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Manufacturing

Engineering of regulatory T cells by means of mRNA electroporation in a GMP-compliant manner

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ABSTRACT

Regulatory T cells (Tregs) are crucial in inducing and maintaining tolerance. This unique capacity of Tregs, in combination with proof-of-principle in preclinical studies, highlights the potential clinical use of Tregs for the treatment of autoimmunity and transplant rejection. Although proven to be safe and well tolerated in the first clinical trials, only modest clinical results were observed. In this regard, it has been hypothesized that current challenges lie in the development of antigen-specific Tregs.

Here, we present an innovative, good manufacturing practices (GMP)-compliant manufacturing protocol for Tregs applicable in a clinical-grade setting, allowing efficient and safe redirection of Treg specificity. First, a soluble polymer conjugated with antibodies to CD3 and CD28 and high amounts of exogenous IL-2 for *in vitro* Treg expansion resulted in a >70-fold and 185-fold increase of a pure population of CD4⁺CD127⁺CD25^{hi} Tregs and CD4⁺CD127⁺CD25⁺CD45RA⁺ Tregs, respectively. Next, as a proof-of-principle, expanded Tregs were engineered by means of TCR-encoding mRNA electroporation to generate antigen-specific Tregs. This resulted in an expression of the newly introduced TCR in up to 85% of Tregs. Moreover, we did not observe a negative effect on the phenotype of Tregs, as demonstrated by the expression of FOXP3, Helios, CTLA-4 and CCR4, nor on the TSDR methylation status. Importantly, mRNA-engineered Tregs were still able to induce *in vitro* suppression of effector T cells and produced anti-inflammatory, but not pro-inflammatory, cytokines when activated.

In conclusion, our findings demonstrate that high numbers of stable and functional Tregs can be obtained with high purity and successfully engineered for gain of function, in a GMP-compliant manner. We envisage that this clinical-grade protocol will provide solid basis for future clinical application of mRNA-engineered Tregs.

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Introduction

Regulatory T cells (Tregs) are the most potent immunosuppressive cells in the human body and play a pivotal role in the delicate but crucial balance between immunity and tolerance. For instance, Tregs can directly interact with and downmodulate self-reactive T cells, thereby regulating self-tolerance and ultimately preventing the development of autoimmunity [1]. Tregs are CD4⁺ T cells characterized by the expression of high levels of the interleukin (IL)-2

receptor α chain (IL-2R α , also called CD25) and the expression of the master regulator forkhead box P3 (FOXP3) transcription factor [2]. FOXP3 orchestrates the transcriptional machinery of Tregs by binding >1400 genes and acting as both a transcriptional repressor and an activator of the expression of genes associated with the function of Tregs, including *IL2RA* and cytotoxic T lymphocyte-associated protein 4 (*CTLA-4*) [3]. Although the importance of FOXP3 in Tregs has long been undisputed [4,5], activated conventional T cells can also express FOXP3 as a negative feedback mechanism, albeit temporarily [6], and FOXP3-independent maintenance of the human Treg identity has been shown in FOXP3-ablated Tregs [7]. Stable FOXP3 expression is directly linked to high demethylation of the Treg-specific demethylation region (TSDR), resulting in increased accessibility of the *FOXP3* gene and, therefore, increased FOXP3 transcription [8]. Indeed, demethylation of CpG motifs results in

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relaxation of chromatin, leading to increased accessibility and binding of specific transcription factors. Conversely, methylated CpG motifs lead to condensation of chromatin and therefore low or unstable FOXP3 expression [8]. However, for *ex vivo* identification of Tregs, the staining of FOXP3 involves destroying the integrity of cells, which is not suitable for the isolation of living cells. Because stable FOXP3 expression is inversely correlated with the expression of IL-7R (CD127) [9], the combination of positive expression of CD25 and negative expression of CD127 is a commonly used strategy to flow cytometrically isolate viable Tregs [10].

Current insights define Tregs as a heterogeneous mixture of cellular subphenotypes with a high degree of phenotypic complexity reflecting distinct developmental states, methods of suppression, homing properties and suppression targets [11]. For instance, CD4⁺CD127[−]CD25^{hi} Tregs are characterized by high expression of the Treg master regulator FOXP3 [12], whereas CD4⁺CD127[−]CD25⁺CD45RA⁺ Tregs, expressing the naivety marker CD45RA, are superior for expansion purposes [13].

To induce tolerance, Tregs exploit a broad spectrum of suppressive mechanisms, including cell contact-dependent mechanisms involving CTLA-4 and the secretion of immune regulatory cytokines such as IL-10 and transforming growth factor β (TGF- β). These mechanisms are influenced by the surrounding microenvironment, the type of immune reaction and the target cell [3]. Furthermore, Tregs can transfer suppressor activity to conventional CD4⁺ T cells. This process, termed “infectious tolerance,” creates a local tolerogenic environment in which naïve T cells convert into an induced Treg phenotype [14,15]. In addition, Tregs are responsible for “bystander suppression” by inducing tolerance to cells without direct interaction [16].

Although Tregs are present throughout the body, the most practical source of these cells is peripheral blood [17]. Nonetheless, Treg frequencies comprise only 5% to 7% of CD4⁺ T cells in the periphery [2]. Consequently, a broad range of Treg isolation and expansion protocols have been developed [17], aiming to mitigate the need for high Treg numbers while preserving the desired Treg characteristics. The most used activation reagent for *ex vivo* expansion of Tregs partially mimics the interaction with antigen-presenting cells, using anti-CD3 and anti-CD28 monoclonal antibodies covalently linked to magnetic beads [18,19]. To date, a plethora of clinical-grade isolation and expansion methods have been used in >50 active and completed clinical studies testing the feasibility, safety and efficacy of the clinical use of autologous peripheral blood-derived Tregs in the context of both autoimmunity and transplantation [20–22]. While safe and feasible, only modest clinical efficacy was observed in these studies [3,20–22]. This could be, at least in part, because polyclonal Tregs are used, collectively targeting a broad mix of antigens, not all disease-related, and therefore potentially weakening their clinical effect.

This prompted the field to move into a more antigen-specific approach to generate Tregs, ultimately fostering a durable patient-tailored cell therapy without the risk for general immunosuppression. Indeed, preclinical studies demonstrate increased potency of antigen-specific Tregs compared with polyclonal Tregs in models of type 1 diabetes [23,24] and transplantation [25,26]. Most research in inducing antigen specificity in Tregs involves the introduction of transgenic T cell receptors (TCRs) or chimeric antigen receptors into Tregs [2]. To date, both technologies are finding their way for genetic engineering of Tregs and are being tested in animal models of different autoimmune diseases and transplantation [3]. Different genetic engineering technologies, including retro- and lentiviral transduction as well as nonviral transfection methods, such as DNA-based transposons, CRISPR/Cas9 technology or direct transfer of *in vitro* transcribed messenger RNA (mRNA), have been explored to introduce the expression of TCRs or chimeric antigen receptors into Tregs [27]. Transduction of cells using viral vectors is the most used method resulting in high transfection efficiency. However, a number of limitations have been attributed to the use of viral vectors, including host

immune responses against the viral vector, potential insertional mutagenesis, the maximum insert size, variability of infection potencies, the laborious viral vector production and introduction, and the elevated laboratory costs because of the requirement for a higher bio-safety level [28]. Compared with viral delivery of genes, mRNA represents a safer alternative without the risk of insertional mutagenesis and with lower immunogenicity [29,30]. Additionally, mRNA has some advantages over DNA, mostly because no translocation to the nucleus is needed, with no risk of integration into the genome, while expression levels are easily adjusted with the amount of supplied mRNA, expression is almost instant, and it does not rely on promoter strength [30,31]. Moreover, introduced mRNA results in transient gene expression, being subjected to the natural decay of mRNA, providing an accurate system to control the synthesis of exogenous proteins [30]. We previously demonstrated the superiority of mRNA transfer by means of electroporation, in terms of efficiency of gene delivery, compared with passive pulsing or lipofection [32–34]. Electroporation is currently emerging as the transfection method of choice thanks to its safety, versatility and relatively easy clinical translation.

Here, we present a new, good manufacturing practices (GMP)-compliant manufacturing protocol for Tregs applicable in a clinical setting and overcoming current challenges in terms of feasibility, efficiency and safer redirection of Treg specificity. For this, *in vitro* Treg expansion is induced using antibodies targeting CD3 and CD28 conjugated to a soluble polymer nanomatrix and high amounts of exogenous IL-2, whereas Tregs are engineered to introduce TCR expression by means of mRNA electroporation. The protocol was proven to be effective for both CD4⁺CD127[−]CD25^{hi} and CD4⁺CD127[−]CD25⁺CD45RA⁺ Tregs without affecting the expression of FOXP3, Helios, CTLA-4 or CCR4; the demethylation status of the TSDR of FOXP3; the secretion of anti-inflammatory, but not pro-inflammatory, cytokines; and the ability of Tregs to suppress the proliferation of effector T cells (Teffs).

