FULL-LENGTH ARTICLE

Translational Research

HLA-A*02:01-directed chimeric antigen receptor/forkhead box P3-engineered CD4+ T cells adopt a regulatory phenotype and suppress established graft-versus-host disease

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ABSTRACT

Background aims: To investigate the feasibility of using CD4+ T cells genetically modified to express an allo-HLA directed CAR and FOXP3 to suppress T cell proliferation and cytokine secretion in GvHD. **Methods:** Human CD4+ T cells from A*02:01 negative donors were transduced to express A*02 CAR and FOXP3 and co-cultured in mixed lymphocyte reaction assays to demonstrate T cell suppression. A*02- CAR/FOXP CD4+ T cells were then injected into mice engrafted with allogeneic T cells in a GvHD mouse model.

Results: CD4+ T cells genetically modified to express allo-HLA-directed CAR and FOXP3 proliferate rapidly, downregulate CD127 and interferon- γ , express high CD25 and Helios and convert to a stable antigen-dependent suppressive phenotype. In mixed lymphocyte reaction assays, these cells potently suppressed T-cell proliferation and secreted IL-10. In a graft-versus-host disease model, A*02-CAR/FOXP3 CD4+ T cells outperformed polyclonal Tregs by reducing liver and lung inflammation, inhibiting pro-inflammatory cytokine production and limiting grafted CD3+ T-cell expansion.

Conclusions: CD4+ T cells expressing allo-antigen directed HLA-specific CAR and FOXP3 act as potent, specific and stable suppressors of inflammation that out-perform their Treg counterparts both *in vitro* and *in vivo*.

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Introduction

Regulatory T cells (Tregs; CD4+CD25+) play an indispensable role in controlling immune responses, and disruption of the key transcription factor forkhead box P3 (FOXP3) results in lethal immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome phenotype in men [1]. When FOXP3 expression is silenced by promoter hypermethylation, Tregs lose their suppressive ability and are more prone to adopting effector-like functions [2,3], whereas ectopic FOXP3 expression restores regulatory capacity in Tregs, with disrupted or silenced FOXP3 expression [4,5].

The use of polyclonal or chimeric antigen receptor (CAR) Tregs is being explored for the treatment of autoimmune and inflammatory conditions [6–9]. Polyclonal Treg therapies have been shown to limit autoimmunity in type 1 diabetes [10], reduce graft rejection following allotransplantation [11,12] and prevent graft-versus-host disease (GVHD) in patients receiving hematopoietic stem cell transplantation (HSCT) [7,8]. Several pre-clinical

models have demonstrated that efficacy can be further improved using antigen-specific Tregs, including CAR Tregs [12–18].

Recently, the UK Medicines and Healthcare products Regulatory Agency authorized first-in-human studies of CAR Treg therapy for solid organ transplant. However, several factors complicate the use of Tregs for cell therapy. First, Tregs are plastic and can adopt an effector-like state that might exacerbate disease [2,19–21]. Second, immunosuppressive therapy frequently limits Treg numbers [22–25] and may hamper autologous CAR Treg approaches. Third, Tregs proliferate at a significantly reduced rate compared with non-regulatory CD3+ T cells [5,26,27]. Combined, these factors call into question the appropriateness of engineering Tregs as therapeutic agents.

Non-Treg CD4+ T cells therefore represent an attractive alternative to Treg-based therapies, from a manufacturing perspective, because of their larger circulating numbers and considerably greater proliferation rates [26,27]. Moreover, non-Treg CD4+ T cells genetically modified to express an allergen-specific T-cell receptor and FOXP3 suppress T-cell effector function in models of allergy [28]. Focusing on the context of GVHD, the authors herein demonstrate that CD4+ T cells can be converted to an antigen-specific, stable regulatory phenotype via sustained expression of a donor-specific CAR and the FOXP3 gene, independent of

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the endogenous *FOXP3* promoter. These cells can also be expanded efficiently because of their higher rate of proliferation. Using this A*02-CAR/*FOXP3* format, the authors demonstrate the conversion of generic CD4⁺ T cells to an A*02-CAR/*FOXP3*⁺ CD4⁺ Treg phenotype in which they act as antigen-restricted, stable, potent suppressors of inflammatory reactions and outperform their polyclonal Treg counterparts in *in vitro* and *in vivo* GVHD models.

