

A METHOD OF MANUFACTURING A CELLULAR THERAPY PRODUCT AND RESPECTIVE KITS AND DEVICES

CROSS-REFERENCE TO RELATED APPLICATION

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The present application claims the benefit of priority of EP Patent Application No. 23204394.3 filed 18 October 2023 and of EP Patent Application No. 23206668.8 filed 30 October 2023, the content of each of which is hereby incorporated by reference in its entirety for all purposes.

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FIELD OF THE INVENTION

The present invention relates to a method of manufacturing a cellular therapy product, wherein the cellular therapy product comprises a first cellular surface receptor which is able to bind to a drug target molecule (on the surface) of a drug target cell and/or wherein the cellular therapy product produces and/or secretes a polypeptide which is able to bind to a drug target molecule either soluble or associated with a drug target cell. The invention also relates to a kit, a device and a tubing to be used in the method.

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BACKGROUND OF THE INVENTION

Current manufacturing strategies of cellular therapy products such as CAR T cells or T cells expressing recombinant T cell receptors (TCR) are unfavorably time- and cost-intensive due to the complexity of the manufacturing process and extensive product release testing and by that occasionally interfering with the treatment of late-stage cancer patients for whom every treatment day can make a crucial difference (1). Also, a rather high logistic burden is associated with the centralized manufacturing at specialized facilities involving multiple manual handling as well as massive in vitro cultivation, manipulation and expansion. The resulting high manufacturing costs cause concerns regarding the affordability of such therapies to the health system and the patients.

Currently huge ethically concerns arise as many patients cannot be treated due to an unavailability of the products because of the laborious and time-consuming non-scalable manufacturing. This makes the therapy not available for patients in need of the highly efficient and lifesaving therapy. In addition, this laborious non-scalable manufacturing prevents extending cellular therapy to new indications and therapeutical fields (auto-immune diseases, infectious diseases, etc.). While CD19 CAR T cells have been shown to be highly effective in systemic lupus erythematosus (2), currently, such therapeutic areas are not addressed due to the limitations above.

10 A strategy providing time and cost benefit is the so-called "allogeneic approach" that takes advantage of novel gene editing methods to generate universal donor T cells from third parties in a way that they would not be rejected by the patient's immune system but also would not induce a graft-versus-host reaction in the patient (3). This requires excessive genetic engineering of the therapeutically active cells to circumvent
15 both rejection (host-vs-graft) or anti-host (host-vs-graft) responses. In theory, this approach may enable the manufacturing of hundreds of doses per batch, but it possesses an inherent risk of high clinical toxicity associated with unmet immunological hurdles (4). Thus, after the death case in 2021 during the UCARTCS1A trial from Cellectis the FDA halted all clinical trials using universal donor cells due to
20 safety concerns (5).

The in vivo generation of manipulated cells (T cells as an example in this context) via systemic or localized application of viral or non-viral gene delivery systems carrying relevant gene(s) is an alternative novel strategy to reduce the complexity of cell
25 manufacturing and avoid the in vitro cultivation step (6). Such delivery systems or nanocarriers can be produced by standardized methods rather easily, enabling a broader worldwide clinical application as compared to conventional cellular therapies. Recent studies show some anti-tumoral responses in humanized mouse models but also method associated side effects comprising high immunogenicity of gene
30 engineering delivery systems and the cargo, fast hepatic and renal clearance of administered delivery systems from body, potentially pre-existing anti-viral vector immunity, limited distribution of agents within the body which may require multiple injections and limit the overall therapeutic efficiency. Also, evidence for a certain degree of unspecific targeting of the gene delivery system towards non-T cells (as a

result of systemic application) impacting dramatically the potential safety profile of the treatment (7-9).

Thus, automated manufacturing of cellular products such as CAR T cells remains of
5 interest.

As one such approach, international patent applications WO 2019/217964 A1 and WO 2022/072885 A1 describe a closed-loop system for the modification of cells from patients, wherein a cell containing sample, e.g. a blood sample, from the patient enters
10 the system and is first separated in a cell separation module. Within this cell separation module cells separated from the initial sample could optionally be enriched. After passing the cell separation module the cells are modified using modifying agents, e.g. therapeutic drugs. While no experimental data are reported, WO 2019/217964 A1 and WO 2022/072885 A1 describe that thereafter the modified cells can be enriched and
15 then are reinfused to the patient. WO 2019/217964 A1 and WO 2022/072885 A1 also describe that these CAR T-Cells are to be activated, for example, by CD3 and CD28 costimulation prior to administration to the patient.

The international patent application publication WO 2023/122277 A2 discloses a rapid
20 method of generating CAR T cells by simultaneous activating the T cells of a mononuclear cell sample and exposing the mononuclear cell sample to a viral vector for transducing T cells with a CAR construct.

There is however still a need for alternative methods that allow easy manufacturing
25 patient derived cellular therapy products such as genetically modified CAR T cells.

SUMMARY OF THE INVENTION

This object is accomplished, inter alia, by the method, the use, the kit, and the device
30 for manufacturing a cellular therapy product having the features of the independent claims.

According to a first aspect, the invention provides a method of manufacturing a cellular therapy product, wherein the cellular therapy product comprises a first cellular surface

receptor which is able to bind to a drug target molecule (on the surface) of a drug target cell, and/or wherein the cellular therapy product produces and/or secretes a polypeptide which is able to bind to a drug target molecule either soluble or associated with a drug target cell, comprising

5 a) enriching via reversible immobilization (in a holding step) a cellular therapy starting cell obtained from a sample comprising the cellular therapy starting cell, using at least one second cellular surface receptor of the cellular therapy starting cell, and

10 b) genetically modifying the cellular therapy starting cell using one or more reagent(s) comprising a gene construct for expression of the first cellular surface receptor and/or for expression and/or secretion of the polypeptide, thereby generating a precursor of the cellular therapy product,

wherein the method takes place in a closed system.

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According to a second aspect, the invention provides a use of a set of reagents for genetically modifying a cellular therapy starting cell, wherein the cellular therapy starting cell is immobilized on a stationary phase or a receptor binding reagent during the genetic modification or the use of the set of reagents for genetic modification of the
20 a cellular therapy starting cell, wherein the gene modification takes place before eluting the cellular therapy starting cell from the stationary phase.

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According to a third aspect, the invention provides a kit for manufacturing a cellular therapy product, wherein the cellular therapy product comprises a first cellular surface
25 receptor which is able to bind to a drug target molecule (on the surface) of a drug target cell, and/or wherein the cellular therapy product produces and/or secretes a polypeptide which is able to bind to a drug target molecule either soluble or associated with a drug target cell, the kit comprising

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a) one or more reagent(s) comprising a gene construct for expression of the first cellular surface receptor or of the polypeptide of the cell therapy product, and

b) a receptor binding reagent comprising a binding site B and a binding partner C,

- wherein the binding site B comprised in the receptor binding reagent is capable of specifically binding to the second cellular surface receptor on a cellular

- therapy starting cell and wherein the bond between the binding site B of the receptor binding reagent and the second cellular surface receptor has a dissociation constant (K_D) is of low affinity or in the range from about 10^{-3} to about 10^{-7} M,
- 5 - wherein the binding partner C comprised in the receptor binding reagent is capable of reversibly binding to a binding site Z of an affinity reagent and the reversible bond between the binding partner C of the receptor binding reagent and the binding site Z of the affinity reagent has a dissociation constant (K_D) in the range from about 10^{-2} to about 10^{-13} M, and
- 10 - a stationary phase, the stationary phase having the affinity reagent immobilized thereon,
- wherein the affinity reagent comprises one or more binding sites Z, wherein said binding site Z forms a reversible bond with the binding partner C comprised in the receptor binding reagent, and wherein the binding site B of the receptor binding reagent binds to the second cellular surface receptor on the cellular therapy starting cell, to allow reversibly immobilizing the cell
- 15 therapy starting cell on the stationary phase.

According to a fourth aspect, the invention provides a device for manufacturing a cellular therapy product, comprising an operating device and a tubing-set, wherein the

20 tubing set is a closed and single-use tubing-set, for use in above mentioned method of manufacturing a cellular therapy product.

According to a fifth aspect, the invention provides a tubing having an affinity reagent immobilized on an inner surface of the tubing, wherein the affinity reagent comprises

25 one or more binding sites Z, wherein said binding site Z forms a reversible bond with a binding partner C comprised in the receptor binding reagent, and wherein the binding site B of the receptor binding reagent binds to a (second) cellular surface receptor on a cellular therapy starting cell, thereby allowing reversibly immobilizing the cellular

30 therapy starting cell on the tubing acting as stationary phase.

According to a sixth aspect, the invention is directed to a use of the tubing of the fifth aspect in a method of manufacturing a cellular therapy product.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will be better understood with reference to the detailed description when considered in conjunction with the non-limiting examples and the accompanying drawings. The figures illustrate embodiments of methods of the invention.

Figure 1 is an illustration of the inventive CAR T cell generation strategies. Respective facilities, as well as the whole duration of CAR T cell generation including potential manufacturing steps until product infusion are depicted per strategy. Conventional in vitro CAR T manufacturing strategy using a centralized manufacturing facility as applied for the commercial production compared to the inventive process envisioned strategy for the targeted extracorporeal vector gene therapy and subsequent controlled in vivo CAR T generation.

Figure 2 depicts an illustrative example of the inventive method using the inventive device for an extra-corporal fully automated cell/gene therapy. In this illustrative example, after system setup and obtaining a bodily sample from the patient such as whole blood sample, the process runs automated on a dedicated device using a single-use set and consists of the following steps: 1) collection of blood starting material (e.g., whole blood) directly from the patient, 2) short target cell specific hold-step via dedicated cell surface markers, 3) release and wash of target cells; 4) targeted genetic modification (e.g., non-viral CRISPR/Cas editing via e.g. electro-/mechanoporation, incubation with loaded lipid nanoparticles), 5) in-process analytics (e.g., non-invasive analytics like viable cell number); 6) removal of residuals and re-infusion.

Figure 3 depicts a further illustrative example of the inventive method using the inventive device for an extra-corporal fully automated cell/gene therapy. In this illustrative example, after system setup and obtaining a bodily sample from the patient such as whole blood sample, the process runs automated on a dedicated device using a single-use set and consists of the following steps: 1) collection of blood starting material (e.g., whole blood) directly from the patient, 2) short target cell specific hold-step via dedicated cell surface markers and targeted genetic modification during the hold-step (e.g., non-viral CRISPR/Cas editing via incubation with loaded lipid

nanoparticles), 3) release and wash of target cells; 4) in-process analytics (e.g., non-invasive analytics like viable cell number); 5) removal of residuals and re-infusion.

Figure 4 depicts the results of an example of the enrichment of CD3+ cells via reversible immobilization from whole blood starting material. CD3+ T cells were retained from 200 ml whole blood using a device that is commercially available on the market (n=10). **Fig. 4A** depicts cell counts (Mio) in the starting whole blood material and in the enriched positive material. **Fig. 4B** is showing CD3 target cell depletion, **Fig. 4C** is showing purity and **Fig. 4D** is showing yield. Depletion is calculated on target cells in starting material vs. target cells in negative fraction. Yield is calculated on target cells in starting material vs. target cells in positive fraction. **Fig. 4E** shows flow cytometry data for two donors for whole blood starting material and positive fraction. Purity is measured via Flow Cytometry and pre-gated on single living CD45 cells.

Figure 5 depicts a TRAC Knock Out in selected CD3 T cells. The graphic is showing the TRAC Knock Out using an RNP complex, composed of Cas9 Nuclease and gRNA directed against the TRAC locus for up to 12 days after mechanoporation of the selected and non-activated T cells. CD3- signal is measured via Flow Cytometry and pre-gated on single living CD45 cells. n=5 individual runs are shown.

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Figure 6 depicts a T cell transfection using transgene LNPs (GFP as mRNA cargo). CD3+ T cells were retained from whole blood and transfected with transgene LNPs. **Fig. 6A** depicts Flow cytometry gating strategy for measuring transgene expression on LNP incubated and non-activated T cells. **Fig. 6B** is showing the control where selected T cells are incubated in the incubator for 4 hours without LNPs. **Fig. 6C** is showing transgene expression after 4 hours incubation with LNPs on column and subsequent elution of transfected cells. **Fig. 6D** is showing transgene expression after 4 hours incubation with LNP after T cell selection and subsequent elution. **Fig. 6E** is showing transgene expression after 4h LNP incubation and on day 1 (after 24h), day 3, day 5, day 7 and day 9 after LNP incubation as well as viable T cells in %. All data are pre-gated on single living CD45 cells and gated separately on CD4 T cells and CD8 T cells.

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Figure 7 shows potent in vivo tumor cell killing resulting from the inventive cellular therapy product. **Fig. 7A** shows a flow-diagram depicting an example of the inventive ultra-short process for extra-corporally generated CD19 CAR T cells comprising a brief CD3 hold/enrichment step from a 200ml whole blood draw, washing, CD19 CAR CRISPR/Cas KO/KI and subsequent infusion. **Fig. 7B** shows in vivo expansion and functionality of the inventive ultra-short extra-corporally generated CAR T cells that were analyzed in a clinically relevant mouse model of lymphoma. 6-8 week old NSG-SGM3 (NSGS) mice were injected intra venously with 0.5×10^6 Raji-fluc-GFP CD19+ tumor cells and subsequently intra peritoneally injected with CAR+ cells prepared according to the invention two days later (a total of 1×10^6 cells was administered as flat dose). Conventionally manufactured CD19 CAR T cells using a standard lentivirus (LV) transfection and expansion process served as positive controls while mice only injected with PBS served as negative control group. Tumor growth/rejection was evaluated using IVIS bioluminescence. Mice treated with CAR+ cells manufactured according to the inventive method (TQ process) and standard LV control cells showed rapid expansion and high tumor reactivity with complete tumor clearance post day 20 (**Fig. 7B** middle and right columns). In contrast, control groups treated with PBS only showed uncontrolled tumor progression (**Fig. 7B** left column) with mice succumbing to the tumor starting around day 14. **Fig. 7C** shows that TRAC-CAR knock-in and expression was confirmed after extended parallel in vitro culture by flow cytometry. **Fig. 7D** shows a survival benefit of mice receiving the inventive cell products compared to PBS control. All mice that received the inventive cells survived and controlled the tumor while all control mice succumbed to the tumor starting around day 14. These data illustrate that ultra-short extra-corporally generated CAR cells (that fully develop/mature within the body of the recipient of the precursor of the cell therapy product) are highly potent in controlling aggressive lymphoma comparable to conventionally manufactured and expanded CAR cells (representative of clinically used CAR T cell products).