Material and Methods

Human blood samples and ethical statement

Buffly coats from anonymous healthy donors were provided by the Blood Service of the Flemish Red Cross (Mechelen, Belgium). This study was approved by the Ethics Committee of the University of Antwerp and the Antwerp University Hospital (Belgium) under reference number EC 18/18/236. Information, if known, of the healthy donors used in this study is depicted in Supplementary Table 1.

T cell isolation

Human peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation (Ficoll-Paque PLUS, GE Healthcare, Diegem, Belgium). Subsequently, CD4⁺ cells were positively isolated from 400×10^6 PBMCs using human CD4 MicroBeads for magnetic-activated cell sorting (MACS, Miltenyi Biotec, Leiden, Netherlands), according to the manufacturer's instructions. Isolated CD4⁺ cells were stained with fluorochrome-conjugated monoclonal antibodies (mAbs) (Supplementary Table 2). CD25^{hi} Tregs were sorted as CD3⁺, CD4⁺, CD127[−] and CD25^{hi}; naïve Tregs were sorted as CD3⁺, CD4⁺, CD127[−], CD25⁺ and CD45RA⁺; and effector CD4⁺ T cells were sorted as CD3⁺, CD4⁺, CD127⁺ and CD25[−] (Figure 1A). Remaining CD8⁺ T cells, monocytes, natural killer (NK) cells and B cells were excluded from the sorting gate using a dump channel consisting of anti-CD8, -CD14, -CD16 and -CD19. Sorting was performed by flow cytometry using a FACSARIA II device (BD Biosciences). After flow cytometric cell sorting, an aliquot of the sorted cells was used to confirm purity.

Figure 1. Multiparametric flow cytometry–based sorting of CD45RA⁺ and CD25^{hi} Tregs and confirmation of their phenotype and functionality. (A) Gating strategy for FACS sorting of CD4⁺CD127⁺CD25⁺CD45RA⁺ and CD4⁺CD127⁺CD25^{hi} Tregs after CD4⁺ magnetic bead enrichment from PBMCs. Tregs were sorted, from left to right, as lymphocytes (FSC-A/SSC-A), single cells (FSC-A/FSC-H), living cells (LIVE/DEAD Fixable Aqua Dead Cell Stain negative population) and CD4⁺ T cells (CD3⁺CD4⁺), and further elimination of irrelevant cell subsets based on dump channel (CD8⁺CD14⁺CD16⁺CD19⁺) and CD127⁺CD25⁺ cells, whereas effector CD4⁺ T cells were sorted as CD127⁺CD25⁺ cells. Selection of the naïve Tregs was based on expression of CD45RA, and CD25^{hi} Tregs were sorted as CD45RA⁺CD25^{hi}. (B) Representative overlay of dot plot and histogram of FOXP3 expression in CD127⁺CD25⁺ effector T cells (black), CD45RA⁺ Tregs (green) and CD25^{hi} Tregs (red). FMO was used as control (gray). FOXP3 expression was analyzed by flow cytometry in CD45RA⁺ (green) and CD25^{hi} (red) Tregs after sorting and compared to control CD127⁺CD25⁺ effector T cells (black). (C and D) *In vitro* analysis of suppression of autologous effector T cell proliferation was performed using FACS-sorted CD45RA⁺ (C) and CD25^{hi} (D) Tregs at different Teff:Treg ratios. All ratios were performed in duplicate, and the mean division index was used to calculate the suppression percentage: $100 - (DI_{\text{condition of interest}}/DI_{1:0}) \times 100$. Results are shown as median $\pm$ interquartile range for four independent donors. Statistical analysis was performed using the nonparametric Friedman test with Dunn's multiple comparisons test: **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Ex vivo expansion of Tregs

Tregs were expanded *ex vivo* in complete medium, consisting of Iscove's modified Dulbecco's medium (IMDM; Life Technologies) supplemented with 5% human AB serum (hAB; Life Technologies) and

500 IU/mL IL-2 (ImmunoTools GmbH, Friesoythe, Germany). Treg activation was achieved by using T cell TransAct (1:100 dilution, Miltenyi Biotec) on day 0. Reactivation was performed on days 7 and 14. Complete medium was replenished twice daily, and cell counts were monitored routinely using an automated hemocytometer (ABX

Micros 60; Horiba, Diegem, Belgium). On days 7, 14 and 19, purity was assessed using fluorochrome-conjugated mAbs (Supplementary Table 2) and measured using a CytoFLEX flow cytometer (Beckman Coulter, Analis, Suarlée, Belgium). A fluorescent-minus-one (FMO) control was used as a negative control. The proportion of viable cells was assessed by means of propidium iodide (PI, Thermo Fisher Scientific, Life Technologies, Merelbeke, Belgium) staining. For analytical flow cytometry, 10,000 events were recorded per sample.

Vector construction and *in vitro* mRNA transcription

The sequence of the TCR recognizing HLA-DR2–restricted myelin basic protein (MBP)_{85–99} peptide was kindly provided by Prof. Dr. David W. Scott of the Uniformed Services School of Health Sciences (USUHS) in Bethesda, MD [35]. Sequences encoding the TCR α - and β -chains were linked with the 2A sequence from porcine teschovirus-1 (P2A) [36]. The sequences were cloned into the *SpeI*-*XhoI* site of the pST1 plasmid (kindly provided by Dr. Ugur Sahin, Johannes Gutenberg University, Mainz, Germany) [37] under the control of a T7 promoter and with the addition of a poly(A) tail, and subjected to codon-optimization (GeneArt, Thermo Fisher Scientific). Additionally, a DNA plasmid encoding enhanced green fluorescent protein (eGFP; pGEM4Z/EGFP/A64 vector [38]) was kindly provided by Dr. Eli Gilboa (Duke University Medical Center, Durham, NC) [39]. All plasmids were propagated in *Escherichia coli* SoloPack Golden supercompetent cells (Agilent Technologie, Machelen, Belgium), and plasmid DNA was purified using a NucleoBond Xtra Midi EF kit (Macherey-Nagel, Düren, Germany). Next, purified plasmid DNA was linearized by *SapI* digestion (Thermo Fisher Scientific) for the MBP_{85–99}-specific TCR plasmid and by *SpeI* digestion (Thermo Fisher Scientific) for the eGFP plasmid. Subsequently, linearized plasmid DNA was used as a DNA template for *in vitro* transcription with a mMessage mMachineT7 *in vitro* transcription kit (Ambion, Life Technologies), according to the manufacturer's protocol. mRNA quality was assessed by agarose gel electrophoresis and Nanodrop (Thermo Fisher Scientific). All mRNA constructs were stored at -20°C at a concentration of $1\ \mu\text{g}/\mu\text{L}$.

mRNA electroporation

On day 19 of Treg expansion, Tregs were electroporated with mRNA encoding the MBP_{85–99}-specific TCR, as described previously [40]. In brief, Tregs were washed twice and resuspended in cold serum-free Opti-MEM I medium (Gibco Invitrogen) at a concentration of 25×10^6 cells/mL. 200 μL of the cell suspension was transferred to a 4.0-mm electroporation cuvette (Cell Projects, Kent, United Kingdom), and $1\ \mu\text{g}/10^6$ cells of *in vitro*-transcribed mRNA was added to the cuvette. Electroporations were performed with a Gene Pulser Xcell device (Bio-Rad, Temse, Belgium) using a square wave pulse of 500 V for 5 ms. As a positive control, cells were electroporated under the same conditions using eGFP-encoding mRNA, while for the negative control no mRNA was added (mock electroporation). Immediately after electroporation, cells were replenished in IMDM supplemented with 10% hAB serum and rested for a minimum of 20 min in a humidified 5% CO_2 incubator at 37°C before further analysis.

For the evaluation of transfection efficiency, kinetics of the expression of eGFP and of the MBP_{85–99}-specific TCR using an anti-TCR V β 2-phycoerythrin (PE) antibody (Beckman Coulter) was evaluated 0, 4, 24, 48, 72, 96, 120, 144, 168 and 192 h after electroporation using a CytoFLEX flow cytometer. The proportion of viable cells was assessed by means of PI staining (Thermo Fisher Scientific). For analytical flow cytometry, 10,000 events were recorded per sample.

Cryopreservation

Remaining PBMCs, not used for CD4⁺ isolation using MACS, and transfected Tregs, used for post-cryopreservation kinetics of the

transgenic TCR, were washed and resuspended in cryopreservation medium consisting of fetal bovine serum (FBS; Life Technologies) supplemented with 10% DMSO (Sigma-Aldrich, Diegem, Belgium). Aliquots were stored in a -80°C freezer. When needed, cells were thawed in prewarmed IMDM supplemented with 10% hAB serum.

