Title page

Automated manufacturing of CAR-T cells for Adoptive Immunotherapy using CliniMACS Prodigy

Running Title (max 50 characters):

Automated T cell transduction for GMP-manufacturing

Ulrike Mock[1,*], Lauren Nickolay[1,*], Brian Philip[2], Gordon Weng-Kit Cheung[2], Hong Zhan[1], Ian CD Johnston[3], Andrew D Kaiser[3], Karl Peggs[2], Martin Pule[2], Adrian J Thrasher[1] Waseem Qasim[1]

[1]Institute of Child Health, Molecular and Cellular Immunology Unit, UCL, London WC1N 1EH, United Kingdom.

[2]UCL Cancer Institute, University College London, 72 Huntley Street, London, WC1E 6DD UK [3]Research & Development, Miltenyi Biotec GmbH, Friedrich-Ebert-Strasse 68, 51429 Bergisch Gladbach, Germany

* These authors contributed equally to this work

Corresponding Author: Lauren Nickolay - l.nickoay@ucl.ac.uk

Abstract

Novel cell therapies derived from human T lymphocytes are exhibiting enormous potential in early phase clinical trials in patients with haematological malignancies. *Ex-vivo* modification of T cells is currently limited to a small number of centres with the required infrastructure and expertise. The process requires isolation, activation, transduction, expansion and cryopreservation steps. To simplify procedures and widen applicability for clinical therapies, automation of these procedures is being developed. The CliniMACS Prodigy (Miltenyi Biotec) has recently been adapted for lentiviral transduction of T cells and here we analyse the feasibility of a clinically compliant T-cell engineering process for the manufacture of T cells encoding chimeric antigen receptors (CAR) for CD19 (CAR19), a widely targeted antigen in B-cell malignancies. Using a closed, single-use tubing set we processed mononuclear cells from fresh or frozen leukapheresis harvests collected from healthy volunteer donors. Cells were phenotyped and subjected to automated processing and activation using TransAct, a polymeric nanomatrix activation reagent incorporating CD3/CD28-specific antibodies. Cells were then transduced and expanded in the CentriCult-Unit of the tubing set, under stabilised culture conditions with automated feeding and media exchange. The process was continuously monitored to determine kinetics of expansion, transduction efficiency and the phenotype of the engineered cells in comparison to small-scale transductions run in parallel. We found that transduction efficiencies, phenotype and function of CAR19 T cells were comparable to existing procedures and overall T-cell yields sufficient for anticipated therapeutic dosing. The automation of closed-system T-cell engineering should improve dissemination of emerging immunotherapies and greatly widen applicability.

(250 words)

Key Words: Automation, CAR19, chimeric antigen receptors, CliniMACS Prodigy, GMP, Immunotherapy, T cells, T cell transduction

Optional: Highlights (3-5 bullet points, max 85 characters per point incl space)

- Demonstration of feasibility of CliniMACS Prodigy for T cell transduction
- Successful automated generation of CD19-CAR+ T cells in clinically relevant doses
- Confirmation of in vitro and in vivo efficacy of automatically generated product

Introduction

Genetically modified T cells are exhibiting enormous potential for the treatment of relapsed and refractory haematopoietic malignancies. In particular, CD19-positive B-cell malignancies such as acute lymphoblastic leukaemia (ALL), chronic lymphocytic leukaemia (CLL) or B cell non-Hodgkin lymphomas (NHL) have been shown to be an excellent target for adoptive immunotherapy with T cells expressing CD19-specific chimeric antigen receptors (CARs) [1-3]. These CARs comprise fusions of antibody derived antigen-recognition domains and various T-cell receptor (TCR) signalling domains, capable of inducing T-cell activation, proliferation and effector functions upon antigen recognition. To achieve long-lasting anti-tumour effects CAR-T cells are primarily generated by lenti- or γ-retroviral vector mediated transduction. Although these procedures are well established, clinical application is limited to a small number of manufacturing facilities with the required infrastructure, containment approval and expertise for Good Manufacturing Practice (GMP). The manufacturing of genetically engineered T cells is a complex process which can be separated into the following steps (Figure 1A): Isolation of mononuclear cells from human subjects, activation of T cells to induce mitosis for enhanced susceptibility for viral transduction, expansion of cells following transduction and finally the harvest of the product followed by formulation and/or cryopreservation. Various culture vessels, including G-Rex flasks, cell differentiation bags and bioreactors have proven suitability for T-cell manufacturing [4-6]. Current procedures involve a variety of hands-on steps and transfer of cells between cultivation vessels, each bearing the risk of contamination, human errors and cell loss. To simplify the protocol and minimise potential risks during the process, the CliniMACS Prodigy [7] has been adapted to automate clinical-scale manufacturing of cell products [8-10]. This platform allows for magnetic bead based cell separation followed by activation, transduction, multiple media-exchanges, and washing steps with a single-use, closed tubing-system (Figure 1A, Figure

1B). Programming flexibility incorporates an "activity matrix" that is user-defined and determines the chronological order of different cultivation steps, feed schedules and duration of culture. It is highly adaptable and can be changed during the process to adjust to individual sample requirements. Here we demonstrate the feasibility of the automated T-cell transduction process on the CliniMACS Prodigy and evaluate the generated T-cell products on a phenotypic and functional level (*in vitro* and *in vivo*) alongside manually processed cells.

Materials and Methods

Automated T-cell transduction (CliniMACS Prodigy)

Automated T-cell transduction (TCT) was performed on the CliniMACS Prodigy using the Tubing Set TS520 and the T-cell transduction process. Where not specified otherwise, reagents and materials were obtained from Miltenyi Biotec. An overview of the general procedure is depicted in Figure 1D. In brief, leukapheresis from healthy donors were either obtained fresh (donor #15-01 and #15-03 and #15-GMP-03) or frozen (#15-04/6, #15-07, #15-GMP-01 and #15-GMP-02) (Caltag). Frozen cells were thawed on d-1 of the procedure, washed with TexMACS GMP Medium supplemented with 10%HS (GemCell Human Serum AB, premium grade (Seralab)) in the presence of at least 50U/ml Benzonase-Nuclease (Sigma-Aldrich) and rested overnight at 37°C in TexMACS, 3%HS at a density of $2x10^6$/ml. Fresh cells were washed in PBS to remove residual donor-serum. Cells were transferred into a 150ml Transfer Bag and attached to the TS520 by sterile welding. Cultivation was initiated with a SYSMEX based cell count of $7.45x10^7$–$1x10^8$ lymphocytes in a total volume of 70ml TexMACS, 3%HS + 20ng/ml MACS GMP Human Recombinant IL-2 in the CentriCult-Unit. Cells were activated with MACS GMP TransAct CD3/CD28 Kit at a final dilution of 1:200 (CD3 Reagent) and 1:400 (CD28 Reagent. Cells were transduced on d1 or d2 using an MOI of 5 with a self-inactivating third generation lentiviral vector encoding a CAR specific for CD19, under the control of EF1α internal promoter and including a mutated woodchuck post-regulatory element (WPRE) and HIV central polypurine tract (cPPT). Vector was pseudotyped with vesicular stomatitis virus (VSV) and concentrated by ultracentrifugation. Titres were calculated by flow cytometric assessment of 293T cells and Jurkat T cells transduced with serial vector dilutions. GMP vector stocks were subjected to additional release assays as described previously [11]. The vector was diluted in 10mls of media in a

150ml transfer bag which was then attached to the CliniMACS Prodigy by sterile welding. The vector was automatically transferred in the culture chamber and the vector bag was further rinsed with 20ml of media to bring the total culture volume to 100ml. Sampling was performed daily for monitoring purposes starting at d3. Monitoring included SYSMEX-based cell count and flow-cytometry Residual TransAct was removed by an automated "culture wash" on d4 or d5 followed by automated activation of the shaker (type 1). More vigorous shaking was initiated depending on cell density (type 2: density > $3x10^6$/ml or type 3: density > $4x10^6$/ml). For the final harvest, cells were automatically formulated in 0.9% Sodium-Chloride solution or CliniMACS PBS/EDTA supplemented with 0.5% HAS (Zenalb20 – Bio Product Laboratory) and transferred into a bag.

