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Feasibility of manufacture of chimeric antigen receptor-regulatory T cells from patients with end-stage renal disease

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Abstract

Background Gene-modified cell therapy with regulatory T cells (Tregs) is a promising approach to prevent graft rejection and induce immunological tolerance in organ transplantation. We are developing a cell therapy comprising autologous naïve Tregs that are isolated from leukapheresate, transduced with lentiviral vector encoding a chimeric antigen receptor (CAR) recognising human leukocyte antigen class I molecule A*02 (HLA-A*02), and expanded ex vivo before cryopreservation as resultant drug product (TX200-TR101). In an ongoing first-in-human study (NCT04817774), kidney transplant recipients will receive a single infusion of TX200-TR101 2–3 months after transplantation. The phase 0 study described here evaluated the feasibility of manufacture of TX200-TR101 for the target population, i.e., end-stage renal disease (ESRD) necessitating kidney transplantation. Participants in this study did not receive an infusion of drug product.

Methods Four patients with ESRD and HLA-A*02 negative typing underwent leukapheresis to collect starting material for manufacture of TX200-TR101. Manufacturing success criteria were predefined as a batch of CAR-Tregs with cell quantity in each batch ≥ 10^4 cells/kg body weight, cell viability ≥ 70%, transduction efficiency ≥ 20% and hypomethylation of the FoxP3 gene (Treg-specific demethylated region [TSDR]) ≥ 80%. Other manufacturing variables included Treg identity and maturation by phenotyping, residual bead count, vector copy number, endotoxin level, sterility, and presence of mycoplasma. The characteristics of leukapheresate starting material and drug product from patients with ESRD were compared with those from commercially purchased leukapheresate from 10 healthy donors.

Results No safety issues were identified during leukapheresis collections. Batches of drug product were manufactured from all 4 patients with ESRD and met the predefined success criteria. There was some variability in leukapheresate starting material in terms of volume of apheresis and total leukocyte counts between patients with ESRD and healthy donors, but percentage differential white blood cell counts were comparable. The quality, quantity and functional activity of manufactured CAR-Tregs were similar between ESRD patients and healthy donors. CAR-Treg drug product from one patient with pre-existing lymphopenia had similar high quality but reduced cell quantity compared with batches from the other patients with ESRD, although yield was still above the predefined target minimum number of cells.

Conclusions Manufacture of high-quality naïve CAR-Tregs from patients with ESRD is safe and feasible.

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Keywords CAR-Tregs, Manufacturing, End-stage renal disease, HLA-A*02 mismatch, Immunotherapy

Background

Kidney allotransplantation is the treatment of choice for patients with ESRD but requires lifelong immunosuppressive treatment to prevent rejection and failure of the allograft [1, 2]. Successful management requires a balance between the risk of allograft rejection from too little immunosuppression versus the drug-related toxicities of overimmunosuppression, such as the burden of infectious complications and de novo malignancies [1, 2].

With rapidly growing knowledge on regulatory T cells (Tregs) as key mediators of immune homeostasis, there is increasing interest in the use of Tregs for adoptive cell therapy in transplantation medicine [3–6]. We are developing a Treg therapy (TX200-TR101) comprising autologous naïve Tregs ($CD4^+$/$CD45RA^+$/$CD25^+$/$CD127^{low/neg}$) to prevent immune-mediated graft rejection and induce immunological tolerance following human leukocyte antigen class I molecule A*02 (HLA-A*02)-mismatched kidney transplantation [7]. HLA-A*02 was chosen as a target antigen because it is frequently linked to donor (HLA-A*02-positive) to recipient (HLA-A*02-negative) transplant incompatibility, with reports of 21–28% of kidney transplant recipients in Europe and the United States receiving an HLA-A*02-mismatched kidney transplant [8–12].

In clinical practice, it is intended that white blood cells (WBCs) will be collected by leukapheresis up to 6 months prior to planned transplant surgery from an HLA-A*02-negative recipient designated to receive a living-donor kidney transplant from an HLA-A*02-positive donor (Fig. 1). The naïve Tregs will be isolated from leukapheresate, then genetically modified with a lentiviral vector encoding for a chimeric antigen receptor (CAR) specific to the mismatched donor HLA-A*02 antigen and expanded ex vivo to produce TX200-TR101 [7]. The transplant recipient will receive a single intravenous infusion of TX200-TR101 between 2 and 3 months after transplant surgery.

The manufacturing process for the drug product was established using leukapheresates collected from healthy HLA-A*02-negative donors. Due to the scarcity of naïve Tregs in the starting material, together with the stringent isolation process to obtain a highly pure population, the quantity of target cells to be seeded for the expansion steps for TX200-TR101 manufacture is low. In contrast to conventional CAR-T cell products, for which the percentage of conventional T cells (Tconv) ($CD4^+$ and $CD8^+$) represents a high proportion of the total WBC population in the starting material (leukapheresate), the proportion of cells that are naïve Tregs ($CD4^+$/$CD45RA^+$/$CD25^+$/$CD127^{low/neg}$) in the leukapheresate is

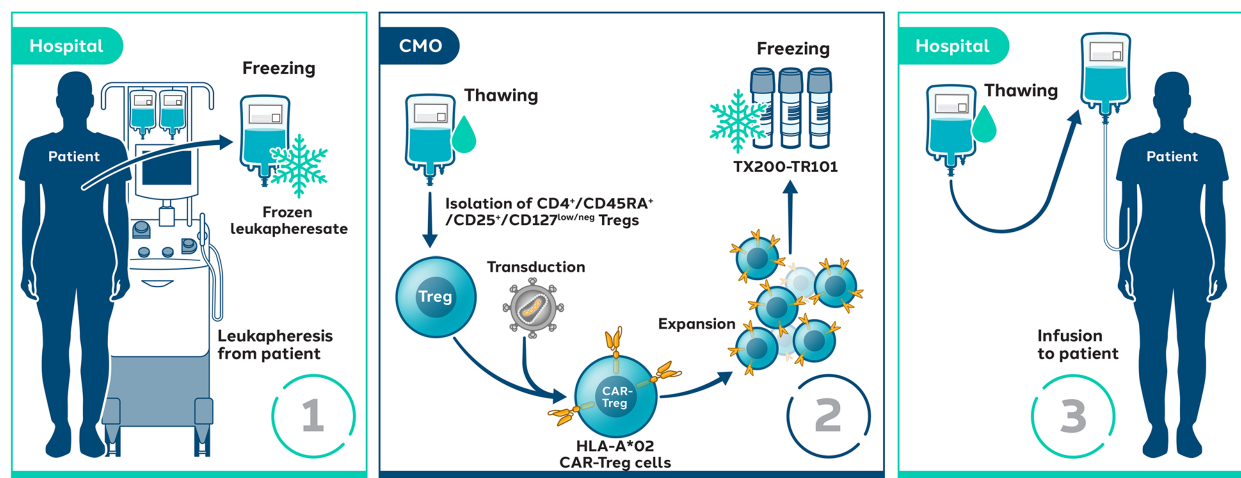

Fig. 1 Manufacture of TX200-TR101 for use in kidney transplantation. (1) HLA-A*02-negative kidney transplant recipients designated to receive a mismatched HLA-A*02–positive organ undergo leukapheresis to collect white blood cells. (2) Naive Tregs ($CD4^+$/$CD45RA^+$/$CD25^+$/$CD127^{low/neg}$) are isolated, transduced with a lentiviral vector encoding the HLA-A2-CAR and expanded ex vivo before cryopreservation of the drug product (TX200-TR101). (3) The transplant recipient will receive an intravenous infusion of the autologous HLA-A2-CAR-Tregs between 2 and 3 months after kidney transplantation. CAR, chimeric antigen receptor; CD, cluster of differentiation; CMO, contract manufacturing organisation; HLA-A*02, human leukocyte antigen class I molecule A*02; Tregs, regulatory T cells

approximately 30-fold lower (*in-house unpublished data*). Furthermore, the factors that determine the quantity and behaviour of circulating naïve Tregs under chronic renal replacement therapy have not been studied. Thus, this manufacturing feasibility study was designed to investigate the manufacturing process of TX200-TR101 for the target population of patients with ESRD necessitating kidney transplantation, before initiating a first-in-human clinical trial (STEADFAST) [7]. Patients participating in this manufacturing feasibility study did not receive an infusion of TX200-TR101.

