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Manufacturing and Process Development

Feeder cell-free production of CAR-NK cells via activation of PKC and calcium signaling pathways

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A B S T R A C T

Background: Ex vivo manufacturing of CAR-NK cells is a complex, multistep process involving NK cell activation, genetic modification, and cellular expansion. Currently, tumor cell-derived feeder cells (e.g., K562) are commonly used to manufacture CAR-NK cells. However, the feeder cell-based (FCB) method presents several risks to the CAR-NK product, including contamination, residual impurities, immunogenicity, and lot-to-lot variability. Additionally, establishing and maintaining feeder cells increases costs, complicates scaling up production, and requires rigorous quality control to ensure safety. In contrast, the feeder cell-free (FCF) method can mitigate these risks.

Methods: We used phorbol ester (PMA) and calcium ionophore (ionomycin) to develop a novel FCF method to manufacture CAR-NK cells and compared their critical quality attributes (CQAs) with those produced using a traditional FCB method.

Results: In small-scale production, most CQAs were comparable between the two methods, except for CAR expression, which was lower in the FCF method. However, in large-scale production, CAR expression was comparable between the two methods, and the FCF method significantly improved CAR-NK potency while reducing CAR-NK-mediated myeloid cell activation.

Conclusion: Our data suggest that the FCF method provides a robust and scalable alternative for producing safer and high-quality CAR-NK cells.

Key Words: CAR-NK, feeder-cells, feeder-free, immunotherapy, manufacturing, safety.

Introduction

Natural killer (NK) cells engineered to express a chimeric antigen receptor (CAR) have potential to treat many human diseases. Due to their ability to kill target cells via multiple mechanisms in an HLA-independent manner, favorable safety profile, and feasibility for allogeneic off-the-shelf treatment, CAR-NK cells are being widely developed as advanced therapeutics [1,2]. Although CAR-NK cells have great potential to be effective therapeutics against numerous human diseases, the *ex vivo* manufacturing of CAR-NK cells is a complex process with multiple steps that can span days to weeks [3]. CAR-NK cell manufacturing typically starts with obtaining NK cells from various sources, such as peripheral blood, cord blood, or through the differentiation of stem cells. NK cells are then subjected to activation, genetic modification to express the CAR transgene, and cellular expansion. Once the CAR-NK cell number is sufficient to meet the target dose, cells are harvested, washed, and formulated for infusion [3–5].

During the *ex vivo* manufacturing of CAR-NK cells lentiviral vectors are commonly used for CAR expression as they offer efficient transduction and stable CAR gene integration. However, efficient transduction with lentiviral vector requires NK cell activation [6]. Furthermore, activation is also required for CAR-NK cell expansion. For activation, NK cells are typically co-cultured with irradiated tumor-derived cell lines, such as K562, as feeder cells in the presence of various cytokines [7,8]. Feeder cells secrete various cytokines, provide cell-to-cell interactions, and enhance expression of NK cell activation receptors such as NKG2D, which all facilitate NK cell activation [9]. However, feeder cell-based activation presents significant challenges, including risk of CAR-NK product contamination with adventitious agents from the feeder cells, safety issues due to presence of residual feeder cells in the final drug product and variability in drug product quality due to lot-to-lot variability of feeder cells. Thus, use of feeder cells requires extensive quality control measures to ensure the safety, quality, and potency of CAR-NK drug products. Moreover, establishing, qualifying, and maintaining these feeder cells as cell banks is costly and time-consuming [1].

Due to the various risks and challenges associated with the feeder cell-based system, considerable efforts have been made to develop

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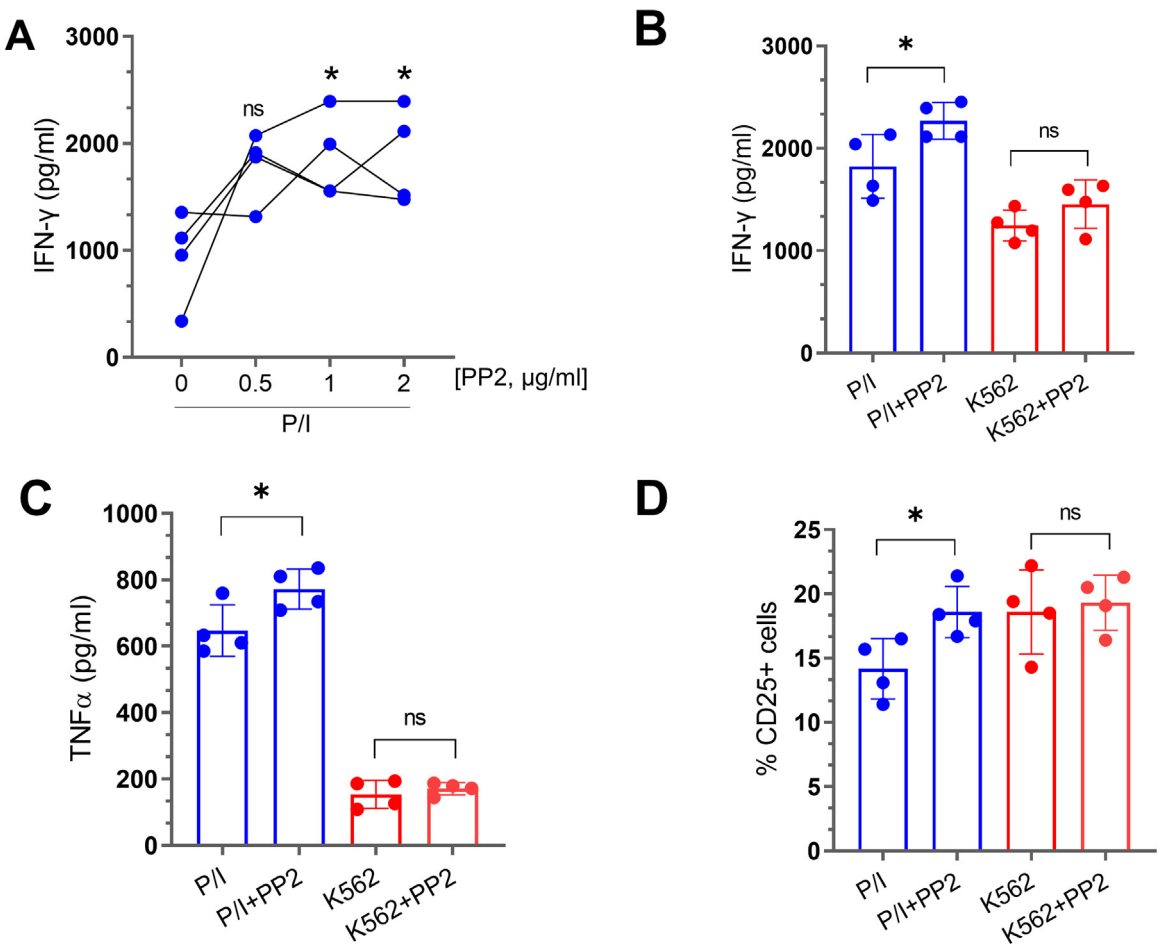

Fig. 1. Src kinase inhibitor enhances feeder cell-free activation of NK cells. Primary NK cells isolated from human healthy donors were treated with Src kinase inhibitor (PP2) for 16 hours and activated using PMA/ ionomycin (P/I) or co-cultured with irradiated K562 (E:T = 1:1) for 24 hours. (A) IFNγ released by NK cells following treatment with various concentration of PP2 and activation with P/I. (B) IFNγ (C) TNFα and (D) CD25 expression (measured by %CD25+ve cells) in NK cells following activation with P/I or co-culture with K562 cells with or without 1μg/ml of PP2. For panel A, data represents the average of 4 donors, and for panels B-D, each dot presents a donor and average with standard deviation is shown. $^*P < 0.05$. ns = not significant. (Color version of figure is available online.)

feeder cell-free systems for NK cell activation. One study found that stimulating NK cells through the NKG2C receptor using plate-bound agonistic antibodies combined with IL-15 resulted in robust activation and proliferation of NK cells [10]. Specialized media supplemented with cytokines and serum have also been used to activate and expand CAR-NK cells instead of feeder cells [11]. Similar strategies have been also used to activate T cells. For example, synthetic beads functionalized with peptide-MHC complexes or antibodies targeting CD3, mimicking synthetic artificial antigen presenting cells, are also effective in activating T cells [12]. These cell-free systems have been shown to provide better and more uniform stimulation signal to target cells, decreasing variability and improving product quality.

