THERAPEUTIC POTENTIAL OF GENE-MODIFIED REGULATORY T CELLS

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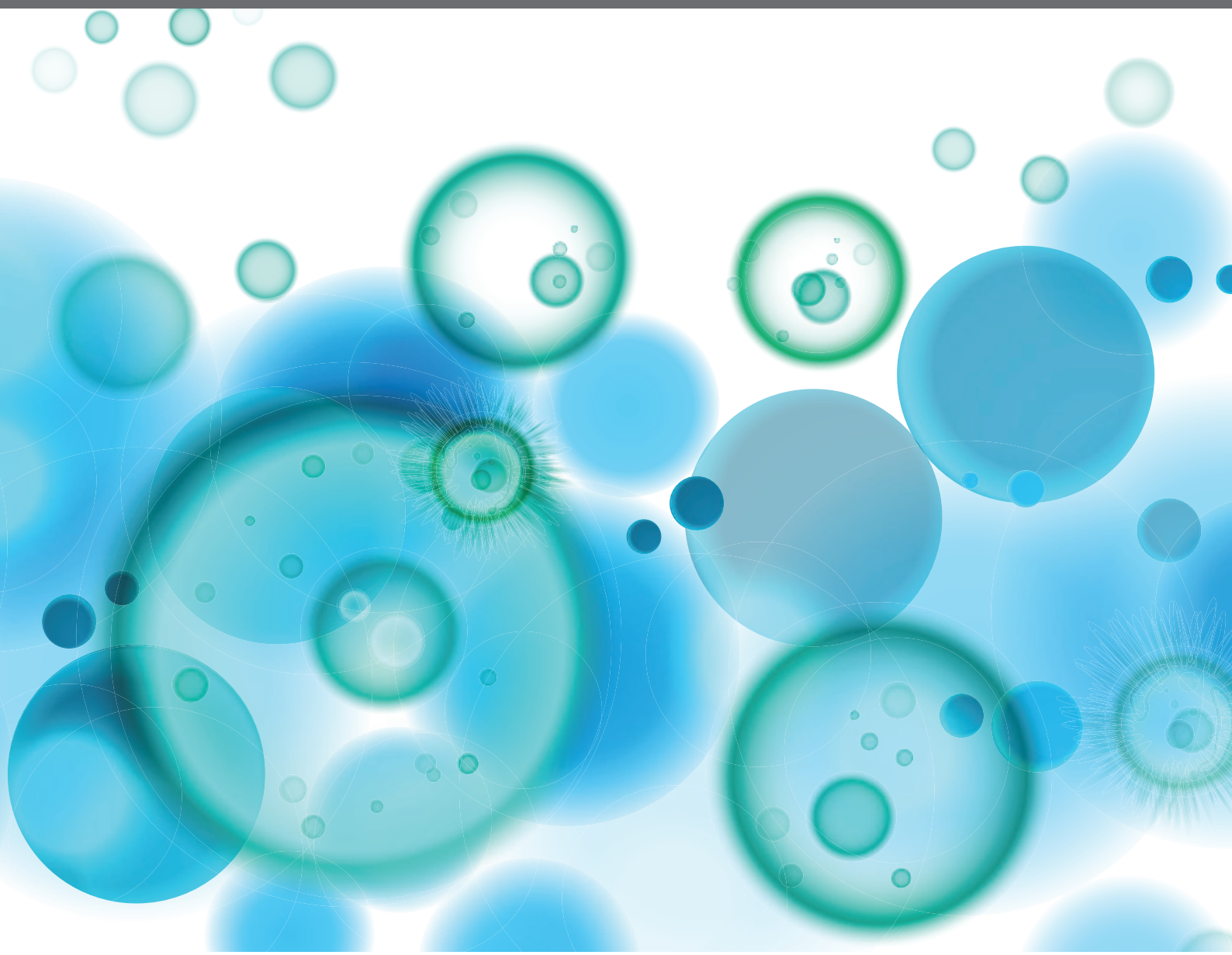

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THERAPEUTIC POTENTIAL OF GENE-MODIFIED REGULATORY T CELLS

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Chimeric Antigen Receptor-Redirected Regulatory T Cells Suppress Experimental Allergic Airway Inflammation, a Model of Asthma

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Cellular therapy with chimeric antigen receptor (CAR)-redirected cytotoxic T cells has shown impressive efficacy in the treatment of hematologic malignancies. We explored a regulatory T cell (Treg)-based therapy in the treatment of allergic airway inflammation, a model for asthma, which is characterized by an airway hyper-reactivity (AHR) and a chronic, T helper-2 (Th2) cell-dominated immune response to allergen. To restore the immune balance in the lung, we redirected Tregs by a CAR toward lung epithelia in mice upon experimentally induced allergic asthma, closely mimicking the clinical situation. Adoptively transferred CAR Tregs accumulated in the lung and in tracheobronchial lymph nodes, reduced AHR and diminished eosinophilic airway inflammation, indicated by lower cell numbers in the bronchoalveolar lavage fluid and decreased cell infiltrates in the lung. CAR Treg cells furthermore prevented excessive pulmonary mucus production as well as increase in allergen-specific IgE and Th2 cytokine levels in exposed animals. CAR Tregs were more efficient in controlling asthma than non-modified Tregs, indicating the pivotal role of specific Treg cell activation in the affected organ. Data demonstrate that lung targeting CAR Treg cells ameliorate key features of experimental airway inflammation, paving the way for cell therapy of severe allergic asthma.

Keywords: allergic asthma, chimeric antigen receptor, regulatory T cells, adoptive cell therapy, ovalbumin mouse model

INTRODUCTION

Asthma is a very common chronic respiratory disease which affects more than 300 million people worldwide (1). It is characterized by airway inflammation, airway hyper-reactivity (AHR), and reversible airway obstruction. Asthma covers several distinct clinical and biological phenotypes whereof allergic asthma is a leading subtype (2). Treatment with anti-inflammatory and broncho-spasmolytic drugs succeeds to control symptoms in most patients (3), however, leaving 10–20% of patients refractory to any therapy (4). Apart from symptomatic treatment no curative therapy is available, which makes new approaches mandatory, especially for patients with severe and uncontrolled asthma.

Allergic asthma is associated with an overwhelming T helper-2 (Th2) cell-dominated immune response to allergens. Physiologically, airway inflammation is counteracted by inhibitory molecules and suppressor cells including $CD4^+$ regulatory T cells (Tregs) (5, 6) which becomes visible upon Treg depletion which causes spontaneous asthma-like airway pathology (7). Patients suffering from allergic asthma have reduced numbers of Tregs that are furthermore impaired in their suppressive capacity (8–11). Some currently applied therapies aim at enhancing Treg cell number and function (8, 12), whereas adoptive transfer of Tregs can suppress both the priming and the effector phase of allergic airway inflammation in experimental models of murine asthma (13–15).

Antigen-specific Tregs seem to be superior in ameliorating an inflammatory disease compared with polyclonal Tregs (16–18). However, the translation to clinical application is limited by the very small number of antigen-specific Tregs in the peripheral blood which can be identified, isolated, and amplified for therapeutic purposes. The tremendous diversity of asthma eliciting antigens makes the identification of such specific Tregs extremely laborious with the risk that no specific cell clone can be identified. To provide pre-defined specificity, Tregs can be engineered *ex vivo* with a chimeric antigen receptor (CAR) (19) that harbors an antibody-derived binding domain for antigen and activates the engineered T cell upon binding independently of MHC recognition (20). While CAR engineered pro-inflammatory T cells are extensively studied and produced impressive efficacy in the treatment of hematologic malignancies in early phase trials (21), CAR engineered Tregs were only sporadically evaluated in animal models, e.g., for the treatment of colitis (22) and multiple sclerosis (23).

In this study, we assessed whether antigen-specifically redirected Tregs are capable to control experimentally induced airway inflammation in a clinically relevant mouse model mimicking the human situation. In order to target to the lung and to initiate Treg cell activation in the targeted tissue, we redirected Tregs by a CAR which recognizes carcinoembryonic antigen (CEA), a glycoprotein present on the surface of adenoepithelia in the lung and gastrointestinal tract. We demonstrate that CAR Tregs accumulate and are activated in the inflamed lung of asthmatic CEA transgenic (CEAtg) mice where they control key symptoms of allergic inflammation more efficiently than non-modified Tregs. The results imply a Treg cell-based strategy for the treatment of patients with severe allergic asthma.

MATERIALS AND METHODS

Mice

Carcinoembryonic antigen transgenic C57BL/6 mice were obtained from the Patterson Institute, Manchester, UK. The CEAtg mouse colony was bred by back-crossing with a colony of C57BL/6N mice (Charles River, Wilmington, MA, USA). Offspring mice were genotyped using the primer oligonucleotides 5′-CTGCAGCTGTCCAATGGC-3′ and 5′-CCTGGGACTGACC GGGAG-3′. C57BL/6 wild-type (wt) mice were used as controls. CEA-specific CAR transgenic (CEA-CARtg) C57BL/6 mice were generated by the laboratory of Prof. Abken (unpublished data). Briefly, embryonic stem cells were transfected with the Cre/loxP rosa26 vector containing a CD4 promoter-driven expression cassette coding for the fully murine SCA431scFv-mIgG-CD4(tm)-CD28–CD3ζ CAR. Blood T cells express the CEA-specific CAR on the cell surface. All experimental protocols were approved by the local animal welfare committee Agency for Nature, Environment and Consumer Protection of the State North Rhine-Westphalia (LANUV) and performed according to their guidelines.

Cell Isolation and Flow Cytometry Analysis

Single cell suspensions from spleens and tracheobronchial lymph nodes (LNs) were obtained by meshing organs through a 70 µm cell strainer (BD, Franklin Lakes, NJ, USA), followed by lysis of erythrocytes and filtering through 30 µm cell strainer (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were stained with the anti-mouse monoclonal antibodies (mAb) CD4-FITC/clone GK1.5 (Southern Biotech, Birmingham, AL, USA), CD25-PerCP-Cy5.5/clone PC61 (BD), PE-conjugated anti-mouse CD25 mAb clone 7D4 (Miltenyi), FITC-labeled anti-mouse CD4 mAb clone GK1.5 (Southern Biotec), AF488-labeled anti-FoxP3/clone MF-14 (BioLegend, San Diego, CA, USA), RPE-conjugated anti-IgG_1 that binds to the CAR extracellular Fc domain (Southern Biotech), anti-latency-associated peptide (LAP)/clone TW7-16B4 (BioLegend), and "FoxP3 Staining Buffer Set" (Miltenyi Biotec). Data were acquired on FACS Canto II flow cytometer (BD) and analyzed using FlowJo software (FlowJo, LCC, Ashland, OR, USA).

In Vitro Assays

T effector cells (Teffs) and Tregs were isolated from the murine spleens using the "Pan T Cell Isolation Kit II" and the "$CD4^+CD25^+$ Regulatory T Cell Isolation Kit" (Miltenyi Biotec), respectively, or using the "autoMACS" (Miltenyi Biotec). CAR Teffs were stained with PKH26 (Sigma-Aldrich, St. Louis, MO, USA) and stimulated for proliferation either by the immobilized agonistic mAb anti-CD3/clone 145-2C11 and anti-CD28/clone 37.51, or the anti-idiotypic CAR-activating mAb BW2064/36 directed against the IgG_1 spacer domain of the CAR (5 µg/ml coating concentration each). Teffs (10^5 cells/well) were incubated with or without CAR Tregs (5×10^4 cells/well) for 48 h. Intensity of PKH26 dye was recorded by flow cytometry using FACS Canto II and the proliferation rate of Teffs was calculated. For the LAP expression analysis, Treg cells were stimulated by incubation on plates coated with the anti-CD3 and anti-CD28 mAb, whereas anti-CEA CAR Tregs were incubated on plates coated with the BW2064/36 (anti-CAR mAb) for 48 h (5×10^4 cells/well). Mock-coated plates (w/o) were used for control. LAP on the surface was detected using LAP-specific mAb using FACS Canto II. For IL-10 expression assay, CAR Tregs were cultured in triplicates in microtiter plates (PolySorp; Nalge Nunc, Rochester, NY, USA) precoated with the BW2064/36 mAb or an IgG_1 control mAb (Southern Biotech). After 72 h, IL-10 was measured in culture supernatants with the "mTh1/Th2/Th9/Th17/Th22/Treg Cytokine Panel 17-plex" (eBioscience/Thermo Fisher Scientific, Waltham, USA).

In Vivo Cell Tracking with Bioluminescence Imaging

CAR Tregs and non-modified (wt) Tregs were retrovirally transduced to express Gaussia Luciferase (GLuc) and intravenously (i.v.) injected to ovalbumin (OVA)-sensitized CEAtg mice (24). 36 h later, Gluc-labeled cells were visualized by intraperitoneal (i.p.) injection of 100 µg benzyl-coelenterazine (PJK GmbH, Kleinblittersdorf, Germany). Lungs, spleen, stomach, and kidney were isolated and in the Petri dish recorded with 300 s exposure time using the Photon Imager (Biospace Lab, Nesles-la-Vallée, France). The threshold of bioluminescence signals was automatically determined using the Photo Vision software (Biospace Lab) and filtered against the background noise. Regions of interest were defined as regions above threshold and automatically gated by pre-defined program tools.

Induction of Allergic Airway Inflammation and Adoptive Transfer of Tregs

Ovalbumin (Grade V, Sigma-Aldrich) was used as model allergen (25). Lipopolysaccharide was removed from OVA by the Detoxi-Gel endotoxin removing gel and columns (Thermo Fisher Scientific). Clearance from endotoxin was confirmed by the Limulus amebocyte lysate test (Lonza, Basel, Switzerland). CEAtg mice were sensitized with two i.p. applications of 20 µg OVA in saline solution, adsorbed to 2 mg of aluminum hydroxide and magnesium hydroxide solution (alum; Inject alum adjuvant, Thermo Fisher Scientific), followed by intranasal (i.n.) exposures with 20 µg OVA at four consecutive days. Control mice were likewise treated with solutions without OVA. CAR Tregs for adoptive transfer were isolated from the spleens of the CEA-CARtg mouse or the C57BL/6 wt mouse using the "$CD4^+CD25^+$ Regulatory T cell Isolation Kit" (Miltenyi Biotec). Seven days after the first sensitization with allergen, CAR Tregs or wt Tregs were i.v. injected (1×10^6 cells per mouse). One day after the last i.n. challenge, mice were sacrificed by asphyxiation with isoflurane (Baxter, Deerfield, IL, USA).

Measurement of AHR

Airway hyper-reactivity was defined as an increase in dynamic lung resistance (R) in response to β-methacholine (MeCh; Sigma-Aldrich). One day after the last challenge with allergen, mice were deeply anesthetized with ketamine (Albrecht GmbH, Aulendorf, Germany) and xylazine (Rompun, Bayer Vital GmbH, Leverkusen, Germany), intubated, and mechanically ventilated *via* the flexiVent system (Scireq, Montreal, QC, Canada) using a tidal volume of 12 ml/kg at a frequency of 150 breaths/min. For baseline measurements mice were exposed to 0.9% (w/v) NaCl, aerosolized in the Aeroneb nebulizer (Inspiration Medical, Bochum, Germany). Subsequently, mice were provoked with increasing concentrations of MeCh (10, 20, and 30 mg/ml). After deep inflation of the lungs, single-frequency forced oscillations were performed; four peak values of R were analyzed for each MeCh concentration. AHR data were calculated as the change from baseline response.

Histological Stainings

Cryostat sections were stained with the biotin-conjugated anti-CEA mAb clone CB30 (Ancell, Bayport, MN, USA), streptavidin-horseradish peroxidase (BioLegend), and DAB chromogen substrate (Biozol, Eching, Germany). Sections were additionally stained with hematoxylin–eosin (H&E; Carl Roth, Karlsruhe, Germany) and analyzed using the Axiovert 400 M laser scan microscope (Carl Zeiss, Oberkochen, Germany). The left lung was fixed in 4% (w/v) formalin and embedded in paraffin. Tissue slices were stained with H&E (Merck, Darmstadt, Germany) or periodic acid-Schiff reagent (PAS; Sigma-Aldrich). Whole lung sections were scanned using the Keyence microscope BZ-9000 (Keyence, Osaka, Japan) at 100× magnification. Sum of H&E- or PAS-positive signal (pixels) per lung slice was quantified using in-house developed imaging software (25). For the immuno-histological detection of Tregs, lung and spleen cells were embedded in "Tissue-Tek O.C.T. Compound" (Sakura Finetek Europe B.V., Alphen aan den Rijn, Netherlands) and 5-µm cryostat sections were fixed in ice-cold acetone. Sections were stained for CD4 and FoxP3 expression with the fluorochrome-conjugated antibodies specific for mouse CD4-AF594/clone GKL1.5, FoxP3-AF488/clone 150D (BioLegend), and DAPI (4,6-diamidino-2-phenylindole dihydrochloride) for nuclear counterstain. For the detection of the CAR expression on the surface of Tregs, sections were incubated with the hybridoma-derived anti-idiotypic antibody BW2064/36 previously biotinylated by the Sulfo-NHS-LC-Biotin kit (Thermo Fisher Scientific). Following incubation with the primary antibody, antibody binding was visualized using AF-647 labeled streptavidin (BioLegend). Magnification was set to 60×. Slides were analyzed using the Olympus FV 1000 microscope (Olympus corporation, Tokio, Japan).

Analysis of Bronchoalveolar Lavage Fluid (BALF)

To obtain BALF, the right lung was flushed three times with 0.4 ml 2 mM EDTA in PBS. The total cell number was determined using the Cedex HiRes automated cell analyzer (Roche, Basel, Switzerland). To determine the cell types, cytospin slides were made with the CytoSpin 4 Cytocentrifuge (Thermo Fisher Scientific) and stained with May-Grünwald/Giemsa (Merck). Cell types were determined using standard microscopic criteria and counted in a blinded manner at 1000× magnification (Axiovert 40 CFL, Zeiss, Oberkochen, Germany).

Quantification of OVA-Specific Immunoglobulins (Ig) and Cytokines

Peripheral blood was clotted at room temperature for 20 min and the serum was collected. OVA-specific IgE, IgG_1, and IgG_{2a} were recorded by ELISA. Serum samples were incubated overnight in microtiter plates (Nalge Nunc) that were previously coated with 10 µg/ml OVA in bicarbonate buffer (pH 9.6). Specific binding was detected with respective goat anti-mouse horseradish peroxidase-conjugated anti-IgE antibodies (Southern Biotech), anti-IgG_1, or anti-IgG_{2a} antibodies (Bethyl Laboratories, Inc., Montgomery, TX, USA) and 3,3′,5,5′-tetramethylbenzidine

as a colorimetric substrate. Optical density was determined at 450/630 nm using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). OVA-specific IgE concentration in samples was determined using a standard calibration curve (AbD Serotec, Kidlington, UK). The titers of OVA-specific IgG_1 and IgG_{2a} were calculated by logarithmic regression as the reciprocal dilution of the sera. Cytokines in sera or cell culture supernatants were measured using the bead-based "Mouse 17-plex Bio-Plex multiplex system" and the Luminex xMAP device (Bio-Rad) or FlowCytomix (eBioscience/Thermo Fisher Scientific) and FACS Canto II.

Gene Expression Analysis

The presence of CAR Tregs in tissues after adoptive transfer was additionally analyzed by RT-PCR. RNA was isolated from 4 to 5 million cells with "RNeasy Mini Kit" and "RNase-Free DNase Set" (Qiagen, Venlo, Netherlands). CAR cells were detected using OneStep RT-PCR Kit (Qiagen) and CAR specific primer oligonucleotides (5′-AAACAAACTGGAATGGATGGGCTACA-3′ and 5′-AACGTGGGATAACTACTCCACTGAT-3′).

Statistical Analysis

Unpaired two-tailed Student's *t*-test, one-way, or two-way ANOVA test with Bonferroni's multiple comparison post-test (95% confidence interval) was performed using GraphPad Prism (San Diego, CA, USA). All data represent the mean ± SEM. Differences were considered statistically significant at $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

RESULTS

CAR Tregs Suppress the Proliferation of Teff Cells

The anti-CEA CAR was composed of the anti-CEA single chain variable fragment (scFv) for binding, the IgG_1 hinge-CH2CH3 as an extracellular spacer, the CD4 trans-membrane domain, and the intracellular CD28 and CD3ζ signaling domains for T cell activation upon binding to CEA (**Figure 1A**). $CD4^+CD25^+$ Tregs and $CD4^+CD25^-$ Teffs with anti-CEA CAR were isolated from the spleens of the anti-CEA CARtg mice. CAR Tregs stained positive for CD4, CD25, high level FoxP3 (**Figure 1B**), and the engineered CAR (not shown). To demonstrate the suppressive activity of $CD4^+CD25^+$ Tregs, cells were co-cultured with PKH26-labeled $CD3^+CD25^{low}$ CAR Teffs. CAR Tregs effectively repressed the proliferation of $CD3^+CD25^{low}$ CAR Teffs when stimulated through TCR/CD28 by the agonistic anti-CD3 and anti-CD28 mAb or through the CAR by the CAR-binding antibody BW2064/26 (**Figure 1C**). We conclude that the $CD4^+CD25^+$ CAR Treg cells constitute functionally active Tregs capable to suppress CAR Teff amplification. Treg cells, stimulated through the CAR, suppressed Teff cell proliferation more efficiently than after stimulation through CD3/CD28. This is likely due to differences in the CAR versus CD3/TCR-mediated T cell activation, i.e., the CD28ζ CAR provided much stronger CD28/CD3ζ signals than CD3/CD28 stimulation. Since the activation of Treg cells depends on the strength of the CD28 stimulation (19), stimulation through the CD28–CD3ζ CAR is likely superior to

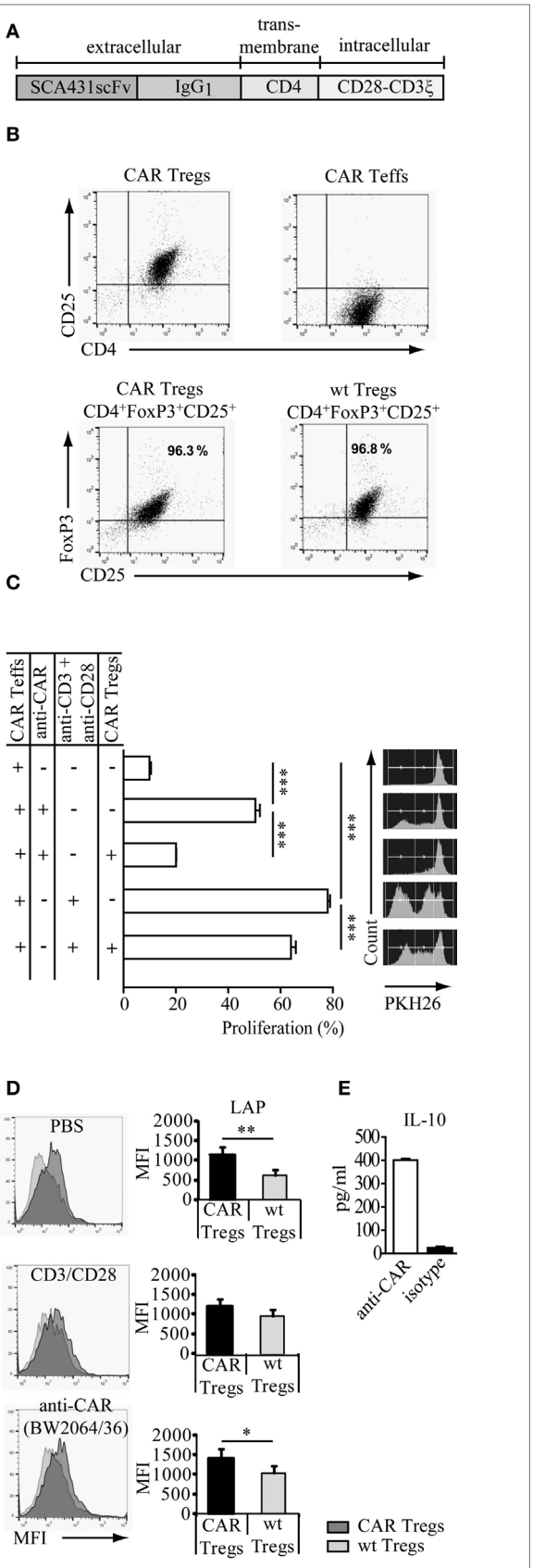

FIGURE 1 | Continued

FIGURE 1 | Continued
Regulatory T cells (Tregs) engineered with a carcinoembryonic antigen (CEA)-specific chimeric antigen receptor (CAR) specifically redirect their suppressive capacity. **(A)** Modular composition of the CEA-specific CAR with the anti-CEA scFv binding domain and the combined CD28–CD3ζ signaling domains. **(B)** $CD4^+CD25^+$ Tregs and $CD4^+CD25^-$ T effector cells (Teffs) with anti-CEA CAR were isolated from the spleens of the anti-CEA CAR transgenic mice. Tregs isolated from CEA transgenic mice or wild-type (wt) C57BL/6 mice stained positive for CD4, CD25, and FoxP3. **(C)** CAR Tregs suppressed the amplification of PKH26-labeled CAR Teffs that were stimulated by the agonistic anti-CD3/anti-CD28 antibodies directed against the TCR/CD28 or by the BW2064/26 antibody (anti-CAR) directed against the CAR. Samples were measured in triplicates. Statistical analyses were performed by the one-way ANOVA test. $^{***}p < 0.001$. **(D)** Latency-associated peptide (LAP) (TGF-β1) is increased on the surface of CAR engineered $CD4^+CD25^+FoxP3^+$ Treg cells upon specific stimulation. The assay was performed in triplicates. Statistical analyses were performed by the Student's *t*-test ($^*p < 0.05$, $^{**}p < 0.01$). **(E)** CAR Tregs were stimulated by the BW2064/36 monoclonal antibodies (mAb) (anti-CAR) as surrogate antigen or by an IgG_1 isotype matched control mAb. IL-10 released into the supernatant was recorded by a bead-based immunoassay. Data are representative for two independent experiments.

CD3/TCR stimulation. In accordance to our conclusion, CAR Treg cells showed higher activation levels than non-modified, wt Treg cells indicated by higher levels of LAP (TGF-β1) expression, as revealed by flow cytometry (**Figure 1D**). Furthermore, IL-10 production by CAR Tregs increased upon CAR stimulation through a specific antibody compared with the incubation with an isotype antibody of irrelevant specificity as control (**Figure 1E**). We concluded that antigen engagement by the CAR improved Treg cell activation in a specific fashion.

Anti-CEA CAR Tregs Home to the Lung of CEAtg Mice

We used the immunocompetent CEAtg mice as recipients to evaluate in a clinically relevant model the immune-modulating effect of adoptively transferred CEA-specific CAR Tregs during induced allergic airway inflammation. The CEAtg mice express CEA under the control of the human CEA promoter on the luminal surface of the pulmonary (**Figure 2A**) and the gastrointestinal tract epithelia, closely mimicking the human situation. These mice were pre-sensitized by exposure to OVA; subsequently, one dose of non-preactivated, Gluc-labeled anti-CEA CAR Tregs or non-modified Tregs were applied by i.v. injection. CAR Tregs accumulated in the CEA^+ lung, spleen, and stomach of OVA-treated mice 36 h after i.v. application while wt Tregs without CAR did far less (**Figure 2B**). Accumulation in CEA^+ organs is specific since CAR Tregs did not substantially infiltrate the CEA^- kidneys.

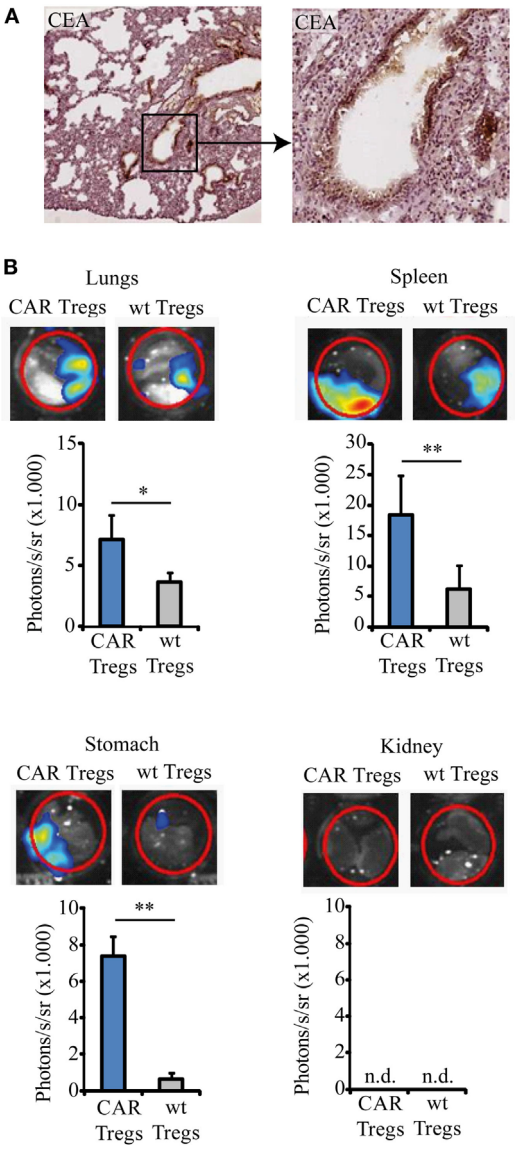

FIGURE 2 | Chimeric antigen receptor (CAR) regulatory T cells (Tregs) home to the lung and spleen. **(A)** Lung slices from the carcinoembryonic antigen transgenic (CEAtg) mouse were stained for CEA (microscope magnifications 5× and 20×, respectively). CEA is expressed in a polarized fashion on the luminal site by the alveolar epithelia. **(B)** Gaussia Luciferase (Gluc)-labeled CAR Tregs and Gluc-labeled non-modified wild-type (wt) Tregs were recorded by bioluminescent imaging in the lungs, spleen, stomach, and kidney in the same mouse 36 h after Treg transfer to the ovalbumin-sensitized mice. One representative mouse out of three from each group is shown. Statistical analyses were performed using the Student's *t*-test. $^*p < 0.05$, $^{**}p < 0.01$; n.d., not detectable.

CAR Tregs Prevent AHR, Eosinophilic Airway Inflammation, Mucus Production, and Th2 Cytokine Production in Mice with Experimental Asthma

We investigated whether adoptive transfer of CAR-redirected Tregs can suppress the clinical symptoms of experimental asthma in the mouse model. By systemic sensitization and local challenges with the model allergen OVA (**Figure 3A**), we induced the typical key features of asthma, such as AHR, eosinophilic airway inflammation, increased Th2 cytokine production, and elevated serum IgE levels. Control animals, inoculated with alum or NaCl solution without OVA, did not show these symptoms.

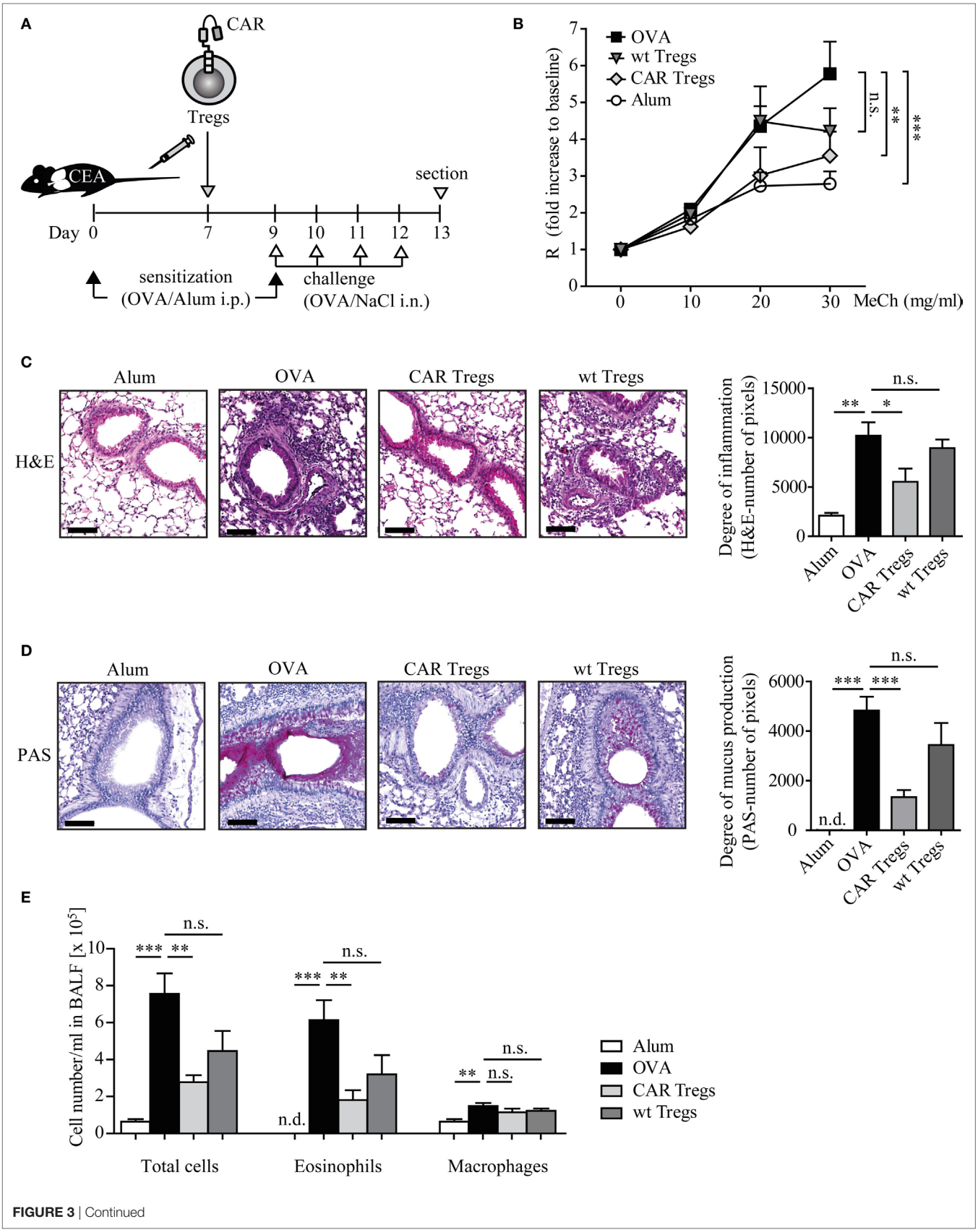

FIGURE 3 | Continued

FIGURE 3 | Continued
Adoptive transfer of chimeric antigen receptor (CAR) regulatory T cells (Tregs) reduces airway hyper-reactivity, inflammation, mucus production, and eosinophilia in mice with induced experimental asthma. **(A)** Schematic outline of the experimental protocol. **(B)** Lung resistance (R) after exposure to increasing concentrations of methacholine (MeCh) was recorded as described in Section "Materials and Methods." Data from $n = 9$–11 mice from three independent experiments are shown. Statistical analyses were performed with the two-way ANOVA test. **(C,D)** Stained whole lung sections were quantified for **(C)** hematoxylin–eosin (H&E) or **(D)** PAS signals (number of pixels), $n = 4$–6 (H&E) and $n = 6$–9 (PAS) each from 3 independent experiments; scale bar = 100 μm. **(E)** Absolute numbers of cells in the bronchoalveolar lavage fluid (BALF); $n = 5$–7 in one of four independent experiments. Alum, mice treated with alum adjuvant only; ovalbumin (OVA), mice treated with OVA in alum adjuvant; CAR Tregs, mice treated with OVA in alum adjuvant and subsequent one dose of CAR Treg cells; wild-type (wt) Tregs, mice treated with OVA in alum adjuvant and subsequent one dose of unmodified wt Treg cells. Statistical analyses were performed by the one-way ANOVA test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; n.s., not significant, n.d., not detectable.