Surface and intracellular staining

To assess intracellular expression of FOXP3, Helios and CTLA-4, membrane markers on effector CD4⁺ T cells and Tregs were stained first (Supplementary Table 2). Next, cells were fixed and permeabilized using the eBioscience FOXP3/Transcription Factor Staining Buffer Set, according to the manufacturer's instructions (Thermo Fisher Scientific). Subsequently, cells were intracellularly stained with anti-FOXP3-Alexa Fluor 488 mAb (BD Biosciences), anti-Helios-Alexa Fluor 647 (BioLegend) and anti-CD152 (=CTLA-4)-BV421 (BioLegend) (Supplementary Table 2). A FMO and CD4⁺ T cells were used as a negative control. For analytical flow cytometry, 50,000 lymphocytes, gated on light scatter characteristics, were measured using a Novocyte Quanteon (Agilent).

DNA methylation analysis of human Treg-specific demethylation regions

Dry pelleted cell samples (0.5×10^6 cells; centrifuged at 480g for 5 min) of CD127⁺CD25[−] effector CD4⁺ T cells and CD45RA⁺ or CD25^{hi} Tregs were collected before and after *ex vivo* expansion and mRNA electroporation, stored at -80°C and shipped on dry ice to EpigenDx (Hopkinton, MA) to perform TSDR methylation analysis (assay ID ADS783-FS2). The analysis covered nine CpG sites spanning positions -2263 to -2330 (upstream from the ATG start codon) of FOXP3.

Briefly, pelleted cell samples were lysed using ZymoResearch M-digestion buffer and 20 mg/mL protease K (ZymoResearch, Irvine, CA), and incubated at 65°C for a minimum of 2 h. Next, supernatants from the sample lysate were bisulfite modified using EZ-96 DNA Methylation-Direct kit (ZymoResearch) as per the manufacturer's protocol with minor modifications. Polymerase chain reactions (PCRs) were performed using 1 μL of the bisulfite-treated DNA and 0.2 μM of each primer (EpigenDx's proprietary information). One primer was biotin labeled and HPLC purified to purify the final PCR product using Sepharose beads. PCR product was bound to Streptavidin Sepharose HP (GE Healthcare Life Sciences), after which the immobilized PCR products were purified, washed, denatured with a 0.2- μM NaOH solution, and washed again using the Pyrosequencing Vacuum Prep Tool (Pyrosequencing, Qiagen), as per the manufacturer's protocol. Next, 0.5 μM of sequencing primer was annealed to the purified single-stranded PCR products, and 10 μL of the PCR products were sequenced by Pyrosequencing on the PSQ96 HS System (Pyrosequencing, Qiagen) following the manufacturer's instructions.

The methylation status of each CpG site was determined individually as an artificial C/T single-nucleotide polymorphism (SNP) using QCpG software (Pyrosequencing, Qiagen). The methylation level at each CpG site was calculated as the percentage of the methylated alleles divided by the sum of all methylated and unmethylated alleles. The mean methylation level was calculated using methylation levels of all measured CpG sites within the targeted region of each gene. Each experiment included non-CpG cytosines as internal controls to detect incomplete bisulfite conversion of the input DNA. In addition, a series of unmethylated and methylated DNA were included as controls in each PCR. Furthermore, PCR bias testing was performed by mixing unmethylated control DNA with *in vitro* methylated DNA at different ratios (0%, no methylated DNA; 5%; 10%; 25%; 50%; 75%; and 100%, only methylated DNA), followed by bisulfite modification, PCR, and Pyrosequencing analysis. For female donors, this ratio was corrected with a factor 2 since one of the two TSDR alleles is methylated because of X-inactivation [41–43].

Suppression assay

Effector CD4⁺ T cells were stained using a CellTrace Violet Cell Proliferation kit (Thermo Fisher Scientific), which allows tracking of cell division, according to the manufacturer's instructions. Subsequently, stained effector CD4⁺ T cells were stimulated with Treg Suppression Inspector beads (Miltenyi Biotec) in a 1:1 ratio, providing optimal polyclonal stimulation of effector CD4⁺ T cells based on anti-CD2, anti-CD3 and anti-CD28, while incremental numbers of Tregs were added to the cell culture. More specifically, the different effector CD4⁺ T cell/Treg ratios were 2:0, 1:0, 1:1, 2:1, 4:1, 8:1 and 16:1. Cells were co-cultured in a 96-well U-bottom plate in IMDM (Life Technologies) supplemented with 5% FBS (Life Technologies) for 5 days in a humidified 5% CO₂ incubator at 37°C. After 5 days, 10,000 lymphocytes, based on light scatter characteristics, were measured using a FACSARIA II flow cytometer (BD Biosciences) (Supplementary Figure 1 and Table 2). The unstimulated responder T cell condition, was used as a reference, after which the division indices of the stimulated responder T cells alone condition (1:0 ratio) and the conditions containing different ratios of Tregs were obtained. The division index (DI), given by FlowJo software, represents the average number of divisions a cell in the starting population has undergone. Subsequently, the percentage suppression for each condition was calculated by the division index method: $100 - (DI_{\text{condition of interest}}/DI_{1:0}) * 100$ [44].

Multiplex cytokine analysis

Expanded Tregs were cultured (0.5×10^6 cells/mL) in IMDM supplemented with 5% hAB serum (*i.e.*, control, not activated) or in complete medium with TransAct (1:100 dilution), mimicking TCR activation. Subsequently, these cell cultures were incubated in a humidified 5% CO₂ incubator at 37°C. As a positive control, thawed cryopreserved autologous effector CD4⁺ cells were cultured in the same conditions. As a negative control, cell-free IMDM supplemented with 5% hAB serum and complete medium was used. After 4 days, a volume of 500 μ l culture medium of each condition was extracted for the simultaneous quantitative determination of both natural and recombinant human interferon- γ (IFN- γ), IL-2, IL-4, IL-5, IL-10, IL-13 and tumor necrosis factor α (TNF- α) using a chemiluminescence-based assay from Meso Scale Discovery (Human TH1/TH2 10-Plex Tissue Culture Kit, MSD, Gaithersburg, MD), according to the manufacturer's instructions. The plate was washed and read with MSD reading buffer on the QuickPlex SQ 120 (MSD). All conditions were measured in duplicate and run at the same time. Background measurements of nonactivated cells were deducted from the measurements of corresponding activated cells.

TCR functionality

The functionality of the MBP₈₅₋₉₉-specific TCR was assessed using two TCR-deficient cell lines, 2D3 and SKW-3. 2D3 cells were generated from TCR-deficient Jurkat 76 cells (human acute T cell leukemia), as described before [45]. SKW-3 cells were purchased from cell bank of German Collection of Microorganisms and Cell Cultures. Exponential growth was maintained by culturing the cells in RPMI 1640 (Gibco Invitrogen) supplemented with 10% FBS. After electroporation of the cells with mRNA encoding the MBP₈₅₋₉₉-specific TCR (5×10^6 cells, 1 μ g mRNA/ 10^6 cells) as described above, cells were stimulated using TransAct, mimicking TCR activation, 6 h after electroporation. Mock electroporation and nonstimulated cells were used as a control. For 2D3 cells, containing a plasmid vector with the eGFP gene under the control of a nuclear factor of activated T cell (NFAT)-dependent promoter [45], TCR signaling can be measured directly by eGFP expression. For SKW-3, TCR-dependent cell activation was assessed by measuring the expression of activation markers CD69 and CD137

(Supplementary Table 2). For analytical flow cytometry, 10,000 lymphocytes, gated on light scatter characteristics, were measured using a CytoFLEX flow cytometer.

Data analysis

FACS data were analyzed using FlowJo software version 10.5.3 (TreeStar, Ashland, OR), and multiplex data were analyzed using Discovery Workbench 4.0 software. Results were analyzed using Prism software version 8 (GraphPad, San Diego, CA), and given as mean values $\pm$ standard deviation (SD). Statistical analysis was performed using nonparametric Kruskal–Wallis test or Friedman test, followed by a *post hoc* Dunn's multiple comparison test where applicable. For transgenic TCR expression over time, mixed-models test with the Geisser–Greenhouse correction, followed by a *post hoc* Dunnett's multiple comparisons test, was used. Any *P* value <0.05 is considered statistically significant.