We previously found that T cell receptor (TCR)-independent activation of human T cells in the presence of a Src-kinase inhibitor (PP2) significantly enhanced distal T cell signaling through NFAT1 signaling [13]. We also found that TCR-independent activation during CAR-T cell manufacturing in the presence of PP2 resulted in CAR-T cell products with improved quality attributes [14]. In both studies, TCR-independent activation was achieved by stimulating T cells using phorbol 12-myristate 13-acetate (PMA) and calcium ionophore (ionomycin).

PMA activates protein kinase C (PKC) and ionomycin activates calcium-dependent signaling pathways [15]. Since previous studies have shown that PKC and calcium signaling play an important role in NK cell activation [16–18], we investigated PMA/ionomycin as a potential feeder cell-free alternative to activate and manufacture CAR-NK cells. In this study, CAR-NK cells were manufactured following PMA/ionomycin activation and expansion (feeder cell-free method), and critical quality attributes were compared with CAR-NK cells following conventional manufacturing with irradiated K562 feeder cells (feeder cell-based method).

Results

Src-kinase inhibitor enhances feeder cell-free activation of human NK cells

Previously, we found that PP2, a Src-kinase inhibitor, enhanced NFAT1-mediated signaling in human T cells following T cell receptor (TCR)-independent activation with PMA/ionomycin (P/I) [13] and manufacturing CAR-T cells in the presence of PP2 following TCR-independent activation improved CAR-T cell product attributes [14].

To determine whether Src-kinase inhibitor has similar effects on NK cells, primary human NK cells were treated with PP2 at various concentrations and stimulated with P/I. NK cell activation was assessed by measuring IFNγ released in the supernatant. We found that PP2 enhanced P/I-mediated activation of NK cells in a dose-dependent manner (Figure 1A). Optimal activation was achieved at 1μg/ml of PP2 concentration and was used in subsequent experiments. Next, we assessed effect of PP2 on two different methods of NK cell activation. NK cells were activated by co-culturing with irradiated K562 cells (FCB method) or by stimulating with P/I (FCF

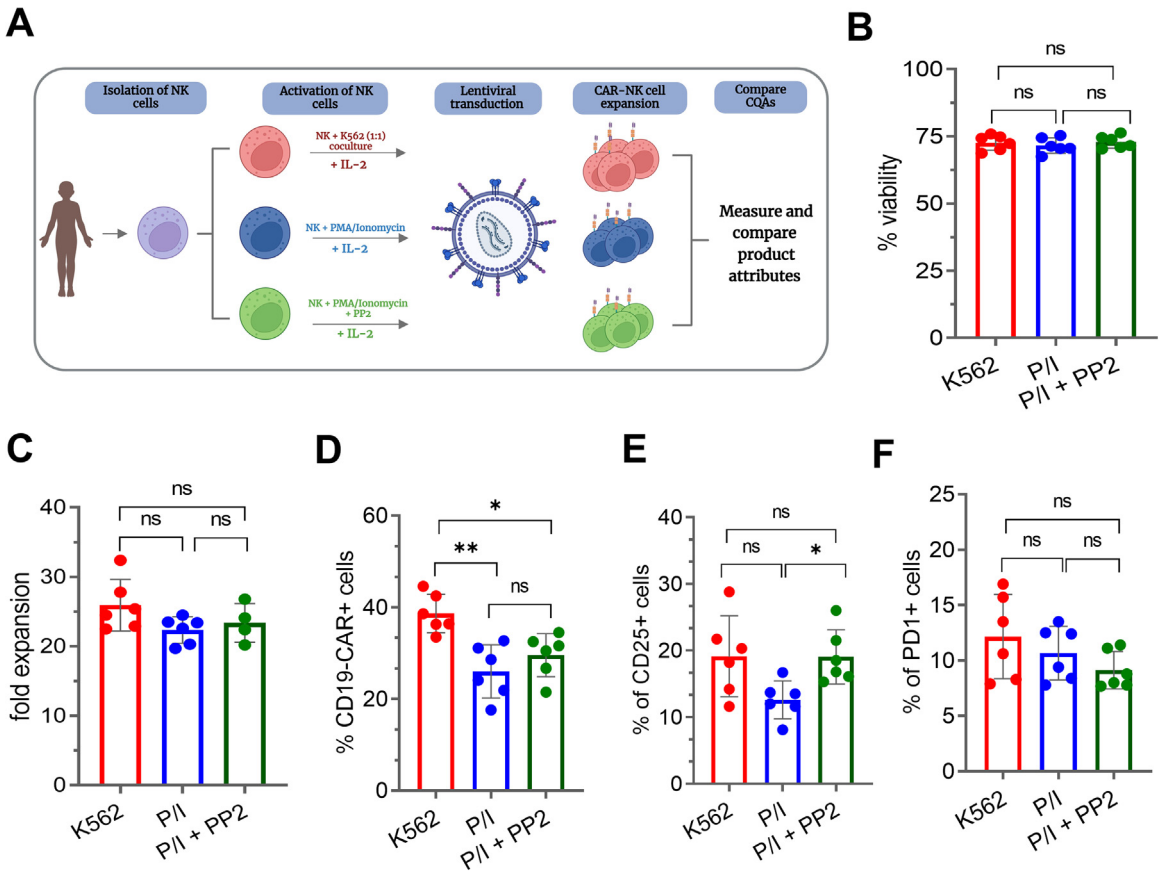

Fig. 2. Effect of Src kinase inhibitor on CAR-NK cell product attributes. (A) Schematic of three different processes used to manufacture and compare CAR-NK cells. CAR-NK cells were analyzed for (B) viability, (C) fold expansion, and (D) %CD19-CAR+ve, (E) %CD25+ve and (F) %PD1+ve cells. Data represents the average with standard deviation, and each dot presents a donor. $^{*}P < 0.05$, $^{**}P < 0.01$. ns = not significant. (Color version of figure is available online.)

method) in presence of PP2 (1μg/ml) and activation was assessed by measuring IFNγ and TNFα levels in the supernatant by ELISA and CD25 expression by flow cytometry. We found that PP2 significantly enhanced NK cell activation when stimulated with P/I (FCF method); however, PP2 did not affect NK cell activation when stimulated with irradiated K562 cells (FCB method) (Figure 1B,C,D). This observation aligns with our previous findings in human T cells, where PP2 enhanced P/I-mediated TCR-independent activation but inhibited anti-CD3/CD28-mediated TCR-dependent activation [13]. Together, these data suggest that the PP2 enhances FCF but not FCB activation of human NK cells.

