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Development of an optimized closed and semi-automatic protocol for Good Manufacturing Practice manufacturing of tumor-infiltrating lymphocytes in a hospital environment

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ABSTRACT

Background aims: Several studies report on Good Manufacturing Process (GMP)-compliant manufacturing protocols for the *ex vivo* expansion of tumor-infiltrating lymphocytes (TILs) for the treatment of patients with refractory melanoma and other solid malignancies. Further opportunities for improvements in terms of ergonomy and operating time have been identified.
Methods: To enable GMP-compliant TILs production for adoptive cell therapy needs, a simple automated and reproducible protocol for TILs manufacturing with the use of a closed system was developed and implemented at the authors' institution.
Results: This protocol enabled significant operating time reduction during TILs expansion while allowing the generation of high-quality TILs products.
Conclusions: A simplified and efficient method of TILs expansion will enable the broadening of individualized tumor therapy and will increase patients' access to state-of-the-art TILs adoptive cell therapy treatment.

Introduction

The prognostic significance of tumor-infiltrating lymphocytes (TILs) in solid cancers has been known for several decades [1,2]. Many studies focusing on the characterization of tumor biopsies demonstrate that a high density of TILs within tumors is mostly associated with a favorable outcome and a longer overall survival, especially in patients exhibiting a high expression of CD8+ T cells [3–13]. However, despite the favorable presence of TILs, some patients may encounter tumor progression and resistance to state-of-the-art conventional therapeutics. Indeed, the tumor microenvironment and expression of specific receptors (e.g., PD-1, CTLA-4) may allow immune escape and impair the potential tumoricidal activity of TILs, hence promoting malignant tumor growth and distant metastasis [14,15].

To overcome this limitation, adoptive cellular therapy (ACT) using autologous *ex vivo*-expanded TILs (TIL-ACT) has been studied in clinical trials involving cancer patients [1,2,16–19]. TIL-ACT demonstrates a high efficiency in patients with advanced carcinoma who have failed prior standard chemotherapy and/or immune checkpoint blockade regimens [18,20]. To date, basic research and clinical trial outcomes have helped pave the way for a safe and efficient clinical use of TILs in cancer therapy. Indeed, the benefit of pre-conditioning chemotherapy (non-myeloid lymphodepletion) prior to TILs infusion has been clearly demonstrated [21–23]. Moreover, the repeated injection of high-dose IL-2 following TILs infusion has been shown to greatly improve the objective and complete response rates in several types of solid tumors [19]. In

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addition, some studies have demonstrated that TILs can be detectable in peripheral blood more than a year after infusion, confirming the potential long-term effect of TIL-ACT [24].

To date, only a few TIL-ACT programs have been developed worldwide. Indeed, the complexity and high labor intensity associated with Good Manufacturing Practice (GMP)-grade TILs manufacturing require highly specialized staff and adequate facilities. Donia *et al.* [25] first reported the development of a simplified protocol, known as rapid expansion protocol (REP), that leads to significant workload reduction during the second step of T-cell expansion and can be integrated into routine manufacturing of TILs. Despite the significant progress made with regard to the clinical use of TILs, one limiting factor for the induction of an efficient targeted anti-tumor immune response remains the number of functional T cells infused. In the present article, the authors report on the first manufacturing process approved by the Swiss regulatory authority, Swissmedic, for the clinical use of *ex vivo*-expanded TILs in cancer treatment. The authors describe a GMP-compliant, reproducible and efficient manufacturing approach, performed in closed and semi-automatic systems, enabling the generation of functional TILs from various solid tumor biopsies associated with melanoma; colorectal, lung and cervical carcinoma; mesothelioma and uterine leiomyosarcoma.

Interestingly, the authors' manufacturing process helped obtain a higher TILs yield with a similar cell viability compared with conventional *in vitro* TILs expansion approaches based on traditional culture plates. Moreover, T-cell subset content (i.e., CD8+ and CD4+ T cells) and phenotype (expression of activation markers) remained comparable to conventional protocols. In addition, the authors demonstrated the high functional avidity, strong effector profile, autologous tumor cell recognition ability and cytolytic potential of TILs generated with the manufacturing protocol. Finally, the manufacturing approach, based on closed systems with reduced hands-on operator time and integrated quality control (QC) strategy, was approved by Swissmedic. Together, the quality of the manufacturing process, reduced operator time and phenotype and functional testing analysis confirm the potential therapeutic use of the authors' manufacturing process for further TIL-ACT clinical trials.

Methods

Patient characteristics

Samples from 47 patients with histologically confirmed metastatic solid tumors referred to the Lausanne University Hospital Department of Oncology were used for this study. For protocol development and validation, a total of 17 REP and 53 pre-REP procedures were performed. In addition, the authors present TIL expansion data from 24 pre-REP and eight REP procedures performed in line with a phase 1 clinical trial (see supplementary Figure 1).

Sample procurement, transport and storage

The work described was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the ethics committee of the canton of Vaud, Switzerland. All patients gave written informed consent before collection of samples under protocols 2016-02094, 2016-02166, 2016-02189 and 2018-01532 and collection and transportation of tumor samples and leukapheresis bags were performed according to local standardized operating procedures.

Solution and media preparation

TILs were manufactured in the GMP facility at the Lausanne University Hospital Center of Experimental Therapeutics by either the process development unit or the GMP production unit. During clinical manufacturing for a phase 1 clinical study by the Center of Experimental Therapeutics GMP production unit, manufacturing was performed under European Union (EU) GMP conditions following standardized operating procedures generated per requirements described in Annexes 1, 2 and 13 of the EudraLex Vol 4, with the TILs product being classified under EC 1394/2007 as an advanced therapy medicinal product. Clinical manufacturing, including a strict air pressure cascade, was performed in a grade D clean room under aseptic conditions according to GMP EU Annex 1. The handling of TILs starting material, raw materials and consumables with direct exposure to the environment was performed in a grade A isolator (GMP EU Annex 1), providing the first level of containment.

Pre-rapid expansion protocol

Tumor specimens (fresh or cryopreserved) were cut into small fragments (1–3 mm^3), which were placed in a complete medium, consisting of RPMI 1640/CTS (Gibco), 0.025 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Invitrogen), 55 μmoL/L 2-mercaptoethanol (Gibco) and 10% human AB serum (Biowest), supplemented with high-dose IL-2 at 6000 IU/mL (Proleukin; Novartis Pharma). For 24-well-based cultures, tumor specimens were seeded at one fragment per well. For G-Rex-based cultures, 30–60 tumor fragments were seeded per G-Rex 100M flask (Wilson Wolf Manufacturing Corporation). When pre-REP was initiated using cryopreserved tissue in 10% dimethyl sulfoxide (DMSO) cryopreservation solution, the tissue was thawed at 37°C in complete media supplemented with Pulmozyme. Media changes and cell counts were performed on day 11 (± 2 days) and day 21 (± 3 days). When cultures reached ≥50–100 × 10^6 cells, TILs were harvested and cryopreserved in a dry-phase liquid nitrogen tank until later use using CryoMACS DMSO (Miltenyi Biotec) supplemented with 90% human AB serum. Culture time for pre-REP was extended up to 35 days. On day 35, based on the cell count, the decision was taken to harvest TILs or to stop the protocol because of insufficient growth of TILs (Figure 1).

During phase 1 clinical trials, pre-REP TILs were cryopreserved to allow for logistical coordination with the clinical teams prior to initiating the REP and for QC testing (Figure 1). Furthermore, at the pre-REP harvest stage, QC testing included sterility, *Mycoplasma*, endotoxins and cell phenotype to ensure the manufactured product complied with quality and safety standards.

Rapid expansion protocol

After thawing of pre-REP TILs and a 1- to 3-day rest in TIL complete media, TILs were seeded in G-Rex 100M flasks (Wilson Wolf Manufacturing Corporation), using a target of 5.0 × 10^6TILs per G-Rex, with 400 mL of 50:50 media (50% TIL complete media and 50% CTS AIM V supplemented with IL-2 at 3000 IU/mL), anti-CD3 (OKT3; Miltenyi Biotec) 30 ng/mL and feeder cells, using a target of 1.0 × 10^9 feeder cells per G-Rex and a 1:200 TIL:feeder ratio (acceptable ratio, 1:10 to 1:200). The mixture was then placed in an incubator at 37°C and >90.0% humidity with 5.0% carbon dioxide.