Cell culture

For each process performed on the CliniMACS Prodigy, cells from the same donor were activated, transduced and expanded on a reduced scale for comparison.. For each condition, $4x10^6$ washed lymphocytes were either re-suspended to $1x10^6$/ml in TexMACS 3%HS + 20ng/ml IL-2 and activated with TransAct as per Prodigy protocol, or re-suspended in X-Vivo 15 (Lonza) 5% HS + 20ng/ml IL-2 and activated at a 1:1 ratio with human T-Activator CD3/CD28 Dynabeads (Dynal). Cells were cultured in 48-well plates at a density of $1x10^6$/ml in a total volume of 700µl,a 100 fold reduction of culture conditions compared to the CliniMACS Prodigy. On d1 or d2 cells were divided into untransduced controls or transduced with CD19-CAR vector at an MOI of 5.The vector was added to each well in a total volume of 300µl to have a total culture volume of 1ml. To mimic the culture wash on d4 or d5, TransAct activated cells were washed once with TexMACS by centrifugation cell culture media and re-suspended in 40mls fresh TexMACS 3%HS + 20ng/ml IL-2 and transferred to a G-Rex 10 (Wilson Wolf Manufacturing) for expansion mimicking the prodigy culture chamber. Similarly, Dynabead activated cells were harvested, topped up to 40mls of X-Vivo

3%HS + 20ng/ml IL-2 and transferred to a G-Rex 10. Small scale activated cells were harvested at the same time point as cells on the CliniMACS Prodigy and cryopreserved. SupT1 cells and CD19+ SupT1 cells, that had previously been engineered to express recombinant CD19, were used for further functional studies and were cultured in RPMI 10% FCS. This cell line was authenticated using the ATCC Cell Line Authentication Service.

Flow Cytometry

To phenotype expanded cells, cells were stained with the following primary antibodies (from Miltenyi Biotec unless otherwise stated) CD3-PE Vio770, CD4-Vio Blue, CD8-PE, CD45-VioGreen, CD45RA – PE Vio770, CCR7-PE, CD62L-APCCy7 (Biolegend), CD95-BV421 (Biolegend), CD14-APC, CD20-APC Vio770, CD56-PE Vio770. To assess the efficiency of CD19-CAR transduction, cells were stained using a Biotin-SP (long spacer) AffiniPure F(ab') Fragment Goat Anti-Mouse IgG, F(ab') Fragment Specific antibody (Jackson Immunoresearch) followed by Streptavidin-APC or Streptavidin-FITC (Biolegend). Cells were acquired on a 4-laser BD LSRII and FACS analysis performed using FlowJo v10.

Cytokine secretion assay

CD19-CAR transduced cells were thawed, washed and re-suspended in RPMI 10% FCS at a concentration of $1x10^6$/ml CAR19+ cells. CD19- SupT1 cells and CD19+ SupT1 cells were counted and also adjusted to a concentration of $1x10^6$/ml. Cells were mixed at a 1:1 ratio in 24 well tissue culture plates and incubated at 37°C. After 24h supernatants were harvested and stored at -80˚C. Levels of cytokine in the stored supernatants were quantified using the $T_H1/T_H2/T_H17$ cytometric bead array kit (BD Biosciences) as per the manufacturers' protocol.

Cytotoxicity Assay

Cytotoxic activity of CAR19-cells was assessed by chromium (^{51}Cr) release assay. For this $5x10^3$ ^{51}Cr-labeled CD19-positive SupT1-target cells were incubated with CD56-depleted (CD56-MicroBeads, Miltenyi Biotec, 130-050-401) effector cells at increasing effector-to-target ratios (E:T ratios) in 96-well microplates for 4h at 37°C and ^{51}Cr-release measured in a microplate scintillation counter.

Anti-tumour effectivity *in vivo*

NSG mice injected with $2.5x10^5$ CD19+ Raji tumour cells expressing Luciferase were treated on d2 with $4x10^6$ prodigy transduced cells (CAR+) or a similar number of non-transduced (NT) cells expanded in a G-Rex flask. *In vivo* imaging of bioluminescence was performed on d1, d6, d9 and d13 (Figure 4A). Bone marrow samples for flow cytometric analysis were prepared by lysing of the red blood cells followed by a blocking step using FcR Blocking reagent, mouse (Miltenyi Biotec) and supernatant from the 2.4G2 hybridoma. Samples were stained with CD4-BV650, CD8-PE-CY7, CD20-PerCPcy5.5, CD19-APC and CD38-eFluour450. Samples were fixed overnight in 0.2% PFA prior to flow cytometry acquisition on a BD LSR-Fortessa cytometer.

Statistics

An unpaired Student's t test was used to assess the significance of activating cells with Dynabeads in XV5% or with TransAct in TM3%. For comparisons of cells produced in automated or manual processes a one way analysis of variance with Bonferroni's post-test was used. All statistical analysis was performed using GraphPad Prism software version 5.01.

Results

Comparison of activation reagents and media for automated T-cell transduction

Most existing T-cell manufacturing protocols use either soluble or magnetic-bead (Dynabead) bound anti-CD3/CD28-antibodies in media such as X-Vivo10 or 15 supplemented with interleukin-2 (IL2). In contrast, the automated T cell transduction TCT protocol for the CliniMACS Prodigy adopts TransAct, a soluble, colloidal reagent with covalently attached anti-CD3/CD28-antibodies [12], in combination with TexMACS GMP medium. TransAct can be removed from the culture by washing cells and obviates the need for a bead-removal step required for CD3/CD28-Dynabeads. To test the suitability of these reagents for expanding and transducing T cells with 3rd generation lentiviral vector encoding a CAR19 receptor with 41BB and CD3ζ activation domains, small-scale transductions of PBMCs activated with TransAct were performed alongside standard conditions using CD3/CD28 Dynabeads. Cell expansion, phenotype and transduction efficiency were evaluated. As depicted in Figure 2A, cells activated with Dynabeads showed a significantly higher expansion rate (mean fold expansion 63.6±21.3) compared to TransAct-activated cells (37.7±14.9) ($P<0.04$). However, transduction efficiency was greater for cells activated with TransAct compared to cells activated with Dynabeads ($P<0.03$) (Figure 2B) and cells activated with TransAct showed increased transduction efficiency of CD8+ T cells in comparison to cells activated with Dynabeads (Figure 2C). The increased transduction efficiency of cells activated with TransAct led to a comparable yield of CD19-CAR+ T cell between the two activation conditions (Figure 2D). Overall, these validations confirmed the potential of TransAct and TexMACS to support a sufficient yield of CAR19 engineered cells for clinical therapies.

Automated T-cell transduction using CliniMACS Prodigy

To validate the automated TCT protocol on the CliniMACS Prodigy, large scale experiments were performed using either cryopreserved or fresh (steady-state) leukapheresis (Fig 1C) and the Tubing Set TS520. Transduction was performed with vector added by sterile welding of the supply bag to access tubing. In comparison to conventional manufacturing processes using the WAVE bioreactor, which can require over 24 hours of 'hand-on' time, operator time was greatly reduced using the CliniMACS Prodigy (Table S1). The following parameters were assessed: expansion rate, transduction efficiency, viability, purity, immuno-phenotype and functionality. Small-scale controls with either TransAct/TexMACS (TransAct) or Dynabeads/X-vivo (Dynabead) were run in parallel.