Src-kinase inhibitor does not improve CAR-NK cell product attributes

As stated above, previously we found that Src-kinase inhibitor PP2 enhanced T cell activation and improved CAR-T cell product attributes following TCR-independent activation with P/I [14]. Since PP2 also enhanced P/I-mediated, FCF activation of human NK cells, we assessed the effect of PP2 on CAR-NK cell product attributes. NK cells were isolated from the peripheral blood of healthy human donors and activated in three different ways: (i) co-culture with irradiated K562 cells (FCB method), (ii) stimulation with P/I (FCF method), and (iii) stimulation with P/I (FCF method) in the presence of PP2. Since PP2 did not affect K562-mediated NK cell activation (Figure 1B,C,D), this condition was not evaluated. Following activation, NK cells were transduced with a lentiviral vector to express anti-CD19 CAR and expanded for 14 days in the presence of IL-2. At the end of expansion, cells were harvested, washed, and formulated in cryopreservation media (Figure 2A). We found that CAR-NK cell viability (Figure 2B) and cell expansion (Figure 2C) were comparable across the three activation methods. However, anti-CD19 CAR expression was significantly lower in NK cells activated with P/I compared to those activated with K562 cells (Figure 2D, Supp. Figure 3A). The addition of PP2 did not improve CAR expression in P/I-activated NK cells (Figure 2D, Supp. Figure 3A), suggesting that unlike in CAR-T cells, PP2 does not enhance CAR-NK cell product attributes. As observed in Figure 1D, CD25 expression was enhanced by addition of PP2 in FCF (P/I) activated CAR-NK cells (Figure 2E). However, there were no differences in CD25 or PD-1 expression between FCB and FCF activated CAR-NK, indicating that cellular activation and exhaustion is comparable between these two methods (Figure 2E,F).

Next, we assessed the effect of PP2 on CAR-NK cell potency by evaluating CAR-NK cell-mediated target cell lysis. Anti-CD19 CAR-NK cells were co-cultured with CD19+ Nalm6 cells at various effector-to-target (E:T) ratios, and Nalm6 cell lysis was assessed after 18 hours. We found that CAR-NK cell-mediated lysis of Nalm6 cells was comparable between CAR-NK cells manufactured using the three activation methods (Figure 3A). CAR-NK cell potency was further assessed by measuring IFNγ release following co-culture with CD19+ Daudi cells (E:T= 1:1). Although, IFNγ levels were comparable between CAR-NK cells manufactured using the FCB and FCF methods (Figure 3B), addition of PP2 significantly improved IFNγ release by CAR-NK cells manufactured using the FCF method. This finding is consistent with our earlier observation in Figure 1B, where PP2 enhanced IFNγ release following P/I activation (FCF method).

Activation of bystander myeloid cells and the release of inflammatory cytokines, such as IL-6 and IL-1β, are hallmark features associated with CAR-T cell-mediated inflammatory toxicities [19,20]. To determine whether different NK cell activation methods affect CAR-NK-mediated bystander myeloid cell activation, supernatants from

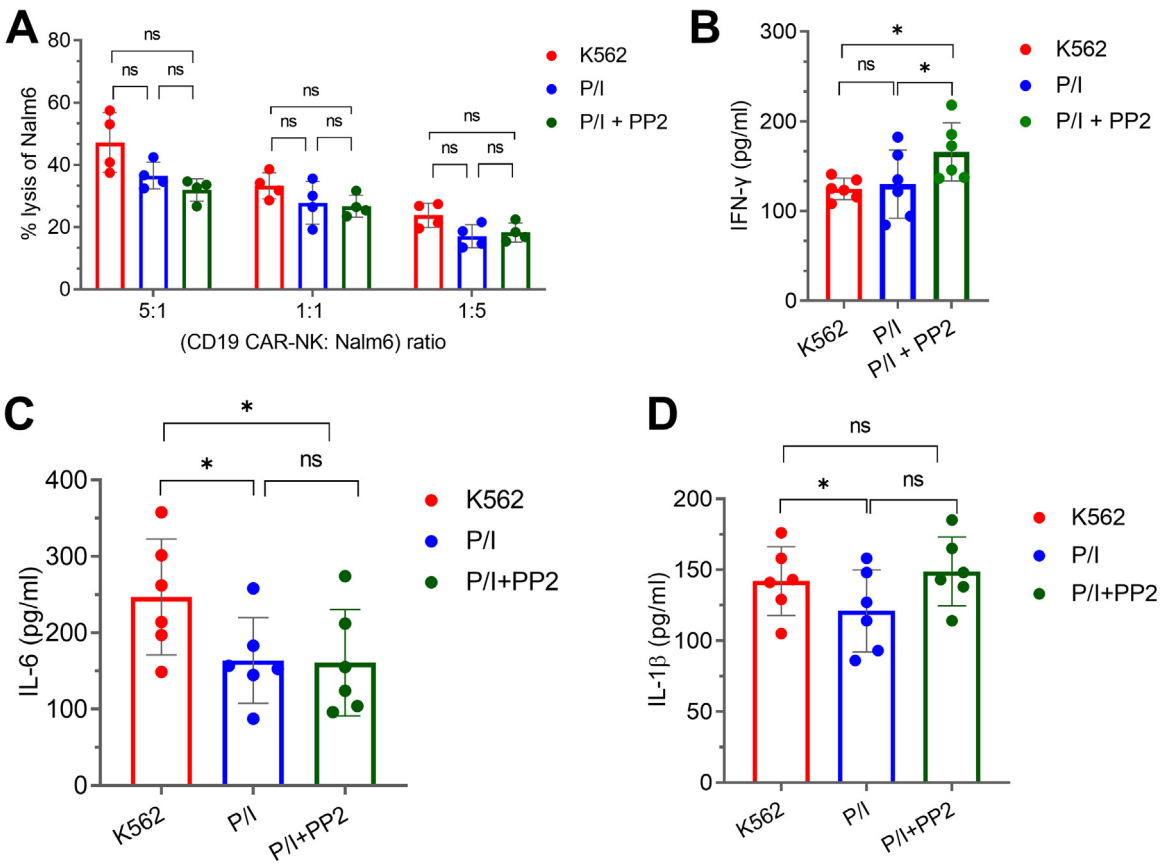

Fig. 3. Effect of Src kinase inhibitor on CAR-NK cell potency and inflammatory toxicity. (A) CD19 CAR-NK (effector) cells manufactured using the three different methods were co-cultured with CD19+ Nalm6 (target) cells and cytotoxicity (lysis of Nalm6 cells) was measured at various effector (E) to target (T) ratios. (B) CD19 CAR-NK cells were co-cultured with CD19+ Daudi cells at E:T= 1:1, and IFNγ released by CAR-NK cells was measured. (C) IL-6 and (D) IL-1β released by THP-1 cells following incubation with supernatants obtained from CAR-NK and Daudi co-culture (E:T= 1:1). Data represents the average with standard deviation, and each dot presents a donor. *$P < 0.05$. ns = not significant. (Color version of figure is available online.)

CAR-NK (manufactured using the three activation methods) and Daudi co-cultures (E:T = 1:1) were incubated with myeloid cells (THP-1). Myeloid cell activation was assessed by measuring IL-6 and IL-1β secretion by THP-1 cells via ELISA. We found that IL-6 and IL-1β were significantly reduced when supernatants from P/I activated CAR-NK cells were incubated with THP-1 cells compared to supernatants from K562 activated CAR-NK cells (Figure 3C,D). IL-6 and IL-1β levels were comparable between CAR-NK cells manufactured following P/I activation with or without PP2 (Figure 3C,D), suggesting PP2 does not affect CAR-NK cell-mediated myeloid cell activation.