To obtain feeder cells, leukapheresis material (autologous from patients or allogeneic from volunteers) was thawed and washed using the LoVo spinning membrane filtration system (Fresenius Kabi). Feeder cells were irradiated at 2 × 30 Gy prior to being seeded in the G-Rex flasks. As a control when using allogeneic feeders, 0.5–1.5 × 10^9 allogeneic irradiated feeders were seeded in flasks without TILs. On day 5 (± 1 day), 200 mL of 50:50 media supplemented with IL-2 at 3000 IU/mL final concentration was added to the G-Rex flasks. On day 7, TILs and feeder cells were transferred from the G-Rex flasks (Wilson Wolf Manufacturing Corporation) to a Xuri cell expansion system W25 (GE Healthcare Life Sciences) 10-L bag containing CTS AIM V supplemented with 3000 IU/mL IL-2 (Figure 1).

From this point, and until cell harvest, cell counts were performed to maintain TILs, initially at a target of 2.0 × 10^6 cells/mL (range, 1.5–2.5 × 10^6 cells/mL), by progressively adding culture media until the volume reached 5.0 L and then by media perfusion, with the

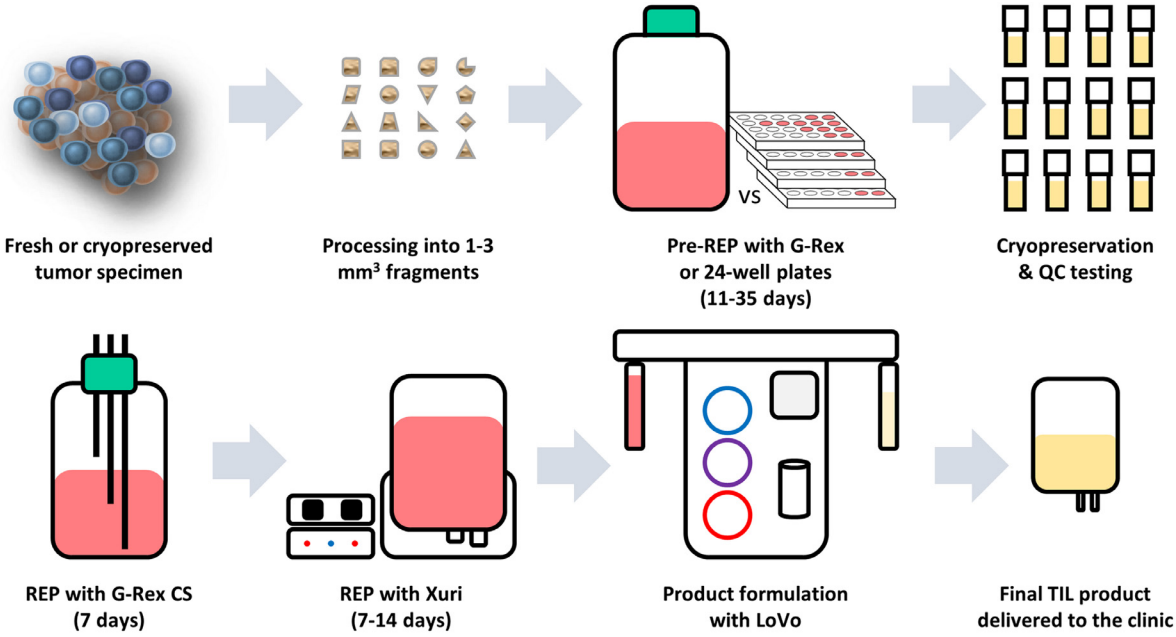

Fig. 1. Description of GMP-compliant manufacturing process for personalized *ex vivo* TILs expansion. CS, closed system.

perfusion rate based on cell concentration. REP harvest was performed on approximately day 14 (i.e., between day 12 and day 20), during which the Xuri 10-L perfusion bag and final product bag were connected to the LoVo spinning membrane filtration system using TSCD welding to perform an automated washing step under sterile conditions (Figure 1).

Final product formulation

TILs were formulated in 200–300 mL 0.9% sodium chloride (B. Braun Medical) and 2% albumin (Albunorm; Octapharma) and supplemented with 300 IU/mL IL-2. QC sampling was collected, and the final product bag was sealed until transport to the clinic (Figure 1). QC sampling was performed to assess appearance, sterility, *Mycoplasma*, endotoxins, cell count, viability and phenotype. For clinical manufacturing, to enable release of a fresh product the day of final formulation, QC testing (sterility, *Mycoplasma*) was also performed on the Xuri 10-L bag a minimum of 2 days prior to harvest.

Phenotype analyses by flow cytometry

For phenotype analyses, $0.5–1.5 \times 10^6$ fresh TILs were washed and stained at 4°C for 20 min with the following antibody cocktail at the respective concentrations: Zombie UV Indo(Violet) viability test at 1/200 (#423108; BioLegend), CD45RA ECD at 1/333 (RRID:AB 10640553, #IM2711U; Beckman Coulter), CD3 BV711 at 1/100 (RRID: AB 2744392, #563725; BD Biosciences), CD27 APC-eF780 at 1/100 (RRID:AB 1272040, #47-0279-42; eBioscience), CD45 PE-Cy5 at 1/50 (RRID:AB 314398, #304010; BioLegend), CD4+ BV605 at 1/50 (RRID:AB 11218995, #317438; BioLegend), CD8-KrO at 1/50 (#PN B00067; Beckman Coulter), PD-1 (CD279) BV421 at 1/50 (RRID:AB 10960742, #329920; BioLegend), TIM-3 APC at 1/50 (RRID:AB 1964725, #FAB2365A; R&D Systems), CD137 AF700 at 1/50 (RRID:AB 2207742, #309816; BioLegend), CD56 PERCPCY5.5 at 1/33 (RRID:AB 893389, #318322; BioLegend), CD197 (CCR7) PE/CY7 at 1/25 (RRID:AB 11125576, #353816; BioLegend), CD25 BB515 at 1/25 (RRID:AB 2744340, #564467; BD Biosciences), CD127 PE at 1/25 (RRID:AB 131301, #IM1980U; Beckman Coulter). Cells were then washed with phosphate-buffered saline (PBS) and resuspended in BD CellFIX before acquisition on a BD LSR Fortessa flow cytometer. Analyses were performed using FlowJo 10.5.2 software.

Derivation of autologous tumor cell lines and single cell digest

Upon receipt of the fresh tumor, a biopsy sample was cut in a Petri dish into fragments of 1–3 mm^3in size. Ten tumor fragments were digested in a mix of collagenase type I (#C-0130-1G; Sigma-Aldrich) and DNAse (#04536 282 001; F. Hoffmann-La Roche) in 20 mL of R10 medium (RPMI 1640 GlutaMAX; Gibco) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (BioConcept). After incubation at 37°C for 1 h 30 min, mixing regularly, the cell suspension was harvested and passed through a cell strainer to remove potential remaining tissue pieces. The cell suspension was centrifuged (600 × g for 5 min at room temperature) and washed once using RPMI. Cells were resuspended in R10, plated in a 25-mm^2 flask and then incubated at 37°C with 5% carbon dioxide. Cell growth was monitored, and the culture medium was changed every 2 days. Passages were performed when the cell layer reached 80% of confluence.

Autologous tumor recognition by flow cytometry

The day before the assay, autologous tumor cells were cultured in R10 medium until a sufficient number was reached. Cells were then detached, washed with PBS and plated at a concentration of $1.5–2.0 \times 10^6$ cells/well overnight in a 24-well plate with R10. TILs were thawed and rested overnight in R8 medium containing IL-2 (6000 U/mL for pre-REP and 3000 U/mL for REP) at a concentration of $1.5–2.0 \times 10^6$ TILs/well in a 24-well plate. Cytokine production was not assessed in this experiment; therefore, cells could be cultured in a medium supplemented with IL-2 without leading to substantial bias in the results.

R8 medium consisted of RPMI 1640 GlutaMAX supplemented with 8% human AB serum (Biowest), 1% penicillin/streptomycin (BioConcept), L-glutamine 2 mM (Thermo Fisher Scientific), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid 10 mM (Thermo Fisher Scientific AG), 2-beta-mercaptoethanol 0.05 mM (Thermo Fisher Scientific), sodium pyruvate 1 mM (Thermo Fisher Scientific) and minimum essential medium non-essential amino acids 1/100 (Thermo Fisher Scientific).