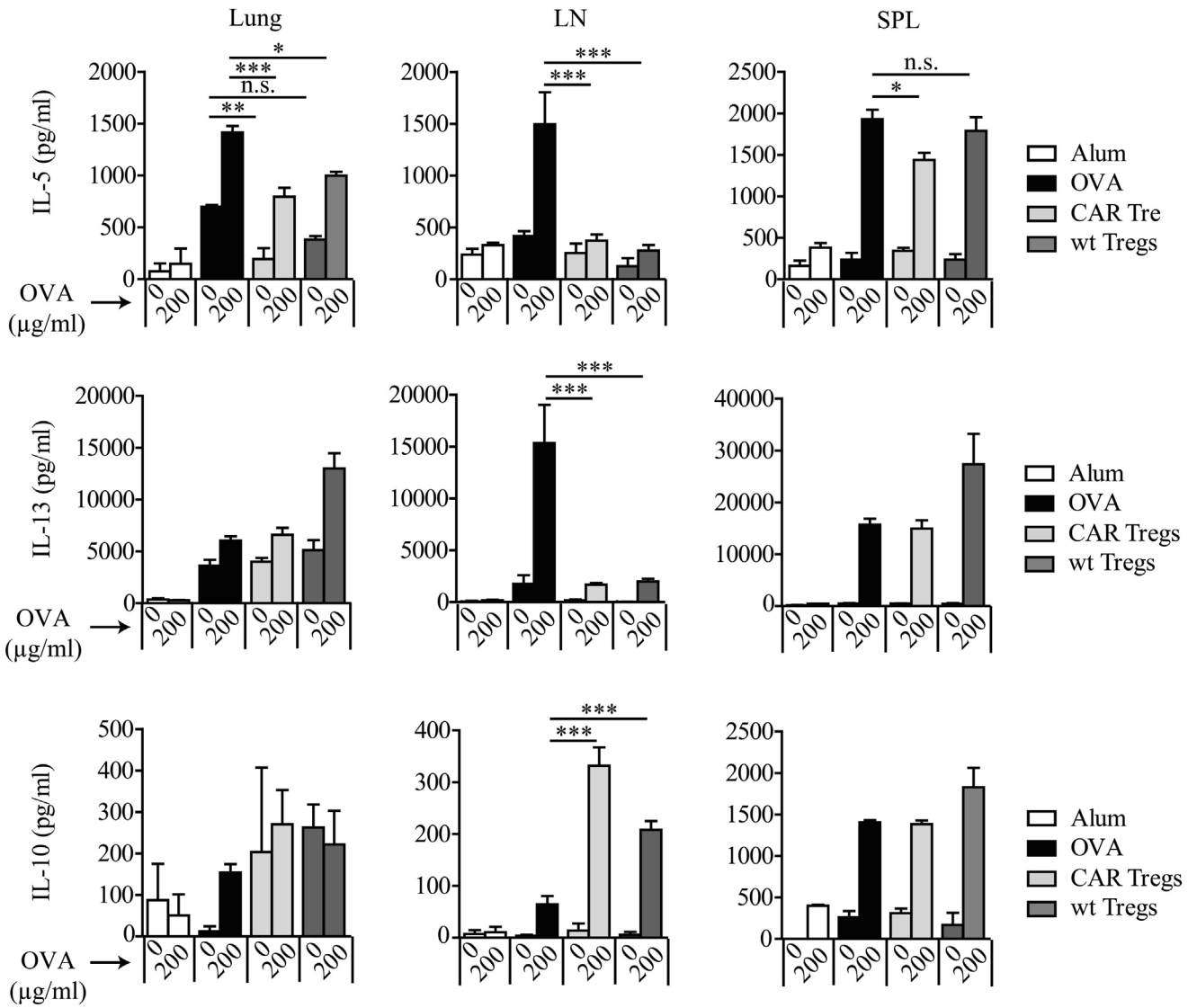

FIGURE 4 | Chimeric antigen receptor (CAR) regulatory T cells (Tregs) reduce antigen-specific T helper-2 cytokine production by lung cells and splenocytes. IL-5, IL-13, and IL-10 levels were measured by bead-based assay in lung, spleen (SPL), and tracheobronchial lymph node (LN) cell culture supernatants after *in vitro* restimulation with 200 μg/ml ovalbumin (OVA). Data represent three independent experiments including pooled samples from more than 6 mice per group. Statistical analyses were performed by the one-way ANOVA test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Adoptive transfer of CAR Tregs by one dose of i.v. injection to OVA-challenged CEAtg mice almost completely prevented the development of MeCh-induced AHR, whereas unmodified Tregs were less efficient as determined by invasive lung function measurements (**Figure 3B**). Histological examination of the lung sections revealed a substantial reduction of infiltrating inflammatory immune cells in the CAR Treg-treated mice compared with the non-treated asthmatic mice (**Figure 3C**). Treatment with Tregs without CAR did not substantially reduce airway inflammation demonstrating the superior therapeutic efficacy of redirecting Tregs by a CAR toward the affected organ.

Likewise, mucus production, one of the major hallmarks of asthma, was significantly suppressed upon CAR Treg cell therapy as revealed by PAS staining of the lung tissue (**Figure 3D**). In this respect, Tregs without CAR showed no substantial therapeutic effect.

Adoptive transfer of CAR Tregs reduced the number of eosinophils in the BALF by about threefold as compared with mice without Treg treatment while the number of macrophages was not altered (**Figure 3E**). Mice which received unmodified Tregs showed a less pronounced decrease in the number of total BALF cells and particularly of eosinophils.

Allergic asthma is characterized by a Th2-dominated immune response. To determine whether adoptive cell therapy with CAR Tregs modulates Th2 cytokine levels in OVA-challenged mice, we analyzed IL-5, IL-13, and IL-10 production, indicating progression of the disease. The superior suppression of asthma-like phenotype by CAR Treg treatment in relation to wt Treg injection was again demonstrated by significant reduction of antigen-specific IL-5 levels in cell culture supernatants of lung cells and splenocytes after *in vitro* restimulation with OVA (**Figure 4**).

Adoptive Transfer of CAR Tregs Prevents the Allergen-Induced Increase of IL-5 and IgE

We further examined IL-5 levels in mouse sera. CAR Treg-treated mice displayed significantly reduced IL-5 levels compared with untreated mice or mice treated with unmodified Tregs (**Figure 5A**). The effect was most prominent as early as 3 days after transfer of CAR Tregs, i.e., at day after second OVA inoculation.

OVA induced experimental asthma was accompanied by an increase of OVA-specific Ig levels in serum (**Figure 5B**). Adoptive CAR Treg transfer reduced the OVA-specific IgE levels, while non-modified Tregs did not. Essentially the same results were observed for IgG_{2a}, while IgG_1 levels were similarly reduced by Tregs with and without CAR (**Figure 5B**).

The pronounced effect of CAR Tregs compared with non-modified Tregs was due to CAR-mediated Treg cell activation. Transfer of CAR Tregs to OVA-treated wt C57BL/6 mice without CEA expression expectedly showed reduction in cell numbers in BALF compared to non-treated mice. However, the CAR Tregs were equally potent as the non-modified Tregs due to the lack of CAR-mediated Treg activation (**Figure 5C**). Accordingly, the levels of OVA-specific IgE were similarly reduced after Treg treatment with or without CAR (**Figure 5D**). We conclude that the suppressor activity of adoptively transferred Treg cells was substantially improved by anti-CEA CAR signaling through engagement of endogenous CEA.

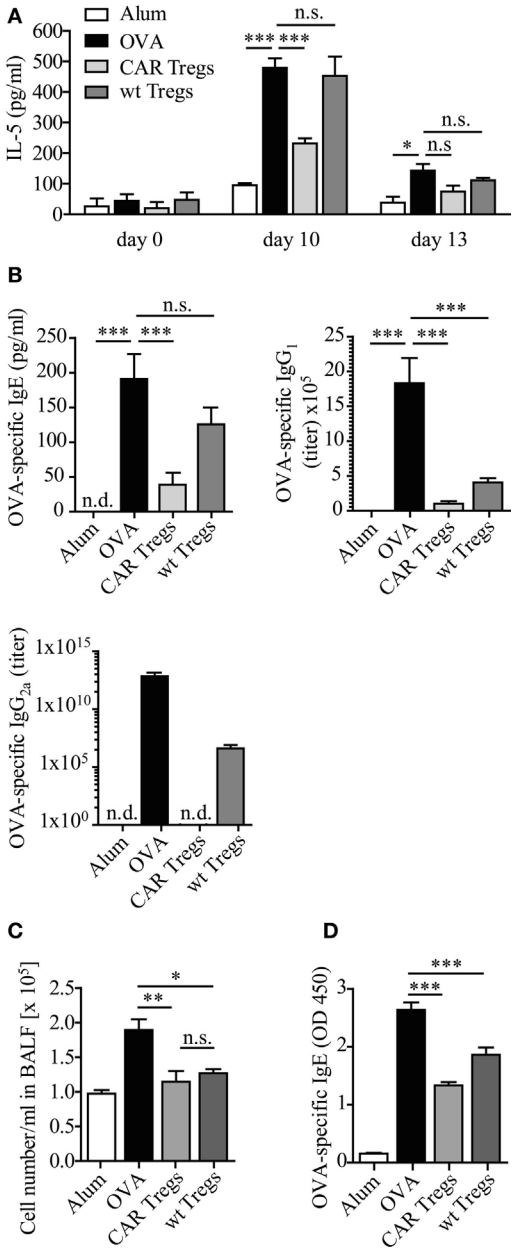

FIGURE 5 | IL-5 and allergen-specific IgE levels are diminished upon chimeric antigen receptor (CAR) regulatory T cell (Treg) treatment of carcinoembryonic antigen transgenic (CEAtg) mice. **(A)** IL-5 levels in sera from CEAtg mice were determined at different times of the experimental protocol (**Figure 3A**), $n = 3$ mice per group per time point; statistical analysis was performed using the two-way ANOVA test. **(B)** The amount of ovalbumin (OVA)-specific IgE ($n = 6$–8 mice), IgG_1 ($n = 6$–9), and IgG_{2a} ($n = 6$–17) in sera of CEAtg mice. Data are pooled from two to three independent experiments. **(C)** Total number of bronchoalveolar lavage fluid (BALF) cells in wild-type (wt) C57BL/6 mice without CEA expression, treated as described in Section "Materials and Methods." **(D)** OVA-specific IgE at day 12 in sera of C57BL/6 mice was recorded by ELISA. Statistical analysis was performed by the one-way ANOVA test, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

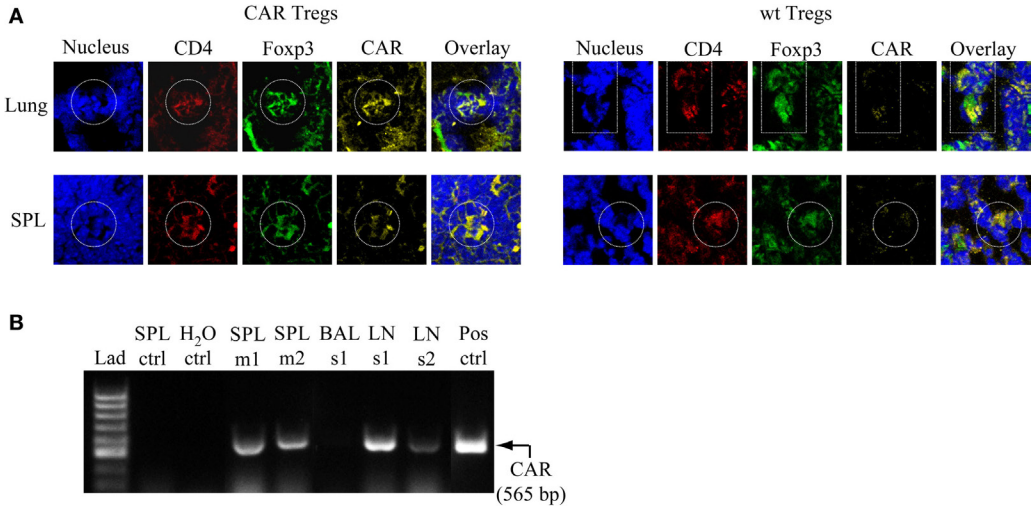

FIGURE 6 | Chimeric antigen receptor (CAR) regulatory T cells (Tregs) accumulate in the lung, spleen (SPL), and tracheobronchial lymph nodes (LNs) after challenge with antigen. **(A)** Lung and SPL cells were isolated at day 13 from carcinoembryonic antigen transgenic mice treated with CAR Tregs or non-modified [wild-type (wt)] Tregs and fluorescently stained for nuclei (DAPI), and CD4, FoxP3, and CAR expression. One representative mouse out of three per group is shown. **(B)** Detection of CAR Tregs in the SPL and tracheobronchial LNs by RT-PCR at the end of experimental protocol (day 13). The specific RT-PCR CAR fragment is 565 bp in size. As expected, tissues from mice which received non-modified Tregs (SPL ctrl) did not show a CAR-derived signal. Bronchoalveolar lavage fluid (BALF) did not contain detectable CAR Treg cells. Lad, ladder of DNA fragments of different sizes; H_2O ctrl, RT-PCR in the absence of RNA; Pos ctrl, RT-PCR with RNA from purified CAR T cells; m, mouse; s, sample consisting of cells that are pooled from five to six mice.

CAR Tregs Accumulate in the Lung, Spleen, and Regional LNs after Antigen Challenge

To examine the presence of CAR Tregs in CEAtg mice after the last antigen challenge at day 12, lung and spleen cells were fluorescently stained with specific anti-CD4, anti-CAR, and anti-FoxP3 antibodies. At this time point, $CD4^+CAR^+FoxP3^+$ cells were still present in the lung and spleen of the CAR Treg treated animals, whereas tissues from mice treated with non-modified Tregs did not stain positive for the CAR signal, as expected (**Figure 6A**). RT-PCR analysis confirmed the immunostaining data and furthermore revealed that the CAR Tregs also accumulated in the tracheobronchial LNs (**Figure 6B**).

DISCUSSION

We show in an established mouse model that CAR-redirected Tregs effectively suppress the pathophysiological hallmarks of allergic airway inflammation. Targeted $CD4^+CD25^+$ Tregs significantly reduced AHR, airway eosinophilia, mucus hypersecretion, Th2 cytokine production, and allergen-specific IgE after sensitization with a model allergen. CAR Tregs efficiently reduced proliferation of CAR Teffs *in vitro* after stimulation by their TCR or by the CAR through binding to cognate antigen. Concomitantly, LAP (TGF-1β) and IL-10 expression was increased after CAR activation. The CAR amplifies Treg cell function by providing strong activation through CD28–CD3ζ CAR signaling and by trapping the cells in the target tissue through binding to cognate antigen (20). At least two processes are taking place to improve CAR Treg function during chronic inflammation. (i) Airway inflammation results in some disruption of the lung epithelial layer with the consequence that those epithelial cells lose the luminal CEA expression and expose CEA also to the stromal site; by contrast, intact lung epithelia express CEA exclusively to the luminal site. Consequently, CAR Tregs in the inflamed lung are more strongly activated by CEA^+ lung epithelia with de-polarized CEA expression while Tregs without CAR do not receive such activation signals. (ii) Tregs with anti-CEA CAR without engagement of cognate antigen are preactivated on a higher level, as indicated by increased LAP expression compared with Tregs without CAR (**Figure 1D**). This is likely due to the "tonus" of the anti-CEA CAR providing antigen-independent Treg cell activation. However, CEA engagement of cognate antigen further improved Treg cell activation, as indicated by a further increase in LAP. In contrast to CAR-redirected Tregs, the same number of unmodified Tregs did not show the striking therapeutic effect potentially due to the lack of activation. Once activated, Tregs suppress excessive immune responses in an antigen-independent fashion and can promote expansion of other Treg cells with different antigen specificities (26, 27). Our results are in line with the observation that preactivated Tregs, but less non-activated Tregs, suppress the airway inflammation in allergen-sensitized mice (28). The establishment of a protective Treg cell effect requires continuous antigen stimulation (29, 30). This is provided by the CEA^+ lung epithelia in the CEAtg mouse model which is also expected to be the case in the human situation. In mice lacking transgenic CEA as source of continuous antigen stimulation, CAR Tregs were similarly efficient as non-modified Tregs in the suppression of inflammation and reduction of OVA-specific IgE levels (**Figures 5C,D**). Antigen-dependent Treg activation as trigger

for the suppression of chronic inflammation was also reported for other disease models, such as multiple sclerosis (16), colitis (18), autoimmune diabetes (17), and gastritis (31).

The higher therapeutic efficacy of antigen-specific Tregs implies that a lower cell number is sufficient for the therapeutic effect than using polyclonal Tregs with diverse specificities. As previously shown, transfer of 5×10^6 polyclonal, *in vitro* expanded $CD4^+CD25^+$ Tregs successfully suppressed airway inflammation in an allergic asthma model (14), whereas a 10-fold lower number of allergen-specific Treg cells were needed for the similar effect (13). In our study, one dose of 10^6 CAR Tregs significantly ameliorated experimental asthma, and even lower cell numbers may be equally efficient.

Accumulation of Tregs at the site of inflammation is essential for their immunosuppressive function (32). In our model, redirecting of Tregs by an antigen-specific CAR sustained their recruitment to the targeted tissue. Accordingly, anti-CEA CAR Tregs accumulated in the CEA^+ lung and spleen at higher levels than unmodified Tregs as revealed by bioluminescence monitoring. CAR Tregs, moreover, accumulated in the tracheobronchial LNs, lung, and spleen, even after repetitive challenge with antigen. Rapid and efficient trafficking to these tissues likely enables Tregs to modulate the ongoing chronic inflammation in the affected tissues.

T helper-2 cell-associated asthma arises from a complex interplay between innate and adaptive immune cells (2) and it is mediated by cytokines that induce AHR, recruitment of eosinophils and mast cells to the airways, and the production of allergen-specific IgE (33). Here, we show that CAR Tregs suppressed the production of IL-5 which is predominantly produced by Teffs (34) and which drives pulmonary eosinophilia and AHR (35). Accordingly, airway inflammation, eosinophilia, and AHR were suppressed upon the application of CAR Tregs. Suppression by Tregs in the allergic asthma model is likely mediated by IL-10 released by activated Tregs in high levels (13); other mechanisms may likewise apply such as induced cell death of responder T cells (36) and inhibition of T cell proliferation by upregulating cyclic adenosine monophosphate (37) or by CD39- and CD73-dependent peri-cellular generation of adenosine (38). The suppression of airway inflammation by Tregs may be due to a combination of these and other suppressive mechanisms (27, 39).

While the data indicate that genetically redirected Tregs are of therapeutic benefit in the treatment of severe asthma, the choice of the targeted antigen to redirect and activate Treg cells remains crucial. One option to redirect Tregs may be targeting of the specific allergen by the engineered CAR which, however, is frequently not known. Alternatively, CARs may be used to activate Tregs at the site of inflammation independently of the specific allergen in order to redirect their suppressive activity to the diseased lesion.

Cell therapy with Tregs has lately been tested in the treatment of refractory autoimmune diseases and high-risk allograft recipients (40). In this context, the stability of the Treg cell suppressive phenotype is a major concern. In particular, in the inflammatory environment Tregs can convert into Th17 cells (41) which could furthermore exacerbate the asthma symptoms (42). Several options were proposed to improve survival and function, including co-administration of IL-2 (43), all-trans retinoic acid (44), rapamycin (45), or ectopic FoxP3 expression in Tregs (46).

In our experimental model, adoptively transferred CAR Tregs preferentially accumulate in the lung, tracheobronchial LNs, and spleen where they efficiently suppress airway inflammation and other hallmarks of allergic asthma. Our results encourage further studies to explore adoptive cell therapy with genetically redirected Tregs as an efficacious treatment option for patients suffering from severe asthma.

ETHICS STATEMENT

All experimental protocols were approved by the local animal welfare committee Agency for Nature, Environment and Consumer Protection of the State North Rhine-Westphalia (LANUV) and performed according to their guidelines.

AUTHOR CONTRIBUTIONS

JS, MC, HA, and GH designed the work. JS, MC, CH, AH, and MB acquired and analyzed experimental data. JS, MC, CH, AH, HA, and GH interpreted data. JS, MC, HA, and GH drafted the manuscript. All authors critically revised the work. JS, MC, HA, and GH contributed equally to this work.

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REVIEW
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Human Tregs Made Antigen Specific by Gene Modification: The Power to Treat Autoimmunity and Antidrug Antibodies with Precision

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Human regulatory CD4+ T cells (Tregs) are potent immunosuppressive lymphocytes responsible for immune tolerance and homeostasis. Since the seminal reports identifying Tregs, vast research has been channeled into understanding their genesis, signature molecular markers, mechanisms of suppression, and role in disease. This research has opened the doors for Tregs as a potential therapeutic for diseases and disorders such as multiple sclerosis, type I diabetes, transplantation, and immune responses to protein therapeutics, like factor VIII. Seminal clinical trials have used polyclonal Tregs, but the frequency of antigen-specific Tregs among polyclonal populations is low, and polyclonal Tregs may risk non-specific immunosuppression. Antigen-specific Treg therapy, which uses genetically modified Tregs expressing receptors specific for target antigens, greatly mitigates this risk. Building on the principles of T-cell receptor cloning, chimeric antigen receptors (CARs), and a novel CAR derivative, called B-cell antibody receptors, our lab has developed different types of antigen-specific Tregs. This review discusses the current research and optimization of gene-modified antigen-specific human Tregs in our lab in several disease models. The preparations and considerations for clinical use of such Tregs also are discussed.

Keywords: human regulatory CD4+ T cells, Tregs, hemophilia A, antigen-specific Tregs, experimental autoimmune encephalomyelitis, chimeric antigen receptor, B cell antibody receptors

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INTRODUCTION

Human regulatory CD4+ T cells (Tregs) are a subset of adaptive lymphocytes well characterized for their immunosuppressive functions and maintenance of immunological tolerance. Tregs are broadly grouped into two categories, either natural (i.e., thymus derived) or induced (i.e., peripherally derived). Natural Tregs (nTregs) represent between 2 and 8% of CD4+ T cells in healthy donor peripheral blood, whereas induced Tregs can be generated by expansion of CD4+ T cells in the presence of TGFβ. The importance of Tregs in immune regulation and brokering tolerance has been robustly demonstrated (1–9), and expanded polyclonal Tregs are being developed for clinical applications. In this review, however, we summarize studies in our lab designed to generate antigen-specific nTregs by transduction of specific receptors.

Engineering antigen-specific T cells by gene modification has proven to be an invaluable immunological technology (10). In addition to exogenous T-cell receptors (TCRs), chimeric antigen

receptors (CARs) containing single chain variable fragments (scFv) are also used to redirect polyclonal T cells to a defined specificity. We have also engineered Tregs to express antigens or antigen fragments that can be recognized by B-cell receptors, which we refer to as B-cell antibody receptors (BARs). For BARs, the scFv of the CAR is replaced with an antigen or its domain. The exogenous TCRs are generally cloned from T cells present in diseased tissue, such as tumor-infiltrating lymphocytes, pancreatic islets, or multiple sclerosis (MS) lesions and are human leukocyte antigen (HLA) (11–15). The CARs, which are synthetic molecules, are typically comprised of scFv fused to T cell co-stimulatory proteins and CD3ζ chain. The scFv portion of the CAR can be derived from phage display technology or traditional monoclonal antibody production (15–19). The antibody-derived properties of the CAR free it from HLA restriction. TCRs or CARs have traditionally been used to engineer effector T cells, predominantly $CD8^+$ cytotoxic cells. The multiple design iterations, clinical successes (e.g., against melanoma and acute lymphoblastic leukemia) of TCR- and CAR gene-modified cells have been extensively reviewed by our group (20) and others (21–25). As noted above, our group and Ellebrecht et al. independently designed a novel method of engineering antigen-specific T cells with antigen domains, called BAR in our lab and called chimeric autoantibody receptor by the Payne group (26). This antigen domain targets pathogenic antibody secreting cells or their precursors with specific surface B-cell immunoglobulin (Ig) receptors (BCR). We have adapted these redirecting technologies to human Tregs with the goal of improving future Treg therapy in clinical trials.

Here, we chronologically review the development of antigen-specific human Tregs by gene modification in our lab. Specifically we describe the use of TCR (**Figure 1A**), CAR (**Figure 1B**), and BAR (**Figure 1C**) Treg therapy in the context of disease models for hemophilia A and MS. The important conclusions from our experiments as well as future directions and considerations for gene-modified Tregs as a therapeutic are discussed.

nTregs THERAPY: POLYCLONAL OR SPECIFIC

Phenotypically, peripheral blood nTregs are identified by high surface expression of CD25 (IL-2 receptor α chain), low expression of CD127 (IL-7α receptor), low to negative expression of CD45RA, and expression of the transcription factors, Foxp3 and Helios. Further markers such as the Treg-specific demethylated

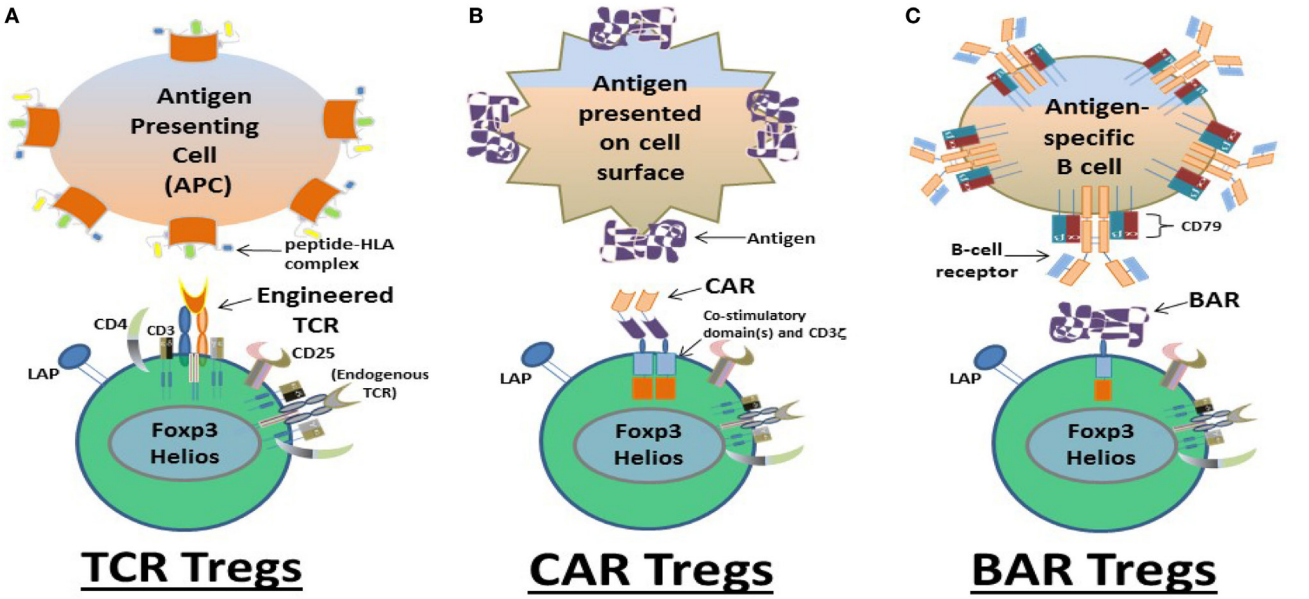

FIGURE 1 | Types of gene-modified antigen-specific human regulatory $CD4^+$ T cells (Tregs). Antigen-specific **(A)** T-cell receptor (TCR), **(B)** chimeric antigen receptor (CAR), and **(C)** B-cell antibody receptor (BAR) Tregs have been designed by the Scott lab as potential therapeutics to ameliorate autoimmune diseases and/or immune responses to biotherapeutics in monogenic diseases, for example. TCR, CAR, and BAR Tregs each have unique properties that can be exploited as treatments geared to the different pathophysiologies of such diseases and/or adverse immune responses. The structure and targeting moieties/cells of TCR, CAR, and BAR Tregs are briefly described and depicted.

region, glycoprotein A repetitions predominant protein (GARP), glucocorticoid-induced TNFR family-related gene (GITR), latency-associated peptide (LAP), CTLA-4, CD27, CD73, and CD39 among others also aid in nTreg identification (27–36).

FoxP3 was identified from early studies with scurfy mice, which have an idiopathic mutation in the Foxp3 gene and develop systemic multi-organ autoimmunity (37, 38). In humans, the importance of Tregs is evident in the debilitating and often fatal polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome which is linked to mutations in the Foxp3 gene (39, 40). The causal link between dysfunctional Tregs and autoimmunity set the stage for using functional Tregs to treat and possibly prevent it. Indeed adoptive Treg therapy to treat animal models of autoimmunity such as experimental autoimmune encephalomyelitis (EAE), arthritis, inflammatory bowel disease, and uveitis among others has proven successful and served as proof of concept for Treg therapy translational use (41–46).

Phase 1 clinical trials using human Tregs have involved participants suffering from acute graft versus host disease (GVHD) following stem cell transplants (47) or type I diabetes (T1D) (48). For the GVHD trial, all participants were infused with umbilical cord blood-derived polyclonal Tregs. No infusion related toxicities or adverse events were reported during the trial period. However, the authors concluded that a randomized control group receiving no Tregs was necessary to properly access treatment efficacy. The T1D trial used autologous peripheral blood-derived polyclonal Tregs. This was a dose escalation phase 1 trial. Treatment efficacy was not accessed, but the escalation protocols and safety profile of this trial has led to a phase 2 trial as of 2017 (49). Further trials using polyclonal Tregs to treat lupus and GVHD from kidney transplants and liver disease have also been initiated.[1]

The majority of these clinical trials have used polyclonal Tregs. While the success of polyclonal Tregs has been promising, the amount of cells needed for infusions is large (believed to be in the 10^9–10^{10} range) and the threat of global immune suppression is possible; indeed, one report cites viral reactivation after infusion of polyclonal Tregs (47) and tumor occurrence/recurrence is of concern given the correlation between Tregs and tumor survival (50, 51). Moreover, polyclonal human Tregs are not a homogenous population which may introduce unwanted variability and a lack of efficacy to their therapeutic potential (36, 52–54). To overcome these drawbacks, we and others believe that using antigen-specific Tregs of a defined homogenous population will require fewer cells to exert their regulatory effects and confer more localized and targeted suppression.

The occurrence of a particular antigen-specific T cell is very low, on the order of 1 in every 10^5–10^7 T cells (55). This greatly hinders the ability to isolate and expand such rare cells. However, in certain disease states or conditions where a target antigen or group of antigens is/are known, the clonal expansion of an antigen-specific T cell facilitates its detection and isolation by molecular methods. Such methods include tetramer-guided epitope mapping and peptide MHC microarrays (56–59). Since the TCR traditionally endows a T cell with its specificity, extracting the TCR cDNA sequence from the expanded cells and cloning it into a viral expression vector allows researchers to engineer antigen-specific T cells.

HUMAN Tregs GENE MODIFIED TO EXPRESS AN FVIII-SPECIFIC TCR

One disease model used in our lab to study the therapeutic potential of antigen-specific Tregs is hemophilia A. Hemophilia A is an X-linked bleeding disorder caused by mutations in the factor 8 (*F8*) gene, which encodes the blood coagulation protein, FVIII. Because of its monogenic etiology, the disorder can be treated with recombinant or plasma derived FVIII replacement therapy. Unfortunately, a large subset of those receiving replacement FVIII develop an antidrug antibody response. These antibodies (referred to as "inhibitors") can neutralize the FVIII, rendering this lifesaving treatment ineffective. Inhibitor formation requires $CD4^+$ T cell help (60, 61), and is largely directed to the A2 and C2 domains of the FVIII protein.

The standard treatment for inhibitors is called immune tolerance induction (ITI). ITI consists of high dose infusions of FVIII for a period of one or more years. Although it has met with some clinical success, ITI does not work for all inhibitor cases. Thus, alternative approaches for inducing tolerance in these unsuccessful cases or preventing inhibitor responses, in the first place, are of clinical importance.

In collaboration with the lab of Dr. Kathleen Pratt, we successfully isolated, cloned and sequenced HLA-DRB1*01:01 (DR1)-restricted TCRs specific for an epitope in the C2 domain of FVIII. The TCRs were isolated from $CD4^+$ T cell clones of a hemophilia A subject at different time points after clonal expansion (62).

As reported in 2015 (63), we sorted human nTregs from healthy donor peripheral blood mononuclear cells (PBMCs) and transduced them with retroviral particles encoding one of these C2 domain specific TCRs, referred to as 17195. Transduced Tregs were then sorted and expanded in the presence of antihuman CD3, autologous γ-irradiated PBMCs, and oligodeoxynucleotides (ODN). Kim et al. have shown that these ODN maintain the Treg phenotype better than inclusion of rapamycin during the critical rapid expansion period (64).

