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Manufacturing

Optimization of therapeutic T cell expansion in G-Rex device and applicability to large-scale production for clinical use

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ABSTRACT

Our center performs experimental clinical studies with advanced therapy medicinal products (ATMPs) based on polyclonal T cells, all of which are currently expanded in standard T-flasks. Given the need to increase the efficiency and safety of large-scale T cell expansion for clinical use, we have optimized the method to expand in G-Rex devices both cytokine-induced killer cells (CIKs) from peripheral or cord blood and blinatumomab-expanded T cells (BETs). We show that the G-Rex reproducibly allowed the expansion of $> 30 \times 10^6$ CD3⁺ cells/cm² of gas-permeable membrane in a mean of 10 to 11 days in a single unit, without manipulation, except for addition of cytokines and sampling of supernatant for lactate measurement every 3 to 4 days. In contrast, 21 to 24 days, twice-weekly cell resuspension and dilution into 48 to 72 T-flasks were required to complete expansions using the standard method. We show that the CIKs produced in G-Rex (CIK-G) were phenotypically very similar, for a large panel of markers, to those expanded in T-flasks, although CIK-G products had lower expression of CD56 and higher expression of CD27 and CD28. Functionally, CIK-Gs were strongly cytotoxic *in vitro* against the NK cell target K562 and the REH pre-B ALL cell line in the presence of blinatumomab. CIK-Gs also showed therapeutic activity *in vivo* in the Ph⁺ pre-B ALL-2 model in mice. The expansion of both CIKs and BETs in G-Rex was validated in good manufacturing practices (GMP) conditions, and we plan to use G-Rex for T cell expansion in future clinical studies.

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Introduction

Over the last 20 years, T cells expanded *in vitro* have become an important therapeutic tool in the armamentarium of oncologists [1, 2]. Our group as well as others have conducted several phase I/II clinical studies using allogeneic cytokine-induced killer cells (CIKs) for the treatment of relapsed leukemia/lymphoma patients (NCT01186809, NCT03821519) [3–6]. CIKs are expanded *in vitro* in the presence of interferon- γ (IFN- γ), anti-CD3 and interleukin (IL)-2. Autologous

blinatumomab-expanded T cells (BETs) are another T cell–based advanced therapy medicinal product (ATMP) generated in our center and are currently being tested for immune-reconstitution purposes in heavily treated immunocompromised leukemia patients after chemotherapy (NCT03823365). BETs are activated polyclonal T cells expanded *in vitro* in the presence of CD19xCD3 bispecific antibody blinatumomab and IL-2, starting from peripheral blood mononuclear cells (PBMCs) of CD19⁺ leukemia or B–non Hodgkin lymphoma (B-NHL) patients [7]. Blinatumomab allows the activation and proliferation of the T cells through their T cell receptor (TCR) and the simultaneous elimination during culture of CD19⁺ leukemic cells [7].

The number of T cells required for treatment of adult patients with CIKs or BETs vary from 5×10^9 to 12×10^9 CD3⁺ cells, depending on patient weight and the specific protocol (NCT03821519, NCT03823365 [4]). This means that about 70 T175 flasks may have to be handled

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during the last phases of production, filling an entire standard 5% CO₂ incubator. This method clearly needs to be improved to decrease the manipulation steps and workload, lessen the risk of microbial contamination during culture and in general facilitate cell production for the relatively large numbers of patients enrolled in clinical trials [4, 5].

In the last 10 to 20 years, different types of bioreactors have been developed to allow reproducible and large-scale expansion of adherent or nonadherent cells in closed systems for clinical use [8–11]. Bioreactors, such as the Xuri (previously Biowave), Quantum-Cell Expansion System or Miltenyi Prodigy, are partially or fully automated, and rely on mechanical rocking or stirring of culture vessels to guarantee adequate distribution of nutrients and gas exchange during cell expansion [8,9,11]. Bioreactors are expensive, however, and usually allow production of only one cell batch at a time, with expansion of each batch lasting 1 to 3 weeks in the case of T cells [9,11]. This implies that cell factories need to have several bioreactors to produce more cell products in parallel, which can be a handicap, especially when urgent, patient-dedicated ATMPs are being produced for gravely ill patients, as in our case [9].

The G-Rex devices were introduced about 10 years ago [12]. They are quite simple closed-culture vessels provided with a flat, gas-permeable silicone membrane forming the base of the vessel [13]. The cells settle by gravity on top of this membrane, so that gas exchange is very efficient and is not compromised by the amount of medium added on top. A large volume of medium can be added from the start of culture, and nutrients in the medium reach the cell layer by simple convection, without the need for agitation, thus avoiding exhaustion of nutrients during cell expansion or the need to manipulate cells to expand the cell volume during culture [14]. G-Rex devices are single-use, good manufacturing practices (GMP)-compliant medical devices that are easily accommodated in standard 5% CO₂ incubators and come in scalable formats with 10-, 100- or 500-cm² membranes and a constant ratio of maximum volume-to-membrane surface area [8,13,14]. The G-Rex devices have been optimized for expansion of different types of T cells for research or clinical use [10,13,15,16]. Thus G-Rex devices have been used to produce antigen-specific T cells [8,12,17,18], tumor-infiltrating lymphocytes (TILs) [19,20], regulatory T cells (Tregs) [21,22], γ/δ T cells [23] and chimeric antigen receptor (CAR) T cells [16,24] as well as CIKs [25].

We present here the results of the optimization, characterization and validation of the G-Rex devices for expansion of CIKs and BETs from different sources and demonstrate that the cells produced have similar functional activity *in vitro* and *in vivo* in animal models compared to the same cells produced in T-flasks.

Methods

Cells and basic culture media

Mononuclear cells (MNCs) were isolated by standard Ficoll-Hypaque gradient centrifugation of freshly collected peripheral blood (PB) (in some cases in the form of buffy coat from the local transfusion unit) or cord blood (CB). In the case of BETs, PB was obtained from B-leukemia/B-NHL patients. The study was approved by the local ethics committee, and donations were collected after informed consent by healthy donors or leukemia patients.

The medium to expand CIKs or BETs consisted of serum-free X-VIVO 15 medium (Lonza, Verviers, Belgium) supplemented with 0.1 mM gentamycin (Fisiopharma, Palomonte, Italy) and 1% heat-inactivated human serum AB (Life Sciences Production, Barnet, UK) (hereafter called complete medium). Additional specific stimuli and cytokines were added to this medium to generate either CIKs or BETs.

The human cell lines REH (pre-B-ALL, CD19⁺) and K562 (erythro-leukemia, CD19⁻) were maintained in RPMI-1640 (Euroclone, Wetherby, West Yorkshire, UK) supplemented with 10% fetal bovine serum (FBS; Euroclone), 2 mM L-glutamine (Euroclone) and 110 μ M gentamycin (PHT Pharma, Milano, Italy).