On the day of the assay, tumor cells adhered to the bottom of the plate, and R10 medium was removed by gentle aspiration. Since it is

well established that CD137 peaks at 6 h in the setting of tumor recognition [26], TILs were co-cultured with tumor cells at an effector-to-target ratio of 1:1 at 37°C for 6 h, and $0.5–1.0 \times 10^6$TILs were kept unstimulated as a background control. After 6 h, cells were harvested from the plate, washed with PBS and stained with the following antibody cocktail at the respective concentrations: live/dead APC at 1/200 (#L10102; Invitrogen), CD4 Pacific Blue at 1/33 (RRID:AB 397037, #558116; BD Biosciences), CD8 FITC at 1/200 (RRID:AB 1877178, #344704; BioLegend) and CD137 PE at 1/20 (RRID:AB 2654986, #130-110-763; Miltenyi Biotec). Cells were then washed with PBS and resuspended in PBS before acquisition on a BD LSRFortessa flow cytometer (BD Biosciences) or BD FACSMelody cell sorter (BD Biosciences), when needed.

Tumor recognition by interferon gamma enzyme-linked immunospot assay

Two days before the assay, TILs were thawed and plated in R8 medium containing IL-2 (6000 U/mL for pre-REP and 3000 U/mL for REP) at a concentration of $1.5–2.0 \times 10^6$ TILs/well in a 24-well plate. Over the next 2 days, half of the medium was changed twice a day (morning and afternoon) and replaced with R8 medium containing no IL-2 to decrease the IL-2 concentration and to limit spontaneous cytokine release (i.e., the assay background) and therefore optimize effect-to-noise ratio.

On the day of the assay, TILs were harvested, counted, washed with PBS and resuspended in R10 at a concentration of 1.0×10^6 cells/mL. Autologous tumor cells grown in R10 were detached, washed with PBS and resuspended in R10 at a concentration of 1.0×10^6 cells/mL. Interferon gamma (IFN-γ) enzyme-linked immunospot assay (#3420-2APT-10; Mabtech AB) was performed according to the manufacturer's recommendations. TILs were co-cultured with tumor cells at a 1:1 effector-to-target ratio. TILs alone were kept as a negative control. Staphylococcal enterotoxin B (#S4881; Sigma-Aldrich) was used as a positive control at a concentration of 125 ng/mL. Anti-HLA-A,B,C (RRID:AB 1877080, #311423; BioLegend) was added at a concentration of 10 μg/mL to check for HLA class I-mediated response. TILs were also tested against a heterologous tumor cell line to ensure a specific response. All conditions were tested for 18 h in triplicate, and the day after, spots were revealed following the manufacturer's instructions. After allowing the plates to dry, spots were counted using an AID automated counter (Autoimmun Diagnostika).

Cytokine measurements

Two days before the assay, TILs were thawed and plated in R8 medium containing IL-2 (6000 U/mL for pre-REP and 3000 U/mL for REP) at a concentration of $1.5–2.0 \times 10^6$ TILs/well in a 24-well plate. Over the next 2 days, half of the medium was changed twice a day (morning and afternoon) and replaced with R8 medium containing no IL-2 to decrease the IL-2 concentration and to limit spontaneous cytokine release (i.e., the assay background) and therefore optimize effect-to-noise ratio.

On the day of the assay, TILs were harvested, counted, washed and resuspended in R10 medium at a concentration of 1.0×10^6 cells/mL. TILs (1.0×10^5) were then plated in a 96-well plate and stimulated with different concentrations of anti-CD3 (#3605-1S; Mabtech AB), ranging from 0.1 ng/mL to 100 ng/mL. The cells were stimulated for 18 h. Supernatants were harvested and cytokine concentrations measured using the V-PLEX pro-inflammatory panel 1 human kit (#K15049D; Meso Scale Discovery) following the manufacturer's instructions. Plates were read using the MESO QuickPlex SQ 120 instrument (Meso Scale Discovery).

Statistical analysis

Statistical analysis included descriptive statistics using Mann-Whitney, Wilcoxon matched-pairs signed rank test, Kruskal-Wallis or *t*-test as appropriate. Statistical analysis was performed using Prism 8.0 (GraphPad Software, San Diego, CA, USA).

Results

Pre-REP optimization process

Pre-TILs grown in G-Rex flasks result in a higher number of cells

Overall, the authors performed 77 pre-REP procedures during the course of process development and in the context of phase 1 clinical trials. During process development, the authors evaluated the use of 24-well plates versus G-Rex 100M flasks for melanoma and solid tumors (Figures 1, 2A–G). In addition, for the pre-REP expansions during the phase 1 clinical trials, all cultures were performed in G-Rex flasks. The use of G-Rex flasks was investigated to help reduce operators' days in the clean room (i.e., repeated media changes) and risks to the product (i.e., manipulation of open plates, in/out isolator) and to improve TILs expansion. For this investigation, 31 pre-REP procedures were performed in parallel in both 24-well plates and G-Rex 100M flasks. The data gathered convincingly demonstrated a beneficial impact of the G-Rex flask in terms of total TILs count at harvest (Figure 2A,B) as well as in TILs expansion when normalized per number of tumor fragments seeded (Figure 2C). TIL viability in the G-Rex cultures was equivalent to TILs viability observed in 24-well plates (Figure 2D). Although a higher total number of TILs was obtained at the end of pre-REP procedures with G-Rex flasks, the overall composition of cultures with regard to the main T-cell subsets remained quite similar compared with TILs grown in 24-well flasks, with a preferential expansion of CD3+ T cells (Figure 2E) and similar frequencies of CD8+ T cells (Figure 2F).

A more comprehensive profiling of pre-REP TILs obtained from cultures in 24-well plates or G-Rex flasks demonstrated comparable CD4+ and CD8+ TILs subsets (Figure 3A,C). Indeed, the pattern of expression of PD1, TIM3, CD25, CD27 and CD137 on CD4+ and CD8+ pre-REP TILs was very similar (Figure 3A,C), and combinatorial analyses confirmed that TILs were mostly composed of PD1+TIM3+ and PD1+TIM3− T cells irrespective of pre-REP culture conditions (Figure 3B,D). Hence, the culture of pre-REP TILs in G-Rex flasks instead of 24-well plates did not enhance expression of exhaustion-related markers [27]. Together, these data indicated that the usage of G-Rex flasks led to an overall higher total number of cells at the end of the pre-REP procedures without affecting the viability, composition or phenotype of the TILs.

Pre-REP TILs can be expanded from cryopreserved and fresh tumors and are functional

Interestingly, the results also demonstrated that pre-REP TILs can effectively be expanded from cryopreserved tumor starting material (Figure 4A). Heterogeneity can be seen when comparing TILs expansion of different patients with the same histology, reinforcing the hypothesis that the efficacy of TILs expansion is patient-dependent. Even though the total TIL count from cryopreserved samples was substantially lower than that observed for fresh tumor samples (data not shown), the authors always achieved the minimal number of 5.0×10^6TILs necessary for REP initiation. The data proved that it was possible to perform successful pre-REP TILs expansion using cryopreserved tumor specimens, thereby allowing for maximum flexibility in GMP manufacturing capacity, as pre-REP initiation could be performed independently of the surgery timetable.