Results

FACS sorting results in pure and functional CD45RA⁺ and CD25^{hi} Tregs

First, we evaluated whether a sufficient number for *ex vivo* expansion of highly pure and functional CD4⁺CD127[−]CD25⁺CD45RA⁺ and CD4⁺CD127[−]CD25^{hi} Treg populations could be isolated using FACS sorting. For this, samples were enriched for CD4⁺ T cells, and CD4⁺CD127[−]CD25⁺CD45RA⁺ and CD4⁺CD127[−]CD25^{hi} Tregs were sorted with an average purity of $94.9 \pm 4.3\%$ and $95.2 \pm 4.2\%$, respectively (Figure 1A). To confirm Treg identity, FOXP3 expression was analyzed (Figure 1B). Both CD45RA⁺ Tregs ($70.6 \pm 2.8\%$; *P* = 0.1573) and CD25^{hi} Tregs ($95.8 \pm 1.5\%$; *P* = 0.0047) showed higher expression of FOXP3 compared with biological control CD127⁺CD25[−] effector T cells ($6.1 \pm 3.0\%$), albeit not significant for CD45RA⁺ Tregs.

To validate the Treg-mediated suppressive capacity of the FACS-sorted Treg populations, we evaluated their suppression on CD4⁺CD127[−]CD25[−] effector T cell proliferation, induced by *in vitro* stimulation with beads coated with anti-CD2, anti-CD3 and anti-CD28 (Figure 1C and D). Tregs were able to suppress effector T cell proliferation at different Teff:Treg ratios compared with effector T cells alone (no suppression of T cells). Indeed, significant inhibition of effector T cell proliferation occurred by CD45RA⁺ Tregs in a Teff:Treg ratio of 1:1 ($81.1\% \pm 11.2\%$ suppression of T cells; *P* = 0.0014) and 2:1 ($76.1\% \pm 13.8\%$ suppression of T cells; *P* = 0.0025). Similarly, CD25^{hi} Tregs significantly suppressed T cell proliferation at a Teff:Treg ratio of 1:1 ($90.7\% \pm 4.7\%$ suppression of T cells; *P* = 0.0008), 2:1 ($84.3\% \pm 13.0\%$ suppression of T cells; *P* = 0.0112) and 4:1 ($80.7\% \pm 14.3\%$ suppression of T cells; *P* = 0.0271). No significant difference in suppression of proliferation rate (*P* = 0.7434) was observed between the 2:0 ratio ($-21.7\% \pm 26.8\%$ suppression of T cells) and the 1:0 ratio, excluding the possibility that proliferation differences occur based on T cell numbers in the cell culture instead of Treg presence. Altogether, our findings confirm that FACS-sorted CD4⁺CD127[−]CD25⁺CD45RA⁺ and CD4⁺CD127[−]CD25^{hi} Tregs display a genuine Treg phenotype and are capable of inducing immunosuppression.

GMP-compliant expansion of Tregs results in a >70-fold and a >185-fold increase of CD25^{hi} and CD45RA⁺ Tregs, respectively

Given the low frequencies of Tregs in the human body, *i.e.*, 5% to 7% of CD4⁺ T cells [2], large-scale expansion is needed to obtain sufficient Treg numbers for clinical application. Therefore, we developed an expansion protocol compliant with GMP using a combination of soluble colloidal polymeric reagent, with covalently attached anti-CD3/CD28-antibodies (T Cell TransAct), and 500 IU/mL IL-2 to activate and expand CD4⁺CD127[−]CD25^{hi} and CD4⁺CD127[−]CD25⁺CD45RA⁺ Tregs. As depicted in Figure 2, an

incremental increase of Treg numbers was observed. Indeed, the number of CD45RA⁺ Tregs (Figure 2A) and CD25^{hi} Tregs (Figure 2B) was significantly higher from day 12 (A, $14.31 \times 10^6 \pm 9.28 \times 10^6$; $P = 0.0143$; B, $4.30 \times 10^6 \pm 1.56 \times 10^6$; $P = 0.0233$), compared with the initial value of $0.28 \times 10^6 \pm 0.06 \times 10^6$ cells and $0.27 \times 10^6 \pm 0.05 \times 10^6$ cells on day 0, respectively. Ultimately, we observed a 186.5 $\pm$ 123.8-fold expansion of CD45RA⁺ Tregs and a 71.4 $\pm$ 50.3-fold expansion of CD25^{hi} Tregs over the course of a 19-day expansion protocol. Assessment of Treg purity at different time points during expansion indicated no outgrowth of contaminating CD127⁺ effector T cells (Figure 2C). Viability assessment indicated 94.9% $\pm$ 1.8% and 93.3% $\pm$ 4.0% viable cells over the course of the 19-day *ex vivo* expansion procedure for CD45RA⁺ and CD25^{hi} Tregs, respectively.

Electroporation of expanded Tregs with TCR-encoding mRNA results in significant amounts of TCR-expressing Tregs

Next, we tested the feasibility of genetically and transiently modifying expanded Tregs using mRNA electroporation. To assess the transfection efficiency of mRNA electroporation, Tregs were electroporated with eGFP-encoding mRNA. On average, we observed 98.5% $\pm$ 0.8% eGFP-expressing CD45RA⁺ Tregs and 98.1% $\pm$ 0.9% eGFP-expressing CD25^{hi} Tregs 24 h after electroporation (Figure S2). Subsequently, we evaluated whether electroporation with mRNA encoding an HLA-DR2–restricted MBP_{85–99}-specific TCR, which comprises a variable $\beta 2$ (V $\beta 2$) TCR chain, would result in effective TCR surface expression in expanded CD45RA⁺ and CD25^{hi} Tregs. The kinetics of MBP_{85–99}-specific TCR surface expression levels were evaluated using a TCR V $\beta 2$ -specific mAb, targeting the TRBV20–1 variable segment, for 8 consecutive days (Figure 2D). The highest TCR V $\beta 2$ expression was observed 24 h after mRNA electroporation in both CD45RA⁺ (85.5% $\pm$ 6.2% TCR V $\beta 2$ ⁺ cells; $P = 0.0035$; Figure 2E) and CD25^{hi} (83.0% $\pm$ 10.0% TCR V $\beta 2$ ⁺ cells; $P = 0.0055$; Figure 2F) Tregs, compared with mock electroporated cells, indicating successful transfection of Tregs with MBP_{85–99}-specific TCR-encoding mRNA. TCR V $\beta 2$ ⁺ expression levels gradually decreased in both Treg subtypes over the evaluated time course. Basal TCR V $\beta 2$ ⁺ levels in mock-electroporated CD45RA⁺ and CD25^{hi} Tregs were 11.5% $\pm$ 0.7% TCR V $\beta 2$ ⁺ and 7.9% $\pm$ 2.6% TCR V $\beta 2$ ⁺, respectively.

Next, CD45RA⁺ and CD25^{hi} Tregs were cryopreserved 4 h after transfection. Upon thawing, the kinetics of TCR V $\beta 2$ expression was assessed. Importantly, no difference in TCR V $\beta 2$ expression could be detected between fresh or cryopreserved Tregs, as demonstrated by similar high expression of the transgenic TCR in cryopreserved CD45RA⁺ Tregs (81.4% $\pm$ 6.8% TCR V $\beta 2$ ⁺; $P = 0.0047$; Figure 2E) and in cryopreserved CD25^{hi} Tregs (77.3% $\pm$ 7.2% TCR V $\beta 2$ ⁺; $P = 0.0020$; Figure 2F) 24 h after mRNA electroporation, and thus 20 h after cryopreservation, compared with mock electroporated cells. In addition, neither mRNA electroporation (94.0% $\pm$ 1.5% and 90.5% $\pm$ 4.2% viable cells) nor cryopreservation (94.7% $\pm$ 0.9% and 96.3% $\pm$ 0.4% viable cells) negatively influenced the viability of CD45RA⁺ and CD25^{hi} Tregs, respectively. At day 8, no significant expression of the V $\beta 2$ TCR chain could be detected in all conditions, compared with the basal TCR V $\beta 2$ ⁺ levels in mock-electroporated CD45RA⁺ and CD25^{hi} Tregs.