Expansion of CIKs in T-flasks

To generate CIKs, PB- or CB-derived MNCs from healthy donors were plated in standard tissue culture T-flasks (Thermo Fisher Scientific, Waltham, MA; or Becton Dickinson, Franklin Lakes, NJ) at 3×10^6 /mL in complete medium, supplemented on day 0 with IFN- γ (1000 IU/mL) and on day 1 with 50 ng/mL anti-CD3 (OKT3; Terumo, Rome, Italy) and 500 IU/mL recombinant human (rh)IL-2 (Clinigen Healthcare, Burton upon Trent, UK). Every 2 to 4 days of culture, cells were counted in a Coulter counter (Beckman Coulter, Nyons, Switzerland) and diluted to a concentration of 1×10^6 /mL in fresh complete medium supplemented with rhIL-2 as above, for 21 days of culture.

Expansion of CIKs in G-Rex devices

To generate CIKs in G-Rex (CIK-G), PB- or CB-derived MNCs were plated at 0.5×10^6 cells/cm² (0.5×10^5 /mL) in G-Rex-10M (or volume-equivalent multiwell G-Rex-6M) or G-Rex 100M devices, in complete medium to the maximum volume capacity of the vessels (100 or 1000 mL, respectively) [13]. IFN- γ was added at 1000 IU/mL on day 0 and 50 ng/mL anti-CD3 mAb OKT3 and 500 IU/mL rhIL-2 were added on day 1. Every 3 to 4 days of culture at 37°C and 5% CO₂, a small aliquot of culture supernatant was collected for lactate measurement, and fresh 500 IU/mL rhIL-2 was added. Lactate was measured using the Lactate Plus reader (Nova Biomedical, Waltham, MA) according to the manufacturer's instructions.

Expansion of BETs in T-flasks

To generate BET in T-flasks, leukemia patient PBMCs were plated in standard tissue culture flasks at 3×10^6 /mL in complete medium, supplemented with 10 ng/mL blinatumomab and 500 IU/mL rhIL-2 (AMG103; Amgen, Thousand Oaks, CA) [7]. Every 2 to 4 days, cells were counted in a Coulter counter (Beckman Coulter) and diluted to a concentration of 1×10^6 /mL in fresh complete medium supplemented with blinatumomab and rhIL-2 at the same concentration as above, until complete disappearance of the B cells, after which only rhIL-2 was added. Cell products were collected after 21 days of culture.

Expansion of BETs in G-Rex

To generate BETs in G-Rex, leukemia patient PBMCs were plated at 0.03×10^6 to 0.3×10^6 CD3⁺ cells/cm² in G-Rex-6M, -10M or -100M devices. Complete medium, supplemented with 5 ng/mL blinatumomab and 500 IU/mL rhIL-2, was added to 40% of the full G-Rex device capacity (*i.e.*, 40 mL for the 6M or 10M devices and 400 mL for the 100M devices). On day 3 or 4, 50% of the supernatant was removed, and fresh medium supplemented with 5 ng/mL blinatumomab and 500 IU/mL rhIL-2 was added to the full capacity of the devices. On day 7, 50% of the supernatant was removed and replenished with fresh complete medium supplemented with 5 ng/mL blinatumomab and 500 IU/mL rhIL-2 as above.

Immunofluorescence analyses by flow cytometry

The phenotypes of MNCs or final products were analyzed by direct immunofluorescence. Major subsets were analyzed using the following antibodies: TCR α/β -FITC (clone WT31), TCR γ/δ -FITC (clone 11F2), CD3-PerCP-Cy5.5 (clone SK7), CD8-APC-H7 (clone SK1), CD4-PerCP or -PE-Cy7 (clone SK3) (all from BD Biosciences, San José, CA). CD45RA-FITC antibodies (BD Biosciences) and CCR7-PE (Miltenyi Biotec, Bergisch Gladbach, Germany) were used for naïve-memory subset analysis. To assess the presence of Tregs, cells were stained with CD4-PerCP-Cy5.5 (clone SK3) and CD25-PE (clone A251) (BD Biosciences) and fixed, permeabilized and stained with FOXP3-APC (clone PCH101) antibody using the FOXP3 staining kit (eBioscience, San Diego, CA). For T helper 1 cell

(Th1), Th2 and Th17 evaluation, cells were treated for 5 h with phorbol myristate acetate (PMA, 50 ng/mL; Sigma-Aldrich, Milan, Italy) and ionomycin (1 μ g/mL; Sigma-Aldrich) in the presence of Golgistop Protein Transport Inhibitor (BD Bioscience). Cells were then fixed and permeabilized using Cytofix/Cytoperm solution and stained intracellularly using the CD4-PerCP-Cy5.5 (clone SK3), IFN γ -FITC, IL-4-APC and IL-17A-PE antibody cocktail according to the manufacturer's instructions (Human Th-1/Th-2/Th17 Phenotyping kit; BD Bioscience).

The following antibodies against markers of T cell activation/costimulation or inhibition were used to stain the CD4⁺ and CD8⁺ subsets: CD27-PE (clone M-T271), CD28-PE (clone L293), CD137-PE (clone 4B4-1), CD154-PFITC (clone TRAP-1), CD272-PE (cloneJ168-540), CD279-APC (clone MIH4), CD244-PE (clone 2-69), NKG2D-PE (clone 1D11), CD11a-FITC (clone G-25.2), CD49d-APC (clone 9F10) (all from BD Biosciences), CD200R-PE (clone OX108; eBiosciences), and CD152-PE (clone L3D10; BioLegend). A FACSCanto II flow cytometer (BD Biosciences) was used to analyze all samples in triple fluorescence.

Cytotoxicity assays

To evaluate the *in vitro* cytotoxicity potential of CIKs or BETs against target cell lines, cell lysis was measured by standard calcein release assays [7,26]. Briefly target cells were labeled for 30 minutes at 37°C with 3.5 μ M Calcein-AM (Fluka, Sigma-Aldrich) and washed twice in complete medium. Labeled cells were then incubated at 37°C, 5% CO₂ in the presence of effector T cells at a 30:1, 10:1, 3:1 or 1:1 effector:target ratio and in the presence or absence of 10 ng/mL blinatumomab. After 4 h, the cells were sedimented by centrifugation, 100 μ L supernatant was collected and calcein release was determined using a fluorescence microplate reader (Greiner Bio-One, Pleidelsheim, Germany), with excitation at 485 nm and emission at 535 nm. The percentage of specific calcein release was calculated as % specific lysis = (test release – spontaneous release) \times 100 \div (maximal release – spontaneous release). Maximal lysis was determined in the presence of 1% Triton X-100.

In vivo immunotherapy

The previously described orthotopic ALL-2 model, derived from a patient with CD19⁺ Philadelphia-positive pre-B-ALL, was used to verify the therapeutic activity of CIK cells [27,28]. *In vivo* passaged cells were inoculated intravenously (iv) at 5 \times 10⁶ per mouse in 5-week-old female CB17.NOD/SCID mice (Charles River Laboratories Italia). On days 4, 11, 18, 25 and 32, 15 \times 10⁶ to 20 \times 10⁶ CIK-G cells were inoculated iv with or without 100 ng blinatumomab. The same dose of bispecific antibody was inoculated iv daily thereafter, for the 4 days after treatment with CIKs + blinatumomab or blinatumomab alone [7,29]. Five animals per group were used for survival determination. When hind leg paralysis was observed, animals were sacrificed and autopsied. Additional control (n = 2) and treated (n = 4) animals were used for analysis of T cell engraftment and tumor control and sacrificed on day 33. PB, spleen and bone marrow (BM) were collected, and cells were disaggregated mechanically and stained with anti-human CD45-PerCP, CD3-PE and CD19-FITC to measure the infiltration of human CIKs or leukemic cells in the different organs. Stained cells were analyzed by flow cytometry on a FACSCanto II instrument.