The authors identified two manufacturing hurdles: the pre-REP TILs expansion kinetics and the number of culture days required to reach a pre-determined threshold of output pre-REP, here established at 100×10^6 TILs. The data presented for the pre-REP expansion of melanoma (Figure 4B) and other solid tumors (Figure 4C)

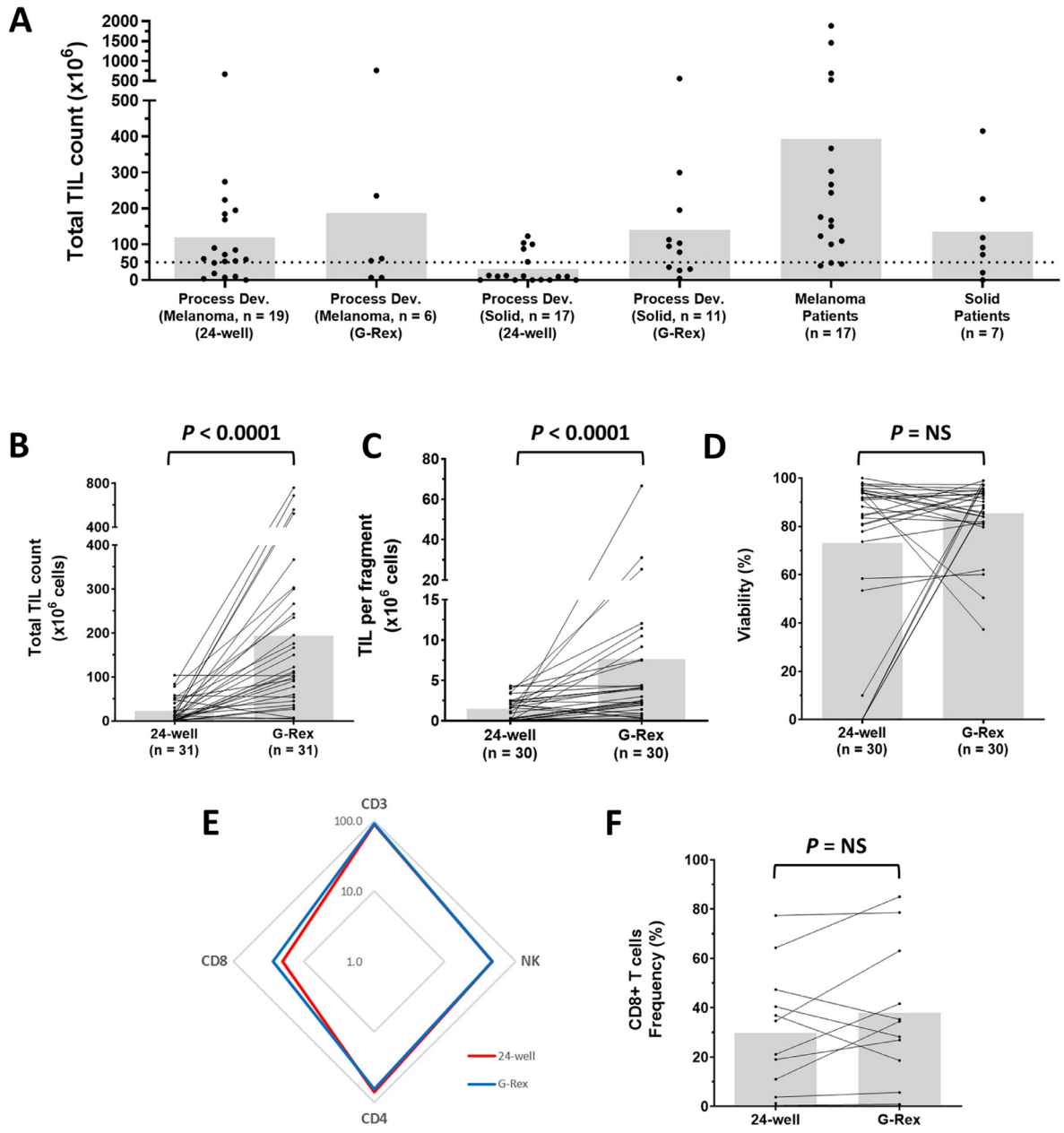

Fig. 2. Pre-REP TILs expansion in 24-well plates or G-Rex flasks. (A) Total TILs number obtained at the end of pre-REP using 24-well plates or G-Rex flasks during process development and phase 1 clinical trials. Dotted line represents the threshold of 50.0×10^6 TILs necessary to initiate two REP procedures with five G-Rex flasks, each seeded with 5.0×10^6 TILs at initiation. Data are plotted as points corresponding to individual cultures, and shaded columns correspond to the mean value. (B) Paired comparison of TILs number obtained at end of pre-REP from 31 samples expanded with 24-well plates or G-Rex flasks. Data are plotted as points corresponding to individual cultures, and shaded columns correspond to the mean value. Wilcoxon matched-pairs signed rank test $P < 0.0001$. (C) Ratio of TILs per fragment seeded. Data are plotted as points corresponding to individual cultures, and shaded columns correspond to the mean value. Wilcoxon matched-pairs signed rank test $P < 0.0001$. (D) Cell viability by Trypan Blue. Data are plotted as points corresponding to individual cultures, and shaded columns correspond to the mean value. Wilcoxon matched-pairs signed rank test $P > 0.05$ (NS). (E) Frequencies of main cell subsets: CD3+, CD8+, CD4+ and NK cells. (F) Frequencies of CD8+ T cells. Data are plotted as points corresponding to individual cultures, and shaded columns correspond to the mean value. Wilcoxon matched-pairs signed rank test $P > 0.05$ (NS). Dev., development; NK, natural killer; NS, not significant.

highlight four distinct kinetics: tumors with (i) rapid (green lines), (ii) moderate (black lines), (iii) slow (blue lines) and (iv) no or minimal TILs expansion potential (red lines).

Together, these results demonstrate that a sufficient number of TILs can be generated from the majority of tumor samples following the authors' pre-REP protocol. Moreover, the data demonstrate that TILs expansion is possible in several types of solid tumors, including cryopreserved tumors, with nonetheless varying expansion kinetics.

The authors next evaluated the functionality and, in particular, the anti-tumoral reactivity of pre-REP TILs. To this end, autologous tumor cell lines (devoid of any CD45+ cells) were established as described previously. Autologous tumor cells were then briefly co-cultured with pre-REP TILs cultures to induce a specific stimulation as evaluated by membrane-bound CD137 (also known as 4-1BB) expression. Of note, when an autologous tumor cell line could not be established in time for the tumor recognition assay, a tumor single-cell digest was used. In this case, the percentage of CD45+ cells was not determined. Although the authors cannot formally exclude potential contamination of the CD137 signal by *ex vivo* TILs present in the tumor samples, this signal is not expected to lead to bias in the evaluation of the extent of tumor

