(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
14 December 2023 (14.12.2023)

WIPO | PCT

(10) International Publication Number
WO 2023/237754 A2

(51) International Patent Classification:
Not classified

(21) International Application Number:
PCT/EP2023/065527

(22) International Filing Date:
09 June 2023 (09.06.2023)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
2208550.0 10 June 2022 (10.06.2022) GB

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CV, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IQ, IR, IS, IT, JM, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, MG, MK, MN, MU, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, CV, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SC, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, ME, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

WO 2023/237754 A2

(54) Title: CYCLOSPORIN H AND USES THEREOF

(57) Abstract: Use of cyclosporin H (CsH) or a derivative thereof for: (a) reducing or preventing T cell exhaustion and/or loss of T cell effector functions; and/or (b) increasing T cell engraftment and/or persistence.

CYCLOSPORIN H AND USES THEREOF

FIELD OF THE INVENTION

The present invention relates to the use of cyclosporin H (CsH) in cell and gene therapy, in particular for reducing or preventing T cell exhaustion and/or loss of T cell effector functions; increasing T cell engraftment and/or persistence; and increasing survival of and/or engraftment by haematopoietic stem and/or progenitor cells (HSPCs).

BACKGROUND TO THE INVENTION

The haematopoietic system is a complex hierarchy of cells of different mature cell lineages. These include cells of the immune system that offer protection from pathogens, cells that carry oxygen through the body and cells involved in wound healing. All these mature cells are derived from a pool of haematopoietic stem cells (HSCs) that are capable of self-renewal and differentiation into any blood cell lineage. HSCs have the ability to replenish the entire haematopoietic system.

Haematopoietic cell transplantation (HCT) is a curative therapy for several inherited and acquired disorders. However, allogeneic HCT is limited by the poor availability of matched donors, the mortality associated with the allogeneic procedure which is mostly related to graft-versus-host disease (GvHD), and infectious complications provoked by the profound and long-lasting state of immune dysfunction.

Gene therapy approaches based on the transplantation of genetically modified autologous HSCs offer potentially improved safety and efficacy over allogeneic HCT. They are particularly relevant for patients lacking a matched donor.

The concept of stem cell gene therapy is based on the genetic modification of a relatively small number of stem cells. These persist long-term in the body by undergoing self-renewal, and generate large numbers of genetically “corrected” progeny. This ensures a continuous supply of corrected cells for the rest of the patient’s lifetime. HSCs are particularly attractive targets for gene therapy since their genetic modification will be passed to all the blood cell lineages as they differentiate. Furthermore, HSCs can be easily and safely obtained, for example from bone marrow, mobilised peripheral blood and umbilical cord blood.

Efficient long-term gene modification of HSCs and their progeny requires a technology which permits stable integration of the corrective DNA into the genome, without affecting HSC function. Accordingly, the use of integrating recombinant viral systems such as γ-retroviruses, lentiviruses and spumaviruses has dominated this field (Chang, A.H. et al. (2007) Mol. Ther.

15: 445-456). Therapeutic benefits have already been achieved in γ-retrovirus-based clinical trials for Adenosine Deaminase Severe Combined Immunodeficiency (ADA-SCID; Aiuti, A. et al. (2009) N. Engl. J. Med. 360: 447-458), X-linked Severe Combined Immunodeficiency (SCID-X1; Hacein-Bey-Abina, S. et al. (2010) N. Engl. J. Med. 363: 355-364) and Wiskott-Aldrich syndrome (WAS; Boztug, K. et al. (2010) N. Engl. J. Med. 363: 1918-1927). In addition, lentiviruses have been employed as delivery vehicles in the treatment of X-linked adrenoleukodystrophy (ALD; Cartier, N. et al. (2009) Science 326: 818-823), and very recently for metachromatic leukodystrophy (MLD; Biffi, A. et al. (2013) Science 341: 1233158) and WAS (Aiuti, A. et al. (2013) Science 341: 1233151).

Nevertheless, although lentiviruses are among the best available platforms for cell transduction, difficulties remain with the methods employed for the genetic modification of cells, in particular haematopoietic stem and progenitor cells. For example, for gene therapy to be efficacious, effective gene transfer into target cells must be reached without inducing detrimental effects on their biological properties.

Chimeric antigen receptor (CAR) T cell therapy has emerged as a potential effective strategy for the treatment of both solid tumor and hematological malignancies (Marofi *et al*, 2021; Zhang *et al*, 2020). However, several limitations still need to be addressed for improving their efficacy. Among these, the tumor microenvironment (TME) represents not only a physical barrier against CAR T cell access to solid tumors, but it also provides immunosuppressive conditions as well as persistent antigen stimulation, which limit CAR T efficacy (Rodriguez-Garcia *et al*, 2020). Metabolic alterations such as increased levels of oxidative stress and reactive oxygen species (ROS) have been demonstrated to be responsible for immune escape of cancer cells (Le Bourgeois *et al*, 2018). In this environment, CAR T cell exhaustion is considered one of the major limitations to their efficacy, and it is associated with poor responses in cancer patients receiving immunotherapy (Delgoffe *et al*, 2021). Moreover, the resting T stem memory compartment that would guarantee long-lived efficacy of the treatment remains difficult to target during CAR T cell generation. Therefore, the development of strategies aimed at mitigating exhaustion, thus maintaining CAR T effector functions and persistence, as well as improving lentiviral transduction efficacy across T cell subsets is important for improving the efficacy and clinical outcomes of CAR T cell therapies.

SUMMARY OF THE INVENTION

The inventors have surprisingly found that CsH provides advantages by improving the quality of cell products due to its capacity to transiently dampen cellular metabolism.

The inventors found that HSC exposed to CsH had upregulation of pathways that are mainly involved in metabolic processes such as Kreb-cycle and fatty acid oxidation. Conversely, genes related to lipid metabolism and T cell receptor signaling pathway were downregulated in cells exposed to CsH as compared to controls. Accordingly, mass spectrometry metabolomics revealed a significant CsH-induced increase in acylcarnitines and free fatty acids at steady state in HSC, thus confirming a potential effect of CsH in inducing relevant metabolic alterations.

Moreover, the inventors found that in human CD34+ HSPC and CD4+ and CD8+ T cells, in which a metabolic reprogramming is important for their activation and differentiation to ultimately mount an efficient response, cells exposed to CsH have significantly reduced levels of both basal and maximal respiration, as well as ATP production. In line with these metabolic changes, genes related to fatty acid β-oxidation and mitochondrial activity such as CPT1a and NRF1, were downregulated. CsH does not impact HSPC and T cell subset distribution and proliferation.

Oxidative stress is proposed to play a major role in impairing HSPC function in specific disease contexts such as Fanconi Anemia (FA). The inventors have also found that, several pathways related to oxidative stress and metabolic pathways were significantly enriched in HSPC from FA patients. Interestingly and in line with its metabolic effects, exposure to CsH mitigated upregulation of these pathways. Thus, CsH may be used to preserve FA HSPC during their ex vivo culture in the context of corrective gene therapy applications.

T cells transduced in the presence of CsH resulted in lower percentages of exhausted cells in culture as compared to controls. This effect was confirmed also in the context of murine T cells. Growing evidence indicates that exhausted T cells undergo metabolic alterations, leading to poor responsiveness to immune-checkpoint-blockade and lower efficacy of CAR T cell therapies.

In one aspect, the invention provides use of cyclosporin H (CsH) or a derivative thereof for: (a) reducing or preventing T cell exhaustion and/or loss of T cell effector functions; and/or (b) increasing T cell engraftment and/or persistence.

In another aspect, the invention provides use of cyclosporin H (CsH) or a derivative thereof for reducing or preventing T cell exhaustion.

In another aspect, the invention provides use of cyclosporin H (CsH) or a derivative thereof for reducing or preventing loss of T cell effector functions.

In another aspect, the invention provides use of cyclosporin H (CsH) or a derivative thereof for increasing T cell engraftment.

In another aspect, the invention provides use of cyclosporin H (CsH) or a derivative thereof for increasing T cell persistence.

In one aspect, the invention provides use of cyclosporin H (CsH) or a derivative thereof for increasing regulatory T (Treg) cell transduction efficiency.

In one aspect, the invention provides use of cyclosporin H (CsH) or a derivative thereof for improving the immunomodulatory profile of a regulatory T (Treg) cell, for example a Treg cell transduced or transfected with a vector. Improved immunomodulatory profile may, for example, comprise increased immunoregulatory cytokine (e.g. IFNγ and/or IL-10, preferably IFNγ and IL-10) production by the Treg cell, and/or decreased proinflammatory cytokine (e.g. TNF) production by the Treg cell.

In another aspect, the invention provides a T cell for use in a method of therapy, wherein the method comprises contacting the T cell with cyclosporin H (CsH) or a derivative thereof.

In one embodiment, T cell exhaustion and/or loss of T cell effector functions is reduced or prevented.

In one embodiment, T cell engraftment and/or persistence is increased.

In one embodiment, the therapy is treatment or prevention of cancer. In one embodiment, the therapy is treatment or prevention of infection.

In one embodiment, the T cell is transduced or transfected with a vector

In one embodiment, the T cell is transduced with a viral vector.

In one embodiment, the vector is a retroviral vector or a lentiviral vector.

In one embodiment, the vector comprises one or more nucleotide sequence encoding a chimeric antigen receptor (CAR)

In one embodiment, the vector comprises one or more nucleotide sequence encoding a T cell receptor (TCR).

In one embodiment, the T cell is a chimeric antigen receptor (CAR) T cell.

In one embodiment, the T cell comprises an exogenous T cell receptor (TCR).

In one embodiment, the T cell is a CD4+ T cell. In one embodiment, the T cell is a CD8+ T cell.

In one embodiment, the T cell is a regulatory T (Treg) cell. In one embodiment, the T cell is a CD4+CD25+ Treg cell. In one embodiment, the T cell is a CD4+CD25+CD127lo Treg cell. In one embodiment, the T cell is a CD4+CD25+FOXP3+ Treg cell.

In one embodiment, the T cell is a stimulated T cell.

In one embodiment, the T cell is a primary T cell.

In one embodiment, the T cell is a T stem memory cell.

In one aspect, the invention provides use of cyclosporin H (CsH) or a derivative thereof for: (a) reducing or preventing NK cell exhaustion and/or loss of NK cell effector functions; and/or (b) increasing NK cell engraftment and/or persistence.

In another aspect, the invention provides use of cyclosporin H (CsH) or a derivative thereof for reducing or preventing NK cell exhaustion. In another aspect, the invention provides use of cyclosporin H (CsH) or a derivative thereof for reducing or preventing loss of NK cell effector functions. In another aspect, the invention provides use of cyclosporin H (CsH) or a derivative thereof for increasing NK cell engraftment. In another aspect, the invention provides use of cyclosporin H (CsH) or a derivative thereof for increasing NK cell persistence.

In another aspect, the invention provides a NK cell for use in a method of therapy, wherein the method comprises contacting the NK cell with cyclosporin H (CsH) or a derivative thereof.

In one embodiment, NK cell exhaustion and/or loss of NK cell effector functions is reduced or prevented.

In one embodiment, NK cell engraftment and/or persistence is increased.

In one embodiment, the therapy is treatment or prevention of cancer. In one embodiment, the therapy is treatment or prevention of infection.

In one embodiment, the NK cell is transduced or transfected with a vector

In one embodiment, the NK cell is transduced with a viral vector.

In one embodiment, the vector is a retroviral vector or a lentiviral vector.

In one embodiment, the vector comprises one or more nucleotide sequence encoding a chimeric antigen receptor (CAR)

In one embodiment, the NK cell is a chimeric antigen receptor (CAR) NK cell.

In another aspect, the invention provides use of cyclosporin H (CsH) or a derivative thereof for increasing survival of and/or engraftment by haematopoietic stem and/or progenitor cells (HSPCs).

In another aspect, the invention provides a haematopoietic stem and/or progenitor cell (HSPC) for use in a method of treating Fanconi Anemia, wherein the method comprises contacting the HSPC with cyclosporin H (CsH) or a derivative thereof.

In one embodiment, the HSPC is transduced or transfected with a vector.

In one embodiment, the HSPC is transduced with a viral vector.

In one embodiment, the vector is a retroviral vector or a lentiviral vector.

In one embodiment, the CsH or derivative thereof is at a concentration of about 1-50 μM. In another embodiment, the CsH or derivative thereof is at a concentration of about 5-50 μM.

In another embodiment, the CsH or derivative thereof is at a concentration of about 1-40 or 5-40 μM. In another embodiment, the CsH or derivative thereof is at a concentration of about 1-30 or 5-30 μM. In another embodiment, the CsH or derivative thereof is at a concentration of about 1-20 or 5-20 μM. In another embodiment, the CsH or derivative thereof is at a concentration of about 1-15 or 5-15 μM.

In another embodiment, the CsH or derivative thereof is at a concentration of about 1-15, 2-14, 3-13, 4-12, 5-11, 6-10 or 7-9 μM. For example, the concentration of CsH may be about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 25, 30, 35, 40, 45 or 50 μM. In a preferred embodiment, the concentration of CsH or a derivative thereof is about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 μM. In a particularly preferred embodiment, the concentration of CsH or a derivative thereof is about 8 μM.

In one embodiment, the T cell or HSPC is contacted with the CsH or derivative thereof at the beginning of the cell culture. In one embodiment, the T cell or HSPC is contacted with the CsH or derivative thereof before transduction or transfection with a vector.

In one embodiment, the T cell or HSPC is contacted twice with the CsH or derivative thereof.

In one embodiment, the T cell or HSPC is:

(a) contacted with the CsH or derivative thereof at the same time as transduction or transfection with a vector; and/or

(b) contacted with the CsH or derivative thereof after transduction or transfection with a vector.

In one embodiment, the T cell or HSPC is cultured for 16 days or less before administration to a subject. In one embodiment, the T cell or HSPC is cultured for 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6 or 5 days or less before administration to a subject.

In another aspect, the invention provides a method of cell therapy comprising the steps of:

(a) contacting a cell with cyclosporin H (CsH) or a derivative thereof; and

(b) administering the cell to a subject.

In one embodiment, the method further comprises the step of transducing or transfecting the cell with a vector.

In another aspect, the invention provides a method of gene therapy comprising the steps of:

(a) contacting a cell with cyclosporin H (CsH) or a derivative thereof

(b) transducing or transfecting the cell with a vector; and

(c) administering the cell to a subject.

In one embodiment, the cell is a T cell. In one embodiment, the cell is a haematopoietic stem and/or progenitor cell (HSPC).

In one embodiment, the T cell is a chimeric antigen receptor (CAR) T cell. In one embodiment, the T cell comprises an exogenous T cell receptor (TCR).

In one embodiment, the T cell is a CD4+ T cell. In one embodiment, the T cell is a CD8+ T cell.

In one embodiment, the T cell is a regulatory T (Treg) cell. In one embodiment, the T cell is a CD4+CD25+ Treg cell. In one embodiment, the T cell is a CD4+CD25+CD127lo Treg cell. In one embodiment, the T cell is a CD4+CD25+FOXP3+ Treg cell.

In one embodiment, the cell is a T cell and wherein:

(a) T cell exhaustion and/or loss of T cell effector functions is reduced or prevented; and/or

(b) T cell engraftment and/or persistence is increased.

In one embodiment, the cell is a HSPC and wherein HSPC survival and/or engraftment is increased.

In another aspect, the invention provides a method of transducing a population of T cells, preferably regulatory T (Treg) cells, comprising the steps of:

(a) contacting the population of T cells with cyclosporin H (CsH) or a derivative thereof; and

(b) transducing the population of T cells with a vector.

The efficiency of transduction may be increased, for example compared to a method of transduction lacking the contacting of the population of T cells with CsH or a derivative thereof.

DESCRIPTION OF THE DRAWINGS

Figure 1 – Impact of CsH on HSPC global proteome. A. Global proteomic profile of cord blood- derived CD34+ cells from three different healthy donors, upon CsH exposure. **B.** EnrichR analysis reveal an upregulation of metabolic pathways and a down regulation of genes belonging to TCR signalling in CsH treated samples, as compared to control (dmso).

Figure 2 – CsH-induced increase in acylcarnitines and free fatty acids at steady state in HSC. Metabolomic analysis on mobilized peripheral blood-derived CD34+ (mPB-CD34+) cells exposed to CsH vs dmso. **A.** General scheme of the transport of free fatty acids from the cytosol to the mitochondrial matrix. Carnitine palmityl transferase (CPT1), Carnitine-acylcarnitine translocase (CACT) **B.** Box plots of the single acylcarnitines and free fatty acids, expressed as normalized levels (normalized to the DMSO groups and autoscaled in MetaboAnalyst v5.0) shown as floating bars (min to max with line at mean). Statistical analysis was performed using 2way ANOVA with Holm-Sidak's multiple comparisons test (*$p<0.05$,**$p<0.01$). Graphs and statistical analysis prepared using GraphPad Prism v9.1.1.

Figure 3 – Metabolic alterations upon CsH exposure in primary HSPC. Oxygen consumption rate (OCR) profile (**A**) and relative quantification of ATP production and Spare respiratory capacity (**B**), evaluated through Seahorse Cell Mito Stress Test on CD34+ HSPC exposed or not to CsH. **C**. UMAP plots of Single-cell RNASeq analysis comparing CD34+ HSPC from healthy donor (HD) and patients affected by Fanconi Anemia (FA). **D**. Heat map

of pathways significantly enriched in FA vs HD HSPC. **E**. Impactof CsH on the enriched pathways in both FA and HD HSPC.

Figure 4 – CsH does not alter HSPC composition or proliferation ex vivo. A. Subpopulation composition of mPB-CD34+ HSPC, measured 24 hours after CsH exposure vs dmso. **B.** Cell cycle distribution of HSPC, expressed as percentage of cells within G0, G1, S and G2/M phases. Data represent the mean of 3 independent experiments. **C.** Proliferation analysis, expressed as mean fluorescence intensity (MFI) of eFluor 670 cell labelling dye, at the indicated time points after CsH exposure.

Figure 5 – Metabolic alterations upon CsH exposure in primary T cells. Oxygen consumption rate (OCR) profile (**A**) and relative quantification of basal and maximal respiration, and ATP production (**B**), evaluated through Seahorse Cell Mito Stress Test on both CD4+ and CD8+ cells, 4 days after T cell transduction, with or w/o CsH. **C.** Gene expression analysis of the most significant metabolic genes downregulated upon CsH exposure, expressed in fold change vs control (dmso), normalized to 18S ribosomal RNA.

Figure 6 – CAR-T cell manufacturing in the presence of CsH partially prevents exhaustion. Exhausted T cells are identified as LAG3+PD1+CTLA4+ or LAG3+PD1+TIM3+ cells in human (**A**) and murine (**B**) primary T cells, acquired from flow cytometry analysis, 4 days after transduction, with or w/o CsH. **C.** Transduction efficiency, expressed as percentage of NGFR+ cells, in T cell stem memory (TSCM) cells, within CD3+ CD4+ and CD8+ populations, transduced with or w/o CsH. TSCM cells are identified as CD62L+CD45RA+ cells. **D.** Proliferation analysis are expressed as mean fluorescence intensity (MFI) of eFluor 670 cell labelling dye, at the indicated time points.

Figure 7 – CsH enhances Treg transduction efficiency. Transduction efficiency of CD19+ CAR Treg cells from 4 independent experiments and 4 HD assessed with FC at day 15 post transduction (LV MOI:10). (a) Representative FC plots show CAR expression (%NGFR+) after gating on CD25+CD127lo cells. (b) Data display %NGFR and gMFI pooled from 4 donors after gating on CD4+ T cells. (c) Data display gMFI NGFR pooled from 4 donors after gating on FOXP3+ T cells.

Figure 8 – CsH enhances PD-1 expression levels of CD19 CAR Tregs. (a) Graphs shows the expansion curve from day 0 to day 14 of both untransduced cells and CAR-Tregs transduced with or without CsH at different MOI (2-5-10). (b) Data display % CD25+ CD127lo cells gating on CD4+; the % and gMFI of FoxP3+, the % of CTLA-4+ and % with gMFI of PD-

1 cells gating on CD25+ CD127lo Treg cells. Results in (b) show data pooled from 4 or 2 HD after transduction with CD19 CAR LV at MOI 10.