An important point with *ex vivo* expansion of human gene-modified Tregs is to determine the activation status of the Tregs during and/or at the end of the expansion. Initial *in vitro* activation of sorted Tregs for 3–5 days is necessary for retro- or lentiviral gene transfer, followed by large-scale expansion for 10–12 days with IL-2, but without TCR or anti-CD3 stimulation. This expansion step generally can be repeated for up to two more cycles. In most cases, successfully expanded gene-modified Tregs do not retain their activation status due to the long-term expansion conditions without cognate/specific antigen (e.g., TCR) or anti-CD3 stimulation. Nonetheless, *in vitro* confirmation of gene-modified Treg activation with specific antigen is mandatory before testing these Tregs *in vivo*. Such confirmation provides a functional estimation of the Treg responsiveness. For this, surface expression of GARP, LAP, and

[1] Available from: https://clinicaltrials.gov/ct2/results?term=adoptive+treg+therapy&Search=Search

CD25 as well as the induction of Foxp3 and Helios are analyzed by flow cytometry at 24–48 h post *in vitro* activation with cognate antigen and PBMCs (63, 65).

Tregs expressing the 17195 TCR proliferated in an antigen-specific manner and, importantly, maintained their Treg phenotype. Moreover, as mentioned above, these cells upregulated the Tregs markers Foxp3, Helios, GARP, LAP, and CD25 when stimulated with specific peptide. This phenotypic response was mirrored by the fact that they were able to prevent FVIII-specific effector cells from proliferating, as demonstrated in an *in vitro* suppression assay. Of clinical note, these Tregs also robustly diminished FVIII antibody production in splenocytes of FVIII-immunized HLA DR1 transgenic hemophilic mice *in vitro* and could prevent anti-FVIII formation *in vivo* in a xenogeneic transfer system.

HUMAN Tregs GENE MODIFIED TO EXPRESS AN FVIII-SPECIFIC CAR

Following the promising results and lessons gleaned from the FVIII-specific TCR gene-modified Tregs, we sought to design a FVIII-specific CAR Treg. CAR Tregs would allow us to test, without HLA restriction, the inhibition of both FVIII-specific antibody production and effector T cell proliferation. In collaboration with the lab of Drs. Anja Schmidt and Christoph Königs, Yoon et al. published results of human FVIII-specific CAR Tregs, referred to as ANS8 CAR Tregs (65). The human scFv region of the CAR was isolated by phage display and confirmed specific for the A2 domain of FVIII by competitive ELISA using known monoclonals against this domain (66). ANS8 CAR Tregs proliferated in response to FVIII and also concomitantly upregulated Foxp3 expression. These CAR Tregs suppressed the proliferation of FVIII-specific effector T cells. Moreover, these CAR Tregs also exhibited bystander suppression as they were able to prevent the proliferation of HLA DR2-restricted T effector cells specific for a myelin basic protein (MBP) peptide in the presence of appropriate antigen-presenting cells. Strikingly, when tested *in vivo*, ANS8 CAR Tregs were able to prevent FVIII antibody titers *prophylactically*, similar to TCR-transduced (17195) Tregs. The prevention of the anti-FVIII response was sustained up to 8 weeks despite the rejections of the transferred human Tregs in immunocompetent mice. This emphasized the potency of the ANS8 CAR and TCR-transduced Tregs and has prompted us to design *in vivo* therapeutic protocols for FVIII antibody prevention.

HUMAN Tregs GENE MODIFIED TO EXPRESS A BAR SPECIFIC FOR FVIII INHIBITORS

To test whether engineered Tregs could directly suppress B cells, we designed a third engineered T cell model that would express antigen and would directly interact with specific B cells *via* their BCR. Thus, our latest gene-modified human Tregs are engineered to express either the immunodominant A2 or C2 domains of FVIII, fused to T cell co-stimulatory and signaling domains, so called "BAR" for B-cell antibody receptor. It has been shown in animal models of autoimmunity and suggested in IPEX patients that Tregs may be able to directly suppress pathogenic B cells (67–70). In light of these studies, we hypothesized that BAR engineered Tregs directly suppress FVIII-specific B cells *via* interaction with their BCR and may possibly suppress other FVIII-specific effector T cells co-localized in the local milieu.

Zhang et al. (71) in our lab showed that A2 and C2 BAR Tregs maintained Treg-specific markers, including Foxp3 and Helios, after long-term expansion *in vitro*. Importantly, we showed that these BAR Tregs also potently suppressed FVIII antibody formation *in vitro* and *in vivo* from FVIII-immunized hemophilic mice, thus providing a third model of specific Tregs. The mechanism of this suppression is discussed below.

HUMAN Tregs GENE MODIFIED TO EXPRESS AN MBP-SPECIFIC TCR

Another important disease studied in our lab is MS. We employ an EAE mouse model for MS. MS is a debilitating autoimmune disorder where effector T cells mediate the attack and destroy the myelin sheath of the central nervous system (CNS). This destruction results in relapsing/remitting symptoms or progressive paralysis, which could result in death in its most severe cases. The etiology of MS is unknown, but certain genetic and environmental factors may play a role (72–75). Current treatment options include immunosuppressive drugs, β-interferon, or Copaxone, a random amino acid copolymer (76–78). Recently, treatment with B-cell depleting antibodies such as ocrelizumab and rituximab (79–83), has been used to relieve symptoms, but their side effects can be severe and also can lead to global immunosuppression (84, 85). Better treatment options thus are clearly warranted. We believe that antigen-specific Tregs targeting CNS antigens implicated in MS can be such an option.

We engineered a construct to express a TCR sequence provided by Dr. Kai Wucherpfennig, who isolated the TCR from an autoreactive $CD4^+$ T cell clone of an MS patient. This TCR, referred to as Ob2F3 (86–88), was specific for MBP epitope 85-99 and was HLA DR15 ("DR2") restricted. PBMC obtained from normal healthy donors were FACS-purified for nTregs, as we had done in the FVIII project, and transduced with the Ob2F3 TCR. These expanded, now MBP-specific, Tregs not only suppressed MBP-specific T-cell proliferation and cytokine production but also they could suppress FVIII-specific responses *in vitro* when both MBP and FVIII peptides were present. Remarkably, Ob2F3 TCR Tregs were also able to reduce myelin oligodendrocyte glycoprotein (MOG 35-55)-induced EAE symptoms in HLA DR2-transgenic mice. This was important because it confirmed that Tregs of one specificity (MBP) could exert bystander suppression of T effectors of another specificity (MOG), presumably in the local milieu. We found that these Ob2F3 TCR Tregs migrated in greater numbers to the CNS than non-specific Tregs and reduced the perivascular infiltrates in the spinal cord. This xenogeneic suppression validates the potency of antigen-specific engineered Tregs.

MECHANISMS OF SUPPRESSION

Understanding the suppression mechanism behind our gene-modified human Tregs is also actively being pursued. Although

it has been shown that Tregs have a diverse repertoire of suppression strategies both contact independent (contactless) and contact dependent (89–95), how these specifically modified Tregs suppress target cells is currently unresolved. While we know that bystander suppression could occur *in vitro* and *in vivo*, it was not clear whether cell-to-cell contact was needed. To investigate contactless and contact-driven mechanisms, our lab used a modified transwell developed by Dr. Kim that consisted of "heat-drilling" holes between microtiter wells so that liquid (and presumably effector suppressive molecules) could mix in the interwell space, dubbed the de-cellularized zone. We found that suppression of effector T-cell proliferation only occurred when specific Tregs *and* specific effector T cells were present together in the adjacent well (96).

We know that both effector and regulatory T cells need IL-2 to grow (97, 98). When we examined Stat5 phosphorylation kinetically, we found that antigen-stimulated effector $CD4^+$ T cells produced and responded to IL-2 with Stat5 phosphorylation starting at 8 h, but that Tregs alone showed minimal Stat5 phosphorylation even at 72 h. However, when cocultured together, Treg Stat5 phosphorylation started as early as 8 h, at which time the $CD4^+$ T cell effector response to IL-2 decreased dramatically. These results suggest that Tregs "co-opt" IL-2 from effector T cells and that a contact-dependent process was initiated with the production of more (long-acting) suppressive moieties.

To understand potential BAR Treg suppression mechanisms in our hemophilia A model, we designed a series of B and T cell coculture assays. Briefly, splenic B and T cells were isolated from A2 and C2 BAR Treg treated or non-specific control BAR Treg-treated FVIII-immunized hemophilic mice. T cells, isolated from A2 and C2 BAR Treg treated mice, were able to cooperate and stimulate antibody formation with B cells from control mice. However, B cells isolated from A2- and C2-tolerized mice failed to be stimulated for anti-FVIII antibody production by control T cells. These observations strongly suggest that A2 and C2 BAR Tregs tolerized the B cell compartment while sparing that of T cells. Further experiments assessing whether A2 and C2 BAR domains are taken up by specific B cells (as exosomes or by trogocytosis?) or whether this tolerization of different compartment has a kinetic component (i.e., T cells become tolerized at a later time point) are underway.

To facilitate further mechanistic studies, we are reversing our trajectory back into murine systems. Our human Tregs are eventually rejected by the mouse immune system so trafficking studies, adoptive transfers and re-challenge experiments are not feasible. In addition, the use of knockout murine cells will aid in completing the mechanistic picture of gene-modified Tregs. These studies are in progress.

Please see **Table 1** for summary of results.

FUTURE DIRECTIONS AND CONSIDERATIONS FOR GENE-MODIFIED HUMAN Tregs

T-cell receptor, CAR, and BAR Treg therapy all provide distinct advantages and (minor) disadvantages as therapeutics. All of these Tregs, while highly specific, can exhibit bystander suppression in the local milieu as demonstrated by their ability to suppress inhibitor formation to the entire FVIII protein *in vitro* and *in vivo*, despite being specific for a single domain or peptide epitopes. TCR gene-modified Tregs allow for targeting specific peptides presented by APC to pathogenic effector cells. The TCR also allows for the physiological activation and regulation of the

TABLE 1 | Types of antigen-specific human Tregs used in the Scott lab.

Gene-modified hTreg	Specificity/target antigen	Disease model	Results
17195 T-cell receptor (TCR) Tregs	Human leukocyte antigen (HLA) DR1-restricted FVIII epitope (C2191–2210)	Hemophilia A	• Expanded in an antigen-specific manner and maintained Treg phenotype following long-term *in vitro* expansion • Suppression of specific T effectors *in vitro* • Suppressed FVIII-specific antibody production *in vitro* and *in vivo* across a xenogeneic barrier • Bystander suppression in the local milieu
ANS8 chimeric antigen receptor (CAR) Tregs	A2 domain of FVIII	Hemophilia A	• Expanded in an antigen-specific manner and maintained Treg phenotype following long-term *in vitro* expansion • Suppression of specific T effectors *in vitro* • Suppressed FVIII-specific antibody production *in vitro* and *in vivo* across a xenogeneic barrier • Bystander suppression in the local milieu
A2 and C2 B-cell antibody receptor (BAR) Tregs	B-cell receptors specific for A2 or C2 domains of FVIII	Hemophilia A	• Expanded in an antigen-specific manner and maintained Treg phenotype following long-term *in vitro* expansion • Suppressed FVIII-specific antibody production *in vitro* and *in vivo* across a xenogeneic barrier • Bystander suppression in the local milieu • Direct suppression of FVIII-specific B cells
OB2F3 TCR Tregs	HLA DR15-restricted myelin basic protein epitope (MBP 85-99)	Multiple sclerosis [experimental autoimmune encephalomyelitis (EAE)]	• Suppressed MOG specific T cells *in vitro* • Suppressed MOG peptide induced EAE across a xenogeneic barrier • Trafficked to brain and spinal cord

The disease models in which they are tested and related results are listed and summarized, respectively.

Tregs. However, the HLA restriction of TCR limits its utility to recipients sharing those HLA class II antigens. This is not as serious with MS, for example, as there is linkage to HLA DR2 (99). However, strong linkage to HLA has not been observed in hemophilia A (100). Nonetheless, there are five to seven most common DR phenotypes in North American Caucasians; conceivably, one could clone the V genes from the T cells of patients with these HLA DRs to create a repertoire of TCRs. Thus, screening for HLA or engineering TCRs *de novo* for each recipient is feasible today.

Chimeric antigen receptor gene-modified Tregs have the advantage of being HLA unrestricted. This greatly increases the universality of their usage as a therapeutic in all patients. These Tregs, like the TCR-transduced Tregs, can exhibit bystander suppression but need to recognize conformational domains in the target antigen. This is likely to occur in the context of cell surfaces, either dendritic or endothelial cells or specific B cells, before uptake. The scFv we have used was obtained by phage display (65). Thus, further scFvs against other domains of FVIII can readily be produced.

B-cell antibody receptor Tregs represent a novel approach for engineering gene-modified antigen-specific cells; these too are not HLA-restricted and only require that specific B cells can bind *via* their surface Ig receptors to the domains expressed on the Tregs. Originally, our lab envisioned this approach for targeting inhibitors in hemophilia A or responses to biotherapeutics in monogenic diseases, but they also can be designed to target pathogenic antibodies in autoimmunity or antidrug antibodies (101, 102). An issue with BARs Tregs (or BAR CD8 killer T cells) is that circulating antibodies may bind to the BAR Treg epitope domains and either neutralize their activity or cause tonic signaling to drive an exhausted phenotype. While a concern, we think this is unlikely since we have found that antibody crosslinking of the BAR can, in some instances, trigger Treg proliferation. In addition, plasmapheresis could be used to remove the circulating antibodies if needed, but these may not possess as high an affinity for the BAR as the isotype-switched memory B cells.

Much remains to be discovered regarding specific Treg suppression mechanisms. We already know that, aside from bystander suppression which occurs locally, the contiguous presence of effector T cells and Tregs can lead to enhanced suppressive activity and contactless suppression of other T cells. This is in part due to the fact that effector cells require much higher amounts of IL-2 to maintain proliferation compared to Tregs, which acquire IL-2 locally and rapidly phosphorylate Stat5 downstream of CD25. How this process activate the Tregs to produce suppressive moieties is unknown but under investigation.

How Tregs modulate antibody formation is not clear. Obviously, suppression of effector (helper) T cell activation is involved. In a preliminary experiment, culture of T and B cells from BAR Treg-tolerized hosts suggests that B cells may be directly targeted (at least by BAR Tregs). We have no evidence at present for direct toxicity of BAR Tregs on B cells, but this remains an open question since human $CD4^+$ T cells can be cytotoxic (103, 104).

A major concern of any gene-modified cellular therapy is safety. Fortunately, technologies such as inducible suicide genes can be applied to gene-modified Tregs (105). For example, this technology would be a protection in the unlikely event in which bystander suppression led to any unintended sequelae.

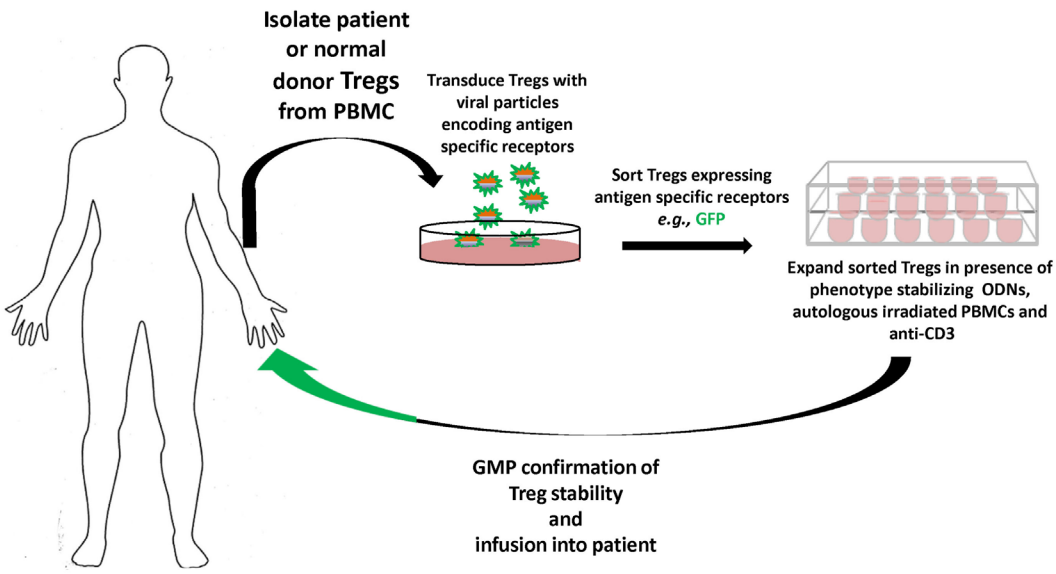

FIGURE 2 | Overview of gene-modified antigen-specific human Treg therapy. Patient (or normal donor) blood is collected, and Tregs are sorted from buffy coat, and virally transduced to express specific receptors [T-cell receptor (TCR), chimeric antigen receptor, or B-cell antibody receptor]. The antigen-specific Tregs are then sorted and expanded in the presence of autologous peripheral blood mononuclear cells (PBMCs), anti-CD3, and oligodeoxynucleotides (ODN), which stabilize Treg functional characteristics during expansion. The antigen-specific Tregs that meet robust GMP standards and Treg phenotype are then infused back into the patient tracking of the Tregs *in vivo* can be performed by deuterium labeling or GFP expression. Safety constructs that trigger the ablation or death of the infused antigen-specific Tregs can also be integrated, and gene editing by CRISPR/Cas9, e.g., used to remove endogenous TCRs or MHC to avoid graft versus host disease or rejection, respectively, of generic donor T cells.

In addition, we would like to create specific Tregs from generic, "off-the-shelf" unrelated donors. While such cells would possess endogenous TCRs and foreign HLA, we plan to apply techniques, such as CRISPR/Cas9 or TALENs (106, 107), to engineer out the endogenous TCRs so that these Tregs would only possess the transduced TCR, CAR, or BAR and would not cause GVHD disease. In addition, deleting HLA class II would also prevent their rejection by an immunocompetent host. Expansion of Tregs and their stable phenotype are also important concerns in advancing gene-modified Treg therapy. It is estimated that a minimal dosage of $1–3 \times 10^6$/kg of Treg may be needed for therapy efficacy (108). As mentioned, the ODN protocol has proven effective in maintaining the human Treg phenotype and function throughout the necessary expansion phase.

Designing treatments for an ongoing and established autoimmune diseases can pose a significant hurdle. Typically, autoimmune patients first visit their physicians after disease symptoms have appeared. The dysregulation and disruption of self-tolerance leading to diabetes, lupus, or MS, for example, typically can occur over an extended asymptomatic or subclinical time period. However, predictive measures in addition to family history are being developed. Therefore, antigen-specific Treg therapy could be administered to these patients to prevent pathological damage. Our data suggest we may even be able to attenuate symptoms once they arise. While there can be multiple (and unknown) target antigens in autoimmune diseases, we have shown in our EAE/MS model that gene-modified antigen-specific Treg therapy can exhibit bystander tolerance for other antigens in the local milieu.

In terms of responses to biotherapeutics, as in hemophilia A or monogenic diseases like Pompe's, the specific antigens are known, as is family history; hence, gene-modified antigen-specific human Tregs can become viable therapies prophylactically, as well as therapeutically. Our results suggest that antigen-specific TCR, CAR, and BAR Tregs each have distinct advantages as therapeutics and, thus, cultivation of each is necessary. Current progress toward good manufacturing practice and economy of scales are now being optimized. The future promise of gene-modified specific human Tregs therapeutics is quickly becoming today's reality (**Figure 2**).

AUTHOR CONTRIBUTIONS

PA and DS: writing organization of manuscript citations and creation of figures. YK, A-HZ, and JY: proofreading and discussion.

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MINI REVIEW
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Forkhead-Box-P3 Gene Transfer in Human CD4+ T Conventional Cells for the Generation of Stable and Efficient Regulatory T Cells, Suitable for Immune Modulatory Therapy

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The development of novel approaches to control immune responses to self- and allogenic tissues/organs represents an ambitious goal for the management of autoimmune diseases and in transplantation. Regulatory T cells (Tregs) are recognized as key players in the maintenance of peripheral tolerance in physiological and pathological conditions, and Treg-based cell therapies to restore tolerance in T cell-mediated disorders have been designed. However, several hurdles, including insufficient number of Tregs, their stability, and their antigen specificity, have challenged Tregs clinical applicability. In the past decade, the ability to engineer T cells has proven a powerful tool to redirect specificity and function of different cell types for specific therapeutic purposes. By using lentivirus-mediated gene transfer of the thymic-derived Treg transcription factor forkhead-box-P3 (FOXP3) in conventional CD4+ T cells, we converted effector T cells into Treg-like cells, endowed with potent *in vitro* and *in vivo* suppressive activity. The resulting $CD4^{FOXP3}$ T-cell population displays stable phenotype and suppressive function. We showed that this strategy restores Treg function in T lymphocytes from patients carrying mutations in *FOXP3* [immune-dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX)], in whom $CD4^{FOXP3}$ T cell could be used as therapeutics to control autoimmunity. Here, we will discuss the potential advantages of using $CD4^{FOXP3}$ T cells for *in vivo* application in inflammatory diseases, where tissue inflammation may undermine the function of natural Tregs. These findings pave the way for the use of engineered Tregs not only in IPEX syndrome but also in autoimmune disorders of different origin and in the context of stem cell and organ transplantation.

Keywords: regulatory T cells, forkhead box P3, tolerance, regulatory T cell-based cell therapy, gene transfer, antigen specificity, autoimmunity, immune dysregulation

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INTRODUCTION

Regulatory T cells (Tregs) are a subset of T lymphocytes devoted to the modulation of immune responses and to the maintenance of immunological tolerance. They control aberrant immune responses toward a wide range of antigens (Ags), including self-, food-Ags, allergens, and tumors (1). Several subsets of Tregs have been identified. Among those, Tregs expressing the forkhead-box-P3

(FOXP3) transcription factor (FOXP3+-Tregs) (2, 3) and the IL-10-dependent T-regulatory-type-1 cells (4) are the best characterized. The latter will be the subject of a review by Gregori et al. in the present Research Topic, whereas the former subset and its application in the clinical practice will be discussed here.

FOXP3+-Tregs can originate either in the thymus [thymic-derived Tregs (tTregs)] or differentiate in the periphery from naïve T cells (pTregs) (5, 6). Regardless of their origin, both subsets are characterized by constitutive expression of FOXP3, a transcription factor critical for their function, as demonstrated by the devastating autoimmunity resulting from mutations of *FOXP3* (7, 8). Impaired Treg function is the key pathogenic event leading to disruption of self-tolerance in patients with immune-dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome (9, 10).

It is now well accepted that although FOXP3 expression is dispensable for thymic development of tTregs, mainly dictated by epigenetic remodeling occurring regardless of FOXP3, its expression becomes fundamental in later stages for the peripheral function and maintenance of Tregs (11). Indeed, high and stable FOXP3 expression allows the acquisition of full suppressive function and stability of the Treg lineage by orchestrating the expression or repression of multiple genes indispensable for Treg suppressive function (12–14).

In addition to FOXP3, the expression of several molecules, including high CD25 (IL2Rα chain) in the absence of CD127 (IL7Rα chain) (15), CTLA-4 (16), GITR (17), CD39 (18), Galectin 10 (19), latency-associated peptide (20), Helios (21), the T-cell immune receptor TIGIT (22), and glycoprotein-A repetitions predominant (23) has been associated with human FOXP3+-Tregs, although none of these molecules is exclusive for this subset, but shared with activated conventional T cells. To date, the most reliable feature unambiguously identifying FOXP3+-Tregs is the epigenetic remodeling of specific genomic regions within the *FOXP3*-locus (CNS2-TSDR) (24) or in Treg-related genes (11).

FOXP3+-Tregs modulate both innate and adaptive immune cells by various mechanisms. The inhibitory activity of Tregs is primarily dependent on contact with target cells, which allows modulation of antigen-presenting cells stimulatory capacity *via* CTLA-4 (25) or the killing of T effector (Teff) cells through the granzyme/perforin axis (26, 27). Additional mechanisms of suppression include the release of inhibitory cytokines, e.g., IL-10 (28), TGF-β (29, 30), and IL-35, at least in murine Tregs (31), cytokine deprivation (32), and generation of immunosuppressive metabolites, i.e., extracellular adenosine (33) and intracellular cAMP (34). FOXP3+-Tregs are not a homogeneous population but are rather constituted by a heterogeneous pool, including specialized subtypes (28, 35–39).

Their great potential as modulators of immune responses, resulting from both preclinical models and clinical evidences, convinced investigators that Tregs could be used as tools to control unwanted immune responses in the context of transplantation or to treat autoimmune/inflammatory diseases (40, 41). A great effort has been devoted to the development of good-manufacturing practice-grade protocols to isolate/expand human Tregs *in vitro* allowing translation of Treg-based cell therapy to the clinical practice (42–45).

In this review, we will give an overview of the clinical trials that applied FOXP3+-Tregs as therapeutics for the control of graft-versus-host disease (GvHD) in the context of hematopoietic stem cell transplantation (HSCT) and for the modulation of autoimmune reactions and the challenges that these trials highlighted. We will discuss the innovative therapeutic approach based on adoptive transfer of engineered Treg-like cells that we are developing for the treatment of IPEX syndrome, whose application could potentially extend to reestablish tolerance in autoimmune diseases of different origin and in transplantation.

Treg-BASED CELL THERAPY IN CLINICAL TRIALS

Several Phase I-clinical trials have been conducted to assess the effect of Treg-based cell therapy on GvHD following allogenic HSCT, organ transplantation, in patients with type 1 diabetes (T1D) and chronic inflammatory diseases. Overall, results obtained with different subsets of Tregs demonstrated favorable safety profiles (46, 47).

Regulatory T cell-based clinical trials in HSCT have preceded other indications because the timing of GvHD onset is known and can be monitored, the time needed for prevention is relatively short, the initial efficacy is likely to provide lifelong protection, and complications of GvHD can be lethal.

Several groups have applied polyclonal CD4+CD25+ Tregs containing a high proportion of FOXP3+ T cells, either freshly isolated or *ex vivo* expanded, with the aim of preventing GvHD after allogenic HSCT for onco-hematological diseases. The results showed that the overall procedure is feasible and safe (48–52). One trial reported decreased incidence of grade II–IV GvHD as compared with historical controls in patients receiving umbilical cord blood-derived Tregs, without increased infections (49). Data were confirmed in a more recent trial from the same group, in which the clinical outcome of patients receiving Treg-based cell therapy was compared with that of control patients who received the same conditioning regimen and immunosuppressive treatment but no Tregs. The incidence of grade II–IV acute-GvHD at 100 days was 9 vs 45% in controls, whereas chronic-GvHD at 1 year was 0 in treated patients (52).

In a third trial patients injected with freshly isolated peripheral Tregs showed low grade GvHD and no development of chronic-GvHD (50). More recently, the same group showed reduced incidence of relapse in Treg-treated patients (53).

These initial reassuring results encouraged a wider application of Tregs as therapy after solid organ transplantation. Several trials are currently ongoing, although final results are not currently available (47). Among those, in The-ONE-Study (http://www.onestudy.org/), a Phase I/II dose-escalation study, several subtypes of Tregs, including *ex vivo* expanded FOXP3+-Tregs, have been infused in patients undergoing kidney transplant with the goal of avoiding lifelong immunosuppression through the induction of active tolerance (NCT02129881) (47, 54). Similarly, a Treg-immunotherapy trial in the setting of liver transplantation, ThRIL (NCT02166177), has been initiated, although safety data are not yet available (44).

FOXP3+-Treg-based therapy was safely tested also in the context of autoimmune diseases. In a trial limited to few patients, *ex vivo* expanded $CD4^+CD25^{hi}CD127^-$ Tregs were administered to children with recent-onset T1D (55), and more recently to new-onset adult T1D patients (43). In both cases, the procedure appeared to be safe, although published data do not allow to draw conclusions on efficacy. Importantly, in the latter trial, safety was demonstrated for transfer of high number of Tregs (up to 2.6×10^9 cells) (43).

Overall, the data available support the feasibility and safety of the approach. These results convinced researchers to pursue adoptive Treg-cell therapy and much effort is currently devoted to address open issues in the field, such as the *in vivo* persistence and stability of the injected product and the need for Ag specificity to increase efficacy.

IPEX SYNDROME: A DISEASE MODEL OF Treg DYSFUNCTION

Immune-dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome is the prototype poly-autoimmune disease caused by mutations in the gene encoding for the transcription factor FOXP3 (8). Affected patients develop early-onset multi-organ autoimmunity, which includes severe enteropathy, T1D, and eczema (9, 56). Beside the severely affected patients, many subjects manifest with a milder form of the disease, which is often misdiagnosed or diagnosed later due to the atypical presentation (57). *FOXP3* mutations result in loss of functional Tregs, which is considered the primary cause of disease. *FOXP3*-mutated Tregs display defective *in vitro* suppressive function (58–60) and unstable behavior in inflammatory conditions, with conversion from a regulatory to an effector (i.e., IL-17-producing) phenotype (61). Defects in peripheral cells other than Tregs have also been described, e.g., conventional T cells (58, 61–63) and B cells (64). Those additional defects are likely to be an indirect consequence of Treg dysfunction, rather than a direct effect of the mutations, thus suggesting that therapies aimed at improving/restoring a functional Treg compartment should be beneficial to IPEX patients.

The treatment of IPEX syndrome currently relies on supportive therapy, immunosuppression, and HSCT. Allogenic HSCT has proven curative (9), but for patients who do not undergo HSCT the treatment is limited to nutritional support, replacement therapy for endocrine organ failure, and to multiple immunosuppressive drugs, with incomplete control of autoimmunity and burdensome side effects in young patients. Therefore, a therapy aimed at restoring Treg functions represents an unmet medical need. Furthermore, experimental evidence in *scurfy* mice, the murine model of FOXP3-deficiency, shows that adoptive Treg transfer improves lifespan (65). On the same line, experience from transplanted patients with partial donor chimerism (66–68) and the presence of a fully wild-type Treg compartment in healthy carriers of *FOXP3* mutations (69) supports the idea that few functional Tregs are sufficient to control disease progression and induce remission.

The latter evidences convinced us that restoration of a functional Treg compartment in IPEX patients is a therapeutic option. We therefore designed an approach to genetically modify autologous T cells for adoptive transfer in these patients.

THE GENERATION OF Treg-LIKE CELLS BY LENTIVIRUS-MEDIATED FOXP3 GENE TRANSFER

The genetic reprogramming of mammalian cells for clinical purposes has recently become an available option, with the completion of clinical trials for the treatment of genetic diseases (70–73) and cancer (74, 75) and their translation in market-authorized therapies (76). Gene-transfer technology has been applied also to the field of Treg-based cell therapy, with the aim of generating high numbers of functional Tregs. Ectopic overexpression of FOXP3 in conventional $CD4^+$ T cells from healthy donors (3, 77–79), ectopic expression of T cell receptors (TCRs) with known specificity in polyclonal Tregs (80–82), and the use of chimeric antigen receptors (CARs) (83–86) are the approaches so far tested in preclinical settings (**Table 1**). While the former approach would maintain the Ag specificity of the starting population, the latter would redirect Treg specificity. In preclinical studies, expression of TCRs specific for tumor-Ags/allergens conferred human Tregs the ability to suppress Ag-specific responses (80, 81). More recently, Tregs-expressing CARs specific for HLA-Ags have proven effective in inhibiting xenogeneic GvHD and allograft rejection in preclinical models (84–86).

With the ultimate goal of controlling the devastating autoimmunity resulting from mutations of *FOXP3* in IPEX syndrome, we envisaged the possibility of performing adoptive transfer of functional autologous Tregs generated *in vitro*. To this aim, the human *FOXP3* coding sequence was cloned under the control of a constitutive promoter in a bidirectional lentiviral vector (LV) construct (88) allowing simultaneous expression of full-length FOXP3 and of a cell-surface marker (ΔNGFR) for the identification/selection of transduced T cells (79) (LV-FOXP3) (**Figure 1A**). Transduction of peripheral $CD4^+$ T lymphocytes with LV-FOXP3 and *in vitro* expansion of transduced cells lead to the generation of a homogeneous pool of T cells constitutively overexpressing FOXP3 (**Figure 1B**). The resulting $CD4^{FOXP3}$ T cells behave as functional and stable FOXP3+-Treg-like cells, with potent *in vitro* suppressive activity, reduced proliferative capacity, and limited cytokine production (79, 87). $CD4^{FOXP3}$ T cells stably express FOXP3 in steady-state and inflammatory conditions, especially when generated from naïve T cells, and maintain inhibitory functions *in vivo* in a model of xenogeneic GvHD (87). Furthermore, we demonstrated that fully functional $CD4^{FOXP3}$ T cells can be generated from T cells of IPEX patients (87), regardless of the underlying *FOXP3* mutation and co-expression of mutated protein, thus demonstrating the feasibility of our approach and paving the way for the development of alternative therapies based on the adoptive transfer of autologous genetically modified Treg-like cells for the control of autoimmunity in IPEX syndrome.

The fact that $CD4^{FOXP3}$ T cells can be obtained from $CD4^+$ T cells renders the manufacturing process easy and cost-effective. $CD4^{FOXP3}$ T cells do not require extensive *in vitro* expansion with high cytokine concentration. The current preclinical small-scale

TABLE 1 | *In vitro* generation of genetically engineered human regulatory T cells (Tregs).

Cell type	Starting cell population	Gene-transfer platform/ transgene	Marker gene	Ag specificity	*In vitro* suppression	*In vivo* function	Disease indication	Reference
CEA-CAR Tregs	Bead-sorted CD4+ $CD25^{hi}$ Tregs	RV/CEA-CAR	No	CEA	–	CEA + CD15A3 cell tumor model	–	(83)
A2-CAR Tregs	FACS-sorted naïve CD4+ $CD25^{+}$ Tregs	Bidirectional LV/A2-CAR	Yes (ΔNGFR)	HLA-A2	Yes	Xeno-GvHD model	Transplantation	(84)
MHC-I-allospecific-CAR Tregs	Bead-purified $CD25^{+}$ Tregs	LV/A2-CAR	Yes (eGFP)	HLA-A2	Yes	Skin xenograft transplant	Transplantation	(85)
A2-CAR Tregs	FACS-sorted CD4+$CD25^{hi}$ $CD127^{low}$ Tregs	RV/A2-CAR	Yes (ΔNGFR)		Yes	*In vivo* MLR; Hu-mice rejection model; Skin xenotransplant	Transplantation	(86)
Tyr-TCR Tregs	FACS-sorted CD4+$CD25^{hi}$ $CD127^{low}$ naïve Tregs	Multicistronic LV/Tyr-specific TCR chains	Yes (GFP)	Tyrosinase	Yes	EL-4-HLA-A2/K tumor model	–	(80)
Islet Ag-specific Tregs	FACS-sorted CD4+$CD25^{hi}$ $CD127^{low}$ Tregs	Multicistronic LV/Islet Ag TCR chains	Yes (mCherry)	IA2, insulin	Yes	–	T1D	(82)
Betv1-TCR-Tregs	Bead-sorted CD4+ $CD25^{-}$ T cells	RV/Betv1-specific TCR chains and FOXP3	No	Betv1	Yes	–	Allergy	(81)
$CD4^{FOXP3}$	Bead-sorted CD4+ T cells	Bidirectional LV/FOXP3	Yes (ΔNGFR)	Polyclonal	Yes	Xeno-GvHD model	IPEX syndrome	(79, 87)

CAR, chimeric antigen receptor; A2, HLA-A2; GvHD, graft-versus-host disease; LV, lentiviral vector; RV, retroviral vector; CEA, carcinoembryonic antigen; MLR, mixed lymphocyte reaction; FOXP3, forkhead-box-P3; IPEX, immune-dysregulation, polyendocrinopathy, enteropathy, X-linked; T1D, type 1 diabetes; TCR, T cell receptor.

method for the generation of $CD4^{FOXP3}$ T cells leads up to 10-fold expansion in 3/4-week culture. This guarantees the feasibility of the production for infusion into patients, taking into consideration that the starting conventional CD4+ T cells can be available in large numbers. In addition, the clinical use of LV platforms does not pose a limitation, since it has proven to be safe in cancer patients and pediatric patients who received HSC gene therapy (72, 73, 89, 90).