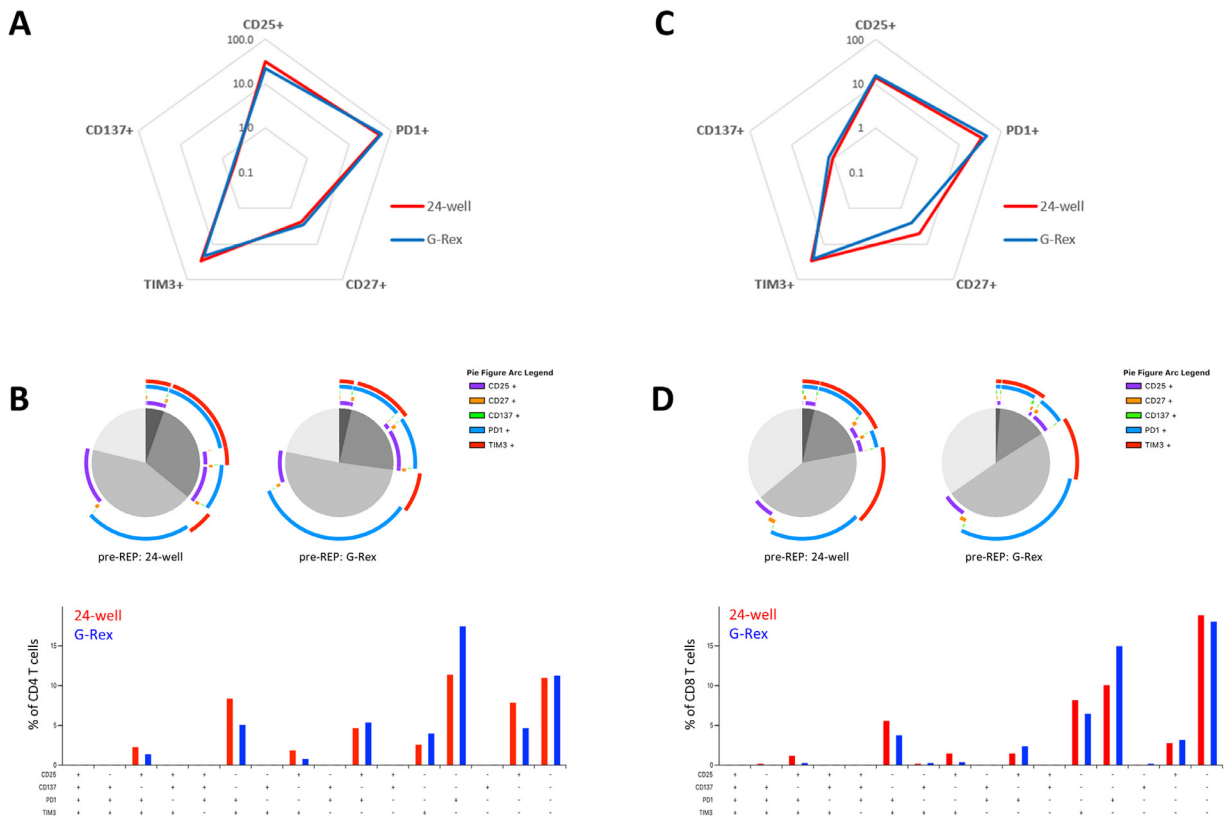

Fig. 3. Detailed phenotype characterization by flow cytometry of pre-REP TILs expanded in 24-well plates or G-Rex flasks. Data are plotted as mean value for eight different patients. Red line and red bars represent pre-REP TILs expanded in 24-well plates. Blue line and blue bars represent pre-REP TILs expanded in G-Rex flasks. (A) Detailed phenotype of CD4+ T-cell subset regarding CD25, CD137, PD-1, CD27 and TIM3 expression (n = 8). (B) Pie chart and diagram representing the percentage of CD4+ TILs co-expressing CD137, PD1, TIM3 or CD27 (n = 8). (C) Detailed phenotype of CD8+ T-cell subset regarding CD25, CD137, PD-1, CD27 and TIM3 expression (n = 8). (D) Pie chart and diagram representing the percentage of CD8+ TILs co-expressing CD137, PD1, TIM3 or CD27 (n = 8).

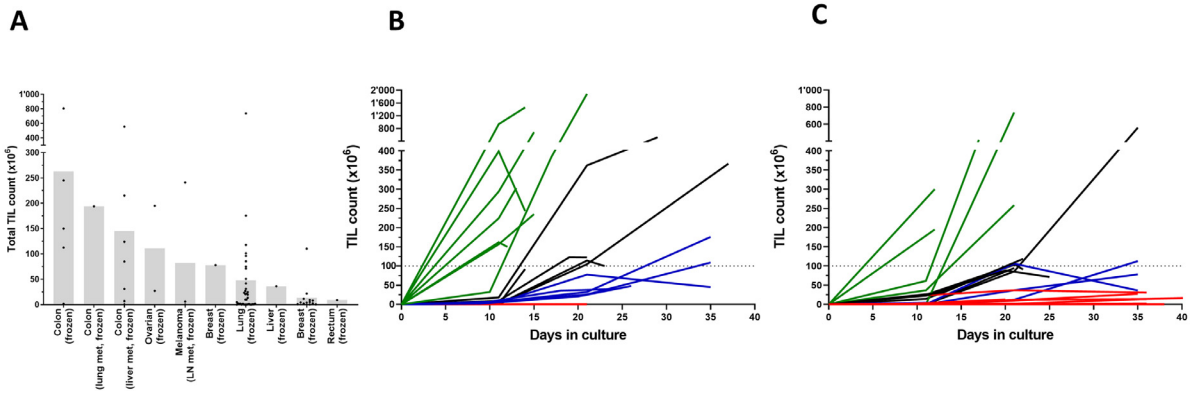

Fig. 4. (A) Total TILs number obtained at the end of pre-REP initiated using cryopreserved samples with different histologies. Data are plotted as points corresponding to individual cultures. Shaded columns correspond to the mean value. Dotted line represents the pre-determined threshold of 100.0 × 10^6 TILs for pre-REP output. Overall growth kinetics of pre-REP TILs (full-scale clinical data) from (B) melanoma and (C) solid tumors (other than melanoma). LN, lymph node; met, metastasis.

recognition by cultured TILs. As shown in the representative example pictured in supplementary Figure 2, tumor-specific reactivity (i.e., CD137 upregulation) was observed for pre-REP TILs CD4+ and CD8+ T-cell subsets when exposed to autologous tumor cells.

REP TILs optimization process

GMP-compliant REP expansion protocol using G-Rex and closed systems

Having established that pre-REP TILs grown in G-Rex contained tumor-reactive T cells, the authors then evaluated their ability to expand during a REP procedure using, sequentially, the G-Rex and Xuri cell expansion systems. Cryopreserved pre-REP TILs were thawed, rested and then seeded in several G-Rex flasks. After 7 days in culture in G-Rex flasks, the TILs were transferred into a Xuri bioreactor for further cell expansion (Figure 1). Out of 25 REP procedures performed, 25 effectively produced TILs that could potentially be used for therapy. At final product harvest, more than 20 × 10^9TILs were obtained in most instances (Figure 5A,B), with an average overall TILs fold expansion rate of 1681 (range, 58–7090) (see supplementary Figure 1). Overall, full-scale REP procedures allowed the harvest of a mean of 41.3 × 10^9 viable TILs (range, 11.0 × 10^9 to 71.5 × 10^9) (Figure 5A,B). Additionally, viability of TILs at the final product step (i.e., after final washing in LoVo) remained

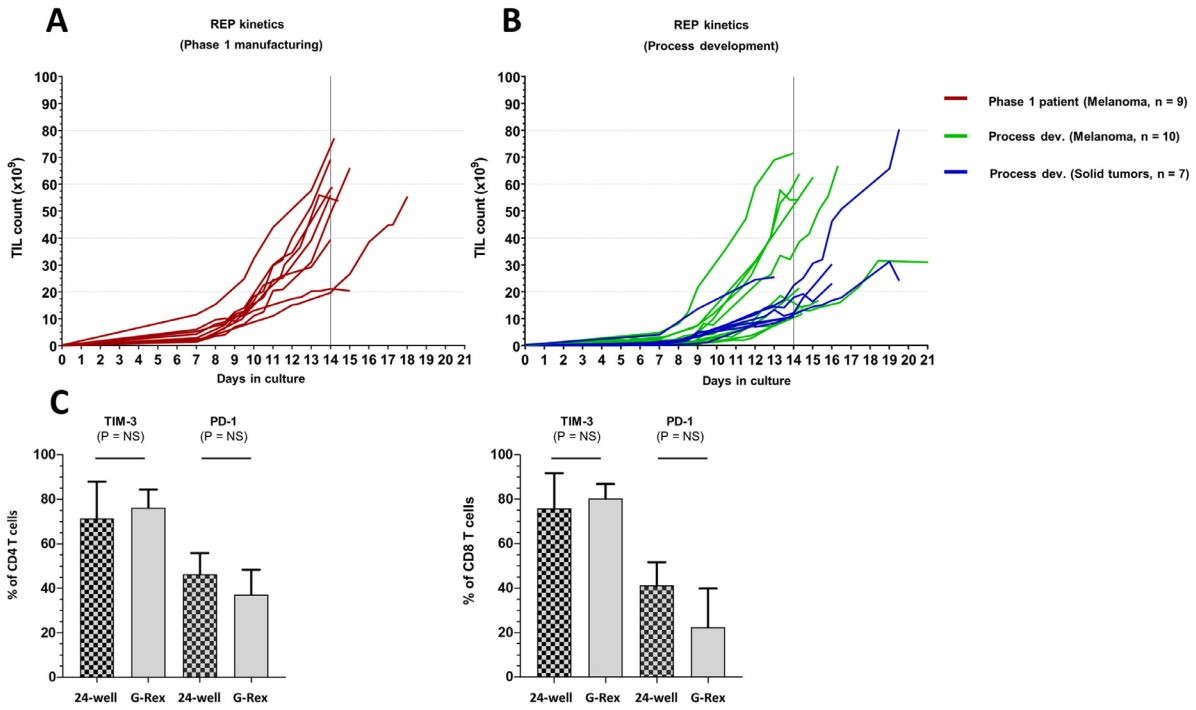

Fig. 5. Overall growth kinetics and characterization of REP TILs (full-scale process development data). Number of cells ($\times 10^9$ TILs) following (A) REP initiated with clinical samples from frozen pre-REP TILs melanoma and solid tumors (phase 1 patients, red lines) and (B) REP from process development based on frozen pre-REP TILs melanoma (green lines) and frozen pre-REP TILs non-melanoma solid tumors (blue lines). Vertical gray lines at 14 days represent planned REP harvest day (in daily practice, between day 12 and day 20). (C) Phenotype of REP TIL CD4+ and CD8+ T-cell subsets regarding TIM-3 and PD-1 expression. Wilcoxon matched-pairs signed rank test $P > 0.05$ (NS). Column "24-well" represents REP initiated with pre-REP TILs grown in 24-well plates. Column "G-Rex" represents REP initiated with pre-REP TILs grown in G-Rex flasks. Data are plotted as the mean value for four different patients in all conditions represented (shaded columns), together with SEM. Dev., development; NS, not significant; SEM, standard error of the mean.

consistently high (90.0% ± 0.05%), suggesting the high quality of the TILs. Furthermore, REP TILs demonstrated a comparable pattern of PD1+ and TIM3+ expression for both CD4+ and CD8+ T-cell subsets irrespective of pre-REP culture conditions (i.e., 24-well plate or G-Rex flask) (Figure 5C). Based on this assessment, the authors did not observe significant increased expression of exhaustion-related markers with the proposed manufacturing process.

