FULL-LENGTH ARTICLE

Manufacturing

A serum-free protocol for the *ex vivo* expansion of Cytokine-Induced Killer cells using gas-permeable static culture flasks

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ABSTRACT

Cytokine-Induced (CIK) cells represent an attractive approach for cell-based immunotherapy, as they show several advantages compared with other strategies. Here we describe an original serum-free protocol for CIK cell expansion that employs G-Rex devices and compare the resulting growth, viability, phenotypic profile and cytotoxic activity with conventional culture in tissue flasks. CIK cells were obtained from buffy coats, seeded in parallel in G-Rex and tissue flasks, and stimulated with clinical-grade IFN- γ , anti-CD3 antibody and IL-2. G-Rex led to large numbers of CIK cells, with a minimal need for technical interventions, thus reducing the time and costs of culture manipulation. CIK cells generated in G-Rex showed a less differentiated phenotype, with a significantly higher expression of naive-associated markers such as CD62L, CD45RA and CCR7, which correlates with a remarkable expansion potential in culture and could lead to longer persistence and a more sustained anti-tumor response *in vivo*. The described procedure can be easily translated to large-scale production under Good Manufacturing Practice. Overall, this protocol has strong advantages over existing procedures, as it allows easier, time-saving and cost-effective production of CIK effector cells, fostering their clinical application.

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Introduction

Adoptive cell therapy (ACT) is based on the administration of *ex vivo* expanded, activated and/or genetically modified effector cells. This approach has shown excellent clinical results against B-cell malignancies, in particular against acute lymphoblastic leukemia and diffuse large B-cell lymphoma following the infusion of CD19 chimeric antigen receptor T cells [1,2]. However, technical and safety hurdles associated with the isolation, modification and expansion of lymphocytes to obtain a therapeutic dose of cells represent an important disadvantage for ACT therapies.

Cytokine-Induced Killer (CIK) cells are a heterogeneous population of *ex vivo* expanded and activated T lymphocytes, which acquire the expression of CD56 during expansion and show several functional and phenotypic properties of both T and natural killer (NK) cells. CIK cells present several advantages compared with other cell-based therapies. In particular, they are easily obtained *in vitro* from peripheral blood mononuclear cells (PBMCs) and cord blood [3,4] with the timed addition of recombinant human interferon- γ , anti-CD3 monoclonal antibody and recombinant human interleukin-2 (rhIL-2) [5]. Importantly, CIK cells do not require antigen-specific stimuli to recognize tumor cells [6] and exert potent major histocompatibility complex-unrestricted antitumor activity against both solid and hematologic malignancies, but not healthy tissues [7,8]. Pre-clinical data have highlighted the low risk of acute graft-versus-host disease by using CIK cells compared with T cells [9,10], a feature representing an important advantage for clinical translation. In contrast to NK

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cells, CIK survival and activity *in vivo* do not rely on IL-2 co-administration, thus limiting the risk of multi-organ toxicity [6,11].

The reported CIK cell therapeutic dose ranges between 10^8 and 10^{10} cells/infusion [12–17]. The expansion of such a high number of cells exhibits several limitations when conventional tissue culture flasks (T-flasks) are used, such as the need for frequent culture manipulations to adjust cell concentration and provide fresh medium, which greatly increases the risk of culture contamination. Alternative expansion protocols for large-scale production of CIK cells have been developed that employ gas-permeable culture bags [16,18–20] or bioreactors [21]. However, culture bags require large amounts of fresh medium and frequent cell density adjustments; similarly, bioreactors are complex to handle, bulky and expensive.

Gas-permeable rapid expansion G-Rex culture devices have been developed for the *ex vivo* expansion of many cell types, such as cytotoxic T lymphocytes [22], tumor-infiltrating lymphocytes [23,24], regulatory T cells [25] and NK cells [26]. In contrast to other devices, the system takes advantage of convection of nutrients in the medium instead of diffusion. Indeed, the structure of the G-Rex flasks allows increased depth of the medium above cells, which provides virtually unlimited oxygen and nutrients and dilutes waste. In fact, O_2 and CO_2 gas exchange occurs through a silicone permeable membrane placed at the base of the device, thus optimizing cell proliferation and survival [22,26,27]. Several protocols for CIK cell expansion require the addition of human derivatives, such as AB serum, frozen plasma or platelet lysate [28]. These supplements, besides raising the risk of infection, increase the variability of the expanded final product since the composition of serum and plasma is highly batch-dependent.

