manufactured as described previously.

### Sampling Time

Cell viability testing was performed before freezing and after freezing CAR-T cells. All other tests were performed on cryopreserved CAR-T cells following thaw.

### Cell Count and Viability

Cell count and viability were determined using automated cell counter (Countess II, ThermoFisher).

### Flowcytometry

Cryopreserved CAR-T cells were thawed and washed with PBS and stained with antibodies at 4°C for 1 hour and washed in PBS. A small aliquot of cells was used to determine CAR expression by staining with FITC conjugated Protein L (AcroBio). For T cell subset analysis following antibodies were used: human CD2 (FITC or APC), CD4 (PE), CD8 (V450), CCR7 (AF647), CD45RA (A700). For activation and exhaustion, CD25 (APC-Cy7), PD-1 (APC), and CD27 (APC-H7) were used. All antibodies were obtained from BioLegend or BD Biosciences. Data was collected using LSR II (BD Biosciences) and analyzed using FlowJo software (Tree Star). Flow cytometry gating strategy and representative panels are shown in Supplemental Figures 1 (Supplemental Digital Content 1, <http://links.lww.com/JIT/A642>) and 2 (Supplemental Digital Content 2, <http://links.lww.com/JIT/A643>).

### Cytotoxicity Assay

Assessment of target cell killing by CAR-T cells (cytotoxicity) is a direct measure of CAR-T cell function. Luminescence based cytotoxicity assay is widely used to assess CAR-T cell function during preclinical and clinical studies.<sup>59</sup> Here to assess cytotoxicity of CAR-T cells, CD19<sup>+</sup> Nalm6 cells (target) expressing firefly luciferase were co-cultured with the CAR-T cells (effector) at an effector to target ratio (E:T) of 1:1, 5:1, and 10:1 in 100 μL of complete RPMI 1640 media in a 96-well plate. A total of 5000 target

cells were co-cultured with either 5000, 25,000 or 50,000 CAR-T cells. As a negative control Nalm6 cells were plated alone, and as a positive control, Nalm6 cells were treated with 10% Triton-X. Following 24 hours, an equal volume of Bright Glo luciferase reagent (100 μL) (Promega) was added to the wells and luminescence was measured using GloMax plate reader (Promega, Madison, WI) in a white walled plate. Cytotoxicity was calculated as Cytotoxicity (% lysis)=100-[(Luminescence for co-culture sample/Luminescence for Nalm6 alone)×100].

### ELISA

Cytokines in the co-culture supernatant was assessed using IFN-γ and GM-CSF ELISA kits (BD Biosciences) following manufacturer's instructions.

### Statistical Analysis

All statistical analyses were performed with Prism 8 software (GraphPad). Paired *t* test or Wilcoxon matched pairs signed rank test was used to compare results between groups. *P*-values <0.05 were considered statistically significant.

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### Conflicts of Interest/Financial Disclosures

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