Figure 8 shows a quantification of tumor growth/control, survival and in vivo expansion of CAR T cells as an exemplary inventive cellular therapy product that can be manufactured using the invention. **Fig. 8A** shows quantification of tumor growth measured by average radiance [p/s/cm²/sr] post luciferin treatment is shown over time using Living image software (Perkin Elmer). **Fig. 8B** shows that besides quantification

of expanding transferred CAR T cells, residual Raji tumor cells were measured in blood using flow-cytometry. **Fig. 8C-G** shows engraftment and in vivo expansion of total transferred T cells and CAR+ transferred T cells. For these experiments mice were bled weekly for ex vivo analysis using flow cytometry and staining for T cell subsets and CAR expression. **Fig. 8C** shows a measurement of the total CD3+ CAR+ T cells, wherein the percentage of CAR+ cells in the blood reaches a maximum at day 14 post CAR T cell injection. **Fig. 8D** shows the percentage of CD4+ CAR+ T cells in the blood and **Fig. 8E** shows the percentage of CD8+ CAR+ T cells in the blood. Both cell types had their maximum at day 14 post CAR T cell injection. **Fig. 8F** shows the percentage of CD4+ cells in the blood. The percentage of CD4+ T cells showed a decrease at day 14 post CAR T cell injection and reached a maximum at day 21 post CAR T cell injection. **Fig. 8G** shows the percentage of CD8+ T cells in the blood. Mice injected with the CAR T cells prepared according to the invention showed an increase of percentage of CD8+ T cells in the blood post CAR T cell injection. Whereas for the LV positive control the percentage of CD8+ T cells in the blood was unchanged throughout the monitored time. All data shown are pre-gated on living CD45+ cells. Black is PBS control, white is inventive processed cells (TQ process), grey is LV transduced expanded cells (positive control). n=4 for all tested cohorts receiving CAR T cells and n=3 for untreated PBS control. Together these data demonstrate a complete control of the tumor as a result of the potent anti-tumor response of transferred CAR T cells (in particular of the relevant CD8+ subset) resulting in a marked survival benefit of treated mice.

Figure 9 shows a schematic drawing of an example of the inventive device including an operating device, a single-use tubing set, process buffers and a set of reagents for gene modification. The operating device will incorporate all non-disposable components needed to automatically operate the inventive method. The device will only get operational via insertion of a single-use tubing set to the operating device. The tubing set will comprise tubing, the cell retention reservoir as well empty bags for whole blood-, waste- and blood cell preparation collection. At the day of treatment, the tubing set will be connected to separately delivered liquid-containing bags as the respective reagents and process buffers. The process unit operation with respect to associated process steps are depicted in the schematic.

DETAILED DESCRIPTION OF THE INVENTION

As explained above, in a first aspect the invention is directed to a method of manufacturing a cellular therapy product, wherein the cellular therapy product
5 comprises a first cellular surface receptor which is able to bind to a drug target molecule (on the surface) of a drug target cell and/or wherein the cellular therapy product produces and/or secretes a polypeptide which is able to bind to a drug target molecule either soluble or associated with a drug target cell. This method comprises
10 a) enriching via reversible immobilization (in a holding step) a cellular therapy starting cell obtained from a sample comprising the cellular therapy starting cell, using at least one second cellular surface receptor of the cellular therapy starting cell, and
b) genetically modifying the cellular therapy starting cell using one or more reagent(s) comprising a gene construct for expression of the first cellular surface receptor and/or for expression and/or secretion of the polypeptide, thereby generating a precursor of
15 the cellular therapy product. As explained above, the method takes place in a closed system.

Thus, in a first alternative the manufactured cellular therapy product comprises a first cellular surface receptor which is able to bind to a drug target molecule (on the surface)
20 of a drug target cell.

In a second alternative, the manufactured cellular therapy product produces and/ or secretes a polypeptide which is able to bind to a drug target molecule either soluble or associated with a drug target cell. The polypeptide is an amino acid chain having at
25 least three amino acids connected by peptide bonds. The polypeptide may be, as non-limiting examples, a peptide hormone, an insulin, or a coagulation factor. The cellular therapy starting cell for genetic modification for production and/or secretion of such a polypeptide is preferably a B cell.

30 Furthermore, it may be also possible that the manufactured cellular therapy product comprises the first and the second alternative.

It has been surprisingly found in the present application that enriching a cellular therapy starting cell via such reversible immobilization allows to manufacture a cellular therapy

product or a precursor of a cellular therapy product without the need for prior cell separation, in particular without the need of prior cell separation of a bodily fluid sample such a blood sample in a cell separation module that comprises a cell separator that produces a fraction of the bodily sample enriched in nucleated blood cells. It has been
5 surprisingly found herein that this enrichment step results in a 99 % pure CD3 lymphocyte cell population that is then subjected to genetic modification (see Example 1, Enrichment of cellular therapy starting cells using a liquid-handling device, **Figure 4**). Also, a comparable enrichment of B cells is possible (see Example 1). Or in other words, it has been found herein, that this reversible immobilization can be carried out
10 as a single enrichment step and so obtained enriched sample (which comprise the cellular therapy starting cell) can still be subjected to genetic modification that results in the functional precursor of the cell therapy product - this precursor, once administered to a patient, will mature and develop into the functional cellular therapy product (see Example 3). To the surprise of the inventors this enrichment step (holding
15 step) even allows to use a whole blood sample obtained from a patient to be used for genetic modification without any prior treatment of the blood sample. This again has significant advantages, as it allows a) to produce the precursor of the cellular therapy product within an extremely short time frame of, for example, about 1.5 hours, about 2 hours, about 3 hours, about 4 hours, or about 5 hours, or about 6 hours, or about 7
20 hours, or between about 1.5 hours and about 16 hours after the sample has been collected from the patient and b) to simplify the design of the respective device since it does not need a cell separation, leukapheresis or apheresis module as it is described in WO 2019/217964 or WO 2022/072885, for example. This means a patient such as critically ill patient can obtain his autologous precursor cell therapy product within a
25 very short time such as about 90 minutes (or a bit longer, for example about 2 hours) and thus on-site in a hospital or doctor's office. This reduces the costs and administrative efforts of manufacturing a cellular therapy product significantly and thus allows to make cellular therapy available for any patient for whom cellular therapy is indicated as medically beneficial. In this context, it is noted that it has also been
30 surprisingly found that there is no need to stimulate the precursor of the cell therapy product, for example via CD3 and CD28 co-stimulation as it is conventionally done and also described in WO 2019/217964 A1 or WO 2022/072885 A1 but that the non-activated genetically modified cell (precursor of the cellular therapy product) such as a T cell, B cell or NK cells can be administered to the patient without such conventional

activation. Also, this shortens the manufacturing time and simplifies the manufacturing process and respective closed-loop device.

In this context, the very high purity of the sample of the cellular therapy starting cell
5 after the reversible immobilization of the present invention should be addressed here. The enrichment of CD3+ cells with a purity of 99% from whole blood samples, without prior leukapheresis, using step a) of the method of the invention, especially with Strep-Tactin® coated beads as shown in Example 1, is in particular surprising, because similar experiments reported in the prior art showed a much lower purity. A comparable
10 enrichment is possible using B cells. For example, WO 2013/124474 A1 describes an example in which CD8+ cells were enriched by column chromatography using Sephadex coupled Strep-Tactin® which carried a CD8 binding Fab fragment as receptor binding reagent. In this experiment peripheral blood mononuclear cells (PBMCs) were first isolated from whole human blood samples and thereafter loaded
15 these suspensions on columns. The so enriched CD8+ cells had a purity of only 68%, cf. Example 1 of WO 2013/124474 A1. WO 2021/152178 A1 reports a cell isolation experiment that uses a liquid-handling device (no closed system) and in which biotin coated beads were incubated with strep-Tactin®/dextran polymers loaded with CD3 binding Fab fragments equipped with a streptavidin binding peptide (Strep-Tag®) in a
20 column. Thereafter, PBMCs were added, the column was closed on both sides and incubated with rotating. After washing CD3+ cells were eluted with a purity of 79% (cf. Example 9 of WO 2021/152178 A1). In view of these two experiments that both used peripheral blood mononuclear cells, it is surprising that reversible enrichment as used herein is able to achieve an enrichment of CD3+ cells with a purity of 99% from whole
25 blood samples. While not wanting to be bound by, the inventors believe that the reason for this high purity of CD3+ cells, stems from the use of fresh whole blood samples instead of separated PBMCs. The inventors reasoned that such fresh blood cells may not be sticky, meaning they show much less unspecific adhesion to surface than processed PMBCs, which may also result in more specific binding the anti CD3 Fab
30 fragments presented on the surface of the streptavidin muteins such as the commercially available "Strep-Tactin®" used as solid support for the reversible enrichment. The conditions used herein for the enrichment may also contribute to the very high purity. As described in Example 1 herein, the enrichment was made using beads in a reservoir with shaking and not with beads packed in a column. The same

procedure using preferably fresh blood sample can also be used for the enrichment of B cells as further cellular therapy starting cell.

5 The term “closed system” or “closed-loop system” as used herein means the “closed system” as defined in Guidelines on Good Manufacturing Practice specific to Advanced Therapy Medicinal Products of 22.11.2017, Glossary No. 12 stating “*A process system designed and operated so as to avoid exposure of the product or material to the room environment. Materials may be introduced to a closed system, but the addition must be done in such a way so as to avoid exposure of the product to the room environment*
10 *(e.g. by means of sterile connectors or fusion systems). A closed system may need to be opened (e.g., to install a filter or make a connection), but it is returned to a closed state through a sanitization or sterilization step prior to process use.*”

As mentioned above, the inventive method allows manufacturing a cellular therapy
15 product in a very short time period. The duration of the inventive method may, for example, be between about 1.5 hours to about 16 hours. However, it is also envisaged here that with further process optimization and automation, it may be possible to manufacture a cellular therapy product within less than about 1.5 hours.

20 The drug target molecule, as mentioned above, can be any molecule on the surface of the drug target cell, provided it is able to get in contact with the first cellular surface receptor. The drug target molecule can however also be a soluble molecule or a drug target cell associated molecule, which could be targeted by the polypeptide secreted by the cellular therapy product. Preferably the drug target molecule is a molecule
25 relevant in a human or animal disease. One non-limiting example of such drug target molecules are cluster of differentiation (CD) molecules, especially CD19. To mention only a few, further examples of suitable drug target molecules include, but are not limited to, Claudin 6, IL13RA2, EGFRv3, a disialoganglioside GD2, prostate-specific membrane antigen, or CLDN18, as described below, or checkpoint inhibitors. The drug
30 target molecule may also be a coagulation factor as a non-limiting example of a soluble (secretory) drug target molecule.

The drug target cell can be any cell related to a disease, in particular the cell expresses a suitable drug target molecule on their surface. Typically, the drug target cell may be

a human or an animal cell. The drug target cell may be a cell that is diseased, for example, a cancer cell, a cell affected by an autoimmune disease, or a cell infected by a virus or a bacterium. Exemplary cancers from which the cancer cell is derived are solid tumors or hematological tumors. Examples of solid tumors include, but are not limited to, germ cell tumors, epithelial ovarian cancer, desmoplastic small round cell tumor, gastric adenocarcinoma, colorectal cancer, endometrial carcinoma, breast cancer, glioblastoma, and neuroblastoma. Examples of hematological tumors are, but are not limited to, acute or chronic myeloid leukemia, lymphoblastic leukemia, and lymphoma. Examples of autoimmune diseases include, but are not limited to, systemic lupus erythematosus, diabetes, psoriasis, or rheumatoid arthritis to name only a few. Also, cells relating to an infectious disease are possible as drug target cell. Furthermore, the drug target cell may be a cell physiologically participating in blood coagulation or be a cell physiologically producing insulin.

It is noted here that recently the results of a phase 1/2 clinical trial using CAR T cells with and without an RNA vaccine was published. In this trial an anti Claudin 6 (CLDN6) CAR was used for retroviral infection of autologous peripheral blood mononuclear cells (PBMCs) derived from leukapheresis of patient blood samples. The RNA vaccines contained a CLDN6 coding RNA optimized for translation in antigen presenting cells. Claudin 6 is a tight junction protein physiological only expressed during fetal organogenesis, but also expressed from several solid tumor cells rendering Claudin 6 as a suitable drug target for cancer therapy. Claudin 6 expression was observed especially in germ cell tumors, epithelial ovarian cancer and endometrial carcinoma. The patients enrolled to this trial had germ cell tumors, epithelial ovarian cancer, desmoplastic small round cell tumor, gastric adenocarcinoma, colorectal cancer, endometrial carcinoma, and breast cancer. The authors concluded that "*CLDN6 is a safe target for cell therapy in solid tumors and does not raise concerns for either combining CAR-T cells with CARVac or for exploring higher doses of CLDN6 CAR-T cells.*" (10).

As evident, the method, kit, device, and tubing of the present invention are also suitable for manufacturing the above described CLDN6 CAR-T cells from autologous blood samples easier, faster and in a closed system compared the method described in (10).

The same is, for example, also true for CAR-T cells using, for instance, drug target molecules such as IL13RA2 or EGFRv3 in glioblastoma patients, the disialoganglioside GD2 in neuroblastoma, H3K27M-mutated diffuse midline gliomas, prostate-specific membrane antigen in metastatic prostate cancer, or CLDN18.2 in
5 patients with gastrointestinal cancers (10).

The cellular therapy starting cell as mentioned herein can be a nucleated animal or human cell. The cellular therapy starting cell may be a human white blood cell, for example a T cell, a B cell, or a NK cell. If such cells are used as starting material for
10 the cellular therapy starting, these cells may be a non-activated T cell, a non-activated B cell or a non-activated NK cell. Thus, the method described here has the advantage that it does not actively/deliberately activates lymphocytes. This is in contrast to the conventional methods and protocols for gene modifying of lymphocytes such as T cells or B cells. For example, WO 2023/122277 A2 discloses a rapid method for producing
15 CAR T cells, wherein the used T cells are mandatory activated before gene modification. Also, Xin et al. did not mention in the actual review regarding CAR T cells the use of explicit non-activated T cells (see references 5, cited above). Notably, in the recent article from Mackensen et al. the use of non-activated T cells is also not mentioned (reference 10).

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The cellular therapy starting cell is obtained in a first step by enrichment via reversible immobilization from a sample comprising the cellular therapy starting cell. The sample can be any cell containing sample as long as the desired cellular therapy starting cell used in the method is comprised in the sample.

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Typically, the sample comprises a human bodily fluid. The sample may comprise nucleated blood cells, the sample can, for example, be a whole blood sample or the sample can comprise or consist of white blood cells.