Figure 9 – CsH increases both IL-10 and IFNγ production while reducing TNF expression in CAR Tregs. Production of proinflammatory and immunoregulatory cytokines from 4 independent experiments and 4 HD assessed with FC at day 15 post transduction (LV MOI:10). (a) Graphs show % of IFNγ, IL-10 and TNF after gating on CD4+. (b) Data display % of double-positive IFNγ, IL-10 cells pooled from 4 donors after gating on CD4+ T cells.

Figure 10 – The effect of CsH on Tregs' suppressive function. CsH-treated vs DMSO-treated CD19 CAR Tregs were co-cultured with heterologous PBMC, previously stained with CSFE, in the presence of anti-CD3/CD28-coated beads at various PBMC:Treg ratios. Five days after stimulation, Teff (gated on CD8+) proliferation was assessed by FC. (a) Representative data. (b) Results expressed as suppression % (mean ± standard deviation).

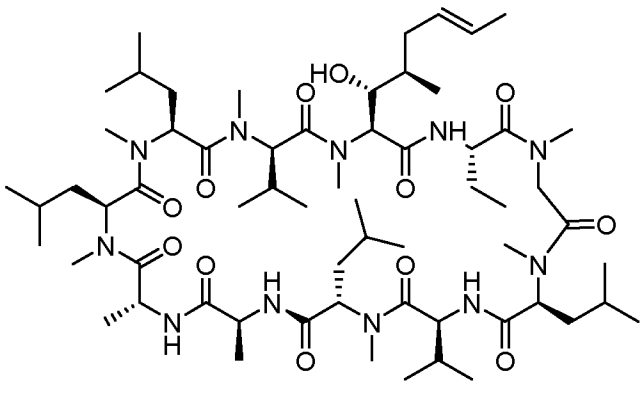
Figure 11 – Effect of CsH on counteracting the generation of exhausted CAR T cells in vitro. The impact of CsH was tested on CAR T cells targeting the GD2 antigen that were exposed to Staphylococcal enterotoxin B (SEB). Results show final average exhaustion with SEB (n=3 subpopulations of exhausted cells at day 7).

DETAILED DESCRIPTION OF THE INVENTION

The terms "comprising", "comprises" and "comprised of" as used herein are synonymous with "including" or "includes"; or "containing" or "contains", and are inclusive or open-ended and do not exclude additional, non-recited members, elements or steps. The terms "comprising", "comprises" and "comprised of" also include the term "consisting of".

Cyclosporin H

Cyclosporin H (CsH, CAS No. 83602-39-5) is a cyclic undecapeptide having the following structure:



CsH is known to selectively antagonise the formyl peptide receptor, however unlike cyclosporin A (CsA), CsH does not bind cyclophilin to evoke immunosuppression. CsA mediates immunosuppression as a complex with the host peptidyl-prolyl isomerase cyclophilin A (CypA). This inhibits the Ca^{2+}-dependent phosphatase calcineurin and consequent activation of pro-inflammatory cytokines such as IL-2 (Sokolskaja, E. et al. (2006) Curr. Opin. Microbiol. 9: 404-8).

Solutions of CsH for use in the present invention may be prepared using routine methods known in the art.

The concentration at which CsH or a derivative thereof is applied to a population of cells may be adjusted for different vector systems to optimise the effect disclosed herein. A skilled person may therefore select a suitable concentration of CsH or a derivative thereof to maximise the effect disclosed herein while minimising any toxicity.

The present invention encompasses the use of CsH and derivatives of CsH. The CsH derivatives of the present invention are those which reduce or prevent T cell exhaustion, and/or increase T cell engraftment and/or persistence; or increase survival of and/or engraftment by haematopoietic stem and/or progenitor cells (HSPCs).

CsH derivatives of the present invention may have been developed for increased solubility, increased stability and/or reduced toxicity.

CsH derivatives of the invention are preferably of low toxicity for mammals, in particular humans. Preferably, CsH derivatives of the invention are of low toxicity for haematopoietic stem and/or progenitor cells; and/or T cells.

Exhaustion and persistence

Cancer immunotherapy may utilise the ability of T cells to recognize and kill their targets and generate antigen-specific memory T cells. Adoptive T cell therapy (ACT) with lymphocytes genetically engineered to express a tumor-specific T-cell Receptor (TCR) or a chimeric antigen receptor (CAR) has shown promising results in clinical trials, although short T cell persistence and T cell exhaustion may limit the efficacy of ACT.

Once tumor-specific T cells infiltrate the tumor, chronic antigen stimulation drives T cell exhaustion, a dysfunctional state characterized by a loss of effector functions. A hallmark of exhaustion is the upregulation of a panel of inhibitory receptors (IRs) including PD-1, CTLA-4, LAG-3, Tim-3 and 2B4. In this context, exhaustion may refer to increased expressed of one

or more of PD-1, CTLA-4, LAG-3, Tim-3 and 2B4 (for example LAG-3, PD-1 and CTLA-4; or LAG-3, PD-1 and Tim-3).

In some embodiments, the uses and methods of the invention reduce the number of LAG-3+ PD-1+CTLA-4+ cells in a population of T cells. In some embodiments, the uses and methods of the invention reduce the number of LAG-3+PD-1+Tim-3+ cells in a population of T cells. The reduction may be, for example, a reduction of at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80% or 90%.

Inhibitory receptors (IRs) are a variety of molecules expressed by T cells, involved in the regulation of motility, cytokine production and effector functions, thus orchestrating peripheral self-tolerance maintenance and acting as breaks for T cell responses. Their ligands are widely upregulated on a broad spectrum of tumor cells and other cells of the tumor microenvironment, thus the interaction between ligands and inhibitory receptors dampens anti-tumor immunity and results in T cell exhaustion and tumor escape. The co-expression of several IRs such as PD-1, LAG-3 or Tim-3, in the absence of activation markers, on antigen-specific T cells represents a hallmark of T cell exhaustion. Physiologically, co-stimulatory and co-inhibitory molecules co-evolved to regulate T cell activation upon TCR triggering. Pivotal initial studies in mice demonstrated the importance of IRs in modulating immune responses. CTLA-4 knock-out mice develop lymphoproliferative disorders, that cause extensive tissue damage and death in the first month of age. PD-1 deficient mice develop autoimmune diseases. These evidences showed that CTLA-4 plays a role in T cell priming, while PD-1 regulation is important in effector T cells in order to prevent excessive tissue damage. Phenotypic characterization of human T cells from healthy subjects point to a dynamic and variegate expression of the IR profile, that varies according to T cell subset, differentiation and activation. For instance, 2B4, KLRG1 and CD160 are highly expressed in effector cells, while BTLA is mainly expressed by naïve T cells. Other IRs, including CTLA-4, Tim-3 and LAG-3, are not expressed by steady state CD8 T cells. Importantly, IRs can be also expressed by early differentiated TSCM, as shown in subjects vaccinated against yellow fever.

Cell survival and engraftment

The term “survival” as used herein refers to the ability of the cells, for example haematopoietic stem and/or progenitor cells to remain alive (e.g. not die or become apoptotic) during in vitro or ex vivo culture. Cells, for example haematopoietic stem and/or progenitor cells may undergo, for example, increased apoptosis following transduction with a vector during cell culture; thus, the surviving cells may have avoided apoptosis and/or cell death.

Cell survival may be readily analysed by the skilled person. For example, the numbers of live, dead and/or apoptotic cells in a cell culture may be quantified at the beginning of culture and/or following culture for a period of time (e.g. about 6 or 12 hours, or 1, 2, 3, 4, 5, 6, 7 or more days; preferably, the period of time begins with the transduction of the cells with a vector). The effect of an agent on cell survival may be assessed by comparing the numbers and/or percentages of live, dead and/or apoptotic cells at the beginning and/or end of the culture period between experiments carried out in the presence and absence of the agent, but under otherwise substantially identical conditions.

Cell numbers and/or percentages in certain states (e.g. live, dead or apoptotic cells) may be quantified using any of a number of methods known in the art, including use of haemocytometers, automated cell counters, flow cytometers and fluorescence activated cell sorting machines. These techniques may enable distinguishing between live, dead and/or apoptotic cells. In addition or in the alternative, apoptotic cells may be detected using readily available apoptosis assays (e.g. assays based on the detection of phosphatidylserine (PS) on the cell membrane surface, such as through use of Annexin V, which binds to exposed PS; apoptotic cells may be quantified through use of fluorescently-labelled Annexin V), which may be used to complement other techniques.

The term “engraftment” as used herein refers to the ability of the cells to populate and/or survive in a subject following their transplantation, i.e. in the short and/or long term after transplantation. For example, engraftment may refer to the number and/or percentages of haematopoietic cells descended from the transplanted haematopoietic stem and/or progenitor cells (e.g. graft-derived cells) that are detected about 1 day to 24 weeks, 1 day to 10 weeks, or 1-30 days or 10-30 days after transplantation. In the xenograft model of human haematopoietic stem and/or progenitor cell engraftment and repopulation, engraftment may be evaluated in the peripheral blood as the percentage of cells deriving from the human xenograft (e.g. positive for the CD45 surface marker), for example. In one embodiment, engraftment is assessed at about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or 30 days after transplantation. In another embodiment, engraftment is assessed at about 4, 5, 6, 7, 8, 9, 10 ,11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23 or 24 weeks after transplantation. In another embodiment, engraftment is assessed at about 16-24 weeks, preferably 20 weeks, after transplantation.

Engraftment may be readily analysed by the skilled person. For example, the transplanted cells may be engineered to comprise a marker (e.g. a reporter protein, such as a fluorescent protein), which can be used to quantify the graft-derived cells. Samples for analysis may be extracted from relevant tissues and analysed ex vivo (e.g. using flow cytometry).

Transduction efficiency

In one aspect, the invention provides use of cyclosporin H (CsH) or a derivative thereof for increasing regulatory T (Treg) cell transduction efficiency.

Increasing the efficiency of transduction refers to an increase in the transduction of the cells (e.g. Treg cells) contacted with an agent (e.g. CsH or a derivative thereof), in comparison to the transduction achieved in the absence of the agent but under otherwise substantially identical conditions. An increased efficiency of transduction may therefore allow the multiplicity of infection (MOI) and/or the transduction time required to achieve effective transduction to be reduced.

In one embodiment, the percentage of cells transduced by the vector is increased (e.g. by at least 5%, 10%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80% or 90%). In another embodiment, the vector copy number per cell is increased (e.g. by at least 1-fold, 2-fold, 3-fold, 4-fold, 5-fold or 10-fold). Preferably both are achieved at the same time.

Methods for determining the percentage of cells transduced by a vector are known in the art. Suitable methods include flow cytometry, fluorescence-activated cell sorting (FACS) and fluorescence microscopy. The technique employed is preferably one which is amenable to automation and/or high throughput screening.

For example, a population of cells may be transduced with a vector which harbours a reporter gene. The vector may be constructed such that the reporter gene is expressed when the vector transduces a cell. Suitable reporter genes include genes encoding fluorescent proteins, for example green, yellow, cherry, cyan or orange fluorescent proteins. Once the population of cells has been transduced by the vector, both the number of cells expressing and not-expressing the reporter gene may be quantified using a suitable technique, such as FACS. The percentage of cells transduced by the vector may then be calculated.

Alternatively, quantitative PCR (qPCR) may be used to determine the percentage of cells transduced by a vector that does not harbour a reporter gene. For example, single colonies of cells may be picked from a semi-solid culture and qPCR may be performed on each colony separately to determine the percentage of vector-positive colonies among those analysed.

Methods for determining vector copy number are also known in the art. The technique employed is preferably one which is amenable to automation and/or high throughput screening. Suitable techniques include quantitative PCR (qPCR) and Southern blot-based approaches.

Immunomodulatory profile

In one aspect, the invention provides use of cyclosporin H (CsH) or a derivative thereof for improving the immunomodulatory profile of a regulatory T (Treg) cell, for example a Treg cell transduced or transfected with a vector.

Improving the immunomodulatory profile may refer to an improvement in the immunomodulatory profile of the cells contacted with an agent (e.g. CsH or a derivative thereof) in comparison to cells not contacted with the agent but under otherwise substantially identical conditions.

Improved immunomodulatory profile may, for example, comprise increased immunoregulatory cytokine (e.g. IFNγ and/or IL-10, preferably IFNγ and IL-10) production by the Treg cell. The immunoregulatory cytokine production may be increased by, for example, at least 1-fold, 2-fold, 3-fold, 4-fold, 5-fold or 10-fold.

Improved immunomodulatory profile may, for example, comprise decreased proinflammatory cytokine (e.g. TNF) production by the Treg cell. The proinflammatory cytokine production may be decreased by, for example, at least 1-fold, 2-fold, 3-fold, 4-fold, 5-fold or 10-fold.

Improved immunomodulatory profile may, for example, comprise both increased immunoregulatory cytokine production and decreased proinflammatory cytokine production by the Treg cell.

Methods for determining levels of cytokine production are well known to the skilled person, and include methods disclosed herein.

T cells

In one embodiment, cell is a T cell.

T cells (or T lymphocytes) are a type of lymphocyte that play a central role in cell-mediated immunity. They can be distinguished from other lymphocytes, such as B cells and natural killer cells (NK cells), by the presence of a T-cell receptor (TCR) on the cell surface.

In one embodiment, the T cells are resting T cells. Resting CD4+ T cells are quiescent. In one embodiment, the T cells are unstimulated T cells. In one embodiment, the T cells are stimulated T cells. Once stimulated, resting T cells proliferate and generate a large clone of antigen-specific cells. In one embodiment, the T cells are CD4+ T cells. In one embodiment, the T cells are CD8+ T cells. In one embodiment, the T cells are CD3+ T cells.

In one embodiment, the T cells are Stem memory T cells; Central Memory T cells; Effector Memory T cells; and/or terminally differentiated effector memory T cells.

In one embodiment, the T cell is a regulatory T (Treg) cell.

Regulatory T cells (Treg cells) are a subpopulation of T cells that modulate the immune system and maintain tolerance to self-antigens. Treg cells are immunosuppressive and may suppress induction and proliferation of effector T cells.

In one embodiment, the T cell is a CD4+CD25+ Treg cell. In one embodiment, the T cell is a CD4+CD25+CD127lo Treg cell. In one embodiment, the T cell is a CD4+CD25+FOXP3+ Treg cell.

Haematopoietic stem and progenitor cells

A stem cell is able to differentiate into many cell types. A cell that is able to differentiate into all cell types is known as totipotent. In mammals, only the zygote and early embryonic cells are totipotent. Stem cells are found in most, if not all, multicellular organisms. They are characterised by the ability to renew themselves through mitotic cell division and differentiate into a diverse range of specialised cell types. The two broad types of mammalian stem cells are embryonic stem cells that are isolated from the inner cell mass of blastocysts, and adult stem cells that are found in adult tissues. In a developing embryo, stem cells can differentiate into all of the specialised embryonic tissues. In adult organisms, stem cells and progenitor cells act as a repair system for the body, replenishing specialised cells, but also maintaining the normal turnover of regenerative organs, such as blood, skin or intestinal tissues.

Haematopoietic stem cells (HSCs) are multipotent stem cells that may be found, for example, in peripheral blood, bone marrow and umbilical cord blood. HSCs are capable of self-renewal and differentiation into any blood cell lineage. They are capable of recolonising the entire immune system, and the erythroid and myeloid lineages in all the haematopoietic tissues (such as bone marrow, spleen and thymus). They provide for life-long production of all lineages of haematopoietic cells.

Haematopoietic progenitor cells have the capacity to differentiate into a specific type of cell. In contrast to stem cells however, they are already far more specific: they are pushed to differentiate into their “target” cell. A difference between stem cells and progenitor cells is that stem cells can replicate indefinitely, whereas progenitor cells can only divide a limited number of times. Haematopoietic progenitor cells can be rigorously distinguished from HSCs only by

functional in vivo assay (i.e. transplantation and demonstration of whether they can give rise to all blood lineages over prolonged time periods).

The haematopoietic stem and progenitor cells of the invention comprise the CD34 cell surface marker (denoted as CD34+).

Haematopoietic stem and progenitor cell (HSPC) source

A population of haematopoietic stem and/or progenitor cells may be obtained from a tissue sample.

For example, a population of haematopoietic stem and/or progenitor cells may be obtained from peripheral blood (e.g. adult and foetal peripheral blood), umbilical cord blood, bone marrow, liver or spleen. Preferably, these cells are obtained from peripheral blood or bone marrow. They may be obtained after mobilisation of the cells in vivo by means of growth factor treatment.

Mobilisation may be carried out using, for example, G-CSF, plerixaphor or combinations thereof. Other agents, such as NSAIDs and dipeptidyl peptidase inhibitors, may also be useful as mobilising agents.

With the availability of the stem cell growth factors GM-CSF and G-CSF, most haematopoietic stem cell transplantation procedures are now performed using stem cells collected from the peripheral blood, rather than from the bone marrow. Collecting peripheral blood stem cells provides a bigger graft, does not require that the donor be subjected to general anaesthesia to collect the graft, results in a shorter time to engraftment and may provide for a lower long-term relapse rate.

Bone marrow may be collected by standard aspiration methods (either steady-state or after mobilisation), or by using next-generation harvesting tools (e.g. Marrow Miner).

In addition, haematopoietic stem and progenitor cells may also be derived from induced pluripotent stem cells.

HSC characteristics

HSCs are typically of low forward scatter and side scatter profile by flow cytometric procedures. Some are metabolically quiescent, as demonstrated by Rhodamine labelling which allows determination of mitochondrial activity. HSCs may comprise certain cell surface markers such as CD34, CD45, CD133, CD90 and CD49f. They may also be defined as cells lacking the expression of the CD38 and CD45RA cell surface markers. However, expression

of some of these markers is dependent upon the developmental stage and tissue-specific context of the HSC. Some HSCs called “side population cells” exclude the Hoechst 33342 dye as detected by flow cytometry. Thus, HSCs have descriptive characteristics that allow for their identification and isolation.

Negative markers

CD38 is the most established and useful single negative marker for human HSCs.

Human HSCs may also be negative for lineage markers such as CD2, CD3, CD14, CD16, CD19, CD20, CD24, CD36, CD56, CD66b, CD271 and CD45RA. However, these markers may need to be used in combination for HSC enrichment.

By “negative marker” it is to be understood that human HSCs lack the expression of these markers.

Positive markers

CD34 and CD133 are the most useful positive markers for HSCs.

Some HSCs are also positive for lineage markers such as CD90, CD49f and CD93. However, these markers may need to be used in combination for HSC enrichment.

By “positive marker” it is to be understood that human HSCs express these markers.

In one embodiment, the haematopoietic stem and progenitor cells are CD34+CD38- cells.

Differentiated cells

A differentiated cell is a cell which has become more specialised in comparison to a stem cell or progenitor cell. Differentiation occurs during the development of a multicellular organism as the organism changes from a single zygote to a complex system of tissues and cell types. Differentiation is also a common process in adults: adult stem cells divide and create fully-differentiated daughter cells during tissue repair and normal cell turnover. Differentiation dramatically changes a cell’s size, shape, membrane potential, metabolic activity and responsiveness to signals. These changes are largely due to highly-controlled modifications in gene expression. In other words, a differentiated cell is a cell which has specific structures and performs certain functions due to a developmental process which involves the activation and deactivation of specific genes. Here, a differentiated cell includes differentiated cells of the haematopoietic lineage such as monocytes, macrophages, neutrophils, basophils, eosinophils, erythrocytes, megakaryocytes/platelets, dendritic cells, T cells, B-cells and NK-

cells. For example, differentiated cells of the haematopoietic lineage can be distinguished from stem cells and progenitor cells by detection of cell surface molecules which are not expressed or are expressed to a lesser degree on undifferentiated cells. Examples of suitable human lineage markers include CD33, CD13, CD14, CD15 (myeloid), CD19, CD20, CD22, CD79a (B), CD36, CD71, CD235a (erythroid), CD2, CD3, CD4, CD8 (T) and CD56 (NK).

Isolation and enrichment of populations of cells

The term "isolated population" of cells as used herein may refer to the population of cells having been previously removed from the body. An isolated population of cells may be cultured and manipulated ex vivo or in vitro using standard techniques known in the art. An isolated population of cells may later be reintroduced into a subject. Said subject may be the same subject from which the cells were originally isolated or a different subject.