Although in principle, the use of CAR-Tregs or TCR-transgenic Tregs would allow the generation of Ag-specific Tregs suitable for the treatment of autoimmune diseases, the Ag target of the autoimmune damage is still unknown in many diseases. The fact that $CD4^{FOXP3}$ originate from polyclonal CD4+ T cells may constitute an advantage for such diseases. Indeed, the CD4+ T cells obtained from a patient suffering with autoimmunity would most likely comprise the pathogenic T cells with TCRs specific for the target Ags. Therefore, in specific disease context, $CD4^{FOXP3}$ cells may find a broader and more effective use, as compared with the TCR-transgenic-/CAR-Tregs.

CHALLENGES IN Treg-BASED IMMUNOTHERAPY FOR IPEX SYNDROME

Despite their promising results, the initial trials of Treg-based cell therapy raised some concern on issues related to FOXP3+-Treg biology. Due to their intrinsic anergic and terminally differentiated phenotype, one open issue is the *in vivo* lifespan of the infused product. Initial data on *in vivo* infused Tregs showed 2-week survival post-injection (49). We obtained similar results when $CD4^{FOXP3}$ T cells were injected in immune-deficient mice (87). Surprisingly, data from a Treg-cell therapy trial in T1D patients demonstrated that, although the majority of *ex vivo* expanded autologous Tregs persists for 2 weeks post-infusion, a fraction of the injected cells is detectable after 1 year, suggesting that Tregs might contribute to tolerance maintenance long term (43).

Several evidences demonstrated that FOXP3+-Tregs are intrinsically plastic and that under inflammatory conditions Tregs can downmodulate FOXP3 and secrete pro-inflammatory cytokines (91–93). Therefore, the risk of loss of regulatory functions by infused Tregs could be worrisome. To address this issue, culture with rapamycin, to favor the generation of stable Treg products, has been developed (94–96). In this context, $CD4^{FOXP3}$ T cells represent the ideal Treg product: constant FOXP3 expression is warranted by a constitutive promoter-driven transcription, and stability has been demonstrated in steady-state and inflammatory conditions, both *in vitro* and *in vivo* (87). Stability is especially maintained when $CD4^{FOXP3}$ T cells are generated from naïve T cells. In the case of memory-derived $CD4^{FOXP3}$ T cells, FOXP3 expression appeared slightly reduced with inflammatory cytokines, resulting in weaker suppressive function and increased proliferation, as compared with naïve T cell-derived products (87), most likely due to posttranscriptional regulatory mechanisms.

Finally, the possibility of a generalized effect of immunosuppression that injection of suppressor cells may cause, as well as concerns about the dose required for injection of polyclonal Tregs has prompted investigators to design more targeted therapies. Methods to expand human Ag-specific Tregs have been developed

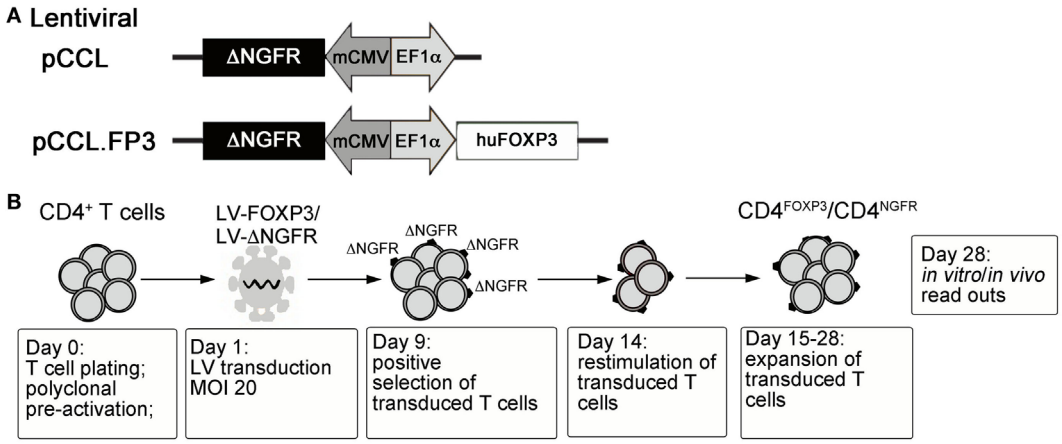

FIGURE 1 | **(A)** Schematic representation of control and FOXP3-expressing lentiviral vector constructs. **(B)** Protocol for the generation of $CD4^{FOXP3}$ Treg-like cells and control transduced T cells ($CD4^{NGFR}$) from conventional $CD4^+$ T cells (either naïve or total $CD4^+$ T cells). MOI, multiplicity of infection; FOXP3, forkhead-box-P3; Treg, regulatory T cell.

(42, 97, 98). These protocols well apply to allo-Ag-specific Tregs. Of note, encouraging safety and efficacy results come from a recently published Treg-based cell therapy trial, in which Tregs induced in the presence of donor-irradiated PBMCs were infused after liver transplantation. Despite low doses of Tregs, in 7/10 patients, immunosuppression was stopped, and operational tolerance to the graft was induced (99). Currently, ongoing trials in solid organ transplantation, which foresee the injection of donor-specific Tregs, will lead to further progresses (47).

We believe that in the case of IPEX syndrome and diseases with multiple autoimmune manifestations, the need for Ag specificity is unlikely to be necessary. The use of patients' Teff cells as source of $CD4^{FOXP3}$ cells will potentially allow the generation of Treg-like cells enriched for autoreactive specificities. Still, the infusion of polyclonal potent suppressor cells may result in a generalized effect of immunosuppression, which could potentially interfere with protective responses to common pathogens. Although the results of the clinical trials using polyclonal Tregs were reassuring, we are currently establishing a protocol to generate $CD4^{FOXP3}$ T cells from Ag-experienced T cells with known specificity, which should restrict their suppressive effect to the target Ag. Briefly, the protocol foresees pre-activation of T cells with a target Ag; Ag-specific T cells activating in response to their cognate Ag will be preferentially transduced. Subsequent *in vitro* expansion allows generation of a T-cell population enriched of FOXP3-overexpressing cells with known Ag specificity (Passerini and Bacchetta, unpublished results). This method could be used to extend the application of the $CD4^{FOXP3}$ T-cell product beyond IPEX syndrome, to treat autoimmune/inflammatory diseases with known target Ags or in the context of transplantation tolerance.

Finally, a relevant open issue on the way to the clinical application of $CD4^{FOXP3}$ T cells is definitely their *in vivo* lifespan, difficult to assess in preclinical models. Short-lived cells would likely be safer, although they may imply clinical protocols with multiple infusions of the therapeutic product. Long-lived $CD4^{FOXP3}$ T cells would allow single infusion but would likely require an additional safety layer, such as addition of a suicide gene in the construct used for their generation. The use of a suicide gene may also be considered as a safety measure to contrast the consequences of possible insertional mutagenesis, although it has been demonstrated that the use of LV-mediated gene transfer is not associated with selective integrations near oncogenes (100). However, for any type of genetically modified cellular product, analysis of the integration sites is recommended during preclinical assessments.

CONCLUDING REMARKS

Thanks to the successfully completed trials, the use of adoptive Treg-cell therapy to control undesired immune responses has become applicable. The next challenge for researchers is the tailoring of the Treg-based therapy for specific diseases. We envisaged an approach based on the use of $FOX3^+$-Treg-like cells electively designed to restore immune regulation in IPEX syndrome. Once safety and proof-of-concept will be completed in IPEX patients, the use of these autologous Treg-like cells could become the future standard of care for certain autoimmune diseases, akin to how CAR-T cells will become the standard of care in hematologic malignancies.

AUTHOR CONTRIBUTIONS

The authors (LP and RB) equally contributed to discuss the topic and write the manuscript.

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Avidity and Bystander Suppressive Capacity of Human Regulatory T Cells Expressing *De Novo* Autoreactive T-Cell Receptors in Type 1 Diabetes

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The ability to alter antigen specificity by T-cell receptor (TCR) or chimeric antigen receptor (CAR) gene transfer has facilitated personalized cellular immune therapies in cancer. Inversely, this approach can be harnessed in autoimmune settings to attenuate inflammation by redirecting the specificity of regulatory T cells (Tregs). Herein, we demonstrate efficient protocols for lentiviral gene transfer of TCRs that recognize type 1 diabetes-related autoantigens with the goal of tissue-targeted induction of antigen-specific tolerance to halt β-cell destruction. We generated human Tregs expressing a high-affinity $GAD_{555–567}$-reactive TCR (clone R164), as well as the lower affinity clone 4.13 specific for the same peptide. We demonstrated that *de novo* Treg avatars potently suppress antigen-specific and bystander responder T-cell (Tresp) proliferation *in vitro* in a process that requires Treg activation ($P < 0.001$ versus unactivated Tregs). When Tresp were also glutamic acid decarboxylase (GAD)-reactive, the high-affinity R164 Tregs exhibited increased suppression ($P < 0.01$) with lower Tresp-division index ($P < 0.01$) than the lower affinity 4.13 Tregs. These data demonstrate the feasibility of rapid expansion of antigen-specific Tregs for applications in attenuating β-cell autoimmunity and emphasize further opportunities for engineering cellular specificities, affinities, and phenotypes to tailor Treg activity in adoptive cell therapies for the treatment of type 1 diabetes.

Keywords: type 1 diabetes, regulatory T cells, T cell receptor, avidity, suppression mechanisms, adoptive cellular therapies, antigen-specific T cells, glutamic acid decarboxylase 65

INTRODUCTION

T-cell receptor (TCR) transgenic regulatory T cells (Tregs) may represent a promising personalized treatment for T-cell-mediated autoimmune diseases such as type 1 diabetes. A curative therapy that targets the underlying immunological cause of disease to restore antigen-specific immunological tolerance represents an essential objective for the preservation of β-cell mass and function in the treatment of type 1 diabetes (1). Non-antigen-specific therapies involving hematopoietic stem

cell transplantation combined with T-cell depletion, *via* high-dose anti-thymocyte globulin (ATG) or fludarabine, plus immunomodulation with cyclosporine and granulocyte-colony stimulating factor (G-CSF) have been shown to preserve β-cell function (2, 3), but the risks associated with these aggressive protocols preclude common clinical use. Comparatively, non-specific polyclonal immunotherapies, including immunoregulatory or depleting agents [e.g., alefacept (human LFA-3/IgG1-Fc fusion protein), teplizumab or otelixizumab (anti-CD3), and rituximab (anti-CD20)], have been better tolerated and offered some temporary efficacy but not long-term induction of tolerance (4–10). Until recently, most antigen-specific tolerance induction efforts have involved mucosal or peripheral administration of autoantigen(s), but thus far, such attempts have yielded limited efficacy in only a subset of patients, again with no indication for long-term tolerance induction (11, 12). Indeed, a safe treatment that controls persistent immune memory and induces long-term tolerance is needed.

Islet cell antigen-reactive Tregs, isolated from BDC2.5 TCR transgenic mice, could be expanded *in vitro*, and following adoptive transfer, migrate to the pancreatic draining lymph node/nodes (13). These Treg prevent and reverse autoimmune diabetes in non-obese diabetic (NOD) mice (14). In contrast, Tregs isolated and expanded from GAD286 TCR transgenic mice could suppress responder T cells (Tresp) *in vitro* but did not proliferate *in vivo* after transfer into recipient animals (14). Moreover, expression of cognate autoantigen is required for efficient trafficking of Tregs to the target organ and suppression of diabetes in NOD mice (15). These preclinical data support the notions that autoantigen-specific Tregs may offer an important therapy for type 1 diabetes, but also that intrinsic factors such as TCR specificity and/or avidity may play an important role in determining the capacity for immunomodulation and efficacy. The need for continued autoantigen expression by the host may render insulin-reactive TCRs less effective in patients with long-standing type 1 diabetes and support a need to investigate additional, potentially bystander, TCRs specific for additional/alternative autoantigen targets such as glutamic acid decarboxylase (GAD). Moreover, antigen localization, density, and persistence in β-cells along with risk of effector cell reprogramming support the use of alternative TCRs (16).

Genetically modified T cells with TCRs specific for tumor or viral antigens have become a valuable tool for the treatment of certain cancers or infections in humans (17–19). We previously demonstrated successful HLA class I-restricted TCR gene transfer in human Tregs using a high-affinity model receptor specific for the melanoma antigen tyrosinase presented by HLA-A*02:01 (20). We also generated a murine form of these tyrosinase-specific Tregs, and when transferred *in vivo*, the cells were capable of suppressing anti-tumor immunity in murine tumor models (20). This prompted us to ask whether candidate TCRs specific for type 1 diabetes-related autoantigens could be used to generate regulatory TCR avatars for human therapy.

Two TCR clones (R164 and 4.13) specific for the same β-cell peptide ($GAD_{555–567}$) presented by HLA-DR4, but with different binding affinities, have been identified from the peripheral blood of subjects with or at-risk for T1D (21–23). Indeed, we recently identified T cells expressing the TCR β-chain complementarity determining region (CDR3β) of the GAD 4.13 clone from tissues of seven organ donors with type 1 diabetes, including the pancreatic islets of one type 1 diabetes subject. Interestingly, for one donor with long-standing disease, the TCR CDR3β was highly enriched in the pLN (>25% of all productive sequences), representing the most prevalent clone in both the Treg and conventional $CD4^+$ T-cell (Tconv) populations (24). Interestingly, 4.13 TCR transgenic HLA-DR4 mice were reported to contain a mixture of Th1 and Tr1 cells capable of producing IL-10 (21). Conversely, R164 TCR transgenic HLA-DR4 mice exhibited greater thymic negative selection, and the T cells that escaped the thymus were skewed toward a Th1 phenotype (21). These observations support the notion that TCR avidity may impart important functional distinctions.

In a recent report by Ali et al., human $CD4^+$ T cells were engineered to express the R164 TCR clone, and importantly, when administered to NSG-Ab^0 DRB*04:01 mice, these R164 cells established long-term engraftment and islet infiltration, up to 12 weeks, without graft versus host disease (GvHD) (25). The creation of these autoreactive T-cell avatars presents the exciting possibility of autologous Treg therapy for type 1 diabetes with the benefit of antigen specificity to potentially enhance Treg trafficking to the target organ and associated draining lymph nodes. These antigen-specific Tregs would likely represent a significant improvement upon autologous polyclonal Treg therapy, which has already been shown to be safe for use in human subjects (26, 27). Indeed, antigen-specific Tregs offer the potential for long-term tolerance to the target antigen and possibly, to other key β-cell epitopes *via* bystander suppression and infectious tolerance (14, 28). To expand on these efforts, we generated primary human Tregs expressing the two $GAD_{555–567}$-reactive TCR clones (R164 and 4.13), and investigated the pre-transfer conditions needed to optimize suppressive activity for potential use in adoptive cell therapy.

RESEARCH DESIGN AND METHODS

Design and Synthesis of Lentiviral Constructs

Lentiviral vectors were generated to express TCR clones 4.13 and R164, both of which react to $GAD_{555–567}$ (21, 25) (**Table 1**). Equimolar expression of TCR α- and β-chains was achieved by inclusion of a multicystronic P2A element, followed by a T2A element and the reporter, enhanced green fluorescent protein (eGFP). The constructs were cloned into pCNFW lentiviral vectors with expression driven by a cytomegalovirus promoter as previously described (25) (**Figure 1A**). Lentiviral vectors containing the Melan-A reactive TCR clone melanoma antigen recognized by T cells 1 (MART-1) were generated as previously described (29) (**Table 1**).

Lentivirus Production

Lentiviral vectors were generated as described (20). Briefly, 55 μg of lentiviral vector and 18.3 μg of each helper plasmid

TABLE 1 | T-cell receptor (TCR) clone information.

TCR (IMGT)		TRA gene			TRB gene				pMHC restriction		Source
S. no.	Clone	V	J	CDR3 AA sequence	V	D	J	CDR3 AA sequence	HLA	Antigen	
1	5	TRAV21	TRAJ6	CAVKRTGGSYIPTF	TRBV11-2	TRBD1	TRBJ2-2	CASSSFWGSDTGELFF	DQ8	InsB (9–23)	Roep, personal communication
2	GSE.20D11[a]	TRAV12-3	TRAJ4	CAILSGGYNKLIF	TRBV02-01*01	TRBD02-01	TRBJ02-05*01	CASSAETQYF	DQ8	InsB (9–23)	(30)
3	GSE.6H9[a]	TRAV26-1	TRAJ40	CIVRVDSGTYKYIF	TRBV7-2	TRBD2	TRBJ2-1	CASSLTAGLASTYNEQFF	DQ8/ DQ8-trans	InsB (9–23)	(30)
4	T1D#3 C8	TRAV17	TRAJ23	CATDAGYNQGGKLIF	TRBV5-1	TRBD2	TRBJ1-3	CASSAGNTIYF	DQ8	InsB (9–23)	(31)
5	T1D#10 C8	TRAV12-3	TRAJ26	CATAYGQNFVF	TRBV4-1	TRBD2	TRBJ2-2	CASSRGGGNTGELFF	DQ8	InsB (9–23)	(31)
6	PM1#11	TRAV35*02	TRAJ54*01	CAGHSIIQGAQKLVF	TRBV5-1*01	TRBD2*02	TRBJ2-1*01	CASGRSSYNEQFF	DRB1*03:01	GAD (339–352)	(32)
7	MHB10.3	TRAV4*01	TRAJ27*01	CLVGDSLNTNAGKSTF	TRBV29-1*01	TRBD2*01	TRBJ2-2*01	CSVEDRNTGELFF	DRB1*03:01	InsB (11–30)	(33)
8	SD32.5	TRAV26-1*01	TRAJ23*01	CIVRVSSAYYNQGGKLIF	TRBV27*01	TRBD2*01	TRBJ2-3*01	CASSPRANTDTQYF	DRB1*04:01	InsA (5–21)	(34)
9	SD52.c1	TRAV4*01	TRAJ27*01	CLVGDSLNTNAGKSTF	TRBV27*01	TRBD1*01	TRBJ1-5*01	CASSWSSIGNQPQHF	DRB1*04:01	PPI (C18–A1)	(34)
10	R164	TRAV19*01	TRAJ56*01	CALSEEGGGANSKLTF	TRBV05-01*01	TRBD02-01*01	TRBJ01-06*01	CASSLAGGANSPLHF	DRB1*04:01	GAD (555–567)	(23)
11	4.13	TRAV19*01	TRAJ44*01	CALSENRGGTASKLTF	TRBV05-01*01	TRBD01-01*01	TRBJ01-01*01	CASSLVGGPSSEAFF	DRB1*04:01	GAD (555–567)	(21)
12	1E6	TRAV12-3	TRAJ12	CAMRGDSSYKLIF	TRBV12-4	TRBD2	TRBJ2-4	CASSLWEKLAKNIQYF	A*02-01	PPI (15–24)	(35)
13	D222D	TRAV17*01	TRAJ36*01	CAVTGANNLFF	TRBV19*01	TRBD1*01	TRBJ2-2*01	CASSIEGPTGELFF	A*02-01	ZnT8 (186–194)	Patent WO2017046335 A1
14	32	TRAV12-1	TRAJ48*01	CVVNILSNFGNEKLTF	TRBV20	TRBD01-01*01	TRBJ2-01*01	CSASRQGWVNEQFF	A*02-01	IGRP (265–273)	(36)
15	MART-1	TRAV12-2	TRAJ23	CAVNFGGGKLIF	TRBV6-4	TRBD2	TRBJ1-1	CASSLSFGTEAFF	A*02-01	Melan-A (27–35)	(37)

For the experiments described herein, T cells were transduced to express TCR clones 4.13 or R164, which were first identified from the peripheral blood or pancreas of a type 1 diabetes patient or an autoantibody positive subject at risk for T1D. CD8⁺ T cells were transduced to express melanoma antigen recognized by T cells 1 (MART-1) TCR. Remaining TCR clones [sourced from the international ImMunoGeneTics information system®, IMGT.org (IMGT)] listed are those with known reactivities to type 1 diabetes-related autoantigen peptides with which we can generate lentivirus constructs to create additional Treg avatars. TCRα (TRA) and TCRβ (TRB) V, D, and J genes as well as complementarity determining region 3 (CDR3) amino acid (AA) sequence, HLA restriction, and antigen target are listed for each clone.

[a]*Intra-islet source material.*

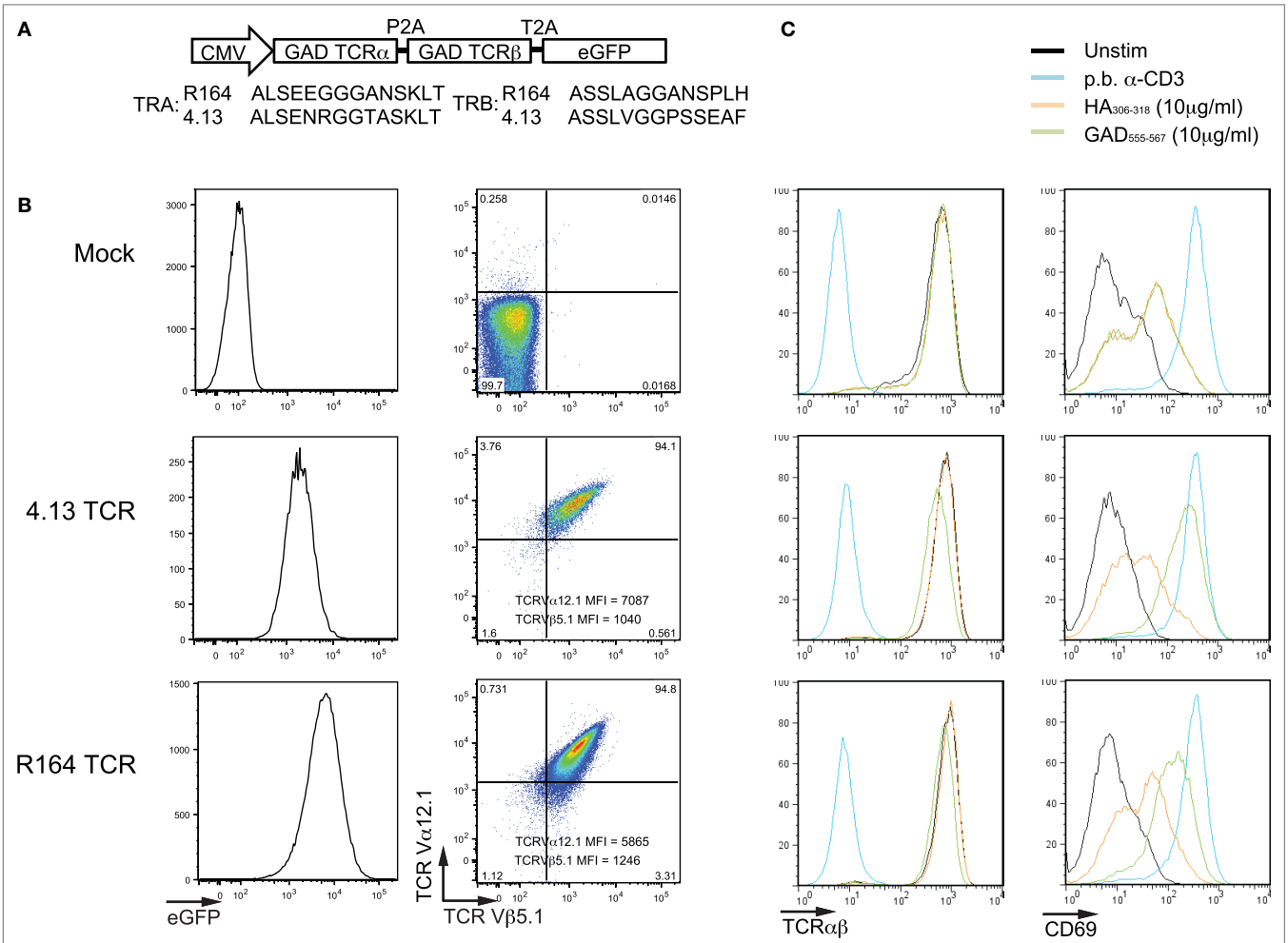

FIGURE 1 | Verifying transfection and activation of Jurkat cells expressing T-cell receptor (TCR) clones. **(A)** Lentiviral constructs were designed containing the TCR α- and TCR β-chain genes (TRA and TRB, respectively) for known glutamic acid decarboxylase (GAD)-reactive clones (R164 and 4.13, additional clone information is listed in **Table 1**). The TRA and TRB coding regions were joined by a multicystronic P2A element, and TRB was linked by a multicystronic T2A element to an enhanced green fluorescent protein (eGFP) reporter. Amino-acid sequences for the complementarity determining region 3 (CDR3) are shown for both clones. **(B)** Jurkat T cells were untransduced (Mock; top), transduced with lentivirus expressing the R164 TCR (middle), and lentivirus expressing the 4.13 TCR (bottom), and expression was confirmed by flow cytometry. Double positivity for TCR Va12.1 and Vβ5.1, which is comparable between both clones, and eGFP indicates successful transduction. Untransduced cells were negative for both markers. **(C)** Mock (top), 4.13 (middle), and R164 (bottom) TCR-transduced cells were unstimulated (black), stimulated with plate bound anti-CD3 (p.b. α-CD3, blue), irrelevant antigen influenza hemagglutinin ($HA_{306-318}$, orange), or cognate antigen ($GAD_{555-567}$, green). TCR expression (left panels) was comparable across all unstimulated, HA-stimulated, and GAD-stimulated cells, and p.b. α-CD3 stimulation induced TCR downregulation. p.b. α-CD3 stimulation also induced the highest level of CD69 expression (right panels), and unstimulated cells exhibited low CD69 expression. These observations were comparable across mock, R164, or 4.13 transduced cells.

were co-transfected in 293T cells using TransIT-2020 transfection reagent (Mirus, Madison, WI, USA). Supernatants were collected 72 h after transfection, filtered through a 0.45-μm filter, and concentrated by ultracentrifugation at 198,000 × *g* for 1.5 h.

Subject Enrollment and T-Cell Isolation

Healthy control blood donors provided written informed consent prior to inclusion in the study in accordance with the Declaration of Helsinki and according to Institutional Review Board-approved protocols at the University of Florida (Protocol no. IRB201600092) and the University of Colorado Denver (Protocol no. COMIRB92-292). T cells where enriched by negative selection from whole blood by Ficoll-Paque density gradient in combination with a total T-cell enrichment cocktail by following manufacturer's instructions (Catalog no. 15061, STEMCELL Technologies, Cambridge, MA, USA). Cells were stained with fluorescently labeled antibodies [CD4-PB (clone RPA-T4), CD8-APC.H7 (SK1), CD25-APC (BC96), CD127-PE (A019D5), and CD45RA-PE-Cy7 (HI100)]. $CD4^+CD25^+CD127^{lo/-}$ Tregs, $CD4^+CD25^-CD127^+CD45RA^+$ naïve Tconv cells, and $CD8^+CD45RA^+$ naïve $CD8^+$ T cells were purified by fluorescence-activated cell sorting (FACS) using a BD FACSAria III (BD Biosciences, San Jose, CA, USA).

Lentiviral Transduction (LV TD) of Human T Cells

Jurkat Cells

Human Jurkat T cells were plated at 2×10^5 cells/well in a 24-well plate and transduced in the presence of protamine sulfate (8 μg/mL; Sigma-Aldrich, St. Louis, MO, USA). Transgene expression was assessed 72 h post-transduction by flow cytometry (**Figure 1**).

Primary Human T Cells

Primary human T cells were transduced as previously described (3). Briefly, FACS-purified $CD4^+$ T cells (total), Tregs, naïve Tconv cells, and naïve $CD8^+$ T cells were plated at 2.5×10^5 cells/well in a 24-well plate. Total $CD4^+$ T cells, naïve Tconv, and $CD8^+$ T cells were activated with anti-CD3 and anti-CD28 dynabeads (Catalog no. 11161D, ThermoFisher Scientific, Waltham, MA, USA), while Tregs were expanded with anti-CD3 and anti-CD28 conjugated microbeads (Catalog no. 130-091-441, Miltenyi Biotec, San Diego, CA, USA) according to the manufacturer's instructions. After 48 h of activation, cells were supplied with protamine sulfate (8 μg/mL) and transduced with 3 TU/cell of lentivirus for TCR expression followed by spinoculation. Total $CD4^+$ T cells were supplied IL-2 (30 IU/mL) every 2–3 days and restimulated with the HLA-DR4 (DRB1*04:01) expressing K562 artificial antigen-presenting cell (aAPC) line and $GAD_{555–567}$ peptide on day 9 and day 16 for serial activation (**Figure 2**). For T-cell subsets, IL-2 (300 IU/mL for Treg; 20 IU/mL for Tconv; 100 IU/mL for $CD8^+$ T cells) was supplied every 1–2 days during expansion (**Figure 3**). K562 aAPCs were kindly provided by Drs. James Riley and Bruce Levine (University of Pennsylvania).

Flow Cytometry

Cells were first stained with live/dead near-IR (Invitrogen) followed by fluorescently labeled antibodies specific for the following surface markers: CD4-PB (clone RPA-T4), CD69-BV711 (FN50), TCR Vα12.1-Alexa Fluor 647 (6D6.6), TCR Vβ5.1-PE (IMMU 157), OX40-APC (ACT35), and CD25-PE (BC96). The TCR Vα12.1 monoclonal antibody was labeled with Alexa Fluor 647 using Zenon labeling kit (ThermoFisher Scientific, Waltham, MA, USA) before staining. Intracellular FOXP3 was stained using a FOXP3-Alexa Fluor 488 (206D) antibody with a FOXP3/transcription factor staining kit (Catalog no. 00-5523-00, ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Flow cytometry data were collected using an LSRFortessa (BD Biosciences) and analyzed with FlowJo software (Tree Star, Ashland, OR, USA).

Treg Suppression Assay

T-cell-receptor-redirected Tregs were FACS-purified based on their eGFP expression and tested for the ability to suppress polyclonal or TCR-transduced Tresp proliferation, as described previously (38). For suppression assays involving polyclonal Tresp, cells were stimulated with 2 μg/mL soluble anti-CD3 (clone Hit3a) and 1 μg/mL soluble anti-CD28 (clone 28.2, BD PharMingen). Proliferation was determined by the incorporation of ^{3}H-thymidine by pulsing cultures with 1 mCi of ^{3}H-thymidine for the final 12–16 h of culture. Plates were harvested on a Packard FilterMate harvester and read on a Packard TopCount Scintillation & Luminescence Counter (Perkin Elmer; Waltham, MA, USA). Interferon-gamma (IFN-γ) was measured from the supernatant by ELISA. For suppression assays involving TCR-redirected Tresp, Tregs expressing the 4.13 TCR were stained with cell proliferation dye eFluor670 (5 μM; Catalog no. 65-0840-85, ThermoFisher Scientific, Waltham, MA, USA), whereas Tresp expressing the 4.13 TCR or a Melan-$A_{27–35}$ reactive MART-1 TCR were labeled with CellTrace Violet (5 μM; Catalog no. C34571, ThermoFisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. Tregs were plated in two-fold serial dilution, co-cultured with Tresp, and activated with the indicated peptide presented by irradiated CD3-depleted peripheral blood mononuclear cells (PBMCs) (HLA-DRB1*04:01 and A*02:01) for 3–4 days. Triplicate cultures were pooled, harvested, stained with live/dead dye and for the surface markers, CD4 and CD8, and then analyzed by flow cytometry as described above. Proliferation was calculated by division and replication index of Tresp cells. Assay conditions are detailed in Table S1 in Supplementary Material.

Statistical Analysis

Data were analyzed by two-way analysis of variance (ANOVA) and graphs prepared using GraphPad Prism version 6 software (La Jolla, CA, USA).