30 The term "enrichment via reversible immobilization" as used herein means that the cellular therapy starting cell is immobilized during enrichment and that this immobilization is or can be reversed after the enrichment of the cellular therapy starting cell took place. Thus, the enrichment or hold step is carried out without prior cell separation, in particular without prior cell separation in a cell separation module

comprising a cell separator configured to produce a fraction enriched in nucleated blood cells.

In one embodiment of the invention enriching via reversible immobilization of the cellular therapy starting cell from the sample comprises

- providing the sample, the sample comprising the cellular therapy starting cell,
- providing a receptor binding reagent comprising a binding site B and a binding partner C,
- wherein the binding site B comprised in the receptor binding reagent is capable of specifically binding to the second cellular surface receptor on the cellular therapy starting cell and wherein the bond between the binding site B of the receptor binding reagent and the second cellular surface receptor has a dissociation constant (K_D) is of low affinity or in the range from about 10^{-3} to about 10^{-7} M,
- wherein the binding partner C comprised in the receptor binding reagent is capable of reversibly binding to a binding site Z of an affinity reagent and the reversible bond between the binding partner C of the receptor binding reagent and the binding site Z of the affinity reagent has a dissociation constant (K_D) in the range from about 10^{-2} to about 10^{-13} M, and
- exposing the sample to chromatography on a suitable solid phase, for example, floating particles or a stationary phase, the solid phase or the stationary phase having the affinity reagent immobilized thereon,
- wherein the affinity reagent comprises one or more binding sites Z, wherein said binding site Z forms a reversible bond with the binding partner C comprised in the receptor binding reagent, and wherein the binding site B of the receptor binding reagent binds to the second cellular surface receptor on the cellular therapy starting cell, thereby reversibly immobilizing the cellular therapy starting cell on the stationary phase,
- providing a competition reagent, the competition reagent comprising a binding site, specifically binding to the binding sites Z of the affinity reagent;
- loading the competition reagent onto the stationary phase, thereby allowing disruption of non-covalent reversible complexes formed between (a plurality of) the receptor binding reagent, the second cellular surface receptor and the affinity reagent;

- recovering an enrichment sample from the eluate of the stationary phase, wherein the enrichment sample comprises the cellular therapy starting cell.

Generally, a chromatographic method according to this and further embodiments is a
5 fluid chromatography, typically a liquid chromatography. The chromatography can be carried out in a flow through mode in which a fluid sample containing the cellular therapy starting cell to be enriched is applied, for example, by gravity flow or by a pump on one end of a column containing the chromatography matrix and in which the fluid sample exists the column at the other end of the column. The column as used herein
10 may also comprise a system or arrangement of columns. The column or at least one column of the system of columns may have one or more obstacle(s). The chromatography can also take place in a tubing or a part of the tubing wherein most of the conditions for the chromatography using the column applies. Alternatively, the chromatography can also be carried out in a batch mode in which the chromatography
15 material (stationary phase or solid phase) is incubated with the sample that contains the cellular therapy starting cell, for example, under shaking, rotating or repeated contacting and removal of the fluid sample. Any material may be employed as chromatography matrix in the context of the invention, as long as the material is suitable for the chromatographic isolation of cells. A suitable chromatography material
20 is at least essentially innocuous, i.e. not detrimental to cell viability (or the viability or stability of the biological entity), when used in a packed chromatography column under desired conditions for cell enrichment. A chromatography matrix as used in the present invention remains in a predefined location, typically in a predefined position, whereas the location of the sample to be enriched and of components included therein, is being
25 altered. Thus, the chromatography matrix is a "stationary phase" in line with the regular understanding of the person skilled in the art that the stationary phase is the part of a chromatographic system through which the mobile phase flows (either by flow through or in a batch mode) and where distribution of the components contained in the liquid phase (either dissolved or dispersed) between the phases occurs. The term "solid
30 phase" means particles which are free floating in e.g. a bag comprising the sample. The terms "chromatography matrix", "solid phase", and "stationary phase" are thus used interchangeable herein.

An example of the "solid phase" are particles such as freely movable beads that are added to a liquid sample, mixed with the sample and are then removed from the sample. A method in which magnetic beads are added to a sample containing the cellular therapy starting cells for immobilization of the cellular therapy starting cells (via
5 a complex formed between the cellular therapy starting cells, the receptor binding reagent and the affinity/multimerization reagent) on such magnetic beads, and the beads are then separated from the sample, for example by temporarily holding the beads in place, while discarding the supernatant, is not a method of the invention. Thus, the use of magnetic beads is not considered in the invention.

10

Typically, the respective chromatography matrix has the form of a solid or semisolid phase, whereas the sample that contains the cellular therapy starting cell to be enriched is a fluid phase. The mobile phase used to achieve chromatographic separation is likewise a fluid phase. The chromatography matrix can be a particulate
15 material (of any suitable size and shape) or a monolithic chromatography material, including a paper substrate or membrane. Thus, the chromatography can be both column chromatography as well as planar chromatography. In addition to standard chromatography columns, columns allowing a bidirectional flow such as PhyTip® columns available from PhyNexus, Inc. San Jose, CA, U.S.A. can also be used for
20 column based/flow through mode based chromatographic separation of cells as described here. Columns generating a non-laminar flow may also be advantageously used. Thus, columns allowing a bidirectional flow and/or a non-laminar flow are also encompassed by the term "chromatography columns" as used herein. If a particulate matrix material is used, the particulate matrix material may, for example, have a mean
25 particle size of about 5 μm to about 200 μm , or from about 5 μm to about 400 μm , or from about 5 μm to about 600 μm . As explained in detail in the following, the chromatography matrix may, for example, be or include a polymeric resin or a metal oxide or a metalloid oxide. If planar chromatography is used, the matrix material may be any material suitable for planar chromatography, such as conventional cellulose-
30 based or organic polymer-based membranes (for example, a paper membrane, a nitrocellulose membrane or a polyvinylidene difluoride (PVDF) membrane) or silica coated glass plates. Throughout the invention the chromatography matrix/stationary phase is a non-magnetic material or non-magnetizable material.

The non-magnetic or non-magnetizable chromatography stationary phase includes a plastic tubing. Further non-magnetic or non-magnetizable chromatography stationary phases that are used in the art, and that are also suitable in the present invention, include derivatized silica or a crosslinked gel. A crosslinked gel (which is typically
5 manufactured in a bead form) may be based on a natural polymer, i.e. on a polymer class that occurs in nature. For example, a natural polymer on which a chromatography stationary phase is based is a polysaccharide. A respective polysaccharide is generally crosslinked. An example of a polysaccharide matrix is an agarose gel (for example, SuperflowTM agarose or a Sepharose[®] material such as SuperflowTM Sepharose[®]
10 that are commercially available in different bead and pore sizes) or a gel of crosslinked dextran(s). A further illustrative example is a particulate cross-linked agarose matrix, to which dextran is covalently bonded, that is commercially available (in various bead sizes and with various pore sizes) as Sephadex[®] or Superdex[®], both available from GE Healthcare. Another illustrative example of such a chromatography material is
15 Sephacryl[®] which is also available in different bead and pore sizes from GE Healthcare.

A crosslinked gel may also be based on a synthetic polymer, i.e. on a polymer class that does not occur in nature. Usually such a synthetic polymer on which a
20 chromatography stationary phase for cell separation is based is a polymer that has polar monomer units, and which is therefore in itself polar. Such a polar polymer is hydrophilic. Hydrophilic ("water-loving") molecules, also termed lipophobic ("fat hating"), contain moieties that can form dipole-dipole interactions with water molecules. Hydrophobic ("water hating") molecules, also termed lipophilic, have a tendency to
25 separate from water.

Illustrative examples of suitable synthetic polymers are polyacrylamide(s), a styrene-divinylbenzene gel and a copolymer of an acrylate and a diol or of an acrylamide and a diol. An illustrative example is a polymethacrylate gel, commercially available as a
30 Fractogel[®]. A further example is a copolymer of ethylene glycol and methacrylate, commercially available as a Toyopearl[®]. In some embodiments a chromatography stationary phase or solid phase may also include natural and synthetic polymer components, such as a composite matrix or a composite or a co-polymer of a polysaccharide and agarose, e.g. a polyacrylamide/agarose composite, or of a

polysaccharide and N,N'-methylenebisacrylamide. An illustrative example of a copolymer of a dextran and N,N'-methylenebisacrylamide is the above-mentioned Sephacryl® series of material. A derivatized silica may include silica particles that are coupled to a synthetic or to a natural polymer. Examples of such embodiments include, but are not limited to, polysaccharide grafted silica, polyvinylpyrrolidone grafted silica, polyethylene oxide grafted silica, poly(2-hydroxyethylaspartamide) silica and poly(N-isopropylacrylamide) grafted silica. Also, polystyrene can be used as a solid phase.

In some embodiments of the enrichment via reversible immobilization of the cellular therapy starting cell, a chromatography matrix is employed as an affinity chromatography matrix. An affinity chromatography matrix itself includes permanently bonded (usually covalently bonded) moieties that are capable to specifically bind a selected target. For example, a conventional affinity chromatography matrix may include an antibody that binds a particular given target. In the art an affinity chromatography matrix is generally designed such that itself is able to specifically bind the analyte or target that is to be enriched. In the present invention, the affinity chromatography matrix itself is not designed to be capable of specifically binding the cellular therapy starting cell that is to be enriched. Rather, in such embodiments the affinity chromatography matrix (stationary or solid phase) used in the present invention comprises an affinity reagent that has at least one or more binding sites Z that are able to specifically bind to a receptor binding reagent that is also employed in the present invention. When the receptor binding reagent is brought into contact with the affinity reagent, a reversible complex via the binding partner C of the receptor binding reagent and the one or more binding sites Z of the affinity reagent is formed. Thus, this complex formation relies on non-covalent interactions between a ligand and its respective binding partner. It is usually sufficient that the affinity reagent contains one binding site Z that is able to form a reversible bond with the binding partner C, as long as the affinity reagent is present/provided on the affinity chromatography matrix in a sufficiently high surface density to cause an avidity effect when the complex between the receptor binding reagent and the affinity reagent is formed via the binding site and the binding partner C. However, it is also possible that the affinity reagent comprises two or more binding sites Z for the binding partner C. In the then non-covalent binding complex formed, two or more receptor binding reagents are immobilized on the affinity chromatography matrix closely arranged to each other such that an avidity effect can

take place if the cellular therapy starting cell having (at least two copies of) a second cellular surface receptor present in the sample, is brought into contact with the receptor binding reagent that have one or more binding sites B being able to bind the particular second cellular surface receptor. Thus, in these embodiments an avidity
5 (multimerization) effect similar to the one described in US patent 7,776,562, US patent 8,298,782 or International Patent application WO02/054065 can take place for allowing a reversible immobilization of the cellular therapy starting cell on the affinity chromatography matrix. Since the bond between the binding sites Z of the affinity reagent (that then may also act as multimerization agent) and the binding partner C of
10 the receptor binding reagent can be disrupted by addition of a competition agent, the cellular therapy starting cell can be subsequently eluted under mild conditions under which the receptor binding reagent completely dissociates from the cellular therapy starting cell, thereby avoiding that the receptor binding reagent affects the functional status of the cellular therapy starting cell. This enrichment of the cellular therapy
15 starting cell via this affinity chromatography method thus does not only have the advantage that it allows for the enrichment of cellular therapy starting cell population (or any other biological entity described herein) without altering the functional status of the cellular therapy starting cell population that is defined by a common specific receptor molecule. Rather, this method also has the added advantage that it entirely
20 abolishes the need to use magnetic beads for cell purification and thereby simplifies any further handling of the cell and opens the way to automatization of the isolation of cellular therapy starting cells.

In other embodiments of a method according to the invention a chromatography matrix
25 is used that has an affinity reagent immobilized thereon. The affinity reagent is able to bind a binding partner C that is included in a receptor binding reagent (see below). Such a chromatography matrix may be an affinity chromatography matrix. It may also be a gel filtration matrix, to which the affinity reagent has been coupled. The chromatography matrix is in some embodiments included in a chromatography column,
30 for example packed therein. By means of the immobilized affinity reagent the chromatography matrix can deplete a mobile phase of the receptor binding reagent. A sample that is contacted with the chromatography matrix, for example, loaded onto a column packed therewith, can likewise be depleted of the receptor binding reagent. In one method according to the invention the receptor binding reagent is included in a

sample that is contacted with a respective stationary phase, i.e. chromatography matrix.

After applying the sample containing the cellular therapy starting cell, the chromatography matrix (regardless of being used for affinity chromatography or for gel permeation) may subsequently be washed with a mobile phase, such as an aqueous medium, e.g. a buffer, in order to remove any matter that has not been immobilized on the chromatography matrix. Dissociation of the above described non-covalent complex, the formation of which immobilizes the cellular therapy starting cell on the affinity chromatography matrix, may then be induced, for example, by a change in conditions. Such a change in conditions may for instance be a change in the ionic strength of an aqueous mobile phase or a change in temperature. In some embodiments a competition reagent is employed in order to induce dissociation of the reversible non-covalent complex between receptor, receptor binding reagent and affinity reagent. The competition reagent is able to associate to the affinity reagent by occupying or blocking the binding site of the affinity reagent for the binding partner included in the receptor binding reagent. By using a competition reagent with a particularly high affinity for the affinity reagent or by using an excess of the competition reagent relative to at least one of the cellular therapy starting cell and the receptor binding reagent (in this case, the competition reagent might also have a lower affinity to the binding site Z of the affinity reagent than the binding partner C of the receptor binding reagent) the non-covalent bonding between the receptor binding reagent and the multimerization reagent may be disrupted. The cellular therapy starting cell is allowed to elute from the chromatography matrix, e.g. from the column into which the chromatography matrix is packed. The eluate is collected and the cellular therapy starting cell thereby collected.