Overall, the data presented here demonstrate that the pre-REP and REP procedures were reproducible and enabled the authors to reach a sufficient number of cells for clinical use using both fresh and cryopreserved tumor samples. Based on the range of results observed with regard to TILs isolation, growth and expansion potential, the data highlight the heterogeneity of TILs expansion between patients.

Functionality of REP TILs and autologous tumor cell recognition

The functionality of the TILs obtained from the REP procedures was confirmed by several experiments. First, the ability of TILs to secrete multiple relevant cytokines was assessed. To this end, TILs were stimulated with different doses of anti-CD3 antibody, and the secretion of IFN-γ, IL-13, IL-4, IL-8 and tumor necrosis factor alpha (TNF-α) was quantified. High concentrations of IFN-γ and tumor necrosis factor alpha were detected (Figure 6A,B), indicating a strong effector profile of REP TILs. Dose responses to anti-CD3 stimulation also indicated high functional avidity of post-REP TILs. The authors next evaluated the ability of REP TILs to recognize an autologous tumor, as was initially shown for pre-REP TILs (see supplementary Figure 2). As shown for two representative examples (i.e., REP-0002 and REP-0007), high levels of tumor-specific T-cell responses were observed after stimulation of post-REP TILs with autologous, but not allogeneic, tumor cells. Furthermore, T-cell responses were mediated by HLA class I-specific T-cell responses, as demonstrated by the loss of cellular reactivity when HLA class I molecules were blocked with antagonist antibody W6-32 (Figure 6C). Following their ability to recognize autologous tumor cells, the potential anti-tumor activity of post-REP TILs was also assessed, ultimately demonstrating their tumoricidal activity (Figure 6D).

The use of Xuri and LoVo equipment resulted in an optimized final product

One of the major innovations in the manufacturing process was the introduction of the LoVo cell processing system in the final product formulation. The LoVo system enabled fast, reproducible and automated cell washing in a closed circuit using only one kit and one machine. The use of the LoVo system significantly facilitated the final TILs product preparation while minimizing operator intervention and overall process time. Consequently, process development experiments were set up to determine the efficacy of the LoVo cell processing system at final product harvest. The data show that the total number of cells and their viability were not impacted (Figure 7A,B). Indeed, the total cell number was constant for most test samples, and the viability of TILs was stable save for one sample (i.e., REP-0004), which showed a decrease of 12% due to an operator malfunction during training. Moreover, the process steps required only one TSCD sterile welding between the Xuri 10-L and LoVo processing kit. The formulation process was also efficient, with an average run time of 90 min. Therefore, the final cell processing step prior to patient infusion (i.e., the LoVo cell processing system) demonstrated no deleterious effect on the cellular fraction. The results regarding total cell count and viability of TILs following REP procedures demonstrate that the *ex vivo* TILs expansion protocol described in this article is consistent with a clinical use of the cells in TIL-ACT protocol.

Clinical-scale manufacturing process and QC strategy

The classification of starting materials, intermediate products, active substances and final product was defined in accordance with European Pharmacopoeia and the local authorities (Swissmedic) as

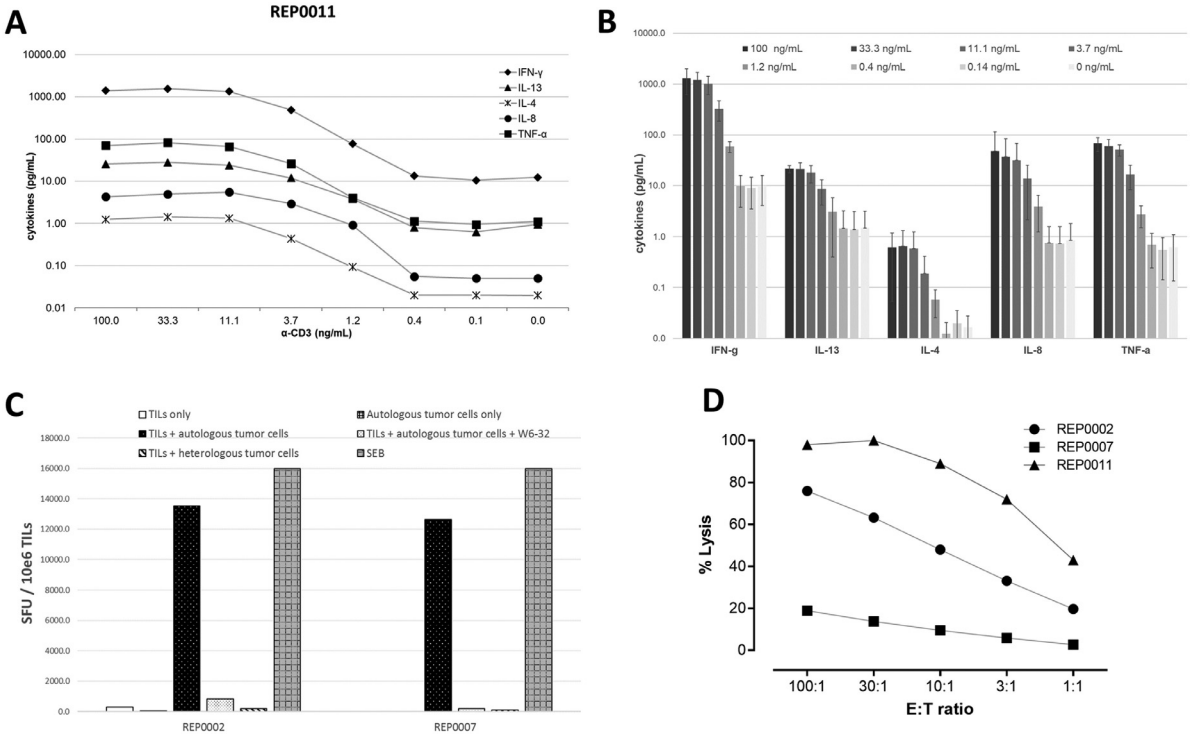

Fig. 6. Functional profiling of post-REP TILs. Representative example (A) and cumulative data (B) showing post-REP TILs secretion of IFN-γ, IL-13, IL-4, IL-8 and TNF-α in response to anti-CD3 stimulation. Basal cytokine secretion of unstimulated post-REP TILs is shown with condition 0 ng/mL. Experiments were performed in duplicate. Data are plotted as points corresponding to the mean value of two measurements for one patient (A) and as bars corresponding to the mean value for three different patients, together with SEM (B). (C) Tumor recognition of post-REP TILs as measured by IFN-γ ELISpot assay for two different patients. TILs were stimulated with autologous tumor cells alone, autologous tumor cells plus anti-human MHC class I antibody (W6-32) or heterologous tumor cells alone. Positive controls were realized by stimulating TILs with SEB. Negative controls were unstimulated TILs. Experiments were performed in triplicate. Data are plotted as bars corresponding to the mean value of three measurements. (D) Autologous tumor killing experiment (cell lysis) by post-REP TILs for three different tumor patients. TILs were added to autologous tumor cells in different ratios (1:1–1:100), and viability of cells was then assessed. Experiments were performed in duplicate. Data are plotted as points corresponding to the mean value of two measurements. ELISpot, enzyme-linked immunospot; E:T, effector-to-target ratio; MHC, major histocompatibility complex; SEB, staphylococcal enterotoxin B; SEM, standard error of the mean; SFU, spot-forming unit; TNF-α, tumor necrosis factor alpha.