GMP-compliant activation, expansion and transfection of CD45RA⁺ and CD25^{hi} Tregs does not affect the stable expression of the Treg master regulator FOXP3

Given the importance of the transcription factor FOXP3 in the phenotype and function of Tregs, we assessed whether GMP-compliant activation, expansion and transfection affected its expression. No difference in FOXP3 expression levels could be found after activation, expansion and transfection of both CD45RA⁺ Tregs (93.9% $\pm$ 4.1% FOXP3⁺ cells after expansion and 92.0% $\pm$ 6.7% FOXP3⁺ cells after

subsequent mRNA electroporation) (Figure 3A) and CD25^{hi} Tregs (94.9% $\pm$ 3.0% FOXP3⁺ cells after expansion and 89.2% $\pm$ 7.7% FOXP3⁺ cells after subsequent mRNA electroporation) (Figure 3B) compared with FOXP3 levels in freshly isolated CD45RA⁺ Tregs (70.6% $\pm$ 2.8% FOXP3⁺ cells) and freshly isolated CD25^{hi} Tregs (95.8% $\pm$ 1.5% FOXP3⁺ cells). Nonetheless, compared with CD127⁺CD25[–] effector T cells (7.0% $\pm$ 2.7% FOXP3⁺ cells), only expanded ($P = 0.0001$) and transfected ($P = 0.0004$) CD45RA⁺ Tregs demonstrated significantly higher expression levels of FOXP3, but not freshly isolated CD45RA⁺ Tregs ($P = 0.2332$), whereas freshly isolated ($P = 0.0026$), expanded ($P = 0.0003$) and transfected ($P = 0.0099$) CD25^{hi} Tregs displayed significantly higher expression levels of FOXP3 compared with effector T cells.

Moreover, stable FOXP3 expression is directly linked to high demethylation of TSDR. Therefore, we analyzed the methylation status of FOXP3, as indicated by the percentage of methylation in nine CpG motifs located in intron 1 of human FOXP3 after GMP-compliant isolation, expansion and transfection of Tregs (Figure 3C and D). Significantly lower methylation percentages were measured in freshly isolated (24.3% $\pm$ 4.9%; $P = 0.0180$ and 13.4% $\pm$ 8.5%; $P = 0.0110$), expanded (31.1% $\pm$ 3.7%; $P = 0.0313$ and 23.4% $\pm$ 8.2%; $P = 0.0370$) and mRNA electroporated (30.0% $\pm$ 6.3%; $P = 0.0376$ and 24.1% $\pm$ 7.2%; $P = 0.0450$) CD45RA⁺ (Figure 3C) and CD25^{hi} (Figure 3D) Tregs, respectively, compared with CD127⁺CD25[–] effector T cells (90.0% $\pm$ 1.5%). No significant differences in methylation status of the CpG motifs were observed between expanded and transfected Tregs and freshly sorted Tregs.

GMP-compliant activation, expansion and transfection of CD45RA⁺ and CD25^{hi} Tregs does not affect CTLA-4 and CCR4 expression but shows a slight decrease in Helios expression by CD25^{hi} Tregs

As FOXP3-independent maintenance of the human Treg identity has been shown in FOXP3-ablated Tregs [7], we assessed the expression of additional Treg-defining markers, including Helios, CTLA-4 and CCR4. We demonstrate high expression of Helios in expanded CD45RA⁺ Tregs (66.5% $\pm$ 17.1% Helios⁺; $P = 0.0019$) and transfected CD45RA⁺ Tregs (65.5% $\pm$ 17.6% Helios⁺; $P = 0.0201$), but slightly lower expression in expanded CD25^{hi} Tregs (31.6% $\pm$ 12.0% Helios⁺; $P = 0.1967$) and transfected CD25^{hi} Tregs (31.8% $\pm$ 10.3% Helios⁺; $P = 0.3017$), compared with autologous CD4⁺ T cells (4.7% $\pm$ 2.5% Helios⁺) (Figure 4A). It should be noted only Helios⁺, and not Helios^{mid}, was considered in the analysis. Nonetheless, high expression of CTLA-4 (Figure 4B) and CCR4 (Figure 4C) was measured in expanded CD45RA⁺ Tregs (95.7% $\pm$ 0.8% CTLA-4⁺; $P = 0.1213$; 92.6% $\pm$ 5.8% CCR4⁺; $P = 0.0444$), transfected CD45RA⁺ Tregs (92.6% $\pm$ 1.4% CTLA-4⁺; $P = 0.4386$; 92.3% $\pm$ 6.4% CCR4⁺; $P = 0.0550$), expanded CD25^{hi} Tregs (97.7% $\pm$ 0.1% CTLA-4⁺; $P = 0.0019$; 94.4% $\pm$ 4.7% CCR4⁺; $P = 0.0495$) and transfected CD25^{hi} Tregs (96.7% $\pm$ 1.4% CTLA-4⁺; $P = 0.0201$; 95.0% $\pm$ 4.3% CCR4⁺; $P = 0.0198$), compared with autologous CD4⁺ T cells (7.7% $\pm$ 2.9% CTLA-4⁺ and 22.7% $\pm$ 5.3% CCR4⁺). In addition, our data indicate no differences in expression of Helios, CTLA-4 and CCR4 between expanded and transfected CD45RA⁺ and CD25^{hi} Tregs, respectively.

GMP-compliant expanded and transfected Tregs are capable of inducing in vitro immunosuppression and produce anti-inflammatory, but not pro-inflammatory, cytokines

Inhibition of Teff proliferation was examined as a measure for Treg functionality after *ex vivo* expansion and genetic engineering via mRNA electroporation (Figure 5A and B). Significant inhibition of Teff proliferation was detected at a Teff:Treg ratio of 1:1 compared with Teffs without the addition of Tregs (1:0 ratio; 0.0% $\pm$ 0.0% suppression of T cells), as indicated by 70.5% $\pm$ 13.3% ($P = 0.0006$) suppression of T cells by expanded CD45RA⁺ Tregs and 79.6% $\pm$ 10.3% ($P = 0.0006$)

Figure 2. A mean 186.5 ± 123.8 -fold expansion of CD45RA⁺ Tregs and 71.4 ± 50.3 -fold expansion of CD25^{hi} Tregs upon 19 days of ex vivo expansion and a >80% expression of a transgenic TCR by means of mRNA electroporation was achieved by using a novel GMP-compatible engineering protocol. Tregs were FACS sorted, cultured in IMDM supplemented with 500 IU/mL IL-2 and 5% hAB serum, and activated using a soluble polymer conjugated with antibodies to CD3 and CD28 (1:100 dilution) on days 0, 7 and 14. Expansion in total cell numbers (top) and fold expansion (bottom) of CD45RA⁺ Tregs (A) and CD25^{hi} Tregs (B) was assessed for 19 days after the initiation of the expansion protocol. Graphs represent the median cell count $\pm$ interquartile range for seven independent donors. Statistical analysis was performed using the nonparametric Friedman test with Dunn's multiple comparisons test. (C) Representative plots of Treg purity assessed by expression of CD127 and CD25 on days 7, 14 and 19 during Treg expansion. (D) Representative histograms of the MBP₈₅₋₉₉-specific TCR expression measured for 8 days after mRNA electroporation using anti-TCR Vβ2-PE antibody in expanded and transfected CD45RA⁺ Tregs (green) (E) and CD25^{hi} Tregs (red) (F). Transgenic TCR kinetics after cryopreservation 4 h after mRNA electroporation (EP) are represented by stripped green or red bars for CD45RA⁺ and CD25^{hi} Tregs, respectively. Baseline TCR Vβ2 expression per day is indicated in black for CD45RA⁺ and CD25^{hi} Tregs. Graphs represent median percentage of Vβ2⁺ Tregs $\pm$ interquartile range for three independent donors. Statistical analysis was performed using mixed-models test with the Geisser–Greenhouse correction; matched values are stacked into a subcolumn, with Dunnett's multiple comparisons test, with individual variances computed for each comparison. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Figure 3. High FOXP3 expression and low methylation levels in engineered CD45RA⁺ and CD25^{hi} Tregs confirm conservation of stable Treg phenotype. FOXP3 expression in CD45RA⁺ Tregs (A) and CD25^{hi} Tregs (B) after sorting, expansion, and MBP₈₅₋₉₉-specific TCR-encoding mRNA electroporation was compared with control CD127⁺CD25⁺ T cells. Percentage of TSDR DNA methylation in FACS-sorted, expanded, and MBP₈₅₋₉₉-specific TCR-encoding mRNA-electroporated CD45RA⁺ (C) and CD25^{hi} (D) Tregs, compared with that of freshly FACS-sorted CD127⁺CD25⁺ T cells. Heatmaps represent the methylation percentages of nine CpG sites located in intron 1 of human FOXP3 gene. Data plots show the summarized data of median percentage FOXP3 (A, B) or median percentage methylation (C, D) $\pm$ interquartile range for four (freshly sorted cells, two male and two female donors) or seven independent donors. Statistical analysis was performed using the nonparametric Kruskal–Wallis test with Dunn's multiple comparisons test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

suppression of T cells by expanded and mRNA-engineered CD45RA⁺ Tregs; $62.6\% \pm 12.4\%$ ($P = 0.0006$) suppression of T cells by expanded CD25^{hi} Tregs and $67.8\% \pm 6.8\%$ ($P = 0.0006$) suppression of T cells by expanded and engineered CD25^{hi} Tregs. Also at a 2:1 Teff:Treg ratio, significant inhibition of Teff proliferation was observed, as indicated by $60.7\% \pm 19.2\%$ ($P = 0.0054$) suppression of T cells by expanded CD45RA⁺ Tregs and $67.5\% \pm 13.0\%$ ($P = 0.0054$) suppression of T cells

by expanded and mRNA-engineered CD45RA⁺ Tregs; $55.9\% \pm 15.1\%$ ($P = 0.0054$) suppression of T cells by expanded CD25^{hi} Tregs and $59.1\% \pm 11.4\%$ ($P = 0.0054$) suppression of T cells by expanded and mRNA-engineered CD25^{hi} Tregs. Additionally, at a 4:1 Teff:Treg ratio, significant inhibition of Teff proliferation was observed, as indicated by $45.6\% \pm 18.7\%$ ($P = 0.0496$) suppression of T cells by expanded CD45RA⁺ Tregs and $54.0\% \pm 12.6\%$ ($P = 0.0334$) suppression of T cells