In this study, we set up an original serum-free expansion protocol for CIK cells in G-Rex devices, comparing the results to the standard culture in conventional T-flasks. Overall, we demonstrated the efficacy and feasibility of this protocol and the advantageous features of the cells obtained with this approach.

Methods

Generation and expansion of CIK cells in conventional T-flasks and G-Rex devices

CIK cells were obtained from anonymized human buffy coats provided by the blood bank of Padua Hospital, Padua, Italy. PBMCs were isolated by means of Lymphoprep (STEMCELL Technologies Inc.) density gradient centrifugation (300 g for 20 min). Cells from each donor were

cultured in parallel in G-Rex devices (Wilson Wolf) or in conventional culture flasks (T-flasks) at 37°C in a 5% CO_2 incubator. With regard to the G-Rex devices, G-Rex6 and G-Rex6M were evaluated. These plates are characterized by the same well surface area (10 cm^2) but media capacity of 40 mL and 100 mL, respectively. On day 0, PBMCs were seeded in both G-Rex6 and G-Rex6M at a density of 2.5×10^5 cells/ cm^2 (2.5×10^6 cells/well) in 40 mL and 100 mL, respectively, of X-VIVO 10 medium (Lonza) supplemented with 1% penicillin/streptomycin (Lonza).

At day 7, cells cultured in G-Rex6 were transferred into a new G-Rex6M (2.5×10^6 cells/well), adding 60 mL of fresh media supplemented with 500 IU/mL of rhIL-2; this culture condition was named G-Rex6–6M. Conversely, cells plated in G-Rex6M did not undergo any cell density adjustment or replenishment of media. For the expansion in conventional T-flasks, PBMCs were seeded at day 0 in T25 non-treated flasks for suspension cell cultures (Nunc) at a density of 5×10^6 cells/mL (50×10^6 cells in 10 mL medium) in either X-VIVO 10 medium supplemented with 1% penicillin/streptomycin (Lonza) or Roswell Park Memorial Institute (RPMI) 1640 medium (Euroclone) containing 10% heat-inactivated fetal bovine serum (FBS) (Gibco), 1% penicillin/streptomycin (Lonza), 1% U-glutamine and 1% HEPES buffer (Lonza). On day 4 and every 3–4 days, the cell density was adjusted to 1.5×10^6 cells/mL and cells were transferred to T75 flasks when needed [3].

In all of the culture conditions, to expand CIK cells the culture medium was supplemented at day 0 with 1000 IU/mL of recombinant human interferon- γ (R&D) and 24 hours later with 50 ng/mL of CD3 pure-functional grade human antibody (Miltenyi). Starting from day 1 and every 3–4 days, 500 IU/mL of rhIL-2 (Proleukin, Novartis) was added to the culture. Growth, viability, phenotypic profile and cytotoxic activity of cells cultured under different conditions were evaluated on days 7, 14, 21 and 28. The expansion protocols are outlined in Figure 1. Viability was assessed by trypan blue exclusion test. Fold expansion was calculated by dividing the number of cells yielded at each time point by the number of cells seeded at day 0.

Phenotype analysis

CIK cell phenotype was analyzed using multi-color flow cytometry. In brief, cells were harvested and stained using the following antibodies: CD3-BV510 (clone UCHT1), CD4-BV650 (clone SK3), CD8-BV421 (clone RPA-T8), NKp30-BV650 (clone P30-15), CD244-BV421 (clone 25235), CD62L-FITC (clone DREG-56), CD45RA-PerCP (clone HI100), CD45RO-BV650 (clone UCHL1) and CCR7-Alexa647 (clone 150503) from BD Bioscience; CD56-PE (clone HCD56), NKG2D-APC

Figure 1. Graphical illustration of CIK cell culture protocols. Four different culture conditions were evaluated: 1) PBMCs were seeded in G-Rex6 plates and transferred 7 days later into a G-Rex6M device; this culture condition was named G-Rex6–6M. 2) PBMCs seeded in G-Rex6M did not undergo any further cell density adjustment or medium replenishment during the culture period. In conventional T-flask, PBMCs were plated in either 3) X-VIVO or 4) RPMI + FBS, and cells were split and supplemented with IL-2 according to our previously published protocol [3]. In all culture conditions, CIK cell generation was stimulated with IFN- γ , CD3 and IL-2, as detailed in Methods.