Methods

Isolation and transduction of CD4⁺ T cells

A*02:01– peripheral blood mononuclear cells (PBMCs) from four donors were isolated from fresh Leukopak (AllCells) using Ficoll density gradient media. CD4⁺ T cells or Tregs were isolated using a CD4⁺ T-cell– isolation kit or CD4⁺CD25⁺ regulatory T-cell isolation kit (Miltenyi) according to the manufacturer's instructions. Post isolation, T cells were cultured at a density of 1×10^6 cells/mL in X-Vivo 15 media supplemented with 5% human A/B serum and 1% penicillin/streptomycin in the presence of CD3/28 Dynabeads (Invitrogen) and 300 units/mL of IL-2 (Miltenyi). After 2 days, cells were transduced with lentivirus (outsourced) at a multiplicity of infection of 5. Cells were cultured in IL-2 for an additional 5 days prior to enrichment.

Phenotyping of CD4⁺ CAR T cells

Cells were stained using antibodies against CTLA-4 (allophycocyanin [APC]) and CD127 (biotinylated primary antibody, followed by streptavidin BV421) (BioLegend) for 30 min on ice and analyzed on FACSCanto (BD Biosciences). CAR expression was monitored by flow cytometry via staining with peptide-major histocompatibility complex tetramers conjugated to a specific fluorophore (phycoerythrin [PE] or APC; BioLegend). For *FOXP3* (PE) and Helios (APC) staining, cells were permeabilized using *FOXP3* fix/perm buffer (BioLegend) prior to staining with antibody. Interferon gamma (IFN γ) staining was performed by activating T cells for 48 h, followed by the addition of 10 μ g/mL of brefeldin A for 18 h. Cells were fixed in intracellular fixation buffer (eBioscience) and stained in 1x permeabilization buffer (eBioscience) and PE anti-human IFN γ antibody (BioLegend) for another 60 min.

A*02 CAR T-cell enrichment

A*02 CAR-transduced T cells were enriched by staining with A*02 monomer conjugated to streptavidin M-280 Dynabeads (Invitrogen) for 30 min. Cells bound to Dynabeads were pulled down using DynaMag (Invitrogen).

Mixed lymphocyte reaction

A*02:01– CD3⁺ T cells from the same donor as CAR T cells (i.e., donor-matched) were isolated from PBMCs using a CD3⁺ negative selection kit from Miltenyi. Post isolation, CD3⁺ T cells were stained with carboxyfluorescein succinimidyl ester (2 μ M) for 15 min at room temperature. LCL721 cells (A*02:01⁺) were treated with mitomycin C at 10 μ g/mL for 30 min at 37°C. CD3⁺ and LCL721 cells were co-cultured at a ratio of 1:0.5 CD3:LCL721 cells. *FOXP3*/CAR T cells were then added to the co-culture at varying ratios. After 4 days, supernatant was collected and stored at –20°C for cytokine analysis. Cells were read on the flow cytometer, and CD3⁺ T-cell proliferation was analyzed using FlowJo. Cytokine analysis was performed using a human CBA Th1/Th2/Th17 kit (BD Biosciences) according to the manufacturer's instructions.

In vivo studies

Next, 5- to 7-week-old female NSG (NOD.Cg-Prkdcscid Il2rgtm1-Sug/SzJ) mice were ordered from The Jackson Laboratory. At week 1,

each animal was injected with 10 million PBMCs isolated from an A2⁺ donor. At 2 weeks, animals were injected via the tail vein with saline, polyclonal Tregs or A*02-CAR/*FOXP3* CD4⁺ T cells. Then, at 4 weeks, a second higher dose of the same cells was injected into these animals via the tail vein. Blood was sampled weekly to measure T-cell engraftment and serum cytokines (BD cytometric bead array; BD Biosciences). At 9 weeks, animals were killed, and lung, liver and spleen were paraffin-embedded, sectioned and stained with hematoxylin and eosin (H&E). All H&E-stained sections were graded using a GVHD severity scale by a pathologist.