A population of cells may be purified selectively for cells that exhibit a specific phenotype or characteristic, and from other cells which do not exhibit that phenotype or characteristic, or exhibit it to a lesser degree. For example, a population of cells that expresses a specific marker (such as CD34) may be purified from a starting population of cells. Alternatively, or in addition, a population of cells that does not express another marker (such as CD38) may be purified.

By "enriching" a population of cells for a certain type of cells it is to be understood that the concentration of that type of cells is increased within the population. The concentration of other types of cells may be concomitantly reduced.

Purification or enrichment may result in the population of cells being substantially pure of other types of cell.

Purifying or enriching for a population of cells expressing a specific marker (e.g. CD34 or CD38) may be achieved by using an agent that binds to that marker, preferably substantially specifically to that marker.

An agent that binds to a cellular marker may be an antibody, for example an anti-CD34 or anti-CD38 antibody.

The term "antibody" refers to complete antibodies or antibody fragments capable of binding to a selected target, and including Fv, ScFv, F(ab′) and $F(ab')_2$, monoclonal and polyclonal antibodies, engineered antibodies including chimeric, CDR-grafted and humanised antibodies, and artificially selected antibodies produced using phage display or alternative techniques.

In addition, alternatives to classical antibodies may also be used in the invention, for example “avibodies”, “avimers”, “anticalins”, “nanobodies” and “DARPins”.

The agents that bind to specific markers may be labelled so as to be identifiable using any of a number of techniques known in the art. The agent may be inherently labelled, or may be modified by conjugating a label thereto. By “conjugating” it is to be understood that the agent and label are operably linked. This means that the agent and label are linked together in a manner which enables both to carry out their function (e.g. binding to a marker, allowing fluorescent identification or allowing separation when placed in a magnetic field) substantially unhindered. Suitable methods of conjugation are well known in the art and would be readily identifiable by the skilled person.

A label may allow, for example, the labelled agent and any cell to which it is bound to be purified from its environment (e.g. the agent may be labelled with a magnetic bead or an affinity tag, such as avidin), detected or both. Detectable markers suitable for use as a label include fluorophores (e.g. green, cherry, cyan and orange fluorescent proteins) and peptide tags (e.g. His tags, Myc tags, FLAG tags and HA tags).

A number of techniques for separating a population of cells expressing a specific marker are known in the art. These include magnetic bead-based separation technologies (e.g. closed-circuit magnetic bead-based separation), flow cytometry, fluorescence-activated cell sorting (FACS), affinity tag purification (e.g. using affinity columns or beads, such biotin columns to separate avidin-labelled agents) and microscopy-based techniques.

It may also be possible to perform the separation using a combination of different techniques, such as a magnetic bead-based separation step followed by sorting of the resulting population of cells for one or more additional (positive or negative) markers by flow cytometry.

Clinical grade separation may be performed, for example, using the CliniMACS® system (Miltenyi). This is an example of a closed-circuit magnetic bead-based separation technology.

It is also envisaged that dye exclusion properties (e.g. side population or rhodamine labelling) or enzymatic activity (e.g. ALDH activity) may be used to enrich for haematopoietic stem cells.

Vector

A vector is a tool that allows or facilitates the transfer of an entity from one environment to another. In accordance with the present invention, and by way of example, some vectors used in recombinant nucleic acid techniques allow entities, such as a segment of nucleic acid (e.g. a heterologous DNA segment, such as a heterologous cDNA segment), to be transferred into

a target cell. The vector may serve the purpose of maintaining the heterologous nucleic acid (DNA or RNA) within the cell, facilitating the replication of the vector comprising a segment of nucleic acid, or facilitating the expression of the protein encoded by a segment of nucleic acid.

Vectors may be non-viral or viral. Examples of vectors used in recombinant nucleic acid techniques include, but are not limited to, plasmids, chromosomes, artificial chromosomes and viruses. The vector may be single stranded or double stranded. It may be linear and optionally the vector comprises one or more homology arms. The vector may also be, for example, a naked nucleic acid (e.g. DNA). In its simplest form, the vector may itself be a nucleotide of interest.

The vectors used in the invention may be, for example, plasmid or virus vectors and may include a promoter for the expression of a polynucleotide and optionally a regulator of the promoter.

Vectors comprising polynucleotides used in the invention may be introduced into cells using a variety of techniques known in the art, such as transformation, transfection and transduction. Several techniques are known in the art, for example transduction with recombinant viral vectors, such as retroviral, lentiviral, adenoviral, adeno-associated viral, baculoviral and herpes simplex viral vectors, Sleeping Beauty vectors; direct injection of nucleic acids and biolistic transformation.

Non-viral delivery systems include but are not limited to DNA transfection methods. Here, transfection includes a process using a non-viral vector to deliver a gene to a target cell. Typical transfection methods include electroporation, DNA biolistics, lipid-mediated transfection, compacted DNA-mediated transfection, liposomes, immunoliposomes, lipofectin, cationic agent-mediated transfection, cationic facial amphiphiles (CFAs) (Nature Biotechnology (1996) 14: 556) and combinations thereof.

The term “vector" includes an expression vector, i.e. a construct capable of in vivo or in vitro/ex vivo expression. Expression may be controlled by a vector sequence, or, for example in the case of insertion at a target site, expression may be controlled by a target sequence. A vector may be integrated or tethered to the cell’s DNA.

Viral delivery systems include but are not limited to adenoviral vectors, adeno-associated viral (AAV) vectors, herpes viral vectors, retroviral vectors, lentiviral vectors and baculoviral vectors.

Nucleotide of interest

The vector may comprise a nucleotide of interest (NOI). Preferably the nucleotide of interest gives rise to a therapeutic effect.

In one embodiment, the vector comprises one or more nucleotide sequence encoding a chimeric antigen receptor (CAR). In one embodiment, the vector comprises one or more nucleotide sequence encoding a T cell receptor (TCR).

The CAR may be, for example, a CD19 CAR or a GD2 CAR.

An example nucleotide sequence encoding a CD19 CAR is:

ACCGGTGTACCGAATTCATGCTGCTGCTGGTGACCTCCCTGCTGCTGTGCGAGCTGCCACACCCAGCC
TTCCTGCTGATCCCCGACATCCAGATGACACAGACCACAAGCTCCCTGTCCGCCTCTCTGGGCGACAG
AGTGACCATCTCTTGTAGGGCCAGCCAGGACATCTCCAAGTATCTGAACTGGTATCAGCAGAAGCCCG
ATGGCACAGTGAAGCTGCTGATCTATCACACCTCTCGCCTGCACAGCGGCGTGCCTTCCCGGTTTAGC
GGCTCCGGCTCTGGCACAGACTACTCTCTGACCATCAGCAACCTGGAGCAGGAGGACATCGCCACATA
TTTCTGCCAGCAGGGCAATACACTGCCATACACCTTTGGCGGCGGCACCAAGCTGGAGATCACAGGCA
GCACCTCCGGCTCTGGAAAGCCAGGCTCCGGAGAGGGCTCTACAAAGGGCGAGGTGAAGCTGCAGGAG
TCCGGACCAGGCCTGGTGGCACCTAGCCAGTCCCTGTCTGTGACATGTACCGTGTCCGGCGTGTCTCT
GCCCGACTACGGCGTGAGCTGGATCAGACAGCCACCTAGGAAGGGCCTGGAGTGGCTGGGAGTGATCT
GGGGCTCCGAGACAACATACTATAATAGCGCCCTGAAGTCCCGCCTGACCATCATCAAGGACAACAGC
AAGTCCCAGGTGTTCCTGAAGATGAATAGCCTGCAGACAGACGATACCGCCATCTACTATTGCGCCAA
GCACTACTATTACGGCGGCAGCTACGCTATGGACTACTGGGGCCAGGGCACATCTGTGACCGTGTCTA
GCTTCGTGCCCGTGTTTCTGCCTGCCAAGCCAACCACAACCCCTGCACCACGCCCACCCACACCAGCA
CCTACCATCGCCTCTCAGCCACTGAGCCTGCGCCCCGAGGCCTGCCGGCCTGCAGCAGGCGGCGCCGT
GCACACCCGGGGCCTGGACTTTGCCTGCGACATCTACATCTGGGCACCTCTGGCCGGCACATGTGGCG
TGCTGCTGCTGTCTCTGGTGATCACCCTGTATTGTAACCACCGCAATCGGAGCAAGAGATCCAGGCTG
CTGCACAGCGACTACATGAACATGACACCTAGACGGCCCGGCCCCACCAGAAAGCACTATCAGCCATA
CGCCCCTCCAAGGGACTTCGCCGCCTATCGCAGCCGGGTGAAGTTCAGCCGGAGCGCCGATGCACCTG
CATATCAGCAGGGACAGAATCAGCTGTACAACGAGCTGAATCTGGGCAGGCGCGAGGAGTACGACGTG
CTGGATAAGAGGCGGGGCCGGGACCCCGAGATGGGAGGCAAGCCAAGGCGCAAGAACCCCCAGGAGGG
CCTGTATAATGAGCTGCAGAAGGACAAGATGGCCGAGGCCTACTCCGAGATCGGCATGAAGGGAGAGC
GGAGAAGGGGAAAGGGACACGATGGCCTGTATCAGGGCCTGAGCACAGCCACCAAGGACACCTACGAT
GCACTGCACATGCAGGCCCTGCCACCTAGGTGACTCGAGAGCGGCCGCGTCGAC

(SEQ ID NO: 13)

An example nucleotide sequence encoding a GD2 CAR is:

ATGGAGTTCGGTCTGAGTTGGCTCTTCCTGGTGGCCATCTTGAAGGGCGTGCAGTGTTCGCGGGACAT
ACTTCTCACCCAGACCCCTTTGTCGCTGCCCGTGTCGCTGGGTGACCAGGCCTCAATTTCTTGCCGTT
CCTCCCAGTCCCTGGTGCACCGCAACGGGAACACGTACCTGCATTGGTACTTGCAGAAGCCCGGACAA
AGTCCAAAGTTGTTAATCCACAAGGTGAGCAACCGCTTCAGCGGCGTGCCCGACCGCTTCTCCGGGTC
TGGCTCTGGCACAGACTTTACTCTCAAGATCTCCCGTGTGGAAGCCGAGGACCTGGGCGTTTACTTCT
GCTCCCAGAGCACTCATGTGCCACCTCTGACCTTCGGAGCTGGCACCAAGCTGGAGCTGAAGCGTGCG
GACGCAGCCCCCACAGTTAGCATCTTCCCTGGTGGGGGCGGTTCCGGGGGAGGGGGCAGCGGCGGGGG
TGGTTCAGGTGGGGGAGGTTCCGAAGTCAAGCTCCAGCAGTCTGGTCCGTCCCTGGTGGAACCCGGCG
CTTCCGTGATGATCTCATGCAAGGCATCTGGCTCCTCGTTCACCGGCTACAACATGAACTGGGTGAGA
CAGAATATCGGCAAAAGCCTGGAGTGGATCGGGGCCATTGACCCGTATTACGGCGGCACCAGCTACAA
CCAGAAATTTAAGGGACGCGCTACCCTGACCGTAGATAAGTCGAGTTCCACCGCGTACATGCACCTGA
AGTCTCTGACCAGCGAGGACTCCGCGGTATACTACTGCGTGAGCGGTATGGAGTATTGGGGCCAGGGC
ACTTCTGTCACCGTCTCGAGCACGCGTACGACGACGCCCGCTCCACGTCCCCCTACCCCCGCACCCAC
CATCGCGAGCCAGCCACTCTCTCTTAGACCTGAGGCTTGTCGCCCCGCGGCCGGCGGAGCCGTGCACA
CTCGCGGCTTGGATTTTGCTTGTGACATCTACATTTGGGCCCCGCTGGCCGGCACCTGCGGGGTCCTG
CTGCTCTCCCTAGTCATCACCCTGTACTGCCGCTCCAAGCGCAGCCGCCTGCTGCACTCCGATTACAT
GAATATGACTCCGCGCAGGCCAGGCCCTACCAGGAAACACTACCAGCCGTATGCTCCCCCGCGCGACT
TTGCGGCGTACCGCTCCCGCGTGAAATTCTCTAGGTCCGCCGACGCTCCAGCCTACCAACAGGGCCAG
AACCAGCTCTACAACGAGTTGAACCTGGGCCGACGTGAGGAGTATGACGTGCTGGACAAGCGCCGCGG
TCGCGATCCTGAGATGGGCGGAAAGCCCCGGCGGAAGAATCCTCAGGAGGGCCTGTACAACGAGCTGC
AGAAGGACAAAATGGCCGAGGCCTACAGCGAGATCGGTATGAAAGGCGAGCGAAGGCGCGGAAAGGGC
CACGATGGCCTTTATCAGGGCCTGTCCACTGCTACCAAGGACACCTACGACGCGCTGCATATGCAGGC
CCTGCCCCCCCGCTGA

(SEQ ID NO: 14)

In one embodiment, the nucleotide sequence encoding a CAR comprises or consists of a nucleotide sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity to SEQ ID NO: 13 or 14.

In one embodiment, the nucleotide sequence encoding a CAR comprises or consists of the nucleotide sequence of SEQ ID NO: 13 or 14.

Suitable NOIs include, but are not limited to, sequences encoding enzymes, cytokines, chemokines, hormones, antibodies, anti-oxidant molecules, engineered immunoglobulin-like molecules, single chain antibodies, fusion proteins, immune co-stimulatory molecules, immunomodulatory molecules, anti-sense RNA, microRNA, shRNA, siRNA, ribozymes, miRNA target sequences, a transdomain negative mutant of a target protein, toxins, conditional toxins, antigens, tumour suppressor proteins, growth factors, transcription factors,

membrane proteins, surface receptors, anti-cancer molecules, vasoactive proteins and peptides, anti-viral proteins and ribozymes, and derivatives thereof (such as derivatives with an associated reporter group). The NOIs may also encode pro-drug activating enzymes.

An example of a NOI is the beta-globin chain which may be used for gene therapy of thalassemia/sickle cell disease.

NOIs also include those useful for the treatment of other diseases requiring non-urgent/elective gene correction in the myeloid lineage such as: chronic granulomatous disease (CGD, e.g. the gp91phox transgene), leukocyte adhesion defects, other phagocyte disorders in patients without ongoing severe infections and inherited bone marrow failure syndromes (e.g. Fanconi anaemia), as well as primary immunodeficiencies (SCIDs).

NOIs also include those useful in the treatment of lysosomal storage disorders and immunodeficiencies.

Pharmaceutical composition

The cells of the invention may be formulated for administration to subjects with a pharmaceutically acceptable carrier, diluent or excipient. Suitable carriers and diluents include isotonic saline solutions, for example phosphate-buffered saline, and potentially contain human serum albumin.

Handling of cell therapy products is preferably performed in compliance with FACT-JACIE International Standards for cellular therapy.

Cell transplantation

The invention provides a population of cells of the invention, which may be used as disclosed herein. The cell(s) may be for use in therapy, for example for use in gene therapy.

The use may be as part of an adoptive cell therapy, for example and adoptive T cell therapy.

The adoptive cell therapy may, for example, be adoptive Treg cell therapy. The therapy (e.g. using Treg cells) may be treatment of an autoimmune disease, such as type 1 diabetes, multiple sclerosis, colitis, vitiligo, organ or tissue transplantation, or a monogenic immune-mediated syndrome (e.g. IPEX or graft versus host disease).

The use may be as part of a haematopoietic stem and/or progenitor cell transplantation procedure.

Haematopoietic stem cell transplantation (HSCT) is the transplantation of blood stem cells derived from the bone marrow (in this case known as bone marrow transplantation) or blood. Stem cell transplantation is a medical procedure in the fields of haematology and oncology, most often performed for people with diseases of the blood or bone marrow, or certain types of cancer.

Many recipients of HSCTs are multiple myeloma or leukaemia patients who would not benefit from prolonged treatment with, or are already resistant to, chemotherapy. Candidates for HSCTs include paediatric cases where the patient has an inborn defect such as severe combined immunodeficiency or congenital neutropenia with defective stem cells, and also children or adults with aplastic anaemia who have lost their stem cells after birth. Other conditions treated with stem cell transplants include sickle-cell disease, myelodysplastic syndrome, neuroblastoma, lymphoma, Ewing's Sarcoma, Desmoplastic small round cell tumour and Hodgkin's disease. More recently non-myeloablative, or so-called "mini transplant", procedures have been developed that require smaller doses of preparative chemotherapy and radiation. This has allowed HSCT to be conducted in the elderly and other patients who would otherwise be considered too weak to withstand a conventional treatment regimen.

In one embodiment, the cell(s) is administered as part of an autologous stem cell transplant procedure.

In another embodiment, the cell(s) is administered as part of an allogeneic stem cell transplant procedure.

By "autologous stem cell transplant procedure" it is to be understood that the starting cell(s) (which may then be genetically engineered) is obtained from the same subject as that to which the final cell(s) is administered. Autologous transplant procedures are advantageous as they avoid problems associated with immunological incompatibility and are available to subjects irrespective of the availability of a genetically matched donor.

By "allogeneic stem cell transplant procedure" it is to be understood that the starting cell(s) (which may then be genetically engineered) is obtained from a different subject as that to which the final cell(s) is administered. Preferably, the donor will be genetically matched to the subject to which the cells are administered to minimise the risk of immunological incompatibility.

In one embodiment, the subject is subjected to a mild myeloablative, reduced intensity or non-myeloablative conditioning regimen before administration of the HSPCs.

Suitable doses of cell(s) are such as to be therapeutically and/or prophylactically effective. The dose to be administered may depend on the subject and condition to be treated, and may be readily determined by a skilled person.

Haematopoietic progenitor cells provide short term engraftment. Accordingly, gene therapy by administering haematopoietic progenitor cells would provide a non-permanent effect in the subject. For example, the effect may be limited to 1-6 months following administration of the haematopoietic progenitor cells. An advantage of this approach would be better safety and tolerability, due to the self-limited nature of the therapeutic intervention.

Such haematopoietic progenitor cell gene therapy may be suited to treatment of acquired disorders, for example cancer, where time-limited expression of a (potentially toxic) anti-cancer nucleotide of interest may be sufficient to eradicate the disease.

The invention (e.g. the haematopoietic stem and/or progenitor cell gene therapy) may be, for example, useful in the treatment of a disease selected from the group consisting of mucopolysaccharidosis type I (MPS-1), chronic granulomatous disorder (CGD), Fanconi anaemia (FA), sickle cell disease, Pyruvate kinase deficiency (PKD), Leukocyte adhesion deficiency (LAD), metachromatic leukodystrophy (MLD), globoid cell leukodystrophy (GLD), GM_2 gangliosidosis, thalassemia, cancer, a genetic disease and a blood disease.

The invention may also be, for example, useful in the treatment of mucopolysaccharidoses disorders and other lysosomal storage disorders.

Method of treatment

It is to be appreciated that all references herein to treatment include curative, palliative and prophylactic treatment. The treatment of mammals, particularly humans, is preferred. Both human and veterinary treatments are within the scope of the invention.

Administration

Although the agents for use in the invention (in particular, the cell(s)) can be administered alone, they will generally be administered in admixture with a pharmaceutical carrier, excipient or diluent, particularly for human therapy.

Dosage

The skilled person can readily determine an appropriate dose of one of the agents of the invention to administer to a subject without undue experimentation. Typically, a physician will determine the actual dosage which will be most suitable for an individual patient and it will

depend on a variety of factors including the activity of the specific agent employed, the metabolic stability and length of action of that agent, the age, body weight, general health, sex, diet, mode and time of administration, rate of excretion, drug combination, the severity of the particular condition, and the individual undergoing therapy. There can of course be individual instances where higher or lower dosage ranges are merited, and such are within the scope of the invention.

Subject

A "subject" refers to either a human or non-human animal.

Examples of non-human animals include vertebrates, for example mammals, such as non-human primates (particularly higher primates), dogs, rodents (e.g. mice, rats or guinea pigs), pigs and cats. The non-human animal may be a companion animal.

Preferably, the subject is a human.

The skilled person will understand that they can combine all features of the invention disclosed herein without departing from the scope of the invention as disclosed.