In vivo graft versus host disease evaluation

To assess the capacity of CIKs to induce graft versus host disease (GvHD), 20 \times 10⁶ unmanipulated PBMCs or CIK-Gs expanded in G-Rex from healthy donors were inoculated iv in NOD/SCID γ chain knockout (NOD.Cg-Prkd^{Scid}, NSG) mice. Mouse weight and appearance were monitored over time, for \leq 47 days. At sacrifice, PB, spleen and BM were collected and analyzed for the presence of T cells by staining with anti-human CD45-PerCP and anti-human CD3-FITC (BD Biosciences). Cells were analyzed by flow cytometry in a FACSCanto II Instrument (BD).

Statistical analysis

The data were analyzed with paired or unpaired Student *t* tests, as appropriate (**P* < 0.05, ***P* < 0.01 and ****P* < 0.001). For *in vivo* experiments, the Mantel-Cox method was used.

Results

Choice of G-Rex devices and their optimization for expansion of CIKs from peripheral blood

Our cell factory has previously generated >100 batches of CIK cells for phase I/II clinical studies (NCT01186809, NCT03821519) [3,4,30]. Current protocols plan to administer to patients three doses of 5 \times 10⁶, 5 \times 10⁶ and 10 \times 10⁶ CD3⁺CD56⁺ CIK cells/kg at 3-week intervals (NCT03821519). This means preparing a mean of 5 \times 10⁹ total nucleated cells (TNCs) per patient, requiring \leq 48 T175 flasks at the end of culture. To decrease the risk of microbiological contamination and workload during GMP production, our group initially tested the automated Xuri bioreactor, composed of a single culture bag placed on a rocking platform and provided with tubing for automated medium and gas perfusion. The Xuri instrument allowed production of CIKs with approximately the same efficiency as flasks and in a similar culture time in a single bag (data not shown) [31]. However, it allows only one batch in production at a time, making the device rather costly for our academic cell factory.

We therefore tested the G-Rex culture devices. We first optimized the culture conditions, starting with different initial seeding densities (0.25 \times 10⁶ to 1 \times 10⁶ PBMCs/cm² of gas-permeable membrane). We then selected 0.5 \times 10⁶ cells/cm² as an optimal density, and this was used thereafter (data not shown). The total volume of medium (100 or 1000 mL) was added to the G-Rex vessels at the start of culture, IFN- γ was added on day 0 and anti-CD3 and rhIL-2 were added on day 1 as in flasks. rhIL-2 was given again every 3 to 4 days. Cell growth in G-Rex was monitored by measuring lactate levels twice a week, so as to avoid cell resuspension, which would take away the advantage of these devices in terms of minimal manipulation and cell disturbance. Pilot experiments were performed by seeding the same number of cells (0.5 \times 10⁶ cells/cm²) in multiwells of the G-Rex 6M device and collecting cells at different times from different wells up to day 17. We observed that TNCs reached a plateau of \sim 500 \times 10⁶ TNCs/100-mL well around day 14 (50 \times 10⁶ TNCs/cm²), and viability decreased to <80% on day 14 and <70% on day 17 (data not shown). Lactate levels during the expansion phase were proportional to cell numbers (data not shown). These pilot experiments showed that optimal cell numbers and viability were obtained on day 10 to 12 of culture and that cultures should be stopped when lactate levels reached \sim 30 mM (data not shown and see below) to recover at least 300 \times 10⁶ viable cells/cm² with >80% viability.

We observed that culture in G-Rex vessels in these conditions allowed more efficient and rapid CIK expansion compared with T-flasks, starting from the same number of cells. Indeed, using 5 \times 10⁶ PBMCs, a mean of 320 \times 10⁶ total cells and 300 \times 10⁶ CD3⁺ T cells were reproducibly obtained in only 10 to 11 days of culture using the G-Rex 10M vessel (100 mL), compared with a mean of 140 \times 10⁶ total cells and 110 \times 10⁶ T cells obtained in 21 days in standard flasks (Fig. 1A and B). The rate of CIK expansion in G-Rex was quite reproducible, as shown by measured lactate levels over time in different batches (Fig. 1C) and cell yields on days 10 and 11 (Fig. 1A and B).

Phenotypic characterization in vitro of CIKs expanded in G-Rex vessels from peripheral blood sources

Having established the best protocol, we went on to characterize the CIKs produced in G-Rex (CIK-Gs) compared with T-flasks from the same PBMC or buffy coat as starting material. We first analyzed

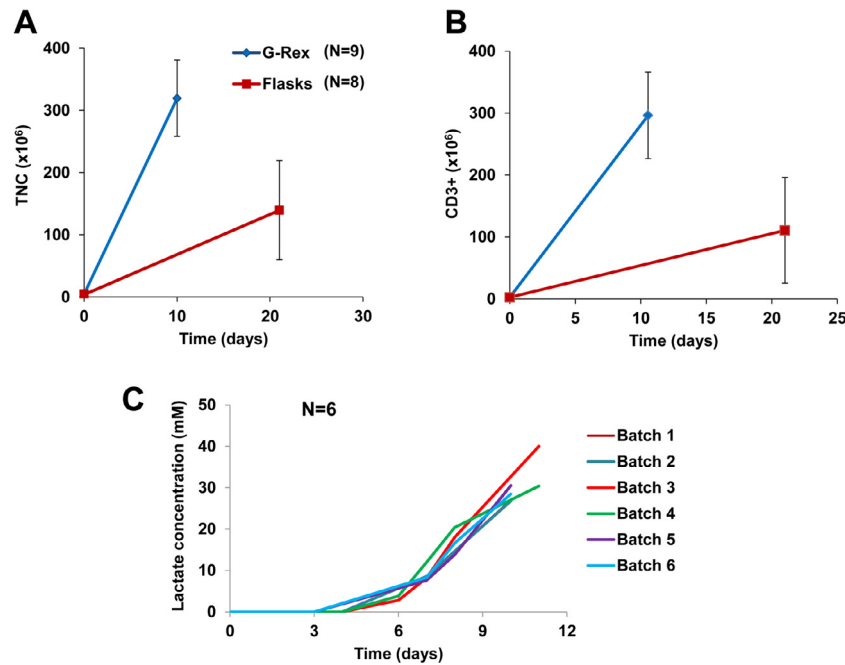


Figure 1. Expansion of CIKs from PB is more efficient in G-Rex devices compared with T-flasks. PBMCs were plated at $0.5 \times 10^6/\text{cm}^2$ in G-Rex vessels (G-Rex-6M, -10M or -100M) or at $3 \times 10^6/\text{mL}$ in T-flasks in CIK conditions. After 10 to 11 days for G-Rex or 21 days for flasks, cells were collected and counted (A). The number of live CD3⁺ was measured by immunophenotyping and flow cytometry (B). Lactate levels in the supernatant of G-Rex cultures were measured at different times to follow cell growth. The results of six representative cultures are shown (C). (Color version of figure is available online.)