The characteristics of leukapheresate starting material and drug product from 4 patients with ESRD were compared with those for commercially purchased leukapheresate from 10 healthy donors. For patients with ESRD, all batches of drug product were manufactured at the sponsor's manufacturing facility. For healthy donors, batches of drug product were either manufactured at the sponsor's manufacturing facility or under good manufacturing practice (GMP) conditions at a contract manufacturing organisation; the characteristics of the drug product manufactured at the two different facilities were similar; thus, data for healthy donors are presented as a single group in this publication.

Methods

Study design

We conducted an open-label, single centre, manufacturing feasibility study (protocol TX200-KT01) to produce a cell therapy (TX200-TR101) by transducing naïve Tregs obtained from leukapheresis material from HLA-A*02 negative ESRD patients with a CAR that would recognize the HLA-A*02 antigen. This was a phase 0 manufacturing feasibility study (no therapeutic intent) and the manufactured cell product was not administered to the study participants.

The primary objective was to evaluate the full manufacturing process and to characterise TX200-TR101 in terms of cell quantity, viability, transduction efficiency and FoxP3 hypomethylation. Secondary objectives were to assess the safety of the leukapheresis procedure in patients with ESRD and to characterise TX200-TR101 in terms of Treg identity and maturation, recovery of viable T cells, residual impurities, endotoxin, sterility, mycoplasma and vector copy number (VCN).

The study was conducted at the Centre Hospitalier et Universitaire de Nantes in collaboration with the Etablissement Français du Sang for leukapheresis and the Unité d'Ingénierie Cellulaire for cryopreservation of leukapheresis material. The study protocol, its amendments, and information provided to patients were reviewed and approved by an independent ethics committee. All patients provided written informed consent before participation.

Patients underwent a screening visit to assess eligibility and a leukapheresis visit up to 21 days later. The leukapheresis procedure followed local centre guidelines. A single course of leukapheresis (~ 2.5 total blood volumes) using the Spectra Optia leukapheresis system (Terumo BCT) was conducted within 21 days after screening. Local guidelines were followed for leukapheresis. One bag of leukapheresate with a volume of ~ 100–250 mL was collected from each patient. Safety assessments (adverse events, laboratory parameters, 12-lead electrocardiogram, physical examination and vital signs) were assessed at screening, pre- and post-leukapheresis. Patients were contacted by phone within 3 days after leukapheresis to check their clinical status.

It was planned to manufacture 6 batches of TX200-TR101, to allow the assessment of naturally occurring variabilities inherent to using a biological starting material. Up to 9 patients were to be enrolled to achieve this aim. The first patient was enrolled in April 2019; enrolment was paused in April 2020 due to the COVID-19 pandemic. At that time, 4 patients had undergone leukapheresis and completed the study as planned. A decision was taken to terminate the study in August 2021 as patients with ESRD continued to be at high risk for severe COVID-19 and the primary objective of the study, to evaluate the manufacturing process for TX200-TR101 from patients with ESRD, had been met with the 4 patients already enrolled.

Study population

Patients aged between 18 and 70 (inclusive) years with ESRD, HLA-A*02 negative typing, body weight ≥ 50 kg, and haemoglobin ≥ 100 mg/L were eligible for the study. Patients had to have full blood count, coagulation screen, biochemistry, and urinalysis without any clinically significant abnormalities except for parameters related to the underlying pathology, normal or non-clinically significant abnormality in electrocardiogram, adequate venous access for apheresis and no other contraindications for leukapheresis. Women of childbearing potential had to have a negative serum pregnancy test at screening and leukapheresis visits.

Manufacturing

Leukapheresate from patients with ESRD was transported to the Unité d'Ingénierie Cellulaire at ambient temperature, where peripheral blood mononuclear cells (PBMCs) were concentrated by centrifugation. The cell pellet was resuspended in 5% human serum albumin and underwent standard analytical testing,

including complete cell count, before cryopreservation in a solution containing 10% dimethyl sulphoxide. The cryopreserved product was shipped in vapour phase liquid nitrogen (≤ 140 °C) to the manufacturing site at Sangamo Therapeutics France SAS. Naïve Tregs were isolated from thawed leukapheresate using an automated cell separation system based on MACS® technology. Isolated Tregs were expanded in vitro prior to transduction with a GMP-Grade third-generation self-inactivating HIV-1 derived lentiviral vector that encodes for an HLA-A*02-specific CAR (produced at Lentigen Technology Inc., Gaithersburg, MD, USA). For information, the HIV-1-derived lentiviral vector used to express the anti-HLA-A2 CAR has been produced in commercially large-scale by Lentigen Technology Inc. The transgene lentiviral backbone and lentiviral vector helpers are Lentigen's proprietary plasmids (confidential). Transduced cells were activated with anti-CD3/anti-CD28 beads, expanded, washed, and harvested to provide TX200-TR101 drug substance. Drug substance was then formulated, finished and cryopreserved as TX200-TR101 drug product.

Cryopreserved leukapheresates from 10 consented healthy human donors were purchased from HemaCare (Charles River). Batches of drug product from 5 healthy donors were manufactured at Sangamo Therapeutics France SAS as described above. The manufacturing process was then transferred to a contract manufacturing organisation in Europe, where batches of drug product from 5 different healthy donors were manufactured under GMP conditions. The full panel of functional assays (see below) was not conducted on every batch of drug product manufactured from healthy donor material.

Characterisation of drug product

Manufacturing success criteria were predefined as a batch of CAR-Tregs with cell quantity in each batch of at least 10^4 cells/kg body weight, cell viability of ≥ 70%, transduction efficiency of ≥ 20%, and hypomethylation of the FoxP3 gene (TSDR) of ≥ 80%. Other manufacturing variables included Treg identity and maturation by phenotyping, count of residual beads and VCN. Compendial tests according to the European Pharmacopeia were also conducted for endotoxin, sterility, and mycoplasma.

Cell quantitation and analysis by flow cytometry was conducted using the MACSQuant® Analyzer 10 (Miltenyi Biotec).

Absolute cell count and cell viability

Absolute cell count was determined volumetrically by flow cytometry immediately after thawing of drug product. Cell viability was determined by adding propidium iodide (PI) (130–093-233, Miltenyi Biotec) to the cell sample immediately before starting the cell count. PI is a fluorescent intercalating agent used to stain DNA inside cells with compromised membrane; it cannot permeate viable cells. Viable cell recovery was determined as the percentage of the total number of viable cells in the thawed drug product compared to the number of cells initially frozen.