Preferred features and embodiments of the invention will now be described by way of non-limiting examples.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of chemistry, biochemistry, molecular biology, microbiology and immunology, which are within the capabilities of a person of ordinary skill in the art. Such techniques are explained in the literature. See, for example, Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory Press; Ausubel, F.M. et al. (1995 and periodic supplements) Current Protocols in Molecular Biology, Ch. 9, 13 and 16, John Wiley & Sons; Roe, B., Crabtree, J. and Kahn, A. (1996) DNA Isolation and Sequencing: Essential Techniques, John Wiley & Sons; Polak, J.M. and McGee, J.O'D. (1990) In Situ Hybridization: Principles and Practice, Oxford University Press; Gait, M.J. (1984) Oligonucleotide Synthesis: A Practical Approach, IRL Press; and Lilley, D.M. and Dahlberg, J.E. (1992) Methods in Enzymology: DNA Structures Part A: Synthesis and Physical Analysis of DNA, Academic Press. Each of these general texts is herein incorporated by reference.

EXAMPLES

EXAMPLE 1

MATERIALS AND METHODS

Primary cells

Human CD34+ hematopoietic stem/progenitor cells (HSPC) were isolated through positive magnetic bead selection according to manufacturer's instructions (Miltenyi) from umbilical cord blood (CB-CD34+), or directly purchased from Lonza or Hemacare (mobilized peripheral blood, mPB-CD34+). HSPCs were cultured at a concentration of 1×10^6 cells per milliliter, in serum-free StemSpan medium (StemCell Technologies) supplemented with penicillin (100 IU/ml), streptomycin (100 µg/ml), 100 ng/ml recombinant human stem cell factor (rhSCF), 20 ng/ml recombinant human thrombopoietin (rhTPO), 100 ng/ml recombinant human Flt3 ligand (rhFlt3), and 20 ng/ml recombinant human IL6 (rhIL6) (all from Peprotech) 24 hours (prestimulation). Cells were then exposed to 8uM Cyclosporyn H (CsH) or to DMSO (as control) for 16 hours, and then collected for proteomic or metabolomics analysis.

Peripheral blood mononuclear cells (PBMCs) were freshly purified from Buffy Coat from healthy donors, using Ficoll by sequential centrifugations. CD3+ T cells were isolated and stimulated using magnetic beads (ratio cell:bead 1:1) conjugated with anti-CD3/anti-CD28 antibodies (Dynabeads human T-activator CD3/CD28, Thermo Fisher). CD4+ and CD8+ T cells were isolated by immune-magnetic separation using CD4 or CD8 T-cell isolation kits (Miltenyi Biotech) according the manufacturer's instructions, and stimulated using Dynabeads. Cells were maintained in RPMI medium supplemented with 10% FBS, 1% penicillin/streptomycin, 2% glutamine, 1X Non-Essential Amino Acids, 1mM Sodium Pyruvate, IL-7 (5 ng/ml; PreproTech) and IL-15 (5 ng/ml; PreproTech). Dynabeads were removed after 3 days of culture and primary T cells were transduced with the given lentiviral vector, at the indicated multiplicity of infection (MOI), in the presence or absence of CsH 8uM, and diluted 1:1 with fresh medium after 16 hours. Cells were collected 4 days after transduction for flow cytometry, molecular and functional analyses. All cells were cultured in a 5% CO_2 humidified atmosphere at 37°C.

Fanconi Anemia samples were provided by Carlo Dufour from Istituto Giannina Gaslini, Genoa, Italy. Bone marrow-derived CD34+ cells (BM-$CD34^+$) from 2 healthy donors and 3 FANCA patients were cultured in CellGenix GMP SCGM serum-free medium, Xeno-free [20802-0500], supplemented with: 1% P/S - 2% Glutamine - 100ng/ml hSCF - 100ng/ml hFlt3 - 100ng/ml hTPO - 20ng/ml IL-3 - 10ug/ml infliximab [anti TNFalpha] - 1mM N-Acetylcistein. After 10 hours of stimulation, cells were exposed to 8uM CsH for 12 hours and then collected for scRNA-seq experiments.

Viral vectors

Lentiviral vectors were produced by transient transfection in 293T cells and were all VSV-g pseudotyped and concentrated by ultracentrifugation as already described (Montini *et al*, 2006). The previously reported Anti-CD19 CAR sequence (J.N. Brudno et al., Nat Med 2020) has been cloned into a bidirectional plasmid, under the control of the human PGK promoter. The NGFR is expressed from the minimal CMV promoter in the opposite orientation.

Flow cytometry analysis

Cytofluorimetric analyses were performed on FACS Canto II (BD Pharmingen) equipped with DIVA Software, and analyzed either with the FSC express software. Fluorescence Minus One (FMO) stained cells were used as controls. Single-color controls CompBeads (BD Pharmingen) were used as compensation beads to set gating parameters and optimize voltages. For surface sample staining, antibodies are: Anti human CD3 Percp5.5 (BioLegend - 300328), Anti human CD4 PB (BD Pharmingen - 558116),Anti human CD8 APC-H7 (BD Pharmingen - 641400), Anti human PD1 APC (BD Pharmingen - 329908), Anti human CD366 (Tim-3) PEcy7 (BD Pharmingen - 345014), Anti human CD223 (Lag-3) PE (BD Pharmingen - 369306), Anti human CD152 (CTLA-4) PEcy7 (BioLegend - 349913), Anti human CD271 (LNGFR) APC (Miltenyi Biotec 130-113-418), Anti human CD34 PEcy7 (BD Pharmingen - 348811), Anti human CD133/2 (293C3) PE (Miltenyi Biotec 130-113-186), Anti human CD90 APC (BD Pharmingen - 559869), Anti human CD62L PE (BioLegend - 304822), and Anti human CD45RA APC (BD Pharmingen - 550855).

Cell Proliferation assay

The proliferation assay was performed with Cell Proliferation Dye eFluor® 670 (Affimetrix, eBioscience), according to the manufacturer's instructions. During cell division, this dye will be distributed equally between daughter cells, and can be measured as successive halving of the fluorescence intensity. Cells were stained and analyzed at flow cytometry at different time points. Rainbow Calibration Particles (BD Pharmingen) were analyzed before every acquisition as an internal control for calibration.

Cell Cycle assay

The cell cycle analysis was performed by Ki67 (BD Pharmingen) and Hoechst (Invitrogen) staining, according to the manufacturer's instructions. Cells were stained and analyzed at flow cytometry, 24 hours after CsH exposure.

RNA extraction, RT-qPCR and gene expression analysis

RNA extraction from cells was performed using the RNeasy Plus micro Kit (QIAGEN), according to manufacturer's instructions. The extracted mRNAs were reverse transcribed (RT) using the SuperScript Vilo kit (Invitrogen). qPCR were performed using Fast Sybr green pcr mastermix (Thermofisher) and run using the Viia7 Real-Time PCR system (Thermofisher). Relative quantification values were calculated as the fold-change expression of the gene of interest over its expression in the reference sample, by the formula 2–ΔΔCt. The expression was normalized using the housekeeping gene 18S ribosomal RNA, whose expression is not affected upon metabolic variation. Primers were designed using Primer3 (version 0.4.0) tool, and their specificity was verified in UCSC genome browser. The sequences are listed below:

hNRF1	F 5'-CGGAATTCCAGTCTCTGTGG-3' (SEQ ID NO: 1)
	R 5'-TGAAACCCTCTGCTTTTGCT-3' (SEQ ID NO: 2)
hNRF2	F 5'-GCGACGGAAAGAGTATGAGC-3' (SEQ ID NO: 3)
	R 5'-TTGGGAATGTGGGCAACCT-3' (SEQ ID NO: 4)
hCPT1a	F 5'-CCAGACGAAGAACGTGGTCA-3' (SEQ ID NO: 5)
	R 5'-ATCTTGCCGTGCTCAGTGAA-3' (SEQ ID NO: 6)
hCPT2	F 5'-GGCTGCCTATTCCCAAACTT-3' (SEQ ID NO: 7)
	R 5'-CATATCAAACCAGGGTCCCG-3' (SEQ ID NO: 8)
hPPARγ	F 5'-CCAACTCCCTCATGGCAATT-3' (SEQ ID NO: 9)
	R 5'-GGCATTATGAGACATCCCCA-3' (SEQ ID NO: 10)
h18S	F 5'-GAGGATGAGGTGGAACGTGT -3' (SEQ ID NO: 11)
	F 5'-TCTTCAGTCGCTCCAGGTCT-3' (SEQ ID NO: 12)

Seahorse assay

Oxygen consumption rate (OCR) was measured on the SeahorseXFe96 Analyzer (Agilent Technologies, Santa Clara,CA, USA) using SeaHorse XF Cell Mito Stress Test, following the manufacturer's instructions. Briefly, on the day of the assay, cells were counted and attached to 96-well Seahorse cell culture microplates, precoated with CorningTMCell-Tak (Sacco, Cadorago, Italy) according to the manufacturer's instructions, at a density of 250000 cells per well. Cells were seeded in eight wells per experimental condition, in XF RPMI Medium pH 7.4 with 1 mM HEPES (Agilent), supplemented with 10 mM glucose, 1 mM sodium pyruvate, and 2 mM L-glutamine. The plates were centrifuge at 1800 RPM for 10 minutes, without brakes, and then incubated at 37°C for 1 h in a non-CO2 incubator. After OCR baseline measurements, oligomycin A, Carbonylcyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), and antimycin A/rotenone were added sequentially to each well, to final concentrations of 1, 0.5, and 0.5 mM, respectively. Results were normalized by cell number,

measured at the end of the experiment using the CyQUANT Cell Proliferation Assays (Thermo FisherScientific). Data are expressed as pmol of oxygen per minute per arbitrary units (pmol/min/a.u.).

Quantitative proteomics analysis

mPB-CD34+ cells from three different healthy donors (100,000 minimum per sample), exposed or not to CsH as described above, were pelleted and flash-frozen in liquid nitrogen for shipment to the Biological Mass Spectrometry Core Facility at University of Colorado Denver. For analysis, cells were lysed and analyzed as previously described (Haudek-Prinz *et al*, 2012). Levels of acylcarnitines and free fatty acids have been normalized to the DMSO groups and autoscaled in MetaboAnalyst v 5.0. Statistical analysis was performed using 2way ANOVA with Holm-Sidak's multiple comparisons test (* $p< 0.05$, ** $p < 0.01$). Graphs and statistical analysis prepared using GraphPad Prism v 9.1.1.

Mass Spectrometry and global proteomic analysis

CB-CD34+ cells from three different healthy donors, exposed or not to CsH as described above, were lysed in RIPA-NP-40 buffer with Phosphatase inhibitor cocktail (PHOSSTOP, Roche, catalogue number 04906837001) and protease inhibitor cocktail (cOmplete™, EDTA-free Protease Inhibitor Cocktail, Sigma Aldrich, catalogue number 11873580001), and examined with Western blotting. The entire lanes were excised from the SDS-gel, cut, and digested. Extracted peptides were dried and purified using the StageTip procedure (Rappsilber *et al*, 2003). Digested samples were injected onto a quadrupole Orbitrap Q-exactive HF mass spectrometer (Thermo Scientific). Peptide separation was achieved on a linear gradient on UHPLC Easy-nLC 1000 (Thermo Scientific). The mass spectrometer was operated in DDA mode as described previously (Matafora *et al*, 2017). All raw files were processed using MaxQuant (Version 1.6.0.16). Statistical analysis was done in Perseus (Tyanova *et al*, 2016) using two-sample t-test with Benjamini-Hochberg correction set at FDR< 0.05. MS experiments were performed at the FIRC Institute of Molecular Oncology (IFOM), Milan.

Single cell RNA-sequencing and analysis

scRNA-seq libraries were generated using a microfluidics-based approach on Chromium Controller (10x Genomics) using the Chromium Single Cell 3′ Reagent Kit v3.1 according to the manufacturer's instructions. The concentration of the scRNA-seq libraries was determined using Qubit v3.0, and size distribution was assessed using an Agilent 4200 TapeStation system. Libraries were sequenced on an Illumina NovaSeq instrument (paired-end, 150-bp

read length). Raw data from scRNA-seq was analyzed and processed as previously described (Giordano *et al*, 2022). Functional enrichment analysis was performed on lists of differentially expressed genes. Heatmaps were generated using the R package pheatmap (v1.0.12).

RESULTS

CsH interferes with metabolic pathways in HSPCs

Hematopoietic stem and progenitor cells (HSPCs) represent the ideal candidates for gene therapy applications thanks to their self-renewal potential, their capability to propagate the entire hematopoietic lineage, and the tolerogenic effect on host immunity (Naldini, 2015). Nevertheless, HSPCs display a low gene manipulation efficiency making it necessary to further enhance *ex vivo* gene transfer efficacy. We have previously described the non-immunosuppressive Cyclosporine H (CsH) as a potent enhancer of lentiviral vector (LV) transduction in human HSPC and T cells (Petrillo *et al.*, 2018). To assess the impact of this molecule on the global proteome of human primary HSPCs, Mass spectrometry (MS)-based proteomics on CB-CD34+ cells, exposed or not with CsH was performed. This analysis allowed us to evaluate the quantitative profiling of the differentially expressed proteins upon CsH exposure (**Figure 1A**). Interestingly, we observed an upregulation of pathways that are mainly involved in metabolic processes such as Kreb-cycle and fatty acid oxidation. Conversely, genes related to lipid metabolism and T cell receptor (TCR) signalling pathway were downregulated in cells exposed to CsH as compared to controls (**Figure 1B**). Together, these observations indicate that besides improving LV transduction, CsH may also have other more metabolic effects on the target cells.

CsH increases steady state levels of Acylcarnitines and many free fatty acids in mPB-CD34+ cells

To characterize the direct effects of CsH on HSPCs metabolism, we performed MS-metabolomics analysis. Notably, we observed increased levels of acylcarnitines and many free fatty acids in HSPCs exposed to CsH as compare to control, at steady state (**Figure 2B**). Following Acetyl-CoA generation from fatty acids, CoA is exchanged for carnitine by carnitine palmityl transferase (CPT1). Acylcarnitine is then transported to the inside of the mitochondria by Carnitine-acylcarnitine translocase (CACT), and beta-oxidation machinery initiates its activity (**Figure 2A**). Thus, the reported accumulation of acylcarnitines and fatty acids in CsH treated cells confirms the potential impact on HSPC metabolism, and more specifically could be in line with an alteration in fatty acids oxidation.

CsH induces metabolic alteration on HSPC from both healthy donors and Fanconi anemia patients

Better deciphering the specific CsH-mediated metabolic effects on HSPC could contribute to improve efficacy of *ex vivo* gene transfer, minimizing cell manipulation. Thus, we explored more in details the impact of CsH on HSPC cell metabolism, performing a seahorse assay in mPB-CD34+ cells exposed or not to CsH. MitoStress Test showed a reduced Oxygen consumption rate (OCR) profile (**Figure 3A**), with a significant reduction of ATP production, as well as Spare respiratory capacity (SRC) in cells exposed to CsH, as compare to controls (**Figure 3B**). Next, we decided to extend our analysis also to a pathological model, in which the isolation and *ex-vivo* manipulation of HSPCs remains challenging due to low recovery and vulnerability of these cells to environmental triggers (Piras & Kajaste-Rudnitski, 2020). In particular, we tested CsH potential effects on Fanconi Anemia (FA), a rare inherited syndrome characterized by the early development of bone marrow failure and increasing predisposition to cancer with age (D'Andrea & Grompe, 1997). To do so, we performed a Single-cell RNASeq analysis comparing BM-CD34+ HSPC from healthy donor and FA patients (**Figure 3C**). As expected, we reported an upregulation of pathways related to FA pathology, such as DNA damage responses and cell cycle progression, in FA HSC as compare to control (**Figure 3D**). Furthermore, enrichment analysis also revealed a vector-independent perturbation of some metabolic pathways in FA-derived cells, which are mitigated by CsH (**Figure 3E**). These results support our previous assumption regarding the impact of CsH on HSPC metabolism, but more interestingly, they allowed us to speculate that CsH could be exploited for preserving FA HSPC during their *ex vivo* culture and, ultimately, improving more stealth gene therapy protocols.

CsH does not alter HSPC proliferation and composition ex vivo

In order to advance *ex vivo* expansion and genetic engineering of HSPCs, it is also crucial to preserve their biological properties. Thus, we performed FACS analysis, evaluating the cell composition of mPB-CD34+ HSPC, exposed or not to CsH. We reported no significant differences in terms of sub populations, in particular the percentage of CD34+CD133+CD90+ cells, that are responsible for long-term multilineage hematopoiesis, is not affected by CsH exposure (**Figure 4A**). Moreover, neither cell cycle nor proliferative capacity displayed any alterations in CsH treated cells as compared to controls (**Figure 4B** and **4C**), further confirming that HSPC maintain their features upon CsH exposure *ex vivo*. These observations are in line with our previous work in which the suitability of CsH as a safe and efficacious transduction enhancer was extensively investigated and no alterations in the in vivo repopulation capacity of bona fide long-term HSPC was observed (Petrillo *et al.*, 2018).

Primary T cells exposed to CsH display a peculiar metabolic profile

As described above, global proteomic analysis has shown the downregulation of genes belonging to TCR downstream signalling (**Figure 1B**). Since CsH can be used as a transduction enhancer also in T cells (Petrillo *et al.*, 2018), we decided to explore its potential effects on T cell biology. T cells rely on different metabolic pathways throughout their lifetime, according to their differentiation and memory status (Pearce, 2010; Shyer *et al*, 2020). Moreover, their capability to mount an efficient response following antigen encounter, or within the tumour microenvironment is strictly connected with metabolic changes (Le Bourgeois *et al.*, 2018). To evaluate the impact of CsH on T cell metabolism, we transduced stimulated CD4+ and CD8+ primary T cells, in the presence or absence of CsH, and performed seahorse assays four days after transduction. MitoStress Test revealed a reduced mitochondrial respiration, measured as Oxygen consumption rate (OCR) in both CD4+ and CD8+ cells exposed to CsH, as compare to controls (**Figure 5A**). Moreover, this profile was associated with a significant reduction of both basal and maximal respiration, as well as ATP production (**Figure 5B**). In line with the reported metabolic alterations, gene expression analysis confirmed the downregulation of genes related to mitochondrial activity and β-oxidation (**Figure 5C**). In particular, reduced levels of CPT1a and CPT2, two important mediators of fatty acids oxidation, could explain the accumulation of acylcarnitynes and free fatty acids observed in HSPCs upon CsH exposure (**Figure 2B**). Metabolomic and lipidomic analysis are currently ongoing to validate our results also in T cells.

CsH partially preserves T cells from exhaustion

T cell exhaustion is a state characterized by sequential phenotypic and functional changes, occurring during many chronic infections and cancer (Feldman *et al*, 2015; Krebs *et al*, 2013). Exhausted T cells display distinctive patterns of cytokine receptors, transcription factors and effector molecules, as well as sustained expression of inhibitory receptors, finally culminated in the loss of T-cell functions (Blackburn *et al*, 2009; Crawford & Wherry, 2009). Growing evidence indicates that exhausted T cells undergo metabolic alterations, associated with distinct signaling cascades and epigenetic landscapes, which leads to poor responsiveness to immune-checkpoint-blockade and lower T cell functionality (Franco *et al*, 2020). Interestingly, we observed that human primary T cells transduced in presence of CsH to express either a CD19-targeting or a GD2-tageting CAR presented a lower percentage of cells positive for markers of exhaustion in culture as compared to controls (**Figure 6A**). This effect was conserved also in murine settings (**Figure 6B**). Moreover, in line with previous observations, we confirmed also in this experimental setting the capability of CsH to enhance lentiviral vector transduction, in the therapeutically relevant T stem memory compartment

(**Figure 6C**). Importantly, we excluded that CsH could affect cell proliferation (**Figure 6D**), thus supporting its specific and direct effects on T cell exhaustion. In a context of CAR T cell therapy, the metabolic reprogramming has been described as an attractive approach to improve CAR T cell anti-tumour activity. Moreover, the inhibition of CAR signalling (or Rest), and the transient inhibition of TCR signalling, were proposed for restoring T cell functionality and potentially preventing exhaustion (Weber *et al*, 2021).

DISCUSSION

Significant efforts to improve LV transduction in HSPC and T cells have led to the development of several transduction enhancers that allow more efficient gene transfer into these relevant therapeutic targets (Heffner *et al*, 2018; Ozog *et al*, 2019; Petrillo *et al*, 2015; Petrillo *et al.*, 2018; Wang *et al*, 2014; Zonari *et al*, 2017). Nevertheless, few studies have addressed the impact of these enhancers on the biological properties of the target cells and in the context of patient cells. We have previously shown that CsH is able to increase LV vector transduction in HSPC and T cells (Petrillo *et al.*, 2018) and have investigated its impact on the biological properties of these clinically relevant target cells in the context of *ex vivo* cell and gene therapy applications.