Expansion and transduction efficiency of T cells using the CliniMACS Prodigy

The expansion of lymphocytes in the CliniMACS Prodigy ranged from 5.4x – 28.4x (mean 16.2x±7.9x) in cultures of 8-10 days (Fig.2A) and was comparable to expansion achieved in small scale controls on the same donors (Fig.2B; TransAct%: 18.1x ± 5.6x, Dynabead: 22.3 ± 12.2x). This resulted in an average yield of 15.8 x 10^8 total lymphocytes from the Prodigy (range: 4 – 23.2 x 10^8, also compare Table 1) from starting numbers of 0.7 - 1.2 x 10^8 (mean 0.95 x 10^8 ± 0.1). Suboptimal feeding of cells during the expansion phase may have contributed to lower yields in 15-01 and 15-03 (Figure 3A) and increasing the volume of media exchange in subsequent runs seem to have supported greater cell expansion. In eight scaled productions, transduction averaged 47.6 ± 14.9% CAR19-positive T cells (range: 23.9 - 66.4%, Figure 2C and Table 1). In small scale comparisons transduction efficiency was comparable for TransAct activated cells (Figure3D, TransAct: 51.8% ± 22.3% CAR19-positive) but lower for Dynabead-activated cells (Figure 3D, Dynabead: 29.5% ± 14.1% CAR19-positive), confirming the results shown in Figure 2B. Thus, although the expansion

of Dynabead-activated cells was greater, the overall yield of CAR19+ T cells was similar as with TransAct and the automatically processed CliniMACS Prodigy product.

Phenotype and purity of final cell product

Cellular composition was analysed by flow cytometry for the presence of CD45+ lymphocytes, CD3+ T cells, CD14+ Monocytes, CD20+ B cells, CD3-CD56+ NK cells and CD3+CD56+ NKT cells. There was no significant difference in the phenotype of expanded cells in the automated or manual processes (Figure 4A) and over eight runs a mean CD3 T-cell purity of 92.3% was achieved in the CliniMACS Prodigy compared to 96.7% (TransAct) and 98.0% (Dynabead) small scale controls. In runs 15-01 and 15-GMP-03 expansion of NK cells was detected (18.3% and 15.1%, respectively) in the automated compared to the manual processes. Interestingly, cells expanded in the CliniMACS Prodigy or in small scale controls with TransAct, had a greater percentage (49%) of CD8+ T cells in the final product compared to cells expanded in small scale controls with Dynabead (36%) although this difference did not reach significance in this limited number of experiments (Figure 4B). Possible T-cell exhaustion after *ex vivo* activation and expansion was determined by the expression of PD-1, a marker of reduced T-cell longevity [13]. The expression of PD-1 did not differ significantly in cells expanded in the CliniMACS Prodigy or in small scale comparison experiments ($p=0.31$), although there was a trend towards higher PD-1 expression in cells activated with TransAct in the latter (Figure 4C). In addition, immunophenotyping of cells expanded in the CliniMACS Prodigy was performed to further characterise memory T cell subpopulations. In order to generate a long-lasting immune response towards the target, memory T cells are thought to be the optimal target for adoptive immunotherapy [14]. Flow cytometric analysis for relevant surface markers was performed as shown and revealed that the majority of cells exhibited a memory phenotype, most being of either a central memory phenotype (T_{CM} CD45-CD62L+) or stem cell memory phenotype (T_{SCM} CD45+CD62L+CD95+) (Figure 4D

and S1) (T_{CM}+T_{SCM} = 82.4% ±7.9%). The proportion of T_{CM} and T_{SCM} populations was comparable between cells expanded in the CliniMACS Prodigy or in small scale controls (T_{CM}+T_{SCM} for TransAct = 80.4% ± 9.6% and Dynabead = 70.4% ± 21.6). Moreover the memory phenotype of cells that were transduced with CD19-CAR were comparable to the phenotype of cells not expressing CD19-CAR in the same culture (Figure S2).

Functional analysis of manufactured CD19-CAR T cells *in vitro*

The final verification of the automated T cell transduction process was to assess whether the automated expanded CAR19 T cells retained the ability to respond to CD19-expressing target cells. To do so, co-cultures were performed with T cells and CD19-expressing SupT1 cells. CAR19+ cells co-cultured with CD19+ SupT1 cells secreted pro-inflammatory cytokines IFN-γ and TNF-α in response to their target antigen whereas cytokine secretion was not initiated upon co-culture with CD19- SupT1 cells (Figure 4E and S3). Cells expanded in the CliniMACS Prodigy produced less IFN-γ in response to CD19+ cells compared to cells expanded with TransAct in small scale controls ($P<0.002$). Secretion of of TNF-α and IL-2 compared to both Dynabead and TransAct small scale controls was also lower, but did not reach significance. Importantly, CAR19 T cells generated in either manner did not produce regulatory cytokine IL-10 and produced cytokines associated with an effector T_H1 phenotype and not those associated with either a T_H2 or T_H17 phenotype [15]. Furthermore, in a ^{51}Cr-release assay, the automated expanded CAR19+ T cells demonstrated specific lysis of CD19 expressing SupT1 target cells (Figure 4F, and Supplementary Figure S4 for CD19 negative SupT1-controls). To assess cytotoxicity purely of expanded CAR19 T cells, the assay was performed on NK cell depleted, cryopreserved cells. Together, our data verify the functionality and specificity of automatically manufactured T cells.

Anti-tumour activity of manufactured CD19-CAR T cells *in vivo*

In vivo efficacy of CD19-CAR engineered T cells was assessed in a human:murine tumour model. Immunodeficient NSG recipients were injected via tail vein with CD19 positive / luciferase expressing Raji cells two days before inoculation with control or CD19-CAR engineered T cells (Figure 5A). Upon *in vivo* imaging, mice that had received CD19-CAR expressing T cells exhibited tumour clearance by 11 days (representative images in Figure 5, full data set in Figure S5). Tumour clearance was not observed in control mice treated with PBS or NT T cells. Furthermore, absence of Raji populations was confirmed by flow cytometric analysis of the bone marrow in mice treated with automatically manufactured CD19-CAR T cells and in contrast to control treated mice (Figure S6).

Discussion

Wider application of adoptive T-cell therapies has been limited by the complexity of manufacturing processes and access to the required infrastructure and expertise. In this report on automated T cell processing on the CliniMACS Prodigy for the generation of CD19-CAR T cells we show that automated manufacturing of T cells, including cell-preparation, activation, transduction using lentiviral vectors, expansion and formulation, using the TCT-program is practicable and adaptable. The majority of CAR19 T cells expanded in the CliniMACS Prodigy exhibited T stem cell memory (T_{SCM}) and T central memory (T_{CM}) T-cell phenotypes, with high expression of CD45RA and CD62L. These are attractive phenotypes for adoptive immunotherapy approaches as less differentiated cells might improve therapeutic responses [16]. Although T effector memory cells (T_{EM}) show higher cytolytic activity, T_{CM} show a greater capacity to persist after adoptive transfer and are believed to further differentiate *in vivo* to support anti-tumour function [17]. In addition, the automatically generated CD19-CAR T cells functioned in an antigen specific manner and showed specific cytokine release, cytolytic activity upon incubation with CD19 SupT1 target cells and anti-tumour activity in a CD19+ humanised tumour mouse model. Elevations of

IFNγ following engagement of CD19-specific cytotoxic T cells produced by CAR T cells were as anticipated [14] but found to be lower in Prodigy manufactured cells compared to smaller scale controls.

Importantly, the final T cell specifications were similar to products manufactured for CAR19-using already established clinical processes (defined as: >10% CAR19+, >70% viability and no growth in sterility tests). Moreover, yields of CAR19-positive T cells generated with the CliniMACS Prodigy ($7.9x10^8$ ± 4.0 CAR+ cells, Table 1) were comparable to previous validations procedures using a WAVE bioreactor preformed in our GMP facility ($3.8x10^8$ ± 4.5 CAR19+ cells, data not shown) and published yields reported in a clinical study for the treatment of ALL with a CD19-CAR T cell product, CTL019 (yield: $2.71x10^8$ ± 2.61 CTL019 cells) [2].