standard T cell populations, including CD3⁺, CD4⁺, CD8⁺, CD3⁺CD56⁺ CIKs and CD3⁻CD56⁺ NK cells. As shown in Fig. 2A, CD4 and CD8 T cell populations and NK cells were not significantly different between G-Rex and T-flasks. In contrast, the percentages of CD3⁺CD56⁺ cells were slightly but reproducibly lower in G-Rex (mean 33%) compared with T-flask (mean 49%) cultures. Among CD4 cells, the percentages of Th1, Th2, Th17 or Treg cells did not differ significantly (Fig. 2B). Also the proportion of naïve (N), central memory (CM), effector memory (EM)

and EMRA among CD8⁺ cells were similar in G-Rex and T-flasks (Fig. 2C). The same results were obtained with CD4⁺ cells (data not shown). We finally analyzed a panel of activation or inhibitory markers expressed by either the CD8⁺ or CD4⁺ populations at the end of culture. As shown in Fig. 2D, most markers were similarly expressed in the CD8⁺ subset, whether expanded in G-Rex devices or T-flasks. Only CD27 and CD28 were more highly expressed in CIK-G compared with products from T-flasks (by 22% and 50%, respectively). A small

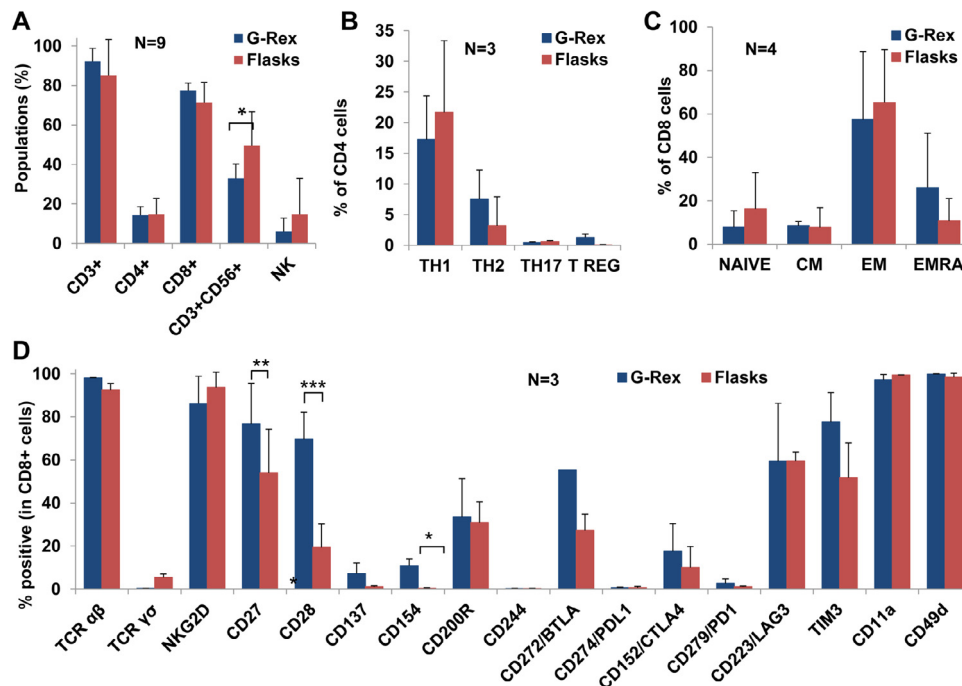


Figure 2. Characterization of CIK products expanded from peripheral blood. CIKs expanded in G-Rex or T-flasks were collected and analyzed by immunophenotyping and flow cytometry. The following parameters were analyzed: CD4, CD8, CD3⁺CD56⁺ and NK cell populations (A); TH1, TH2, TH17 and Treg subpopulations among CD4⁺ cells (B); naïve, central memory (CM), effector memory (EM) and effector memory RA populations (EMRA) in the CD8⁺ subset (C); and extended panels of activation and inhibitory molecules in the CD8⁺ subset (D). The results are the means and standard deviations of three to nine independent experiments. (Color version of figure is available online.)

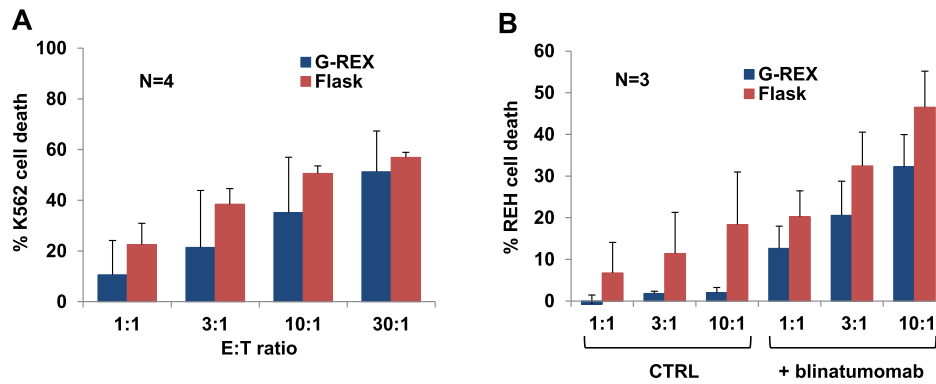


Figure 3. Cytotoxic activity of CIK expanded in G-Rex or T-flasks. CIKs expanded in G-Rex or T-flasks were used as effector cells against the K562 target cell line (A) or against the CD19⁺ B-ALL cell line REH in the presence or absence of 10 ng/mL blinatumomab (B), at the indicated effector-target ratios. Target cell lysis was measured after 4 h of incubation. The results are the means and standard deviations of three to four independent experiments. (Color version of figure is available online.)

difference in CD154 costimulatory molecule was also observed, but the percentages were in any case very low in both products (10.8% and 0.5%, Fig. 2D). Similar results were obtained when analyzing activation or inhibitory markers in the CD4⁺ population (data not shown).

We conclude that CIK cells expanded in G-Rex are phenotypically very similar to those expanded in T-flasks for expression of a quite extensive panel of activation, differentiation and inhibitory molecules. They showed only ~10% lower levels of CD56 and higher expression of CD27 and CD28.

Functional characterization *in vitro* and *in vivo* of CIK cells expanded in G-Rex compared with T-flasks

We next verified the cytotoxic capacity of CIKs expanded in G-Rex or T-flasks against the K562 tumor target. As shown in Fig. 3A, in both cases, significant cytotoxic activity was observed that did not

differ significantly between G-Rex and T-flask cultures, with maximal lysis reaching 51% to 57%. Similarly, both CIK populations were efficiently cytotoxic against the CD19⁺ ALL cell line REH in the presence of blinatumomab (Fig. 3B, maximal lysis 32% to 46%).