Patients with the genomic instability syndrome Fanconi anemia (FA) commonly develop progressive bone marrow failure and have a high risk of cancer. The prominent role of the FA protein family involves DNA damage response and/or repair and ssignificant evidence supports excessive apoptosis of HSPC, induced by oxidative and other sources of stress, as a critical factor in the pathogenesis of bone marrow failure and leukemia progression in FA (Du *et al*, 2008). LV-mediated HSC gene therapy may constitute a new safe and efficient approach for the treatment/prevention of the bone marrow failure (BMF) characteristic of FA patients. Nevertheless, the isolation and ex-vivo manipulation of FA HSCs remains challenging due to low recovery and vulnerability of these cells to environmental triggers (Tolar *et al.*, 2012). We show here that CsH has the capacity to mitigate activation of several pathways potentially harmful for HSC biological integrity such as oxidative phosphorylation DNA damage and repair pathways as well as Myc-driven transcirptional programms that have recently been shown to Promotes Bone Marrow Stem Cell Dysfunction in Fanconi Anemia (Rodriguez *et al*, 2021). Moreover, CsH treated HSPC showed a significant upregulation in free fatty acid content. High fatty acid levels mimicking the bone marrow microenvironment have been shown to enable maintenance of engraftable quiescent HSCs *ex vivo* (Kobayashi *et al*, 2019) and sustained HSC functions (Dong *et al*, 2021). Interestingly, fatty acids have also been show to modulated CD8+ T cell responses and improve cacner immnuotherapies (Luu *et al*, 2021).

Chimeric antigen receptor (CAR)-T cells show great promise in treating cancers and viral infections. However, most protocols developed to expand T cells require relatively long periods of time in culture, potentially leading to progression toward populations of terminally differentiated effector memory cells. Ideally, adoptively transferred T cells should express a less differentiated phenotype because those cell subsets circulate to lymphoid organs and are capable of robust expansion (Redeker & Arens, 2016). In animal studies and human clinical trials, it is apparent that less differentiated T cells, defined as cells expressing the lymphoid homing molecules CD62L and CCR7, better contribute to engraftment and long-term persistence (Klebanoff *et al*, 2012). In clinical trials, a failure of infused T cells to persist has been correlated with the absence of $CD4^+$ T cells in the $CD8^+$ T cell product (Patel *et al*, 2016). The use of PBMCs in the production of the CAR-T cells in this protocol allows the final cultures to contain both antigen-specific $CD8^+$ T cells and $CD4^+$ T cells. However, for efficient viral gene transfer, PBMCs are activated with anti-CD3 and anti-CD28 along with interleukin 2 (IL-2) at a relatively high density. Moreover, $CD4^+$ T cells are more refractory to lentiviral transduction as compared to CD8+ T cells (Kerkar *et al*, 2011). In this context, the use of CsH during lentiviral transduction could offer the possibility to generate CAR-T cells achieving gene marking also in the relevant T stem memory compartment (Gattinoni *et al*, 2011) while better preserving their antitumoral capacities.

T cell exhaustion plays a major role in limiting CAR T efficacy, and it is associated with poor responses in cancer patients receiving immunotherapy (Delgoffe *et al.*, 2021). This status can be promoted by the immunosuppressive conditions within the tumor microenvironment, as the increase of reactive oxygen species (ROS) levels, or by excessive CAR signaling itself, as a result of high antigen burden or tonic signaling induced by clustering of CAR molecules (Long *et al*, 2015; Lynn *et al*, 2019). For these reasons, the inhibition of CAR signaling (Rest), as well as T cell metabolic reprogramming, have been proposed to reverse CAR-T exhaustion (Weber *et al.*, 2021). Our results suggest that, upon CsH exposure, T cells undergo a sort of transient metabolic delay, which could be exploited to reduce ROS production, but also to coax the cells into a resting state, thus inhibiting tonic signaling and potentially preventing exhaustion.

Overall, our findings support a role for CsH in regulating both HSPC and T cell metabolic activities in a manner that could benefit the maintenance of their biological properties in the context of *ex vivo* gene and cell therapies. Moreover, CsH would also allow improving transduction efficiencies and enable lowering the vector doses required for clinically relevant gene marking and dramatically reduce manufacturing costs, rendering gene and cell therapies available to all patients.

EXAMPLE 2

RESULTS

CsH enhances LV transduction efficiency in human Tregs.

We established a protocol for the generation of CD19 CAR Tregs starting from CD4+CD25+ Tregs isolated from healthy donors (HD). Tregs were purified from buffy coats using anti-CD4 and anti-CD25 magnetic beads (Treg isolation kit, Miltenyi). Cells were activated with anti-CD3/CD28-coated beads and kept in culture with 1000U/ml of IL-2 and rapamycin (100nM) to foster Treg enrichment. After 30 h of stimulation, Tregs were transduced with the LV vector encoding for CD19 CAR (MOI 10) +/- CsH (8μM). Transduction efficiency was evaluated by FC at 8- and 15-days post transduction by assessing the percentage of NGFR+ cells (**Fig. 7**). Our results show that CsH exposure enhances human Treg transduction efficiency measured both as %NGFR, gMFI on CD4+ (**Fig. 7a-b**) and gMFI on FOXP3+ cells (**Fig. 7c**).

CsH does not alter the survival and growth potential of human CD19 CAR Tregs but increases their PD-1 surface levels.

The effect of CsH on CAR Treg survival was evaluated *in vitro* after transduction with increasing amounts of LV (2, 5 and 10 MOI). As shown in **Fig. 8a**, the expansion of CAR Tregs with CsH was similar to DMSO-treated CAR Tregs. While a slight decrease in the content of CD25+ CD127lo Treg cells was observed in the end-product in the presence of CsH, no differences in the % and gMFI of FOXP3 and CTLA4 were observed (**Fig. 8b**). Interestingly, a higher proportion of cells expressed PD-1 on their surface (**Fig. 8b**).

CsH improves the immunomodulatory profile of human CD19 CAR Tregs.

Following fifteen days of expansion, the resulting cells were stimulated briefly, for 3 hours, with LAC (Leukocyte Activation Cocktail with BD GolgiPlugTM) (BD Biosciences) and analysed by flow cytometry for the production of proinflammatory and immunoregulatory cytokines, i.e. IFNγ, IL-10 and TNF. As shown in **Fig. 9**, transduction in the presence of CsH had a significant impact on the cytokine profile of CD19 CAR Tregs: while IFNγ and IL-10 cytokine production increased, TNF reduced (**Fig. 9a**). Interestingly, the fraction of IFNγ+ IL-10+ double producing Treg cells also increased (**Fig. 9b**). This Treg fraction is considered highly suppressive in preclinical models (Trinchieri (2001) J Exp Med 194: F53-57; Zhou et al. (2008) Genome Biol 9: R119).

CsH does not alter the *in vitro* suppressive function of CD19 CAR Tregs.

Next, we performed functional assays to evaluate the *in vitro* functionality of CsH-treated CD19 CAR Tregs. We performed a suppression assay where allogeneic PBMC were stained with a proliferation dye (CFSE) and served as responders. CsH vs DMSO-treated CAR Tregs were added at different PBMC:Treg ratios, and cells were activated with anti-CD3/CD28-coated beads. Tconv proliferation was evaluated after 5 days of culture. CsH-treated CAR-Tregs showed a potent suppressive capacity (expressed as suppression %) similar to DMSO-treated Tregs (**Fig. 10**). This result indicated that LV transduction in the presence of CsH increases CAR expression without impairing Tregs' suppressive properties *in vitro*.

METHODS

Isolation, transduction, and expansion of primary human Treg cells

Human blood samples were obtained from buffy coat of healthy donors following written consent according to protocols approved by the IRCSS San Raffaele Hospital, Milan, Italy. Human PBMCs were obtained by density gradient centrifugation before proceeding with CD4+CD25+ Treg isolation using Human Regulatory T cells isolation kit (Miltenyi Biotec). Tregs were stimulated with anti-CD3/CD28 activation microbeads (DynabeadsTM Human T-Activator CD3/CD28 gibcoTM) (3:1 beads:Treg), in the presence of rIL-2 (1000U/mL) (PROLEUKIN). After 30h stimulation, Tregs were transduced at 10 MOI with the lentiviral vector encoding the CD19 CAR, with or without 8μM of CsH in DMSO. Cell culture was maintained for 15 days by refreshing the culture medium every 2 days with cRPMI, r-IL2 and rapamycin (100nM) (Sigma).

Flow cytometry

On day 8 and 15, transduction efficiency (evaluating the percentage of NGFR+ cells), phenotype, and cytokines' production were assessed by flow-cytometry. Fluorochrome-conjugated monoclonal/polyclonal anti–human FoxP3-FITC, anti-human NGFR-PE, anti-human CD4 PerCP 5.5, anti-human CTLA-4 -APC, anti-human CD127- PeCy7, anti-human CD25- APCCy7, anti-human PD1-PB, anti-human CD3-PO, anti-human IL-10-PE, anti-human TNF – PeCy7, anti-human IFNg-PB anti-human IL-17-PO; were purchased from eBioscience, BioLegend, BD Biosciences or Thermo Fisher Scientific. Intranuclear staining was performed using fixation/permeabilization buffer solution (eBioscience), according to the manufacturer's instructions. Stained cells were analyzed on a FACSCanto II (BD Biosciences), and data were analyzed with Diva software (BD Biosciences) and FlowJo software.

Cytokine production

For intracellular cytokine staining, CAR Treg cells were stimulated with 1:100 LAC (Leukocyte Activation Cocktail with BD GolgiPlugTM) (BD Biosciences) for 3 hours at 37°C with 5% of CO_2. Then, intracellular cytokine production was stained and analyzed by flow cytometry, as previously described (Milardi et al. (2022) Eur J Immunol 52: 1171-1189).

Suppression assay

Treg cells were co-cultured with PBMCs cells (labeled with 5μM of carboxy fluorescein succinimidyl ester fluorescent dye (CFSE) (Invitrogen) in PBS at 37°C for 8 minutes) at a ratio of 1:1, 1:2, 1:4, 1:8, 1:16 (PBMCs:Treg cells) for 5 days. The percentage of proliferation cells in the CD8+ T cells was estimated using flow cytometry analysis.

Statistic

Statistical analyses were performed using an unpaired t-test (two-tailed) with GraphPad Prism version 5 software. All data are presented as mean ± SEM. Values at $P < 0.05$ were considered statistically significant.

EXAMPLE 3

CsH treatment dampens CAR T cell exhaustion upon polyclonal stimulation

To further test the potential of CsH in counteracting the generation of exhausted CAR T cells in vitro, we tested its impact of CAR T cells targeting the GD2 antigen (Long et al. (2015) Nat Med 21: 581-590) that were exposed to Staphylococcal enterotoxin B (SEB), a polyclonal and strong T cell activator (**Fig. 11**). CsH exposure led to a reduction in the % of CAR T cells, both CD4 and CD8, positive for the three exhaustion markers Lag3, Tim3 and PD-1, indicating the exposure to CsH may help preserve CAR T cells under strong polyclonal stimulation.

Materials and Methods

Cryopreserved peripheral blood mononuclear cells (PBMCs), previously purified from Buffy Coat using Ficoll by sequential centrifugations, were thawed and rest over a day in RPMI medium (Corning). CD4+ and CD8+ cells were isolated by immune-magnetic separation using CD4 or CD8 T-cell isolation kit (Miltenyi Biotech) according to the manufacturer's instructions, and stimulated using magnetic beads (ratio cell:bead 1:1) conjugated with Dynabeads human T-activator CD3/CD28 (Day 0). Cells were maintained in RPMI medium supplemented with 10% FBS, penicillin (100 IU/ml), streptomycin (100μg/ml), 2% glutamine, Non-Essential Amino Acids (NEAA), Sodium Pyruvate and IL-7 (5 ng/mlPreproTech) and IL-15 (5 ng/ml; PreproTech). Dynabeads were removed after 3 days of culture and cells were transduced with

GD2 CAR-T vector as previously described (Long et al. (2015) Nat Med 21: 581-590), in the presence of CsH (8uM), or DMSO as vehicle control. After a 16 hours incubation, the medium was diluted 1:1 to reduce CsH-induced toxicity. A second hit of CsH treatment was performed after two days, Staphylococcal enterotoxin B (SEB) was also added overnight, to boost T cell activation. Cytofluorimetric analyses were performed at day 7 on FACS Canto II (BDPharmingen), and analyzed with the FSC express software. Exhausted cells were identified as the triple positive population for LAG3, TIM3 and PD1 surface markers.

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EMBODIMENTS

Various features and embodiments of the invention will now be described with reference to the following numbered paragraphs:

1. Use of cyclosporin H (CsH) or a derivative thereof for: (a) reducing or preventing T cell exhaustion and/or loss of T cell effector functions; and/or (b) increasing T cell engraftment and/or persistence.

2. A T cell for use in a method of therapy, wherein the method comprises contacting the T cell with cyclosporin H (CsH) or a derivative thereof.

3. The T cell for use according to paragraph 2, wherein:

(a) T cell exhaustion and/or loss of T cell effector functions is reduced or prevented; and/or

(b) T cell engraftment and/or persistence is increased.

4. The T cell for use according to paragraph 2 or 3, wherein the therapy is treatment or prevention of cancer or infection.

5. The use or T cell for use according to any preceding paragraph, wherein the T cell is transduced or transfected with a vector, optionally a viral vector.

6. The use or T cell for use according to paragraph 5, wherein the vector is a retroviral vector or a lentiviral vector.

7. The use or T cell for use according to paragraph 5 or 6, wherein the vector comprises one or more nucleotide sequence encoding a chimeric antigen receptor (CAR) and/or a T cell receptor (TCR).

8. The use or T cell for use according to any preceding paragraph, wherein the T cell is a chimeric antigen receptor (CAR) T cell and/or comprises an exogenous T cell receptor (TCR).

9. The use or T cell for use according to any preceding paragraph, wherein the T cell is a CD4+ and/or CD8+ T cell.

10. Use of cyclosporin H (CsH) or a derivative thereof for increasing survival of and/or engraftment by haematopoietic stem and/or progenitor cells (HSPCs).

11. A haematopoietic stem and/or progenitor cell (HSPC) for use in a method of treating Fanconi Anemia, wherein the method comprises contacting the HSPC with cyclosporin H (CsH) or a derivative thereof.

12. The use or HSPC for use according to paragraph 10 or 11, wherein the HSPC is transduced or transfected with a vector, optionally a viral vector.

13. The use or HSPC for use according to paragraph 11 or 12, wherein the vector is a retroviral vector or a lentiviral vector.

14. The use, or T cell or HSPC for use according to any preceding paragraph, wherein the CsH or derivative thereof is at a concentration of about 1-50 μM.

15. The use, or T cell or HSPC for use according to any preceding paragraph, wherein the T cell or HSPC is contacted twice with the CsH or derivative thereof.

16. The use, or T cell or HSPC for use according to any preceding paragraph, wherein the T cell or HSPC is:

(a) contacted with the CsH or derivative thereof at the same time as transduction or transfection with a vector; and/or

(b) contacted with the CsH or derivative thereof after transduction or transfection with a vector.

17. The use, or T cell or HSPC for use according to any preceding paragraph, wherein the T cell or HSPC is cultured for 16 days or less before administration to a subject.

18. A method of cell therapy comprising the steps of:

(a) contacting a cell with cyclosporin H (CsH) or a derivative thereof; and

(b) administering the cell to a subject.

19. The method of paragraph 18, further comprising the step of transducing or transfecting the cell with a vector.

20. A method of gene therapy comprising the steps of:

(a) contacting a cell with cyclosporin H (CsH) or a derivative thereof

(b) transducing or transfecting the cell with a vector; and

(c) administering the cell to a subject.

21. The method of any one of paragraphs 18-20, wherein the cell is a T cell or a haematopoietic stem and/or progenitor cell (HSPC).

22. The method of any one of paragraphs 18-21, wherein the T cell is a chimeric antigen receptor (CAR) T cell and/or comprises an exogenous T cell receptor (TCR).

23. The method of any one of paragraphs 18-22, wherein the T cell is a CD4+ and/or CD8+ T cell.

24. The method of any one of paragraphs 18-23, wherein the cell is a T cell and wherein:

(a) T cell exhaustion and/or loss of T cell effector functions is reduced or prevented; and/or

(b) T cell engraftment and/or persistence is increased.

25. The method of any one of paragraphs 18-20, wherein the cell is a HSPC and wherein HSPC survival and/or engraftment is increased.

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the disclosed agents, uses and methods of the invention will be apparent to the skilled person without departing from the scope and spirit of the invention. Although the invention has been disclosed in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the disclosed modes for carrying out the invention, which are obvious to the skilled person are intended to be within the scope of the following claims.

CLAIMS

1. Use of cyclosporin H (CsH) or a derivative thereof for: (a) reducing or preventing T cell exhaustion and/or loss of T cell effector functions; and/or (b) increasing T cell engraftment and/or persistence.

2. A T cell for use in a method of therapy, wherein the method comprises contacting the T cell with cyclosporin H (CsH) or a derivative thereof.

3. The T cell for use according to claim 2, wherein:

(a) T cell exhaustion and/or loss of T cell effector functions is reduced or prevented; and/or

(b) T cell engraftment and/or persistence is increased.

4. The T cell for use according to claim 2 or 3, wherein the therapy is treatment or prevention of cancer or infection.

5. The use or T cell for use according to any preceding claim, wherein the T cell is transduced or transfected with a vector, optionally a viral vector.

6. The use or T cell for use according to claim 5, wherein the vector is a retroviral vector or a lentiviral vector.

7. The use or T cell for use according to claim 5 or 6, wherein the vector comprises one or more nucleotide sequence encoding a chimeric antigen receptor (CAR) and/or a T cell receptor (TCR).

8. The use or T cell for use according to any preceding claim, wherein the T cell is a chimeric antigen receptor (CAR) T cell and/or comprises an exogenous T cell receptor (TCR).

9. The use or T cell for use according to any preceding claim, wherein the T cell is: (a) a CD4+ and/or CD8+ T cell; or (b) a regulatory T (Treg) cell.

10. Use of cyclosporin H (CsH) or a derivative thereof for increasing survival of and/or engraftment by haematopoietic stem and/or progenitor cells (HSPCs).

11. A haematopoietic stem and/or progenitor cell (HSPC) for use in a method of treating Fanconi Anemia, wherein the method comprises contacting the HSPC with cyclosporin H (CsH) or a derivative thereof.

12. The use or HSPC for use according to claim 10 or 11, wherein the HSPC is transduced or transfected with a vector, optionally a viral vector.

13. The use or HSPC for use according to claim 11 or 12, wherein the vector is a retroviral vector or a lentiviral vector.

14. The use, or T cell or HSPC for use according to any preceding claim, wherein the CsH or derivative thereof is at a concentration of about 1-50 μM.

15. The use, or T cell or HSPC for use according to any preceding claim, wherein the T cell or HSPC is contacted twice with the CsH or derivative thereof.

16. The use, or T cell or HSPC for use according to any preceding claim, wherein the T cell or HSPC is:

(a) contacted with the CsH or derivative thereof at the same time as transduction or transfection with a vector; and/or

(b) contacted with the CsH or derivative thereof after transduction or transfection with a vector.

17. The use, or T cell or HSPC for use according to any preceding claim, wherein the T cell or HSPC is cultured for 16 days or less before administration to a subject.

18. A method of cell therapy comprising the steps of:

(a) contacting a cell with cyclosporin H (CsH) or a derivative thereof; and

(b) administering the cell to a subject.

19. The method of claim 18, further comprising the step of transducing or transfecting the cell with a vector.

20. A method of gene therapy comprising the steps of:

(a) contacting a cell with cyclosporin H (CsH) or a derivative thereof

(b) transducing or transfecting the cell with a vector; and

(c) administering the cell to a subject.

21. The method of any one of claims 18-20, wherein the cell is a T cell or a haematopoietic stem and/or progenitor cell (HSPC).

22. The method of any one of claims 18-21, wherein the T cell is a chimeric antigen receptor (CAR) T cell and/or comprises an exogenous T cell receptor (TCR).