RESULTS

Validation of TCR Expression and Activation in Human Jurkat Cells

Two lentiviral constructs with identical backbone each contained the TCR α- and TCR β-chain genes (TRA and TRB, respectively) for the $GAD_{555–567}$-reactive clones R164 or 4.13 followed by an eGFP reporter sequence as shown in **Figure 1A**. Multi-cystronic and equal molar expression of TCR α- and β-chains is achieved by including P2A and T2A elements between TRA, TRB, and the eGFP reporter. We used lentivirus carrying these constructs to transduce human Jurkat cells and express one of the two *de novo* TCRs. As expected, untransduced cells did not express eGFP, TCR α-chain V gene family 12.1 (TCRVα12.1), and TCR β-chain V gene family 5.1 (TCRVβ5.1), which are common to both R164 and 4.13 clones (**Figure 1B**). Over 94% of Jurkat cells transduced with either the R164 or 4.13 TCR lentiviral construct were double positive for both TCRVα12.1 and Vβ5.1 with comparable mean fluorescence intensity (MFI) (**Figure 1B**). To verify stable transfection and antigen-specific activation of Jurkat cell lines, we stimulated mock ($eGFP^–$), R164, and 4.13 transduced cells with K562 aAPCs loaded with cognate antigen ($GAD_{555–567}$) and evaluated for TCR and CD69 expression levels. Positive and negative controls included stimulation of transduced cells with plate bound anti-CD3 or K562 aAPCs loaded with "irrelevant" antigen influenza hemagglutinin ($HA_{306–318}$), respectively. Compared with unstimulated cells, anti-CD3 induced TCR downregulation concurrent with high expression of the activation marker CD69

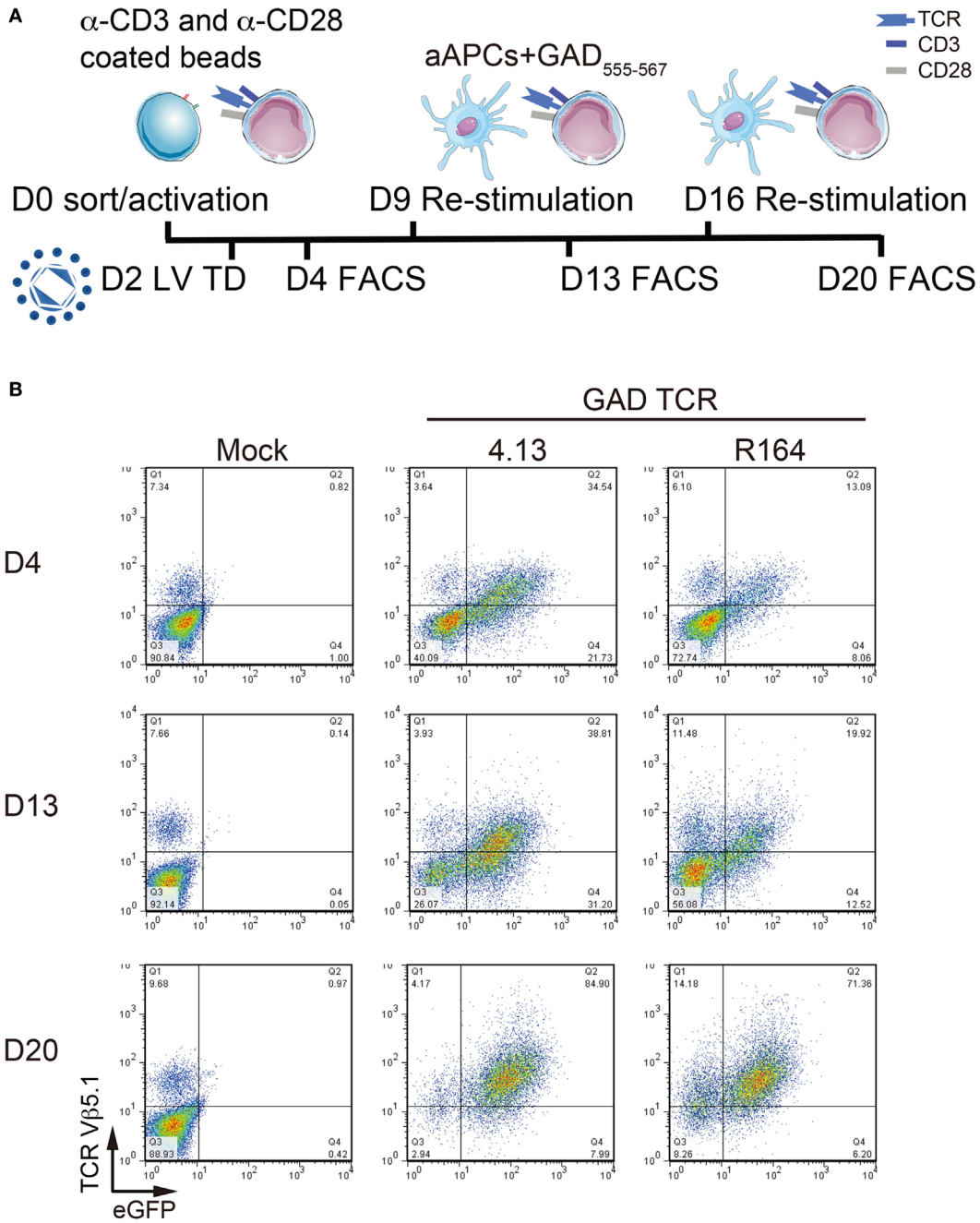

FIGURE 2 | Serial activation increases transduction efficiency. **(A)** Primary CD4+ T cells remain untransduced (Mock) or transduced with lentivirus (LV TD) expressing T-cell receptor (TCR) clones 4.13 or R164 were activated with α-CD3/α-CD28 coated beads on day 0 (D0). Cells were restimulated with artificial APC (aAPCs; K562 cell line expressing HLA-DR4) and $GAD_{555–567}$ peptide for an additional two rounds on day 9 (D9) and day 16 (D16). IL-2 (30 IU/mL) was given every 2–3 days. Transduction efficiency was detected by fluorescence-activated cell sorting (FACS) every 4 days after stimulation (D4, D13, and D20). **(B)** TCR Vβ5.1 and enhanced green fluorescent protein (eGFP) reporter were assessed by flow cytometry on day 4 (D4, top), day 13 (D13, middle), and day 20 (D20, bottom). At each time point, a portion of untransduced cells were positive for TCR Vβ5.1, but no eGFP was observed. TCR Vβ5.1 and eGFP positivity was observed for 4.13 and R164 transduced cells at each time point, and the proportion of dual positive cells increased with time.

in each of the three cell lines (**Figure 1C**). $GAD_{555–567}$ stimulation of both R164 and 4.13 cell lines resulted in high CD69 expression without TCR downregulation, whereas irrelevant antigen resulted in only modest upregulation of CD69, likely due to interaction with the costimulatory molecule, CD80 constituently expressed by the aAPCs (**Figure 1C**). These data support both surface receptor expression and activation in the presence of the cognate peptide presented by HLA-DR4.

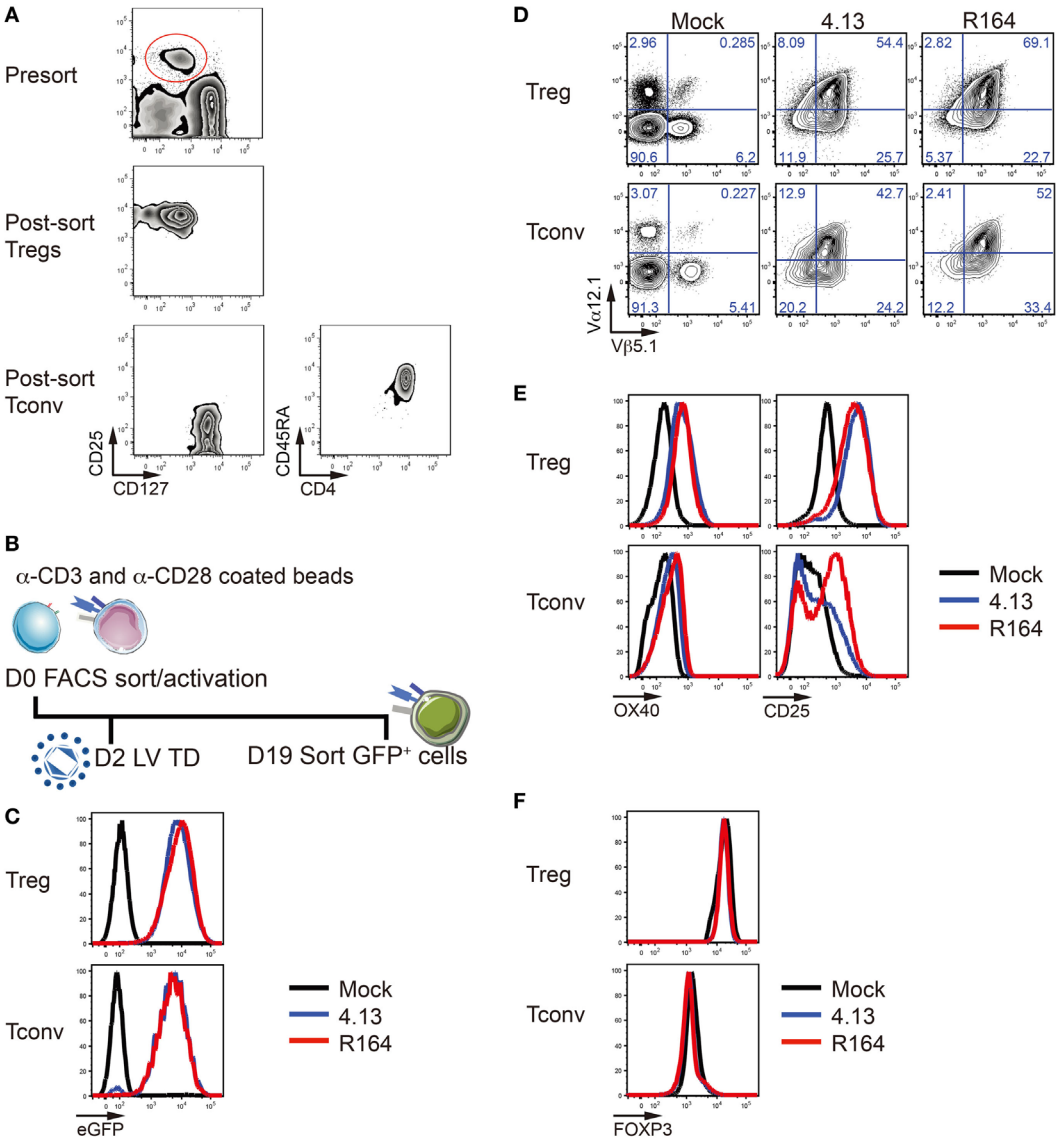

FIGURE 3 | Verification of T-cell receptor (TCR) overexpression and stability of transfected regulatory T cells (Tregs). **(A)** $CD25^{+}CD127^{lo/-}$ Tregs (top and middle) and $CD25^{lo/-}CD127^{+}CD4^{+}CD45RA^{+}$ naïve conventional T cells (Tconv, bottom) were purified from adult peripheral blood by fluorescent-activated cell sorting (FACS). **(B)** Tregs were activated with α-CD3/α-CD28 coated beads on day 0 (D0). Lentiviral transduction (LV TD) was performed on day 2 (D2). Successfully transduced cells expressing enhanced green fluorescent protein (eGFP) were FACS-purified on day 19 (D19) for further analysis. **(C)** Treg (top) and Tconv cells (bottom) were untransduced (Mock; black), transduced with lentivirus containing the expression vector for the GAD-reactive TCR clone 4.13 (blue) or R164 (red), and eGFP expression among TCR transduced cells was confirmed by flow cytometry. **(D)** Mock (left), 4.13 (middle), and R164 (right) transduced Tregs (top) and Tconv cells (bottom) were stained for the TCRVα12.1 and TCRVβ5.1 chains that comprise the TCR clones 4.13 and R164, and overexpression was confirmed for TCR transduced cells. **(E)** Cell activation markers OX40 and CD25 were measured after co-culturing T cells with HLA-DR4 expressing K562 artificial antigen-presenting cells (aAPCs) loaded with GAD $GAD_{555–567}$ peptide for 1 day. **(F)** The majority of Tregs in all conditions (non-transduced, 4.13, or R164) maintain FOXP3 expression (top left). Low frequency of FOXP3 expression was observed in Tconv (bottom left).

Optimizing TCR Expression in Primary Human $CD4^{+}$ T Cells

We next transduced primary human peripheral blood $CD4^{+}$ T cells to express the GAD-reactive 4.13 and R164 TCRs and assessed transduction efficiency. Cells were stimulated with anti-CD3/anti-CD28 coated beads on day 0, transduced on day 2, and restimulated on days 9 and 16 with K562-DR4 aAPCs loaded with $GAD_{555–567}$ peptide (**Figure 2A**). As expected, a portion of untransduced cells expressed TCRVβ5.1 but not eGFP (**Figure 2B**). In addition, 35% of GAD 4.13 and 13% of GAD R164 cells were $TCRV\beta5.1^{+}eGFP^{+}$ on day 4, and by day 20, 85 and 71% of 4.13 and R164 cells, respectively, were double positive for TCRVβ5.1 and eGFP (**Figure 2B**) suggesting that serial activation resulted in enriched T-cell avatars.

TCR Expression in Primary Human Regulatory and Conventional T-Cell Subsets

$CD4^+CD25^+CD127^{lo/-}$ Tregs and $CD4^+CD25^-CD127^+CD45RA^+$ naïve Tconv were FACS-purified from peripheral blood (**Figure 3A**). We then generated primary human Tregs and Tconv expressing the GAD 4.13 and GAD R164 TCRs and expanded them with anti-CD3/CD28-coated beads for 19 days (**Figure 3B**). Again, compared with untransduced cells, 4.13 and R164 cells were confirmed to express high levels of eGFP (**Figure 3C**) as well as the Vα12.1 and Vβ5.1 chains of the GAD-reactive TCRs as measured by flow cytometry (**Figure 3D**). The activation markers OX40 and CD25 were upregulated on 4.13 and R164 transduced Tregs compared with mock transduced Tregs 1 day post co-culture with HLA-DRB1*04:01 expressing K562 aAPC loaded with cognate peptide (**Figure 3E**). Similarly, OX40 was slightly upregulated on the surface of 4.13 and R164 transduced Tconv following aAPC-antigen activation, while CD25 upregulation was more pronounced for R164 Tconv compared with 4.13 Tconv (**Figure 3E**) (39). After transduction and anti-CD3/28 stimulation, Tregs maintained FOXP3 positivity whereas Tconv cells showed low/intermediate expression of FOXP3 (**Figure 3F**) indicating transduction affected neither Treg differentiation nor development.

Suppressive Capacity of R164 and 4.13 Treg Avatars

The capacity to impact type 1 diabetes progression prior to symptomatic onset (i.e., in the context of multiple autoantibody positive high-risk individuals) or at the time of symptomatic disease will likely require the capacity to control a polyclonal memory T-cell response. Depletion of these cells is one potential approach but would require broad targeting resulting in a period of immunosuppression. We hypothesize that tissue targeting and dominant suppression of a broad repertoire by TCR-redirected Tregs may confer persistent tolerance. Therefore, we sought to understand if Treg avatars functionally suppress Tresp in an antigen-specific and/or bystander manner. We first demonstrated that LV TD does not affect Treg capacity to suppress polyclonal Tresp using well-described *in vitro* suppression assays (38). Both proliferation and IFN-γ production by polyclonal stimulated Tresp were comparable between R164, 4.13, and mock transduced Treg groups (Figure S1 in Supplementary Material). Then, we assessed Treg suppressive capacity in both antigen-specific and bystander mechanisms with or without Treg activation (**Figure 4A**). At physiological ratios, Tregs showed excellent suppression of Tresp against cognate antigen by culturing both $CD4^+$ Tresp and Tregs engineered to express a GAD-reactive TCR clone 4.13 and activated with cognate $GAD_{555–567}$ peptide (**Figures 4B,C**, Ag-specific; Table S1 in Supplementary Material). Specifically, Tresp division was significantly blunted, and Treg percent suppression was significantly greater than in settings of bystander suppression (**Figure 4C**).

Importantly, $CD8^+$ T cells are thought to drive type 1 diabetes pathogenesis *in vivo* through the direct killing of β-cells (40). We therefore sought to understand whether Treg avatars are capable of suppressing $CD8^+$ T cells in a bystander manner in the islets or periphery. We tested the capacity of GAD-specific Tregs to suppress MART-1 $CD8^+$ T cells recognizing the tumor antigen Melan-A, with or without Treg activation. While unactivated 4.13 Tregs exhibited limited suppression of MART-1 $CD8^+$ Tresp proliferation, GAD-activation of 4.13 Tregs resulted in significantly reduced Tresp proliferation and increased suppression of MART-1 $CD8^+$ Tresp (**Figures 4B,C**). This supports two notions: first, that Treg activation is required for functional suppression and second, that TCR transgenic Treg avatars are capable of both antigen-specific and bystander suppression.

Finally, we examined if TCR avidity affects Treg suppressive ability with the advantage of using two $GAD_{555–567}$-reactive TCR clones, R164 and 4.13, where R164 exhibits higher avidity. Either GAD R164 or 4.13 Tregs were cultured with R164 $CD4^+$ Tresp in the presence of peptide presented by CD3-depleted APCs from HLA-DRB1*04:01/A*02:01 individuals. We normalized the Treg suppression capacity against reporter eGFP MFI allowing us control for potential variation in TCR expression levels. Indeed, cells expressing the high-avidity R164 TCR were significantly more suppressive than cells expressing the lower avidity 4.13 TCR (**Figure 5**). These data support the notion that Treg avatars engineered to receive a higher affinity signal through the TCR are more efficient suppressors of bystander T-cell responses. It remains to be investigated how costimulatory signals will impact suppressive activity, a notion that may be particularly important for assessing signaling through CAR-T vectors if expressed by Tregs.

DISCUSSION

For tolerogenic adoptive cell therapy, autologous polyclonal Tregs can be expanded from peripheral blood which provides an attractive Treg source given the abundant cell numbers, allowing for repeat dosing if needed, and the safety associated with autologous cell therapy (26, 27). Concerns remain, however, regarding the lack of antigen specificity by administrating polyclonal Tregs. Indeed, Tregs have a highly diverse repertoire (24), which indicates the precursor frequency of autoreactive Tregs will likely be quite low in peripheral blood, especially considering that Tregs do not enrich to the extent that is observed for Tconv during conversion to effector T cells and expansion. Hence, we expect polyclonal Treg therapy to confer potentially limited efficacy and trafficking to the pancreas or PLN to induce immunological tolerance for β-cell antigens. To address this, we utilized LV TD to generate primary human T-cell avatars expressing two $GAD_{555–567}$-reactive TCRs (R164 and 4.13) originally identified from the peripheral blood of subjects with or at risk for type 1 diabetes (21–23). These clones, which differ by only 10 amino acids in TRAV and TRBV genes and only three amino acid charge differences in the CDR3 region (**Table 1**), exhibit different binding affinities for their cognate antigen peptide (21).

Regulatory T cell avatars maintained FOXP3 positivity, indicating that LV TD did not impair Treg stability. Functionally, 4.13 Treg avatars effectively suppressed antigen-specific 4.13 $CD4^+$ Tresp. Beyond this, 4.13 Treg avatars exhibited a moderate ability to suppress MART-1 $CD8^+$ Tresp in a bystander suppressive

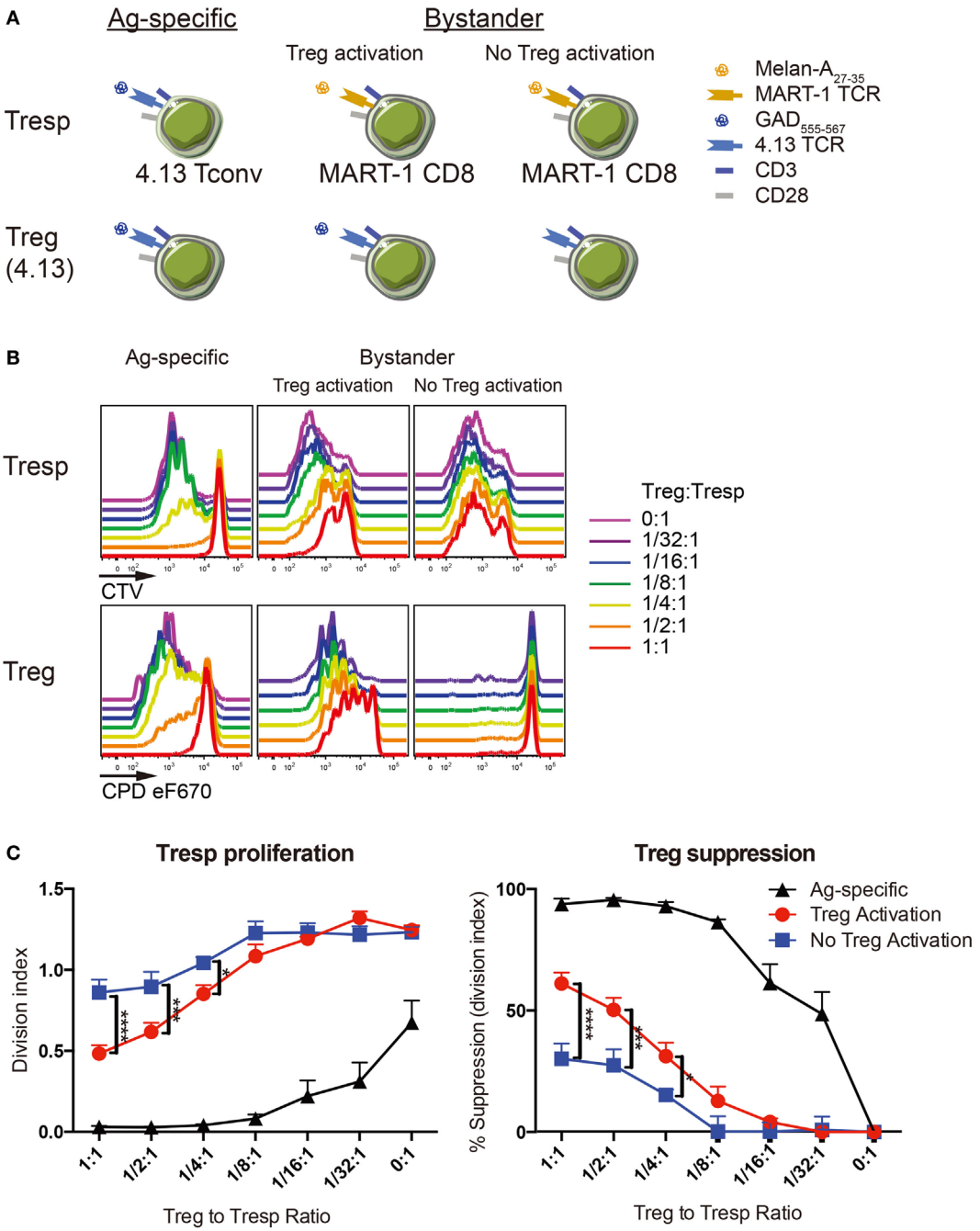

FIGURE 4 | Regulatory T-cell (Treg) suppression is optimal with activation. **(A)** Antigen-specific suppression by 4.13 Tregs was tested on 4.13 T-cell receptor (TCR) transduced conventional T cells (Tconv) *in vitro* (left). Bystander suppression by 4.13 Tregs was assessed on $CD8^+$ T cells expressing the melanoma antigen recognized by T cells 1 (MART-1) TCR, with (middle) or without (right) Treg activation. **(B)** Tregs were isolated from adult peripheral blood and transduced to express glutamic acid decarboxylase (GAD) 4.13 TCR. Transduced Tregs were sorted, labeled with cell proliferation dye (CPD) eFluor670, and plated in decreasing proportions with GAD 4.13 TCR transduced $CD4^+$ responder T cells (Tresp) (Ag-specific) or MART-1 transduced $CD8^+$ Tresp (Bystander) stained with cell trace violet (CTV) dye. For Ag-specific suppression, GAD 4.13 Tresp and Treg were activated with cognate $GAD_{555–567}$ peptide presented by CD3-depleted peripheral blood mononuclear cell (PBMC) from an HLA-DR4 individual. For bystander suppression, MART-1 $CD8^+$ Tresp and GAD 4.13 Tregs were activated with Melan-$A_{27–35}$ with or without $GAD_{555–567}$ peptide, again presented by CD3-depleted PBMC from an HLA-DR4 individual. Cell proliferation was evaluated *via* dye dilution for Tresp (top) and Tregs (bottom). Tresp proliferation decreased as the Treg to Tresp ratio increased only when Tregs were activated, and suppression was most effective when Treg activation was antigen-specific. Unactivated Tregs exhibited little to no proliferation. **(C)** Suppression was evaluated by Tresp division index (left) and percent (%) suppression (right). Tresp division index was significantly lower and percent suppression of Tresp proliferation was significantly greater in antigen-specific settings (Ag-specific, black) followed by bystander suppression when Tregs were activated (red). Two-way analysis of variance (ANOVA) (*$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$).

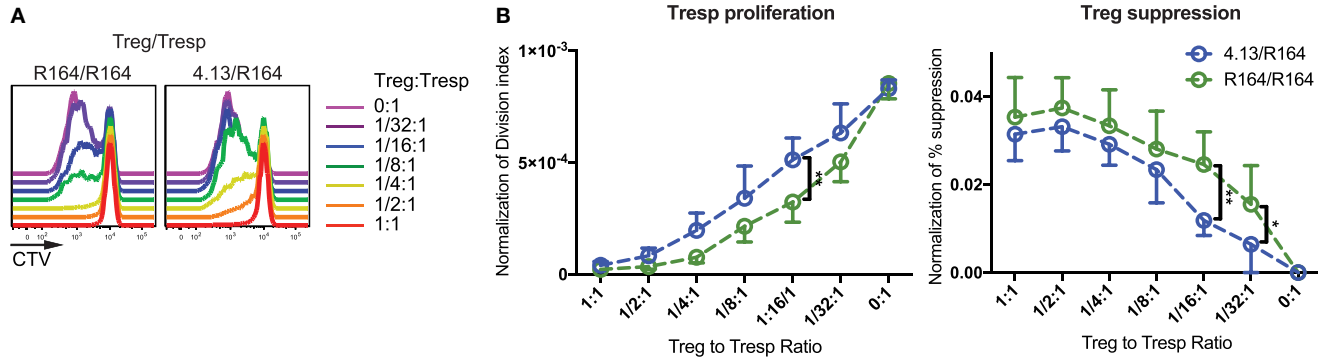

FIGURE 5 | High-avidity T-cell receptor (TCR) activation augments regulatory T-cell (Treg) suppression. **(A)** Tregs expressing the high-avidity $GAD_{555–567}$-reactive R164 TCR (left) or the low-avidity $GAD_{555–567}$-reactive 4.13 TCR (right) were activated with their cognate antigen. Activated Tregs were plated in decreasing proportions with Tresp expressing the R164 TCR and stained with cell trace violet (CTV) dye as indicated in the figure. Tresp proliferation was evaluated *via* dye dilution. Both Treg clones were able to suppress Tresp proliferation at 1:1 cell ratio, but R164 Tregs were more effective in suppressing Tresp proliferation at lower ratios (1/2:1–1/16:1). **(B)** Both Tresp proliferation and Treg suppression were normalized to the reporter enhanced green fluorescent protein (eGFP) mean fluorescence intensity (MFI) to control for potential variation in TCR expression levels. The Tresp division index (left) was significantly lower and percent (%) suppression (right) based on replication index was significantly greater for suppression assays using high-avidity R164 Tregs (green) compared with 4.13 Tregs (blue).

mechanism that required Treg activation. Interestingly, when the high-avidity R164 or lower avidity 4.13 Treg avatars were cultured with R164 $CD4^+$ Tresp in the presence of $GAD_{555–567}$ peptide presented by CD3-depleted HLA-DRB1*04:01/HLA-A*02:01 APCs, R164 TCR were significantly more suppressive. This suggests that Treg TCR avidity affects suppressive ability and importantly, that the optimal avidity of TCR or CAR signals may be required for effective Treg avatar cellular therapy. Importantly, however, there is the potential for heterologous TCR chain pairing with the endogenous receptors, and further experiments are needed to empirically determine this for each receptor. Recent developments in gene editing technologies could be used to correct for this potential caveat. Specifically, knockout of endogenous TCR α- and β-chains *via* CRISPR/Cas9, silencing of endogenous TCR *via* shRNA with expression of a codon optimized *de novo* TCR, or the domain-swap approach described by Bethune et al. (41) could be applied.

Although preproinsulin (PPI) and alternative forms of this antigen (e.g., hybrid insulin peptides, alternative mRNA transcripts) (42, 43) are considered key type 1 diabetes autoantigens, we anticipate continued expression of cognate antigen will be imperative for Treg survival and trafficking to the target organ (15, 44). Hence, we focused our efforts on the development of Tregs against GAD65, which exhibits a high autoantigen density in T1D (45) and is the target of persistent autoimmunity, as evidenced by maintenance of autoantibodies (46). We anticipate that adoptive cell therapy with GAD-specific Tregs will lead to bystander suppression and infectious tolerance (47) with the hope for inducing long-term antigen-specific tolerance to GAD as well as other β-cell antigens. A recent report by Hull et al. described the generation and *in vitro* characterization of peripheral blood-derived human Tregs expressing TCRs specific for insulinoma-associated protein-2 (IA-2) and insulin (48). Although the authors did not conduct functional comparisons of TCR avidity, *in vivo* investigations of these and the GAD-specific clones generated herein are certainly warranted to determine the optimal clone(s) for tolerogenic cell therapy as we move forward toward clinical testing.

Chimeric antigen receptor (CAR) Treg therapy should also be considered given the promising outcomes observed from CAR effector T cells in cancer immunotherapy (49, 50). CAR Treg therapy could be particularly advantageous given that CAR T cells are not constrained by HLA restriction, hence, offering the opportunity for off-the-shelf clinical utility. However, the need for surface expression of the target antigen on islets or β-cells represents a clear limitation compared with TCR gene transfer, which allows for recognition of intracellular antigens in the context of class II HLA. An additional approach to potentially address this challenge could involve the use of a CD8-restricted TCR that functions independently of the CD8 co-receptor. In fact, this type of activity has been demonstrated previously with a high-affinity melanoma antigen tyrosinase-reactive TCR expressed by $CD4^+$ T cells (20). Yet an additional approach could involve the identification of a CAR capable of recognizing an islet epitope in the context of HLA-A2, given the observation that beta cells hyperexpress class I HLA in settings of type 1 diabetes (51).

RNA TCR or CAR gene transfer has been demonstrated as one potential approach to confer T-cell antigen-specificity (52), and could be further explored in the context of tolerogenic adoptive Treg therapy for type 1 diabetes. Specifically, mRNA encoding the TCR or a CAR of choice can be introduced to T cells *via* electroporation, thereby eliminating the need for LV TD and associated safety requirements. This approach would, of course, be transient with transgene expression lasting only a few days (53), but might be accomplished with multiple autologous dosings. Temporary transgene expression presents lower risk of off-target effects such as bystander suppression of anti-tumor or anti-infection immunity. However, lentivirus transduced Treg avatars likely offer greater potential for long-term efficacy in

clearing islet infiltration/inflammation and leading to persistent engraftment.

Importantly, adoptive cell therapy with polyclonal autologous peripheral blood Tregs has been demonstrated to be safe in Phase I clinical trials (26, 27). While we anticipate a similar safety profile with TCR transgenic Treg therapy, tolerogenic cell therapies always carry with them possible associations with increased risk of infection or cancer due to bystander suppression and infectious tolerance mechanisms. Thus, there is a need to perform Phase I safety studies and simultaneously, investigate co-transfection of suicide genes for inducible apoptosis of TCR transgenic Tregs—a biological "off-switch" (54).

We recently demonstrated that cryopreserved umbilical cord blood Tregs (cryoCB Tregs) could be isolated and expanded efficiently while retaining their naïve phenotype as well as suppressive capacity (55). Beyond the possibility for polyclonal autologous cryoCB Treg therapy, these cells offer the potential to generate antigen-specific Treg avatars from precursors with an optimal naïve phenotype and without the need for a large-volume peripheral blood draw and leukapheresis, which is generally contraindicated in pediatric patients. This is a goal currently being pursued by our lab and others. Additional optimization, such as further genetic manipulation of TCR transgenic Tregs, could be implemented to correct intrinsic single-nucleotide polymorphisms (SNPs) with putative implications for Treg function and known associations with type 1 diabetes as identified by genome-wide association studies (GWAS) (56). Beyond this, there is potential for delivery of tissue repair factors directly to the pancreas *via* production by antigen-specific Tregs or *via* conjugation to the Treg surface using poly lactic-co-glycolic acid (PLGA) nanoparticles (57–59). For these approaches to be successful, we expect that Treg survival *in vivo* and trafficking to the target organ will depend largely upon TCR specificity. Hence, we anticipate the functional effects of TCR avidity on human Treg phenotype and function, as demonstrated herein, will be extremely important as we refine adoptive cell therapies to reverse autoimmunity in type 1 diabetes.

ETHICS STATEMENT

Healthy control blood donors provided written informed consent prior to inclusion in the study in accordance with the Declaration of Helsinki and according to Institutional Review Board-approved protocols at the University of Florida (Protocol no. IRB201500059) and the University of Colorado Denver (Protocol no. COMIRB92-292).

GUARANTOR STATEMENT

As the guarantor of this work, Todd Brusko assumes responsibility for ethical completion of the study, integrity of the data, and accuracy of the data analysis reported herein.

AUTHOR CONTRIBUTIONS

W-IY researched and analyzed the data and wrote the manuscript. HS and BN researched the data and reviewed/edited the manuscript. AP and FM contributed to discussion and wrote the manuscript. AM and CM contributed to discussion and reviewed/edited the manuscript. JB conceived of the study and reviewed/edited the manuscript. TB conceived of the study, researched the data, and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

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FIGURE S1 | Comparable regulatory T-cell (Treg) avatar suppression of polyclonal stimulated responder T cells (Tresp). **(A)** Tregs were transduced with GAD-reactive TCR clones (R164 or 4.13) or remained untransduced (Mock) and cultured with autologous polyclonal Tresp cells in decreasing proportions for 4 days with soluble anti-CD3 (2 μg/mL) and anti-CD28 (1 μg/mL) stimulation. Tresp proliferation was assessed by ^{3}H-thymidine incorporation (left), and the percent suppression was determined by upon Tresp division index relative to the proliferation of Tresp when no Tregs were present (right). **(B)** IFN-γ production by polyclonal T cells was inhibited by Treg cells with or without TCR transduction. The levels of IFN-γ were measured from the supernatant by ELISA.

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frontiers in Immunology

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Engineered Tolerance: Tailoring Development, Function, and Antigen-Specificity of Regulatory T Cells

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Regulatory T cells (Tregs) are potent suppressors of immune responses and are currently being clinically tested for their potential to stop or control undesired immune responses in autoimmunity, hematopoietic stem cell transplantation, and solid organ transplantation. Current clinical approaches aim to boost Tregs *in vivo* either by using Treg-promoting small molecules/proteins and/or by adoptive transfer of expanded Tregs. However, the applicability of Treg-based immunotherapies continues to be hindered by technical limitations related to cell isolation and expansion of a pure, well-characterized, and targeted Treg product. Efforts to overcome these limitations and improve Treg-directed therapies are now under intense investigation in animal models and pre-clinical studies. Here, we review cell and protein engineering-based approaches that aim to target different aspects of Treg biology including modulation of IL-2 signaling or FOXP3 expression, and targeted antigen-specificity using transgenic T cell receptors or chimeric antigen receptors. With the world-wide interest in engineered T cell therapy, these exciting new approaches have the potential to be rapidly implemented and developed into therapies that can effectively fine-tune immune tolerance.