The fluid phase used as the mobile phase in chromatography may be any fluid suitable for preserving the biological activity of the cellular therapy starting cell. Typically, the fluid is a liquid. In some embodiments the respective liquid is or includes water, for example in the form of an aqueous solution. Further components may be included in a respective aqueous solution, for example dissolved or suspended therein. As an illustrative example an aqueous solution may include one or more buffer compounds. Numerous buffer compounds are used in the art and may be used to carry out the

various processes described herein. Illustrative examples of buffers include, but are not limited to, solutions of salts of phosphate such as phosphate buffered saline (PBS), carbonate, succinate, carbonate, citrate, acetate, formate, barbiturate, oxalate, lactate, phthalate, maleate, cacodylate, borate, cell culture medium, N-(2-acetamido)-2-aminoethanesulfonate (also called (ACES), N-(2-hydroxyethyl)-piperazine-N'-2-ethanesulfonic acid (also called HEPES), 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid (also called HEPPS), lactated ringers solution, piperazine-1,4-bis(2-ethanesulfonic acid) (also called PIPES), (2-[Tris(hydroxymethyl)-methylamino]-1-ethansulfonic acid (also called TES), 2-cyclohexylamino-ethanesulfonic acid (also called CHES) and N-(2-acetamido)-iminodiacetate (also called ADA). Any counter ion may be used in these salts; ammonium, sodium, and potassium may serve as illustrative examples. Further examples of buffers include, but are not limited to, triethanolamine, diethanolamine, zwitterionic buffers such as betaine, ethylamine, triethylamine, glycine, glycyglycine, histidine, tris-(hydroxymethyl)aminomethane (also called TRIS), bis-(2-hydroxyethyl)-iminotris(hydroxymethyl)-methane (also called BIS-TRIS), and N-[Tris(hydroxymethyl)-methyl]-glycine (also called TRICINE), to name only a few. The buffer may further include components that stabilize the cellular therapy starting cell to be enriched, for example proteins such as (serum) albumin, growth factors, trace elements and the like. The choice of the suitable mobile phase is within the knowledge of the person of average skill in the art and can be carried out empirically.

The strength of the binding between the receptor binding reagent and a second cellular surface receptor on the cellular therapy starting cell may not be essential for the reversibility of the binding of the cellular therapy starting cell to the affinity reagent via the receptor binding reagent. Rather, irrespective of the strength of the binding, meaning whether the dissociation constant (K_d) for the binding between the receptor binding reagent via the binding site B and the second cellular surface receptor is of low affinity, for example, in the range of a K_d of about 10^{-3} to about 10^{-7} M, or of high affinity, for example, in the range of a K_d of about 10^{-7} to about 1×10^{-10} M, the cellular therapy starting cell can be reversibly stained as long as the dissociation of the binding of the receptor binding reagent via the binding site B and the second cellular surface receptor occurs sufficiently fast. In this regard the dissociation rate constant (k_{off}) for the binding between the receptor binding reagent via the binding site B and the second

cellular surface receptor may have a value of about $3 \times 10^{-5} \text{ sec}^{-1}$ or greater (this dissociation rate constant is the constant characterizing the dissociation reaction of the complex formed between the binding site B of the receptor binding reagent and the second cellular surface receptor on the surface of the cellular therapy starting cell).

5 The association rate constant (k_{on}) for the association reaction between the binding site B of the receptor binding reagent and the second cellular surface receptor on the cellular therapy starting cell may have any value. In order to ensure a sufficiently reversible binding between second cellular surface receptor and receptor binding reagent it is advantageous to select the k_{off} value of the binding equilibrium to have a

10 value of about $3 \times 10^{-5} \text{ sec}^{-1}$ or greater, of about $5 \times 10^{-5} \text{ sec}^{-1}$ or greater, such as about $1 \times 10^{-4} \text{ sec}^{-1}$ or greater, about $1.5 \times 10^{-4} \text{ sec}^{-1}$ or greater.

The second cellular surface receptor that is located on the cellular therapy product starting cell surface (or an accessible surface of a biological entity) may be any

15 molecule as long as it remains covalently or noncovalently bonded to the cell surface during a chromatographic separation process in a method according to the invention. The second cellular surface receptor is a molecule against to which a receptor binding reagent may be directed. In some embodiments the second cellular surface receptor is a peptide or a protein, such as a membrane receptor protein. In some embodiments

20 the second cellular surface receptor is a lipid, a polysaccharide or a nucleic acid. A second cellular surface receptor that is a protein may be a peripheral membrane protein or an integral membrane protein. It may in some embodiments have one or more domains that span the membrane.

25 In some embodiments the second cellular surface receptor may be an antigen defining a desired cell population or subpopulation, for instance a population or subpopulation of blood cells, e.g. lymphocytes (e.g. T cells, T-helper cells, for example, CD4+ T-helper cells, B cells or natural killer cells), monocytes, or stem cells, e. g. CD34-positive peripheral stem cells or Nanog or Oct-4 expressing stem cells.

30 Examples of T-cells include cells such as CMV-specific CD8+ T-lymphocytes, cytotoxic T-cells, memory T-cells and regulatory T-cells (Treg). An illustrative example of Treg are CD4+CD25+CD45RA Treg cells and an illustrative example of memory T-cells are

CD62L+CD8+ specific central memory T-cells. The receptor may also be a marker for a tumor cell.

Typical second cellular surface receptors used herein are CD3, CD4, CD8 (T-cell),
5 CD45 (Leukocytes), CD56 (NK cell), CD27 (B and T-Cell), CD28 (B and T-Cell), CD19 (B-cell), CD62L, and CD25 (T cell).

In some embodiments the binding partner C that is included in the receptor binding reagent includes biotin and the affinity reagent includes a streptavidin analog or an
10 avidin analog that reversibly binds to biotin. In some embodiments the binding partner C that is included in the receptor binding reagent includes a biotin analog that reversibly binds to streptavidin or avidin, and the affinity reagent includes streptavidin, avidin, a streptavidin analog or an avidin analog that reversibly binds to the respective biotin analog. In some embodiments the binding partner C that is included in the receptor
15 binding reagent includes a streptavidin or avidin binding peptide and the affinity reagent includes streptavidin, avidin, a streptavidin analog or an avidin analog that reversibly binds to the respective streptavidin or avidin binding peptide. In some embodiments the binding partner that is included in the receptor binding reagent may include a streptavidin-binding peptide Trp-Ser-His-Pro-Gln-Phe-Glu-Lys (SEQ ID NO.
20 10) and the affinity reagent may include a streptavidin mutein having the amino acid sequence Val⁴⁴-Thr⁴⁵-Ala⁴⁶-Arg⁴⁷ (SEQ ID NO. 7) at sequence positions 44 to 47 of the wildtype streptavidin sequence (as set forth in SEQ ID NO: 9) or a streptavidin mutein having the amino acid sequence Ile⁴⁴-Gly⁴⁵-Ala⁴⁶-Arg⁴⁷ (SEQ ID NO. 8), at sequence positions 44 to 47 of the wildtype streptavidin sequence (as set forth in SEQ ID NO:
25 9). Such muteins are, for example, described as mutein "1" and "2" in US patent 6,103,493 and are commercially available from IBA Lifesciences GmbH, Göttingen, Germany under the trademark StrepTactin®. The streptavidin binding peptides might, for example, be peptides such as the "Strep-tag®" with one binding module as described in US patent 5,506,121, for example, or streptavidin binding peptides having
30 a sequential arrangement of two or more individual binding modules as described in International Patent Publication WO 02/077018 or US patent 7,981,632. Such streptavidin binding peptides having a sequential arrangement of two individual binding modules are also offered under the trademark Twin-Strep-tag® by IBA Lifesciences GmbH, Göttingen, Germany.

Further suitable streptavidin muteins that can be used in the present invention are described in International patent applications WO 2014/076277 A1 or WO 2017/186669 A1. Such streptavidin muteins are commercially available from IBA
5 Lifesciences GmbH Göttingen under the name Strep-Tactin® XT. The streptavidin muteins of WO 2017/186669 may (a) have a Cys residue at sequence position 127 with reference to the amino acid sequence of wild-type streptavidin (as set forth in SEQ ID NO: 9) and (b) comprises at least one mutation in the region of the amino acid positions 115 to 121 with reference to the amino acid sequence of wild type streptavidin
10 as set forth in SEQ ID NO: 9. These muteins could additionally have one of the above mentioned mutations SEQ ID NO. 7 or 8.

In some embodiments the binding partner C comprised in the receptor binding reagent comprises an affinity tag, and wherein the affinity reagent comprises a suitable binding
15 partner for the affinity tag, which may be an antibody or an antibody fragment, which is capable of binding to the affinity tag.

Non-limiting examples of such affinity tags are an oligohistidine, a polyhistidine, an immunoglobulin domain, a maltose-binding protein, a glutathione-S-transferase (GST),
20 a chitin binding protein (CBP), a thioredoxin, a calmodulin binding peptide (CBP), a FLAG'-peptide, a HA-tag, a VSV-G-tag, and a HSV-tag. Further suitable affinity tags are known by the skilled person.

In some embodiments the affinity reagent is an oligomer or a polymer of streptavidin
25 or avidin or of any analog of streptavidin or avidin. The binding site Z is the natural biotin binding of avidin or streptavidin. The respective oligomer or polymer may be crosslinked by a polysaccharide. In one embodiment oligomers or polymers of streptavidin or of avidin or of analogs of streptavidin or of avidin may be prepared by the introduction of carboxyl residues into a polysaccharide, e. g. dextran, essentially
30 as described in Noguchi, A, et al., (11) in a first step. Then streptavidin or avidin or analogs thereof may be linked via primary amino groups of internal lysine residue and/or the free N-terminus to the carboxyl groups in the dextran backbone using conventional carbodiimide chemistry in a second step. Nevertheless, cross-linked oligomers or polymers of streptavidin or avidin or of any analog of streptavidin or avidin

may also be obtained by crosslinking via bifunctional molecules, serving as a linker, such as glutardialdehyde or by other methods described in the art.

In the method of the invention the one or more binding sites of the receptor molecule
5 binding reagent, which specifically binds to the second cellular surface receptor, may
for instance be an antibody, a fragment thereof and a proteinaceous binding molecule
with antibody-like functions. Examples of (recombinant) antibody fragments are Fab
fragments, Fv fragments, 5 single-chain Fv fragments (scFv), a divalent antibody
fragment such as an (Fab)₂'-fragment, nanobodies, diabodies, triabodies (12),
10 decabodies (13) and other domain antibodies (14). In some embodiments one or more
binding sites of the receptor molecule binding reagent may be a bivalent proteinaceous
artificial binding molecule such as a dimeric lipocalin mutein that is also known as
"duocalin". In some embodiments the receptor binding reagent may have a single
second binding site, i.e., it may be monovalent. Examples of monovalent receptor
15 binding reagents include, but are not limited to, a monovalent antibody fragment, a
proteinaceous binding molecule with antibody like binding properties or an MHC
molecule. Examples of monovalent antibody fragments include, but are not limited to
a Fab fragment, a Fv fragment, and a single-chain Fv fragment (scFv), including a
divalent single-chain Fv fragment.

20

Especially preferred are an immunoglobulin, a functional fragment of an
immunoglobulin, a proteinaceous binding molecule with immunoglobulin-like functions,
an aptamer and an MHC molecule as receptor binding reagent.

25 In some embodiments of the invention the reversible immobilization is carried out via
two or more types of the second cellular surface receptor, for example, by sequential
immobilization via the cell surface receptor CD3 as first type of second cellular surface
receptor and CD4 as second type of second cell surface receptor.

30 In further embodiments enriching via reversible immobilization of the cellular therapy
starting cell is carried out as single enrichment step, without prior cell separation, in
particular without prior cell separation in a cell separation module comprising a cell
separator configured to produce a fraction enriched in nucleated blood cells.

In some embodiments the affinity matrix is bound/covalently coupled to beads and the beads are incubated with the sample comprising the cellular therapy starting cell in a reservoir in which the beads are moved. Such a reservoir may be a column capped on both sides, which is incubated on a shaker. The advantage of such a reservoir is that
5 the beads are movable within the reservoir and more cells could be bound by the receptor binding reagent via the second cellular surface receptor.

In some embodiments the affinity reagent is immobilized on the inner surface of the tubing, or a portion thereof, by (means of) a covalent bond, whereby this part of the
10 tubing may be columnar extended. Such a portion is advantage as it could be used like a known column but within the closed system. Furthermore, higher purity of enriched cellular therapy starting cells may be achieved compared to other methods.

In a second step of the inventive method the cellular therapy starting cell is genetically
15 modified using one or more reagent(s) comprising a gene construct for expression of the first cellular surface receptor and/or for expression and/or secretion of the polypeptide, thereby generating a precursor of the cellular therapy product.

This means that the enriched cellular therapy starting cell (see above) is genetically
20 modified, such that the cell after the gene modification is capable of expressing the first cellular surface receptor and/or for expression and/or secretion of the polypeptide. After the gene modification the cell is termed "precursor of the cellular therapy product" or "precursor cell of the cellular therapy product", because directly after gene modification the cell does not express the modified gene. Only after some time the cell
25 expresses the modified gene and so the precursor of the cellular therapy product changes to the cellular therapy product, the cell expressing the first cellular surface receptor and/or the polypeptide. Thus, the used cell has three different terms depending on the process step of the inventive method.

30 In several embodiments the one or more reagent(s) used for the gene modification is a set of reagents for gene modification. The set of reagents comprise a viral gene delivering system or a non-viral gene delivering system.

The viral gene delivering system can be any suitable viral gene delivering system known to the skilled person. Typical but not limiting examples of such viral gene delivering system are systems which use a retroviral transduction, a lentiviral transduction, or an adenoviral transduction. Such systems are commercially available.

5

The non- viral gene delivering system can be any suitable non-viral gene delivering system known to the skilled person. Typical but non-limiting examples of such non-viral gene delivering system are electroporation, lipid nanoparticles, mechanoporation, ultrasound treatment, optical gene delivery, exosomes, amphiphilic peptides or a combination thereof. Especially preferred is the use of lipid nanoparticles, which is shown in Example 2.

10

In some embodiments the set of reagents comprises nucleic acids coding for proteins which facilitate the gene transfer, e.g. nucleases. Additional or instead of the nucleic acid the proteins themselves can be part of the set of reagents. Typical non-limiting examples are a nucleic acid encoding a Transcription activator-like effector nuclease (TALEN), a Zinc-finger nuclease (ZFN), CRISPR and Cas, or transposases such as sleeping beauty, piggyback, Tn5 transposases. Exemplary proteins are a Transcription activator-like effector nuclease (TALEN), a Zinc-finger nuclease (ZFN), Cas.

15

20

In some embodiments the gene construct as part of the set of reagents comprising a nucleic acid encoding the first cellular surface receptor and/or the polypeptide, wherein the nucleic acid can be DNA or RNA. Throughout the present application the term "DNA" includes any possible type of DNA, e.g. single stranded DNA, double stranded DNA. The term "RNA" includes any possible type of RNA.

25

In one preferred embodiment the gene construct comprises a nucleic acid encoding a chimeric antigen receptor (CAR), or a recombinant T cell receptor (TCR), and/or switch receptors or a bi-specific T cell engager (Bite), which is able to bind to the drug target molecule. It is known to the skilled person how to construct the chimeric antigen receptor (CAR) and the recombinant T cell receptor.

30

In a further preferred embodiment, the gene construct comprises a nucleic acid encoding the polypeptide, which is able to bind to a drug target molecule either soluble or associated with a drug target cell.