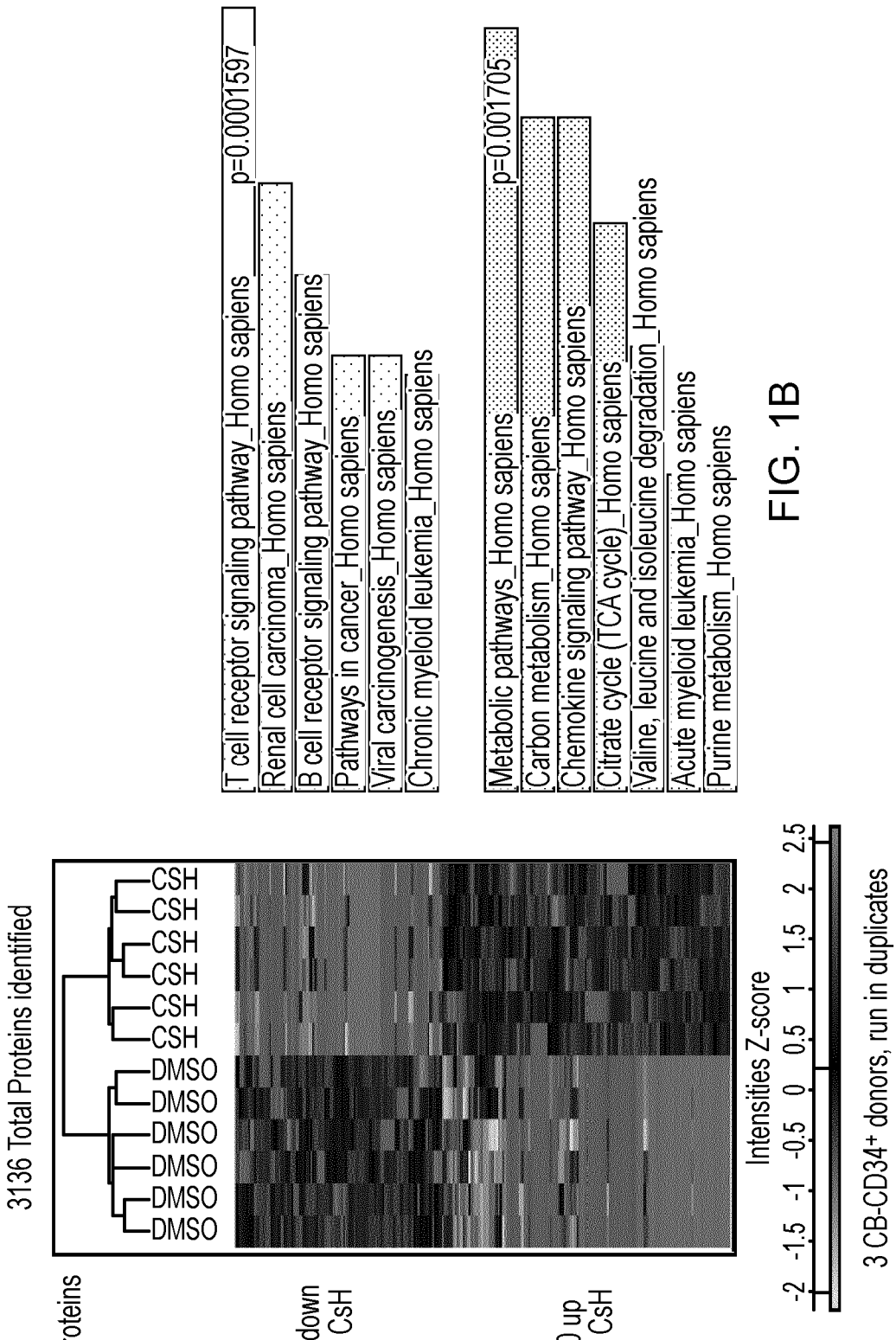
23. The method of any one of claims 18-22, wherein the T cell is: (a) a CD4+ and/or CD8+ T cell; or (b) a regulatory T (Treg) cell.

24. The method of any one of claims 18-23, wherein the cell is a T cell and wherein:

(a) T cell exhaustion and/or loss of T cell effector functions is reduced or prevented; and/or

(b) T cell engraftment and/or persistence is increased.

25. The method of any one of claims 18-20, wherein the cell is a HSPC and wherein HSPC survival and/or engraftment is increased.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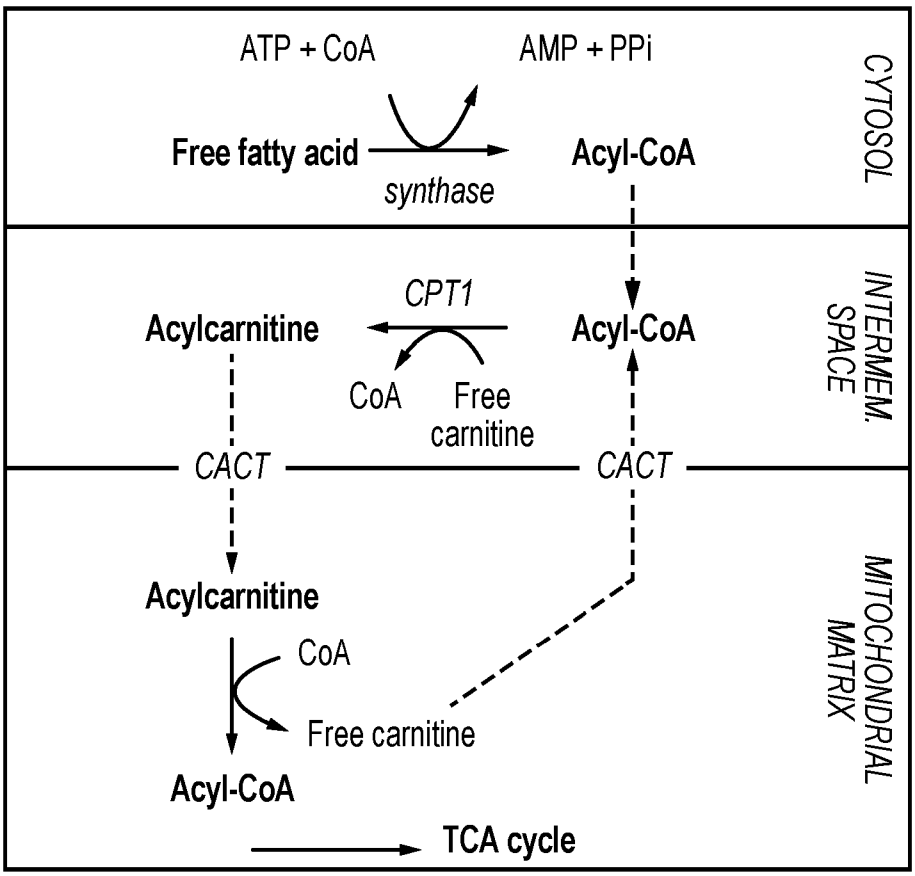
Significant proteins
T-test
FDR<0.05
100 down
with CsH
140 up
with CsH
CSH
DMSO
Intensities Z-score
-2 -1.5 -1 -0.5 0 0.5 1 1.5 2 2.5
3 CB-CD34+ donors, run in duplicates
T cell receptor signaling pathway_Homo sapiens p=0.0001597
Renal cell carcinoma_Homo sapiens
B cell receptor signaling pathway_Homo sapiens
Pathways in cancer_Homo sapiens
Viral carcinogenesis_Homo sapiens
Chronic myeloid leukemia_Homo sapiens
Metabolic pathways_Homo sapiens p=0.001705
Carbon metabolism_Homo sapiens
Chemokine signaling pathway_Homo sapiens
Citrate cycle (TCA cycle)_Homo sapiens
Valine, leucine and isoleucine degradation_Homo sapiens
Acute myeloid leukemia_Homo sapiens
Purine metabolism_Homo sapiens

FIG. 1A

FIG. 1B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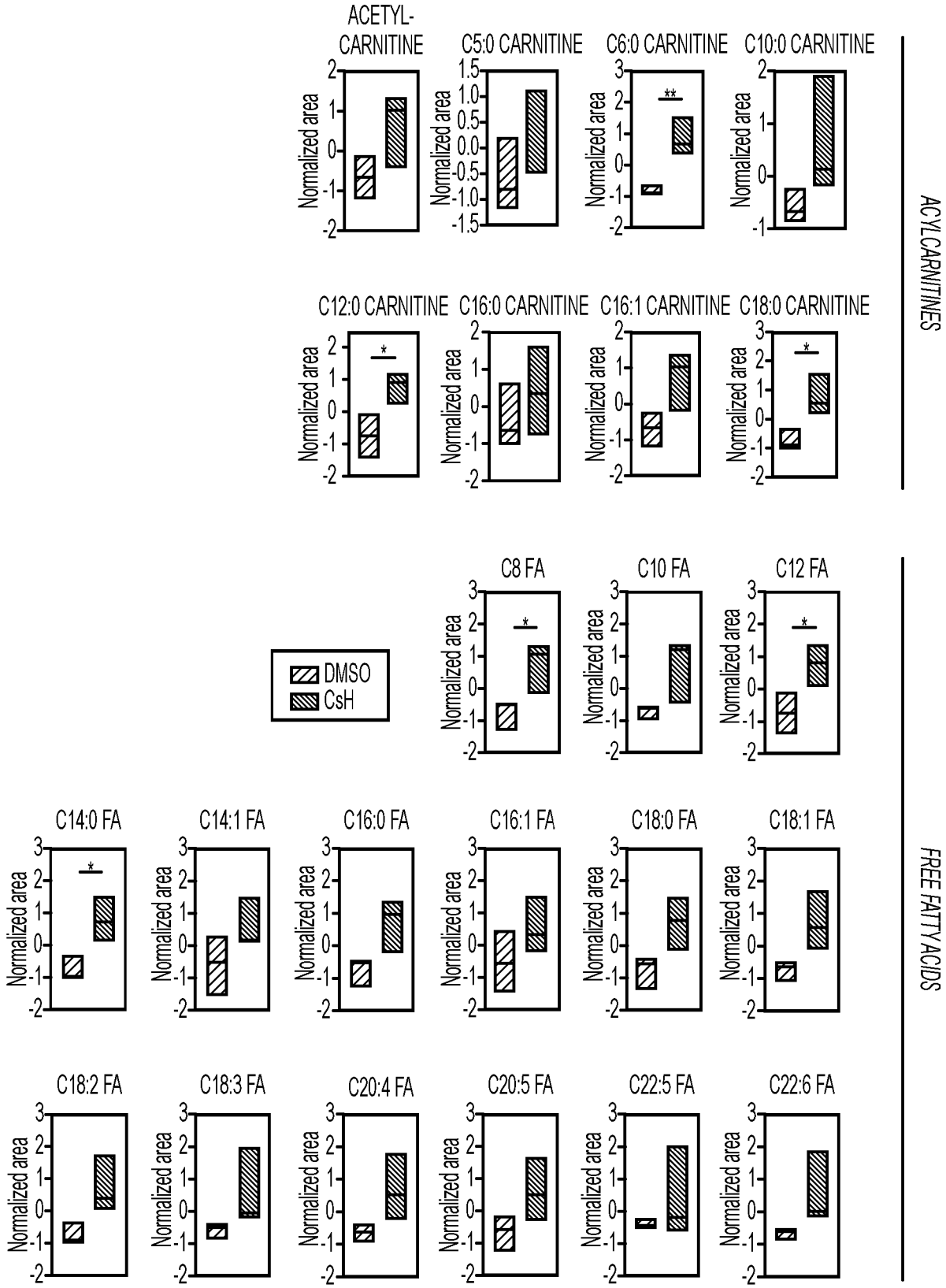
AMP + PPi
Free fatty acid
synthase
Acyl-CoA
CYTOSOL
CPT1
Acylcarnitine
Acyl-CoA
CoA
Free carnitine
INTERMEM. SPACE
CACT
CACT
Acylcarnitine
CoA
Free carnitine
Acyl-CoA
MITOCHONDRIAL MATRIX
TCA cycle

FIG. 2A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C16:0 CARNITINE
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C18:0 CARNITINE
ACYLCARNITINES
DMSO
CsH
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C12 FA
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C14:1 FA
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C16:1 FA
C18:0 FA
C18:1 FA
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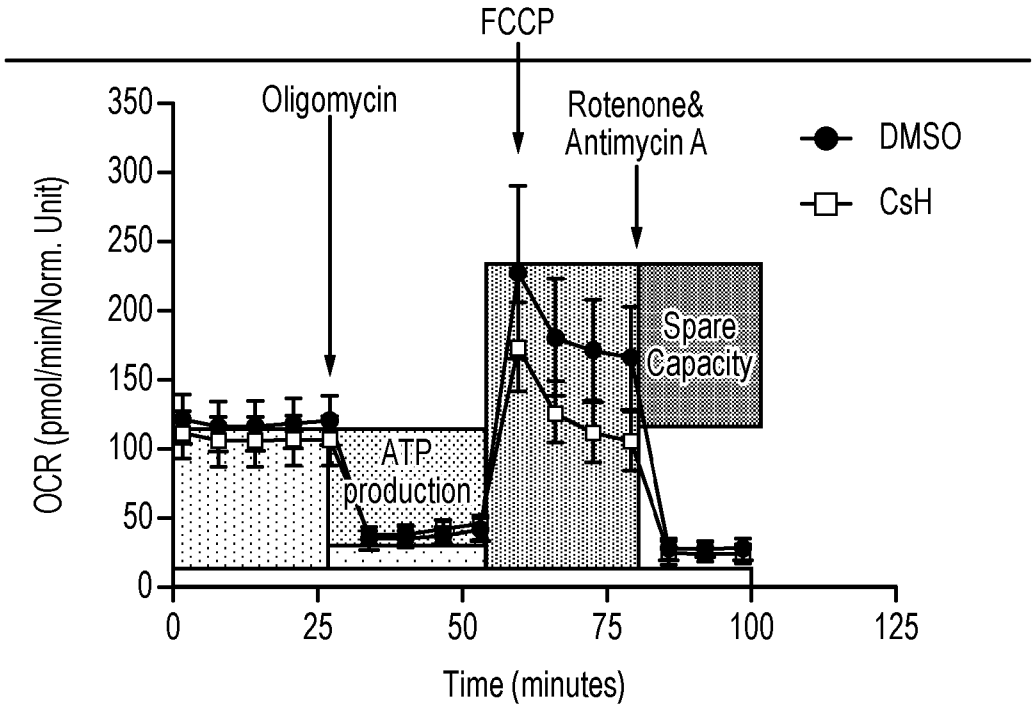
C22:5 FA
C22:6 FA
FREE FATTY ACIDS
Normalized area

FIG. 2B


FCCP
Oligomycin
Rotenone&
Antimycin A
DMSO
CsH
Spare
Capacity
ATP
production
OCR (pmol/min/Norm. Unit)
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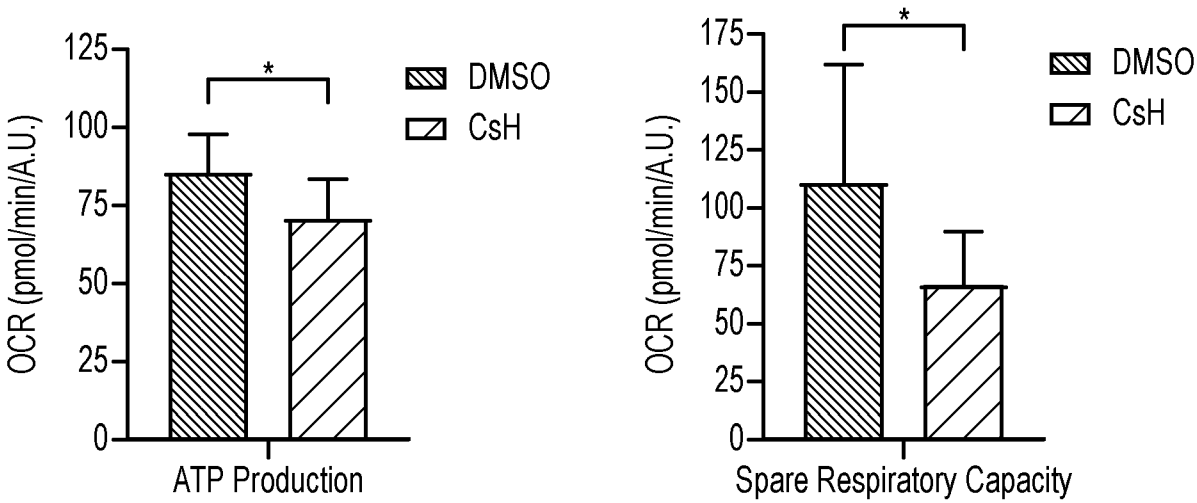
300
250
200
150
100
50
0
0
25
50
75
100
125
Time (minutes)

FIG. 3A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DMSO
CsH
OCR (pmol/min/A.U.)
125
100
75
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25
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ATP Production
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100
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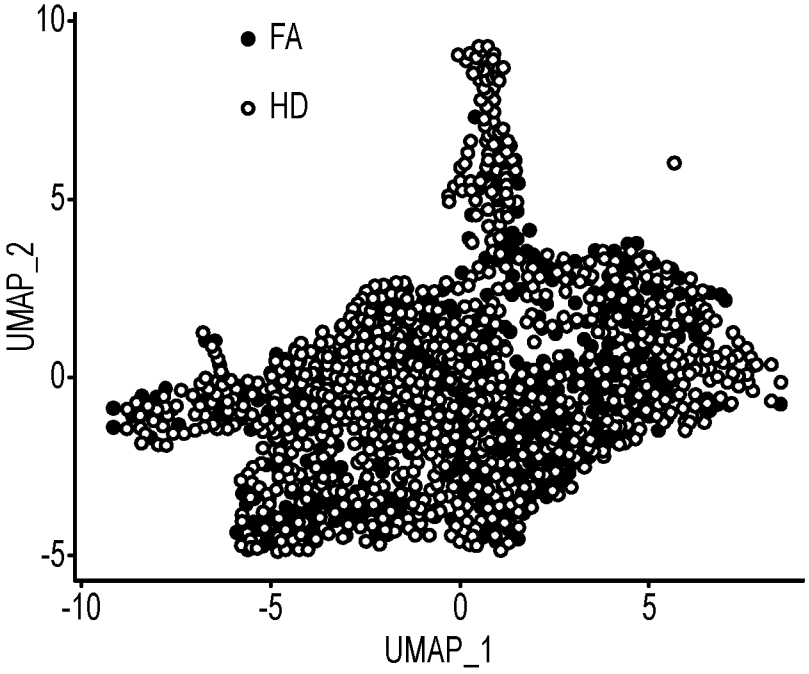
50
25
0
Spare Respiratory Capacity

FIG. 3B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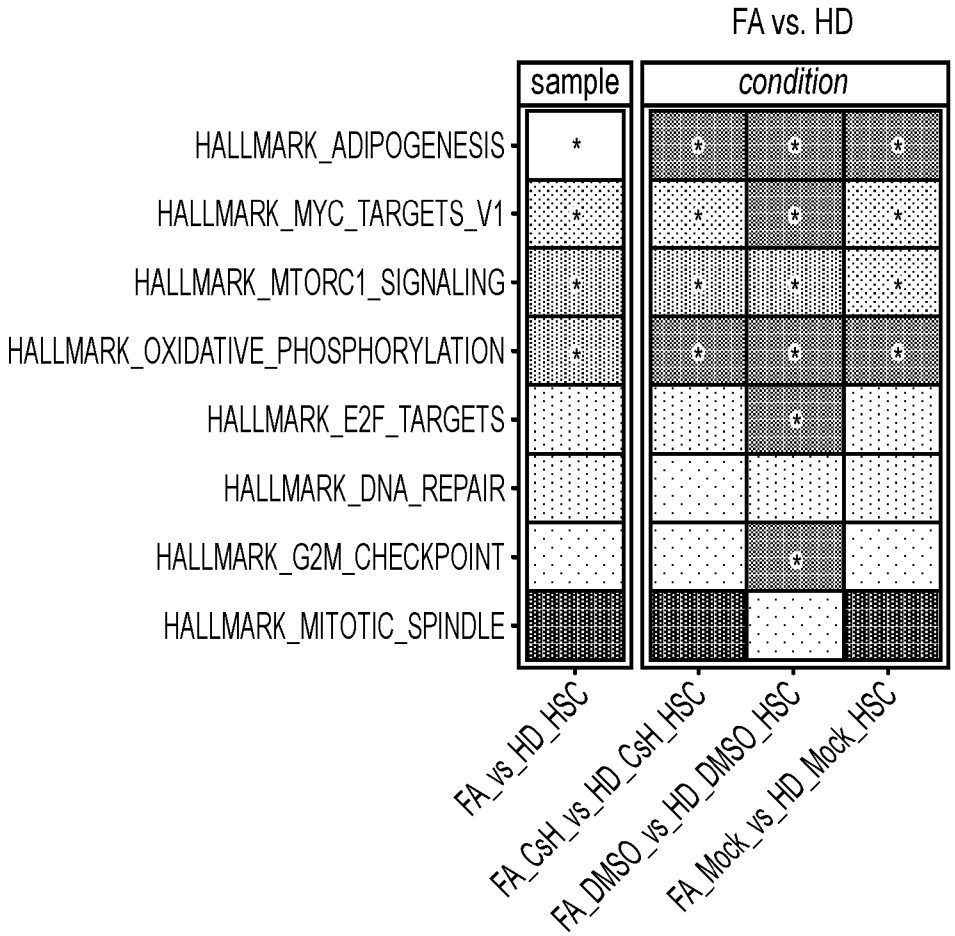
UMAP_1