Also *in vivo*, CIK-G cells alone showed a measurable therapeutic activity in the CD19⁺ ALL-2 pre-B-ALL orthotopic model in mice (median survival time of 45 days with CIK-G alone compared with 36 days with vehicle only, Fig. 4A, $P < 0.01$). Furthermore, this activity was significantly increased by addition of blinatumomab, as expected, with a median survival time of 55 days for animals treated with CIK-G + blinatumomab (Fig. 4A, $P < 0.01$ versus vehicle and $P < 0.05$ versus CIK-G alone). These data are similar to previous data using CIKs from PB or CB expanded in T-flasks in the same ALL-2 model *in vivo* [28]. Interestingly, we could verify the presence of human CD3⁺ cells in the PB, BM and spleen of animals treated with CIK-G or CIK-G + blinatumomab (Fig. 4B, left panels). In contrast,

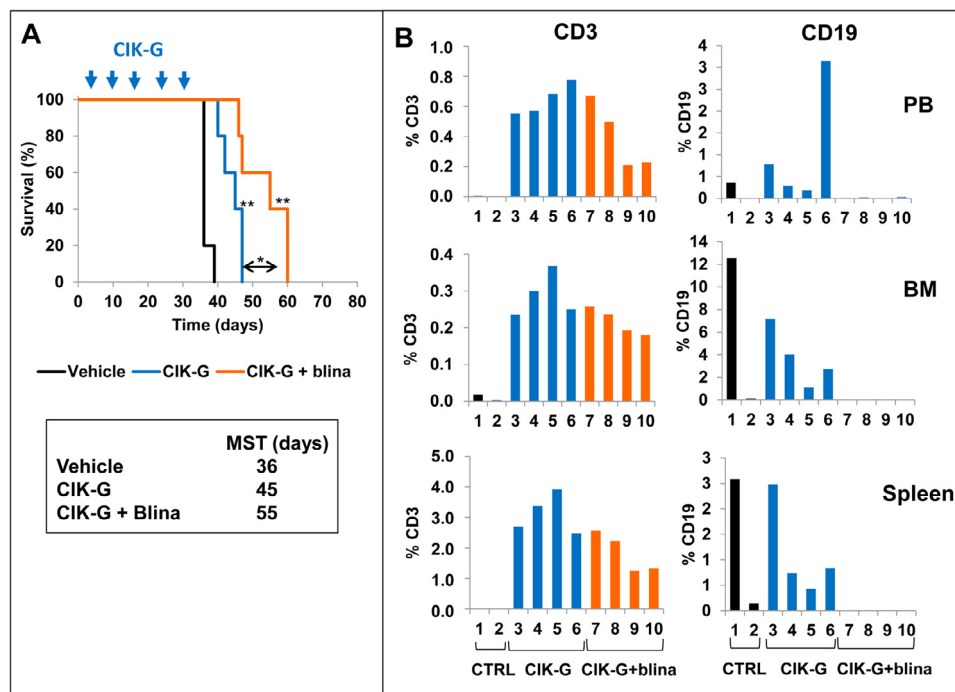


Figure 4. Therapeutic efficacy and persistence of CIK-Gs in pre B-ALL mouse model. 5×10^6 *in vivo* passaged ALL-2 cells were inoculated *iv* in NOD-SCID mice. 15×10^6 to 20×10^6 CIK-Gs were administered *iv* at days 4, 11, 18, 25 and 32 in the presence or absence of blinatumomab, in groups of five mice to measure survival (A). Median survival times (MSTs) for each group are shown in the lower table. Another two to four mice were set up in the same way to measure CD3⁺ CIK infiltration and CD19⁺ leukemic cells in the PB, BM and spleen. On day 33, these mice were sacrificed, the organs were collected and immunophenotyping by flow cytometry was performed with anti-human CD45, CD3 and CD19 antibodies (B). The percentages of CIKs and leukemic cells in the PB, BM and spleens of single animals treated with either PBS (n = 2, black bars), CIK-Gs (n = 4, blue bars) or CIK-Gs + blinatumomab (n = 4, orange bars) are shown (B). (Color version of figure is available online.)

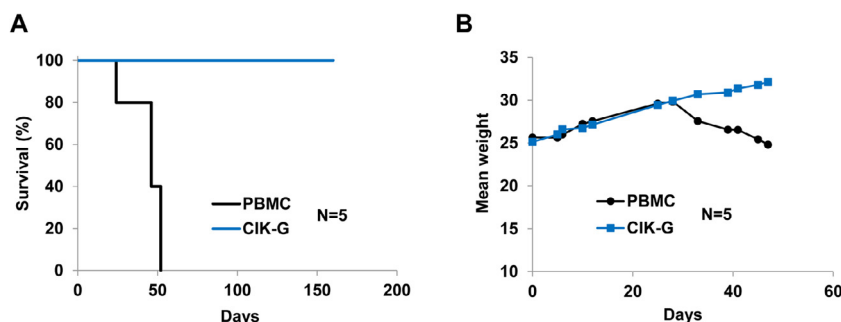


Figure 5. CIK-Gs do not induce GvHD in NSG mice. 20×10^6 PBMCs or CIK-Gs were administered iv to groups of five NSG mice. Survival was recorded up to day 160 (A), and the weight of the animals was measured twice a week up to day 50 (B, showing means of five animals). (Color version of figure is available online.)

human CD19⁺ tumor cells were detectable in the same tissues in untreated controls and in animals treated with only CIK-G, but not in animals treated with CIK-G + blinatumomab, confirming the efficacy of the strategy of CIK-G combined with blinatumomab in eliminating CD19⁺ leukemic cells *vivo* (Fig. 4B, right panels).

Lack of GvHD induction in mice treated with CIK-G compared with unmanipulated PBMC

CIKs, unlike unmanipulated human T cells, do not induce GvHD in NSG mice [5,32]. To verify that human CIKs expanded in G-Rex still lack GvHD potential, we performed an experiment in NSG mice. Groups of mice were inoculated with 20×10^6 PBMCs or CIK-Gs, and mouse weight and survival were monitored over time. As shown in Fig. 5A, unmanipulated PBMCs induced GvHD, leading to the death of all animals within 51 days after inoculation. This was accompanied by weight loss in the animals from day 28 onwards (Fig. 5B). The animals inoculated with PBMCs had strong infiltration of human T cells in all tested organs, including spleen, PB and BM, as expected (data not shown). In contrast, animals treated with the same number of CIK-Gs survived for > 150 days after inoculation without any external sign of disease and did not lose weight during the time frame tested (≤ 50 days, Fig. 5B). All animals treated with CIK-Gs were healthy at the end of the experiment (day 160). These data suggest that CIK-Gs do not induce significant GvHD *in vivo*.

CIKs from CB can also be rapidly expanded in G-Rex

Our cell factory has also developed CIKs from CB (CB-CIKs) for clinical use [30]. We therefore analyzed whether these cells can also be expanded using G-Rex vessels. Using the same protocol as CIKs from PB, and starting from freshly isolated CB MNCs, we indeed demonstrated more rapid and more efficient expansion of CB-CIK-Gs, compared with those obtained in T-flasks performed in parallel (Fig. S1A). Similarly to CIK-Gs from PB, expansion of CB-CIK-Gs in G-Rex lasted a mean of 9.7 days compared with 21 days in T-flasks. The CB-CIK-Gs had similar cellular composition as CB-CIKs produced in flasks, with again a similar proportion of CD4, CD8 and NK cells but a lower percentage of CD3⁺CD56⁺ cells, a pattern already observed in CIKs from PB (Fig. S1B). CB-CIK-Gs showed a capacity to lyse CD19⁺ leukemic cells (REH) in the presence of blinatumomab similar to that of CB-CIKs expanded in flasks (Fig. S1C). Altogether, these data show that G-Rex devices can be used to rapidly expand functional CB-CIKs.