Keywords: regulatory T cells, chimeric antigen receptors, T cell receptor, IL-2, autoimmunity, transplantation, inflammatory bowel disease, immunotherapy

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INTRODUCTION

Regulatory T cells (Tregs) are essential to maintain self-tolerance and dampen immune responses during infection (1, 2). The best characterized subset of Tregs is defined by high expression of CD25 and FOXP3, the master-regulator of their phenotype and suppressive function (3). The critical role of FOXP3 in controlling Treg development and function is illustrated by the study of Tregs from patients with immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome (4). Depending on the specific mutation, IPEX patients may or may not have circulating FOXP3$^+$ T cells, but even if FOXP3$^+$ T cells are present, they are functionally defective due to inadequate FOXP3 transcriptional function (5–7).

Mechanistically, Tregs suppress the proliferation and function of many immune cells, even at very low Treg:effector cell ratios (2). In terms of suppressive pathways, multiple possibilities have

Abbreviations: CEA, carcinoembryonic antigen; EAE, experimental autoimmune encephalomyelitis; IPEX, immunodysregulation polyendocrinopathy enteropathy X-linked; HSCT, hematopoietic stem cell transplantation; GVHD, graft-versus-host disease; MOG, myelin oligodendrocyte glycoprotein; T1D, type 1 diabetes; Th, T helper; TNP, 2,4,6-trinitrophenol; Treg, regulatory T cell; Tconv, conventional T cell.

been described, such as immunosuppressive cytokines, contact-dependent cytotoxicity, metabolic disruption, and suppression of antigen presenting cells *via* co-inhibitory molecule expression. Focusing on human Tregs, there is a dominant role for CTLA-4 and TGF-β. Monogenic mutations affecting CTLA-4 or proteins in its pathway affect Treg function (8, 9) and antibodies that block activation of TGF-β by human Tregs prevent their ability to control xenogeneic graft-versus-host disease (GVHD) (10). An additional aspect of Treg mechanisms is their ability to take on characteristics of other T helper (Th) cells (11, 12) resulting in sub-specialization and enhanced suppression of the Th cell subset they mirror (13). Whether or not these sub-specialized Tregs have unique suppressive mechanisms or are simply better able to traffic to the relevant sites of inflammation remains to be defined.

The immunosuppressive properties of Tregs make them attractive candidates for cellular therapy, particularly for application in conditions such as hematopoietic stem cell transplantation (HSCT), solid organ transplantation, and autoimmunity. However, harnessing Tregs for this purpose has not been trivial due to limitations related to cell isolation and expansion. In this review, we summarize the current state of Treg therapy in the clinic and discuss how engineering strategies can be used to improve upon current approaches.

CURRENT Treg CLINICAL TRIALS

There are two main approaches to increase Treg numbers and function: *in vivo* "boosting" using small molecules or proteins and adoptive cellular therapy. To date, the most successful strategy to "boost" Treg *in vivo* is the use of low-doses of IL-2. When given in limiting concentrations, IL-2 preferentially expands $CD25^{hi}$ Tregs without significantly affecting cells expressing low-levels of CD25, such as resting conventional T (Tconv) cells or NK cells. This concept was first tested for treatment of hepatitis-C-virus-induced vasculitis where low doses of IL-2 induced an increase in circulating Tregs and clinical improvements in 8 of 10 patients (14). Subsequently, the beneficial effect of low-dose IL-2 therapy was also observed in GVHD, alopecia areata, type 1 diabetes (T1D), and systemic lupus erythematosus (15–19). However, a cautionary note is that in one study of T1D where IL-2 therapy was combined with rapamycin, there was an unexpected expansion of NK cells and worsening of disease (20). Thus, this approach may need further refinement to reduce the risk of expanding non-Tregs. Low-dose IL-2 and other strategies for *in vivo*-boosting of Tregs are discussed extensively in Zhang et al. and Boyman et al. (21, 22).

An alternate to *in vivo*-boosting is adoptive therapy with *ex vivo*-enriched, often expanded, Tregs. This method aims to overcome defective or low numbers of Tregs by transfer of a large number of Tregs to re-set the Treg:Tconv cell balance. Adoptive Treg therapy has been applied in the clinic for many years. The first successful study reported that chronic GVHD patients treated with Tregs had a significant reduction in clinical symptoms and immunosuppression (23). Subsequently, Treg therapy has been tested in several other GVHD cohorts, overall showing that infusion of autologous or third party (partially HLA-matched) Tregs is well tolerated, does not inhibit graft-versus-leukemia, and may be protective from GVHD (24, 25).

Adoptive transfer of Tregs has also been applied successfully in autoimmunity and organ transplantation. Children with T1D who received Tregs showed slowed disease progression and long-term preservation of residual beta-cells (26, 27). Adoptive transfer of Tregs in adults with T1D is also well tolerated, with evidence that the cells persist long term (>1 year) (28). A clinical trial of *in vitro*-expanded naïve Tregs is also underway in Crohn's Disease, the first application of $FOXP3^+$ Treg immunotherapy for inflammatory bowel disease (IBD) (ISRCTN97547683) (29). In addition, several clinical trials are testing autologous polyclonal or antigen-expanded expanded Tregs in kidney or liver transplantation; these trials are reviewed extensively in Ref. (30–33). To date, all of these studies have shown that adoptive Treg therapy in humans is feasible and safe, and initial data suggest that this approach may also be effective.

ENGINEERING IL-2

With the early success of low-dose IL-2 therapy as an approach to expand Tregs *in vivo*, there are now several efforts to improve upon this approach by modulating the way IL-2 interacts with its receptors. One strategy to modulate IL-2 is to use IL-2/anti-IL-2 monoclonal antibody (mAb) combination therapy to form "IL-2 complexes" that enhance the half-life of IL-2 after intravenous injection and provide preferential selection of certain immune cell subsets. For example, IL-2 in complex with anti-IL-2 mAbs, JES6-1A12 (mouse), or 5344 (human), preferentially expands Tregs, but not other IL-2-dependent cells such as $CD8^+$ T and NK cells (34). This approach enriches Tregs and treats disease in several different mouse models (22, 34). In 2015, Spangler et al. solved the crystal structure of IL-2/JES6-1A12, showing that this IL-2 complex preferentially binds cells with the trimeric IL-2R (CD25, CD122, and common gamma chain) and not dimeric complexes (CD122 and common gamma chain), thus selecting for Tregs because of their constitutive CD25 expression (35).

Another approach to modulate IL-2 is to directly mutate IL-2 itself to change how it interacts with its receptor complex. Specifically, IL-2 "muteins" have alterations in the CD25-binding domain, thus decrease affinity for CD25, and enabling preferential binding to dimeric IL-2R complexes and activation of NK and $CD8^+$ T cells (36–38). There is also much commercial interest in making IL-2 muteins with the opposite effect: IL-2 muteins that preferentially activate Tregs have led to a $400 million investment from Eli Lilly to Nektar Therapeutics and $300 million from Celgene to Delinia to develop this technology (39).

A final approach to modulate IL-2 signaling is to change IL-2R's affinity for IL-2. Specifically, it is well established that single nucleotide polymorphisms in the *CD25* locus are associated with autoimmunity (40–43). Considering the power of CRISPR/Cas9 technology, in the future it could be possible to edit risk alleles of CD25 into protective alleles or otherwise engineer IL-2 signaling pathways to optimize therapeutic Treg function (44).

ENGINEERING Tregs WITH FOXP3

A hurdle in Treg therapy is generating sufficient numbers for clinical application (33). Since activated Tconv cells also express CD25 and FOXP3, and downregulate CD127, isolating Tregs on the basis of CD25 and CD127 alone introduces the risk of co-purifying and co-expanding non-Tregs. One way to overcome this limitation is to isolate naive $CD45RA^{+}CD25^{hi}$ cells from blood to enrich for a more homogeneous population (45, 46). However, this also significantly decreases the number of cells with which a culture can be started. Another potential solution to this problem is to isolate Tregs directly from the thymus for application as a third party cell therapy (47).

An additional approach is to find a way to engineer the desired Treg product. Indeed, the possibility of engineering Tregs *via* over-expression of FOXP3 has been considered since its discovery, with multiple studies showing that viral-mediated overexpression of FOXP3 in mouse or human T cells can induce suppressive function (48). Notably, in order to re-program human T cells into Tregs, FOXP3 has to be expressed at high and stable levels (49, 50); Treg suppressive capacity can be quickly reversed upon removal of FOXP3 (51).

Although FOXP3 is the master Treg transcription factor, evidence that its over-expression alone does not fully recapitulate the Treg gene signature led to the search for other co-factors and the discovery that co-expression of other transcription factors is important for full lineage specification (52). A consideration is whether studies which found that FOXP3 expression alone is not sufficient to induce a complete Treg gene signature considered the time that may be required for epigenetic re-programing to take place. Epigenetic modification and the consequent change in expression of other transcription factors is necessary to stabilize Treg phenotype and function (3). Since these epigenetic changes may require multiple rounds of cell division, re-programing Tconv cells into Tregs may not take place in short-term culture. The first application of FOXP3-engineered Treg therapy will likely happen as gene therapy for IPEX. $CD4^{+}$ T cells from IPEX patients can be efficiently converted into functional and stable Tregs by FOXP3 gene transfer *in vitro* (53, 54). Testing these cells *in vivo* will rigorously determine if they have acquired sufficient Treg function to treat the severe autoimmunity in these patients.

ENGINEERING ANTIGEN-SPECIFICITY

Antigen-specific Tregs have the benefit of being directed toward desired therapeutic antigens, thus increasing their potency up to 100-fold compared to polyclonal Tregs (55). Not only would fewer antigen-specific Tregs need to infused but they would also carry a lower risk of off-target suppression (55, 56). However, antigen-specific Tregs are extremely rare and must undergo significant *in vitro* expansion to achieve clinical doses. Despite this technical barrier, the testing of antigen-specific Tregs is already underway in the clinic in the context of organ transplantation (31).

Engineering antigen-specific Tregs by genomic modification to confer expression of desired transgenic T cell receptors (TCR) or by chimeric antigen receptors (CARs) represents an exciting approach to solve the challenge of the rarity of antigen-specific Tregs (57). Attempts to re-program the specificity of Tregs have been underway for several years. The first application in human Tregs involved gene transfer of a melanoma-specific, MHC Class I-restricted, TCR (58). These human TCR-transduced Tregs proliferated in response to antigen and suppressed antigen-specific Tconv cells *in vitro* and *in vivo*. Similarly, human Tregs transduced with a factor VIII (VIII)-specific TCR suppressed FVIII-specific Tconv cells and anti-FVIII antibody production from primed splenocytes (59). Human Tregs transduced with an islet antigen-specific TCR suppressed antigen-stimulated T cell responses. However, they were less efficient than Tregs expressing a viral antigen-specific TCR (60), possibly due to Treg-specific TCR affinity requirements (61). On the other hand, another study of human Tregs in which multiple class I-restricted TCRs recognizing the same peptide-MHC complex, but with affinities varying up to 3,500-fold, were tested, found TCR affinity had no effect on antigen-specific suppressive function (62). Thus, a consideration for future development of this approach is to find TCRs with an MHC restriction and specificity that would make them applicable in multiple patients, and which possess an optimal affinity for Tregs. TCRs which meet these requirements are most likely to be found in autoimmunity where there are well-known and relatively common MHC-peptide complexes that could be targeted.

CHIMERIC ANTIGEN RECEPTORS

Another approach to engineer antigen-specific Tregs is to use a CAR technology, an idea borrowed from cancer immunotherapy. CARs were first described by Eshhar et al. in 1993 (63) and now being applied in humans for cancer immunotherapy (64–66). CARs give T cells the B-cell-like ability to bind to antigen in an MHC-independent manner. Additionally, the modular design of CARs allows for customization of specific regions, such as the signaling domains, to tailor the desired response from the engineered cell (67).

Over the last decade, a number of publications demonstrated the utility of CARs in Tregs (56). All reports used a standard second-generation design and included the CD28 co-stimulatory domain (**Table 1**) (68). Beginning with mouse models in 2008, Elinav et al. used Tregs from a mouse expressing a transgene for a hapten 2,4,6-trinitrophenol (TNP)-specific CAR (69). They found that transgenic TNP-specific CAR Tregs mediated antigen-specific suppression of effector T cells *in vitro* as well as *in vivo* resistance to colitis. The same group then demonstrated that the TNP-CAR could be introduced into mouse Tregs using retroviral-mediated gene transfer, giving these cells the ability to protect from disease *in vivo* in a dose-dependent manner (70). In a similar system, mouse CAR Tregs specific for a different model antigen, carcinoembryonic antigen (CEA), prevented disease in a model of colitis better than CAR Tregs specific for an irrelevant antigen. Importantly, these CEA-CAR Tregs homed to the location of the antigen (71).

Apart from these studies in the context of IBD, there is currently only one other report of mouse CAR Tregs. Specifically, in 2012, Fransson et al. developed a CAR specific for myelin

TABLE 1 | Summary of salient details from the current chimeric antigen receptor (CAR) regulatory T cells (Treg) publications.

Antigen and model disease	CAR structure	Species and expression system	Effects of CAR Treg therapy and points of significance	Reference
TNP Colitis	Hinge: CD28 TM: CD28 Co-stim: CD28 ITAMs: FcRγ	Mouse Transgene	– Protect from TNBS colitis – Bystander suppression of oxazolone-induced colitis – CD28 signaling required for CAR Treg function – *In vivo* imaging of Treg trafficking to site of inflammation	(69)
TNP Colitis	Hinge: CD28 TM: CD28 Co-stim: CD28 ITAMs: FcRγ	Mouse Retrovirus	– *Ex vivo* expansion through cognate antigen – Protect from TNBS colitis	(70)
Carcinoembryonic antigen (CEA) Sarcoma	Hinge: IgG Fc[a] TM: CD28 Co-stim: CD28 ITAMs: CD3ζ	Human Retrovirus	– Suppression of CEA-specific antitumor response in humanized mouse model	(72)
Myelin oligodendrocyte glycoprotein Experimental autoimmune encephalomyelitis (EAE)	Hinge: IgG Fc[a] TM: CD3ζ Co-stim:CD28[b] ITAMs: CD3ζ	Mouse Lentivirus	– Dual expression system of FOXP3 and CAR – Reversal of EAE clinical symptoms, given at peak of disease	(73)
CEA Colitis	Hinge: IgG Fc[a] TM: unknown Co-stim: CD28 ITAMs: CD3ζ	Mouse Retrovirus	– Protect from CEA-CAR T effector cell induced colitis – *In vivo* imaging of Treg trafficking to site of inflammation – Presence of CAR-specific antibodies correlated with disappearance of CAR Tregs	(71)
HLA-A2 Transplant rejection	Hinge: CD8α TM: CD28 Co-stim: CD28 ITAMs: CD3ζ	Human Lentivirus	– CAR-stimulated Tregs maintain stable phenotype – Suppression of alloantigen-specific T cells *in vitro* – Prevention of xenogeneic GVHD *in vivo*	(74)
HLA-A2 Transplant rejection	Hinge: CD28 TM: CD28 Co-stim: CD28 ITAMs: CD3ζ	Human Lentivirus	– Prevention of skin allograft rejection in humanized mouse model – Partial effect of CAR-lacking CD28 and CD3ζ intracellular signaling domains	(75)
HLA-A2 Transplant rejection	Hinge: CD8α TM: CD8 Co-stim: CD28 ITAMs: CD3ζ	Human Retrovirus	– Prevention of skin allograft rejection in humanized mouse models – CAR specificity tested against a panel of HLA-typed cells	(76)
Factor VIII Hemophilia A	Hinge: IgG Fc[a] TM: CD28 Co-stim: CD28 ITAMs: CD3ζ	Human Retrovirus	– CAR directed against clinically-relevant soluble antigen – Suppression of recall antibody responses – Direct comparison between CAR and T cell receptor engineered Tregs	(77)

A summary of the key features of the types of CARs that have been tested in Tregs. To date all CARs have utilized the CD28 co-stimulatory domain, but there are variations in the hinge and transmembrane (TM) regions employed. CARs containing Immune Tyrosine Activation Motifs (ITAMs) either from the FcRγ or CD3ζ proteins have been tested. All studies report superior effects of antigen-specific CAR Tregs compared to polyclonal or non-specific CAR Tregs.

[a]*Hinge region presumed to be derived from IgG Fc.*

[b]*This CAR encoded CD3ζ amino-terminal to CD28.*

oligodendrocyte glycoprotein (MOG), the disease-causing agent for experimental autoimmune encephalomyelitis (EAE) (73). In this study, instead of isolating $CD25^+FOXP3^+$ Tregs, lentivirus was used to ectopically express FOXP3 and enforce a Treg phenotype. The resultant MOG-specific CAR Tregs suppressed responder T cell expansion *in vitro* and reversed symptoms of EAE. Overall, these publications provided important proof-of-concept data supporting the development of CAR Tregs for use in human cells.

Several publications have demonstrated the application of CAR technology to human Tregs. Three reports investigated the utility of expressing a CAR specific for HLA-A*02:01 (A2) to test whether CAR Tregs could be a new approach to control alloreactive T cells that cause rejection in HSCT and solid organ transplantation (74–76). The first publication showed that A2-CAR Tregs are activated and proliferate when stimulated through the CAR *via* coculture with A2-expressing cells (74). Additionally, A2-CAR Tregs prevented engraftment of $A2^+$ PBMCs and development of xenogeneic GVHD in a humanized mouse model. Two other groups confirmed this approach, showing that A2-CAR Tregs suppress alloimmune responses better than polyclonal Tregs in humanized mouse models of $A2^+$ skin xenografts (75, 76). A2 is an ideal antigen to target with CAR Tregs because it is broadly applicable in the transplant setting due to its high allelic frequency, meaning that a significant proportion of organ transplants could potentially benefit from this therapy (74). Moreover, HLAs in general are likely good targets for CAR Tregs since they are a membrane-bound protein specifically expressed on the transplanted tissues.

Yoon et al. reported the characterization of human CAR Tregs that target FVIII, the protein lacking in hemophilia which is immunogenic in patients receiving FVIII replacement therapy (77). Of specific interest from this study is the finding that a CAR specific for soluble antigens is suitable for use in Tregs, widening the possible antigen-targets that could be considered. This study also demonstrated that both T cell and antibody responses can be controlled by CAR Tregs. Also of note is that this study directly compared the effects of TCR versus CAR-engineered Tregs, finding that antibody recall responses were more effectively controlled by TCR-transgenic Tregs. More research is required to explore similarities and differences between TCR- and CAR-activated Tregs to better understand the affinity requirements and limitations of each approach (**Table 2**).

HOW MIGHT CAR Treg BEHAVE IN HUMANS?

Many of the fundamental properties of Tregs are similar to Tconv cells so it may be possible to predict some aspects of *in vivo* Treg behavior on the basis of findings from CAR Tconv cells used in the oncology field. However, Tregs also have many unique properties, such as their strict dependence on other cells for IL-2 and constitutive expression of inhibitory proteins such as CTLA-4 and TGF-β. Thus, there is a need for more detailed studies in animal models to fully appreciate the similarities and differences between the two cell types. For example, will CAR Tregs be able to persist long term even if their antigen is not available? Some research has shown that Tregs have different activation requirements than Tconv cells (62, 78), meaning that optimal proliferation and long-term persistence may require Treg-specific CAR design. Will CAR Tregs traffic to the necessary locations and mediate tolerance? CAR Tconv cells have been found to traffic to the lungs before moving to secondary lymphoid organs and disease sites, delaying their tumor-killing effect (79, 80). If there is similar phenomenon with Tregs then regional cell delivery may be preferred (79). Will CAR Tregs induce tolerance, and if yes, what molecular mechanisms will be necessary? CAR-activated Tregs upregulate CTLA-4, LAP, GARP, and CD39 (74), but it is unknown which pathway(s) are necessary for CAR Treg-mediated suppression. Further, what is the primary target of CAR Treg-mediated suppression? It is unknown whether CAR Tregs suppress immune cells at the site of inflammation, in secondary lymphoid organs, or both. Dissecting the mechanisms important to CAR Treg function may also provide clues as to their primary mode and location of immune suppression. Many of these questions are ideally suited for study in models of transplantation where similar questions with polyclonal or transgenic Tregs have been addressed (55).

NEXT STEPS: WHERE WILL ENGINEERED Treg THERAPIES GO FROM HERE?

Many clinical trials with low-dose IL-2 therapies, expanded polyclonal and antigen-specific Tregs for use in autoimmune diseases, HSCT and solid organ transplantation are underway (18, 31, 33). While initial reports from these trials show that the treatments are well tolerated, the aggregate safety and efficacy data from each approach will greatly inform future studies. Notably, the possible long-term effects, and in particular the potential risk of cancer and infection, of these treatments will not be known for a significant period of time.

We predict that in the next ~5 years there will be a rapid transition from the rather crude current approaches with unmodified IL-2 and/or polyclonal Tregs to engineered approaches that enable precise control over the desired effect (81). It is likely that, as for low-dose IL-2 and polyclonal Treg therapy, transplantation will lead the way in testing these new engineered approaches. HSCT is a setting with a wealth of experience in using engineered T cells for cancer and it would be a natural transition to test engineered Tregs in this context. Moreover, in solid organ transplantation allogeneic HLA antigens represent an ideal target for antigen-specific Tregs because they are only expressed on the transplanted

TABLE 2 | Comparison of the benefits and limitations of engineering regulatory T cells (Tregs) to express a defined T cell receptor (TCR) versus chimeric antigen receptor (CAR), see also Harris and Krantz (57).

TCR	CAR
Pros:	*Pros:*
✓ "Natural" protein; engineered cells should not be immunogenic	✓ MHC-independent antigen detection of soluble or cell-surface antigens
✓ Recall responses of TCR-transgenic Tregs may be more effective than CAR Tregs	✓ Modular design enables more precise control over the type of antigen-stimulated response
✓ Designed to detect intracellular antigens	✓ Hinge region provides flexibility, allowing CARs to bind antigen in a variety of orientations
✓ Low affinity but high antigen sensitivity; fewer number of antigens required for TCR activation than CAR activation	✓ Higher antigen affinity than TCRs
Cons:	*Cons:*
– MHC-dependent peptide detection; each TCR complex has limited patient applicability	– "Unnatural" peptide sequence; construct may be immunogenic and limit ability to administer repeat doses
– May require a large library of several TCR genes to adequately cover MHC/peptide complexes for one disease	– Ability to detect cell-surface antigens may be blocked by the presence of competing soluble antigen
– Mispairing with endogenous TCRs could create new specificities and reduce efficacy	

issue, minimizing the risk of off-target suppression (56). Additionally, since solid organ transplant donors and recipients are usually not HLA-matched, there is a large pool of patients that could benefit from this treatment. CAR targets for autoimmunity will be more difficult to identify because there are few truly organ and/or cell-specific antigens that would be suitable CAR targets. This challenge is similar to that faced in oncology, where off-target effects of CAR T cells can have devastating consequences (67, 82). The field of engineered Tregs will benefit greatly from the huge resources being invested into solving this problem in oncology (64–66), creating an ideal landscape to support the rapid development of this next generation of Treg therapies.

AUTHOR CONTRIBUTIONS

ND, JV-S, and ML reviewed the literature and wrote and revised the manuscript.

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Engineering Specificity and Function of Therapeutic Regulatory T Cells

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Adoptive therapy with polyclonal regulatory T cells (Tregs) has shown efficacy in suppressing detrimental immune responses in experimental models of autoimmunity and transplantation. The lack of specificity is a potential limitation of Treg therapy, as studies in mice have demonstrated that specificity can enhance the therapeutic potency of Treg. We will discuss that vectors encoding T cell receptors or chimeric antigen receptors provide an efficient gene-transfer platform to reliably produce Tregs of defined antigen specificity, thus overcoming the considerable difficulties of isolating low-frequency, antigen-specific cells that may be present in the natural Treg repertoire. The recent observations that Tregs can polarize into distinct lineages similar to the Th1, Th2, and Th17 subsets described for conventional T helper cells raise the possibility that Th1-, Th2-, and Th17-driven pathology may require matching Treg subsets for optimal therapeutic efficacy. In the future, genetic engineering may serve not only to enforce FoxP3 expression and a stable Treg phenotype but it may also enable the expression of particular transcription factors that drive differentiation into defined Treg subsets. Together, established and recently developed gene transfer and editing tools provide exciting opportunities to produce tailor-made antigen-specific Treg products with defined functional activities.

Keywords: regulatory T cells, gene therapy, immunotherapy, chimeric antigen receptor, T cell receptor, autoimmunity

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INTRODUCTION

Inherent checkpoints ensure that an immune response normally only occurs in response to genuine threats from pathogens. However, loss of this self-tolerance and resultant autoimmunity does occur, with prevalence as high as 12.5% in developed countries (1). The life-long chronic nature of both disease and treatment, and the high association of comorbidities (2) means the impact of autoimmunity on patients, their families, the health service, and the economy is substantial (3). The ultimate aim of autoimmune therapy would be to restore the lost self-tolerance while retaining the full potential of the immune system to respond to infection.

Regulatory T cells (Tregs) are an essential component of maintaining normal self-tolerance (4). Tregs possess powerful multifaceted suppressive mechanisms capable of controlling a broad range of innate and adaptive immune cells. Importantly, Treg-mediated suppression is exerted in a targeted antigen-specific manner, allowing for suppression of the immune response when appropriate without interfering with productive immunity when required (5). A number of approaches have been explored to boost Treg number and function in order to treat autoimmune disease. One of the most promising and actively explored of these at present is the adoptive transfer of Tregs. Augmenting Treg numbers by transferring an activated/expanded population of Tregs can ameliorate autoimmunity

(6–8). However, the ability of disease-targeted Tregs to reverse ongoing autoimmunity, where high doses of polyclonal Tregs failed, is a strong indication that merely boosting numbers will not be sufficient to control disease (9–12). Appropriate disease-targeted antigen specificity is important to ensure that Tregs are localized and activated at the site of disease (13).

Achieving antigen specificity in a clinically applicable setting has been a major challenge in translating promising pre-clinical results to therapy. Treg specificity is determined by the T cell receptor (TCR) expressed on their surface. While it may be possible to expand the rare Treg clones with appropriate specificity to suppress disease, this process is prolonged, expensive, and has a number of conceptual issues, not least whether the appropriate disease-suppressing clones are present in autoimmune patients. To circumvent these problems, we, and others, have explored redirecting the specificity of bulk Treg populations by the gene transfer of a disease-relevant TCR (9). This process involves the genetic engineering of Treg with genes encoding TCR or chimeric antigen receptors (CARs) to target Treg specificity to antigens that are present at the sites of autoimmunity and absent in healthy tissues. This approach provides a mechanism to achieve disease-specific immune suppression while retaining systemic immune competence.

REDIRECTING THE SPECIFICITY OF Tregs USING GENE THERAPY

We were among the first laboratories to use TCR gene therapy to generate antigen-specific primary Treg with the capacity to mediate immune suppression *in vivo*. Murine $CD4^+$ $CD25^+$ Tregs were engineered to express a TCR that recognized a fragment of the ovalbumin (OVA) protein. When TCR-transduced Tregs were cultured with dendritic cells presenting OVA, engineered cells were capable of suppressing proliferation and IL-2 production by conventional T cells activated by a different antigen. These findings were validated *in vivo* using a model of autoimmune inflammatory arthritis showing that the presence of OVA, a non-disease causing antigen, in the knee was required for OVA-specific Treg to suppress inflammation caused by pathogenic T cells specific for arthritic antigens (9). The capacity of antigen-specific Treg to locally suppress pathogenic T cells with different specificities provides a strategy to treat autoimmune disease even when the target antigens that are recognized by the autoimmune T cells are unknown. Studies of human cells have shown that Tregs transduced with a TCR recognizing factor VIII, a clotting factor that often stimulates immune responses in hemophilia patients treated with recombinant protein, were able to suppress factor VIII-specific helper T cell responses (14). Similarly, TCR-transduced Treg specific for a pancreatic islet cell antigens were shown to suppress responses by pathogenic T cells with greater potency than polyclonal Treg *in vitro* (15).

As an alternative to the use of TCR gene transfer, a number of groups have explored transfer of CARs. CARs are a man-made alternative to TCR, made up of the antigen-binding domain of a specific antibody linked *via* an extracellular stalk to intracellular signaling motifs required for T cell activation. While TCR have the ability to recognize any cellular proteins when processed and presented by MHC molecules, CARs recognize only cell surface proteins. However, CARs have the advantage that recognition is independent of MHC and, therefore, applicable to patients irrespective of their MHC genotype. The intracytoplasmic portion of CARs contains signaling domains derived from molecules that are involved in T cell activation such as CD3ζ, CD28, 41BB, OX40, and others. In the setting of cancer immunotherapy, various combinations of signaling domains have been tested in second- and third-generation CAR constructs (16). At present, there is little experimental data about which combination of signaling domains may stimulate optimal Treg function, and it is not known whether anti-cancer effector T cells and suppressive Treg will require CARs with distinct intracellular signaling domains.

The efficacy of CAR-Treg has been demonstrated in studies of murine intestinal inflammation. Two groups have shown successful generation of CAR-Treg that maintain their phenotype when expanded, traffic to the gut and suppress inflammation in an antigen-dependent manner independent of MHC (17, 18). More recent studies have shown that factor VIII-specific human CAR-Treg function comparably to factor-VIII-specific TCR engineered Treg (19) and that human CAR-Treg specific for alloantigens can prevent graft rejection (20) and development of graft-versus-host disease (21) in xenogeneic transplantation models.

STRATEGIES TO IDENTIFY THE MOST APPROPRIATE CELL FOR GENE ENGINEERING

It has become apparent that Treg heterogeneity extends beyond the well-defined thymic and peripherally induced subsets and represents populations of suppressive cells with multiple functions, niches, and genetic landscapes. FOXP3 is considered a master transcriptional regulator of Treg function because humans and mice that lack this gene also lack a functional Treg compartment and go on to develop an autoimmune-like disease (4). However, it has become clear that FOXP3 expression is not sufficient to imprint a stable and fully functional Treg phenotype. The discovery of 300 uniquely demethylated regions in Treg genes, known as the Treg-specific demethylated regions (TSDRs) offered fundamental insights into how a Treg phenotype is established. TSDRs were found to be specific to natural Treg (nTreg); the same markers were absent in *in vitro* generated induced-Treg, in $FOXP3^+$ conventional T cells and in various helper T cell subsets (22, 23). This suggests that TSDRs have a Treg-specific role independent of FOXP3 expression. Interestingly, this TSDR profile was identified in a subset of cells from scurfy mice, a naturally occurring FOXP3-deficient strain, and it was found that these $TSDR^+$ cells failed to suppress T cell responses *in vitro* but were less likely than $TSDR^-$ cells to contribute to autoimmunity when adoptively transferred *in vivo* (24). Thus, a functional and stable Treg must express FOXP3 and have a distinct hypomethylation profile.

Examination of Treg markers, function, and hypomethylation led to the identification of three subsets of FOXP3-expressing cells in the peripheral blood of humans (25) (**Figure 1**). $FOXP3^{hi}$ $CD45RA^-$ Tregs have been described as activated-Treg. These

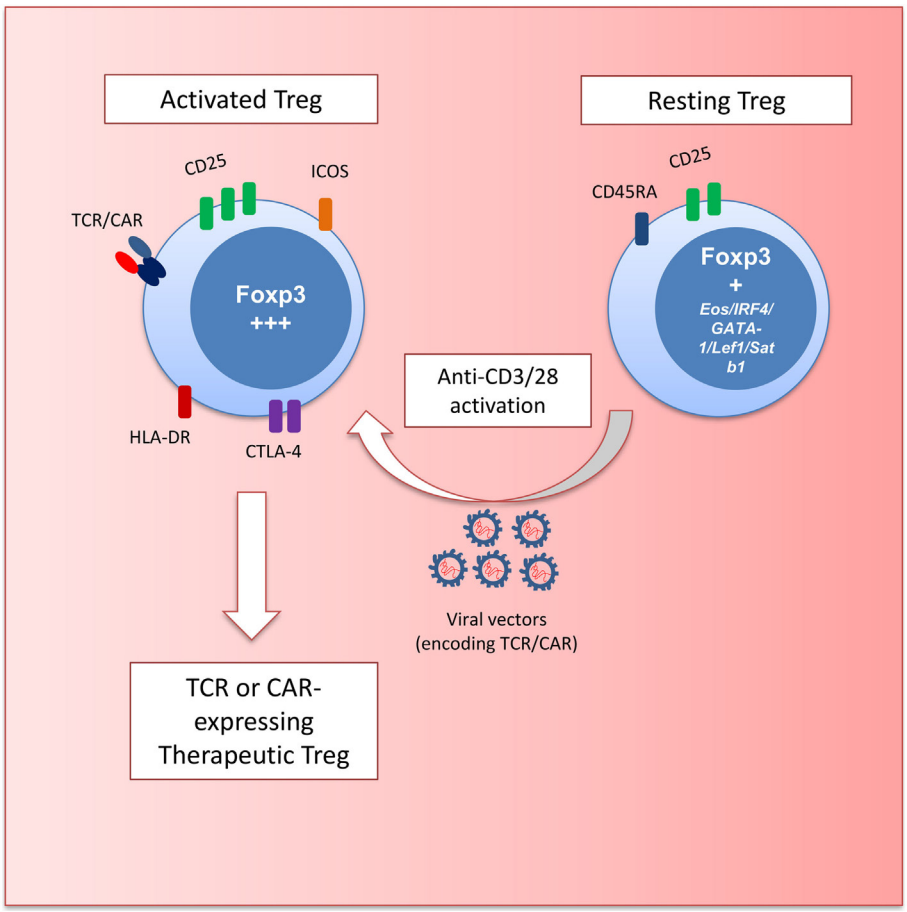

FIGURE 1 | Schematic depicting regulatory T cell (Treg) gene engineering. Resting Treg (CD45RA$^+$FOXP3$^+$) are activated with anti-CD3 and anti-CD28 antibodies to transduce cells with retro- and lentiviral vectors encoding T cell receptors (TCRs) or chimeric antigen receptors (CARs). These engineered Treg have a defined specificity and an activated effector phenotype (HLA-DR$^+$ICOS$^+$CTLA-4^{hi}CD25^{hi}FOXP3^{hi}) with potent suppressive potential.

cells can be terminally differentiated and prone to apoptosis but are hypomethylated and highly suppressive. FOXP3^{lo} CD45RA$^+$ cells are considered resting Treg. These cells also bear the Treg hypomethylation pattern and differentiate into an activated-Treg when stimulated. FOXP3^{lo} CD45RA$^-$ cells are non-Tregs that do not display TSDR hypomethylation or suppressive function and produce inflammatory cytokines upon stimulation.