Results

It has been clear for some time that Tregs are essential for self-tolerance and that *FOXP3* expression is necessary to maintain both thymic-derived and peripheral CD25⁺CD4⁺ Tregs distinct from other CD4⁺ T cells. Treg markers, including Helios, CTLA-4 and *FOXP3*, were expressed at very high levels in the CD25⁺CD4⁺ population (see supplementary Figure 1A), and, as expected, these CD25⁺CD4⁺ Tregs expanded more slowly than CD4⁺ T cells after anti-CD3/anti-CD28 bead stimulation (see supplementary Figure 1B). The authors designed a CAR construct with an HLA-A*02:01-binding single-chain fragment antigen binding format linked to a CD8 hinge, the CD28 transmembrane domain, the CD28 cytosolic tail and the CD3zeta cytosolic domain. Since maintenance of *FOXP3* expression in mature Tregs is critical for Treg-mediated suppression, this construct was further modified by the addition of a T2A cleavage site and full-length h*FOXP3* (Figure 1A). Both CD25⁺CD4⁺ Tregs and CD4⁺ T cells were engineered to express CAR/*FOXP3* and enriched to >90% CAR⁺ cells (see supplementary Figure 2). The authors observed a slower rate of proliferation of both untransduced Tregs and A*02-CAR/*FOXP3* Tregs. Surprisingly, anti-CD3/anti-CD28 and IL-2 treatment induced proliferation in A*02-CAR/*FOXP3* CD4⁺ T cells equivalent to untransduced CD4⁺ T cells (Figure 1B; also see supplementary Figure 1B). Compellingly, these CAR/*FOXP3* CD4⁺ T cells expressed *FOXP3* and adopted a Treg phenotype expressing CD25 at high levels and downregulating CD127 expression (Figure 1C). Intracellular staining revealed a marked reduction in intracellular IFN γ expression at levels below that observed in polyclonal Tregs derived from the same donor (Figure 1C).

Here the authors show that CD4⁺ T cells derived from many normal donors and transduced with a CAR construct containing *FOXP3* converted to a regulatory phenotype and proliferated at rates comparable to those of untransduced CD4⁺ T cells after anti-CD3/anti-CD28 bead stimulation. Together, these data emphasize that converted A*02-CAR/*FOXP3* CD4⁺ T cells may be superior candidates for the scalable manufacturing of anti-inflammatory therapies because of their abundance, robust growth profiles and insensitivity to significant *FOXP3* expression.

To investigate the suppressive capacity of these cells, the authors tested their ability to limit autologous CD3⁺ T-cell proliferation driven by allogeneic reactions directed against HLA-mismatched LCL721 cells in a mixed lymphocyte reaction (MLR) (Figure 2B,C). CD4⁺ T cells, isolated from freshly thawed HLA-A*02– PBMCs, were transduced with A*02-CAR/*FOXP3* and sorted using magnetic beads coated with A*02 NY-ESO peptide-major histocompatibility complex to enrich for CAR⁺ T cells (Figure 2A). *FOXP3* levels were then quantified by flow cytometry (see supplementary Figure 3), revealing sustained expression of *FOXP3* after enrichment. Furthermore, the authors found that A*02-CAR/*FOXP3* CD4⁺ T cells maintained their *FOXP3* expression when cultured in inflammatory conditions (see supplementary Figure 4). These observations were reproducible between experiments and across donors and, when taken together, illustrate that exogenous *FOXP3* is stable in A*02-CAR/*FOXP3* CD4⁺ T cells.

Next, CD3⁺ T cells were isolated from the same A*02– donor and co-cultured with HLA-mismatched LCL721 (A*02⁺) cells. To suppress the MLR response, either A*02-CAR or A*02-CAR/*FOXP3* CD4⁺

Figure 1. Characterization of CAR/FOXP3 transduced CD4+ T cells: (A) CAR construct with WT FOXP3 used in this experiment. Expression was controlled via a EF1a promoter (not shown above). (B) Transduced and enriched CD4+ T cells, A*02-CAR/FOXP3 CD4+ T cells, A*02-CAR/FOXP3 Tregs, and Tregs were cultured in X-Vivo media supplemented with 100 U/mL of hIL-2 for 5 days following CD3/28 stimulation. (C) Cells were phenotyped using antibodies against FOXP3 (PE), CD25 (APC), and CD127 (APC). Cells were activated with CD3/28 beads for 2 days and Brefeldin A was added for 24 hours before cells were stained for intracellular IFN- γ . Experiments shown in "B" and "C" above were repeated with 3 additional donors (for a total of 4 different donors); data shown above are representative of replicates from different donors. scFab, single-chain fragment antigen binding domain; stim, stimulation.

Figure 2. FOXP3/CAR-T cells suppress autologous CD3+ proliferation in MLR (A) CD4+ T cells were activated with CD3/28 beads and transduced with lentivirus at a MOI of 5 and CAR+ cells were magnetically enriched to obtain > 95% CAR+ and > 70% FOXP3+ T cells. (B) 7 days post enrichment and expansion, FOXP3/CAR-T cells were co-cultured with CFSE-labeled autologous CD3 cells (A*02:01-) and growth arrested LCL721 cells (A*02:01+) in an MLR assay. Cells were co-cultured for 4 day and CD3 proliferation was quantified using flow cytometry. (C) Supernatants from the MLR experiment were harvested and cytokine production was quantified using Cytometric Bead Array assay. Experiments were repeated with 2 additional donors (for a total of 3 different donors); data shown above are representative of replicates from different donors.