Optimization of BET expansion in G-Rex

Autologous BETs are currently tested for the immune reconstitution of severely immunodeficient leukemia patients after chemotherapy, and the current dose is 12×10^9 CD3⁺ cells per infusion (NCT03823365) [7]. This translates into ≤ 72 T175-flasks at the end of

culture, making this ATMP a good candidate for simplified expansion in G-Rex. The starting PBMCs from patients contain variable percentages of CD3⁺ and CD19⁺ leukemic cells, the latter being eliminated during culture by the T cells activated with blinatumomab. During the G-Rex culture optimization phase, we therefore decided to define plating cell density on the basis of the number of CD3⁺ cells in the starting PBMC samples, plating 0.03×10^6 , 0.1×10^6 or 0.3×10^6 CD3⁺ cells/cm² in the multiwell G-Rex-6M vessels (corresponding to 0.3×10^6 , 1×10^6 and 3×10^6 CD3/100-mL cultures). Lactate concentration in the supernatant, total viable cell numbers, and CD3⁺ and CD19⁺ cells were analyzed on days 7, 10, 14 and 17. The results show that in all cases, $\geq 400 \times 10^6$ TNCs were recovered from each G-Rex vessel ($> 40 \times 10^6$ /cm²), but cultures with the higher CD3 starting doses (0.1×10^6 or 0.3×10^6 CD3⁺ cells/cm²) reached this level faster (in ~ 10 days) than the wells with lower number of cells seeded (0.03×10^6 CD3⁺ cells/cm²), which required 14 days to reach this cell yield (Fig. 6A). The same results were obtained when considering absolute CD3 counts during culture (Fig. 6B). CD3⁺ cells were in all cases >95% from day 7 onwards (data not shown). Viability was >80% at days 7 to 10 but started to diminish on days 14 and 17 (data not shown), explaining the decline in absolute number of TNC and CD3⁺ cells recovered at day 17 (Fig. 6A and B). The time course of lactate concentration followed cell numbers quite closely during the expansion phase and reached a plateau just above 30 mM from day 14 (Fig. 6C). On the basis of these pilot experiments, we selected 0.1×10^6 to 0.3×10^6 CD3⁺ cells/cm² as a standard starting dose for BET expansion and, as in CIK cultures, we defined the threshold of 30 mM lactate as a good surrogate marker to plan cell collection (Fig. 6C).

We then performed several experiments in these standard conditions in G-Rex and T-flasks in parallel. The fold increase of CD3⁺ cells in G-Rex versus flasks is shown in Fig. 6D, to normalize the data for the different starting cell numbers in different cultures. The data show that BETs were at least as efficiently expanded in G-Rex as in flasks, but equivalent yields were obtained in a mean of 10.8 days in G-Rex (Sd 1.6) compared with 21 days in flasks (Fig. 6D). Furthermore, final yields of CD3⁺ cells were less variable in G-Rex than in T-flasks (Fig. 6D). Thus, $\geq 320 \times 10^6$ CD3⁺ cells could be reproducibly obtained in six different 100-mL G-Rex cultures (range 320×10^6 to 455×10^6), corresponding to a yield of BETs of $\geq 32 \times 10^6$ CD3⁺ cells/cm² (Fig. 6D and data not shown).

The purity and viability of T cells and depletion of CD19⁺ leukemic cells in the final products were equivalent in T-flasks or G-Rex (Fig. 7A). Finally composition of CD3⁺ cells at the end of culture, in terms of CD4 and CD8 populations, was also similar (Fig. 7B).

GMP validations of CIK and BET expansion in G-Rex

The expansion of CIKs from healthy donors and BETs from B-NHL patient PB was also validated in GMP. The results are shown in Tables 1 and 2, respectively. Three batches of each ATMP were

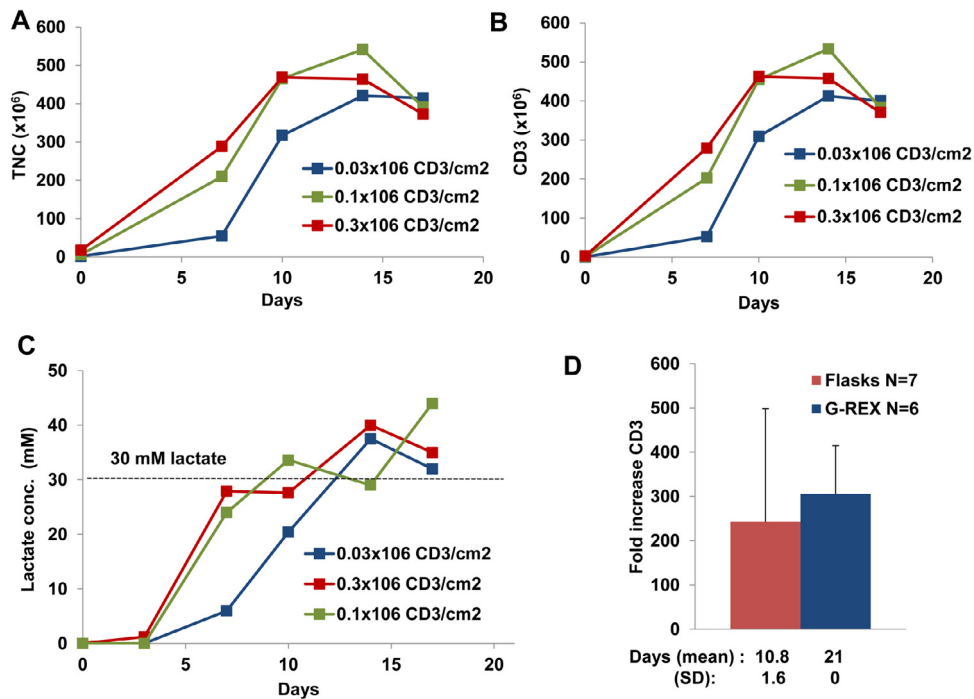


Figure 6. Optimization of BET expansion in G-Rex. (A–C) PBMCs from a leukemia patient were plated at 0.03, 0.1 and 0.3 $\times 10^6$ CD3⁺ cells/cm² in a G-Rex-6M multiwell device (100 mL/well) in the presence of blinatumomab and rhIL-2. The total numbers of cells (A) and CD3⁺ (B) were counted on days 7, 10, 14 and 17. Lactate concentration in the supernatant was measured twice weekly from day 3 onwards (C). (D) PBMCs from B-cell leukemia or lymphoma patients were plated at 0.1 to 0.3 $\times 10^6$ CD3⁺ cells/cm² in G-Rex devices or at 3 $\times 10^6$ TNC/mL in T-flasks in the presence of blinatumomab and rhIL-2. The fold increases in CD3⁺ cells obtained in six G-Rex and seven T-flasks cultures are shown. The mean number of days and standard deviation required to reach the observed cell yield is shown below the bars. (Color version of figure is available online.)

produced in 10M or 100M devices, and quality control was performed on the final products. All expansions were successful, with $>3 \times 10^9$ CD3⁺ cells obtained per liter of G-Rex cultures in 10 to 11 days, equivalent to $>30 \times 10^6$ CD3⁺ cells/cm². In the case of BETs, the starting material contained a relatively high percentage of CD19⁺ cells (61.9% to 85.3%). Nonetheless a mean of 11×10^9 BETs could have been obtained starting from a mean 25.5 mL PB, had the whole starting material been used (Table 2, note that not all post-Ficoll CD3⁺ were seeded). This shows that even high CD19⁺ cell contamination in the starting material does not inhibit BET expansion in G-Rex. The viability of both CIK and BET products was well over 80% in all cases and all product attributes, including cytotoxic potential, endotoxin contamination, and sterility and mycoplasma were compliant with the specifications (Tables 1 and 2). We conclude that CIKs and BETs can be conveniently and safely expanded in G-Rex devices for clinical purposes.