The most promising starting population for Treg engineering is the Foxp3-expressing CD45RA$^+$ cells. These cells can efficiently expand *in vitro* (26) while maintaining their suppressive function (27). Currently used gene transfer protocols with retro- or lentiviral vectors involve stimulation with beads coated with anti-CD3 and anti-CD28 antibodies to trigger T cell proliferation that is required for efficient gene transfer (**Figure 1**). Thus, the ability of CD45RA$^+$ Tregs to proliferate without losing functional activity provides a strong rationale for using these cells for genetic engineering.

In addition, use of rapamycin, an inhibitor of the mammalian target of rapamycin (mTOR) pathway, may improve the production of therapeutic Treg. Several publications have shown that rapamycin promotes the expansion of stable Treg subsets *in vitro*, maintaining hypomethylation at TSDRs over multiple rounds of expansion (28, 29). Data from our lab have previously shown successful reduction of mTOR activation in T cells engineered to express the proline-rich Akt substrate of 40 kDa (PRAS40), a negative regulator of the mTOR pathway (30). Genetic modification of Treg to express PRAS40 could be employed to ensure the maintenance of a Treg phenotype *in vivo*.

CONVERTING CONVENTIONAL T CELLS INTO Tregs THROUGH GENE ENGINEERING

From the first reports describing Tregs that had been generated from conventional T cells *in vivo*, there have been attempts to replicate this for therapeutic use. The large pool of peripheral T cells makes the proposition of converting these cells into a population of suppressive cells attractive. Unlike protocols that use *in vitro* stimulation to induce Treg, gene therapy offers the prospect of converting cells into a stable population of "Treg-like" cells through genetic reprogramming.

We have previously shown that cotransfer of a FOXP3 gene construct with TCR can redirect the specificity and phenotype conventional T cells in mice (9). In these cells, expression of FOXP3 correlated with the upregulation of Treg-associated markers. Compared to conventional T cells transduced with TCR, cells transduced with TCR and FOXP3 were hypo-responsive to cognate peptide. Examination of suppressive function of these cells *in vitro* and *in vivo* showed that TCR plus FOXP3-converted T cells were able to suppress immune responses by T cells specific for a third party antigen, but that they were less potent than TCR-transduced nTreg.

The difference between engineered nTregs and FOXP3-converted T cells expressing the same TCR may lie in the requirement of non-FOXP3 factors to stabilize the Treg phenotype. Experiments in which conventional T cells were transduced with FOXP3 showed an induction of a partial Treg gene profile that could be stabilized by co-transfection with one of five transcription factors Eos, IRF4, GATA-1, Lef1, or Satb1 (31). It would be interesting to determine if the transfer of genes for FOXP3 plus one of the five transcription factors listed above would make the function of FOXP3-converted T cells comparable to TCR-transduced nTreg.

FUTURE PROSPECTS: ENGINEERING DISEASE-SPECIFIC Tregs

Heterogeneity of Treg is considered to be a relatively new finding but we have known for some time that there is a wide range of suppressive mechanisms utilized by Tregs that may be context dependent. Better understanding of the factors that mediate this heterogeneity could lead to the development of disease-specific Treg that target distinct inflammatory processes.

It is now clear that Treg undergo differentiation into an effector phenotype expressing distinct transcription factors, chemokine receptors, and displaying different antigen recall responses (32). T-bet-expressing Tregs differentiate in parallel with T helper (TH)1 cells; they express CXCR3 and are required for competitiveness at IFNγ-rich sites and for homeostasis of

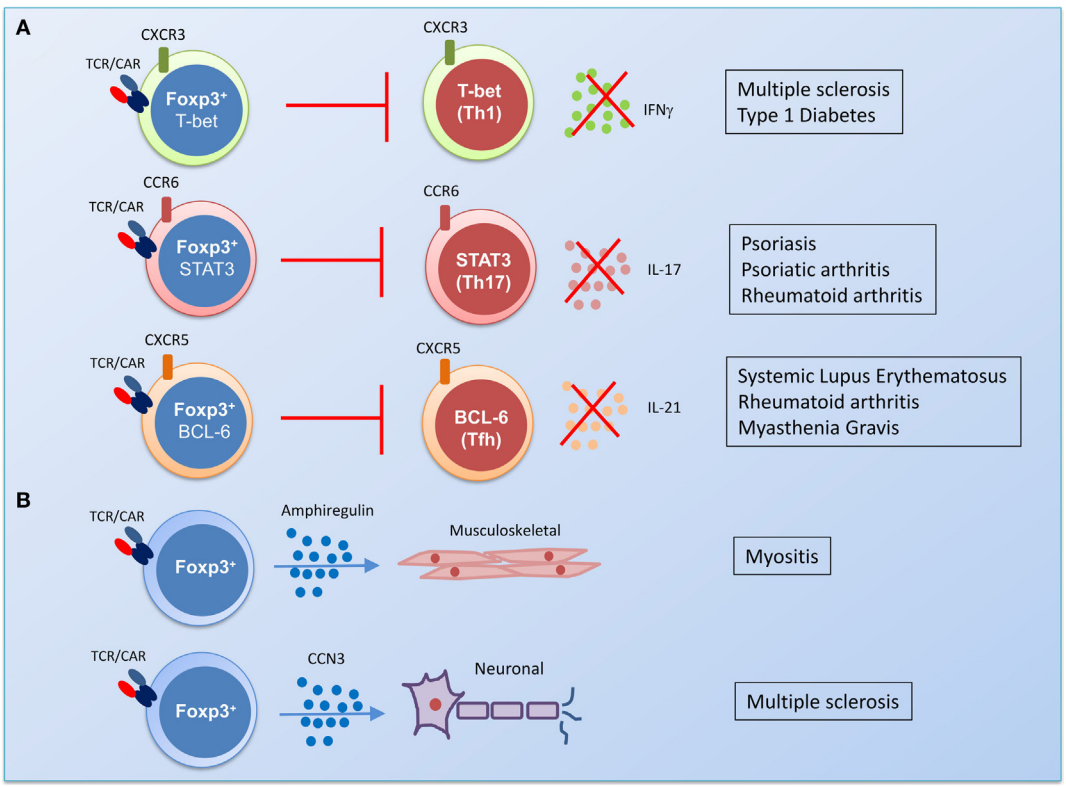

FIGURE 2 | The potential of gene engineering to produce functionally specialized disease-suppressing regulatory T cells (Tregs). **(A)** The identification of transcription factors that drive differentiation of an effector Treg population in parallel with pathogenic T helper (TH) cells could be harnessed by gene therapy. In a predominantly TH1-driven chronic disease such as multiple sclerosis or type I diabetes, transduction of Treg with T cell receptor (TCR) or chimeric antigen receptor (CAR) and the transcription factor T-bet could generate antigen-specific Treg with the capacity to control TH1 responses *in vivo*. In rheumatic diseases, transduction of antigen-specific Treg with STAT3 could promote control of pro-arthritogenic TH17 responses. Antibody-driven diseases, such as systemic lupus erythematosus, rheumatoid arthritis, and myasthenia gravis, could be targeted by antigen-specific Treg that express the transcription factor associated with follicular helper T cells, BCL6. **(B)** Gene therapy could also be used to target the damage caused by chronic inflammation by transducing Treg with genes for factors that promote homeostatic tissue repair. Amphiregulin-producing Treg are enriched in the muscle and have been shown to promote repair of damaged tissue (38–40), while the production of the protein CCN3 by Treg has been shown to promote the repair of the myelin sheath in a mouse model of multiple sclerosis (41).

Treg in a model of TB infection. Similarly, in an experimental transplant model, IFNγ was required for promoting the survival of alloantigen-specific Th1-like Treg (33). Further studies have shown that upregulation of STAT3 expression is associated with increased CCR6 and the control of TH17 responses (34) and BCL6 expression is required for CXCR5 on Treg and suppression of follicular helper T cell responses (35–37). Thus, genetic engineering of Tregs with these transcription factors in the relevant autoimmune setting might facilitate successful trafficking of Treg to the site of inflammation and promote effective control of pathogenic responses (**Figure 2**).

However, a number of papers have highlighted that while Tregs expressing transcription factors associated with TH phenotypes are functional, these cells have a high proportion of inflammatory cytokine-producing T cells (32, 42, 43). Moreover, it has been suggested that these cells are not stable and that TCR stimulation can lead to their conversion into pathogenic effector cells (43, 44). Supporting this finding are data describing an increase in IFNγ-producing Tregs in patients with multiple sclerosis and type-1 diabetes. In these individuals, production of IFNγ by Tregs was associated with expression of T-bet and with an impaired suppressive function compared to non-IFNγ-producing cells (44, 45). Thus, the capacity of Treg to adopt an effector function could potentially contribute to pathogenesis.

Beyond the broad transcriptional approaches mentioned above, a more nuanced approach in which Treg are genetically modified to secrete regulatory effector molecules or made resistant to environmental signals that impair Treg function could contribute to the development of disease-specific therapy. In patients with rheumatoid arthritis, anti-TNF therapy has been incredibly effective in treating disease, response to this therapy is associated with the induction of Treg that can secrete IL-10 and TGFβ to control inflammatory responses (46). Using this insight into the mechanism of action of an established therapy, it might be possible to engineer Treg to secrete IL-10 and TGFβ, which could favor the suppression of arthritic inflammation. By contrast, a major concern of adoptive therapy with engineered Treg is the risk that an inflammatory environment *in vivo* may trigger the conversion into antigen-specific pathogenic effector T cells. To address this, newer technologies such as CRISPR could be used to undertake gene editing; making Treg impervious to inflammatory signals by editing out cytokine receptors such as IL-6 offers an attractive option for such an approach. Moreover, the propensity of Tregs from patients with autoimmunity to produce inflammatory cytokines could be ablated by using CRISPR to remove genes for IFNγ or IL-17 from engineered Treg. An equally novel approach might be to utilize features of tissue-resident Tregs that have recently been shown to contribute to tissue protection (47) and the repair of damaged tissue (38–41) *via* distinct transcription factors (**Figure 2**). This is a tantalizing glimpse of the additional potential of Treg therapy not just to suppress inflammation but to aid in repair of damaged tissue.

CONCLUSION

The rapid development of technology that allows genetic engineering of primary immune cells has opened the door to a world of potential therapeutic interventions. The prospect of producing tailor-made cellular therapies with disease-specific function could revolutionize the treatment of autoimmunity.

AUTHOR CONTRIBUTIONS

JM and GW—research and writing of review, and critical revisions of the manuscript. HS—contributed to research and writing the review. All authors approved the manuscript before submission and agreed to be accountable for the content.

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REVIEW
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Engineered T Regulatory Type 1 Cells for Clinical Application

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T regulatory cells, a specialized subset of T cells, are key players in modulating antigen (Ag)-specific immune responses *in vivo*. Inducible T regulatory type 1 (Tr1) cells are characterized by the co-expression of CD49b and lymphocyte-activation gene 3 (LAG-3) and the ability to secrete IL-10, TGF-β, and granzyme (Gz) B, in the absence of IL-4 and IL-17. The chief mechanisms by which Tr1 cells control immune responses are secretion of IL-10 and TGF-β and killing of myeloid cells *via* GzB. Tr1 cells, first described in peripheral blood of patients who developed tolerance after HLA-mismatched fetal liver hematopoietic stem cell transplantation, have been proven to modulate inflammatory and effector T cell responses in several immune-mediated diseases. The possibility to generate and expand Tr1 cells *in vitro* in an Ag-specific manner has led to their clinical use as cell therapy in patients. Clinical grade protocols to generate or to enrich and expand Tr1 cell medicinal products have been established. Proof-of-concept clinical trials with Tr1 cell products have demonstrated the safety and the feasibility of this approach and indicated some clinical benefit. In the present review, we provide an overview on protocols established to induce/expand Tr1 cells *in vitro* for clinical application and on results obtained in Tr1 cell-based clinical trials. Moreover, we will discuss a recently developed protocol to efficient convert human CD4$^+$ T cells into a homogeneous population of Tr1-like cells by lentiviral vector-mediated IL-10 gene transfer.

Keywords: T regulatory type 1 (Tr1) cells, tolerance, T regulatory cell-based therapy, IL-10, gene transfer

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INTRODUCTION

T regulatory type 1 (Tr1) cells are a subset of adaptive CD4$^+$ T cells that promote immune tolerance and control excessive and/or inappropriate inflammation mediated by effector T cells and antigen-presenting cells (APCs). In contrast to thymic-derived T regulatory cells (Tregs) that constitutively express the transcription factor (TF) FOXP3 (FOXP3$^+$ Tregs) (1, 2), Tr1 cells can only transiently upregulate FOXP3 upon activation (3–7).

Tr1 cells are memory CD4$^+$ T cells that co-express the integrin alpha2 subunit (CD49b) and the lymphocyte-activation gene 3 (LAG-3) (7). Although other cell surface markers, including PD-1, ICOS, TIGIT, CD39, CD73, TIM-3, GITR, OX40, TNFRSF9, and CEACAM-1 (8), have been associated with Tr1 cells, their expression on other cell types precludes them from being defined as Tr1-specific markers. Tr1 cells produce high levels of IL-10 and TGF-β; variable amounts of IFN-γ; and low/no IL-2, IL-4, and IL-17 (6, 7, 9, 10) and have a specific gene signature (7). In addition, Tr1 cells have unique metabolic requirements that distinguish them from FOXP3$^+$ Tregs: Tr1 cells depend on glycolysis and are inhibited by hypoxia and extracellular ATP (11), while peripheral FOXP3$^+$ Tregs depend on fatty acid oxidation (12).

The main mechanism of Tr1-mediated suppression is the secretion of IL-10 and TGF-β. Importantly, Tr1 cells require activation *via* their T cell receptor, thus by their cognate antigen (Ag), to mediate suppression, but, once activated, they mediate bystander suppression against other Ags (6, 9). The expression of granzyme (Gz) B endows Tr1 cells with the ability to specifically kill myeloid APCs (6, 13). Similar to FOXP3$^+$ Tregs, Tr1 cells also inhibit T cell responses *via* CTLA-4/CD80 and PD-1/PDL-1 interactions (14) and metabolic disruption (15) (**Figure 1**). IL-10 signaling is required for maintaining high IL-10 production by Tr1 cells, which in turn is necessary for controlling inflammatory responses. Notably, in the absence of IL-10-mediated signaling, Tr1 cells lose their ability to secrete IL-10, but they still express GzB and CTLA-4 (16). These findings suggest that in the absence of IL-10/IL-10R-mediated signaling, and consequent IL-10 production, Tr1 cells may suppress immune responses *via* alternative mechanisms such as specific killing of APCs and/or cell-to-cell contact-mediated inhibition of effector T cells and APCs (**Figure 1**).

IL-10 is the driving cytokine for Tr1 cell differentiation and function (9, 16). In the past years, it has become evident that activation of CD4$^+$ T cells in the presence of IL-27, key regulator of IL-10 production in T cells (17), promotes the differentiation of Tr1 cells in mice (11, 18–20). In T cells, the downstream effects of IL-10/IL-10R interaction is signaling *via* STAT3 (21), and although no formal proof for the critical role of STAT3 in Tr1 cell

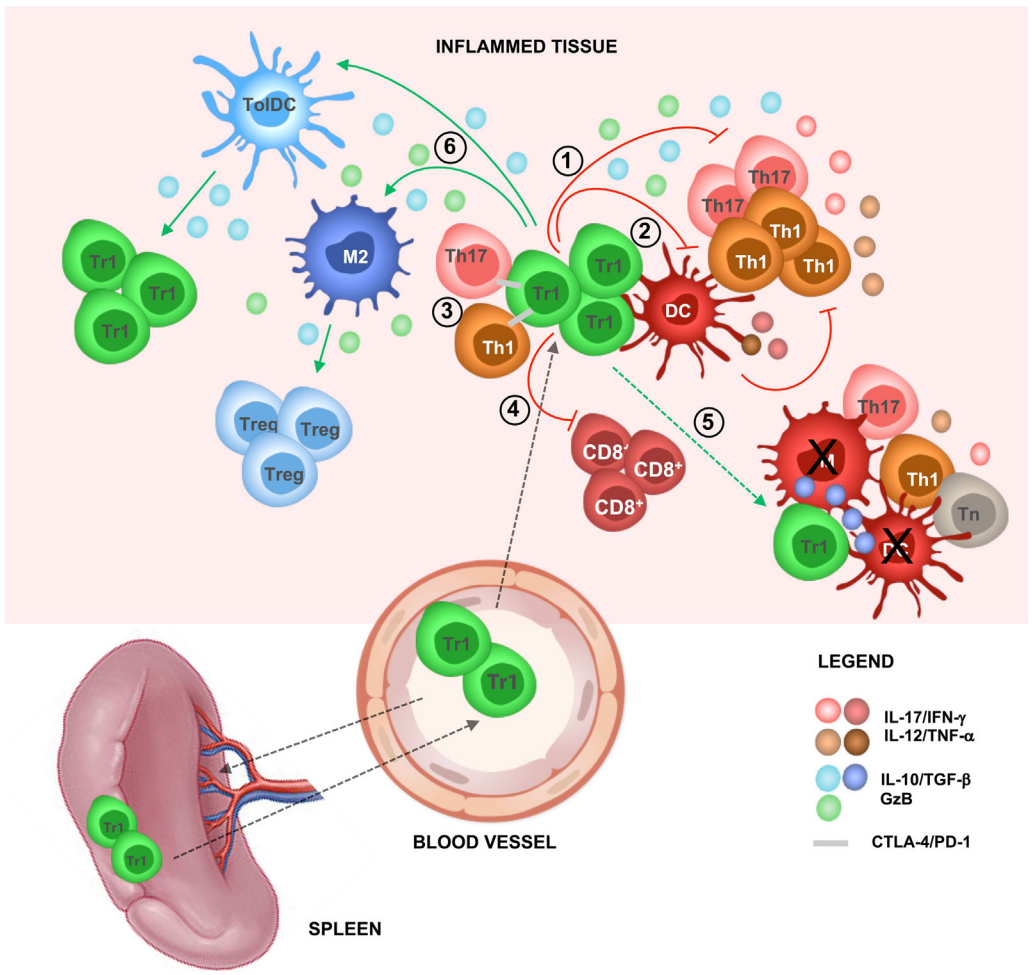

FIGURE 1 | T regulatory type 1 (Tr1)-mediated suppression *in vivo*. In steady-state condition, Tr1 cells reside in the spleen and circulate in the periphery. During inflammation, Tr1 cells are recruited to the site of tissue injury (i.e., after infections, autoimmune reactions, or transplantation) and are activated by professional antigen-presenting cells [APCs; dendritic cells (DCs)] *via* their T cell receptor, thus by their cognate antigen (Ag). Upon activation, Tr1 cells secrete IL-10 and TGF-β and (1) directly inhibit effector T cell (i.e., Th1 and Th17 cells) proliferation and pro-inflammatory cytokines production and (2) indirectly inhibit effector T cells by modulating professional APCs (i.e., downregulation of costimulatory and HLA class II expression and inhibition of pro-inflammatory cytokine secretion). (3) Tr1 cells can suppress effector T cells by cell-to-cell contact-mediated mechanisms, (4) suppress CD8$^+$ T cell responses (i.e., proliferation and IFN-γ production), and (5) mediate bystander suppression by specifically killing professional APCs [DC or macrophages (M)], thus preventing naive T (Tn) cell priming and reactivation of effector T cells (i.e., Th1 and Th17 cells). Concomitantly, (6) Tr1 cells *via* IL-10 and TGF-β promote the induction of tolerogenic DC and anti-inflammatory macrophages (M2), which in turn promote *de novo* induction of Tr1 cells and T regulatory cells (Tregs), restoring tissue homeostasis and promoting long-term tolerance.

differentiation exists, several evidences indicate that it represents the link between IL-10/IL-10R and downstream activation of TFs involved in Tr1 cell induction and functions. Specifically, (i) overexpression of active STAT3 in T cells promotes Tr1 cell induction (22), (ii) IL-27-dependent induction of IL-10 is STAT1 and STAT3 mediated (23), and (iii) STAT3 interacts with the aryl hydrocarbon receptor (AhR) that by inducing HIF-1α degradation leads to the stabilization of the glycolytic metabolism in Tr1 cells (11).

A plethora of TFs have been shown to be involved in driving Tr1 cell differentiation, phenotype, and functions (24). The TFs c-Maf and AhR induced by IL-27 bind together to transactivate the *IL-21* and *IL-10* promoters. While IL-21 maintains c-Maf and AhR expression, the expression of IL-10 is essential for the suppressive function of Tr1 cells. Moreover, IL-27-induced AhR, alone or with an unknown cofactor, promotes GzB expression in Tr1 cells. The latter mechanism allows killing of myeloid APCs (18, 19, 25, 26). Additional TFs have been shown to activate *IL-10* promoter during IL-27-mediated induction of Tr1 cells: the early response gene 2 (27) and B lymphocyte-induced maturation protein-1 (Blimp-1) (28). Based on the above studies, it has been proposed that two transcriptional components activate *IL-10* in Tr1 cells upon IL-27 stimulation: c-Maf and Ahr are required for promoting IL-10 production under certain conditions, whereas Egr-2 *via* STAT3 induces Blimp-1 and IL-10 production (29). More recently, it has been suggested that after hematopoietic stem cell transplantation, Ag presentation in the presence of macrophage-derived IL-27 promotes Tr1 cell differentiation *via* Blimp-1 and eomesodermin (eomes). Eomes enables stable IL-10 production and consequently Tr1 cell induction *in vivo* (30). Moreover, the early induction of IRF1 and BAFT expression has been shown to be essential for IL-27-mediated induction of murine Tr1 cells (31). In the latter study, it was proposed that while BAFT is required for both Th17 and Tr1 cell induction, IRF4 and IRF1 are differentially required for the two cell subsets (31). However, this conclusion is in contrast with the demonstration that activin-A promotes human Tr1 cells *via* activation of IRF4 that along with AhR binds to *IL-10* and *icos* promoters (32). Moreover, ITK signaling *via* Ras/IRF4 pathway regulates the induction and function of both murine IL-27-induced Tr1 cells and human IL-10-induced Tr1 cells (33). ITK kinase activity is indeed critical for AhR, c-MAF, and IRF4 expression in T cells during Tr1 cell differentiation. Overall, despite the increased knowledge on the different pathways involved in promoting IL-10 production in T cells during Tr1 cell induction *via* IL-27 or IL-10, the master TF for Tr1 cells still remains unclear.

In vivo studies demonstrated that Tr1 cells circulate in peripheral blood (7) but are induced and also localized in tissues where IL-10 plays an essential role in maintaining homeostasis, such as the intestinal mucosa (34, 35). Recent observations in preclinical models indicate that Tr1 cells induced in the intestinal mucosa migrate to the periphery and control effector T cell responses and the development of type 1 diabetes (36). Interestingly, *in vitro* induced human Tr1 cells express the gut-homing receptors GPR15 and CCR9, supporting the capacity of Tr1 cells to migrate to the intestinal mucosa (37). Moreover, *in vivo* induced Tr1 cells have been identified in the spleen of tolerant mice (38). It still remains unclear whether Tr1 cells are generated in the spleen or if the spleen represents the *in vivo* natural "reservoir" of Tr1 cells. Moreover, it has not been demonstrated yet whether this organ is also the privileged site for Tr1 cell accumulation in humans. The discovery of CD49b and LAG-3 as specific biomarkers of Tr1 cells (7) renders possible to better study the *in vivo* localization of Tr1 cells in physiological and pathological conditions.

Tr1 CELLS AND THEIR ROLE IN TOLERANCE INDUCTION

A defect in Tr1 cell frequency/function has been consistently demonstrated in a number of autoimmune and inflammatory diseases in preclinical and clinical models, indicating that IL-10-producing Tr1 cells are relevant for disease protection [reviewed in Ref. (10)]. These evidences built the rationale for medical intervention for Tr1 cell boosting *in vivo* to prevent/cure T cell-mediated diseases. Several stimuli have been used to promote Tr1 cell induction *in vivo*. We and other demonstrated the ability of IL-10 or IL-10-inducing agents in combination with immunosuppressive treatments to generate Tr1 cell in *in vivo* models of autoimmunity or allogeneic transplantation. Among others, interesting treatments to promote Tr1 cells *in vivo* are the administration of anti-CD3 monoclonal antibodies or tolerogenic DCs, which in both preclinical models and in humans have been demonstrated to promote Tr1 cells. Alternatively, *in vivo* administration of soluble Ags has been proved to promote repolarization of autoimmune T cells into Tr1 cells in preclinical models [reviewed in Ref. (10)]. Some of these approaches have been translated to treat autoimmune diseases, including type 1 diabetes (T1D) and multiple sclerosis (MS). The first-in-man clinical trials with HLA-DR4-restricted proinsulin peptide or ATX-MS-1647, a cocktail of myelin basic protein-derived peptides, demonstrated the safety of these approaches without the induction/re-activation of pro-inflammatory autoimmune response, but with limited benefit for the patients (39, 40). Interestingly, in T1D patients, proinsulin peptide immunotherapy was associated with the transient appearance of Ag-specific IL-10$^+$ CD4$^+$ T cells (39), and in treated MS patients, a trend toward high levels of IL-10 gene expression associated with reduced Ag-specific T cell proliferation has been observed (40). These preliminary data indicate that peptide immunotherapy in autoimmune diseases may boost *in vivo* Ag-specific Tr1 cells.

T regulatory type 1 cells have been associated with long-term transplantation tolerance, induced or spontaneously established, in preclinical and clinical settings (38, 41, 42). Moreover, after the first demonstration that adoptive transfer of *in vitro* induced Ag-specific Tr1 cells efficiently prevents colitis induced in SCID mice by pathogenic T cells (9), several studies demonstrated that *in vitro* induced Tr1 cells can be used as cellular therapy to treat inflammatory and autoimmune disease as well as to control graft-versus-host disease (GvHD) and to prevent organ rejection [reviewed in Ref. (10)]. These evidences built the rationale for medical intervention with *in vitro* generated Tr1 cells to cure T-cell mediated diseases and to promote transplantation tolerance.

GENERATION OF Tr1 CELL MEDICINAL PRODUCTS

Several good manufacturing practice (GMP) compatible protocols have been established to generate human Ag-specific Tr1 cells. Originally, we induced alloAg-specific Tr1 cells by culturing human PBMCs (or purified CD4$^+$ T cells) with allogeneic monocytes in the presence of exogenous human IL-10 [mixed lymphocytes reactions (MLR)/IL-10, **Figure 2**] (9). With this culture condition, a population of IL-10-anergized T cells is induced: these bulk populations are anergic in response to the alloAgs used for priming and contain precursors of IL-10-producing alloAg-specific Tr1 cells, as demonstrated by single cell T cell cloning of alloAg-specific Tr1 cells (9, 43). Moreover, after IL-10 anergization, the bulk cultures also contain non-alloAg-specific T cells that respond to other Ags, such as pathogens or third-party alloAgs (44, 45). Being a mixed population of cells, the IL-10-anergized cultures on one hand give rise to alloAg-specific

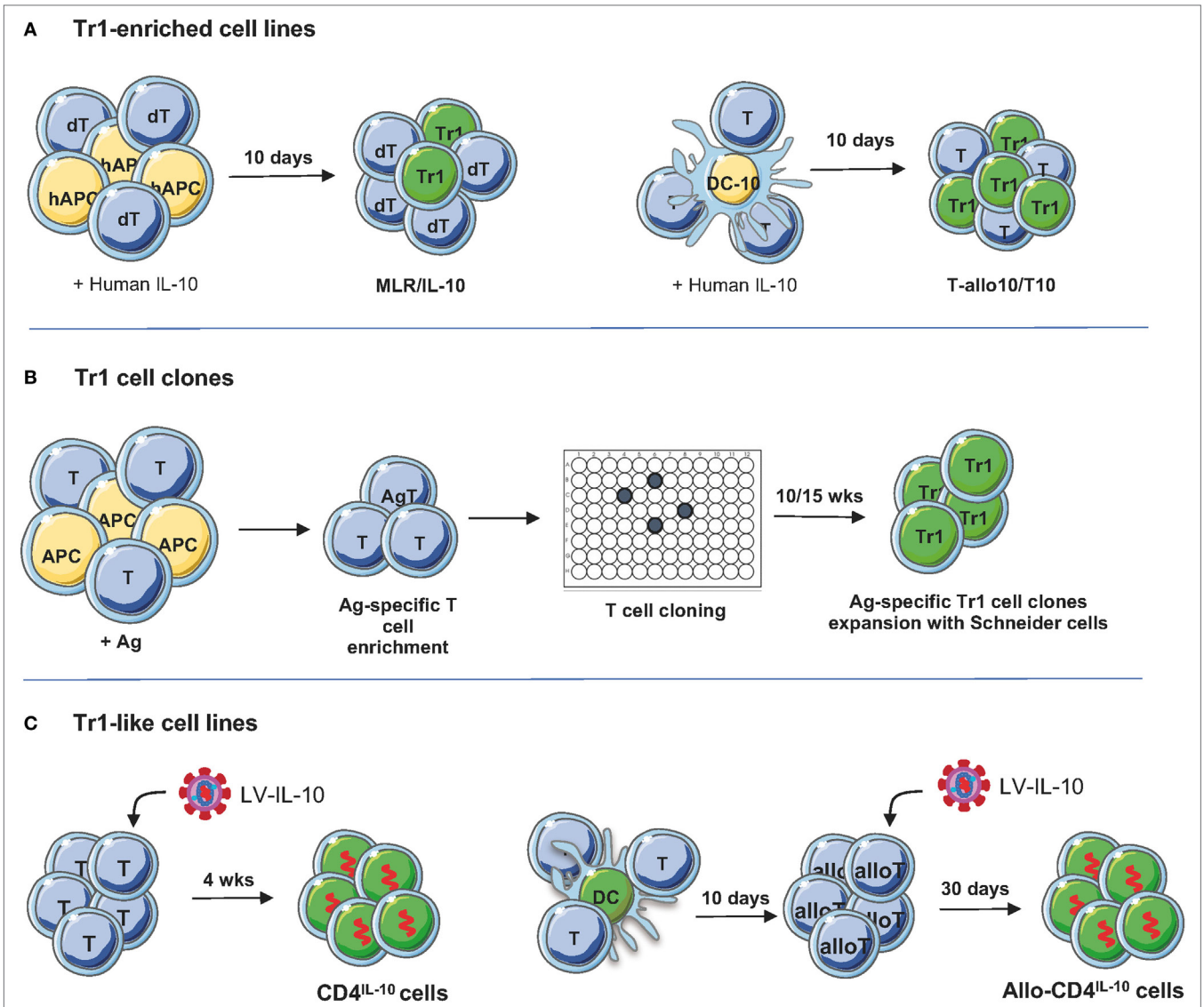

FIGURE 2 | Protocols to generate/expand T regulatory type 1 (Tr1) cells. **(A)** Tr1-enriched cell lines. Donor-derived PBMC or CD4$^+$ T cells are stimulated with host-derived monocytes in the presence of recombinant human IL-10 for 10 days. Alternatively, PBMC or CD4$^+$ T cells are cultured for 10 days with allogenic dendritic cell (DC)-10, differentiated *in vitro* from peripheral blood monocytes with GM-CSF/IL-4/IL-10, in the presence of recombinant human IL-10 for 10 days (45). To generate T-allo10 cells, donor-derived T cells are cultured with host-derived DC-10 (Bacchetta and Roncarolo, unpublished data), whereas to induce host-derived T10 cells, T cells are stimulated with donor-derived DC-10 (46). **(B)** Tr1 cell clones. PBMC are stimulated with antigen (Ag; i.e., ovalbumin or collagen II) in the presence of IL-2 and IL-4 to enrich/expand Ag-specific T cells, followed by T cell cloning and expansion of the T cell clones using Schneider cells (4). **(C)** Tr1-like cell lines. Human CD4$^+$ T cells are preactivated for 48 h with soluble anti-CD3/CD28 mAbs and IL-2 and then transduced with lentiviral vector (LV)-IL-10 overnight. Transduced T cells are isolated and expanded in feeder mixture (47). To generate allospecific IL-10-transduced cells, naive CD4$^+$ T cells are stimulated with allogeneic DC and transduced with LV-IL-10 upon secondary stimulation. After selection, IL-10-trasduced cells are expanded *in vitro* with feeder mixture (48).

Tr1 cells able to induce and sustain tolerance in the absence of immunosuppression, and on the other hand, they contain T cells with the ability to mount an efficacious immune response against infectious agents, when adoptively transferred into an immune-suppressed host. These features offer a strong rationale for the use of IL-10-anergized T cells as cell therapy to improve immunoreconstitution in immunocompromised hosts such as patients after allogeneic hematopoietic stem cell transplant (HSCT) and to modulate responses to alloAgs and promote long-lasting tolerance (**Table 1**).

The discovery of DC-10, a subset of monocyte-derived human DC, which secrete high levels of IL-10 and express the tolerogenic molecules ILT4 and HLA-G (51), offered the possibility to modify the protocol to generate alloAg-specific Tr1 cells (**Figure 2**). Functional assays demonstrated that stimulation of human PBMCs or $CD4^+$ T cells with allogeneic DC-10 induces the differentiation of anergic alloAg-specific IL-10-producing Tr1 cells (45, 51). DC-10, in contrast to monocytes and exogenous IL-10, can promote T cell anergy not only in fully mismatched pairs but also in matched-unrelated pairs (45). Moreover, DC-10-anergized T cells contain up to 15% of already differentiated alloAg-specific $CD49b^+LAG\text{-}3^+$ Tr1 cells (**Figure 2A**, right), whereas, as discussed above, MLR/IL-10 cultures are enriched of IL-10-producing Tr1 cell precursors (**Figure 2B**) (43, 45). The MLR/IL-10 and MLR/DC-10 methods have been validated in GMP grade laboratories.