Together, these data suggest that the FCF method generates CAR-NK with product attributes comparable to those manufactured using the FCB method. Although CAR expression was reduced in the FCF method, NK cell-mediated myeloid cell activation was also reduced, suggesting that the FCF activation method may produce CAR-NK cells with a lower inflammatory profile. Furthermore, addition of PP2 did not improve CAR-NK cell viability, cellular expansion, CAR or PD-1 expression, or cytotoxicity, nor did it reduce myeloid cell activation, indicating that, unlike in CAR-T cells [14], Src-kinase inhibition does not improve CAR-NK cell product attributes.

Effect of IL-2 family cytokines on feeder cell-free activation of CAR-NK cells

Since the Src-kinase inhibitor PP2 did not improve CAR-NK cell attributes, except for CD25 expression and IFNγ secretion, compared to CAR-NK cells manufactured using the FCF method without PP2 (Figs. 2E and 3B), we studied whether IL-2 family cytokines could enhance CAR-NK cell attributes during manufacturing using the FCF method.

The IL-2 family of cytokines, particularly IL-2, IL-15, and IL-21, play crucial roles in the activation, proliferation, and function of NK cells [9,21–23]. IL-2 enhances NK cell cytotoxicity and promotes growth by activating signaling pathways that increase the expression of key receptors and effector molecules. IL-15 is essential for NK cell development and survival, supporting their maintenance in the immune system and cytotoxic functions and IL-21 promotes NK cell proliferation and enhances effector functions by modulating surface receptor expression and increasing cytotoxic granule production.

Since IL-2 alone did not improve CAR-NK cell product attributes in the FCF method compared to the FCB method (Figures 2 and 3), we assessed the combined effects of IL-2 and IL-15, with or without IL-21. CAR-NK cells were manufactured following activation with K562 (FCB method) or P/I (FCF method) in the presence of cytokines, as shown in Figure 4A, and their product attributes were compared.

We found that viability (Figure 4B) and cellular expansion (Figure 4C) were comparable between CAR-NK cells manufactured using the FCF and FCB methods in the presence of IL-2/IL-15 and addition of IL-21 did not significantly change viability or cellular expansion of CAR-NK cells (Figure 4B,C). As observed in Figure 2D, CD19 CAR expression was significantly lower in CAR-NK cells manufactured using the FCF method in the presence of IL-2/IL-15, and supplementation with IL-21 did not improve CAR expression compared to the FCB method (Figure 4D, Supp. Figure 3B). CD25 and PD-1 expression levels were also comparable between CAR-NK cells

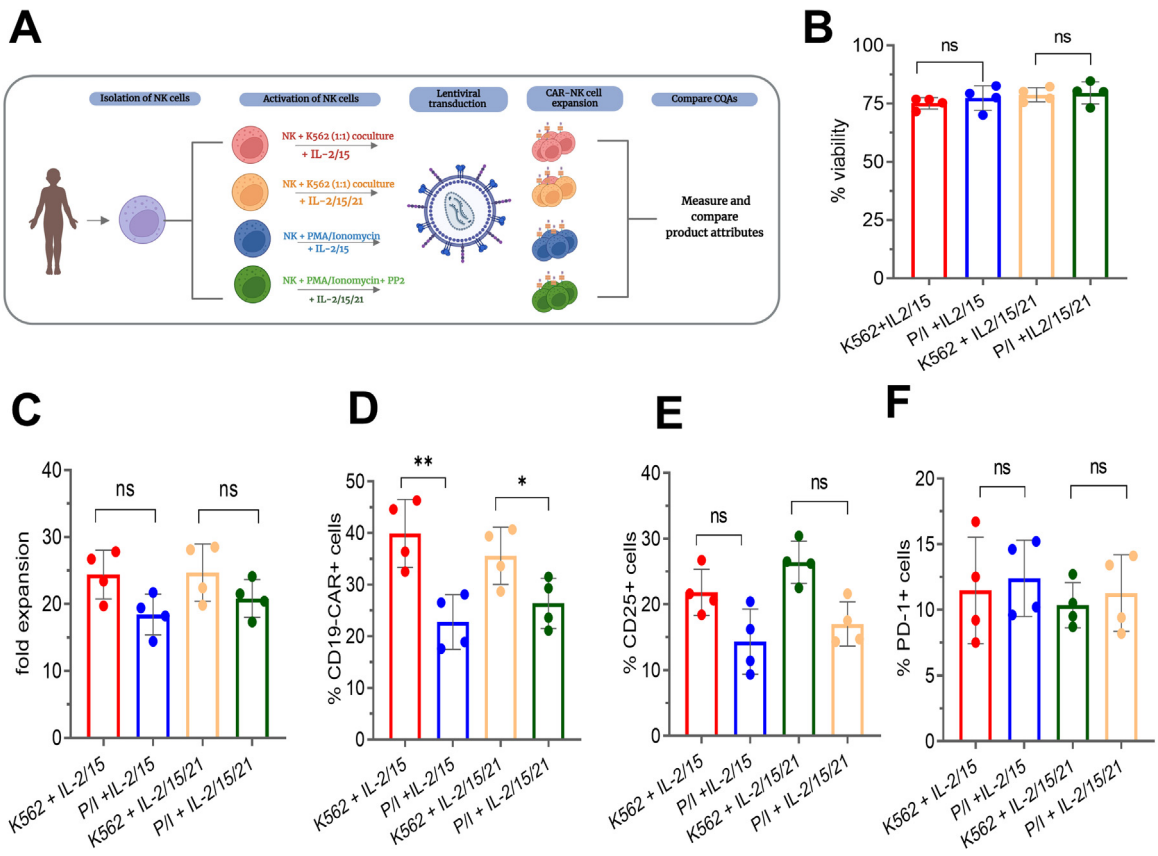

Fig. 4. Effect of IL-2 family of cytokines on CAR-NK cell product attributes. (A) Schematic of four different processes used to manufacture and compare CAR-NK cells. CAR-NK cells were analyzed for (B) viability, (C) fold expansion, and (D) %CD19-CAR+ve, (E) %CD25+ve and (F) %PD1+ve cells. Data represents the average with standard deviation, and each dot presents a donor. *$P < 0.05$, **$P < 0.01$. ns = not significant. (Color version of figure is available online.)

manufactured using both methods, in presence of IL-2/IL-15 with or without IL-21 (Figure 4E,F).

Next, we assessed CAR-NK cell potency by evaluating CAR-NK cell-mediated target cell lysis and IFNγ secretion. CAR-NK cells manufactured using the FCF method in the presence of IL-2/IL-15 exhibited significantly higher Nalm6 lysis (Figure 5A) and IFNγ secretion (Figure 5B) compared to those manufactured using the FCB method. The addition of IL-21 resulted in comparable target cell lysis and IFNγ release between CAR-NK cells manufactured by both methods (Figure 5A).

To determine whether the addition of cytokines affects CAR-NK-mediated bystander myeloid cell activation, supernatants from anti-CD19 CAR-NK and Daudi co-cultures (E:T = 1:1) were incubated with myeloid cells (THP-1). THP-1 secreted IL-6 levels were comparable between CAR-NK cells manufactured using the FCB and FCF methods in the presence of IL-2/IL-15 with or without IL-21 (Figure 5C). IL-1β levels were also comparable between CAR-NK cells manufactured using the FCF and FCB methods in presence of IL-2/IL-15; however, the addition of IL-21 during CAR-NK manufacture with the FCF method significantly reduced IL-1β levels in THP-1 cells (Figure 5D).

Together, these data suggest that the FCF method generates CAR-NK cells with product attributes comparable to those manufactured using the FCB method in the presence of cytokines IL-2, IL-15, and IL-21. While CAR expression was reduced in the FCB method (Figure 4D, Supp. Figure 3B), as previously observed in Figure 2D, CAR-NK cell cytotoxicity (Figure 5A) and IFNγ (Figure 5B) increased in the presence of IL-2/IL-15 in the FCF method, and myeloid cell activation was decreased in the presence of IL-2/IL-15/IL-21 with the FCF method, suggesting the FCF method may help improve CAR-NK cell product attributes in presence of IL-2 family cytokines.