T cells were added to the MLR co-culture at a ratio of 0.5:1, 0.2:1, 0.1:1 or 0.05:1 (CAR-T:CD3), and cells were co-cultured for 4 days. Overall, the authors observed significant suppression of CD3+ T-cell proliferation in co-cultures with A*02-CAR/FOXP3 CD4+ T cells at all CAR-T:CD3 ratios (variability between donors ranged from 50% to

80% suppression at the highest CAR-T:CD3 ratios tested; see supplementary Figure 5 for representative data from another donor). In comparison, CD4+ T cells transduced with A*02-CAR alone and untransduced CD4+ T cells both showed minimal suppression even at high CAR-T:CD3 ratios (Figure 2B). Supernatant was collected at

Figure 3. *In vivo* GVHD study: (A) Schematic of the *in vivo* GVHD study design with indicated dosing regimens. (B) Blood samples were taken at week 2, 3, 4, 5, 6, 8, 9 and cell used for flow cytometric analysis of engraftment of PBMCs in peripheral blood (See methods section detailing flow cytometric analysis details). (C) Kaplan-Meier survival curves based on treatment regimen. (D) For serum cytokine analysis samples were incubated with cytometric bead array (CBA) beads and were read on an FACS CANTO (BD Biosciences) and results analyzed using FlowJo Software (Tree Star). (E) Histological analysis of pulmonary inflammatory infiltrates (left) and GVHD severity (right) in mice that received A*02-CAR/FOXP3 CD4+ cells. Each experimental group in the above figures was composed of 6 mice (data in figure “E” are representative examples from each group). CTRL, control; SEM, standard error of the mean.

the end of the MLR study, and levels of the pro-inflammatory cytokines IL-2 and tumor necrosis factor alpha (TNF α) and the anti-inflammatory cytokine IL-10 were quantified using cytometric bead arrays (Figure 2C). Co-cultures with A*02-CAR/FOXP3 CD4+ T cells showed 4- to 6-fold higher IL-10 secretion compared with FOXP3–CAR T cells. In addition, co-culture with A*02-CAR/FOXP3 CD4+ T cells significantly reduced IL-2. Expression of FOXP3 further reduced TNF α secretion compared with A*02 CAR CD4+ T cells targeting A*02 antigen on LCL721 cells. Interestingly, transduction of polyclonal Tregs with an A*02 CAR construct without added FOXP3 also increased secretion of TNF α post co-culture.

As has been previously reported, instability of FOXP3 and subsequent emergence of effector-like functions present a serious risk [21,29]. These observations suggest that Tregs derived from peripheral blood and engineered to express A*02 CAR unaccompanied by exogenous FOXP3 have the propensity to behave as effector cells. Transduction with FOXP3 protein along with A*02 CAR, however, reduced TNF α secretion, suggesting that additional FOXP3 stabilized these Tregs during co-culture (see supplementary Figure 6). In summary, this work demonstrated that A*02-CAR/FOXP3 CD4+ T cells attenuated T-cell activation with concomitant robust IL-10 production.

To explore the suppressive function of A*02-CAR/FOXP3 CD4+ T cells *in vivo*, the authors used a GVHD model in NSG mice. In this model, 1e7 A*02+ human PBMCs were engrafted in NSG mice, prior to randomization into a control group and two groups that received intravenous injections of either polyclonal Tregs or A*02-CAR/FOXP3 CD4+ T cells (Figure 3A). Body weight and clinical scores were monitored daily, and engraftment and serum cytokines were evaluated weekly. In this model, CD45+ engraftment was evident at 4 weeks, and mice that received A*02-CAR/FOXP3-expressing CD4+ T cells had significantly improved survival compared with those that received polyclonal Tregs (Figure 3C). Compellingly, the group administered A*02-CAR/FOXP3-expressing CD4+ T cells showed limited serum pro-inflammatory cytokines IFN γ and TNF α versus the high levels observed in the PBMC control and polyclonal Treg groups (Figure 3D). The authors did not detect circulating hA2-CAR Tregs, measured from 14 days after injection. Histological analysis showed clear attenuation of pulmonary and hepatic inflammatory infiltrates in mice that received A*02-CAR/FOXP3 CD4+ T cells. When compared with the PBMC alone or PBMC Treg groups, lungs from animals in the A*02-CAR/FOXP3 CD4+ T-cell group showed limited or no inflammatory findings and no evidence of alveolar parenchyma consolidation, in contrast to lung sections from mice in the PBMC alone or PBMC Treg groups, where evidence was observed of severe inflammation coupled with consolidation of alveolar parenchyma, impacting about 30–90% of the lung in all animals investigated (Figure 3E). Again, analysis of liver H&E sections showed that A*02-CAR/FOXP3 CD4+ T-cell-treated animals had reduced liver changes, with generally less than 5% degeneration or necrosis (considered minimal), with inflammation and/or degeneration/necrosis being observed in 25–40% of the sections examined from the PBMC alone or PBMC Treg groups (see supplementary Figure 7). These observations suggest that therapeutic administration of A*02-CAR/FOXP3 CD4+ T cells can markedly attenuate ongoing inflammatory pathology in the GVHD model.