- 5 In some embodiments the gene modification takes place after eluting the cellular therapy starting cell from the stationary phase. This is exemplified in Example 2.

Alternatively, in some embodiments the gene modification takes place before eluting the cellular therapy starting cell from the stationary or solid phase. Also, this is
10 exemplified in Example 2.

Thus, it is possible to gene modify the cell while it is reversible immobilized on the affinity matrix. After the gene modification the cell, now the precursor of the cellular therapy product can be eluted from the affinity matrix.
15

In one preferred embodiment the sample is a whole blood sample, the cellular therapy starting cell is a non-activated T cell and the set of reagents comprises a non-viral gene delivering system, a nucleic acid and/or protein encoding the CRISPR and Cas, and a nucleic acid encoding a chimeric antigen receptor (CAR) or a TCR, which is able to
20 bind to the drug target molecule.

In a further preferred embodiment, the sample is a whole blood sample, the cellular therapy starting cell is an activated or a non-activated B cell and the set of reagents comprises a non-viral gene delivering system, a nucleic acid encoding the CRISPR and Cas, and a nucleic acid encoding the polypeptide.
25

In a preferred embodiment, the sample is a whole blood sample, the cellular therapy starting cell is an activated or a non-activated T cell, B cell or NK cell and the set of reagents comprises a non-viral gene delivering system comprising a lipid nanoparticle.
30

In a second aspect, the invention is directed to a use of a set of reagents for genetically modifying a cellular therapy starting cell as mentioned before and included herein by reference. During the genetic modification or the use of the set of reagents for genetic modification the cellular therapy starting cell is immobilized on a stationary phase or a

receptor binding reagent as described in the first aspect. The precursor of the cellular therapy product is eluted from the stationary phase or the receptor binding reagent after the genetic modification.

- 5 In a third aspect, the invention is directed to a kit for manufacturing a cellular therapy product, wherein the cellular therapy product comprises a first cellular surface receptor which is able to bind to a drug target molecule (on the surface) of a drug target cell, and/or wherein the cellular therapy product produces and/or secretes a polypeptide which is able to bind to a drug target molecule either soluble or associated with a drug
- 10 target cell, the kit comprising
- a) one or more reagent(s) comprising a gene construct for expression of the first cellular surface receptor or of the polypeptide of the cell therapy product, and
 - b) a receptor binding reagent comprising a binding site B and a binding partner C,
- 15 - wherein the binding site B comprised in the receptor binding reagent is capable of specifically binding to the second cellular surface receptor on a cellular therapy starting cell and wherein the bond between the binding site B of the receptor binding reagent and the second cellular surface receptor has a dissociation constant (K_D) is of low affinity or in the range from about 10^{-3} to
- 20 about 10^{-7} M,
- wherein the binding partner C comprised in the receptor binding reagent is capable of reversibly binding to a binding site Z of an affinity reagent and the reversible bond between the binding partner C of the receptor binding reagent and the binding site Z of the affinity reagent has a dissociation constant (K_D) in
- 25 the range from about 10^{-2} to about 10^{-13} M, and
- a stationary phase, the stationary phase having the affinity reagent immobilized thereon,
 - wherein the affinity reagent comprises one or more binding sites Z, wherein said binding site Z forms a reversible bond with the binding partner C
- 30 comprised in the receptor binding reagent, and wherein the binding site B of the receptor binding reagent binds to the second cellular surface receptor on the cellular therapy starting cell, to allow reversibly immobilizing the cell therapy starting cell on the stationary phase.

The one or more reagent(s) comprising a gene construct for expression of the first cellular surface receptor or of the polypeptide of the cell therapy product are described above in the method section; this description is incorporated herein by reference.

- 5 The receptor binding reagent, the stationary phase, the solid phase and the affinity reagent are as described above in the method section; this description is incorporated herein by reference.

- 10 In some embodiments the one or more reagent(s) comprise a viral gene delivering system or a non-viral gene delivering system, a nucleic acid part encoding a Transcription activator-like effector nuclease (TALEN), a Zinc-finger nuclease (ZFN), or CRISPR and Cas, and a gene construct comprising a nucleic acid encoding the first cellular surface receptor or the polypeptide. These reagents are described in more detail above; this description fully applies to the embodiments described in this paragraph.
- 15

In some embodiments the cellular therapy starting cell can typically be a T cell, a B cell, or a NK cell and may be a non-activated T cell or a non-activated B cell.

- 20 In some embodiments the gene construct of the kit comprises a nucleic acid encoding a chimeric antigen receptor (CAR) or TCR, which is able to bind to the drug target molecule. This is also described above in more detail; this description fully applies to the embodiments described in this paragraph.
- 25 In some embodiments the gene construct of the kit comprises a nucleic acid encoding the polypeptide, which is able to bind to a drug target molecule either soluble or associated with a drug target cell. This is also described above in more detail; this description fully applies to the embodiments described in this paragraph.
- 30 In some embodiments the drug target cell can be a diseased cell, e.g. a cancer cell or a cell affected by an autoimmune disease. Also, cells relating to an infectious disease are possible as the drug target cell. This is described in more detail above; this description fully applies to the embodiments described in this paragraph.

In some embodiments the viral gene delivering system of the kit comprises a retroviral transduction system or a lentiviral transduction system. These systems are known to the skilled person and described above; this description fully applies to the embodiments described in this paragraph.

5

In some embodiments the non-viral gene delivering system of the system comprises an electroporation, a lipid nanoparticle, mechanoporation, ultrasound treatment, optical gene delivery, exosomes, amphiphilic peptides or a combination thereof. These systems are known to the skilled person and described above; this description fully applies to the embodiments described in this paragraph.

10

In a preferred embodiment the kit is used in the method described above.

In a fourth aspect the invention is directed to a device for manufacturing a cellular therapy product, comprising an operating device and a tubing-set, wherein the tubing set is a closed and single-use tubing-set, for use in the methods described above (the description is incorporated herein by reference). The term "tubing" and "tubing set" includes the term "cassette".

15

It is to mention that the tubing set is a closed system within the above definition, which fully applies to the embodiments described in this paragraph.

20

A schematic drawing of an example of the device is depicted in **Figure 9**.

In one embodiment the operating device comprises a liquid handling control unit comprising one or more valve(s), a liquid pump, a reservoir holder, and optionally a reservoir rocker. The operating device can further comprise a process unit, for operating process steps, comprising blood collection, second target cell hold-step (cell enrichment), second target cell release/elution, second target cell wash, genetic modification, precursor cell of a cellular therapy product wash and formulation, reinfusion of the precursor cell of a cellular therapy product by optionally controlling the valve and optionally comprising means for input of commands relating to the process steps and means for output of an information relating to the process steps. Thus, the

30

operating device may include every possible part for automatic execution of the inventive method and the above-mentioned process steps, excluding the tubing set.

5 In one embodiment the tubing-set comprises a system for blood withdrawal, a system for reinfusion of cells into the human body, a second target cell retention reservoir, a genetic modification unit, a second target cell washing and formulation unit, a blood reservoir, one or more bag containing process buffers, a bag containing a set of reagents for gene modification as describe above in the method and kit sections (which are incorporated herein by reference), a waste reservoir and a re-infusion reservoir,
10 wherein the reservoirs and bags are fluidly connected by a closed tube system. The closed tube system is a closed system within the above definition. In the case that the gene modification of the cellular therapy starting cell takes place before eluting the cellular therapy starting cell from the stationary phase, the second target cell retention reservoir and the genetic modification unit share the same space. Thus, the second
15 target cell retention reservoir may be a column or a part of a tubing as described elsewhere herein and the genetic modification takes place within the second target cell retention reservoir turning it to the genetic modification unit while being the same column or tubing part.

20 In one embodiment the tubing set of the device is construed as follows:

- the system for blood withdrawal is fluidly connected to the blood reservoir,
- the blood reservoir is fluidly connected to the second target cell retention reservoir,
- the second target cell retention reservoir is fluidly connected to the bag containing process buffers and optionally to the waste reservoir,
- 25 • the second target cell retention reservoir is in a first alternative further fluidly connected with the genetic modification unit, in a second alternative, in the case that the gene modification of the cellular therapy starting cell takes place before eluting the cellular therapy starting cell from the stationary phase, the genetic modification unit is part of the second target cell retention unit in that both units share the same
30 space as described above,
- the genetic modification unit or, in the case that the gene modification of the cellular therapy starting cell takes place before eluting the cellular therapy starting cell from the stationary phase the second target cell retention unit is fluidly connected to the

bag containing the set of reagents for gene modification or the gene therapy product, to the bag containing process buffers and optionally to the waste reservoir,

- the genetic modification unit or, in the case that the gene modification of the cellular therapy starting cell takes place before eluting the cellular therapy starting cell from the stationary phase, the second target cell retention unit is further fluidly connected to the second target cell washing and formulation unit,
- the second target cell washing and formulation unit is fluidly connected to the bag containing process buffers and to the waste reservoir,
- the second target cell washing and formulation unit is further fluidly connected to re-infusion reservoir,
- the re-infusion reservoir is fluidly connected to the system for reinfusion of cells into the human body,
- wherein the fluidly connections comprise tubes and valves, which could be controlled by the process unit.

In a further embodiment of the device the second target cell retention reservoir can be a tubular expansion of the fluidly connection.

In some embodiments an inner side of the second target cell retention reservoir can be a stationary phase as specified and further described above in the method section, this description is incorporated herein by reference.

In a further embodiment the second target cell retention reservoir may comprise one or more sieve(s), for example to hold back particles comprising the affinity matrix. The second target cell retention reservoir may also comprise obstacles such as one or more pins or meshes.

In a fifth aspect the invention is directed to a tubing, which equals to tubing set, having an affinity reagent immobilized on an inner surface of the tubing, wherein the affinity reagent comprises one or more binding sites Z, wherein said binding site Z forms a reversible bond with a binding partner C comprised in the receptor binding reagent, and wherein the binding site B of the receptor binding reagent binds to a (second) cellular surface receptor on a cellular therapy starting cell, thereby allowing reversibly immobilizing the cellular therapy starting cell on the tubing acting as stationary phase.

The meaning and examples relating to the affinity reagent, the binding partner C, the receptor binding reagent, and the binding site B are described in detail in the method section above and fully apply to the embodiments described in this paragraph.

5

In one embodiment the affinity reagent can be immobilized on the inner surface of the tubing by a covalent bond.

In one preferred embodiment the affinity reagent is an avidin, a streptavidin or a streptavidin mutein. These affinity reagents are described above in the method section in detail fully applies to the embodiments described in this paragraph.

In one further preferred embodiment the streptavidin mutein may include the amino acid sequences Val⁴⁴-Thr⁴⁵-Ala⁴⁶-Arg⁴⁷ (SEQ ID NO. 7) or Ile⁴⁴-Gly⁴⁵-Ala⁴⁶-Arg⁴⁷ (SEQ ID NO. 8), and/or a Cys residue at sequence position 127 with reference to the amino acid sequence of wild-type streptavidin (as set forth in SEQ ID NO: 9) and/or (b) may comprise at least one mutation in the region of the amino acid positions 115 to 121 with reference to the amino acid sequence of wild type streptavidin as set forth in SEQ ID NO: 9. Examples of such mutein include the muteins m1-9 or m302 as described in WO 2014/076277.

20

In a sixth aspect, the invention is directed to a use of the tubing of the fifth aspect in a method of manufacturing a cellular therapy product as described above and the disclosure of which fully applies to the embodiments described in this paragraph.

25

The term “a” or “an” or the singular of features like cellular therapy product starting cell means the singular and the plural.

Experimental Examples

30

The present examples use the inventive method of manufacturing a cellular therapy product and show the efficient tumor killing in a mouse model.

Example 1: Cellular therapy starting cell enrichment

For enrichment of CD3+ cells or B cells, the cellular therapy starting cells, from a whole blood sample these cells were selective and reversible immobilized.

5 *Production of the anti-CD3 affinity matrix for reversible immobilization of cells*

For the enrichment of CD3+ cells via reversible immobilization an anti-CD3 affinity matrix was used. The matrix was composed as following (calculated on 20mL matrix): 10mg of multimerized streptavidin mutein (Strep-Tactin® m2 (as described in US 6,103,493), SEQ Id. No. 8) was coupled to 8g initial dry weight epoxy-activated
10 polystyrene resin (CY17030; Cytosorbents) using solvent-exposed primary amine groups, in phosphate buffered saline (PBS) buffer. Afterwards 1.8mg of cellular therapy starting cell selection agent with an anti-CD3 Fab fragment and a streptavidin-binding peptide (Twin-Strep-tag®, Twin-Strep-tag® streptavidin-binding peptide including a sequential arrangement of two streptavidin-binding modules) carboxyl-terminally fused
15 to the heavy chain of the Fab fragment, was added to the Strep-Tactin®-coated 1:1 matrix suspension (total volume vs. matrix bed volume). The matrix suspension was incubated under constant gentle movement at room temperature for 60 min to enable the reversible binding of the anti-CD3 agent to the matrix via the immobilized Strep-Tactin®. 20mL final anti-CD3 functionalized matrix were filled into a reservoir bigger
20 than 20mL to enable movement of the matrix during blood loading, non-target cell washing and release of the hold-back cellular therapy starting cells. Prior to holding back the cellular therapy starting cells, the reservoir including the matrix was equilibrated with wash buffer (PBS with 0.5% HSA). The above mentioned anti-CD3 Fab fragment is peptide-tagged and was produced recombinantly (see International
25 Patent App. Pub. Nos. WO 2013/011011 and WO 2013/124474). The Fab fragment was derived from the CD3 binding monoclonal antibody produced by the hybridoma cell line OKT3 (ATCC® CRL-8001™; see also U.S. Patent No. 4,361,549). It contained the heavy chain variable domain and light chain variable domain of the anti-CD3 antibody OKT3 e. g. described in **Figure 1** in (16).

30

Production of the anti-CD19 affinity matrix for reversible immobilization of cells

For the enrichment of CD19+ cells via reversible immobilization an anti-CD19 affinity matrix is used. The matrix is composed as following (calculated on 20mL matrix): 10mg of multimerized streptavidin mutein (Strep-Tactin® m2 (as described in US 6,103,493),

SEQ Id. No. 8) is coupled to 8g initial dry weight epoxy-activated polystyrene resin (CY17030; Cytosorbents) using solvent-exposed primary amine groups, in phosphate buffered saline (PBS) buffer. Afterwards cellular therapy starting cell selection agent (e.g. 1.8mg) with an anti-CD19 Fab fragment and a streptavidin-binding peptide (Twin-Strep-tag®, Twin-Strep-tag® streptavidin-binding peptide including a sequential arrangement of two streptavidin-binding modules) carboxyl-terminally fused to the heavy chain of the Fab fragment, is added to the Strep-Tactin®-coated 1:1 matrix suspension (total volume vs. matrix bed volume). The matrix suspension is incubated under constant gentle movement at room temperature for 60 min to enable the reversible binding of the anti-CD19 agent to the matrix via the immobilized Strep-Tactin®. 20mL final anti-CD19 functionalized matrix is filled into a reservoir bigger than 20mL to enable movement of the matrix during blood loading, non-target cell washing and release of the hold-back cellular therapy starting cells. Prior to holding back the cellular therapy starting cells, the reservoir including the matrix is equilibrated with wash buffer (PBS with 0.5% HSA). The above mentioned anti-CD19 Fab fragment is peptide-tagged and is developed and produced according to a standard procedure for generating Fab fragments.