Feeder cell-free activation can produce high-quality CAR-NK cells in a G-Rex bioreactor

One of the challenges associated with feeder cell-based (FCB) manufacturing of cellular products is scalability [24]. To manufacture CAR-NK cells using feeder cells, an equal number of feeder cells are typically used to activate NK cells. This necessitates cell culture systems with larger physical spaces, complicating scale up. Additionally, it becomes increasingly difficult to maintain consistent culture conditions across larger surfaces during expansion, e.g., lower gas exchange, which can affect viability, functionality, and lot-to-lot consistency. Thus, a feeder cell-free (FCF) system may help mitigate these challenges.

The G-Rex (Wilson–Wolf) is a large-scale bioreactor system, which supports rapid growth of suspension cells with minimal manipulation and ease of media change as compared to conventional systems such as plates, flasks, or bags. To assess feasibility of FCF method in large scale production of CAR-NK cells, G-Rex bioreactor was used to produce CAR-NK cells using the FCF and FCB methods (Figure 6A). We found that viability (Figure 6B), cellular expansion (Figure 6C), expression of CD19 CAR (Figure 6D, Supp. Figure 3C), CD25 (Figure 6E), and PD-1 (Figure 6F) were comparable between CAR-NK cells manufactured in the G-Rex bioreactor using the FCB and FCF methods. Unlike the observations in smaller cell cultures (Figures 2D, 4D, Supp. Figure 3A,B), CD19 CAR expression was not significantly different between CAR-NK cells produced via the FCB and FCF methods (Figure 6D, Supp. Figure 3C), suggesting that the G-Rex bioreactor may support to manufacture CAR-NK cells using the FCF method.

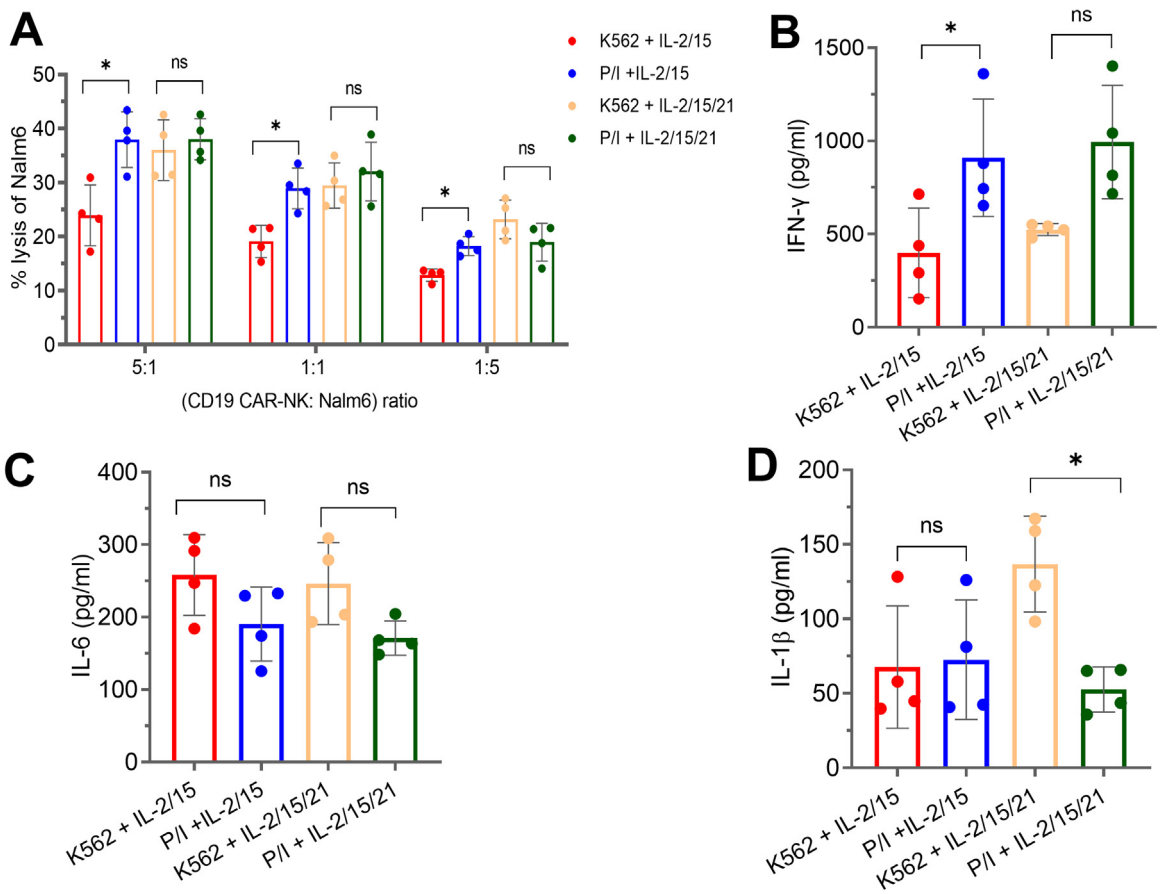

Fig. 5. Effect of IL-2 family cytokines on CAR-NK cell potency and inflammatory toxicity. (A) CD19 CAR-NK (effector) cells manufactured using the four different methods were co-cultured with CD19+ Nalm6 (target) cells and cytotoxicity (lysis of Nalm6 cells) was measured at various effector (E) to target (T) ratios. (B) CD19 CAR-NK cells were co-cultured with CD19+ Daudi cells at E:T= 1:1, and IFNγ released by CAR-NK cells was measured. (C) IL-6 and (D) IL-1β released by THP-1 cells following incubation with supernatants obtained from CAR-NK and Daudi co-culture (E:T= 1:1). Data represents the average with standard deviation, and each dot presents a donor. *$P < 0.05$. ns = not significant. (Color version of figure is available online.)

Next, we assessed potency of CAR-NK cells manufactured using the G-Rex bioreactor. CAR-NK cells produced using the FCF method demonstrated significantly higher cytotoxicity against Nalm6 cells at an effector-to-target (E:T) ratio of 5:1, while cytotoxicity was comparable at lower E:T ratio of 1:1 (Figure 7A). Similarly, IFNγ release following co-culture with Daudi cells was significantly higher in CAR-NK cells manufactured using the FCF method compared to the FCB method at both 1:1 and 5:1 E:T ratios (Figure 7B). Furthermore, CAR-NK cells produced using the FCF method induced lower bystander myeloid cell activation compared to the CAR-NK cells manufactured using the FCB method. IL-6 and IL-1β levels were significantly lower in THP-1 cells following stimulation with supernatant obtained from FCF manufactured CAR-NK and Daudi co-culture (E:T = 1:1) compared to supernatant obtained from FCB manufactured CAR-NK and Daudi cells (Figure 7C,D). At the lower E:T ratio of 1:1, IL-6 and IL-1β secretion by THP-1 cells was comparable between CAR-NK cells produced using the FCB and FCF methods.

Together, these data suggest that the FCF method can produce high-quality CAR-NK cells in G-Rex bioreactors with potentially enhanced potency and safety.