Discussion

Regulatory T cells play a significant role in transplant tolerance, but immunosuppressive therapies given to patients prior to HSCT or allotransplantation compound challenges when manufacturing Treg-based therapies. Traditional regimens—such as those that rely on calcineurin inhibitors like tacrolimus—decrease systemic numbers, proliferative capacity and *ex vivo* viability of Tregs in renal and liver transplant patients [22–25]. Circulating at levels 5- to 10-fold greater than Tregs, CD25–CD4+ T cells that can be converted to a regulatory

phenotype therefore represent superior candidates for scalable anti-inflammatory therapies [25]. Although others have reported that introducing FOXP3 into CD4+/FOXP3– T cells derived from immune dysregulation, polyendocrinopathy, enteropathy, X-linked patients blunts their proliferation [4], the authors show that CD4+ T cells derived from many normal donors and transduced with a CAR construct containing FOPXP3 convert to a regulatory phenotype and proliferate at rates comparable to those of untransduced CD4+/FOXP3– T cells. Proliferation of these A*02-CAR/FOXP3 CD4+ T cells was much more rapid than Treg controls. This discrepancy may be explained in two ways. Passerini *et al.* [4] isolated naive CD25–CD4+ T cells and transduced with a feeder layer of irradiated PBMCs cultured in IL-7. The transduction protocol in this work differed in using transact and that all CD4+ T cells were used, with a high percentage of CD45RO+ cells. Therefore, differentiation is likely to have occurred, and thus different epigenetics and chromatin remodeling may exist [3,20]. A potential explanation could be that Treg cells express different suppressors of cytokine signaling genes compared with other CD4+ T cells, and these are known to have differential effects on cytokine signaling and hence control CD4+ T-cell differentiation and plasticity (see Knosp *et al.* [30] for a review of CD4+ T-cell polarization by suppressors of cytokine signaling). It is possible, therefore, that these cells may proliferate more readily in the presence of IL-2, whereas Treg cells are known to proliferate more slowly than effector T cells.

This article represents the first characterization to date of cells transduced to express both alloantigen-specific CAR and exogenous full-length FOXP3. Although it is conceivable that simultaneous expression of CAR within transduced cells plays a role in ameliorating the growth-inhibiting effects of expressing significant levels of FOXP3, a comprehensive characterization of the underlying mechanism(s) is needed to describe the extent of any such influence in detail. It will also be important to determine if the epigenome differs from a Treg-type epigenome [31].

Collectively, the data show that CD4+ T cells expressing alloantigen-directed, HLA-specific CAR and FOXP3 act as potent, specific and stable suppressors of inflammation that outperform their Treg counterparts both *in vitro* and *in vivo*. Importantly, these cells retain a suppressive phenotype, providing an avenue for cell-based therapy with a significantly improved safety profile that circumnavigates the risks associated with Treg-based therapies, which have a propensity to drift to an effector-like state that can worsen disease. This approach represents a novel antigen-specific strategy for potentially controlling undesired immunity driven by alloantigen reactions—such as that arising from GVHD following HSCT or rejection of allografts—with an opportunity for improved safety that is well suited for scalable manufacturing, regardless of patient status. Together, these data emphasize that converted A*02-CAR/FOXP3 CD4+ T cells may be superior candidates for the scalable manufacturing of anti-inflammatory therapies because of their abundance, robust growth profiles and insensitivity to significant FOXP3 expression.

Declaration of Competing Interest

The authors have no commercial, proprietary or financial interest in the products or companies described in this article.

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Author Contributions

Project conceptualization, planning and experimental design: JC, AM, MD, and JJ; Experimental execution: AM, JC, and MD; Data analysis: JC and AM; Manuscript drafting: JC, AM and JJ.

Supplementary materials

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