Transduction efficiency

Identification and quantification of the HLA-A*02 CAR expressed on the cell surface of the transduced Tregs was based on the use of Dextramer® consisting of a dextran polymer backbone carrying the HLA-A*02 antigen labelled with phycoerythrin (PE) (WB2666-PE, Immudex). For estimation of background noise, Dextramer blank (NI3233-PE, Immudex) labelled with phycoerythrin only was used. Results are expressed as the percentage of anti-HLA-A2 CAR expressing cells among living cells, measured by flow cytometry.

FoxP3 Hypomethylation

The percentage of TSDR within the FoxP3 locus was measured by Precision for Medicine GmbH (Berlin, Germany), using quantitative PCR (qPCR). Briefly, genomic DNA was isolated using the DNeasy blood and tissue Kit (Qiagen). After bisulfite conversion of genomic DNA, PCR products were generated with methyl- and non-methyl-specific primers for FoxP3 TSDR. Amounts of methylated and unmethylated FoxP3 DNA were estimated from calibration curves. The proportion of unmethylated DNA was computed as the ratio of unmethylated FoxP3 TSDR-DNA and the sum of methylated and unmethylated FoxP3 TSDR-DNA.

Treg identity and maturation status phenotyping

For Treg identity phenotyping (expression of FoxP3 and CD62L), the human antibodies VioGreen-conjugated anti-CD4 (130–113-230, Miltenyi Biotec), PE-conjugated anti-CD25 (130–113-282, Miltenyi Biotec), APC-Vio770-conjugated anti-CD127 (130–113-416, Miltenyi Biotec) andPE-Vio770-conjugated anti-CD62L (130–113-621, Miltenyi Biotec) were used for surface membrane staining and AlexaFluor 647-conjugated anti-FoxP3 antibody (560,045, BD Biosciences) was used for intranuclear staining. For Treg maturation status phenotyping (expression of CD45RA and CD27 among $CD4^+$ cells), the human antibodies VioBlue-conjugated anti-CD4 (130–113-219, Miltenyi Biotec), APC-conjugated anti-CD27 (130–113-636, Miltenyi Biotec), and PE-conjugated anti-CD45RA (130–113-366, Miltenyi Biotec) were used for surface membrane staining.

Residual beads count

Cells in the drug product were lysed to release any residual beads. Beads were separated from cell debris using a magnet (DynamagTM-15), resuspended, concentrated and counted by flow cytometry. The lower limit of quantification was 200 beads per 1×10^7 Tregs.

Vector copy number

A duplex quantitative real-time polymerase chain reaction (qPCR) assay was used to detect both the HIV-PSI gene (specific sequence of the lentiviral vector used in the process) and the human albumin gene (housekeeping sequence) in a single reaction tube. Each target was amplified by a different set of primers and a uniquely-labelled probe distinguishing each PCR amplicon. The qPCR amplification was performed using the Bio-Rad CFX96 or equivalent system.

HLA-A2 CAR-Treg functional assays

Activation capacity was determined by measuring the percentage of CD69-expressing cells among $CD4^{pos}$/CAR HLA-A*02^{pos} cells by flow cytometry following activation for 24 h with HLA-A*02^{pos} PBMCs or HLA-A*02^{neg} PBMCs. PE-conjugated anti-CD69 (130–112-613, Miltenyi Biotec) was used for surface membrane staining. Controls were included in the assay, including no activation and polyclonal activation through the T cell receptor (TCR) using anti-CD3/CD28 coated Dynabeads® (40203D, Life Technologies). Results are expressed as the difference in levels of CD69-expressing cells under the activation conditions of HLA-A*02^{pos} PBMCs versus HLA-A*02^{neg} PBMCs.

CAR-Treg proliferation was determined using adenosine triphosphate (ATP) quantitation by bioluminescence (luciferin/luciferase reaction). HLA-A2 CAR-Tregs were stimulated with HLA-A2 Dextramer or Dextramer blank. Positive and negative controls were included in the assay, i.e., polyclonal stimulation through the TCR using anti-CD3/anti-CD28 coated Dynabeads or no activation. The proliferation of HLA-A2 CAR Tregs was measured after 3 days using CellTiter®-Glo 2.0 (G9241, Promega) colorimetric assay. Light output (relative light units) was measured with the GloMax® luminometer (Promega). Results are expressed as the ratio of ATP bioluminescence between HLA-A2 Dextramer and Dextramer blank activation conditions.

Suppressive capacity was determined by measuring the proliferation of allogeneic Tconv ($CD4^+CD25^-$) by flow cytometry when co-cultured with drug product. Tregs were expanded during 24 h in medium without IL-2 to suppress any activation signal; allogeneic Tconv (HLA-A*02 negative) were thawed the same day and maintained in the same condition. At the end of the 24-h expansion without any activation, Tconv were stained with eFluor450 (65–0842-85, eBioscience), a cell proliferation dye used to monitor individual cell divisions. Immediately after staining, the Tconv were preactivated for 24 h through their TCR using anti-CD3/anti-CD28 coated Dynabeads to induce proliferation whilst Tregs were preactivated through their CAR with HLA-A*02 Dextramer or Dextramer blank. After 24 h of activation, Tregs and Tconv were co-cultured for 3 days at Tconv:Treg ratios ranging from 1:1 to 16:1. Cell proliferation was measured by flow cytometry.

Results

Study population of patients with ESRD

Four patients (3 men and 1 woman) aged 41 to 63 years with ESRD were screened for the study, underwent leukapheresis and completed the study as planned. Body weight ranged from 63 to 80 kg, body mass index from 23 to 25 kg/m^2 and estimated glomerular filtration rate [13] from 5 to 20 mL/min/1.73 m^2.

The patients had various medical histories and ongoing illnesses at screening as might be expected for this patient population. All 4 patients had hypertension, dyslipidaemia and anaemia, all medically treated. Causes of ESRD were congenital polycystic kidney disease, type 1 diabetes and hypertensive nephropathy in two patients. Of note, 1 patient had ongoing grade 2 lymphopenia at screening with a lymphocyte count of 0.7×10^9/L (normal range 1.5 to 4×10^9/L) and a leukocyte count of 4.4×10^9/L (normal range 4 to 10×10^9/L). Two of the patients were on dialysis (three-times weekly); for these patients the leukapheresis was conducted on a dialysis-free day.

Safety of the leukapheresis procedure for patients with ESRD

No safety issues were identified during the leukapheresis procedure in any patient. No serious adverse event or abnormality of Common Terminology Criteria for Adverse Events (CTCAE) grade 3 or higher was reported. Twelve procedure-emergent adverse events were reported (Table 1). Post-procedure haematological profiles were consistent with those anticipated with leukapheresis.

Characterisation of leukapheresate starting material from patients and healthy donors

The volume of apheresis and total leukocyte counts in leukapheresates (starting material) were generally higher for the 4 patients with ESRD compared with commercially purchased leukapheresates from 10

Table 1 Summary of procedure-emergent adverse events

MedDRA preferred term	Number of patients with events	Number of events		
		CTCAE grade 1	CTCAE grade 2	Total
Haemoglobin decreased	3	2	1	3
Asthenia	2	1	1	2
Leukopenia	2	2	0	2
Anaemia	1	0	1	1
Paraesthesia	1	1	0	1
Nausea	1	1	0	1
Cough	1	1	0	1
Hypertension	1	0	1	1
Total	**4**	**8**	**4**	**12**

There were no adverse events of CTCAE grade 3 or higher.