FIG. 3C


FA vs. HD
sample
condition
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HALLMARK_MYC_TARGETS_V1
HALLMARK_MTORC1_SIGNALING
HALLMARK_OXIDATIVE_PHOSPHORYLATION
HALLMARK_E2F_TARGETS
HALLMARK_DNA_REPAIR
HALLMARK_G2M_CHECKPOINT
HALLMARK_MITOTIC_SPINDLE
FA_vs_HD_HSC
FA_CsH_vs_HD_CsH_HSC
FA_DMSO_vs_HD_DMSO_HSC
FA_Mock_vs_HD_Mock_HSC

FIG. 3D

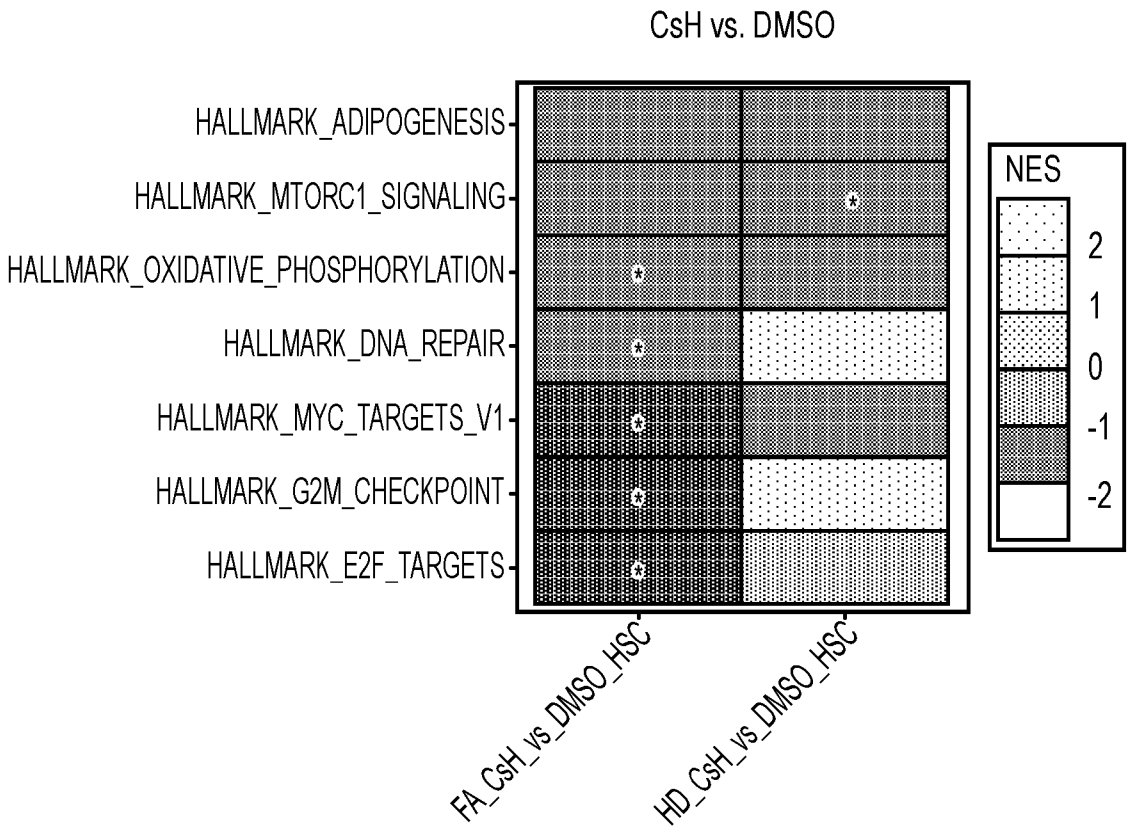

CsH vs. DMSO
HALLMARK_ADIPOGENESIS
HALLMARK_MTORC1_SIGNALING
HALLMARK_OXIDATIVE_PHOSPHORYLATION
HALLMARK_DNA_REPAIR
HALLMARK_MYC_TARGETS_V1
HALLMARK_G2M_CHECKPOINT
HALLMARK_E2F_TARGETS
FA_CsH_vs_DMSO_HSC
HD_CsH_vs_DMSO_HSC
NES
2
1
0
-1
-2

FIG. 3E

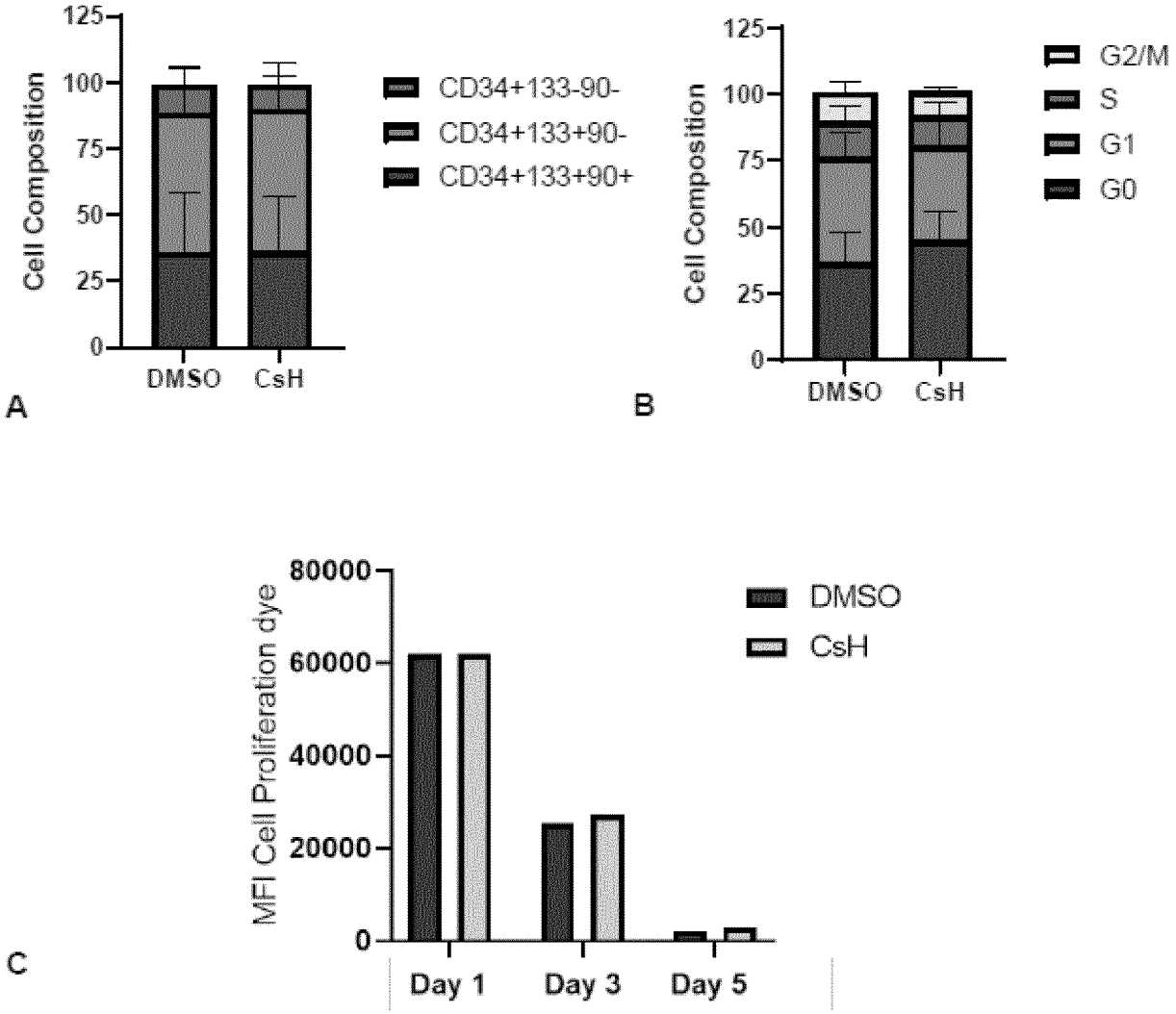

125
100
75
50
25
0
Cell Composition
DMSO
CsH
CD34+133-90-
CD34+133+90-
CD34+133+90+
A
125
100
75
50
25
0
Cell Composition
DMSO
CsH
G2/M
S
G1
G0
B
80000
60000
40000
20000
0
MFI Cell Proliferation dye
Day 1
Day 3
Day 5
DMSO
CsH
C

FIG. 4

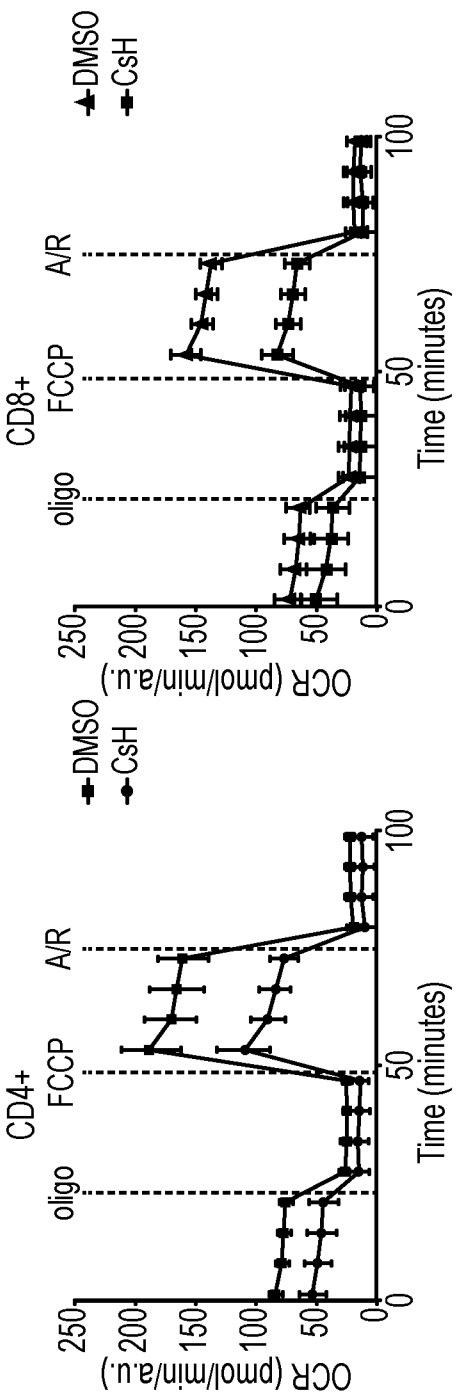

CD4+
CD8+
DMSO
CsH
oligo
FCCP
A/R
OCR (pmol/min/a.u.)
Time (minutes)
0
50
100
150
200
250

FIG. 5A

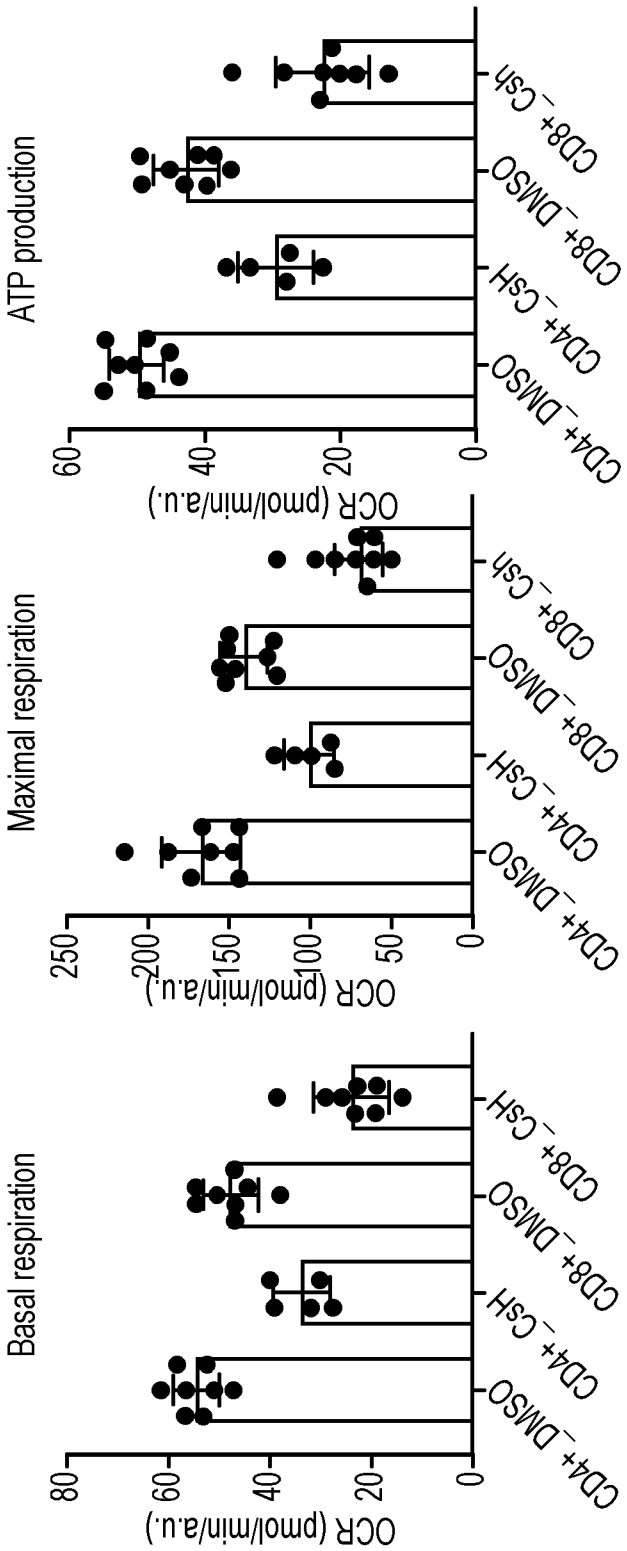

Basal respiration
Maximal respiration
ATP production
OCR (pmol/min/a.u.)
CD4+_DMSO
CD4+_CsH
CD8+_DMSO
CD8+_CsH

FIG. 5B

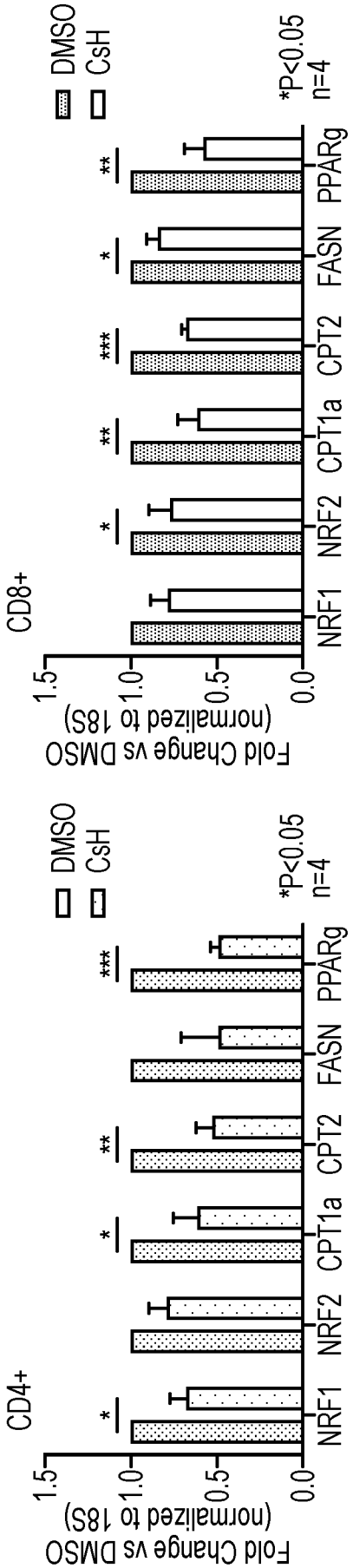
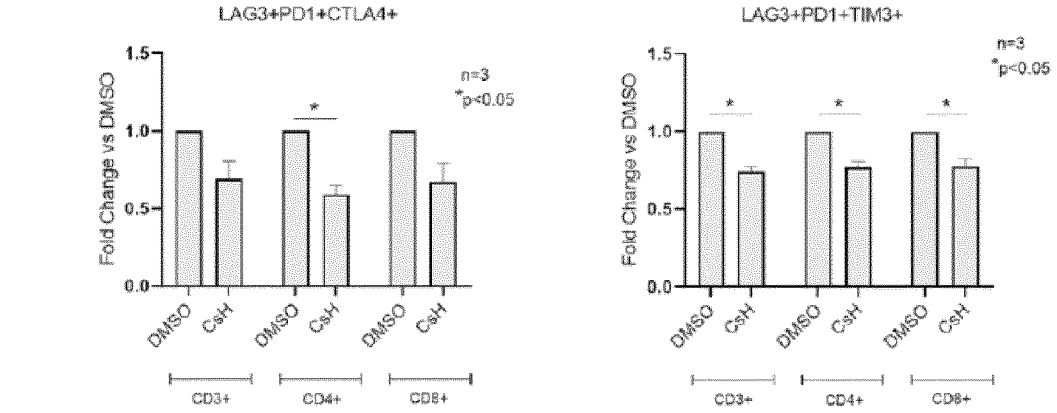
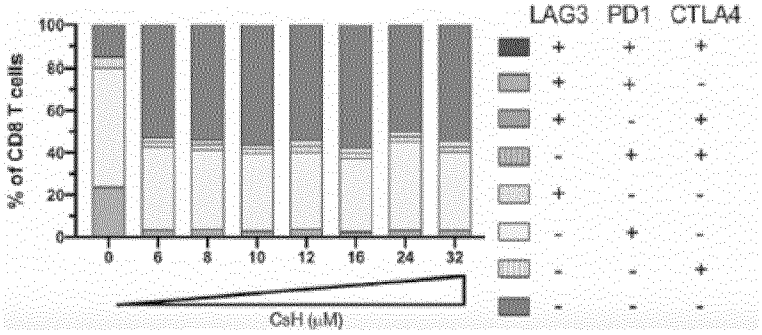

DMSO
CsH
*P<0.05
n=4
CD8+
Fold Change vs DMSO
(normalized to 18S)
1.5
1.0
0.5
0.0
NRF1
NRF2
CPT1a
CPT2
FASN
PPARg
*
**
***
CD4+

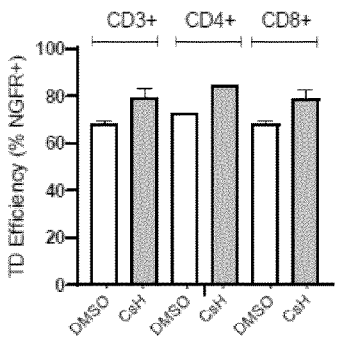
FIG. 5C


LAG3+PD1+CTLA4+
Fold Change vs DMSO
1.5
1.0
0.5
0.0
n=3
*p<0.05
*
DMSO
CsH
CD3+
CD4+
CD8+
LAG3+PD1+TIM3+
n=3
*p<0.05