Figure 4. Helios, CTLA-4 and CCR4 expression in engineered CD45RA⁺ and CD25^{hi} Tregs confirm conservation of stable Treg phenotype. Helios (A), CTLA-4 (B) and CCR4 (C) expression in CD45RA⁺ Tregs (green) and CD25^{hi} Tregs (red) after expansion and MBP₈₅₋₉₉-specific TCR-encoding mRNA electroporation were compared to control CD4⁺ T cells (black). Results are shown as representative dot plot overlay (top), histogram overlay (middle) and data graphs, representing median percentage $\pm$ interquartile range for three independent donors (bottom). Statistical analysis was performed using the nonparametric Friedman test with Dunn's multiple comparisons test: * $P < 0.05$, ** $P < 0.01$.

by expanded and mRNA-engineered CD45RA⁺ Tregs; $47.0\% \pm 21.6\%$ ($P = 0.0334$) suppression of T cells by expanded CD25^{hi} Tregs and $46.4\% \pm 23.3\%$ ($P = 0.0334$) suppression of T cells by expanded and mRNA-engineered CD25^{hi} Tregs. No significant differences ($P > 0.9999$) were found between expanded and mRNA-electroporated Tregs, or between the two different Treg subsets (*data not shown*). No significant difference ($P > 0.9999$) in proliferation rate was observed between the 2:0 ratio ($-11.8\% \pm 12.1\%$ suppression of T cells) and the 1:0 ratio (no suppression of T cells), excluding the possibility that proliferation differences occur based on T cell numbers in the cell culture instead of Treg presence.

Quantitative analysis of secreted cytokines by expanded and mRNA-electroporated Tregs after TCR activation indicates high production of anti-inflammatory, but not pro-inflammatory, cytokines (Figure 5C and D). Indeed, cell-free supernatant of activated expanded CD45RA⁺ Tregs (1564 ± 1090 pg/mL IFN- γ [$P > 0.001$]; 658 ± 166 pg/mL TNF- α [$P = 0.1441$]; 3697 ± 4570 pg/mL IL-2 [$P = 0.0106$]), expanded and mRNA-electroporated CD45RA⁺ Tregs (1649 ± 1259 pg/mL IFN- γ [$P = 0.0003$]; 653 ± 423 pg/mL TNF- α [$P = 0.0679$]; 4222 ± 5503 pg/mL IL-2 [$P = 0.0285$]), expanded CD25^{hi}

Tregs (3333 ± 2051 pg/mL IFN- γ [$P = 0.0446$]; 423 ± 64 pg/mL TNF- α [$P = 0.0003$]; 639 ± 237 pg/mL IL-2 [$P = 0.0035$]) and expanded and mRNA-electroporated CD25^{hi} Tregs (3658 ± 2505 pg/mL IFN- γ [$P = 0.2012$]; 436 ± 193 pg/mL TNF- α [$P < 0.0001$]; 599 ± 311 pg/mL IL-2 [$P = 0.0010$]) contained lower concentrations of pro-inflammatory cytokines, compared with activated CD4⁺ T cells ($50,881 \pm 23,741$ pg/mL IFN- γ ; $11,762 \pm 638$ pg/mL TNF- α ; $22,734 \pm 3081$ pg/mL IL-2), albeit not all significant (Figure 5D). Importantly, it should be noted that complete medium used for activation contained high concentrations of exogenous IL-2, resulting in $19,437 \pm 2857$ pg/mL IL-2 when measured with the multiplex. On the other hand, cell-free supernatant of activated expanded CD45RA⁺ Tregs (5200 ± 4576 pg/mL IL-4 [$P = 0.446$]; 3893 ± 1414 pg/mL IL-5 [$P = 0.4652$]; $14,565 \pm 974$ pg/mL IL-10 [$P = 0.0176$]; 6371 ± 2287 pg/mL IL-13 [$P = 0.0019$]), expanded and mRNA-electroporated CD45RA⁺ Tregs (4430 ± 4143 pg/mL IL-4 [$P = 0.2012$]; 4115 ± 963 pg/mL IL-5 [$P = 0.4652$]; $14,799 \pm 1021$ pg/mL IL-10 [$P = 0.0176$]; 6621 ± 3066 pg/mL IL-13 [$P = 0.0019$]), expanded CD25^{hi} Tregs ($11,389 \pm 3304$ pg/mL IL-4 [$P < 0.0001$]; 4025 ± 1375 pg/mL IL-5 [$P = 0.3613$]; $15,223 \pm 3168$ pg/mL IL-10 [$P = 0.0003$]; $4640 \pm$

Figure 5. *Ex vivo*–expanded and mRNA-electroporated CD45RA⁺ and CD25^{hi} Tregs induce the suppression of effector T cell proliferation *in vitro* and produce anti-inflammatory, but not pro-inflammatory, cytokines when activated. Autologous CD127⁺CD25⁺ T cells were stained using CellTrace Violet and activated with human Treg suppression inspector beads, leading to cell proliferation. Inhibition of CD127⁺CD25⁺ T cell proliferation was obtained by different ratios of expanded CD45RA⁺ Tregs (A) and expanded CD25^{hi} Tregs (B). MBP₈₅₋₉₉-specific TCR-encoding mRNA-electroporated cells are indicated with an asterisk (*). Graphs represent median percentage of suppression ± interquartile range for four independent donors. Each donor was measured in duplicate, and the mean division index was used to calculate the suppression percentage: $100 - (DI_{\text{condition of interest}} / DI_{1:0}) \times 100$. Statistical analysis was performed using the nonparametric Friedman test with Dunn's multiple comparisons test. Pro-inflammatory (C) and anti-inflammatory (D) cytokine production of expanded CD45RA⁺ Tregs (green) and expanded CD25^{hi} Tregs (red), which are MBP₈₅₋₉₉-specific TCR-encoding mRNA-electroporated or not, compared with control CD4⁺ T cells (black). Statistical analysis was performed using the nonparametric Friedman test with Dunn's multiple comparisons test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. EP, mRNA electroporation.

1308 pg/mL IL-13 ($P = 0.0446$), mRNA-electroporated CD25^{hi} Tregs ($10,018 \pm 4518$ pg/mL IL-4 [$P = 0.0010$]; 4239 ± 651 pg/mL IL-5 [$P = 0.2012$]; $14,412 \pm 3731$ pg/mL IL-10 [$P = 0.0106$]; 4426 ± 495 pg/mL IL-13 [$P = 0.3613$]) contained higher concentrations of anti-inflammatory cytokines, compared with activated CD4⁺ T cells (284 ± 219 pg/mL IL-4; 2630 ± 2411 pg/mL IL-5; 1212 ± 277 pg/mL IL-10; 3116 ± 602 pg/mL IL-13), albeit not all significant (Figure 5C). Hence, a genuine Treg phenotype and functionality can be assigned to both expanded and mRNA-engineered Treg subtypes.