(clone 1D11), Nkp44-APC (clone P44-8) and CD27-FITC (clone O323) from BioLegend. Dead cells were excluded using Fixable Viability Stain 780 (FVS780; BD Bioscience), and the positivity to the markers evaluated was determined by gating on CD3⁺CD56⁺ or CD3⁺CD56⁻ cells. Naïve/memory subsets were identified according to their expression of CD45RA and CD62L: naïve, N, CD62L⁺CD45RA⁺; central memory, CD62L⁺CD45RA⁻; effector memory (EM), CD62L⁻CD45RA⁺; effector memory RA⁺ (EMRA), CD62L⁻CD45RA⁺. Flow cytometry was performed using Celesta flow cytometer and DIVA software (BD Bioscience), and data analyses were performed using FlowJo software (Treestar).

Cell lines

Human breast cancer (MDA-MB-468) cell line was cultured in Dulbecco's Modified Eagle's Medium growth medium (Euroclone), while chronic myelogenous leukemia (K562) and Burkitt lymphoma (Raji) cell lines were cultured in RPMI 1640. Both media were supplemented with 10% heat-inactivated FBS, 1% penicillin/streptomycin, 1% HEPES buffer and 1% U-glutamine. All cell lines were authenticated by short tandem repeat sequence analysis [29].

Cytotoxicity assay

CIK cells harvested between days 14 and 21 were challenged against tumor targets using a standard calcein-acetoxymethyl (Sigma-Aldrich) release assay. Specifically, 10⁶ tumor cells were labeled with 3.5 μ M calcein-acetoxymethyl in complete RPMI medium for 30 minutes at 37°C and then washed with Dulbecco's Phosphate-Buffered Saline (Sigma

Aldrich). Tumor cells were then plated in 96-well U-bottom plates and incubated with CIK cells at the indicated E:T ratios at 37°C. Maximum and spontaneous release was obtained by incubating target cells with 3% Triton X-100 (Sigma Aldrich) or complete RPMI medium. After 4-hour incubation, the plates were centrifuged, and 100 μ L of supernatant collected from each well was transferred into an OptiPlate-96 black plate (PerkinElmer) to measure the released fluorescence using the VICTOR multilabel plate reader (PerkinElmer). The results of the cytotoxicity assay are expressed as specific lysis, which is calculated as follows: % specific lysis = [(experimental release – spontaneous release) / (maximum release – spontaneous release)] $\times$ 100.

Statistical analysis

Results were analyzed for statistical significance by one-way or two-way analysis of variance with Bonferroni correction for multiple comparisons as appropriate. Histograms represent mean value $\pm$ standard deviation. Box plots represent 25th and 75th percentiles and median value, and their whiskers go from minimum to maximum values. Statistical analysis was performed using GraphPad Prism 7 software (* P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001).

Results

CIK cells efficiently expand in gas-permeable culture G-Rex devices using serum-free medium

In this study, four different culture conditions were set up and evaluated. PBMCs were seeded in either G-Rex6 plates and

Figure 2. Assessment of CIK cell expansion and viability. At days 7, 14, 21 and 28, CIK cells cultured in T-flasks ($n = 7$ in RPMI + FBS; $n = 19$ in X-VIVO), G-Rex6–6M ($n = 19$) and G-Rex6M ($n = 13$) were sampled and counted to evaluate (A) cell expansion (the statistics reported refer to day 28; for all other statistical comparisons, see supplementary Table 1) and to calculate (B) the fold increase. (C) Cell viability was calculated as percentage of viable cells at the different time points. Data were analyzed by one-way (A) or two-way (B and C) analysis of variance with Bonferroni correction. * P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001.

transferred 7 days later into a G-Rex6M device (G-Rex6–6M protocol) or G-Rex6M without any further cell density adjustment. In T-flasks, PBMCs were plated in either X-VIVO or RPMI + FBS, and cells were split with fresh media according to our previously published protocol (Figure 1) [3].