The present study shows the effectiveness of the automation of T-cell manufacturing for CAR T cell immunotherapy. Using the CliniMACS Prodigy, clinically relevant therapeutic doses of CD19-CAR T cells could be generated with minimal manipulation and a reduction in staff time compared to current T-cell processing protocols (Table S1). In the first instance the procedures have been adopted for the transduction of healthy donor T cells for programmed donor lymphocyte infusions in patients undergoing allo-SCT. Additional pre-selection steps to enrich particular T cell subsets, or post transduction enrichment procedures could be incorporated with relative ease. Currently the TCT program is most suitable for high titre lentiviral vectors and development of transduction processes with γ-retroviral vectors where co-localisation agents such as Retronectin is usually required, is underway. Wider applications for T cell and stem cell engineering procedures are foreseen and rapid dissemination of the technology to centres already using CliniMACS platform should help widen access and applicability.

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Author contribution

Conceived and designed experiments: LN, UM, WQ, AT. Performed experiments: LN, UM. Designed and performed *in vivo* experiments: BP. Analysed the data: LN, UM, WQ. Contributed data / reagents / material / analysis or tools: AT, AK, GC, HZ, IJ, WQ. Wrote the paper: UM, LN, WQ.

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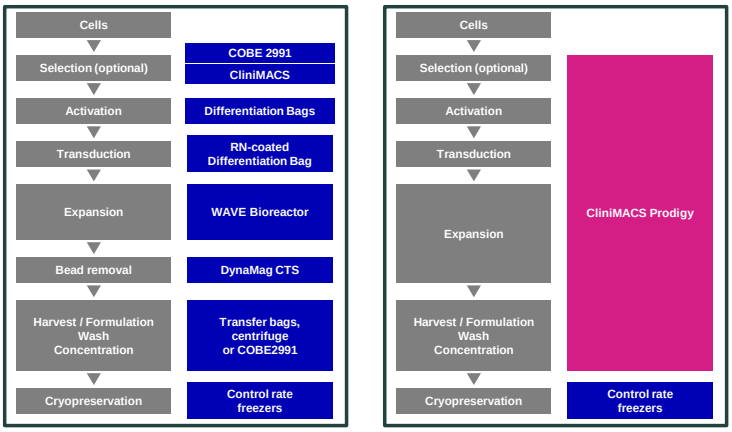
Figures

A



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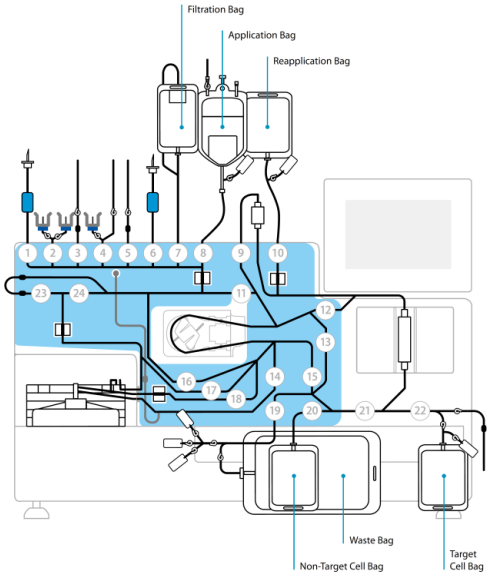

C

Day	Activity modules (Parameter)	Volume (in mL)	User interaction
0	Start of culture (no selection)	70	Yes
1/2	Transduction	100	Yes
3/4	Feed	100-175	No
4/5	Culture Wash	200	Yes
4/5	Activate Shaker (shaker type 1)	200	No
5/6/7/8	Media exchange	250	No
5/6/7/8	Activate shaker (shaker type 2 or 3)	250	No
8-10	End of culture	250	Yes

Figure 1. The T-cell transduction program on the CliniMACS Prodigy. (A) Overview of T-cell manufacturing processes and all equipment required. Left: Established protocol applied in clinical studies for adoptive immunotherapies. Right: Simplified procedure using the CliniMACS Prodigy where the majority of processing steps take place using the CliniMACS prodigy. Adapted from Kaiser *et al* [18]./Zhan *et al*.[5] (B) Tubing Set TS520. Single-use, closed tubing set allows for sterile handling of cell products in a single container. The CentriCult-Unit is an integral part of the tubing set and enables cultivation with stable temperature, gas-mix and the possibility to wash cells during the procedure without removing

them from the device. Reagents, cells and media are connected to the TS520 by sterile welding without compromising sterility of the procedure. The numbers are representative of the numbered valves on the CliniMACS prodigy (C) Example for TCT-work flow. Upon starting the process, an "activity matrix" is programmed by the user by the addition of different activity modules (Transduction, Feed, etc.) into the matrix. This makes the TCT process adaptable depending on the cell source, vector-type and desired expansion period for the respective product. There are three types of shaking that can be implemented in the Centricult unit depending on the cell concentration in the chamber : Type 1 - 100RPM, one direction, recommended > $2x10^6$ cells/ml. Type 2 - 300RPM, one direction, recommended >$3x10^6$ cells/ml. Type 3 – 300RPM, change of direction, recommended > $4x10^6$ cells/ml.

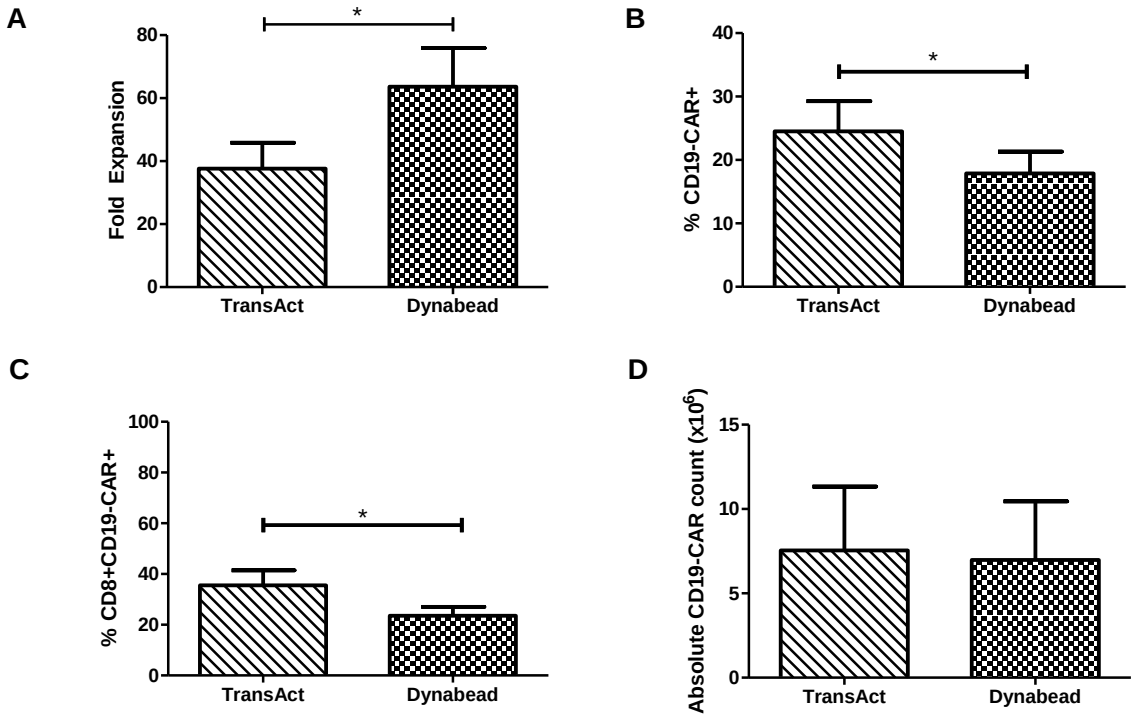

Figure 2 Suitability of TransAct for activation, transduction and expansion of PBMCs in small-scale experiments. (A-D) PBMCs were activated with either TransAct in TexMACS 3% human serum (TA TM3%) or with Dynabeads in X-Vivo 5% human serum (D XV5%) and transduced with a CD19-CAR lentivirus after 48 hours. At the end of 9 days, cultures were assessed for the mean (A) fold expansion (B) percentage of CD3 T cells transduced with CD19-CAR (C) percentage of CD8+CD19-CAR and (D) the absolute CD19-CAR count, as calculated by % of CD19-CAR+ cells x Final cell count. Data shown is from 5 individual healthy controls PBMCs and an unpaired Student's t test was used to calculate data significance whereby * = $p<0.05$