Enrichment of cellular therapy starting cells using a liquid-handling device (CD3+ cells)

CD3+ cells from two independent fresh whole blood samples (2 – 3 h post blood withdrawal) were enriched using a commercially available liquid handling device with a closed system. The liquid handling device includes the operating device and a tubing set wherein the blood reservoir, containing the whole blood sample, is fluidly connected to the second target cell retention reservoir and the second target cell retention reservoir is fluidly connected to the bag containing process buffers and optionally to the waste reservoir.

100-200 mL of whole blood from each independent healthy human donor with anticoagulant were transferred into one bag. Subsequently the bag was connected to a tubing set and the blood was flushed stepwise into a reservoir containing anti-CD3 affinity matrix (prepared as describe above) where cellular therapy starting cells, the target cells, are immobilized for a short time period while non-target cells flow through the affinity matrix into another separate bag. Blood loading was performed with a speed of 20 mL per minute (calculated onto a standard blood tubing) in 15mL pulses, following

a backward-pulse and incubation of blood under gentle moving conditions of the matrix particles after each 15mL pulse. After complete loading of 100mL whole blood non-target cells were washed out with wash buffer (phosphate buffered saline (PBS) buffer with 0,5% human serum albumin (HSA)) with a speed of 40 mL per minute (calculated
5 onto a standard blood tubing) in 40mL pulses using a backward pulse after each wash step and under gentle movement of the matrix. In total 9 washing pulses were performed. After washing out the non-target cells, the short enrichment step of cellular therapy starting cells was finished by flushing a 200 μ M D-Biotin buffer solution (phosphate buffered saline (PBS) buffer with 0,5% human serum albumin (HSA) and
10 200 μ M D-Biotin) into the matrix containing reservoir. Cellular therapy starting cell release was performed with a speed of 75 mL per minute (calculated onto a standard blood tubing) in 30mL pulses using a backward pulse after each step in total 3 times under gentle movement of the matrix. Further cellular therapy starting cell release steps were performed by flushing the matrix with 30mL D-Biotin buffer followed by an
15 incubation step with stronger movement of the matrix for 3 min, repeating this step 7 times for Run 1 and double the amount for Run 2. During cellular therapy cells release step the complete volume was flushed through a device specific membrane including device specific parameters for automated target cell wash into a final volume of 20 ml. Thus, the cellular therapy starting cells were specifically enriched by a reversible
20 immobilization on an anti-CD3 affinity matrix.

The results are shown in **Figure 4**.

Fig. 4A depicts the cell count (total nucleated cell count, TNC) in million cells of the starting whole blood sample and of the enriched CD3+ T cells. **Fig. 4B** shows the depletion, **Fig. 4C** the purity and **Fig. 4D** the yield of CD3+ cells. Depletion was calculated on CD3+ cells in the starting material vs. CD3+ cells in the negative fraction (flow-through, etc.). Yield is calculated on CD3+ cells in the starting material vs. CD3+ cells in the positive fraction (eluate). Purity is measured via Flow Cytometry and
30 pre-gated on single living CD45 cells. The results show a yield of CD3+ cells of about 40 %, with a very high purity of 99,2 %. As discussed herein above, such a very high purity for enrichment/isolation of such cells has not yet reported for cell separation as described in WO 2013/124474 or WO 2021/152178, for example, that use Fab fragments and a streptavidin mutein based system.

Thus, the enrichment step of the inventive method is unique in its ability to enrich CD3+ cells with a high yield and high purity.

- 5 **Fig. 4E** shows flow cytometry analysis of the whole blood sample and the CD3+ cell fraction (eluate) of two independent donors. For each of the two blood samples the CD19+ cells and CD3+ cells (CD19 vs. CD3) were measured. The results show a significant enrichment of CD3+ T cells with a clear reduction of CD19+ cells in the CD3+ cell fraction compared to whole blood sample.

10

For example, the yield of CD3+ cells were 48,5 % in the whole blood sample and 99,5 % in the CD3+ cell fraction whereas the yield of CD19+ cells was reduced from 4,3 % in the whole blood sample to 0,03 % in the CD3+ cell fraction (cf. **Fig. 4E**, donor 2).

- 15 To conclude, the enrichment of CD3+ T cells using a commercially available liquid handling device results in a yield of about 99% CD3+ T cells in the eluate, showing a specific and highly pure T cell fraction for using in the gene modification step of the inventive method.

- 20 *Enrichment process of cellular therapy starting cells using a liquid-handling device (B cells)*

CD19+ cells from a fresh whole blood sample are enriched using a commercially available liquid handling peristaltic pump and commercially available tubing to operate the peristaltic pump (or any other liquid handling systems). The needed tubing set is self-build and includes the blood reservoir, containing the whole blood sample, that is fluidly connected to the target cell retention reservoir and the target cell retention reservoir is fluidly connected to the bag containing process buffers and optionally to the waste reservoir.

- 25 30 100mL of whole blood from a healthy human donor with anticoagulant is transferred into one bag. Subsequently the bag is connected to the tubing set and the blood is flushed stepwise into a reservoir containing anti-CD19 affinity matrix (prepared as describe above) where cellular therapy starting cells, the target cells, are immobilized for a short time period while non-target cells flow through the affinity matrix into another

separate bag. Blood loading is performed with a special speed (e.g. 20 mL per minute, calculated onto a standard blood tubing) in 15mL pulses, following a backward-pulse and incubation of blood under gentle moving conditions of the matrix particles after each 15mL pulse. After complete loading of 100mL whole blood non-target cells are washed out with wash buffer (phosphate buffered saline (PBS) buffer with 0,5% human serum albumin (HSA)) with a speed of 40 mL per minute (calculated onto a standard blood tubing) in 40mL pulses using a backward pulse after each wash step and under gentle movement of the matrix. In total multiple washing pulses will be performed (e.g. 9). After washing out the non-target cells, the short enrichment step of cellular therapy starting cells are finished by flushing a D-Biotin buffer solution (phosphate buffered saline (PBS) buffer with 0,5% human serum albumin (HSA) and e.g. 200 μ M D-Biotin) into the matrix containing reservoir. Cellular therapy starting cell release is performed with a speed of 75 mL per minute (calculated onto a standard blood tubing) in 30mL pulses using a backward pulse after each step in total multiple times (e.g. 3) under gentle movement of the matrix. Further cellular therapy starting cell release steps are performed by flushing the matrix with 30mL D-Biotin buffer followed by an incubation step with stronger movement of the matrix for a time period (3 min, repeating this step multiple times (e.g. 7). After cellular therapy cells release step the complete volume is washed using centrifugation for a final cell wash into a final volume of 20 ml. Thus, the cellular therapy starting cells will be specifically enriched by a reversible immobilization on an anti-CD19 affinity matrix.

Example 2: Gene modification of cellular therapy starting cells

Cellular therapy starting cell wash and re-buffering using CD3+ cells

After the cellular therapy starting cell enrichment and release of cellular therapy starting cells into one bag, the cellular therapy starting cells were washed and transferred into electroporation buffer by using a Counterflow Centrifuge System. A protocol was used to achieve maximal cellular therapy starting cell yield and minimal cellular therapy starting cell output volume with the following parameters: Input cells 300mL, Wash Buffer 1000mL, Load Waste 0mL, Pulsing Buffer 55mL, Air Filter 0mL, Wash waste 0mL, Output 0mL. Cellular therapy starting cells were finally concentrated to 5mL in Electroporation buffer into one small bag (Electroporation buffer: KCl 5mM, $MgCl_2$ 15mM, Sodium Succinate 25mM, D-Mannitol 25mM, Na_2HPO_4 120mM).

Gene modification of cellular therapy starting cells via electroporation using CD3+ cells

After cellular therapy starting cell wash and re-buffering into electroporation buffer the cellular therapy starting cells were immediately electroporated in 5×10^6 cellular therapy starting cells number steps using 14,5 μM of ribonucleoprotein (RNP) complexes with

5 *Streptococcus pyogenes* (*S. pyogenes*) Cas9 and 1 μg of a HDR template per 1×10^6 cellular therapy starting cells comprising 5' scrambled bases, 5' Cas9 target sequence site (CTS), 5' homology arm, gRNA target sequence, 3' homology arm, 3' Cas9 target sequence site (CTS), 5' homology arm, 3' scrambled bases. Additionally, 1 μM XL-413 CDK inhibitor and 1 μM M-3814 DNA-PK inhibitor was added during or after

10 electroporation. The gene modification was done essentially as described in (15). For electroporation 5×10^6 target cells in 100 μl were used in cuvettes and program EH-100 was performed on the Nucleofector device.

The used sequences are:

- 15 • The **target sequence for 5' Cas9** is 5'-
TGGCTCTCTCTCAGCTGGTACACGG-3' (SEQ ID NO. 1).
- The **target sequence for 3' Cas9** is 5'-
CCAGCACCTCTCTCAGCTGGTACACGG-3' (SEQ ID NO. 2)
- The **TRAC-targeting gRNA** is 5'-AGAGTCTCTCAGCTGGTACA-3' (SEQ ID NO.
- 20 3).
- **5' homology arm:** 5'-
CTGCCTTTACTCTGCCAGAGTTATATTGCTGGGGTTTTGAAGAAGATCCTATT
AAATAAAAGAATAAGCAGTATTATTAAGTAGCCCTGCATTTTCAGGTTTCCTTGA
GTGGCAGGCCAGGCCTGGCCGTGAACGTTCACTGAAATCATGGCCTCTTGG
25 CCAAGATTGATAGCTTGTGCCTGTCCCTGAGTCCCAGTCCATCACGAGCAGC
TGGTTTCTAAGATGCTATTTCCCGTATAAAGCATGAGACCGTGACTTGCCAGC
CCCACAGAGCCCCGCCCTTGTCCATCACTGGCATCTGGACTCCAGCCTGGG
TTGGGGCAAAGAGGGAAATGAGATCATGTCCTAACCCTGATCCTCTTGTCCC
ACAGATATCCAGAACCCTGACCCTGCCGTG- 3' (SEQ ID NO. 4)
- 30 • **3' homology arm:** 5'-
TACCAGCTGAGAGACTCTAAATCCAGTGACAAAGTCTGTCTGCCTATTCACCGATTTTG
ATTCTCAAACAAATGTGTCACAAAGTAAGGATTCTGATGTGTATATCACAGACAAAAC
TGTGCTAGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTGGCCTGGAGCAA
CAAATCTGACTTTGCATGTGCAAACGCCTTCAACAACAGCATTATTCAGAAGACACC
35 TTCTTCCCCAGCCCAGGTAAGGGCAGCTTTGGTGCCTTCGCAGGCTGTTTCCTTGCT
TCAGGAATGGCCAGGTTCTGCCCAGAGCTCTGGTCAATGATG - 3' (SEQ ID NO. 5)
- **TRAC exon 1 target site:** 5'-ATTACCGATTTTGATTCTC- 3' (SEQ ID NO. 6)

Following electroporation, the gene modified cellular therapy starting cells, which are now a precursor of the cellular therapy product, were washed and transferred into NaCl buffer.

5 *Mechanoporation (of CD3+ cells)*

The mechanoporation was used for mechanical disruption to permeabilize cell membranes of CD3+ cells for intracellular delivery of the RNP complex.

10 The Gateway system of Portal Biotechnologies was utilized to deliver cargo into purified CD3+ cells using a target cell affinity matrix. The Gateway device squeezes the cells by mechanical pressure through a microchip with pores which leads to a disrupted cell membrane and enables cargo to enter the cell. The process where cells are squeezed by pressure is called "boosting". The aim of the experiments was to knock-out the TRAC (T-cell receptor alpha chain constant) locus of CD3+ cells using
15 the Gateway system. The knock-out was performed by delivering an RNP complex, composed of Cas9 Nuclease and gRNA directed against the TRAC locus, into the cell. In total 5 mechanoporation experiments were performed (**Fig. 5**) using a microchip with pore size of 5 μm .

20 For Mechanoporation, selected CD3+ cells were resuspended in serum-free medium (Opti-MEM). 2 – 5 Mio CD3+ cells in 50 μl were used per boost. To generate the RNP complex, equal amounts of tracrRNA and crRNA were mixed leading to a concentration of 40 μM , respectively. To anneal both RNA fragments the RNA mix is incubated 5 min at 95 °C. The Cas9 nuclease is diluted to a concentration of 21 μM in
25 sterile PBS and slowly added to the gRNA (tracrRNA + crRNA). The mixture is then incubated for 15 min at RT for assembly. 3 μl RNP complex per 1 Mio cells is added to the boosting sample. In total the sample volume being boosted is adjusted to 100 μl . The cells were boosted with 8 psi (pound-force per square inch) and collected in a tube. Between each sample, a wash of the microchip with 100 μl OptiMEM is
30 performed. Pre-warmed complete medium was added to the cells post boost and cells were transferred to a cell culture plate. Cells were cultured for up to 12 days at 37°C and knock-out frequency was analyzed using flow cytometry.

Cellular therapy starting cell wash and re-buffering using B cells

After the cellular therapy starting cell enrichment and release of cellular therapy starting cells, the cellular therapy starting cells are washed and transferred into buffer for genetic modification, e.g. electroporation buffer, by using a Centrifuge System.

5

Gene modification of cellular therapy starting cells via e.g. electroporation using B cells

After cellular therapy starting cell wash and re-buffering into an electroporation buffer the cellular therapy starting cells are immediately electroporated in suitable conditions. A ribonucleoprotein (RNP) complex (composed of Cas9 and guide RNA) is used to
10 knock out the specific gene in the cell. Furthermore, an HDR dsDNA template is added to the electroporation reagents to insert the gene of interest into the IGHG1 locus (e.g. enzyme like insulin $\pm$ GFP reporter). For electroporation of 5×10^6 target cells in 100 μ l in cuvettes a specific program on the Nucleofector device is used. The readout is analyzed by measuring the amount of enzyme secretion and function as well as
15 measurement of the surrogate GFP reporter.