CTCAE Common Terminology Criteria for Adverse Events, *MedDRA* Medical Dictionary for Regulatory Activities

healthy donors (median leukocyte counts: 20.1×10^9 cells for ESRD patients vs 11.0×10^9 cells for healthy donors). However, differential WBC counts, expressed as a percentage of total leukocyte counts, were comparable in leukapheresates from ESRD patients and healthy donors (Fig. 2). Naïve Treg frequencies amongst leukocytes in thawed leukapheresates from patients with ESRD ranged from 0.35% to 0.96%. Naïve Treg counts for leukapheresates from the 10 healthy donors used for this comparison were not analysed, but naïve Treg frequencies in subsequent commercially purchased leukapheresates from other healthy donors ranged from 0.52% to 2.69% (unpublished data from $n = 26$, N Lounnas-Mourey, 2023), with a generally higher range than observed for patients with ESRD.

Characterisation of drug product from patients and healthy donors

Batches of drug product with the quality attributes of a CAR-Treg cell therapy were manufactured for all 4 patients with ESRD and met the predefined success criteria (see *Methods*) for cell quantity, cell viability and FoxP3 hypomethylation. In addition, all four batches met the predefined success criteria for transduction efficiency (> 20%), with no statistically significant difference in the level of transduction efficiency between batches manufactured from ESRD and healthy donors (Tukey–Kramer, $p = 0.38$). The batch of CAR-Tregs from the patient with pre-existing lymphopenia had similar high quality but lower cell quantity than the batches from the other patients with ESRD, although yield was still above the predefined target minimum number of cells.

The naïve Treg population ($CD4^+$/ $CD25^+$/ $CD45RA^+$/ $CD127^{low/neg}$) was ≥ 93% in all batches of TX200-TR101, from both patients and healthy donors (Fig. 3a). The phenotypic markers FoxP3 (intranuclear transcription factor) [14] and CD62L (L-selectin adhesion molecule expressed on the surface of naïve T cells) [15] were used to confirm the purity of the manufactured Tregs. The $CD4^+$/$CD25^+$/$FoxP3^+$

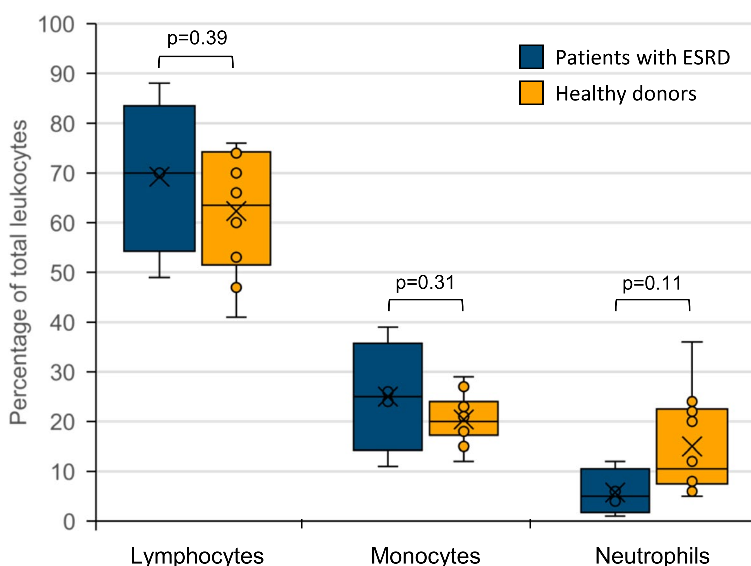

Fig. 2 Differential white blood cell counts in leukapheresates. Boxplots of differential cell counts (expressed as percentage of total leukocytes) in fresh leukapheresate from patients with ESRD ($n = 4$) and commercially purchased leukapheresate from healthy donors ($n = 10$). The box represents the interquartile range; the median value divides the box in two parts; whiskers represent minimum and maximum values; X represents the mean value. Individual data points (other than minimum and maximum values) are shown as open circles. No statistically significant differences in the means between the two populations (ESRD patients vs healthy donors) were observed