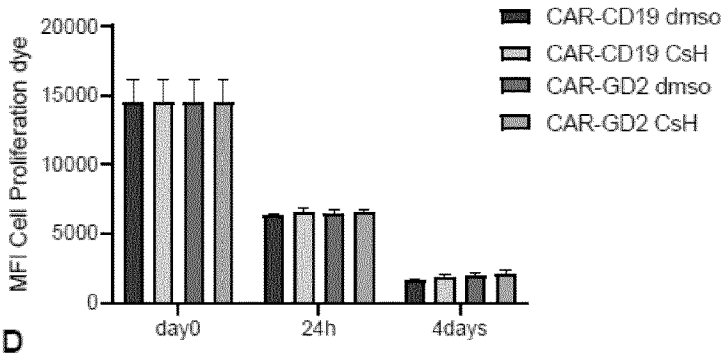
A


LAG3 PD1 CTLA4
% of CD8 T cells
100
80
60
40
20
0
0
6
8
10
12
16
24
32
CsH (µM)

B


CD3+
CD4+
CD8+
TD Efficiency (% NGFR+)
100
80
60
40
20
0
DMSO
CsH

C


MFI Cell Proliferation dye
20000
15000
10000
5000
0
day0
24h
4days
CAR-CD19 dmso
CAR-CD19 CsH
CAR-GD2 dmso
CAR-GD2 CsH

D

FIG. 6

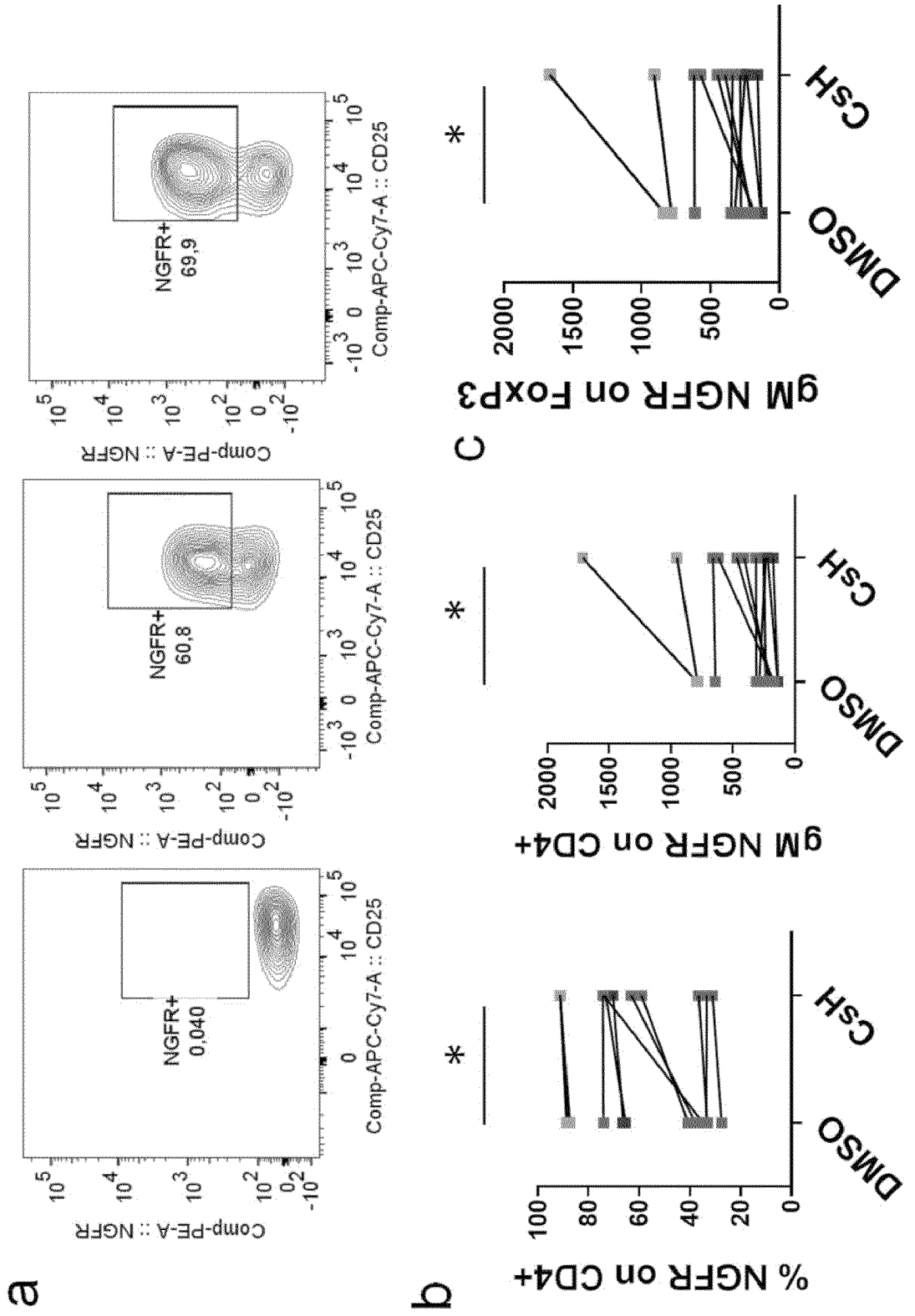

a
Comp-PE-A :: NGFR
Comp-APC-Cy7-A :: CD25
NGFR+
0,040
NGFR+
60,8
NGFR+
69,9
b
% NGFR on CD4+
gM NGFR on CD4+
c
gM NGFR on FoxP3
DMSO
CsH
*

FIG. 7

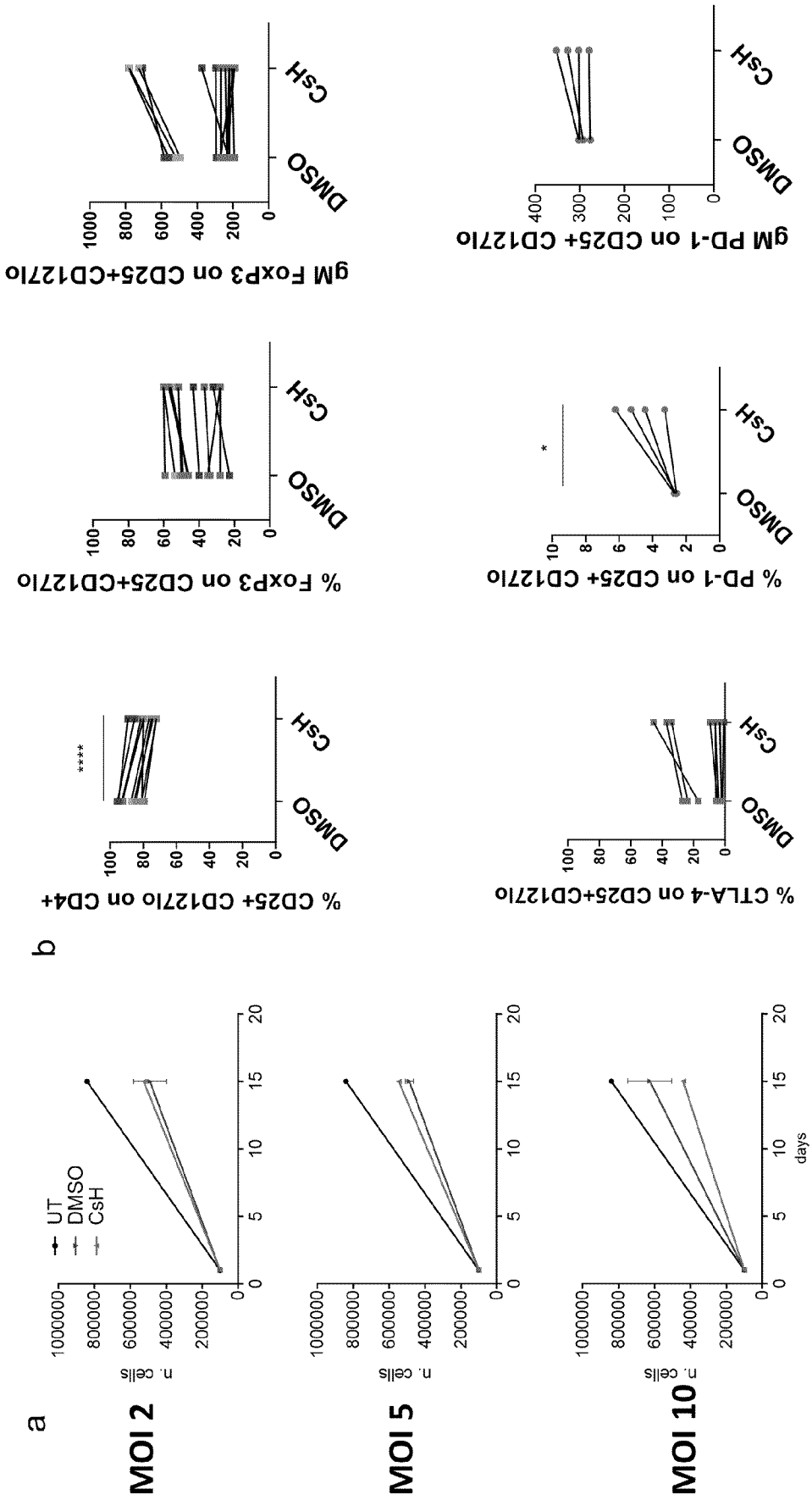

a
MOI 2
MOI 5
MOI 10
UT
DMSO
CsH
n. cells
days
b
% CD25+ CD127lo on CD4+
% FoxP3 on CD25+CD127lo
gM FoxP3 on CD25+CD127lo
% CTLA-4 on CD25+CD127lo
% PD-1 on CD25+ CD127lo
gM PD-1 on CD25+ CD127lo
DMSO
CsH

FIG. 8

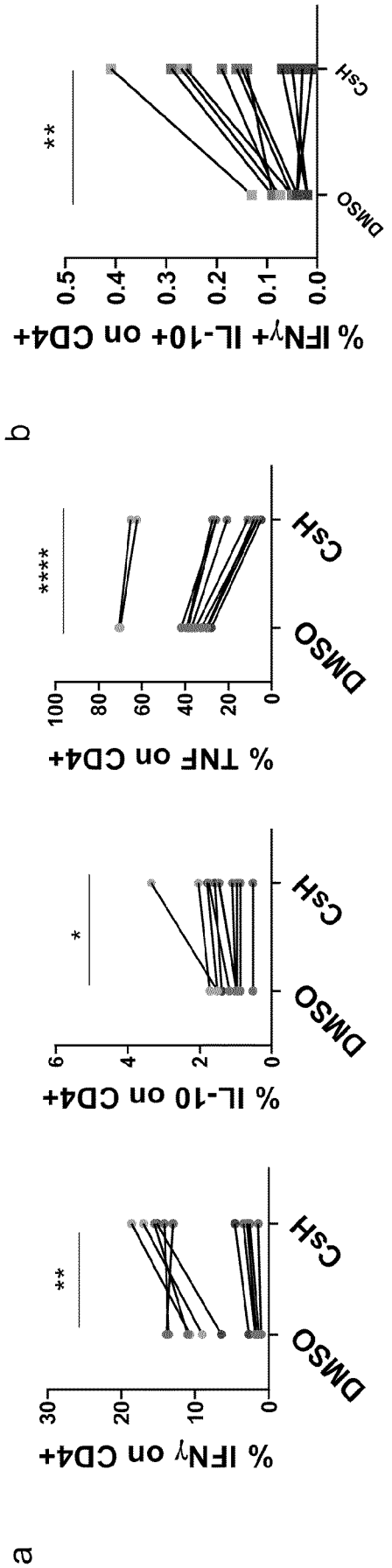

a
% IFNγ on CD4+
30
20
10
0
**
DMSO
CsH
% IL-10 on CD4+
6
4
2
0
*
DMSO
CsH
% TNF on CD4+
100
80
60
40
20
0
****
DMSO
CsH
b
% IFNγ+ IL-10+ on CD4+
0.5
0.4
0.3
0.2
0.1
0.0
**
DMSO
CsH

FIG. 9

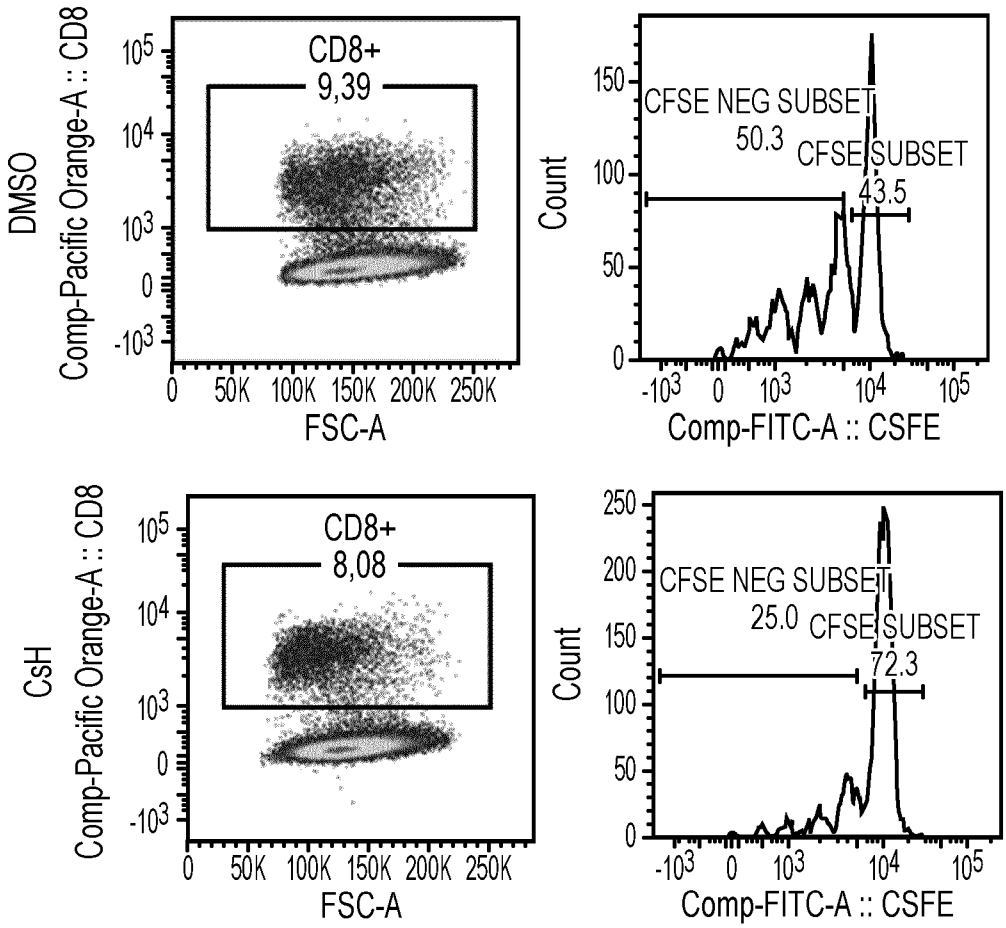


FIG. 10A

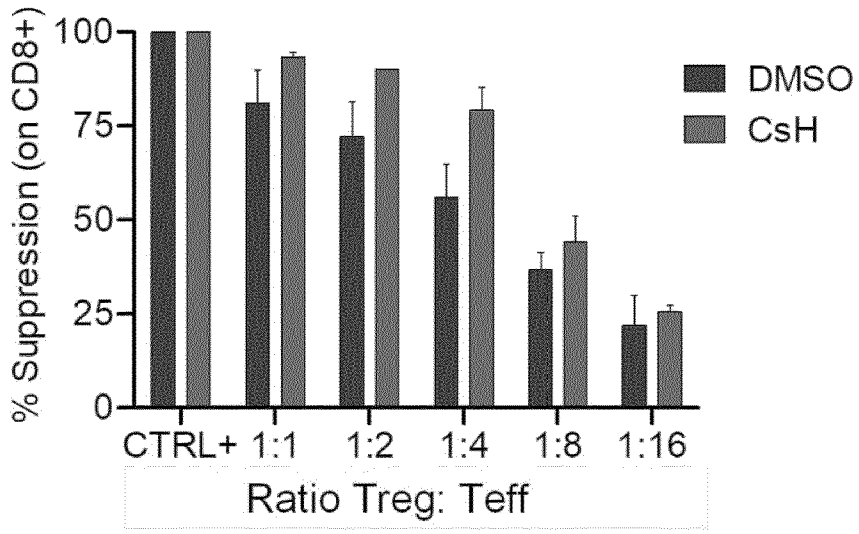


FIG. 10B

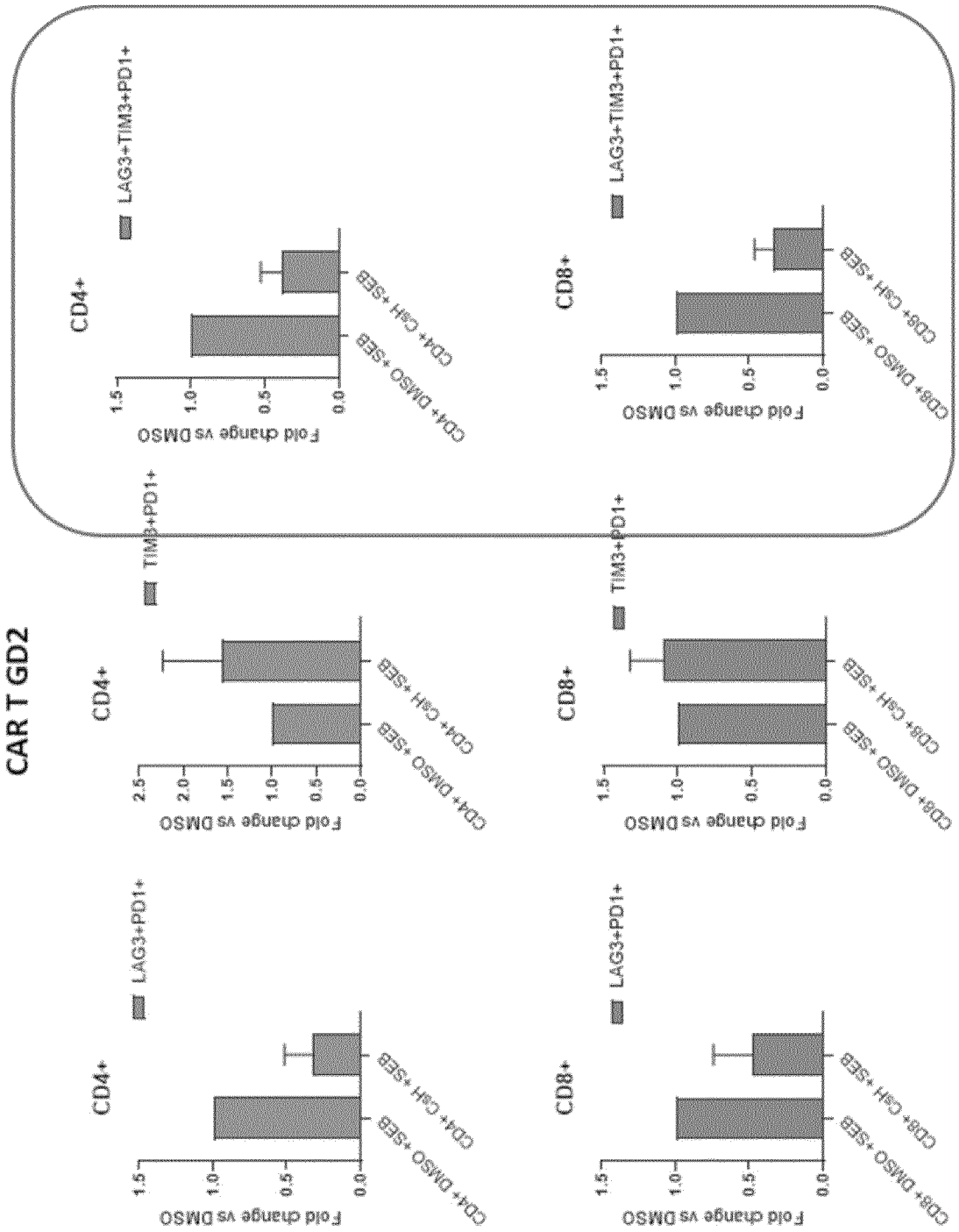

CAR T GD2
CD4+
CD8+
LAG3+TIM3+PD1+
TIM3+PD1+
LAG3+PD1+
Fold change vs DMSO
CD4+ DMSO +SEB
CD4+ CAR +SEB
CD8+ DMSO +SEB
CD8+ CAR +SEB

FIG. 11