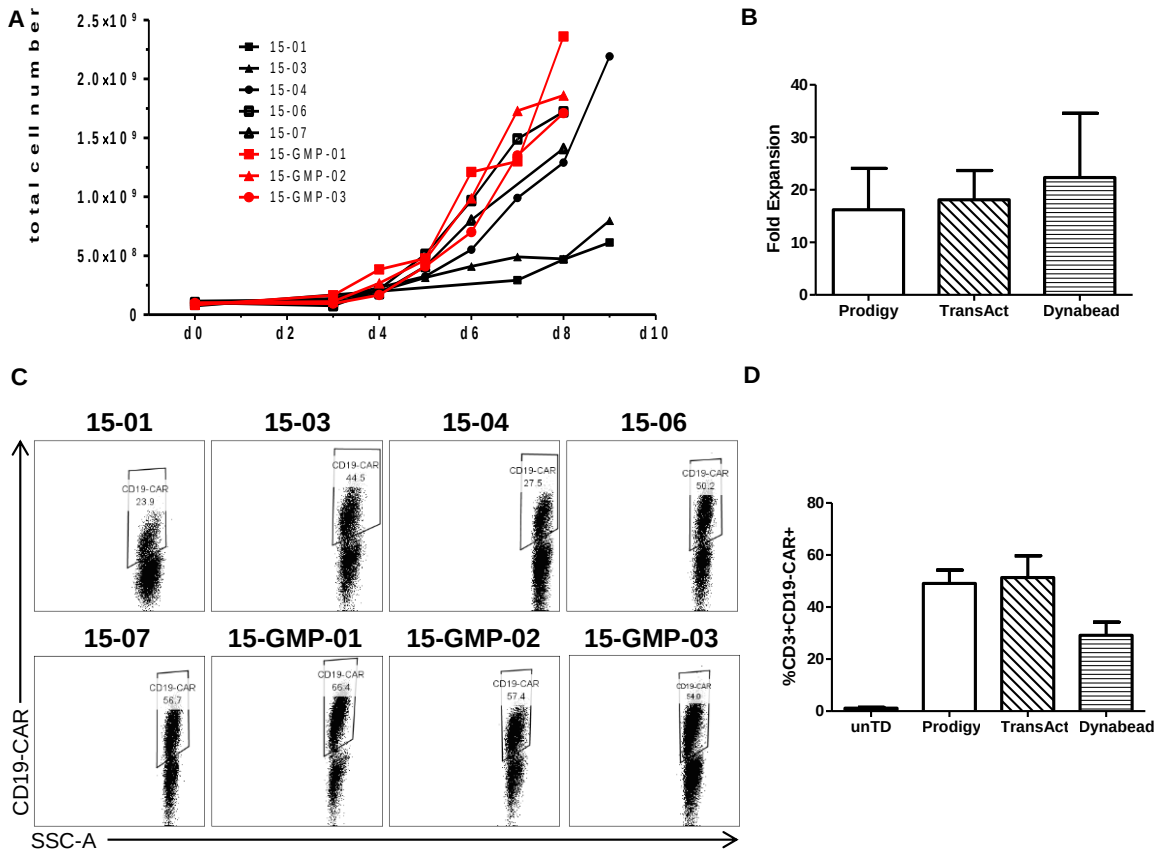

Figure 3 Automated expansion and transduction of T cells in the CliniMACS Prodigy. (A) The expansion of transduced T cells in the CentriCult unit of the CliniMACS Prodigy was monitored over a 8 day expansion period for eight independent, healthy donors. The processes performed in the GMP facility are shown in red (B) The average fold expansion of T cells in the CliniMACS Prodigy was compared to small scale manual controls activated under the same conditions as in the CliniMACS Prodigy with TransAct in TexMACS 3% human serum (TransAct) or under standard methods of activation using αCD3αCD28 Dynabeads in X-vivo 5% human serum (Dynabead). (C) Cells were transduced in the CliniMACS Prodigy with a CD19-CAR lentiviral vector at an MOI of 5. Transduction of T cells was assessed by flow cytometry staining for CD3, CD4, CD8 and FAB staining. Gates were set on mock-transduced cells that were expanded in parallel. (D) The percentage of automated CD19-CAR transduction was compared to cells transduced in small scale controls.