Discussion

While CAR-NK cells have vast therapeutic potential, manufacturing challenges persist, and robust quality control measures are essential throughout the CAR-NK cell manufacturing process to ensure quality, safety, and potency of these products [25–27]. To manufacture CAR-NK cells, feeder cells are commonly used to activate and expand NK cells; however, use of feeder cells presents significant challenges [28,29]. Feeder cells can introduce adventitious agents in the drug product or residual feeder cells can increase immunogenicity risks of the drug product. Feeder cells are also subjected to lot-to-lot variability, which can affect drug product quality and consistency. Furthermore, maintaining feeder cells as cell banks, and ensuring quality, safety and stability of these cell banks requires additional procedures adding to the overall manufacturing cost. Thus, to ensure quality, safety and potency of CAR-NK cell products manufactured using the feeder cells, extensive quality control measures are required increasing manufacturing complexity and costs [29–33]. Alternatively, feeder cell-free systems to manufacture CAR-NK cells can greatly improve safety, quality, and potency of the product in a cost-effective manner.

PMA (Phorbol 12-myristate 13-acetate) and ionomycin are commonly used to activate immune cells, including T cells, NK cells, and monocytes [34–36]. PMA acts as a diacylglycerol (DAG) analog that activates protein kinase C (PKC), a critical pathway involved in T cell and NK cell activation. Ionomycin is a calcium ionophore that increases intracellular calcium levels, activating calcium-dependent signaling pathways, enhancing cellular activation [37]. Previously, we found that activation of T cells using PMA/ionomycin (P/I) in presence of PP2, a Src-kinase inhibitor, enhanced NFAT1 signaling in human T cells [13], and manufacturing CAR-T cells following P/I activation in presence of PP2 significantly improved product attributes [14]. Here we found that, similar to T cells, PP2 enhanced NK cell activation following P/I stimulation. However, unlike CAR-T cells, PP2 did not improve CAR-NK cell product attributes following P/I activation

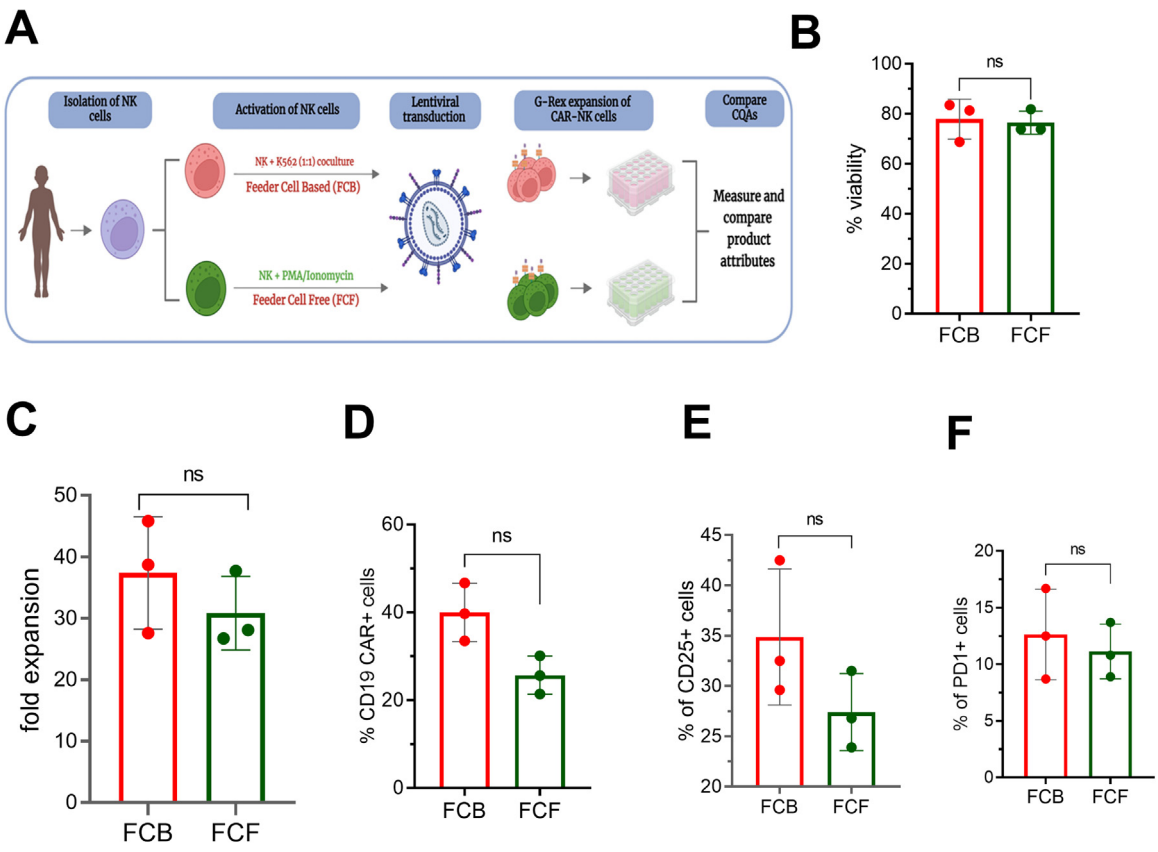

Fig. 6. Effect of feeder cell-based and feeder cell-free manufacturing on CAR-NK cell product attributes in a large-scale bioreactor. (A) Schematic of feeder cell-based (FCB) and feeder cell-free (FCF) processes used to manufacture and compare CAR-NK cells. CAR-NK cells were analyzed for (B) viability, (C) fold expansion, and (D) %CD19-CAR+ve, (E) %CD25 +ve and (F) %PD1+ve cells. Data represents the average with standard deviation, and each dot presents a donor. ns = not significant. (Color version of figure is available online.)

and expansion compared to CAR-NK cells manufactured with P/I activation without PP2. This suggests that although inhibition of Src-kinases enhances NK cell activation, it does not affect CAR-NK cell product attributes. Interestingly, we also found that CAR-NK cells manufactured following irradiated K562 feeder cell-based (FCB) activation were comparable to CAR-NK cells manufactured following feeder cell-free (FCF) activation with P/I except for CAR and CD25 expression. CD25 is the IL-2 receptor alpha chain (IL-2Rα) and is upregulated following activation of lymphocytes. IL-2Rα combines with IL-2Rβ, and the common gamma chain to form the high-affinity IL-2 receptor complex required for optimal IL-2 signaling. Lower expression of CD25 on CAR-NK cells manufactured following P/I suggests FCF method does not result in excessive cellular activation. In activated NK cells, lentiviral vector-mediated transduction is significantly better compared to resting NK cells [38], thus, lower CAR expression following FCF method of activation is likely due to lower activation of NK cells compared to the FCB method.

The IL-2 family of cytokines such as IL-12, IL-15, and IL-21 play an important role in promoting NK cell activation, expansion, and cytotoxicity [21,22,38,39]. We found that CAR-NK cells manufactured following FCF activation in presence of IL-2 and IL-15 had lower CAR expression but had higher potency compared to CAR-NK cells manufactured following FCB activation. This suggests that IL-2 and IL-15 have potential to improve potency of CAR-NK cells manufactured using the FCF method even though it may result in lower CAR expression. Improving potency of CAR-NK cells may be beneficial to treat diseases such as solid tumors, or tumors with lower antigenicity, which has been challenging with currently available CAR-NK cell therapies. Only after addition of IL-21 with IL-2 and IL-15, potency of CAR-NK cells manufactured using the FCB method was comparable to the FCF method. This suggests that FCF method can produce highly potent CAR-NK cells without need for additional IL-21 cytokine supplementation. Reducing use of IL-21 can help reduce the cost of manufacturing and may also improve CAR-NK life span by reducing apoptosis [40]. In the FCB method, we did not observe significant improvement in CAR-NK cell potency following addition of IL-15 and IL21 compared to IL-2 (Supp. Figure 1). This suggests that a short-term (24-hours) cytotoxicity assay may not be able to identify differences in CAR-NK cell potency manufactured using the FCB method in presence of IL-2 family cytokines.