Enrichment process of cellular therapy starting cells using a liquid-handling device and subsequent genetic modification while the cellular therapy starting cells are immobilized on the stationary phase (B cells)

20 A whole blood sample (e.g. 200ml) is collected into one bag and directly flushed into a reservoir containing anti-CD19 affinity matrix where cellular therapy starting cells, the target cells, are immobilized for a short time period while non-target cells flow through the affinity matrix into another separate bag. After complete loading of the whole blood non-target cells are washed out with wash buffer. After washing out the non-target
25 cells, the cellular therapy cells are immediately genetically modified while staying on the anti-CD19 affinity matrix using e.g. LNPs. The LNPs are flushed onto the anti-CD19 affinity matrix and incubated with the CD19 cells on the matrix. Incubation of LNPs with the cellular therapy cells on the anti-CD19 affinity matrix can be either done with a co-incubation with an activation reagent or without an activation reagent. After the
30 incubation period the genetically modified cells are released from the anti-CD19 affinity matrix by flushing D-Biotin buffer into the matrix containing reservoir. After release of the cellular therapy cells, they are washed and reinfused into the human body after an optional in-process-analytics step.

Transfection of CD3+ cells using liquid nanoparticles (LNP) after elution from the stationary phase

CD3+ cells were selected from whole blood using a cell affinity matrix. To transfer GFP mRNA into the cell cytoplasm LNPs containing the desired information were generated
 5 by Pantherna Therapeutics GmbH. Immediately after cell selection and elution from the matrix, 1 Mio CD3+ cells were resuspended in cell culture medium without antibiotics and 15 µg LNPs were added to the cells. The total sample volume was adjusted to 500 µl per well. Cells were then incubated for at least 4 h up to 9 d. Analysis (cell number, viability, GFP expression, phenotype) was performed on different time
 10 points, starting with the earliest time point at 4 h post transfection and the last time point on day 9. T cells for data in **Fig. 6B** and **Fig. 6D** are analyzed after 4 hours LNP incubation by flow cytometry, the data are also shown in Table 1. T cells in **Fig. 6E** are analyzed after 4h incubation and on day 1 (after 24h), day 3, day 5, day 7 and day 9 after LNP incubation.

15

Transfection of CD3+ cells immobilized on the stationary phase using liquid nanoparticles (LNP)

CD3+ cells were selected from 1 ml whole blood using a cell affinity matrix in a pipette tip. To transfer GFP mRNA into the cell cytoplasm LNPs containing the desired
 20 information were generated by Pantherna Therapeutics GmbH. Immediately after cell selection while staying on the matrix in the pipette tip, CD3+ cells were flushed with cell culture medium without antibiotics and 15 µg LNPs were added to the pipette tip in 60 µl volume. Cells were then incubated for 4 h in the incubator while staying on the pipette tip. After 4 h cells were eluted from the resin in the pipette tip by using 0,1 mM
 25 D-Biotin and analyzed. Analysis (cell number, viability, GFP expression, phenotype) was performed and data are shown in **Fig. 6C** and Table 1.

Table 1

	4h Transfection		WBC
	GFP+ CD4 T cells	GFP+ CD8 T cells	
CTR	1.14%	1.67%	80.31%
on column	24.13%	19.05%	4.37%
eluted cells	71.64%	59.96%	80.64%

30 Table 1 summarizes the % values for transgene expression on CD4 and CD8 T cells for the control, on-column transduction and transduction on selected and eluted cells

as graphically depicted in **Fig. 6B**, **Fig. 6C**, and **Fig. 6D** after 4 hours incubation (WBC = white blood cells).

Example 3: Determination of functionality of the cellular therapy product *in vivo*

5 For determining the functionality of the genetically modified target cells (CD3+ cells) *in vivo*, the expression and expansion of the target cells was studied in a clinically relevant humanized murine lymphoma model.

6-8 week old NSG-SGM3 (NSGS) mice were injected intravenously with 0.5×10^6 Raji-fluc-GFP CD19+ tumor cells. Two days later these mice were intraperitoneally injected with the precursor of the cellular therapy product. A total of 10×10^6 precursor cells of the cellular therapy product were administered as flat dose. TRAC-CAR knock-in and expression was confirmed after extended parallel *in vitro* culture by flow cytometry (cf. **Fig. 7C**). Conventionally manufactured CD19 CAR T cells using a standard lentivirus (LV) transfection and expansion process served as positive controls while mice only injected with PBS served as negative control group. All groups consisted of 3-4 mice. The mice were monitored each 7 days by tumor growth imaging using IVIS bioluminescence and measuring *in vivo* expansion of the cellular therapy product in blood. Therefore, mice were bled weekly for ex vivo analysis using flow cytometry.

20

Mice treated with precursor of the cellular therapy product and standard LV control cells showed rapid expansion and high tumor reactivity with complete tumor clearance post day 20 (**Fig. 7B** middle and right columns). In contrast, control groups treated with PBS only showed uncontrolled tumor progression (**Fig. 7B** left column) with mice succumbing to the tumor starting around day 14. As shown in **Fig. 7D**, depicting a Kaplan-Meier analysis, mice injected with PBS control died around 14 days post tumor cell injection whereas mice injected with the precursor of the cellular therapy product (TQ process 1) or standard LV control cells survived until the end of the experiment (measured until day 21). These data illustrate that ultra-short extra-corporally generated CAR cells (that fully develop within the body) are highly potent in controlling aggressive lymphoma comparable to conventionally manufactured and expanded CAR cells (representative of clinically used CAR T cell products).

30

Using the fluc gene of the injected Raji-fluc-GFP CD19+ cells, the tumor growth was measured via luciferin treatment using Living image software (Perkin Elmer). The tumor size was measured as average radiance [p/s/cm²/sr] at different time point using the software after luciferin treatment. The results are shown in **Fig. 8A**. The diagram
5 shows that both, the cellular therapy product (TQ process) manufactured according to the inventive method and the standard lentiviral transduced CD19 CAR T cells are able to reduce tumor growth compared to the PBS negative control.

Overall tumor bearing mice injected with the precursor of the cellular therapy product
10 and standard LV control cells had a survival benefit compared to PBS injected negative control.

Also, the residual Raji tumor cells were measured in blood using flow-cytometry. The results are shown in **Fig. 8B**. In the mice with PBS control, the amount of tumor cells
15 is high compared to the tumor cell amount in mice treated with the precursor of the cellular therapy product (TQ process) or standard LV control cells.

For further measuring the engraftment and *in vivo* expansion of the gene modified cells mice were bled weekly for ex vivo analysis using flow cytometry and staining for T cell
20 subsets and CAR expression.

As shown in **Fig. 8C** the amount of CAR cells reached a maximum at day 14 after CAR cell injection. In this diagram all CAR+ transferred T cells (CD3+CAR+ T cells) are shown. There is no mark difference between the inventive cellular therapy product (TQ
25 process) and the standard LV control.

In **Fig. 8D** and **Fig. 8F** only CD4+ T cells were measured. The result shown in **Fig. 8F**, is also true for CAR+ CD4+ T cells (see **Fig. 8D**). The CAR+, CD 4 + T cells plateaus around day 14 after CAR cell injection, whereas the total CD4+ T cells rose until end
30 of experiment (21 days post CAR cell injection).

In **Fig. 8E** and **Fig. 8G** only CD8+ T cells were measured. The result shown in **Fig. 8G**, is also true for CAR+ CD8+ T cells (see **Fig. 8E**). The CAR+, CD 8 + T cells plateau around day 14 after CAR cell injection. The percentage of total CD8+ T cells in the

blood in the experiment with CAR T cells prepared according to the invention rose until end of experiment (21 days post CAR cell injection), whereas the percentage of CD8+ T cells of the positive control experiment is unchanged.

- 5 All data shown were pre-gated on living CD45+ cells. Black bars PBS control, gray bars squares LV transduced expanded cells (positive control). n=4 for all tested cohorts receiving CAR T cells and n=3 for untreated PBS control.

- 10 Together these data demonstrate a complete control of the tumor as a result of the potent anti-tumor response of transferred CAR T cells (in particular of the relevant CD8+ subset) resulting in a marked survival benefit of treated mice.

- 15 The listing or discussion of a previously published document in this specification should not necessarily be taken as an acknowledgement that the document is part of the state of the art or is common general knowledge.

- 20 The invention illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, for example, the terms "comprising", "including," containing", etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various
25 modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by exemplary embodiments and optional features, modification and variation of the inventions embodied therein herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the
30 scope of this invention.

The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or

negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

The amino acid sequences and nucleic acid sequences disclosed in the present application are shown in Table 2 below. If any doubts will arise the sequences in Table 2 prevails over the sequence listing.

Table 2

Seq No.	ID	Sequence	Description
1		5'-TGGCTCTCTCTCTCAGCTGGTACACGG-3'	target sequence for 5' Cas9
2		5'-CCAGCACCTCTCTCAGCTGGTACACGG-3'	target sequence for 3' Cas9
3		5'-AGAGTCTCTCAGCTGGTACA-3'	TRAC-targeting gRNA
4		5'- CTGCCTTTACTCTGCCAGAGTTATATTGCTGGGG TTTTGAAGAAGATCCTATTAAATAAAAGAATAAGC AGTATTATTAAGTAGCCCTGCATTTTCAGGTTTCCT TGAGTGGCAGGCCAGGCCTGGCCGTGAACGTTT ACTGAAATCATGGCCTCTTGGCCAAGATTGATAG CTTGTGCCTGTCCCTGAGTCCCAGTCCATCACGA GCAGCTGGTTTCTAAGATGCTATTTCCCGTATAAA GCATGAGACCGTGACTTGCCAGCCCCACAGAGC CCCGCCCTTGTCCTCACTGGCATCTGGACTCCA GCCTGGGTTGGGGCAAAGAGGGAAATGAGATCA TGTCCTAACCCCTGATCCTCTTGTCCCACAGATATC CAGAACCCCTGACCCTGCCGTG-3'	5' homology arm
5		5'- TACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTG TCTGCCTATTCACCGATTTTGATTCTCAAACAAATGTG TCACAAAGTAAGGATTCTGATGTGTATATCACAGACAA AACTGTGCTAGACATGAGGTCTATGGACTTCAAGAGC AACAGTGCTGTGGCCTGGAGCAACAAATCTGACTTTG CATGTGCAAACGCCTTCAACAACAGCATTATTCCAGAA GACACCTTCTTCCCAGCCCAGGTAAGGGCAGCTTTG GTGCCTTCGCAGGCTGTTTCTTGCTTCAGGAATGGC CAGGTTCTGCCCAGAGCTCTGGTCAATGATG-3'	3' homology arm
6		5'-ATTCACCGATTTTGATTCTC- 3'	TRAC exon 1 target site
7		Val ⁴⁴ -Thr ⁴⁵ -Ala ⁴⁶ -Arg ⁴⁷ (VTAR)	streptavidin mutein m1
8		Ile ⁴⁴ -Gly ⁴⁵ -Ala ⁴⁶ -Arg ⁴⁷ (IGAR)	streptavidin mutein m2
9		DPSKDSKAQVSAAEAGITGTWYNQLGSTFIVTAGA DGALTGTYESAVGNAESRYVLTGRYDSAPATDGSG	wild type streptavidin

	TALGWTVAWKNNYRNAHSATTWSGQYVGGAEARI NTQWLLTSGTTEANAWKSTLVGHDTFTKVKPSAAS IDAAKKAGVNNGNPLDAVQQ	
10	WSHPQFEK	streptavidin- binding peptide

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Claims

What is claimed is:

1. A method of manufacturing a cellular therapy product, wherein the cellular
5 therapy product comprises a first cellular surface receptor which is able to bind
to a drug target molecule (on the surface) of a drug target cell, and/or wherein
the cellular therapy product produces and/or secretes a polypeptide which is able
to bind to a drug target molecule either soluble or associated with a drug target
cell, comprising
 - 10 a) enriching via reversible immobilization (in a holding step) a cellular therapy
starting cell obtained from a sample comprising the cellular therapy starting
cell, using at least one second cellular surface receptor of the cellular therapy
starting cell, and
 - 15 b) genetically modifying the cellular therapy starting cell using one or more
reagent(s) comprising a gene construct for expression of the first cellular
surface receptor and/or for expression and/or secretion of the polypeptide,
thereby generating a precursor of the cellular therapy product,
wherein the method takes place in a closed system.
- 20 2. The method of claim 1, further comprising c) formulating the cell therapy product
in a buffer suitable for subsequent administration of the cellular therapy product
to a patient.
3. The method of any of claims 1 or 2, wherein the duration of the method is between
25 about 1.5 hours to about 16 hours.
4. The method of any of the preceding claims, wherein the sample comprises a
bodily fluid.
- 30 5. The method of claim 4, wherein the sample comprises nucleated blood cells.
6. The method of claim 4 or 5, wherein the sample is a whole blood sample or
wherein the sample comprises or consists of white blood cells.