The cell yield obtained at the end of the expansion differed considerably among culture conditions, as clearly indicated by the cell growth curve in Figure 2A. The culture of CIK cells in X-VIVO serum-free medium using G-Rex6–6M led to a remarkably more efficient cell expansion compared with all of the other culture protocols, yielding a mean of $1.88 \pm 1.77 \times 10^9$ total cells in 28 days of culture, starting from only 2.5×10^6 cells, which corresponds to a 752-fold increase (Figure 2B). The expansion of CIK cells in X-VIVO serum-free medium appeared significantly reduced when G-Rex6M or T-flasks were employed. Indeed, in both latter cases the cell growth curve quickly reached a plateau starting from day 14 (Figure 2A), leading to a reduced overall fold increase of 124 and 2.3, respectively (Figure 2B). The use of RPMI medium supplemented with FBS led to a higher cell yield in the first 14 days, reaching a peak on day 21. The number of cells then dramatically decreased on day 28, with a mean of $0.42 \pm 0.28 \times 10^9$ total cells obtained from 50×10^6 cells seeded, corresponding to an 8.4-fold increase. Additionally, cells cultured according to the G-Rex6–6M protocol showed an improved long-term viability over the entire culture period, whereas increased cell death was observed in G-Rex6M and X-VIVO T-flasks, especially at the last time point tested ($P=0.008$ and $P=0.031$, respectively) (Figure 2C).

CIK cells expanded in G-Rex exhibit a more naïve phenotype

Multi-color flow cytometry analysis was performed to characterize in detail the phenotype of CIK cells cultured under different conditions. In the first 2 weeks of culture, the percentage of $CD3^+CD56^+$ cells was comparable among the four different culture systems, but this subpopulation expanded more efficiently in G-Rex6–6M, resulting in the highest percentage of $CD3^+CD56^+$ CIK cells at day 21 (mean: $45.19 \pm 14.53\%$) and day 28 (mean: $40.01 \pm 17.38\%$) (Figure 3A). $CD3^-CD56^+$ NK cells, typically present in the bulk cultures, did not expand under conventional T-flask culture conditions [3]. Nonetheless, we observed that in the gas-permeable static culture device the percentage of NK cells was even lower compared with T-flasks, in particular compared with T-flasks with X-VIVO medium (Figure 3B). In both G-Rex6–6M and G-Rex6M, the $CD8^+$ component within the $CD3^+CD56^+$ subset appeared more enriched than in T-flasks, starting from day 7 up to the end of the culture (Figure 3C). Conversely, the $CD4^+$ CIK fraction progressively decreased in G-Rex cultures but remained constant in X-VIVO T-flasks (Figure 3D). NKG2D was highly expressed on CIK cells throughout the culture period and conditions even though it appeared to decrease in G-Rex6M from day 21 (Figure 3E). Nkp44 appeared to be less expressed on cells cultured in G-Rex6M (Figure 3F), whereas with regard to other activating receptors—namely, Nkp30 (Figure 3G) and CD244 (Figure 3H)—differences in expression did not seem correlated with the culture condition.

At the end of the expansion period (day 28), CIK cells were stained for CD62L and CD45RA to identify the four main subpopulations related to memory and effector functions—namely, naïve (N, $CD62L^+CD45RA^+$), central memory (CM, $CD62L^+CD45RA^-$), EM ($CD62L^-CD45RA^-$) and effector memory RA^+ (EMRA, $CD62L^-CD45RA^+$)—within both $CD3^+CD56^+$ and $CD3^+CD56^-$ cell subsets (Figure 4A). CIK cell cultures from G-Rex6–6M and G-Rex6M contained a significantly higher proportion of $CD3^+CD56^+$ naïve cells ($34.6 \pm 12.4\%$ and $42.1 \pm 19.2\%$, respectively) compared with X-VIVO T-flasks, which comprised a mean of $14.7 \pm 7.1\%$ naïve $CD3^+CD56^+$ cells ($P=0.0286$ and $P=0.0016$, respectively) (Figure 4B). Likewise, the phenotype of the $CD3^+CD56^-$ T cell counterpart appeared to be

less differentiated; in particular, in G-Rex6–6M and G-Rex6M cultures the $CD62L^+CD45RA^+$ naïve cell subset accounted for $59.9 \pm 19.4\%$ and $50.5 \pm 16.4\%$ of total T cells, respectively (Figure 4B). Conversely, $CD3^+CD56^+$ CIK cells cultured in RPMI + FBS T-flasks appeared mostly as fully differentiated EM ($82.3 \pm 8.4\%$), whereas the percentage of this subset was significantly reduced in X-VIVO T-flasks ($42.7 \pm 15.8\%$; $P < 0.0001$) (Figure 4B).

These results were confirmed by the expression of the lymphoid homing marker CCR7, which is associated with a naïve or early memory phenotype. Indeed, CCR7 was significantly more expressed on $CD3^+CD56^+$ cells cultured in G-Rex6–6M and G-Rex6M as compared with both protocols employing T-flasks (Figure 4C). Accordingly, the antigen-experienced cell marker CD45RO was more expressed in cells expanded in conventional T-flasks with either RPMI + FBS or X-VIVO (Figure 4D), whereas the co-stimulatory marker CD27 was significantly higher in G-Rex6–6M and G-Rex6M (Figure 4E).