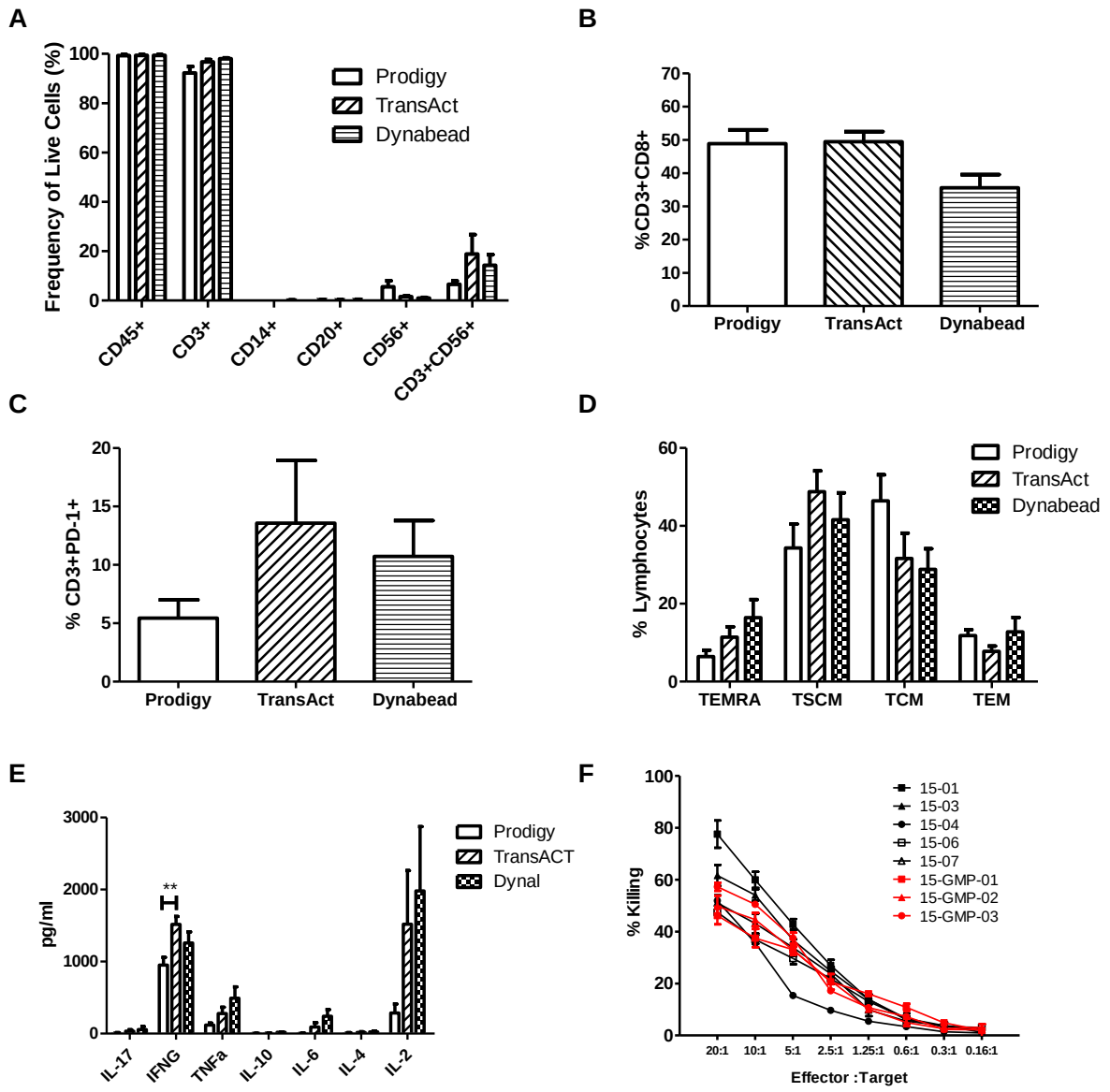

Figure 4 Phenotype and functionality of T cells activated, transduced and expanded in the CliniMACS Prodigy. (A-D) Immunophenotyping. At the end of the process, the phenotype of cells expanded in the CliniMACS Prodigy or in manual processes was assessed by flow cytometry to identify the percentage of (A) lymphocytes (CD45+), T cells (CD3+), Monocytes (CD14+), B Cells (CD20+), NK cells (CD3-CD56+) and NKT cells (CD3+CD56+); (B) CD3+CD8+ T cells and (C) the total PD-1 expression on expanded T cells. (D) The percentage of memory T cell markers on cells expanded in the CliniMACS Prodigy. Results shown as mean ± SD for three independent healthy donors (n=3). (E+F) Functionality. (E) Cytokine production of CAR19 T cells when co-cultured with CD19+

SupT1 target cells as measured by CBA. Results are shown for 6 TCT-processes, not including 15-01 or 15-04 where small scale control cells were unavailable. (F) Cytotoxic activity. Killing efficiency was measured in a ^{51}Cr-release assay with CD19-positive SupT1 target cells in increasing effector-to-target ratios (E:T ratios) post NK-cell depletion. Results for 8 TCT-processes are shown as mean ± SEM from triplicates.

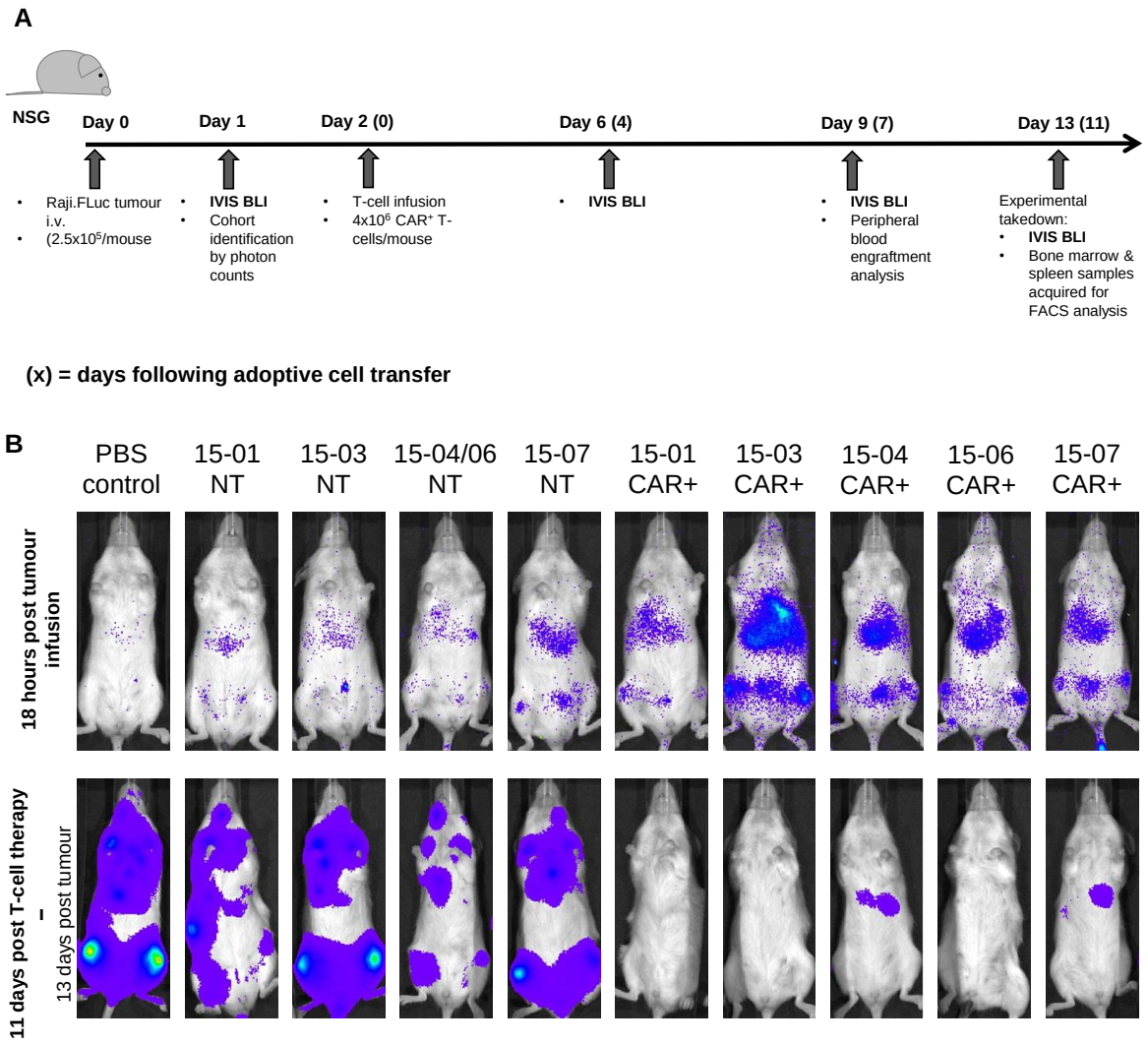

Figure 5 Anti-tumour effect of automatically manufactured CD19-CAR T cells

(a) Schematic of mouse model. CD19+ Raji cells expressing Luciferase were injected into NSG mice and tumour engraftment monitored on d1. On d2, CAR19 T cells and the respective non-transduced controls were injected and tumour growth assessed on d6, d9 and d13. (b) IVIS imaging post tumour injection at d1 (top row) and d13 with CAR19 T cells at d13 (bottom row, 5 mice on the right). Whereas non-transduced cells did not show anti-tumour activity, CAR19 T cells cleared the CD19+-tumour *in vivo*. Representative pictures for 5 different manufacturing processes shown. Additional data can be found in the supplementary figures.

Table 1 Summary of automated manufacturing of CAR19 T cells

Run	Yield Total lymph ($x10^8$)	% CAR19+ T cells	Yield CAR19 + T cells ($x10^8$)	Target met */** ($\geq 3.5x10^8$ CAR19+ T cells)	Length of Process (Days)
15-01	4.00	23.9	0.96	X	10
15-03	8.08	44.5	3.6	✓	9
15-04	23.2	27.5	6.4	✓	9
15-06	18.5	50.2	9.3	✓	8
15-07	17.1	56.7	9.7	✓	8
15-GMP-01	20.0	66.4	13.3	✓	8
15-GMP-02	18.6	57.4	10.7	✓	8
15-GMP-03	17.1	54.0	9.2	✓	8
mean	**15.8 ±6.4 $x10^8$**	**47.6 ± 14.9%**	**7.9 ±4.0 $x10^8$**		