Discussion

The use of bioreactors greatly facilitates production of ATMPs for clinical studies. Several platforms exist to expand T cells, such as rocking motion, hollow fiber, stirred vessels or Prodigy, all of which generally incorporate semi-automated or automated cell culture sampling and medium replacement, as well as closed system options. The characteristics of these platforms and advantages and disadvantages for T cell and NK cell production in GMP have been nicely summarized in a recent review [9].

The G-Rex vessels are different from the above systems in that they are disposable, single-use culture vessels that can easily be implemented at low cost in an academic laboratory already equipped with standard 5% CO₂, 37°C incubators. G-Rex vessels come in different formats, provided with 10-, 100- or 500-cm² gas-permeable membranes, allowing easy scaling-up from development to clinical

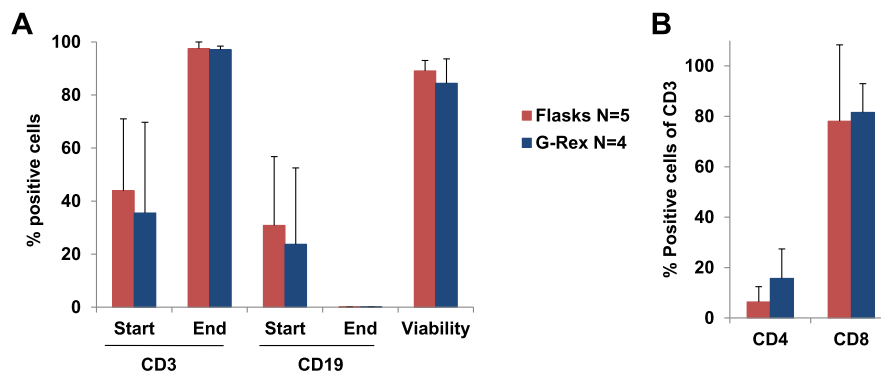


Figure 7. Phenotypic characterization of BETs expanded in G-Rex versus T-flasks. PBMCs from B-cell leukemia or lymphoma patients were plated at 0.1 to 0.3 $\times 10^6$ CD3⁺ cells/cm² in G-Rex devices or at 3 $\times 10^6$ TNC/mL in T-flasks in the presence of blinatumomab and rhIL-2. The percentages of CD3⁺ and CD19⁺ cells at the beginning and end of culture in G-Rex versus T-flasks is shown, together with the percentage viability of the cell products (A). The composition of CD3 cells in terms of CD4⁺ and CD8⁺ populations at the end of BET expansions in G-Rex or T-flasks is shown (B). The results are the means and standard deviations of four to five independent experiments. (Color version of figure is available online.)

Table 1
GMP validation of CIK expansion in G-Rex-10M devices.

Batch no.CIK	PBMC input $\times 10^6$	Days	Cell product									
			TNCs $\times 10^6$	Fold increase TNCs	% Viability	%CD3 ⁺	% CD3 ⁺ /CD56 ⁺	%NK	% Cytotoxicity (E:T 30:1 on K562)	Endotoxin (EU/mL)	Sterility	Mycoplasma
CIK 230-G	5	10	301	60.2	92.4	93.7	35.9	2.3	55.4	<7	sterile	absent
CIK 231-G	5	11	460	92.0	89.0	95.0	40.4	1.4	46.3	<3.5	sterile	absent
CIK 232-G	5	11	331	66.2	90.3	82.0	35.4	16.7	70.8	<3.8	sterile	absent
Specification	NA	NA	≥ 200	≥ 40	≥ 80	≥ 75	≥ 20	ND	≥ 30	<7	sterile	absent
Mean	5	11	364	72.8	90.6	90.2	37.2	6.8	57.5	NA	NA	NA
SD	0	0.6	84.5	16.9	1.7	7.2	2.8	8.6	12.4	NA	NA	NA

NA, not applicable.

runs from 100 mL to 1000- and 5000-mL cultures, respectively [13]. The constant proportion between gas-permeable surface area and maximal volume of culture medium means that the scale-up is straightforward and reproducible. In our hands, we observed that the conditions set up to expand CIKs or BETs in the small vessels (10M single vessels or 6M multiwell vessels accommodating a maximum of 100 mL) could indeed be scaled up to the 1-liter 100M vessels, just applying the same plating density and using medium and additives in proportion to total volume [13]. The plating density that we found to be optimal for CIK expansion (0.5×10^6 MNCs/cm²) was the same as that recommended by the manufacturers [13] and in line with that published by other groups for T cells [12,14,19,25].

Cells in G-Rex grow in a static manner and close to each other, which has been reported to be particularly favorable for T cell cultures that tend to grow in clusters [9,13]. This, together with the efficient gas exchange and ready availability of abundant nutrients in culture medium, may explain the much more rapid and efficient expansion in G-Rex compared with T-flasks. G-Rex has been used by other groups for the optimal expansion of several types of blood cells, including tumor-infiltrating lymphocytes (TILs) [19,20], antigen-specific T cells [8,12,17,33–35], $\gamma\delta$ T cells [23], Tregs [21,22], NKs [15], CIKs [25], CAR-Ts [16,24], megakaryocytes [36] and red blood cells [37] (reviewed in [9]). In most of these published cases, medium addition or exchange, as well as culture splitting, was performed with cell counting at various phases of the culture to define optimal dilution and collection times. In contrast, in our case, cell manipulation during expansion needed to be reduced to a minimum to define GMP-compliant, large-scale CIK or BET production. We used lactate levels as an indicator of cell growth, a method also used by others to follow cell expansion in bioreactors [10,16]. Glucose consumption is also sometimes used to estimate cell growth, but in our culture conditions, lactate levels were more reliable than glucose (data not shown). We observed that levels of lactate paralleled cell expansion and that 30 mM lactate indicated that cell collection could be carried out and would guarantee a yield of total cells $>30 \times 10^6$ /cm² and viability $>80\%$. Clearly, optimal lactate concentration may vary according to culture conditions and cell type and would need in any case to be verified in each laboratory. Additional metabolites may also be implemented for a more accurate measurement of cell expansion and cell death [9].

An advantage of G-Rex is that several vessels can be inserted in a single standard incubator, potentially allowing parallel expansions of multiple products with just one instrument. In contrast, other platforms require specific and quite expensive bioreactors in addition to single-use culture bags or vessels, and the machinery generally needs to be multiplied to be able to perform more than one expansion run in parallel [8,9,11]. This of course increases the costs significantly, which may not be sustainable for small or academic laboratories.