TCR-dependent stimulation of cells electroporated with MBP₈₅₋₉₉-specific TCR-encoding mRNA leads to cell activation

Finally, two TCR-deficient cell lines were electroporated with MBP₈₅₋₉₉-specific TCR-encoding mRNA, which results in TCR and CD3 surface expression (Figure S3). Subsequently, 6 h after electroporation, both cell types were stimulated with TransAct, mimicking TCR activation, to assess TCR functionality. As a negative control, unstimulated and stimulated mock-electroporated cells and unstimulated mRNA-electroporated cells were used. Flow cytometric analyses from both cell types, by either eGFP production (Figure 6A) or expression of activation markers CD69 and CD137 (Figure 6B), indicated TCR-dependent activation. Indeed, only stimulated mRNA-electroporated 2D3 cells expressed eGFP ($60.9\% \pm 1.4\%$ eGFP⁺), compared with unstimulated mock 2D3 ($0.02\% \pm 0.02\%$ eGFP⁺; $P < 0.0001$), stimulated mock 2D3 ($0.04\% \pm 0.03\%$ eGFP⁺; $P < 0.0001$) and unstimulated

mRNA-electroporated 2D3 cells ($3.3\% \pm 0.1\%$ eGFP⁺; $P = 0.0880$). Similarly, only stimulated mRNA-electroporated SKW-3 cells expressed activation markers ($91.1\% \pm 2.0\%$ CD69⁺; $20.1\% \pm 2.5\%$ CD137⁺) compared with unstimulated mock 2D3 ($2.8\% \pm 1.4\%$ CD69⁺ [$P < 0.0001$]; $0.3\% \pm 0.2\%$ CD137⁺ [$P = 0.0005$]), stimulated mock 2D3 ($2.8\% \pm 0.6\%$ CD69⁺ [$P = 0.0003$]; $0.3\% \pm 0.1\%$ CD137⁺ [$P = 0.0009$]) and unstimulated mRNA-electroporated 2D3 cells ($3.7\% \pm 1.4\%$ CD69⁺ [$P = 0.0077$]; $0.3\% \pm 0.2\%$ CD137⁺ [$P = 0.0006$]) (Figure 6C). In conclusion, TCR-encoding mRNA-electroporation leads to expression of a functional TCR.

Discussion

The therapeutic landscape for autoimmune diseases and transplant rejection is constantly advancing. However, despite progress in targeted biologic and pharmacologic interventions, none of the currently available treatments results in permanent stabilization of disease, and most of the current treatments indiscriminately suppress the immune system [3]. Moreover, general immune modulation may be accompanied by undesired adverse events, such as opportunistic infections and secondary autoimmunity. Breakthroughs in cell and molecular biology have enabled the development of cell-based vaccines [46]. The potential of Tregs as an adoptive cell therapy for the treatment of autoimmune diseases and transplant rejection seems undisputed. Indeed, Tregs can suppress not only CD4⁺ T cells, but also CD8⁺ T cells and many other immune cells when recruited to an

Figure 6. MBP₈₅₋₉₉-specific TCR-encoding mRNA-electroporated 2D3 and SKW-3 cells indicate TCR-dependent activation following TransAct stimulation. 2D3 (A) and SKW-3 (B) cells were MBP₈₅₋₉₉-specific TCR-encoding mRNA-electroporated and unstimulated (gray) or TCR-specific stimulated using TransAct (black) 6 h after mRNA electroporation. NFAT-dependent GFP expression in 2D3 cells and expression of activation markers CD69 and CD137 in SKW-3 cells were analyzed 12 h after activation. Results are represented as data plot and histogram overlay for unstimulated and stimulated electroporated cells. (C) The data graph represents median percentage $\pm$ interquartile range for eight independent experiments, representing unstimulated and stimulated, mock and electroporated cells. Statistical analysis was performed using the nonparametric Friedman test with Dunn's multiple comparisons test: ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

identical antigen-presenting cell [3,47,48]. Nonetheless, a lot of challenges remain, for instance identifying the most potent Treg subtype and developing an effective expansion and engineering procedure that is applicable in a clinical-grade setting [2]. To date, ambiguity regarding the optimal Treg expansion method remains. Different approaches using different activation methods, and concentrations of IL-2 and human serum result, make comparisons difficult [17]. For this, reporting guidelines to include “minimum information about regulatory T cells” have been generated, ultimately driving process reproducibility and harmonization [49].

Here, we present a novel GMP-compliant and easy-to-use protocol for the expansion and genetic engineering of two different Treg subtypes. We observed a 186.5 ± 123.8 -fold expansion of CD45RA⁺ Tregs and a 71.4 ± 50.3 -fold expansion of CD25^{hi} Tregs over the course of a 19-day expansion protocol based on anti-CD3 and anti-CD28 signaling in combination with high amounts of exogenous IL-2. For this, we made use of TransAct, a clinical-grade colloidal reagent consisting of iron oxide crystals embedded into a biocompatible polysaccharide matrix with a diameter of ~ 100 nm. Agonistic anti-CD3 and anti-CD28 antibodies are coated onto the nanomatrix. In contrast to the stimulation reagents commonly used, the use of TransAct is less complex, omitting the need for extensive cell testing during the cell manufacturing process [50–52]. Indeed, to activate Tregs, soluble anti-CD3 and anti-CD28 antibodies require accessory cells such as antigen presenting cells, often resulting in variable T cell expansion depending on the quality of the “feeders” used. Alternatively, large beads (2 to 5 μ m) coated with agonistic anti-CD3/CD28 antibodies are used successfully. However, cell manufacturing processes using such reagents entail manipulations that are suboptimal to implement cell therapy for large numbers of patients, whereas TransAct can be removed easily by centrifugation, thereby overcoming the hurdles of magnetic bead removal [19,53]. Also, one of the biggest setbacks when expanding Tregs *in vitro* is the outgrowth of contaminating effector T cells [54,55]. In our hands, assessment of Treg purity at different time points during expansion indicated no outgrowth of contaminating CD127⁺ effector T cells.

Next, we investigated whether Tregs can be engineered to introduce the expression of an antigen-specific TCR by using nongenotoxic RNA-based electroporation of Tregs. Our findings demonstrate that mRNA electroporation of CD45RA⁺ and CD25^{hi} Tregs was efficient, as evidenced by a $>95\%$ expression of the reporter gene, eGFP, and up

to 85% expression of TCR V β 2, indicating expression of the HLA-DR2–restricted MBP₈₅₋₉₉-specific TCR. To the best of our knowledge, this is the first time Tregs are demonstrated to be engineered using mRNA electroporation. This is especially interesting, since some cell types have been shown to be refractory to electroporation [30]. The introduction of an antigen-specific TCR has the potential to increase the suppressive capacity of Tregs by focusing their arsenal in the same targeted direction. Moreover, it is hypothesized that fewer Tregs are needed for clinical application when antigen-specific Tregs are used. Whereas doses used in clinical trials range from 0.1 up to 100×10^6 polyclonal Tregs/kg per patient [19], depending on the disease setting, state and activity, preclinical experiments underscore the capacity of reduced numbers of antigen-specific Tregs [23,56]. Furthermore, TCR-dependent transcriptional programs are crucial for the activation and the suppressive activity of Tregs [57–59] and are required for maintaining $\sim 25\%$ of the Treg transcriptional signature [54]. Corroborating observations reported by others [60,61], transgenic TCR expression after mRNA electroporation peaked at 24 h and gradually decreased over time, reverting to background levels 1 week after electroporation. Additionally, our findings demonstrate higher efficiency and unaltered high viability, compared with reports of Tregs engineered using viral methods [62–65]. However, the biggest concern of mRNA-based approaches is the stability and durability of expression, mainly because of the unstable structure of mRNA and ubiquitous presence of RNases. In contrast, transduction with viral vectors results in permanent engineered cells, which could be sorted to increase purity of engineered cells. Nonetheless, mRNA has other significant virtues, compared with viral transduction: (i) introducing antigen specificity by means of TCR-encoding mRNA electroporation has a safer genetic engineering profile over transduction using viral vectors, as these can integrate viral DNA into the host genome, potentially leading to insertional mutagenesis [30,66]; (ii) moreover, protein translation takes place almost immediately because of its functionality in the cell's cytoplasm, while expression levels are easily adjusted with the amount of supplied mRNA [30]; (iii) host immune responses due to immunogenic reaction of Toll-like receptor (TLR)-activated mRNA are weaker than TLR9 recognition of unmethylated CpG motifs of DNA [67]; (iv) host cell transfection using mRNA is more facile and relatively efficient compared with plasmid DNA, due to its far smaller construct, lack of requirement for a high biosafety level during production and circumventing the need

of selecting a specific promoter [28,30,67]; and (v) the transient nature, being subjected to the natural decay of mRNA, provides an accurate system to control the synthesis of exogenous proteins [30] and avoids long-lasting toxicities in the event of unanticipated transgenic TCR specificities [66,68]. More importantly, it is believed not to interfere with the clinical effect. Indeed, infectious tolerance, a phenomenon when tolerance induction is transferred from one cell type to another, is thought to play an important role in the beneficial effects of cell therapy [15]. This is achieved via the production of inhibitory cytokines by activated Tregs, creating a suppressive microenvironment in which effector T cells undergo apoptosis, naïve T cells convert into a Treg phenotype and dendritic cells become tolerogenic. In doing so, Tregs can induce tolerance to cells involved in the immune reaction, even without direct interaction, setting a tolerance-inducing cascade in motion. The superiority of electroporation in terms of efficiency, compared with other transfection methods, has already been shown by our group [32–34]. This method's efficiency depends on only two factors, the electrical field type and strength, and the pulse time, which can easily be optimized. Therefore, electroporation is currently emerging as the transfection method of choice thanks to its safety and relatively easy clinical translation [30,69]. The latter is further underscored by our data, indicating no negative effect on transgenic TCR expression over time after cryopreservation.