In our studies, we utilized unmodified K562 cells for NK cell activation and expansion at 1:1 effector to target (E: T) ratio in presence of IL-2 family cytokines and observed CAR-NK cell expansion ranging from 30 to 50-fold. Other studies have used various other types of cells including modified K562 cells expressing membrane bound IL-21 or IL-15 with or without other stimulatory ligands at various E: T ratios [41,42]. Although, many factors including culture conditions, cytokines, E:T ratios etc. can impact cellular expansion, we noticed that CAR-NK expansion observed by previous studies at E:T 1:1 ratio over 14 days is comparable to our observations [41].

Manufacturing cellular products using feeder cells can also present challenges to scale up production needed for large clinical studies [24]. Use of feeder cells during manufacturing necessitates large cell culture systems with bigger physical spaces, difficult to maintain consistent culture conditions across larger surfaces during expansion, which can affect viability, functionality, and lot-to-lot consistency. The Gas Permeable Rapid Expansion (G-Rex) system, developed by Wilson Wolf Manufacturing, is a specialized platform designed for the scalable production of immune cells such as CAR-T and CAR-NK cells. The G-Rex bioreactor utilizes a gas-permeable membrane that

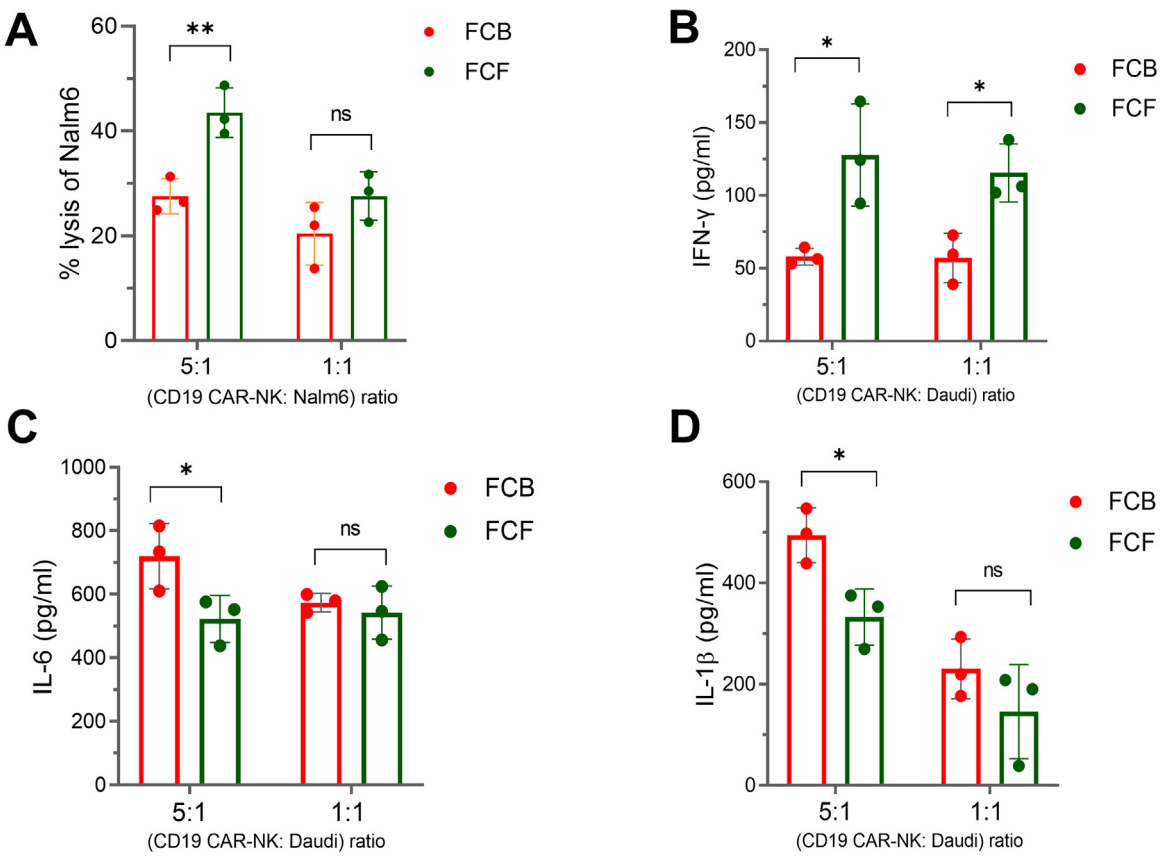

Fig. 7. Effect of IL-2 family cytokines on CAR-NK cell potency and inflammatory toxicity. (A) CD19 CAR-NK (effector) cells manufactured using the feeder cell-based (FCB) or the feeder cell-free (FCF) methods were co-cultured with CD19+ Nalm6 (target) cells and cytotoxicity (lysis of Nalm6 cells) was measured at 5:1 and 1:1 effector (E) to target (T) ratios. (B) CD19 CAR-NK cells were co-cultured with CD19+ Daudi cells at 5:1 and 1:1 E:T ratios, and IFNγ released by CAR-NK cells was measured. (C) IL-6 and (D) IL-1β released by THP-1 cells following incubation with supernatants obtained from CAR-NK and Daudi co-culture at 5:1 and 1:1 E:T ratios. Data represents the average with standard deviation, and each dot presents a donor. $^{*}P < 0.05$, $^{**}P < 0.01$. ns = not significant. (Color version of figure is available online.)

allows efficient gas exchange while supporting large media volumes. G-Rex bioreactors are available in various sizes, enabling linear scalability from small-scale research to large-scale clinical production and can accommodate cell expansions ranging from millions to billions of cells in a single device. We found that the FCF method produced high quality CAR-NK cells in G-Rex system with significantly improved potency and reduced ability to cause inflammatory toxicity compared to the FCB method. This suggests that FCF method can be a viable option to produce large number of high-quality CAR-NK cells in bioreactors.

In conclusion, our study found that PMA/ionomycin-mediated NK cell activation and expansion during CAR-NK cell manufacturing as an alternative to feeder cell-based activation and expansion results in high-quality CAR-NK cells with potentially improved potency and reduced ability to cause inflammatory toxicity. Thus, this feeder cell-free method of manufacturing CAR-NK cells may be a viable strategy to manufacture CAR-NK cells for research, preclinical and clinical studies. Future studies should further characterize CAR-NK cells manufactured using the feeder cell-free method, and assess its safety and efficacy in relevant model systems.

Materials and Methods

Primary NK cells: Buffy coats from healthy donors were obtained from the National Institutes of Health (NIH) Blood Bank. All donors provided written consent for their blood products to be used in research projects and all blood samples were deidentified. This study was exempted by the FDA's IRB, Research Involving Human Subject Committee (RIHSC). PBMCs were isolated using Ficoll gradient centrifugation, washed in 1X PBS and resuspended in complete RPMI 1640 media supplemented with 2mM L-glutamine, 100 IU/mL penicillin, 10 μg/mL streptomycin and 10% heat-inactivated FBS, and incubated overnight at 37°C, 5% CO2. The following day, cells in suspension were harvested, and adherent cells were discarded. NK cells were isolated from the cell suspension using NK cell isolation kit (Miltenyi Biotec). Cell purity was assessed by flow cytometry by staining for NK cells (CD56 and CD16). We routinely obtained over 90% NK cell purity in our experiments (Supp. Figure 2). Primary NK cells were cultured in NK MACS Medium (Miltenyi Biotec) supplemented with 5% human AB serum and IL-2 (50 IU/ml).