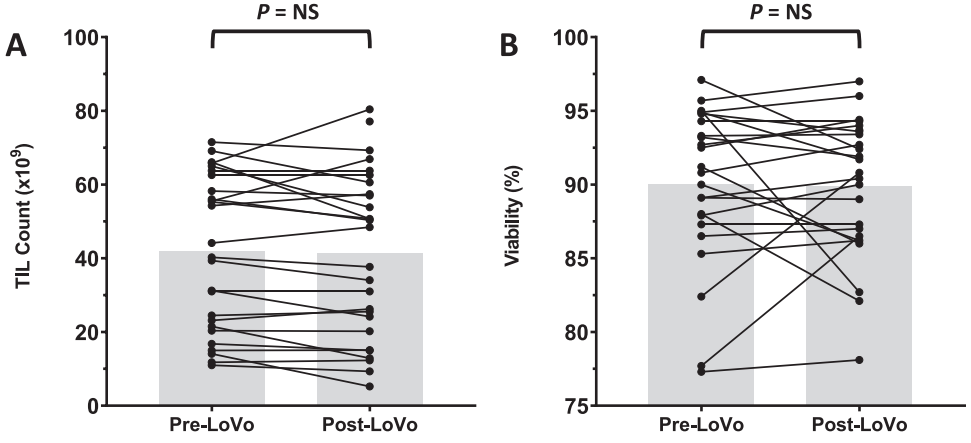

Fig. 7. TILs characteristics following LoVo processing at harvest. Efficiency of LoVo as the equipment to perform final product formulation in terms of total cell count (A) and viability (B) before (Pre-LoVo) and after (Post-LoVo) processing. Data are plotted as points corresponding to individual cultures. Shaded columns correspond to the mean value. $P > 0.05$ NS. NS, not significant.

described in Table 1. The QC strategy (see supplementary Figure 3) relied on European Pharmacopoeia-approved methods. The specifications of the in-process controls and final product release testing are described in Table 2 and Table 3. In addition to these tests, constitutional karyotyping (G-banding) was performed on Xuri TILs to assess chromosome stability following long-term culture. No chromosomal anomalies were detected at the end of the REP culture for this complementary testing (data not shown).

Following final product formulation and packaging, the final product bag was kept in the grade D clean room on an oscillating rocker at room temperature. Once the product was released for use by the local responsible person, the final labeled product bag was transported to the site of administration at ambient temperature. The stability of the final product was evaluated at a maximum of 24 h, with products being delivered to the bedside within 6 h post-formulation.

Table 1
Definitions of TILs starting materials, intermediate products, active substances and final product.

Name	Definition
Starting material	Patient tumor-derived biopsy obtained following surgery that serves to isolate and expand TILs
Intermediate product	Cryopreserved *ex vivo*-isolated TILs
Active substances	TILs and autologous PBMCs
Final product	TILs
Final reconstituted dose	Individual TILs doses formulated in 0.9% NaCl and 2.0% albumin supplemented with 300 IU/mL of IL-2

NaCl, sodium chloride; PBMCs, peripheral blood mononuclear cells.

Discussion

In the past decades, the complex production process used for first TIL-ACT attempts was a substantial limitation to patient therapy [28]. For this reason, in 2008, Tran *et al.* [29] simplified and standardized the TILs production process by generating "young" TILs for therapy. These minimally manipulated young TIL cultures, as used in our *ex vivo* TILs expansion protocol, consist of bulk lymphocytes rather than microcultures, and the batch-specific assay for tumor recognition is eliminated. Over the course of TIL-ACT development, metastatic melanoma was identified as the most appropriate tumor type because of its high level of immunogenicity as well as the relative ease of deriving autologous tumor cell lines. Indeed, retrospective analysis in metastatic melanoma revealed that non-responders to anti-CTLA4 (ipilimumab) or IL-2-based therapy had an objective response rate to TIL-ACT similar to that seen in ipilimumab-naive patients. Moreover, no additional toxicities to TILs therapy were highlighted following immune checkpoint blockade treatment [20]. In this article, the authors' data complement previously published data on melanoma, as additional data on solid tumors are provided and the potential clinical use of TILs in other types of solid tumors (i.e., breast, non-small cell lung, colorectal, ovarian, head and neck, cervical and renal cell carcinoma) is shown [30–36]. Together, the benefit of autologous TILs for the treatment of advanced solid cancers refractory to conventional therapeutics (i.e., chemotherapy and radiotherapy) and

Table 2
In-process controls (IPC).

Sample	Test	Analytical procedure	Specification	Test result
Transport media	Safety sterility	BacTEC (aerobic, anaerobic, mycosis)	No growth	IPC
Supernatant at first media change of pre-REP	Safety sterility	BacTEC (aerobic, anaerobic, mycosis)	No growth	IPC
TILs during pre-REP	Cell count	Manual count with Trypan Blue exclusion assay	IPC for determination of fold expansion during pre-REP	IPC
TILs at pre-REP harvest	Cell count	Manual count with Trypan Blue exclusion assay	$\geq 100.0 \times 10^6$ viable TILs at pre-REP harvest	IPC
	Cell viability	Manual count with Trypan Blue exclusion assay	$\geq$70.0% viability	IPC
	Safety sterility	BacTEC (aerobic, anaerobic, mycosis)	No growth	IPC
	Safety *Mycoplasma*	MycoSEQ	Negative	IPC
	Safety endotoxins	Endosafe	Target limit $\leq$1.17 EU/mL	IPC
	Cell purity/identity	Flow cytometry	e.g., detailed T-cell phenotype, $CD45^{pos}$ vs $CD45^{neg}$	IPC
Leukapheresis material at collection	Safety sterility	BacTEC (aerobic, anaerobic, mycosis)	No growth	IPC
Pre-REP TILs at thawing (day 3) Leukapheresis at thawing (day 0) Pre-REP TILs at REP seeding (day 0)	Cell count	Manual count with Trypan Blue exclusion assay	Sufficient numbers of pre-REP TILs and leukapheresis material at REP seeding	IPC
Pre-REP TILs at REP seeding (day 0)	Safety sterility	BacTEC (aerobic, anaerobic, mycosis)	No growth	IPC
	DNA fingerprinting	DNA fingerprinting	Pre-REP TILs (reference control), feeder cells (control for allogeneic alleles), pool from G-Rex with TILs + feeders (control for allogeneic and autologous alleles)	IPC (DNA fingerprinting assay only performed when using allogeneic feeders)
TILs during REP expansion (several days)	Cell count	Manual count with Trypan Blue exclusion assay	IPC for determination of fold expansion during REP	IPC
TILs during REP expansion (allogeneic feeder only control flask)	Cell count	Manual count with Trypan Blue exclusion assay	Quantification of irradiated allogeneic leukapheresis material viability: 0% viability	IPC for quantification of allogeneic feeder cell viability
TILs during REP expansion (day 7)	Safety sterility	BacTEC (aerobic, anaerobic, mycosis)	No growth	IPC
TILs during REP expansion (day 11 or at least 7 days prior to final product infusion)	Visual inspection	Manual count with Trypan Blue exclusion assay	Healthy-looking cells	IPC to start chemotherapy
	Visual inspection	Manual count with Trypan Blue exclusion assay	Healthy-looking cells	IPC to start chemotherapy
TILs at REP harvest	Cell purity/identity	Flow cytometry	e.g., detailed T-cell phenotype, $CD45^{pos}$ vs $CD45^{neg}$	IPC
	DNA fingerprinting	DNA fingerprinting	TILs from Xuri to show no residual feeder cells	IPC (DNA fingerprinting assay only performed when using allogeneic feeders)
	Karyotype analysis	Constitutional karyotyping	TILs from Xuri show similarity with PBMC control	IPC

PBMCs, peripheral blood mononuclear cells.

Table 3
Final product specifications for batch release and certification.

Specimen	Test	Analytical procedure	Specification
REP aliquot minimum of 2 days prior to harvest	Safety sterility	BacTEC (aerobic, anaerobic, mycosis)	Negative to date/no growth
REP aliquot minimum of 2 days prior to harvest	Safety *Mycoplasma*	MycoSEQ	Negative
Final product aliquot	Cell count	Manual cell count	No minimum, $\leq 150.0 \times 10^9$ viable cells
Final product aliquot	Cell viability	Manual cell count	$\geq$70.0% viability
Final product aliquot	Appearance	Visual inspection	Color: white/beige/milky; physical aspect: absence of clotting /aggregates
Final product aliquot	Safety endotoxins	Endosafe	$\leq$1.17 EU/mL
Final product aliquot	Cell purity/identity	Flow cytometry	$\geq$90.0% live CD3+ T cells
Final batch certification (aliquot)	Safety sterility	BacTEC (aerobic, anaerobic, mycosis)	Negative/no growth
Final batch certification (aliquot)	Safety *Mycoplasma*	MycoSEQ	Negative

immunotherapies such as immune checkpoint inhibitors has been clearly established [20,37].