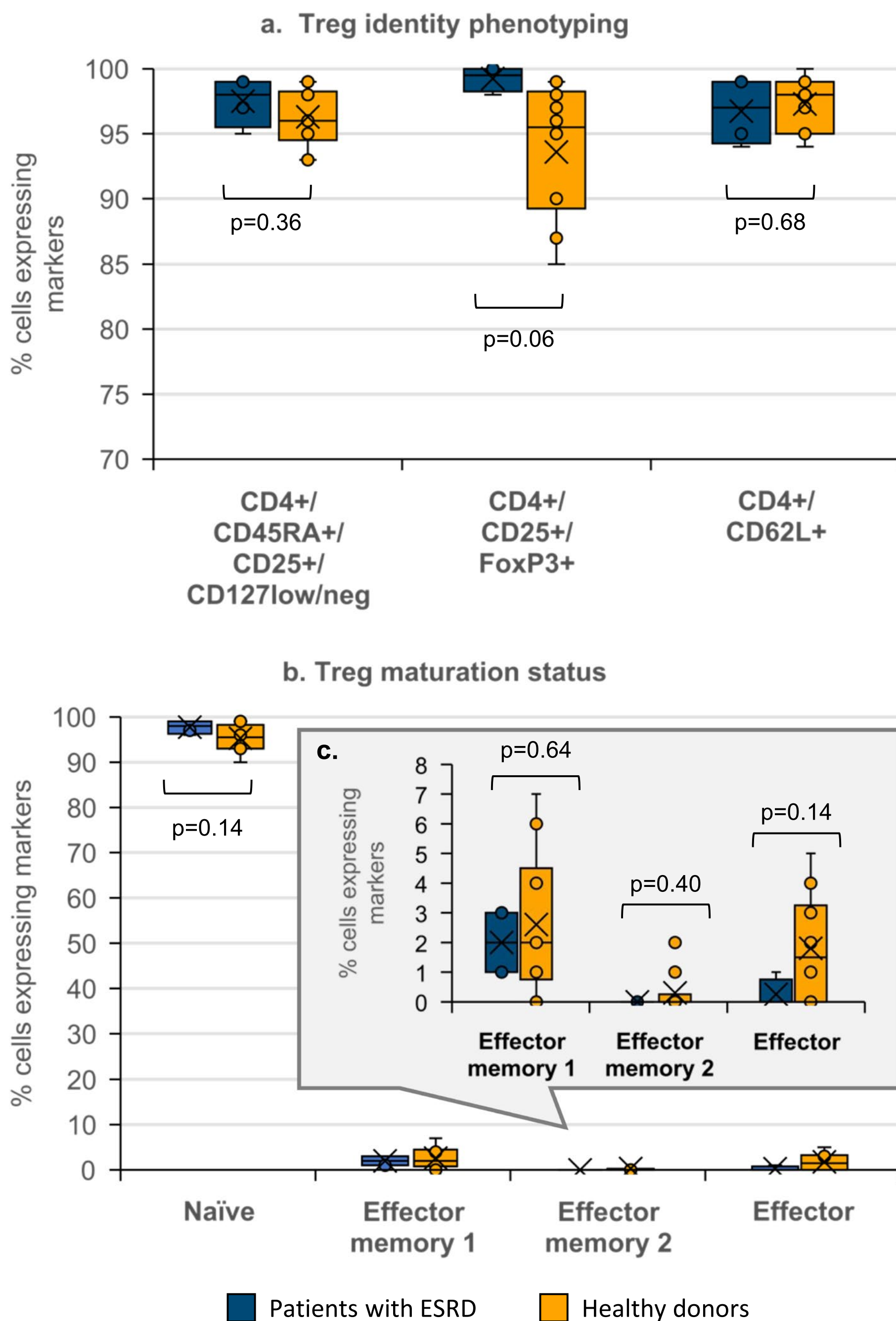

Fig. 3 Treg identity and maturation status phenotyping. **a** Percentage of cells expressing the combination of specified markers in batches of TX200-TR101 manufactured from patients with ESRD ($n=4$) and healthy donors ($n=10$). **b** Percentage of naïve (CD4$^+$/CD45RA$^+$/CD27$^+$), effector memory 1 (CD4$^+$/CD45RA$^-$/CD27$^+$), effector memory 2 (CD4$^+$/CD45RA$^-$/CD27$^-$) and effector (CD4$^+$/CD45RA$^+$/CD27$^-$) cells in batches of TX200-TR101 manufactured from patients with ESRD ($n=4$) and healthy donors ($n=10$). **c** The inset panel shows the same data as panel b for effector memory 1, effector memory 2 and effector cells plotted on a larger y-axis scale. For each boxplot, the box represents the interquartile range; the median value divides the box in two parts; whiskers represent minimum and maximum values; X represents the mean value. Individual data points (other than minimum and maximum values) are shown as open circles. No statistically significant differences in the means between the two populations (ESRD patients vs healthy donors) were observed

population was ≥ 85% and the $CD4^+/CD62L^+$ population was ≥ 94% in all batches from patients and healthy donors (Fig. 3a), demonstrating a highly pure Treg population.

At the start of the manufacturing process, naïve Tregs were isolated based on the expression of $CD4^+/CD45RA^+/CD25^+/CD127^{low/neg}$. To show the stability of the naïve profile of cells at the end of the manufacturing process, we evaluated co-expression of CD45RA and CD27 among $CD4^+$ cells, as markers of naïve Tregs [16]. We confirmed that Tregs were predominantly still present in naïve status ($CD4^+/CD45RA^+/CD27^+$) at the end of the manufacturing process (Fig. 3b). In contrast, percentages of effector memory 1 (central memory; $CD4^+/CD45RA^-/CD27^+$), effector memory 2 ($CD4^+/CD45RA^-/CD27^-$) and effector ($CD4^+/CD45RA^+/CD27^-$) Tregs were low (≤ 7%) in all batches of drug product from ESRD patients and healthy donors.

The stability of the naïve status of CAR-Tregs on freeze/thawing was evaluated for 4 batches of drug product manufactured from healthy donor material. No loss of naïve phenotype was observed after storage at ≤ -140 °C for up to 12 months.

All batches of drug product from patients met release criteria for visual inspection (no visible particles), mycoplasma testing (negative), bacterial and fungal sterility (no growth), endotoxin (≤ 5 EU/mL), vector copy number (< 5 copies/CAR-Treg) and residual beads (below lower limit of quantification of 200 beads/10^7 Tregs). The batches of drug product manufactured from patient material had quality attributes that were consistent with batches manufactured from healthy donor material.

Functional activity of drug product from patients and healthy donors

By introducing an HLA-A2 CAR into naïve Tregs, our therapeutic hypothesis is that the CAR-Tregs will engage the target antigen HLA-A*02 in the kidney graft and produce an immunoregulatory effect. Engagement of the HLA-A2 CAR with HLA-A*02 PBMCs in vitro consistently induced the activation of the CAR-Tregs, as demonstrated by upregulation of cell surface expression of the activation marker CD69 [17] above the level of expression of CD69 on cells that were stimulated with control (HLA-A*02^{neg} PBMCs). Activation capacity (defined as the difference in the level of expression of CD69 between cells stimulated with HLA-A*02^{pos} PBMCs versus HLA-A*02^{neg} PBMCs) was similar for cells manufactured from material from ESRD patients and healthy donors (median values of 84.5% and 80.0%, respectively) (Fig. 4a).

Activation of HLA-A2 CAR-Tregs through the CAR by the HLA-A*02 antigen consistently induced CAR-Treg proliferation in vitro, as demonstrated by ATP production measured with a bioluminescence assay (Fig. 4b). This assay indicates the presence of metabolically active cells and is directly correlated to cell proliferation [18]. Median values for the ratio of ATP production for cells stimulated with HLA-A2 Dextramer versus blank Dextramer were 1.50 and 1.58 for batches of drug product manufactured from ESRD patient material and healthy donor material, respectively, demonstrating similar levels of induced proliferation in vitro.

The suppressive capacity of the HLA-A2 CAR-Tregs was measured as their ability to suppress in vitro proliferation of Tconv ($CD4^+/CD25^-$). For all manufactured batches of TX200-TR101, it was consistently shown that Tregs, pre-activated through the CAR with HLA-A2 Dextramer, exerted a dose-dependent suppressive effect on Tconv proliferation in vitro (Fig. 5). Median percentage suppression for CAR-Tregs manufactured from ESRD patients was lower than that observed for healthy donors (28.5% vs 54% at 1:1 ratio of Tconv:Treg). However, the assays for ESRD patients and healthy donors were performed at different times using different batches of reagents. The batch of HLA-A2 Dextramer used in assays with ESRD patient material consistently gave lower suppression values, even with CAR-Treg material from other healthy donors tested during the same period.

Statistical analysis

All cell populations were analysed by one way analysis of variance using SAS JMP v16.1, to assess mean differences between batches manufactured from patients with ERSD and healthy donors with the confidence quantile set to a limit of 95% ($\alpha = 0.05$). Tukey–Kramer analysis was performed on all pairs and no statistically significant differences in the means between the two populations were observed.

Discussion

This study was conducted to ensure that manufacture of clinical grade cell therapy product from the target population of patients with ESRD was safe and feasible by assessing full-scale manufacture for TX200-TR101 drug product (HLA-A2 CAR-Tregs) using leukapheresis from patients with ESRD awaiting kidney transplantation.

We found that the leukapheresis procedure was well tolerated in patients with ESRD and that it could be fitted around the patients' routine dialysis schedule. There was some variability in volume of apheresis and total leukocyte counts in leukapheresates obtained from patients with ESRD and commercially purchased leukapheresates from healthy donors, but differential WBC counts in starting materials were comparable.

We successfully obtained highly purified, naïve Tregs from patients with ESRD and confirmed the feasibility of the subsequent manufacturing process for production