CIK cells cultured in G-Rex display cytotoxic activity comparable to cells cultured in T-flasks

Cytotoxic activity of CIK cells cultured in G-Rex devices was evaluated against different tumor cell lines and compared with lytic activity from CIK cells cultured in T-flasks in either RPMI + FBS or X-VIVO (Figure 5). G-Rex6–6M CIK cells showed cytotoxicity that was overall similar to CIK cells cultured in all other conditions and appeared slightly reduced against only K562 target cells as compared with CIK cells cultured in X-VIVO in T-flasks (12.5:1 ratio: $40.62 \pm 17.24\%$ versus $59.27 \pm 25.12\%$, $P=0.042$). By contrast, G-Rex6M CIK cells showed decreased lytic activity compared with X-VIVO T-flask effector cells against K562 and MDA-MB-468 targets (Figure 5).

Discussion

CIK cells represent an attractive approach for cell-based immunotherapy, as they do not require antigen-specific priming for tumor cell recognition, they exert major histocompatibility complex-unrestricted cytotoxicity against malignant cells and can be efficiently and rapidly expanded *in vitro* [30,31]. Although CIK cells have been studied since the 1990s in both pre-clinical studies and clinical trials, there is not yet a standard protocol to produce them. For example, CIK cells have been obtained from peripheral blood, leukocytapheresis or cord blood [3,4,28] and have been stimulated with different concentrations of anti-CD3 monoclonal antibody and IL-2, ranging from 50 to 100 ng/mL and from 300 to 500 IU/mL, respectively [3,7,28]. Different cytokines have also been added in culture, such as IL-1, IL-6, IL-7, IL-12 and IL-15 [28,32–34]. Moreover, several groups have expanded CIK cells together with dendritic cells, and several clinical trials have assessed the efficacy of this combination [35,36]. Finally, to translate CIK cells from the pre-clinical setting to clinical practice, the addition of alternative supplements to replace FBS in sustaining cell growth, such as human serum or plasma and platelet lysate, has been proposed [28,37]. However, these additives have several limitations, such as availability, variability between different batches and risk of viral transmission [37].

In this study, CIK cells were expanded from peripheral blood, seeding PBMCs in G-Rex in X-VIVO 10 serum-free medium, and stimulated with clinical-grade cytokines. The protocol led to large numbers of CIK cells, with a minimal need for technical interventions, thus reducing the time and risks associated with culture manipulation. Indeed, whereas the expansion in T-flasks requires harvesting and centrifuging the entire culture volume twice a week, the protocol we have developed in G-Rex6–6M needs a single cell density adjustment at day 7 and the addition of IL-2 every 3–4 days.

Recently, the characterization of the phenotype of ACT products has gained great attention, as their *ex vivo* expansion potential and subsequent clinical responses have been strongly correlated with the

Figure 3. Phenotypic profiling of CIK cell cultures. CIK cells expanded according to the different culture protocols were analyzed by flow cytometry at days 7, 14, 21 and 28 for their phenotypic profile. (A) Percentages of CD3⁺CD56⁺ CIK cells and (B) CD3⁺CD56⁺ NK cells were assessed within the bulk cultures. The expression of (C) CD8, (D) CD4, (E) NKG2D, (F) NKP30, (G) NKP44 and (H) CD244 markers was evaluated within the CD3⁺CD56⁺ subset. Results show the mean expression ± standard deviation. Data were analyzed by two-way analysis of variance with Bonferroni correction. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

Figure 4. Analysis of naïve/memory phenotype of CIK cell cultures. Cells were stained for CD3, CD56, CD62L and CD45RA to identify N, CM, EM and EMRA within both CD3⁺CD56⁺ and CD3⁺CD56⁻ cells (n = 7). (A) One representative dot plot of each culture condition and subpopulation is reported. Data are graphically shown in (B) and reported in supplementary Table 2. For all statistical comparisons, see supplementary Table 3. CD3⁺CD56⁺ and CD3⁺CD56⁻ cells were also evaluated for the expression of (C) CCR7, (D) CD45RO and (E) CD27. Histograms show the mean expression $\pm$ standard deviation. Data were analyzed by one-way analysis of variance with Bonferroni correction. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. CM, central memory; EMRA, effector memory RA⁺; N, naïve.