Another advantage of G-Rex is that the time of CIK production is ~ 10 days compared with 21 days for T-flasks. In the context of

clinical trials involving treatments with cell products dedicated to single, severely ill patients [4–6, 30], speed of production and release of ATMPs are crucial elements for the success of therapy, so reducing time of expansion by ~ 10 days is important. The lower risk of microbial contamination during culture, thanks to the reduced manipulation, may facilitate early release of products in case of urgent clinical need, before full quality control data have been obtained. Finally the safety of G-Rex vessels for CIK expansion can be increased further using CS devices, which are complete sterile tubings allowing the collection of samples for lactate measurement and addition of IL-2 and other substances in a fully closed manner using a sterile welder [13]. These devices are now available with more extended certifications for clinical manufacturing purposes.

We have shown here that the same method could be used to successfully expand CIK cells from CB. This is important, since qualified and HLA-identified cryopreserved CB units not used for transplantation purposes are becoming a useful source of material to expand T cells with therapeutic potential [28,38,39].

CIK cells expanded in G-Rex showed a T cell subset composition similar to that of CIKs from flasks, in particular CD4, CD8, TH1, TH2, TH17, Treg, $\alpha\beta$ and $\gamma\delta$ as well as naïve/memory subsets. They also expressed a similar phenotype for most activation markers, adhesion molecules and checkpoint inhibitors tested. Significant differences were observed only for CD27 and CD28, which were more highly expressed in CIK-G compared with CIKs from T-flasks. Both CD27 and CD28 are costimulatory receptors associated with T cell activation and induction of long-term memory [40–42]. Increased CD27 and CD28 in CIK-G may therefore favor CIK activity and permanence *in vivo*, although this needs to be demonstrated. CIK-G also had a reproducibly lower expression of CD56 than CIKs from flasks, whether they were expanded from PB or CB. CD56 is a differentiation marker of CIKs [43] that is induced late during culture. CD56⁺ cells are mostly responsible for degranulation and for their cytotoxic activity against NK tumor targets such as K562, whereas CD56⁻ cells are more immature and more highly proliferating cells that give rise to CD56⁺ cells during culture [43]. The lower expression of CD56 suggests therefore that CIK-Gs may be slightly less differentiated than CIKs from T-flasks. Our data are consistent with previous data showing that CD56 increases during culture [25]. Despite small differences in phenotype, CIKs expanded in G-Rex showed strong cytotoxic activity *in vitro* against the K562 and REH leukemia targets (in presence of blinatumomab). There was apparently only a small difference in cytotoxic activity, cells expanded in G-Rex being slightly less cytotoxic than those in flasks. The difference was not statistically significant but was observed quite consistently for CIKs from PB (Fig. 3) or CB (Fig. S1C), against either K562 or REH targets. We believe that this small difference may reflect the fact that CIKs in G-Rex are less differentiated than those expanded in T-flasks, as shown by lower CD56 expression and higher CD27 and CD28, as mentioned above.

Table 2
GMP validation of BET expansion in G-Rex-100M devices, starting from PB of three CLL patients.

Batch	Blood volume (mL)	%CD19 ⁺	CD3 ⁺ × 10 ⁶ post Ficoll	CD3 ⁺ × 10 ⁶ seeded	Days	TNCs × 10 ⁶	%CD3 ⁺	CD3 ⁺ × 10 ⁶	Fold increase CD3 ⁺	%CD4 ⁺	%CD8 ⁺	%CD19 ⁺	% Viability	Endotoxin (EU/mL)	Sterility	Mycoplasma	Theoretical CD3 ⁺ × 10 ⁶ yield using whole starting material
BET-G#4	24.5	61.9	79.5	13.3	10	4152	97.5	4048	304.4	29.0	68.1	0.0	89.5	<3.5	sterile	absent	24287
BET 20-G	26.0	85.3	17.2	10.3	11	3310	98.2	3250	315.5	8.6	90.3	0.1	87.6	<3.5	sterile	absent	6501
BET 21-G	26.0	88.4	10.1	10.1	10	3312	98.2	3252	322.0	79.6	17.9	0.0	94.0	<3.5	sterile	absent	3252
Specification	NA	NA	NA	NA	NA	NA	NA	>3000	>300	NA	NA	<0.5	>80%	<7	sterile	absent	NA
Mean	25.5	78.5	36.1	11.2	10.3	3591	98.0	3517	314	39.1	58.8	0.03	90.3	NA	NA	NA	11346
SD	0.9	14.5	37.7	1.8	0.6	485	0.4	460	8.9	36.5	37.1	0.06	3.3	NA	NA	NA	11324

NA: Not applicable

Also *in vivo*, CIK-Gs showed therapeutic activity in the CD19⁺ orthotopic pre-B acute leukemia model ALL-2. Finally CIK-G cells did not induce any GvHD activity in NSG mice, like CIK cells expanded in standard T-flasks ([32] and data not shown). In contrast, unmanipulated MNCs did induce GvHD as expected. These data altogether suggest that CIK-Gs are functional *in vitro* and *in vivo* and do not induce GvHD, similar to CIK cells expanded in T-flasks.

The G-Rex system was also successfully used in our hand to expand BETs from leukemia patients having 9.4% to 88.5% contaminating CD19⁺ leukemic cells in the starting PB sample. In the case of BETs, we defined the doses of CD3⁺ rather than total mononuclear cells to be plated/cm² of G-Rex vessel (0.1 × 10⁶/cm²), because the percentage of contaminating B cells is highly variable between samples. We also substituted half the medium twice during culture, to remove excess cell debris derived from lysed B cells. Also in this case, expansion was very reproducible, with >3 × 10⁹ T cells obtained per liter of culture in a mean of 11 days (>30 × 10⁶ T cells/cm²). Thus, a single G-Rex 500 vessel would be expected to yield ≥15 × 10⁹ BETs. In contrast, ≥72 flasks are needed to obtain 12 × 10⁹ BETs, as required by our current clinical protocol (data not shown). The BET products at the end of G-Rex culture had a similar composition of T cell subsets as BETs expanded in T-flasks. Thus BET expansion in G-Rex is clearly advantageous compared with standard flasks in terms of yield, reduced manipulation and therefore increased safety and shorter culture times.

The method to expand CIK-Gs and BET-Gs was validated in GMP. The X-VIVO medium used is manufacturing grade, free of animal-derived components and compatible with GMP production for human use [13]. Although not tested here, cell expansion in G-Rex could easily be connected to other devices, using a sterile welder. We are indeed presently setting up a direct connection of G-Rex devices to the Gatherex cell harvesting and LOVO cell processing instruments, for washing and medium exchange at the end of the expansion, in a fully closed system [13]. We are preparing an IMPD for CIK cells expanded in G-Rex for clinical use.

Conclusions

In summary, the authors have optimized a GMP-compliant method in disposable G-Rex devices to rapidly and reproducibly expand large numbers of CIK cells from healthy donors' peripheral and cord blood and BETs from leukemia patients, with minimal manipulation. The cell products were characterized phenotypically, in comparison with the same cell products expanded in standard T-flasks. In presence of blinatumomab, CIK cells expanded in G-Rex were cytotoxic against CD19⁺ leukemia cell both *in vitro* and *in vivo* and lacked GvHD activity, supporting their use for clinical immunotherapy studies.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at [doi:10.1016/j.jcyt.2021.11.004](https://doi.org/10.1016/j.jcyt.2021.11.004).

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