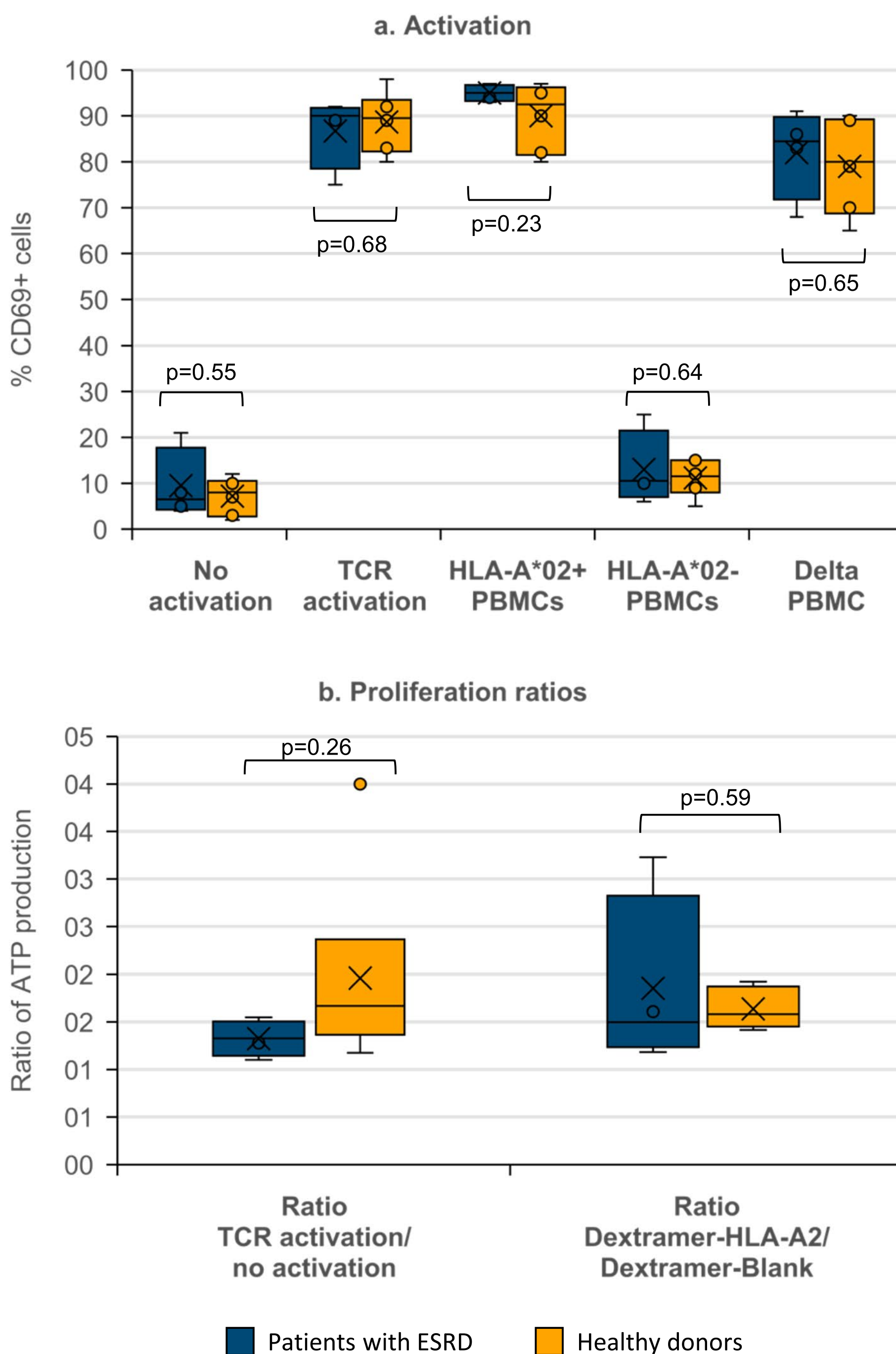

Fig. 4 Functional activity of HLA-A2 CAR-Tregs through the target antigen HLA-A*02. **a** Percentage of CD69 + cells among CAR-Tregs under conditions of no activation (negative control), polyclonal activation through the T cell receptor (TCR) with anti-CD3/anti-CD28 (aCD3/aCD8) coated beads (positive control), specific activation via the CAR using HLA-A*02^{pos} PBMC or control (incubation with HLA-A*02^{neg} PBMC). Delta PBMC is the difference in percentage of CD69^{+} cells under activation conditions of HLA-A*02^{pos} PBMCs versus HLA-A*02^{neg} PBMCs. **b** Ratio of ATP production measured by bioluminescence assay for HLA-A2 CAR Tregs activated through their TCR (positive control) versus no activation (negative control) or HLA-A2 CAR Tregs activated with the target antigen HLA-A*02 using HLA-A2 Dextramer versus Dextramer blank (control). Boxplots show data for batches of TX200-TR101 manufactured from patients with ESRD ($n = 4$) and healthy donors ($n = 6$)[1]. The box represents the interquartile range; the median value divides the box in two parts; whiskers represent minimum and maximum values; X represents the mean value. Individual data points (other than minimum and maximum values) are shown as open circles. No statistically significant differences in the means between the two populations (ESRD patients vs healthy donors) were observed. [1]There was insufficient final product available from 4 of the healthy donors for all of the tests; consequently, the activation assay and the proliferation assay were only conducted on 6 out of the 10 batches manufactured from healthy donor material