7. The method of any of the preceding claims, wherein the cellular therapy starting cell is a T cell, a B cell, or a NK cell.
8. The method of claim 7, wherein the cellular therapy starting cell is a non-activated T cell, a non-activated B cell or a non-activated NK cell.
9. The method of any of the preceding claims, wherein the drug target cell is a diseased cell, preferably in an infectious disease or a cancer cell or an autoreactive (autoimmune) cell causing an autoimmune disease.
10. The method of any of the preceding claims, wherein enriching via reversible immobilization of the cellular therapy starting cell from the sample comprises
- providing the sample, the sample comprising the cellular therapy starting cell,
 - providing a receptor binding reagent comprising a binding site B and a binding partner C,
 - wherein the binding site B comprised in the receptor binding reagent is capable of specifically binding to the second cellular surface receptor on the cellular therapy starting cell and wherein the bond between the binding site B of the receptor binding reagent and the second cellular surface receptor has a dissociation constant (K_D) is of low affinity or in the range from about 10^{-3} to about 10^{-7} M,
 - wherein the binding partner C comprised in the receptor binding reagent is capable of reversibly binding to a binding site Z of an affinity reagent and the reversible bond between the binding partner C of the receptor binding reagent and the binding site Z of the affinity reagent has a dissociation constant (K_D) in the range from about 10^{-2} to about 10^{-13} M, and
 - exposing the sample to chromatography on a suitable solid phase, for example, floating particles or a stationary phase, the solid phase, for example, the solid phase and/or the stationary phase having the affinity reagent immobilized thereon,
 - wherein the affinity reagent comprises one or more binding sites Z, wherein said binding site Z forms a reversible bond with the binding partner C comprised in the receptor binding reagent, and wherein the binding site B of the receptor binding reagent binds to the second cellular surface receptor on

- the cellular therapy starting cell, thereby reversibly immobilizing the cellular therapy starting cell on the solid phase,
- providing a competition reagent, the competition reagent comprising a binding site, specifically binding to the binding sites Z of the affinity reagent;
 - 5 - loading the competition reagent onto the stationary phase, thereby allowing disruption of non-covalent reversible complexes formed between (a plurality of) the receptor binding reagent, the second cellular surface receptor and the affinity reagent;
 - 10 - recovering an enrichment sample from the eluate of the stationary phase, wherein the enrichment sample comprises the cellular therapy starting cell.
11. The method of claim 10, wherein the receptor binding reagent is selected from the group of an immunoglobulin, a functional fragment of an immunoglobulin, a proteinaceous binding molecule with immunoglobulin-like functions, an aptamer
- 15 and an MHC molecule.
12. The method of any one of claims 10 or 11, wherein the binding partner C comprised in the receptor binding reagent comprises one of biotin, a biotin analogue, a streptavidin binding peptide and an avidin binding peptide, and
- 20 wherein the affinity reagent comprises streptavidin, a streptavidin mutein, avidin an avidin mutein or a mixture thereof.
13. The method of claim 12, wherein the streptavidin mutein includes the amino acid sequence Val⁴⁴-Thr⁴⁵-Ala⁴⁶-Arg⁴⁷ (SEQ ID NO. 7) or Ile⁴⁴-Gly⁴⁵-Ala⁴⁶-Arg⁴⁷ (SEQ
- 25 ID NO. 8 in the region of the amino acid positions 44 to 47 with reference to the amino acid sequence of wild type streptavidin as set forth in SEQ ID NO. 9,
14. The method of any one of claims 10 or 11, wherein the binding partner C comprised in the receptor binding reagent comprises an affinity tag, and wherein
- 30 the affinity reagent comprises a suitable binding partner for the affinity tag, preferably an antibody or an antibody fragment, which is capable of binding to the affinity tag.

15. The method of claim 14 wherein the affinity tag is selected from a group comprising an oligohistidine, a polyhistidine, an immunoglobulin domain, a maltose-binding protein, a glutathione-S-transferase (GST), a chitin binding protein (CBP), a thioredoxin, a calmodulin binding peptide (CBP), a FLAG'-peptide, a HA-tag, a VSV-G-tag, and a HSV-tag.
16. The method of any one of claims 10 to 15, wherein the stationary phase is comprised in a reservoir such as bag, a tubing, a column or is a planar stationary phase.
17. The method of any one of claims 10 to 16, wherein the stationary phase or the solid phase comprises or consists of one of a plastic tubing, a cellulose membrane, a plastic membrane, a polysaccharide gel, a polyacrylamide gel, an agarose gel, polysaccharide grafted silica, polyvinylpyrrolidone grafted silica, polyethylene oxide grafted silica, poly(2-hydroxyethylaspartamide) silica, poly(N-isopropylacrylamide) grafted silica, polystyrene particles, a styrene-divinylbenzene gel, a copolymer of an acrylate or an acrylamide and a diol, a copolymer of a polysaccharide and N,N'-methylenebisacrylamide and a combination of any two or more thereof.
18. The method of any of preceding claims, wherein the second cellular surface receptor on the cellular therapy starting cell is selected from the group of CD3, CD4, CD8, CD45, CD56, CD27, CD28, CD19, CD62L, CD25.
19. The method of any of the preceding claims 10 to 18, wherein the reversible immobilization is carried out via two or more types of the second cellular surface receptor, for example, by sequential immobilization via the cell surface receptor CD3 as first type of second cellular surface receptor and CD4 as second type of second cell surface receptor.
20. The method of any of the preceding claims, wherein enriching via reversible immobilization of the cellular therapy starting cell is carried out as single enrichment step, without prior cell separation, in particular without prior cell

separation in a cell separation module comprising a cell separator configured to produce a fraction enriched in nucleated blood cells.

21. The method of any of the preceding claims, wherein the one or more reagents is
5 a set of reagents for gene modification.
22. The method of claim 21, wherein the set of reagents comprises a viral gene delivering system or a non-viral gene delivering system.
- 10 23. The method of claim 22, wherein the non-viral gene delivering system comprises an electroporation, a lipid nanoparticle, mechanoporation, ultrasound treatment, optical gene delivery, exosomes, amphiphilic peptides or a combination thereof, wherein a lipid nanoparticle is preferred.
- 15 24. The method of claim 23, wherein the viral gene delivering system comprises a retroviral transduction, a lentiviral transduction, or an adenoviral transduction.
25. The method of any one of the claims 22 to 24, wherein the set of reagents comprises a nucleic acid encoding a Transcription activator-like effector nuclease
20 (TALEN), a Zinc-finger nuclease (ZFN), or CRISPR and Cas, transposases such as sleeping beauty, piggyback, Tn5 transposases or wherein the set of reagents comprises a Transcription activator-like effector nuclease (TALEN), a Zinc-finger nuclease (ZFN), or CRISPR and Cas.
- 25 26. The method of any of the claims 22 to 25, wherein set of reagents comprises the gene construct comprising a nucleic acid encoding the first cellular surface receptor and/or the polypeptide, wherein the nucleic acid can be DNA or mRNA.
27. The method of claim 26, wherein the gene construct comprises a nucleic acid
30 encoding a chimeric antigen receptor (CAR), a recombinant T cell receptor (TCR), switch receptors, which is able to bind to the drug target molecule.

28. The method of any one of the claims 21 to 27, wherein the gene modification takes place after eluting the cellular therapy starting cell from the stationary phase.
- 5 29. The method of any one of the claims 21 to 27, wherein the gene modification takes place before eluting the cellular therapy starting cell from the stationary phase.
- 10 30. The method of any one of the preceding claims, wherein the sample is a whole blood sample, the cellular therapy starting cell is the non-activated T cell and the set of reagents comprises the non-viral gene delivering system, the nucleic acid encoding the CRISPR and Cas, and the nucleic acid encoding the chimeric antigen receptor (CAR) or the TCR, which is able to bind to the drug target molecule.
- 15 31. The method of any one of claims 1 to 29, wherein the sample is a whole blood sample, the cellular therapy starting cell is an activated or the non-activated B cell and the set of reagents comprises the non-viral gene delivering system, the nucleic acid encoding the CRISPR and Cas, and the nucleic acid encoding the polypeptide.
- 20 32. The method of any one of claims 1 to 29, wherein the sample is a whole blood sample, the cellular therapy starting cell is an activated or the non-activated T cell, B cell or NK cell and the set of reagents comprises the non-viral gene delivering system comprising the lipid nanoparticle.
- 25 33. A use of a set of reagents for genetically modifying a cellular therapy starting cell according to any one of claims 1, 21 to 27 or 29 to 32, wherein the cellular therapy starting cell is immobilized on a stationary phase or a receptor binding reagent according to any one of claims 10 to 20 during the genetic modification or the use of the set of reagents for genetic modification of the a cellular therapy starting cell, wherein the gene modification takes place before eluting the cellular therapy starting cell from the stationary phase.
- 30

34. A kit for manufacturing a cellular therapy product, wherein the cellular therapy product comprises a first cellular surface receptor which is able to bind to a drug target molecule (on the surface) of a drug target cell, and/or wherein the cellular therapy product produces and/or secretes a polypeptide which is able to bind to a drug target molecule either soluble or associated with a drug target cell, the kit comprising
- a) one or more reagent(s) comprising a gene construct for expression of the first cellular surface receptor or of the polypeptide of the cell therapy product, and
 - b) a receptor binding reagent comprising a binding site B and a binding partner C,
 - wherein the binding site B comprised in the receptor binding reagent is capable of specifically binding to the second cellular surface receptor on a cellular therapy starting cell and wherein the bond between the binding site B of the receptor binding reagent and the second cellular surface receptor has a dissociation constant (K_D) is of low affinity or in the range from about 10^{-3} to about 10^{-7} M,
 - wherein the binding partner C comprised in the receptor binding reagent is capable of reversibly binding to a binding site Z of an affinity reagent and the reversible bond between the binding partner C of the receptor binding reagent and the binding site Z of the affinity reagent has a dissociation constant (K_D) in the range from about 10^{-2} to about 10^{-13} M, and
 - a stationary phase or a solid phase, the stationary phase or solid phase having the affinity reagent immobilized thereon,
 - wherein the affinity reagent comprises one or more binding sites Z, wherein said binding site Z forms a reversible bond with the binding partner C comprised in the receptor binding reagent, and wherein the binding site B of the receptor binding reagent binds to the second cellular surface receptor on the cellular therapy starting cell, to allow reversibly immobilizing the cell therapy starting cell on the stationary phase.
35. The kit of claim 34, wherein the one or more reagent(s) comprise a viral gene delivering system or a non-viral gene delivering system, a nucleic acid part encoding a Transcription activator-like effector nuclease (TALEN), a Zinc-finger

nuclease (ZFN), or CRISPR and Cas, and a gene construct comprising a nucleic acid encoding the first cellular surface receptor or the polypeptide.

- 5 36. The kit of claim 34 or 35, wherein the cellular therapy starting cell is a T cell, a B cell, or a NK cell.
37. The kit of claim 36, wherein the cellular therapy starting cell is a non-activated T cell or a non-activated B cell.
- 10 38. The kit of any one of claims 34 to 37, wherein the gene construct comprises a nucleic acid encoding a chimeric antigen receptor (CAR) or TCR, which is able to bind to the drug target molecule or wherein the gene construct comprises a nucleic acid encoding for the polypeptide.
- 15 39. The kit of any one of claims 34 to 38, wherein the drug target cell is a diseased cell, preferably in an infectious disease or a cancer cell or an autoreactive (autoimmune) cell causing an autoimmune disease.
- 20 40. The kit any one of claims 35 to 39, wherein the non-viral gene delivering system comprises an electroporation, a lipid nanoparticle, mechanoporation, ultrasound treatment, optical gene delivery, exosomes, amphiphilic peptides or a combination thereof.
- 25 41. The kit of any one of claims 35 to 39, wherein the viral gene delivering system comprises a retroviral transduction or a lentiviral transduction.
42. The kit of any one of claims 34 to 41, wherein the kit is used in the method of any of claims 1 to 32 and/or the use of claim 31.
- 30 43. A device for manufacturing a cellular therapy product, comprising an operating device and a tubing-set, wherein the tubing set is a closed and single-use tubing-set, for use in the methods of any of claims 1 to 32 and/or the use of claim 33.

44. The device of claim 43, wherein the operating device comprises a liquid handling control unit comprising one or more valve(s), a liquid pump, a reservoir holder, and optionally a reservoir rocker.
- 5 45. The device of any one of claims 43 or 44, wherein the operating device further comprises a process unit, for operating process steps, comprising blood collection, second target cell hold-step, second target cell release/elution, second target cell wash, genetic modification, second target cell wash and formulation, reinfusion of a gene modified cell by optionally controlling the valve and optionally
10 comprising means for input of commands relating to the process steps and means for output of an information relating to the process steps.
46. The device of any one of claims 43 to 45, wherein the tubing-set comprises a system for blood withdrawal, a system for reinfusion of cells into the human body,
15 a second target cell retention reservoir, a genetic modification unit, a second target cell washing and formulation unit, a blood reservoir, one or more bag containing process buffers, a bag containing a set of reagents for gene modification according to any one of claims 1, 21-30, a waste reservoir and a re-infusion reservoir, wherein the reservoirs and bags are fluidly connected by a
20 closed tube system, in the case that the gene modification of the cellular therapy starting cell takes place before eluting the cellular therapy starting cell from the stationary phase, the second target cell retention reservoir and the genetic modification unit share the same space.
- 25 47. The device of claim 46, wherein
the system for blood withdrawal is fluidly connected to the blood reservoir,
the blood reservoir is fluidly connected to the second target cell retention reservoir,
the second target cell retention reservoir is fluidly connected to the bag
30 containing process buffers and optionally to the waste reservoir,
the second target cell retention reservoir is further fluidly connected with the genetic modification unit, or, in the case that the gene modification of the cellular therapy starting cell takes place before eluting the cellular therapy

starting cell from the stationary phase, the genetic modification unit is part of the second target cell retention unit in that both units share the same space, the genetic modification unit or, in the case that the gene modification of the cellular therapy starting cell takes place before eluting the cellular therapy starting cell from the stationary phase the second target cell retention unit is fluidly connected to the bag containing the set of reagents for gene modification or the gene therapy product, to the bag containing process buffers and optionally to the waste reservoir,

the genetic modification unit or, in the case that the gene modification of the cellular therapy starting cell takes place before eluting the cellular therapy starting cell from the stationary phase, the second target cell retention unit is further fluidly connected to the second target cell washing and formulation unit, the second target cell washing and formulation unit is fluidly connected to the bag containing process buffers and to the waste reservoir,

the second target cell washing and formulation unit is further fluidly connected to re-infusion reservoir,

the re-infusion reservoir is fluidly connected to the system for reinfusion of cells into the human body,

wherein the fluidly connections comprise tubes and valves, which could be controlled by the process unit.

48. The device of any one of claims 46 or 47, wherein the second target cell retention reservoir is a tubular expansion of the fluidly connection.
49. The device of any of claims 46 to 48, wherein an inner side of the second target cell retention reservoir is a stationary phase as defined in claims 10 to 20.
50. A tubing having an affinity reagent immobilized on an inner surface of the tubing, wherein the affinity reagent comprises one or more binding sites Z, wherein said binding site Z forms a reversible bond with a binding partner C comprised in the receptor binding reagent, and wherein the binding site B of the receptor binding reagent binds to a (second) cellular surface receptor on a cellular therapy starting cell, thereby allowing reversibly immobilizing the cellular therapy starting cell on the tubing acting as stationary phase.

51. The tubing of claim 50, wherein the affinity reagent is immobilized on the inner surface of the tubing by a covalent bond.
- 5 52. The tubing of claim 50 or 51, wherein the affinity reagent is, avidin, streptavidin or a streptavidin mutein.
53. The tubing of claim 52, wherein the streptavidin mutein includes the amino acid sequence Val⁴⁴-Thr⁴⁵-Ala⁴⁶-Arg⁴⁷ (SEQ ID NO. 7) or Ile⁴⁴-Gly⁴⁵-Ala⁴⁶-Arg⁴⁷ (SEQ
10 ID NO. 8) in the region of the amino acid positions 44 to 47 with reference to the amino acid sequence of wild type streptavidin as set forth in SEQ ID NO. 9.
54. A use of a tubing as defined in any of claims 50 to 53 in a method of manufacturing a cellular therapy product as defined in any of claims 1 to 32.
- 15