percentage of naïve cells within the cell product, especially in chimeric antigen receptor T therapy [38,39]. Interestingly, CIK cells cultured under G-Rex6–6M protocol showed a pronounced immature phenotype, with a significantly higher expression of naïve-associated

markers such as CD62L, CD45RA and CCR7, all features that can explain their remarkable expansion potential. Conversely, the higher proportion of more differentiated subsets observed in T-flasks, which are characterized by a limited proliferative potential, correlates with

Figure 5. Analysis of CIK cell lytic activity against tumor cell targets. CIK cells were challenged against K562, Raji and MDA-MB-468 tumor cell lines. Lytic activity was measured by calcein-acetoxymethyl release assay between days 14 and 21 of cultures. Results show mean values $\pm$ standard deviation of the specific lysis at different E:T ratios of CIK cell cultured in T-flasks (n = 8 in RPMI + FBS; n = 20 in X-VIVO), G-Rex6–6M (n = 17) and G-Rex6M (n = 14). Data were analyzed by two-way analysis of variance with Bonferroni correction. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

their decline in cell growth. As demonstrated for T-cell therapies, *in vivo* killing following ACT is more complex: the more differentiated EM and terminal effector cells, which are endowed with a high lytic activity, are supposed to mediate the primary attack after infusion. As they become anergic, the less differentiated naïve and central memory cells, which are characterized by self-renewal capacity, would then act as a reservoir by differentiating and providing a second line of effector cells that persist longer and mediate more successful and sustained anti-tumor responses [39–41]. In the context of CIK cell cultures, the CD3⁺CD56[−] T cell subset is a highly proliferating population that gives rise to further CD3⁺CD56⁺ effector cells by differentiation [42]. Thus, the higher proportion of naïve and central memory CD3⁺CD56[−] T cells in the final CIK cell product, which we demonstrate to occur in the G-Rex cultures, could ensure a source of new post-infusion CIK effector cells that may possibly contribute to higher survival rates and long-term clinical responses.

Interestingly, the adjustment of cell density and the transfer of the culture from the G-Rex6 to the G-Rex6M device introduced in the G-Rex6–6M protocol remarkably increased the cell yield compared with the G-Rex6M protocol. The G-Rex6–6M protocol allowed the production of $\sim 10^9$ total cells in 28 days of culture, starting from just 2.5×10^6 cells, which corresponds to an outstanding 752-fold increase, suggesting that a lower cell density during the early phases of culture promotes a higher CIK cell expansion potential. Indeed, the cells produced with the G-Rex6–6M protocol continued to appear in the log phase of the growth curve at day 14 and therefore are potentially able to further expand *in vivo*, which would also be consistent with their less differentiated phenotype. Moreover, almost 5×10^8 cells were already available at day 14, which means that even a small volume of peripheral blood can lead to a sufficient number of cells to repeatedly treat a 70-kg patient when administered at a concentration of 5×10^6 cells/kg [43]. Additionally, if more effectors are required—for example in an allogeneic setting to treat several patients simultaneously—the method can easily be scaled up by employing more than one G-Rex6M for each donor or by using the G-Rex100M, which is a larger device characterized by a surface area of 100 cm² and a volume capacity of 1 L of culture medium.

In conclusion, we have developed a serum-free method of *ex vivo* CIK cell expansion based on the use of gas-permeable flasks, which drastically reduces culture manipulations and can be easily reproduced under Good Manufacturing Practice conditions. The procedure does not depend on the use of human derivatives to supplement the medium, thus also reducing the risk of contamination. Taken together, results demonstrate that the G-Rex6–6M protocol is a reliable and optimized procedure for CIK cell expansion in terms of cell growth, viability, phenotype and lytic activity. In particular, remarkably higher percentages of less differentiated effector cells can be obtained, which could contribute to long-lasting therapeutic responses and *in vivo* persistence. Overall, this protocol represents an improvement over the existing procedures, as it provides a more simplified, time-saving and cost-effective approach for the production of clinically compliant CIK cells and can represent a boost for their clinical application.

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Declaration of competing interest

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

Author contributions

Conception and design of the study: EC, AR, RS, and PP. Acquisition of data: PP, ADP, RS, EC and AV. Analysis and interpretation of data: PP, ADP, RS, EC, AV, GA, KC, CV, MCT, OP and MR. Drafting or revising the manuscript: PP and EC. All authors have approved the final article.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:[10.1016/j.jcyt.2020.05.003](https://doi.org/10.1016/j.jcyt.2020.05.003).

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