However, a generalized limiting step in this therapeutic approach remains the ergonomy of a GMP-compliant manufacturing process (operator time) in relation to the regulatory requirements (raw material, clean room infrastructure, QC and quality assurance). Several TILs expansion processes adapted to the use of GMP-compliant raw material (i.e., buffers, cytokines) have already been published [25,38,39]. Noticeable improvements can be made in terms of the manufacturing process, pharmacoeconomics and GMP compliance needs. Here the authors presented an optimized TILs expansion process in a closed system with low manufacturing footprint, illustrating improvements in terms of manufacturing process and GMP compliance needs. The commonly used two-step TILs expansion protocol (i.e., pre-REP and REP) was optimized by the authors' team to produce sufficient cells with the required quality attributes at reduced operator time. For instance, the authors show that pre-REP cultures can be initiated using fresh or cryopreserved tumor, which significantly increases manufacturing flexibility. Fresh and cryopreserved tumor samples are commonly available following tumor surgical resection or biopsy, which makes them a perfect choice as starting materials.

The expansion of TILs was performed in G-Rex flasks and the Xuri cell expansion system. The results showed that, for the pre-REP expansion, the process using G-Rex flasks instead of 24-well plates enabled the overall expansion of more TILs without impacting their phenotype or functionality. Moreover, substantial operating time was saved during cell culture thanks to fewer media change steps. All arguments are therefore in favor of using G-Rex flasks compared with standard 24-well plates. For tumor specimen dissociation, the authors deliberately chose to use a manual dissociation method since this technique has been extensively published, with very reproducible results, and represents a quick approach to starting the pre-REP procedure without additional manufacturing time to obtain a single-cell digest by enzymatic digestion [25]. Moreover, the authors chose not to use automated mechanical single-cell digest, as this was considered an additional GMP step (i.e., equipment, consumables and buffers). Indeed, as manual dissection of the initial tumor was required to cut the tumor into small pieces—for instance, unwanted parts of the tumor biopsy (i.e., fat, necrotic tissues) must be manually removed by the operator—there was no benefit identified with regard to an additional process step to obtain a single-cell digest. Together, the authors' manual dissociation method was validated in terms of TILs generation efficiency and was shown to overcome the issue of tumor heterogeneity, as indicated by the 60 fragments collected on each biopsy and grown *ex vivo* in one G-Rex. Manual dissociation of tumor material is therefore an interesting approach in terms of ergonomy, reagent reduction, decreased equipment qualification and cost and time efficiency.

In addition, the use of the Xuri system, in combination with a closed-system G-Rex during the REP expansion, allowed for TILs expansion with minimal operator intervention using GMP-compliant reagents and a TSCD-II-compatible tubing system. It was previously reported that the efficiency of the expansion process yields overall TIL expansion of 1000- to 5500-fold [25,40,41]. The authors' data are comparable to these results. Indeed, for the 25 REP procedures performed by the authors' team, an overall fold expansion of 1785 $\pm$ 1575, with a minimum and maximum fold expansion of 58 and 7090 (data not shown), respectively, was obtained. These results show the great heterogeneity that exists among patients. Thus, optimal conditions for TILs expansion protocol and reproducibility in terms of quality remain critical to ensure that patients grow sufficient autologous TILs *ex vivo* to receive such treatment.

In terms of process ergonomy and safety, a key point of the authors' manufacturing system is the integration of the LoVo processing system. The LoVo processing system was used to wash the leukapheresis material and to wash and volume-reduce the TILs at the REP harvest step. In both cases, cells were directly transferred to the LoVo processing system using TSCD sterile welding. Indeed, the LoVo processing system offered yields at REP harvest >95%, with high viability. Consequently, the optimized TILs expansion manufacturing process described herein enables the preparation of GMP-compliant materials to be used for therapeutic purposes in TIL-ACT. Overall, the use of closed systems for cell culture is highly advantageous from a GMP standpoint, as it considerably reduces the risk of sterility loss and potential contamination. Furthermore, in clean rooms with a grade D environment where open steps are performed under a grade A isolator, using closed systems and TSCD sterile connections greatly facilitates operator manipulations while reducing time spent working under the isolator.

To assess the reliability and therapeutic potential of the *ex vivo*-expanded TILs obtained using the authors' manufacturing protocol, three different methods were used: cytokine secretion following anti-CD3 stimulation, autologous tumor recognition and autologous tumor cell lysis assays. The data confirmed the functionality of *ex vivo*-expanded TILs as described in previous publications [17,37,42] and hence support the use of minimally cultured TILs in adoptive cell therapy. Moreover, the autologous tumor recognition and tumoricidal activity of the TILs obtained after REP procedures using the Xuri expansion system and LoVo processing system were confirmed. In addition, no significant increase in the expression of exhaustion-related markers was observed with the proposed manufacturing process. These results led the authors to the conclusion that G-Rex flasks and Xuri bags are suitable for GMP TILs expansion, as they do not impair cell phenotype and functionality. Additionally, the LoVo processing step in a closed system allows the formulation of a ready-to-use TILs dose for subsequent clinical use.

The major regulatory advantages with regard to the use of TILs in the clinical setting are the safety tests, such as sterility, *Mycoplasma*

and endotoxin tests. In the authors' approach, the QCs performed to detect the presence of endotoxins (Endosafe; release criteria, ≤1.17 EU/mL) and *Mycoplasma* (MycoSEQ; release criteria, negative) and test sterility (BD Bactec; release criteria, no growth) passed for all the batches tested (data not shown).

Despite the advantages and high reproducibility of the authors' GMP-compliant TILs expansion protocol, some improvements still need to be made. For instance, the culture time is relatively long, at up to 35 days for the pre-REP procedures and up to 21 days for the REP procedures. Additionally, it would be advantageous to limit even more the quantity of raw materials. For instance, the authors are striving to remove human serum from future processes, in line with recommendations from the local authorities. Additionally, the authors are always striving to improve yield. Finally, operator time and cost remain important points of improvement. With regard to operator time, the authors found that paper-based quality-compliant documentation is a major time constraint. As such, the authors' group will integrate documentation software for the quality assurance and production units. With regard to the cost of TIL-ACT, it may presently appear elevated because of product manufacturing and hospitalization costs. However, the real costs of managing the toxicity of immune checkpoint blockade treatment (e.g., anti-CTLA-4/PD-1 combination) are often higher than expected. Indeed, clinical efficacy, toxicity, quality of life and pharmacoeconomic endpoints must also be taken into consideration. Previous economic analysis has demonstrated a more favorable role for TILs therapy than ipilimumab alone [43]. Besides increased efficiency and effectiveness, the authors' optimized manufacturing protocol also helped reduce the financial burden of full-scale TILs expansion, hence enabling the development of further TIL-ACT at a more competitive cost.

Altogether, in this article, the authors demonstrated the generation of functional TILs from solid tumors based on an improved GMP-compliant manufacturing process. The authors' process, with low manufacturing footprint, was optimized to be GMP-compliant as well as time-effective. The authors believe this competitive, robust and standardized production process can be used as a reference for the preparation of TIL-ACT materials in a hospital-based GMP environment.

Conclusions

In this article, the authors presented a manufacturing process for *ex vivo* TILs expansion from solid tumor samples. The feasibility of using a GMP-compliant manufacturing process was demonstrated for use in a grade D clean room environment using a grade A isolator for open steps. This reproducible and standardized TILs production process could be used as a reference for TILs production. Indeed, the use of automated features for TILs expansion (REP) should facilitate technical transfer between manufacturing centers independently of the classification of their clean rooms (grade D to A). The TILs production process was approved by Swissmedic, and two ongoing phase 1 clinical trials utilizing TILs expanded using the authors' protocol are currently recruiting metastatic cancer patients eligible for TIL-ACT based on several indications (i.e., melanoma and other solid tumor samples).

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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: AH, POG, KEL, GC and LEK. Acquisition of data: POG, CM, AA, OR, CB, PB, BNR, TN, SR, KEL, LG, SZ, LT, CS and ND. Analysis and interpretation of data: AH, POG, CM, AA, OR, CB, PB, BNR, TN, SR, VZ, LG, EMI, GC and LEK. Drafting or revising the manuscript: AH, POG, GC, VZ and LEK. All authors have approved the final article.

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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.2020.07.011.

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