Supplementary Figures

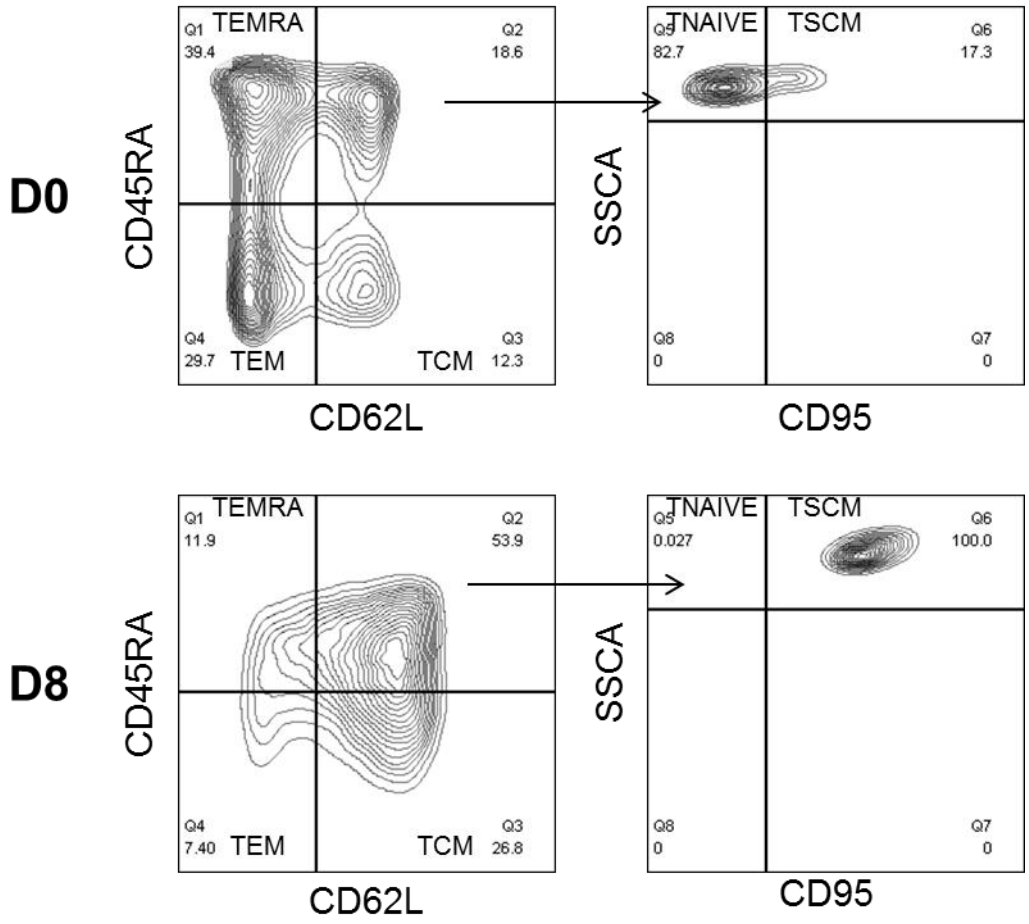

Figure S1. Gating Strategy for Memory T cell populations.

Memory T cell populations were analysed by gating on lymphocytes based on their FSC-A/SSC-A profile and excluding any doublets. Memory T cell populations were assessed by their expression of CD45RA, CD62L and CD95 as follows: CD45RA+CD62L- – TEMRA, CD45RA+CD62L+CD95- – TNAIVE, CD45RA+CD62L+CD95+ – TSCM, CD45RA-CD62L+ – TCM and CD45RA-CD62L- – TEM. All FACS gates were set on cells of the same donor stained prior to activation on D0 as shown.

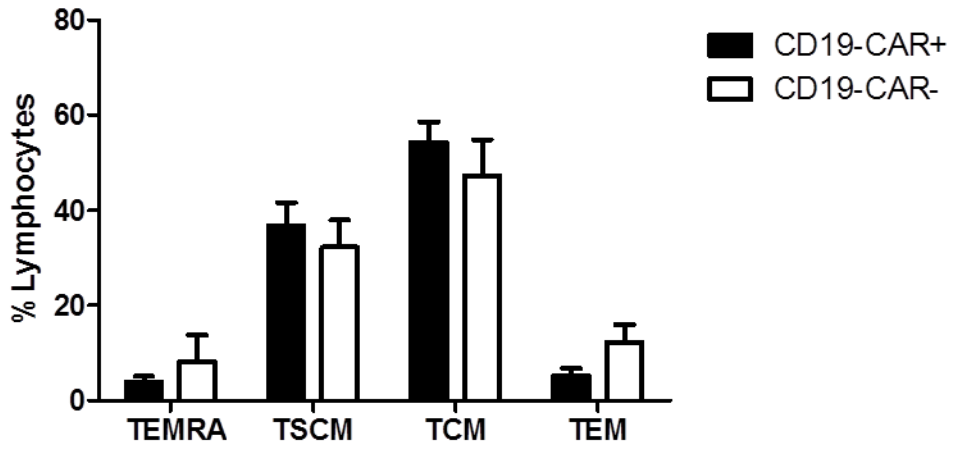

Figure S2. Transduction with CD19-CAR lentiviral vector does not effect the memory T cell compartment.

The total lymphocyte populations, based on their FSC-A/SSC-A profile, were gated on transduced CD19-CAR+ or untransduced CD19-CAR-T cells. The memory T cell compartment were assessed based on expression of CD45RA, CD62L and CD95 in Figure S2. Results shown are from 8 TCT processes on the CliniMACS Prodigy.

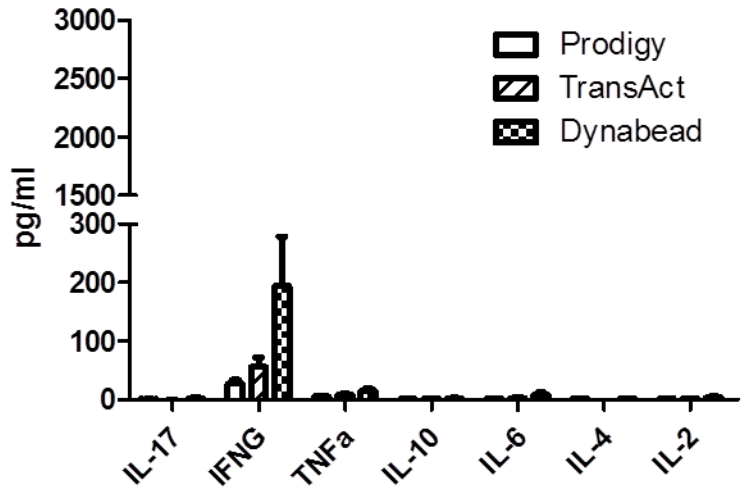

Figure S3. Negative control for cytokine release assay measured by CBA.

CAR19+ T cells manufactured as described were co-cultured with CD19- SUPT1 cells and cytokine release in the cell supernatant was measured by CBA assay. Results shown are from 6 TCT process excluding donors 15-01 and 15-04.

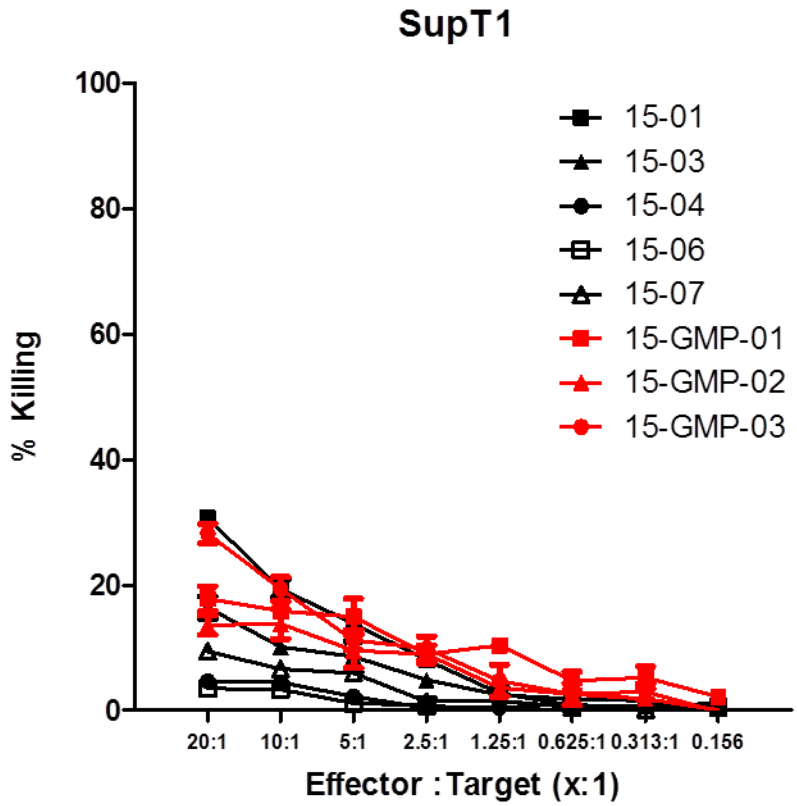

Figure S4. Negative control for cytotoxicity assay.