Cell Lines: Nalm6 cells expressing GFP/luciferase with beta-2 microglobin (B2M) knock out were previously described [14]. Daudi, THP-1 and K562 cells were purchased from ATCC and were maintained at 37^{0}C with 5% CO2. Nalm6, Daudi and K562 cells were cultured in RPMI media supplemented with 2mM GlutaMax, 100 IU/ml penicillin, 10μg/ml streptomycin, and 10% heat-inactivated fetal bovine serum (HI-FBS, Atlanta Biologicals). THP-1 cells were cultured in IMDM media (ATCC) with 100 IU/ml penicillin, 10μg/ml streptomycin, and 10% HI-FBS. HEK293T cells were obtained from Cell Genesys (Foster City, CA) and were cultured in DMEM media (Lonza) supplemented with 100 IU/ml penicillin, 10μg/ml streptomycin, 2mM GlutaMax (Thermo), and 10% heat-inactivated FBS. K562 cells were irradiated with 100 Gy in a Cs-137 irradiator, and lack of cell proliferation was confirmed.

Lentiviral vector: Production of lentiviral vector encoding anti-CD19 CAR (anti-CD19 scFv-CD28-41BB-CD3z) has been previously

described [14]. Briefly, plasmids encoding VSV-G envelope, CD19 CAR and packaging plasmid pCD/NL-BH*DDD were mixed with PEI-Max (Polysciences) in Opti-MEM media (Gibco) for 15 minutes and added to HEK293T cells for 6 hours. Cells were washed with 1X PBS and resuspended in complete media for 72 hours at 37°C. Supernatant was filtered using 0.4μm filter (VWR) and concentrated using a 100kDa filter (Millipore Sigma). Concentrated supernatant with lentivirus vector were stored at -80°C. Infectious titer was determined using NK cells by serial dilution. CAR expression on live cells was measured by flow cytometry (BD LSR Fortessa) using PE conjugated Protein L (AcroBio). Vector titer was calculated as T = (P*N)/(D*V), where T = titer (TU/ml); P = % PE positive cells, N = number of cells at the time of transduction, D = dilution factor and V = total volume of viral inoculum.

CAR-NK cells: Primary NK cells (4×10^6) from each donor were split into multiple groups as needed per experiment, and CAR-NK cells were manufactured following feeder cell-based (FCB) or feeder cell-free (FCF) activation. FCB activation was achieved following co-culture with irradiated K562 cells at an effector to target (E:T) ratio of 1:1 and FCF activation was achieved following stimulation with phorbol 12-myristate 13-acetate (50ng/mL) and ionomycin (1μg/mL), which activates protein kinase C (PKC) and calcium signaling pathways [13,14]. For experiments which evaluated effects of Src-kinase inhibition on NK cell activation, cells were treated with Src-kinase inhibitor (PP2) (SelleckChem) at 1μg/mL for 16 hours, followed by activation with PMA/ionomycin. Following 24 hours of activation, cells were transduced with a lentiviral vector by spinoculation, multiplicity of infection of 10. Cells were spun at 1800 rpm for 60 minutes at 25°C in 6-well non tissue culture treated plate coated with RetroNectin (50μg/ml, Takara Bio). After spinoculation, cells were incubated at 37°C for at least 6 hours, after that media was exchanged, and cells were expanded for 14 days in T-75 tissue culture flasks. CAR-NK cells were expanded following media changes every three days with NK MACS media (Miltenyi Biotec) in the presence of specific cytokines; IL-2 (500 IU/ml; Miltenyi Biotec), IL-15 (5 ng/ml; Peprotech) and IL-21 (25 ng/ml; Miltenyi Biotec). For expansion in G-Rex bioreactors, transduced NK cells (4×10^6) were seeded into a G-Rex 6-well formatted plate (Wilson Wolf P/N 80240M) and co-cultured for 14 days in 40 ml media. Media in G-Rex was changed every 3 days, and fresh cytokines were supplemented with each media change. In both T-75 flaks and G-Rex, cells were monitored daily, and cell density was maintained around 0.5×10^6 cells/mL. Following 14 days of expansion, CAR-NK cells were harvested, washed twice in 1X PBS and cryopreserved in cell freezing media (90% FBS, 10% DMSO). CAR-expressing cells were assessed for viability, CD19-CAR expression (Protein L), NK cell purity (Supp. Figure 2) and endotoxin (ToxinSensor Chromogenic LAL Endotoxin Assay Kit, Genscript).

CAR-NK cell potency: Anti-CD19 CAR-NK cell potency was measured by assessing cytotoxicity against CD19+ Nalm6 cells and IFNγ release following co-culture with CD19+ Daudi cells.

- *Cytotoxicity assay:* Cytotoxicity assay was performed as previously described [14]. Briefly, CD19+ Nalm6 cells (target) expressing firefly luciferase were co-cultured with anti-CD19 CAR-NK cells (effector) at effector-to-target (E:T) ratios of 1:5, 1:1, or 5:1 in 100 μL of complete RPMI media in a 96-well white-walled plate. A total of 50,000 target cells were seeded and co-cultured with varying numbers of CAR-NK cells to achieve the desired E:T ratios. Nalm6 cells plated alone served as the negative control, while cells treated with 10% Triton X-100 served as the positive control. After 24 hours of co-culture, 100 μL of Bright-Glo luciferase reagent (Promega) was added to each well, and luminescence was measured using a Varioskan plate reader (Thermo Scientific). Cytotoxicity was calculated using the formula: Cytotoxicity (% lysis) = 100 - [(Luminescence of co-culture sample / Luminescence of Nalm6 alone) $\times$ 100].
- *IFNγ release assay*: Anti-CD19 CAR-NK cells were co-cultured with CD19+ Daudi cells at various E:T ratios, and following 24 hours, IFNγ released in the supernatant was measured by ELISA. IFNγ released by Daudi and Nalm6 cells were also measured to demonstrate these cells do not secrete IFNγ after activation (Supp. Figure 4).

Myeloid cell activation assay: The human monocytic cell line, THP-1, was used to assess myeloid cell activation. First, anti-CD19 CAR-NK cells were co-cultured with CD19+ Daudi cells at 5:1 or 1:1 E:T ratios. Following 24 hours, supernatants from co-culture were added to THP-1 cells (1×10^6 cells/ml) and incubated overnight. Myeloid cell activation was assessed by measuring the release of IL-6 and IL-1β by THP-1 cells by ELISA.

Cell Count and Viability: Cell count and viability were determined using Trypan Blue and an automated cell counter (Countess II, ThermoFisher).

Flow cytometry: Cryopreserved CAR-NK cells were thawed and washed with 1X PBS and stained with antibodies at 4°C for 1 hour and washed two times in 1X PBS. Antibodies used: Protein-L (PE) from AcroBio and CD25 (FITC) and PD-1 (APC) from BD Biosciences. Data was collected using LSR Fortessa (BD Biosciences) and analyzed using FlowJo software (Tree Star). At least 10,000 events were collected in the live gate.

ELISA: IFNγ, TNFα, IL-6 and IL-1β were quantified using ELISA kits (BD Biosciences) following manufacturer's instructions and plates were read using Varioskan Lux (Thermo Scientific) plate reader.

Statistics: Student t test was used to compared between two groups, and one-way or two-way ANOVA was used to compare results from multiple groups. *P* values <0.05 were considered statistically significant. GraphPad PRISM (GraphPad Software Inc.) was used for statistical analysis.

Declaration of competing interest

The authors have no commercial, proprietary, or financial interest in the products or companies described in this article.

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Author Contributions

SK, AB and NB were involved in the conception, design of experiments, data analysis, interpreting results, and drafting this manuscript. SK and AB performed the experiments. NB is accountable for all aspects of this work.

Supplementary materials

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