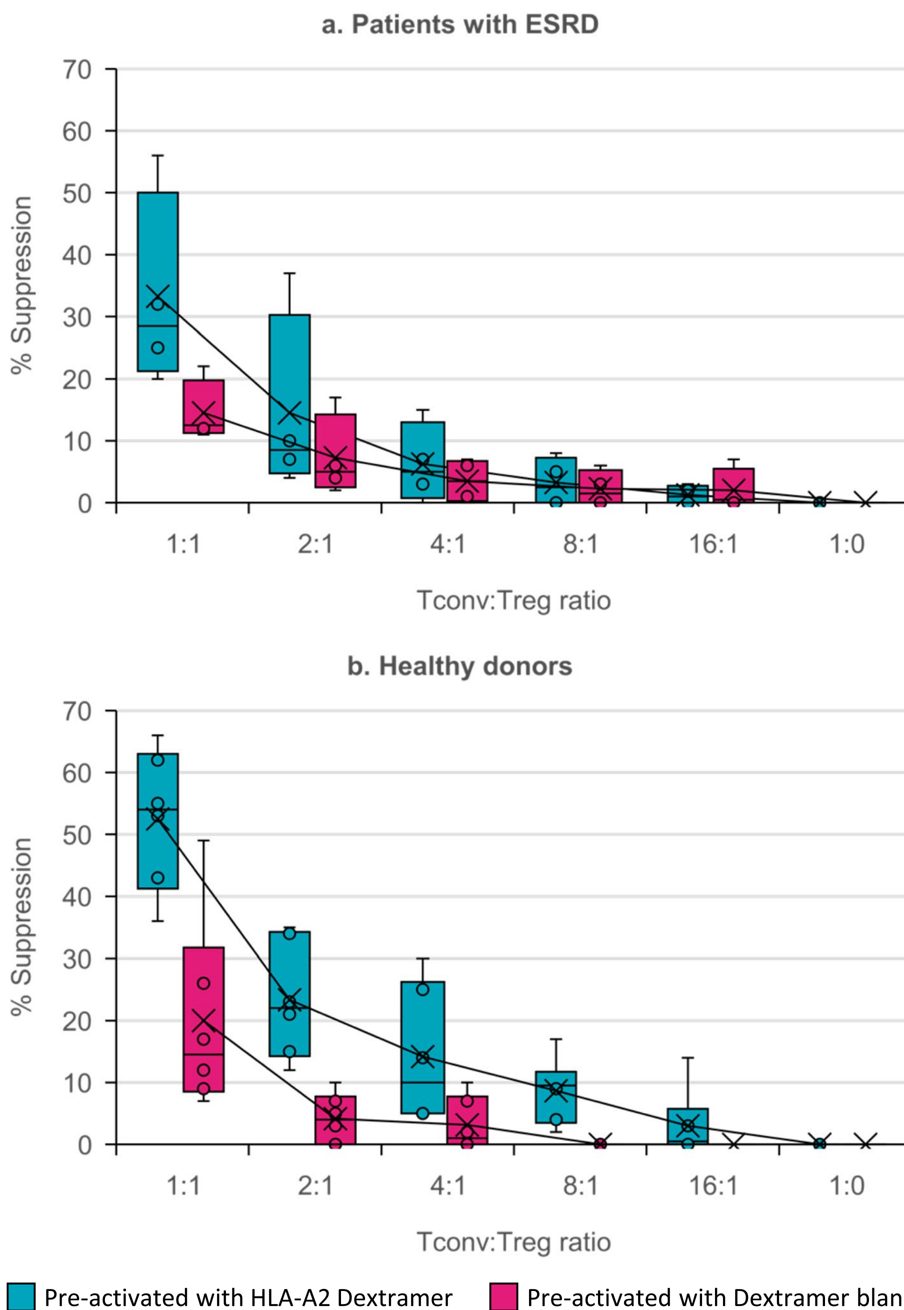

Fig. 5 Suppression of Tconv proliferation by HLA-A2 CAR-Tregs pre-activated by HLA-A2 Dextramer or Dextramer blank (control). Percentage suppression of conventional T cell (Tconv) proliferation at different ratios of Tconv:Treg under different pre-activation conditions using batches of TX200-TR101 manufactured from patients with ESRD ($n = 4$) or healthy donors ($n = 6$)[1]. The box represents the interquartile range; the median value divides the box in two parts; whiskers represent minimum and maximum values; X represents the mean value. Individual data points (other than minimum and maximum values) are shown as open circles. No statistically significant differences in the means between the two populations (ESRD patients vs healthy donors) were observed. [1]There was insufficient final product available from 4 of the healthy donors for all of the tests; consequently, the suppression assay was only conducted on 6 out of the 10 batches manufactured from healthy donor material

of the antigen-specific drug product (HLA-A2 CAR-Tregs). The underlying condition of patients with respect to concurrent illness, concomitant medications or dialysis did not impact on the ability to manufacture good quality CAR-Tregs and all batches of drug product met prespecified release criteria. It was possible to manufacture a batch of drug product from all 4 patients, including a patient with underlying lymphopenia, although

the resulting CAR-Treg cell quantity was lower for this patient. Phenotypic markers demonstrated the high purity of Tregs obtained from patient material and maturation status markers confirmed that Tregs were predominantly present in a naïve status. The stability of the naïve phenotype of the CAR-Tregs was maintained on freeze/thawing cycle. The characteristics of TX200-TR101 were broadly comparable regardless of the source of starting material (patient or healthy donor).

We showed that the engineered HLA-A2 CAR-Tregs were functional in vitro. They could be successfully activated and were shown to proliferate upon engagement with their target antigen, HLA-A*02. Furthermore, HLA-A2 CAR-Tregs, preactivated with HLA-A*02, exerted a dose-dependent suppressive function on Tconv proliferation in vitro. The observed percentage suppression was lower for CAR-Tregs derived from patient material compared with healthy donor material, but we consider this more likely due to variability of the assay and different lots of reagents used in the two sets of assays rather than a true difference in functional activity.

We have previously published data on the in vivo efficacy of the HLA-A2 CAR-Tregs in a xenogeneic graft-versus-host disease (GvHD) model [19]. We showed that mice injected only with HLA-A*02+PBMCs displayed significant GvHD, quantified with increased GvHD scores, whilst GvHD scores remained low in mice co-injected with TX200-TR101 CAR-Tregs.

In clinical practice, it is hypothesized that after intravenous infusion of TX200-TR101 to HLA-A*02 negative transplant recipients who have received a transplant from a HLA-A*02 positive donor, the HLA-A2 CAR-Tregs will migrate into the HLA-A*02 positive graft tissue, where they are expected to activate upon binding to the HLA-A*02 MHC class I molecules expressed exclusively in the allograft [7]. Upon activation, the Tregs are anticipated to proliferate and expand within the allograft and acquire their full immunosuppressive capacities [7]. The immunosuppressive function of Tregs is expected to dampen effector and cytotoxic T cell activation responsible for rejection of the graft whilst educating dendritic cells to present alloantigen to donor naïve T cells in a non-immunogenic fashion and thus establish immunologic tolerance. It is proposed that these effects will, in turn, allow for the gradual reduction and potential cessation of immunosuppressive therapy, which would enable patients to avoid the cumulative toxicities associated with such therapies.

The tolerability and safety of Treg therapies have been demonstrated in early phase clinical studies in transplantation and autoimmune disease [20–26]. In 2020, the ONE Study consortium published data from a series of studies performed in living-donor kidney transplant recipients treated with regulatory cell therapies, including polyclonal and donor-reactive Tregs, that showed first evidence that immune cell therapy is a potentially useful therapeutic approach [24]. They showed that regulatory cell therapies are safe and that living-donor kidney transplant recipients receiving immune cell therapy have fewer infectious complications compared with a reference group of kidney transplant recipients on standard-of-care immunosuppressive therapy. Most of the patients on cell therapy, in whom minimization of immunosuppression was attempted, could be successfully weaned to monotherapy within the first year post-transplantation without an increased risk of rejection [24]. Immune monitoring of peripheral blood leukocyte populations showed a return towards a state of immune homeostasis, providing evidence that cell therapy has positive systemic immunological effects [24]. The two individual studies included in the ONE Study that used polyclonal, autologous Tregs observed excellent allograft survival 4 years after kidney transplantation, along with substantial tapering of immunosuppressive treatments [25, 26].

Most Treg clinical studies performed to date have used polyclonal cells expanded ex vivo. The current study demonstrates the feasibility of collecting leukapheresate from patients with ESRD and manufacturing a Treg cell therapy product engineered to have specificity to a single antigen, thereby greatly increasing the potency of the Tregs to exert their tolerogenic effects upon encountering the target antigen, compared to antigen-nonspecific, polyclonal Tregs.

Participants in this manufacturing feasibility study did not receive an infusion TX200-TR101 after successful manufacture of drug product. However, a first-in-human dose-ranging study to assess TX200-TR101 in living-donor kidney transplant recipients is now ongoing (NCT04817774; STEADFAST) [7]. The STEADFAST study represents the next frontier in adoptive cell therapies, being the first clinical study to our knowledge to administer a CAR-Treg therapy to humans. For living-donor transplantation, the starting material (leukapheresate) for manufacture of TX200-TR101 will be collected up to 6 months before the planned date of transplantation [7]. Previous studies have shown that pharmacological immunosuppression and previous acute rejection episodes in kidney transplant recipients do not specifically alter the frequency, phenotype, or maturation of natural Tregs in vivo [3, 22]. Thus, patients who have already received allogeneic kidney transplantation may also potentially be candidates for TX200-TR101 autologous Treg therapy in the future.

Conclusions

The manufacture of high-quality naïve HLA-A2 CAR-Tregs from patients with ESRD is safe and feasible. The quality, yield and functional activity of the manufactured drug product, TX200-TR101, were similar between patients with ESRD and healthy donors.