Killing efficiency was measured in a 51Cr-release assay with CD19-negative SupT1 target cells in increasing effector-to-target ratios (E:T ratios) post NK-cell depletion. Results for 8 TCT-processes are shown as mean ± SEM from triplicates.

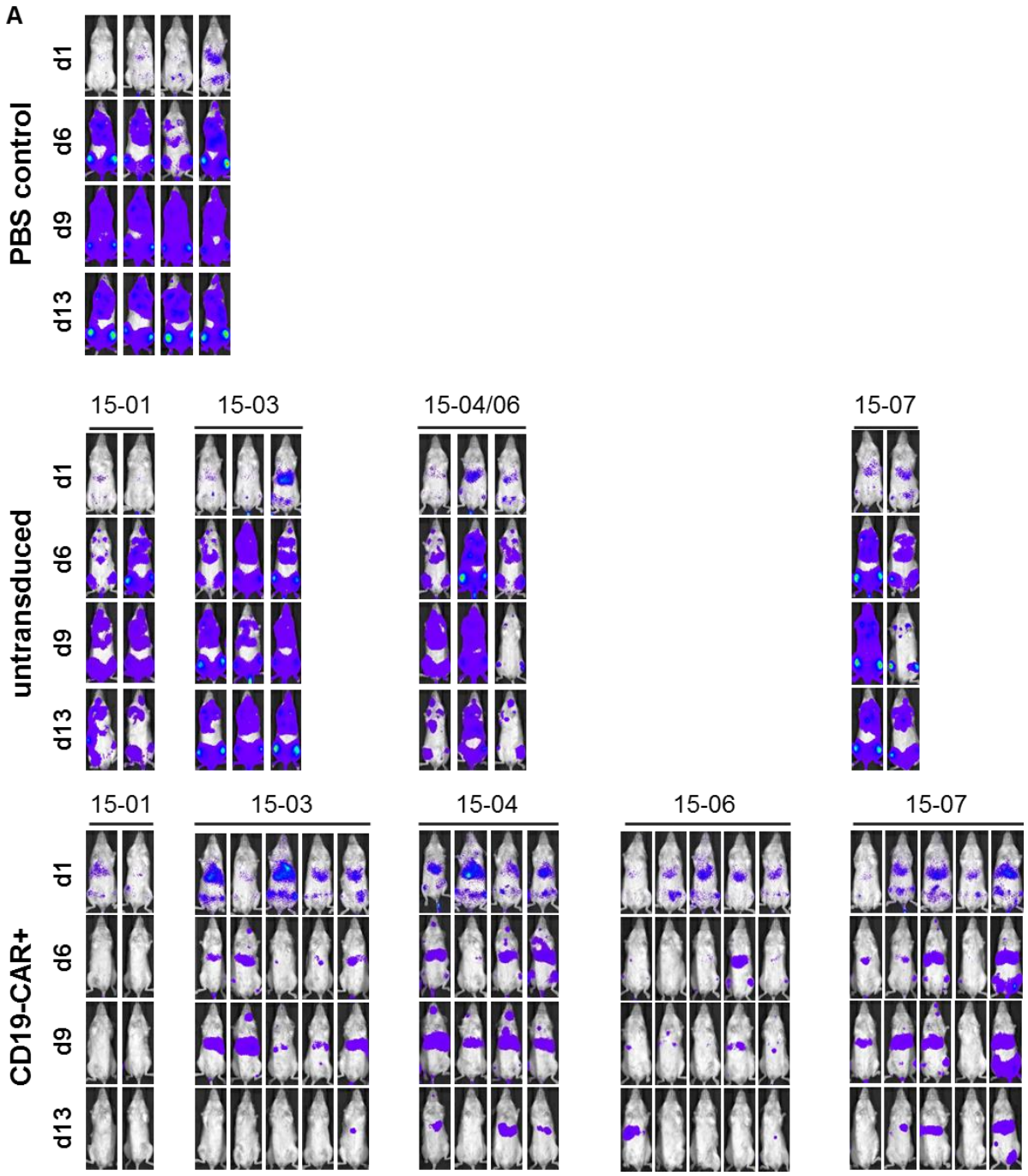

Figure S5 Complete IVIS imaging of in vivo model.

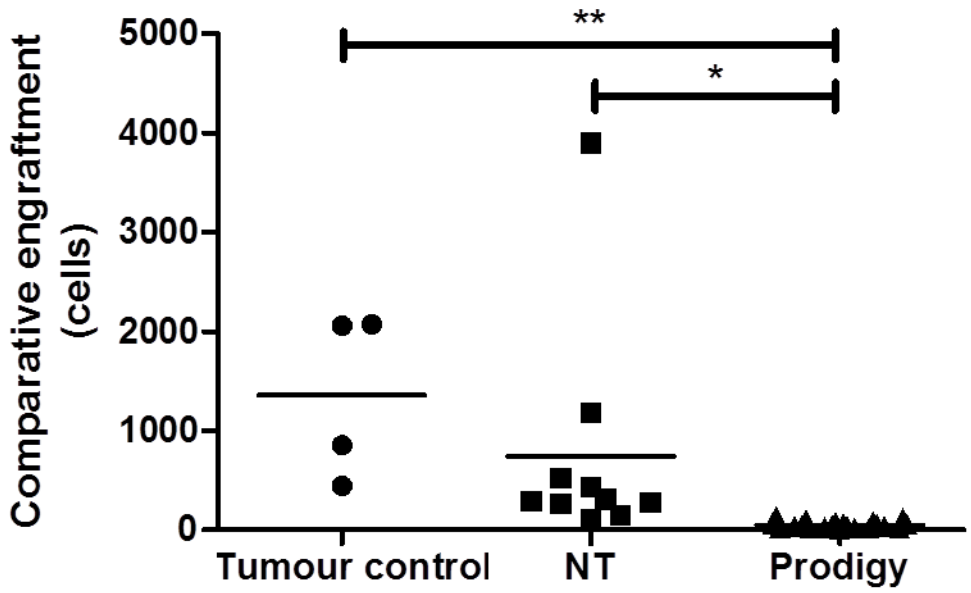

Figure S6. Tumour Engraftment post CD19-CAR T Therapy

Tumour engraftment was assessed by flow cytometry analysis of Raji tumour present in the bone marrow 11 days post T cell transfer. Tumours were identified based on expression of tumour markers CD20 and CD38.

Table S1. Hands-on time during the TCT process

Day of procedure	Action	Steps in detail	Hands-on time
-1	**Preparations**	– Installation of Tubing Set	45 min
0	**Start of run**	– Preparation and connection of media – Preparation and connection of activation reagent – Connection of cells – Programming of activity matrix	90-120 min
1	**Transduction**	– Preparation of vector and connection to the machine – Start Transduction (10min)	30 min
2	**-**		
3	**Sampling**	– Start sampling and detach sampling pouch	10 min
4	**Sampling**	– Start sampling and detach sampling pouch	10 min
5	**Sampling** **Culture Wash**	– Start sampling and detach sampling pouch – Start Culture Wash (confirmation only)	15 min
6	**Sampling**	– Start sampling and detach sampling pouch	10 min
7	**Sampling**	– Start sampling and detach sampling pouch	10 min
8	**Sampling**	– Start sampling and detach sampling pouch	10 min
9	**Sampling** **End of culture**	– Prepare and connect formulation buffer – Start harvest (45 min) – Detach Target Cell bag – De-install Tubing Set	120 min + freezing time
Total Hands on Time			6hrs 20 mins