The HLA-DR2–restricted MBP_{85–99}-specific TCR used in this study was previously obtained from a multiple sclerosis patient–derived T cell clone Ob.2F3 [70], and its preclinical potential has already been investigated by others [35,71]. Although we did not perform an antigen-specificity assessment of the TCR used, antigen-induced proliferation of MBP_{85–99}-specific TCR-transduced T cells was shown by the Scott laboratory [35], confirming its specificity. Here, we confirmed that TCR-encoding mRNA electroporation results in the expression of a functional TCR in two TCR-deficient cell lines. Indeed, TCR-dependent activation led to NFAT-dependent eGFP expression in transfected 2D3 cells and upregulation of activation markers CD69 and CD137 in transfected SKW-3 cells. Current challenges for this form of therapy lie in further identifying disease-associated antigens and in developing these antigen-specific Treg products. In line with this, mRNA electroporation could also be used to introduce proteins or cytokines involved in Tregs' mechanism of action, possibly resulting in gain of function, as previously tested in dendritic cells [72].

We detected transgenic TCR expression via upregulation of TCR V β 2 chain using a monoclonal antibody targeting the TRBV20-1 variable gene segment. Monoclonal antibodies against TCR alpha and beta chains are commonly used to analyze surface levels of introduced TCR. Alternatively, tetramers for antigen-specific detection of transgenic MBP_{85–99}-specific TCR expression could be used. Because coexpression of endogenous and transgenic TCR chains may result in TCR mispairing, it is possible that TCR V β 2 staining also detects heterodimers formed by MBP_{85–99}-specific TCR V β 2 chain mispairing with endogenous TCR alpha chains. For this, basal TCR V β 2 chain expression in mock-electroporated cells was used as a control in this study of which expression levels were in line with basal levels of TCR V β 2 chain expression found in previously published reports [73,74]. Therefore, it is fair to assume that TCR V β 2 chain expression above this threshold results from MBP_{85–99}-specific TCR mRNA electroporation. Nonetheless, TCR mispairing negatively affects transgenic TCR levels and may lead to unpredictable reactivities. Therefore, downregulation or complete disruption of endogenous TCRs is a key subject to be addressed for this type of therapy. Prevention of mispairing between endogenous and transgenic TCR chains can be achieved by different methods, both transient and stable. For example, our group recently developed an RNA-based double sequential electroporation platform whereby Dicer-substrate small interfering RNAs (DsiRNAs) are first introduced to transiently suppress endogenous TCR

expression on a transcript level, followed by electroporation with DsiRNA-resistant TCR mRNA [60]. A genetically stable alternative is based on the use of clustered regularly interspaced short palindromic repeats (CRISPR) [75,76].

Notwithstanding the tremendous potential of engineered Tregs, Tregs also demonstrate plasticity, as evidenced by inherent flexibility in response to changing microenvironments [77,78], including *in vitro* expansion or engineering. Here, we demonstrate that GMP-compliant expansion and mRNA-based genetic engineering of both CD45RA⁺ and CD25^{hi} Tregs did not negatively affect the Treg characteristics of both subtypes. Our data indicate no significant difference in the expression levels of the Treg master regulator FOXP3, expression after activation, expansion and transfection of both CD45RA⁺ Tregs and CD25^{hi} Tregs, albeit that freshly isolated CD45RA⁺ Tregs did not demonstrate significantly higher expression levels of FOXP3 compared with CD127⁺CD25[–] effector T cells. However, a limitation of our study is that we did not investigate the effect of antigen-driven TCR signaling on the Treg directly. Nonetheless, we demonstrated that Tregs are still capable of producing anti-inflammatory cytokines after anti-CD3 and anti-CD28 activation of expanded and mRNA-electroporated Tregs, indicative of the stability of the Treg phenotype and function. Moreover, because FOXP3 expression has been observed in activated conventional T cells as well [6], and FOXP3-independent maintenance of the human Treg identity in FOXP3-ablated Tregs has been shown [7], we also analyzed the methylation levels of the TSDR and expression levels of Treg markers Helios, CTLA-4 and CCR4. First, stable FOXP3 expression is directly linked to high demethylation of the TSDR, resulting in increased accessibility of the FOXP3 gene and consequently its transcription and translation [8]. We observed low TSDR methylation levels for both Treg subtypes, which are significantly lower compared with CD127⁺CD25[–] effector T cells. Similar levels of TSDR methylation were observed by others after retroviral transduction of human Tregs [79]. Next, Helios, an Ikaros family transcription factor, is shown to be upregulated in Tregs, responsible for the binding of the FOXP3 promoter and aiding in its stable expression [80–82]. Generally, it is expressed in ~70% of FOXP3⁺ human cells [80,83]. Alternatively, Helios is shown to be a marker, but not a driver, of human Treg stability [84]. Indeed, lower Helios expression has been linked to *ex vivo* expansion conditions, especially in CD25^{hi} Tregs, but with preservation of equivalent suppressive function and stability [84]. Interestingly, similar conclusions can be made in this study in expanded and mRNA-engineered CD25^{hi} Tregs, but with the preservation of high FOXP3 expression in both subtypes. CD45RA⁺ Tregs, expanded and mRNA electroporated, retained their Helios expression levels. In addition, high expression of CTLA-4, responsible for cell contact–dependent suppression, and CCR4, allowing their migration toward antigen-presenting cells and activated T cells, resulting in inhibition of function of antigen-presenting cells or corresponding T cell suppression [85], further confirmed their genuine Treg phenotype after expansion and mRNA engineering in both subtypes, compared with CD4⁺ cells. On top of that, Tregs remained functional after *in vitro* expansion and mRNA electroporation, as indicated by their capacity to inhibit the proliferation of CD127⁺CD25[–] effector T cells *in vitro* in a ratio-dependent manner, similar to previously published data [86]. Future experiments could include lower Treg:effector T cell ratios, mechanistic studies of Treg suppression and the influence of antigen-driven stimulation of Tregs, as this might positively influence the Treg suppression capacity, given the important role of TCR stimulation in Tregs [57–59]. Altogether, our findings underline the clinical potential of this innovative GMP-compliant Treg manufacturing protocol, without negative alteration of Treg characteristics.

Whereas Tregs are a heterogeneous mixture of complex cellular subphenotypes [11], a compromise between high suppressive functionality and susceptibility to loss of suppression might be needed when identifying the ideal Treg subtype for clinical application. In current study, we selected two distinct Treg subtypes, namely

CD4⁺CD127[−]CD25^{hi} and CD4⁺CD127[−]CD25⁺CD45RA⁺ Tregs, by means of high-purity multiparametric flow cytometric cell sorting. We observed FOXP3 expression in >95% of CD25^{hi} Tregs, in agreement with earlier findings by Trzonkowski and colleagues [12], demonstrating robust FOXP3 expression in the top 2% of CD25⁺ T cells. In our hands, FACS-sorted CD45RA⁺ Tregs showed lower FOXP3 expression compared with the CD25^{hi} Tregs, albeit not significant. In another study, lower FOXP3 expression in CD45RA⁺ Tregs, compared to CD25^{hi} Tregs, has also been reported [87,88]. Nonetheless, both subtypes are genuine Tregs, as indicated by their functional capacity to suppress CD4⁺CD127⁺CD25[−] effector T cell proliferation *in vitro*. Moreover, selecting naïve CD45RA⁺ Tregs has the advantage to be more stable, as confirmed by our results, and less contaminated with effector T cells [87], aiding the *ex vivo* expansion and engineering purposes. Indeed, Hornero *et al.* [87] have shown that CD45RA⁺ Tregs are refractory to loss of TSDR demethylation status, whereas CD25^{hi} Tregs lose TSDR demethylation status following treatment with tacrolimus, an immunosuppressant, potentially resulting in loss of stability of the Treg phenotype [87]. Altogether, selecting the most suitable Treg subtype might be dependent, among others, on disease progression [89], exposure to immunosuppressants [87] or the applied *in vitro* expansion and engineering approaches.

In conclusion, we present a novel, GMP-compliant approach for *ex vivo* expansion and RNA-based engineering of Tregs. Our approach is convenient and robust for transient genetic engineering of different subtypes of Tregs, without affecting Treg phenotype and function. Our findings offer new opportunities for mRNA engineering of Tregs for future clinical applicability, in which this approach can be used for the induction of antigen specificity or evaluating possible gain of function, after introduction of proteins or cytokines involved in Tregs' mechanism of action.

Author Contributions

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All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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Supplementary materials

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