Abbreviations

APC	Allophycocyanin
ATP	Adenosine triphosphate
CAR	Chimeric antigen receptor
CTCAE	Common Terminology Criteria for Adverse Events
ESRD	End-stage renal disease
GMP	Good manufacturing practice
GvHD	Graft-versus-host disease
HLA-A*02	Human leukocyte antigen class I molecule A*02
HLA-A2 CAR	Chimeric antigen receptor specific for HLA-A*02
PE	Phycoerythrin
PI	Propidium iodide
qPCR	Quantitative polymerase chain reaction
Tconv	Conventional T cells
TCR	T cell receptor
Tregs	Regulatory T cells
TSDR	Treg-specific demethylated region
VCN	Vector copy number
WBC	White blood cell

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Authors' contributions

HB, PH and NLM provided input to the concept and/or design of the clinical study. DC was the principal investigator of the clinical study. BLH, KHS, CC, ECS and GFA were involved in the conduct of the study. HB and PH analysed and interpreted the data regarding characterisation of HLA-A2 CAR-Tregs. NLM analysed and interpreted the data regarding characterisation of leukapheresate starting material. BLH and KHS are the sponsor's responsible medical officers. All authors were involved in interpretation of the results, drafting and critical revision of the manuscript and read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are not publicly available due to the proprietary nature of information and patient confidentiality but are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

The protocol, protocol amendments, informed consent form and other relevant study documents were reviewed and approved according to local regulations by an independent ethics committee (Comité de Protection des Personnes Ile-de-France VII, France; reference numbers 2018-A02923-52 and protocol # 18–092 (CPP IDF VII)). All patients provided written informed consent before participation. Commercially purchased leukopheresates from Charles River were collected in U.S. Food and Drug Administration-registered collection centres from healthy human donors who had consented under an institutional review board-approved protocol.

Consent for publication

Not applicable.

Competing interests

The TX200-KT01 study was sponsored by Sangamo Therapeutics. HB, NLM, BLH, KHS, CC, ECS and GFA are full-time employees of Sangamo Therapeutics. PH was formerly a full-time employee of Sangamo Therapeutics when the study was conducted and the manuscript drafted. DC was the principal investigator of the TX200-KT01 study.

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References

1. Birnbaum LM, Lipman M, Paraskevas S, Chaudhury P, Tchervenkov J, Baran D, et al. Management of chronic allograft nephropathy: a systematic review. Clin J Am Soc Nephrol. 2009;4:860–5.
2. Katabathina V, Menias CO, Pickhardt P, Lubner M, Prasad SR. Complications of immunosuppressive therapy in solid organ transplantation. Radiol Clin North Am. 2016;54:303–19.
3. Landwehr-Kenzel S, Zobel A, Hoffmann H, Landwehr N, Schmueck-Henneresse M, Schachtner T, et al. Ex vivo expanded natural regulatory T cells from patients with end-stage renal disease or kidney transplantation are useful for autologous cell therapy. Kidney Int. 2018;93:1452–64.
4. Bluestone JA, Anderson M. Tolerance in the age of immunotherapy. N Engl J Med. 2020;383:1156–66.
5. Ferreira LMR, Muller YD, Bluestone JA, Tang Q. Next-generation regulatory T cell therapy. Nat Rev Drug Discov. 2019;18:749–69.
6. Vaikunthanathan T, Safinia N, Boardman D, Lechler RI, Lombardi G. Regulatory T cells: tolerance induction in solid organ transplantation. Clin Exp Immunol. 2017;189:197–210.
7. Schreeb K, Culme-Seymour E, Ridha E, Dumont C, Atkinson G, Hsu B, Reinke P. Study design: Human leukocyte antigen class I molecule A*02-chimeric antigen receptor regulatory T cells in renal transplantation. Kidney Int Rep. 2022;7:1258–67.
8. Middleton D, Hamilton P, Doherty CC, Douglas JF, McGeown MG. Mismatching for HLA-A, -B antigens and renal graft survival. Clin Nephrol. 1985;23:26–7.
9. Barocci S, Santori G, Fiordoro S, Mossa M, Valente U, Nocera A. Detection and analysis of HLA class I specific alloantibodies in the sera of sensitised dialysis recipients waiting for kidney retransplantation. Riv Ital Med Lab. 2007;3:189–95. https://www.sipmel.it/en/riviste/articolopdf.php/2211. Accessed 12 June 2023.
10. Barocci S, Valente U, Nocera A. Detection and analysis of HLA class I and class II specific alloantibodies in the sera of dialysis recipients waiting for a renal retransplantation. Clin Transplant. 2007;21:47–56.
11. Marrari M, Duquesnoy RJ. Detection of donor-specific HLA antibodies before and after removal of a rejected kidney transplant. Transpl Immunol. 2010;22:105–9.
12. Schnitzler MA, Woodward RS, Brennan DC, Phelan DL, Spitznagel EL, Boxerman SB, et al. Cytomegalovirus and HLA-A, B, and DR locus interactions: Impact on renal transplant graft survival. Am J Kidney Dis. 1997;30:766–71.
13. Levey AS, Stevens LA, Schmid CH, Zhang YL, Castro AF 3rd, Feldman HI, et al. A new equation to estimate glomerular filtration rate. Ann Intern Med. 2009;150:604–12.
14. Fontenot JD, Rasmussen JP, Williams LM, Dooley JL, Farr AG, Rudensky AY. Regulatory T cell lineage specification by the forkhead transcription factor foxp3. Immunity. 2005;22:329–41.
15. Ermann J, Hoffmann P, Edinger M, Dutt S, Blankenberg FG, Higgins JP, et al. Only the CD62L+ subpopulation of CD4+CD25+ regulatory T cells protects from lethal acute GVHD. Blood. 2005;105:2220–6.
16. Ambada GN, Ntsama CE, Nji NN, Ngu LN, Sake CN, Lissom A, et al. Phenotypic characterization of regulatory T cells from antiretroviral-naive HIV-1-infected people. Immunology. 2017;151:405–16.

17. Ziegler SF, Ramsdell F, Alderson MR. The activation antigen CD69. Stem Cells. 1994;12:456–65.
18. Adan A, Kiraz Y, Baran Y. Cell proliferation and cytotoxicity assays. Curr Pharm Biotechnol. 2016;17:1213–21.
19. Proics E, David M, Mojibian M, Speck M, Lounnas-Mourey N, Govehovitch A, et al. Preclinical assessment of antigen-specific chimeric antigen receptor regulatory T cells for use in solid organ transplantation. Gene Ther. 2023;30:309–22.
20. Duggleby R, Danby RD, Madrigal JA, Saudemont A. Clinical grade regulatory CD4+ T cells (Tregs): Moving toward cellular-based immunomodulatory therapies. Front Immunol. 2018;9:252.
21. Todo S, Yamashita K, Goto R, Zaitsu M, Nagatsu A, Oura T, et al. A pilot study of operational tolerance with a regulatory T-cell-based cell therapy in living donor liver transplantation. Hepatology. 2016;64:632–43.
22. Chandran S, Tang Q, Sarwal M, Laszik ZG, Putnam AL, Lee K, et al. Polyclonal regulatory T cell therapy for control of inflammation in kidney transplants. Am J Transplant. 2017;17:2945–54.
23. Mathew JM, H-Voss J, LeFever A, Konieczna I, Stratton C, He J, et al. A phase I clinical trial with ex vivo expanded recipient regulatory T cells in living donor kidney transplants. Sci Rep. 2018;8:7428.
24. Sawitzki B, Harden PN, Reinke P, Moreau A, Hutchinson JA, Game DS, et al. Regulatory cell therapy in kidney transplantation (The ONE Study): a harmonised design and analysis of seven non-randomised, single-arm, phase 1/2A trials. Lancet. 2020;395:1627–39 Erratum in: Lancet. 2020;395:1972.
25. Harden PN, Game DS, Sawitzki B, Van der Net JB, Hester J, Bushell A, et al. Feasibility, long-term safety, and immune monitoring of regulatory T cell therapy in living donor kidney transplant recipients. Am J Transplant. 2021;21:1603–11.
26. Roemhild A, Otto NM, Moll G, Abou-El-Enein M, Kaiser D, Bold G, et al. Regulatory T cells for minimising immune suppression in kidney transplantation: phase I/IIa clinical trial. BMJ. 2020;371:m3734.

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