More recently, two optimized clinical grade compatible protocols for the induction of alloAg-specific Tr1 cells have been established. For a clinical trial in HSCT for hematological malignancies, a GMP compatible protocol in which purified donor-derived $CD4^+$ T cells are cultured with tolerogenic DC-10 of host origin in the presence of IL-10 for 10 days to obtain alloAg-specific Tr1 cells (named T-allo10, Bacchetta and Roncarolo, Clinicalgov identifier NCT03198234) have been established (**Table 1**). In this setting, donor-derived T cells react against patient (host) alloAgs and mediate GvHD; therefore, to suppress GvHD after allo-HSCT, Tr1 cells *ex vivo* generated are donor-derived and specific for patient alloAgs. For a clinical trial in kidney transplant recipients planned under the umbrella of "The ONE study" (52), a GMP-compatible protocol to generate donor-specific Tr1-enriched cell medicinal product [named T10 cells (46)] has been developed (**Table 1**). In this protocol, donor-specific Tr1 cells are induced by culturing $CD4^+$ T cells isolated from patients on dialysis with donor DC-10 in the presence of exogenous IL-10 for 10 days. In the contest of organ transplantation, patient T cells react against transplanted organ and mediate rejection; therefore, to prevent graft rejection, Tr1 cells *ex vivo* induced are patient-derived and specific for donor alloAgs. T10 and T-allo10 medicinal products contain a higher proportion of $CD49b^+LAG\text{-}3^+$ Tr1 cells compared to the IL-10-anergized T cells obtained by *in vitro* stimulation of donor-derived PBMCs with host CD3-depleted PBMCs in the presence of IL-10 [(46) and Bacchetta and Roncarolo, Clinicalgov identifier NCT03198234]. Tr1 cell medicinal products need to meet a number of release criteria for their *in vivo* delivery: (i) quality controls during the manufacturing, i.e., number, purity, and viability of $CD14^+$ cells used for DC-10 differentiation, of $CD4^+$ T cells, and of DC-10; (ii) quality controls of the final products, i.e., number, purity, and viability of T10 or T-allo10 cells, contamination of non-$CD4^+$ T cells, and the percentage of allospecific T cell anergy, i.e., allospecific proliferation of T10 or T-allo10 medicinal product/allospecific proliferation of control cells generated in parallel by culturing $CD4^+$ T cells with mature DC (46).

An alternative method to induce/expand Ag-specific Tr1 cell medicinal product has been developed by the France-based company TxCell.[1] The method includes the use of *Drosophila*-derived artificial APCs (Schneider cells) transfected with a transmembrane form of a murine anti-human CD3 antibody, human CD80, human CD58, human IL-2, and human IL-4 (4). The expansion of Ag-specific Tr1 cell clones requires first stimulation of PBMCs with a specific Ag [i.e., ovalbumin (OVA)] in the presence of IL-2 and IL-4 to enrich for Ag-specific T cells, followed by T cell cloning by limiting dilution and expansion of the T cell clones using Schneider cells (**Figure 1**). This method has been applied to expand a large number of OVA-specific Tr1 cell clones (termed Ovasave®) that have been infused in patients affected with refractory Crohn's disease (CD) (50) (**Table 1**). With a similar procedure, collagen II-specific Tr1 cell clones (Col-Treg) were produced from PBMCs of rheumatoid arthritis patients (5) (**Table 1**).

[1] http://www.txcell.com.

TABLE 1 | Tr1 cells in clinical and preclinical development.

Specificity	Name	Stage of development	References
Tr1 cell products for clinical applications			
Allospecific Tr1 cells	MLR/IL-10	ALT-TEN trial—completed	(49)
	T-allo10 cells	Phase 1 clinical trial—open now recruiting	T-allo10, NCT03198234
	T10 cells	GMP grade medicinal product	(46)
Ag-specific Tr1 cell clones	Ovasave®	CATS1 trial—completed	(50)
	Col-Treg	GMP grade medicinal product	http://www.txcell.com
Tr1 cell products in preclinical development			
Polyclonal Tr1-like cells	$CD4^{IL\text{-}10}$ cells	*In vitro* and *in vivo* functional characterization	(47, 48)
Allospecific Tr1-like cells	Allo-$CD4^{IL\text{-}10}$ cells	*In vitro* functional characterization	(48)

GMP, good manufacturing practice; MLR, mixed lymphocytes reaction; Tr1, T regulatory type 1.

Tr1-BASED CLINICAL TRIALS

Treg cell-based cell therapy was first used to prevent GvHD after allogeneic HSCT. These proof-of-concept clinical trials demonstrated the feasibility and safety of the approach [reviewed in Ref. (53)] and paved the way to a wider application of Tregs as medical products for the treatment of autoimmune and chronic inflammatory disease and the prevention of organ rejection. Several trials are ongoing with different types of Tregs, including $CD25^+$Tregs and Tr1 cells (53). A major difference between the $CD25^+$ Tregs and Tr1 cells is that a pool of polyclonal non-Ag-specific cells are administered in the former, whereas Ag-specific products are infused with the latter.

T regulatory type 1 cell-based therapy mediated fast immune reconstitution and prevented severe GvHD in patients with advanced hematological malignancies undergoing haploidentical HSCT therapy [the ALT-TEN trial (49), **Table 1**]. A high dose (average of 12×10^6/kg $CD34^+$ cells) of haploidentical purified $CD34^+$ hematopoietic stem cells, virtually devoid of T cells ($\leq 2.6 \times 10^4$/kg $CD3^+$ cells), was infused in myeloablated patients. Once there were signs of myeloid engraftment, the donor-derived, host-alloAg-specific IL-10-anergized T cells were infused in patients in the absence of immunosuppression for GvHD prophylaxis. Results show that the treatment was safe and well tolerated. Clinical outcome of patients treated in the ALT-TEN trial suggests that donor-derived IL-10-anergized T cells could sustain immune reconstitution with no severe GvHD and no disease relapse. Moreover, the results suggest that a higher number of Tr1 cells within the total T cell suspension would provide enhanced activity to prevent GvHD. An improved method to generate alloAg-specific Tr1 cells (T-allo10 and T10 cells) using DC-10 has been established (see above). T-allo10 cells are currently tested in a phase I trial in patients with hematologic malignancies receiving un-manipulated HSCT from mismatched related or mismatched unrelated donors with the aim of preventing acute and chronic GvHD (T-allo10, Clinical-gov identifier NCT03198234, **Table 1**). In organ transplantation, as part of The ONE Study,[2] T10 cells will be infused in living donor renal transplant recipients [(46), **Table 1**].

Tr1 cell-based therapy has been also tested to treat patients affected with refractory CD (50). In a phase I/IIa clinical, the CATS1 study, OVA-specific Tr1 cell clones (Ovasave®), generated as described above, were infused in CD patients, who ingested an OVA-enriched diet to activate OVA-specific Tr1 cells migrating to the gut (**Table 1**). Multiple doses of the cell product Ovasave® were injected, and a response was observed in 40% of patients, with a stronger effect in the group of patients who received the lowest Tr1 cell dose (50). The study demonstrated the safety of the approach and showed some clinical benefit. However, the clinical effect was limited, reaching the maximum at 5 weeks after treatment and declining thereafter. Col-Treg will be tested in a clinical study for severe and refractory autoimmune uveitis (see text footnote 1; **Table 1**).

[2]http://www.onestudy.org/.

Overall these first clinical trials showed the feasibility, the safety, and potential clinical benefit of the Tr1 cell-based cell therapy approach. Because of their Ag specificity, Tr1 cells have the potential to be applied in several clinical settings.

IL-10-ENGINEERED $CD4^+$ T CELLS

The limitations to broaden the clinical application of Tr1 cells are as follows: (a) the presence of potential contaminating non-Tr1 cells in the preparation; (b) limited expansion capacity *in vitro*. The discovery of Tr1 cell-specific biomarkers, CD49b and LAG-3 (7), opened the possibility to isolate alloAg-specific Tr1 cells from *in vitro* cultures. Specifically, we showed that FACS-sorted $CD49b^+LAG\text{-}3^+$ T cells from DC-10-induced Tr1 cell populations had higher suppressive capacity compared to the original bulk population (7).

An alternative strategy to generate a large number of Tr1 cells is to induce stable and sustained overexpression of IL-10 into conventional $CD4^+$ T cells. We developed a protocol based on the use of bidirectional LVs co-encoding for human *IL-10* and a marker gene of selection [i.e., GFP (47)]. We demonstrated that LV-mediated human *IL-10* gene transfer converted conventional human $CD4^+$ T cells into Tr1-like cells, termed $CD4^{IL\text{-}10}$ cells (**Figure 2**; **Table 1**). $CD4^{IL\text{-}10}$ cells are phenotypically and functionally similar to Tr1 cells: they secrete high levels of IL-10, suppress T cell responses *via* IL-10 and TGF-β, and prevent xenoGVHD in humanized models (47). More recently, we modified our vector platform by substituting GFP with *ΔNGFR*, as a clinical grade marker gene for selection, and we demonstrated that $CD4^{IL\text{-}10}$ cells acquire not only the potential to suppress T cell responses *in vitro* and *in vivo*, but also the ability to specifically kill myeloid leukemic cell lines and blasts in an HLA class I-dependent but Ag-independent manner (48). $CD4^{IL\text{-}10}$ cells indeed selectively eliminate $CD13^+$ leukemic cells, and for optimal killing of target cells, they require stable CD54/LFA-1-mediated adhesion and CD112/CD226-mediated activation. Importantly, $CD4^{IL\text{-}10}$ cells mediate antitumor and antileukemic effects *in vivo* in humanized mouse models of solid myeloid tumors and leukemia (48). This newly identified antileukemic activity of $CD4^{IL\text{-}10}$ cells is of critical importance, since an active area of investigation is the identification of regimens that prevents GvHD after allo-HSCT without affecting graft-versus-leukemia (GvL) activity of donor T lymphocytes. In a newly developed protocol of GvHD/GvL in humanized mice, we demonstrated that $CD4^{IL\text{-}10}$ cells adoptively transferred *in vivo* prevent xenoGvHD mediated by allogeneic PBMC and collaborate with PBMC in mediating GvL. The LV-hIL-10 platform has been also applied to generate IL-10-engineered alloAg-specific Tr1-like cells, namely allo-$CD4^{IL\text{-}10}$ cells (**Figure 2**; **Table 1**). Allo-$CD4^{IL\text{-}10}$ cells suppress alloAg-specific T cell responses *in vitro* and kill myeloid target cells in an Ag-independent manner. Overall, we showed that enforced IL-10 expression in conventional or allospecific $CD4^+$ T cells promotes their conversion into Tr1-like suppressor cells able to kill myeloid cell lines (48). These findings pave the way for adoptive cell therapy with IL-10-engineered T cells in patients undergoing allogeneic

organ and HSCT transplantation for oncological diseases. Moreover, the antitumor and antileukemic activity of $CD4^{IL\text{-}10}$ cells can be considered for *ad hoc* immunotherapy in relapsing patients.

FUTURE PERSPECTIVES

The discovery that Tr1 cells modulate immune responses led to the idea that they could be developed as a therapeutic product to promote/restore tolerance in transplantation and in inflammatory and autoimmune diseases. The completed clinical trials proved the safety of Tr1 cell-based therapy and indicate potential therapeutic effects. AlloAg-specific Tr1 cells can be generated *in vitro*, and protocols have been translated into clinical practice. It remained a major challenge to expand or generate Ag-specific Tr1 cells suitable for cell-based approaches in autoimmune diseases. Schneider cells have been shown to efficiently sustain Ag-specific Tr1 cell clone expansion for clinical application. However, Tr1 cell clones have limited survival capacity *in vivo* upon chronic activation (50, 54). The discovery that LV-mediated IL-10 gene transfer converts conventional polyclonal and alloAg-specific T cells into Tr1-like cells paves the way for applying this technology to generate a large number of Tr1 cells from Ag-specific T cells isolated from the peripheral blood of patients. Transcriptome analysis of this engineered Tr1-like cells will allow us to identify the key molecules involved in Tr1 cell immunomodulatory function.

AUTHOR CONTRIBUTIONS

SG and MGR designed and wrote the manuscript.

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Therapeutic Potential of Gene-Modified Regulatory T Cells: From Bench to Bedside

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Regulatory T cells (Tregs) are an important subset of adaptive immune cells and control immune reactions for maintaining homeostasis. Tregs are generated upon their encounter with self or non-self-antigen and mediate tolerance or suppress aberrant immune responses. A high level of specificity of Tregs to recognize antigen(s) suggested their instrumental potential to treat various inflammatory diseases. This review will first introduce seminal basic research findings in the field of Tregs over the last two decades pertinent to therapeutic approaches in progress. We will then discuss the previous approaches to use Tregs for therapeutic purposes and the more recent development of gene-modification approaches. The suppressive function of Tregs has been studied intensively in clinical settings, including cancer, autoimmunity, and allotransplantation. In cancer, Tregs are often aberrantly increased in their number, and their suppressor function inhibits mounting of effective antitumor immune responses. We will examine potential approaches of using gene-modified Tregs to treat cancer. In autoimmunity and allotransplantation, chronic inflammation due to inherent genetic defects in the immune system or mismatch between organ donor and recipient results in dysfunction of Tregs, leading to inflammatory diseases or rejection, respectively. Since the recognition of antigen is a central part in Treg function and their therapeutic use, the modulation of T cell receptor specificity will be discussed. Finally, we will focus on future novel strategies employing the therapeutic potential of Tregs using gene modification to broaden our perspective.

Keywords: regulatory T cell, autoimmunity, cancer, gene modification, therapy

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INTRODUCTION

Regulatory T cells (Tregs) are an important T cell subpopulation that maintains immunological homeostasis. In the early 1970s, a few papers provided evidence for an inhibitory role of thymus-derived T cells. Seminal research by Gershon and Kondo introduced the concept of infectious tolerance (1, 2). Over the next decade, these thymus-derived T cells were termed "suppressor T cells." Later, suppressor T cells were renamed as "regulatory T cells," based on their capability to regulate autoimmunity. Since the 1990s, Tregs have been one of the most intense research fields in immunology. The negative regulation of immune responses by Tregs is vital in autoimmune and auto-inflammatory disorders, acute and chronic infection, allergy, metabolic inflammation, transplantation, and cancer. Accordingly, modulation of Tregs holds the therapeutic potential to treat numerous disease classes. The unique history of Tregs has been well-reviewed previously (3).

Identifying Treg Markers and Mechanistic Studies of Treg Function in Mice

Initial attempts to isolate and to identify Tregs for immunological studies used IL-2 receptor α-chain (CD25), which is highly expressed on Tregs. Adoptive transfer of $CD25^+$ Tregs prevented various experimental mouse models of autoimmunity (4–6). Other Treg markers were subsequently identified including lymphocyte activation gene (LAG)-3, CTLA-4, folate receptor-4, latency-associated peptide, and IL-35 (7–10). Often these markers were also expressed in activated effector CD4 T cells, necessitating the identification of more definitive markers of Tregs.

In this regard, one of the most important findings in Treg biology was the discovery of Treg lineage transcriptional factor FOXP3. Mutations in the *FOXP3* gene were identified in Immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) human patients. The murine counterpart of IPEX patients, *scurfy* mice, lacks functional FOXP3 and showed similar phenotypes to IPEX patients (11–13). Two studies demonstrated the importance of FOXP3 in Treg development and function (14, 15). Development of biological tools including Red Fluorescent Protein (RFP) FOXP3 reporter mice, *Foxp3*-Diphtheria Toxin Receptor mice which permitted Treg depletion, and the development of Treg-specific $Foxp3^{YFP\text{–}Cre}$ mice which allowed the conditional deletion of a gene in Tregs, facilitated the understanding of Treg biology in mice (16–19).

$CD4^+FOXP3^+$ Tregs can be induced from peripheral $CD4^+$ naïve T cells in the periphery by many factors such as tolerogenic dendritic cells expressing indoleamine 2,3-dioxygenase (IDO), commensal bacteria, retinoic acid, or transforming growth factor (TGF)-β and are designated peripheral Tregs (pTregs) to distinguish them from the thymic-derived Tregs (tTregs) (20–24). Similar to their tTregs, pTregs regulate immune responses in various types of inflammatory disease environments including spontaneous intestinal tumorigenesis, inflammatory bowel disease, asthma, and experimental autoimmune encephalomyelitis (EAE) (25–28). It has been reported that $FOXP3^+$ Tregs express the immunosuppressive cytokine IL-10. Later, IL-10-expressing Tregs were further dissected into $IL\text{-}10^+FOXP3^+$ Tregs and Foxp3-negative type 1 Tregs (Tr1) that are induced by dexamethasone and Vitamin D (29–31).

Using genetic, biochemical, and molecular biological approaches, functional modules of Foxp3 such as dimerization/oligomerization of the transcriptional factor were identified, and the regulatory mechanism of Foxp3-mediated gene expression in Tregs was extensively studied (32–40). The molecular mechanism of stable FOXP3 expression has been under intense investigation by measuring DNA demethylation at the Treg-specific demethylated region (TSDR), a conserved CpG-rich region within the *Foxp3* locus where methylation maintains stable lineage commitment of Tregs (41, 42). In parallel to the regulation of FOXP3 expression, posttranslational modification by acetylation, ubiquitination, or phosphorylation has an important role in modulating the Foxp3-mediated transcriptional repression that is required for suppressor function (43–48).

Human Tregs in Basic and Clinical Studies

In the past two decades, there has been significant progress in the understanding of regulatory mechanisms of tolerance in humans. Various markers for the identification of human Tregs were found including CD25, FOXP3, and CD127 (IL-7Rα chain) (49–52). Further studies revealed that human conventional T cells transiently express FOXP3 without acquiring suppressive activity (53). Human Tregs are functionally and phenotypically distinguished by their activation status. Suppressive Treg cells are $CD45RA^+FOXP3^{lo}$ in resting state and $CD45RA^-FOXP3^{hi}$ in activated state while $CD45RA^-FOXP3^{lo}$ T cells are non-suppressive. The proportion of the three subpopulations was markedly different between aged individuals, cord blood and patients with immunological diseases (54, 55).

Expansion of Tregs using rapamycin or induction of Tr1 cells has been utilized to induce polyclonal Tregs for clinical intervention (56, 57). Tr1 cells express similar markers to $FOXP3^+$ Tregs such as CTLA-4, PD-1, CD39, and ICOS. Tr1 cells do not express FOXP3 constitutively, but they do express IL-10 and TGF-β once they are activated *via* T cell Receptor (TCR). Tr1 cells show bystander suppressor activity (58). IL-10 and TGF-β from Tregs inhibit effector CD4 T cells proliferation and production of effector cytokines, such as IL-2 and IFN-γ (59). Other than cytokine-mediated suppression, it is known that granzyme B-mediated cell death of myeloid APCs is mediated by the stable adhesion between HLA-class I molecules on Tr1 cells and CD112/CD115 on myeloid APCs (60).

In clinical settings, modification of TCR has been utilized to modulate Treg activity to intervene in various types of inflammatory diseases in an antigen-specific manner (61, 62). Treg-based therapies with freshly isolated or expanded Tregs have been implemented in clinical practice for patients undergoing allogeneic hemopoietic stem cell transplantation to prevent graft-versus-host disease (GVHD) (63), inhibiting rejection in solid organ transplantation and controlling autoimmunity in patients [e.g., Type 1 Diabetes (T1D)] (64). Since Tregs have multiple roles in a variety of clinical settings, the generation of gene-modified Tregs and administration of those Tregs *via* adoptive transfer is a promising approach to treat chronic inflammatory diseases, cancer, or rejection in transplantation medicine.

GENE-MODIFIED Tregs IN CANCER IMMUNOTHERAPY

Regulatory T cells are found at high frequencies in the tumor microenvironment in a variety of cancers (65). Analysis in a variety of human carcinomas suggested that the accumulation of Tregs in the tumor microenvironment is associated with a poor prognosis (65).

Generating Tumor Antigen-Specific CAR^+ Tregs

Over a decade ago, a seminal study proposed the therapeutic potential of genetically engineered T cells bearing a tumor antigen-specific TCR in cancer immunotherapy (66). Overexpression of the α and β chains of a specific TCR has been

used as a traditional approach to engineer T cell specificity. The antigen-specific suppressor function of Tregs on effector T cells was demonstrated by the tumor antigen NY-ESO-1. Depletion of Tregs enabled the activation of NY-ESO-1-specific naïve CD4 T cells in healthy subjects and melanoma patients with NY-ESO-1-expressing tumors (67, 68). TCRs recognizing melanoma antigens have been successfully transduced in human Tregs *in vitro* (69). Interestingly, the affinity of the TCR did not affect the antigen-specific suppressive function. This indicated that tumor antigen-mediated TCR signals do not affect the function of fully differentiated Tregs *ex vivo*.

An alternative strategy is to transduce a chimeric antigen receptor (CAR) into Tregs to generate antigen-specific Tregs. CARs are synthetic proteins generated by fusing an extracellular domain for antigen recognition with transmembrane and signaling domains from the TCR and co-stimulatory receptors (70). The antigen-recognizing domain of a CAR is generated by a single-chain variable fragment (scFv) fusion protein of the complementarity determining regions of the heavy and light chains of a monoclonal antibody. A major advantage of generating scFvs is to avoid the limitation of MHC restriction. This expands the pool of treatable patients compared to the TCR overexpression approach. Expression and engineering of CARs that are specific to tumor antigens is now a primary interest in cancer immunotherapy employing CAR Tregs (71). Further studies for a more diverse set of tumor antigens are warranted to broaden the therapeutic potential of this approach.

Modulating Foxp3 Expression in Tregs

Another approach to inhibit the suppressor function of Tregs is to downregulate FOXP3 expression. Use of lentiviral FOXP3 shRNA delivery inhibited Treg-like leukemia in mice (72). This lentiviral strategy was used to knockdown FOXP3 mRNA in human Tregs, and this approach demonstrated the loss of suppressor function, indicating that it has potential to be used in cancer immunotherapy (73). However, Tregs that are transduced with the lentivirus have not been tested for safety, and thus further research is needed. Stat3 has been reported to play a crucial role to maintain FOXP3 expression in human. Delivery of small interfering RNA (siRNA) for Stat3 into Tregs demonstrated the loss of the suppressor function (74). Recently, it has been reported that siRNA can be delivered in gold nanoparticles, circumventing the issue with a lentiviral system in human patients (75). A stable FOXP3 expression is dependent on posttranslational modification. Genetic or pharmacologic modulation of FOXP3 acetylation *via* the histone/protein acetyltransferases (HATs), p300, and CBP downregulated suppressive function of Treg and promoted antitumor immunity (76).

A recent study demonstrated that the pharmacologic inhibition of a single de-ubiquitination enzyme, Usp7, determines the fate of FOXP3 and Tip60 in Tregs, thus providing a target for therapeutic modulation of Treg function in antitumor immunity (77). It has been shown that selective small molecule inhibitors for the bromodomains of CREBBP/EP300 reduced FOXP3 expression, as well as expression of functional markers in Tregs (e.g., LAG-3, CTLA-4, and TIM-3) (78). It has also been reported that intranuclear interactomic inhibition of FOXP3 could abrogate suppressor function *via* nuclear delivery of FOXP3 (79). These approaches are promising at a preclinical stage, yet assessment of target-specific delivery of siRNA or Protein Transduction Domain-FOXP3 protein, and their side effects have not been assessed. Potential autoimmune responses should be considered when Treg dysfunction is implemented as a therapeutic approach in cancer immunotherapy. An additional concern is that a series of surprising reports found that a high incidence of tumor-infiltrating Tregs is associated with improved prognosis in cancer patients (80–83). Thus, the inhibition of FOXP3 expression needs further study and careful consideration regarding the role of Tregs in a given tumor microenvironment.

GENE-MODIFIED Tregs IN AUTOIMMUNITY

Past successes using genetically enhanced T-cells in the cancer arena have prompted interest in the development of related approaches to suppress unwanted autoimmune responses. Refractory autoimmune disease is associated with a markedly decreased life expectancy urging consideration of intensive therapeutic approaches. Tregs provide an attractive tool for genetic targeting against autoantigens present in the organ(s) of interest.

Modulating Antigen Specificity and CAR Approach in Tregs to Treat Autoimmunity

Therapeutic effect of purified Tregs have been demonstrated in preclinical studies in a range of autoimmune disease models in mice, including Systemic lupus erythematosus (84), T1D, autoimmune hepatitis, inflammatory bowel diseases, and autoimmune encephalomyelitis (85–88). Subsequently, studies in several disease model systems have demonstrated that antigen-specific Tregs were present in diseased animals and more potent in suppressing pathogenic immune responses compared to polyclonal Tregs (9, 89).

Among autoimmune diseases, T1D has been an intense area of development for gene-modified Treg-mediated therapy with islet-specific Tregs. Most recently, it has been demonstrated that lentiviral TCR gene transfer to polyclonal human Tregs achieved human islet-specific and viral-specific CD4 T cell clones. This enabled antigen-specific suppression at increased potency compared to polyclonal Tregs, increasing optimism for the success of this approach (90). However, T cells transduced with islet-specific TCRs were less responsive to cognate antigen than T cells with virus-specific TCRs, suggesting further work in this area is needed. The animal model of multiple sclerosis, EAE, has been instrumental in testing gene-modified Tregs for therapeutic intervention in neurological autoimmune diseases. For example, a lentiviral gene delivery system was used to express a CAR targeting myelin oligodendrocyte glycoprotein with the murine FOXP3 in CD4 T cells. Intranasal administration of these cells diminished ongoing neuronal inflammation *in vivo* (61).

Several other attempts to utilize CAR^+ Tregs to treat autoimmunity have revealed the important fact that activation of Tregs

needs to be antigen-specific; this was found in murine colitis and arthritis models as well as in human Treg activation (91–93). This appears a critical point since in many autoimmune disorders autoantigen(s) that trigger autoimmune responses are unknown. In the case of murine arthritis, naïve $CD4^+$ T-cells were engineered to co-express FOXP3 with HLA-DR1, covalently linked to an immunodominant peptide capable of driving collagen-induced arthritis. HLA-DR1 is associated with human rheumatoid arthritis. By this approach, T-cells were equipped with a bait molecule that allowed them to engage collagen autoreactive $CD4^+$ T-cells in a TCR-dependent manner. In DR1 humanized mice, the engineered T-cells could inhibit the development of autoimmune arthritis more effectively than cells engineered to express FOXP3 alone (94). However, this approach warrants further studies, among other reasons because of the distinct subset of $CD4^+CD25^+$ Tregs expressing HLA-class II in humans (95).

Inducing FOXP3 Expression to Treat Autoimmunity

In addition to TCR modulation, modulation of FOXP3 expression itself is a promising strategy to treat autoimmune diseases. IPEX syndrome is a hereditary immunodeficiency characterized by the loss of function of FOXP3-expressing Tregs (11). A recent study demonstrated the lentiviral delivery of the FOXP3 gene into IPEX-derived CD4 T cells produced a stable Treg population. In this study, CD4 T cells from IPEX patients were converted into FOXP3-expressing Tregs, and they acquired Treg-like phenotypes *in vivo*. When FOXP3 is expressed by lentiviral gene transduction, T-cells express several Treg markers such as CD25, CTLA-4, and GITR. Functionally, the cells resembled Tregs with decreased proliferation, hypo-responsiveness, reduced cytokine release, and suppressive activity similar to purified Tregs (96). This approach for FOXP3 gene transfer with adoptive cell therapy may potentially be a promising approach to treat IPEX patients as well as other autoimmune patients with dysfunctional human Tregs. Further studies regarding the stability of FOXP3 expression in these CD4 T cells and further assessment of the efficacy of this approach in clinical settings are warranted.

Retroviral delivery of the FOXP3 gene into purified $CD4^+CD25^-CD45RO^-$ human T cells showed unstable levels of FOXP3 and Treg-associated phenotypic markers while lentiviral delivery using elongation factor-1α showed reliable expression of CD25 and GITR (97). An alternative approach may be to enforce Treg differentiation using a cell-permeable form of FOXP3 protein with a transduction domain. The introduction of FOXP3 in protein form induced a Treg phenotype in human and mouse T cells, respectively (98, 99). Repeated infusion of FOXP3 with a transduction domain showed amelioration of the *scurfy* phenotype, and inflammatory bowel disease and rheumatoid arthritis mouse models (100, 101). The cost of infusion for protein delivery in a clinical setting for human patients, the stability of a functional Foxp3 protein *in vivo*, and lack of specificity in immunosuppression due to Foxp3 protein delivery to the nucleus awaits further optimization of this approach.

MANIPULATION OF Tregs IN ALLOTRANSPLANTATION AND OTHER DISEASES

Clinical evaluation of adoptive immunotherapy using Tregs is attracting increasing interest. Most experience has been gained using donor-derived Tregs, which have been infused safely in patients treated with allogeneic stem cells (102). These studies have also provided encouraging evidence of efficacy in prevention of GVHD, even in the context of haploidentical stem cell transplantation (102, 103).

Approaches to Generate Alloantigen-Specific Tregs

Similar to improved ability of autoantigen-specific Treg to control autoimmune inflammation, alloantigen-specific Tregs are more effective than polyclonal Tregs at preventing organ or tissue graft rejection. These alloantigen-specific Tregs were enriched by *in vitro* alloantigen-stimulated expansion or the expression of a TCR transgene (104–106). A humanized mouse model of skin graft rejection has also shown the potency of suppressor function of alloantigen-expanded human Tregs (107). Tregs expressing CARs could also be used in the context of transplantation. For example, a CAR approach targeting HLA-A2 has been used to produce alloantigen-specific Tregs (108). CAR-stimulated Tregs showed minimal cytotoxicity. *In vitro*, HLA-A2-CAR Tregs maintained high levels of FOXP3 expression and other Treg markers, and stable demethylation of the TSDR ensured suppressor function. The HLA-A2 approach may have significant advantages in the clinical setting where a sufficient number of APCs are required (107), and the potential loss of FOXP3 after repeated stimulation has been reported (109). With improved stability alloantigen-specific Tregs will have more versatile uses in future transplantation trials.

Other Gene-Modification Approaches for Generating Suppressor Lymphocytes

In vitro generation of Tr1 cells has been developed for clinical purposes. However, a major caveat of clinical use of Tr1 cell therapy is lack of purity. Andolfi et al. showed lentiviral delivery of IL-10 and GFP could generate a homogeneous Tr1 cell population to circumvent this issue (110). These "pure" Tr1 cells showed an anergic phenotype and TGF-β/IL-10 -dependent suppression of allogeneic T-cell responses and successfully controlled GVHD (110). Tr1 cells were generated *in vitro* using genetically modified B cells in an allergy model in an antigen-specific manner. Retroviral transduction of the fusion protein, Derp 2, a major house dust mite allergen, with an endosomal targeting sequence (gp75) was performed in B cells for efficient MHC class II presentation. The engineered B cells were adoptively transferred to the host (BALB/c mice) before or after peptide immunization. The production of IL-10 from these retrogenic B cells and the induction of IL-10 expressing Tr1 cells achieved allergen-specific immune tolerance against asthma (111). Although the result is encouraging, more studies with different types of allergens, or the use of humanized mouse

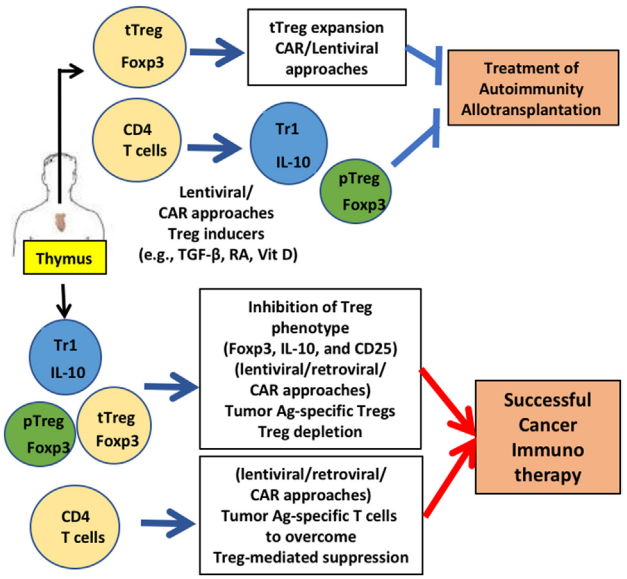

FIGURE 1 | Gene-modified regulatory T cell (Treg)-mediated therapy. Engineering of Tregs aims to generate Tregs that are functionally similar to various types of Tregs that were identified in human: thymic-derived thymic-derived Treg (tTreg), transforming growth factor (TGF)-β-induced peripheral Treg (pTreg), and IL-10 expressing Tr1 cells. CD4 T cells and Tregs are generated from thymus. For treating autoimmunity or allotransplantation, gene-modification approaches aim to acquire a stable Treg phenotype and sufficient numbers *via in vitro* expansion to obtain enough cells for treatment. Generation of antigen-specific Tregs reduces the number of Tregs for therapy significantly. For successful cancer immunotherapy, Treg function needs to be downregulated. Destabilizing Treg functions by inhibiting FOXP3 and other functional Treg proteins (e.g., CD25) by gene modification is under development. Applications for these gene-modified Tregs are currently being expanded in mice (e.g., humanized mice) and men (human Tregs for clinical trials).

models, could be considered to assess the potential scope of this approach.

Corneal allograft failure is mediated by CD4 T cells (112). CD25 in CD4 T cells plays an important role in the induction of corneal graft rejection. CD25-mediated signaling is associated not only with the expression of Treg cytokines (IL-10, TGF-β) but also T helper 1 type cytokines (IFN-γ, IL-1β, and TNF-α). A recent study showed that the use of CD25 siRNA in a corneal transplantation model significantly prolonged graft survival time on Sprague-Dawley rat recipients with Wistar rat donors (113). In this study, neovascularization and maintenance of transparency of the cornea were significantly improved. However, similar studies have not been extended to human patients, and the safety of this approach remains to be tested.

FUTURE PERSPECTIVE IN GENE-MODIFIED Treg THERAPY

Recent technical advances and developments in the field of gene-modified Treg therapy have evolved into a new era. It is clear that the approach is very promising, yet several hurdles need to be overcome before broad clinical implementation. One of the biggest concerns is to ensure the purity of clinical products using GMP-based protocols. There is some concern about the stability of engineered Tregs and the fact that some Tregs might be converted into effector T cells, particularly into Th17 type cells (114, 115). Approaches that may be helpful to maintain FOXP3 expression have been discussed including all-trans-retinoic acid, IL-2, vitamin C or *ex vivo* treatment with rapamycin (116–119). For lentiviral gene transfer approaches, studies in the past showed long-term safety in human immunodeficiency virus patients who received gene-modified T-cells without genotoxic effects such as clonal expansion (120). Development in vector engineering has also achieved enhanced genetic stability and greater stability of transgene expression, providing greater safety (121). In TCR engineering, there is a concern about TCR cross-reactivity which is caused by recognition of low-affinity antigen by a TCR. There were two cases in which T cells were engrafted with an affinity-enhanced TCR selected for the tumor antigen, MAGE A3, and this TCR was found to have cross-reactivity and cardiovascular toxicity (122, 123).

CONCLUSION

The regulatory functions of Tregs and specificity to various types of stimuli triggered intense research efforts to develop these cells for various clinical treatments. For example, CAR-T cells, lentiviral gene transfer, small molecule compounds that regulate FOXP3 expression, and infusion of cell-permeable FOXP3 proteins were developed (**Figure 1**). Potential uncertainties of gene-modified Treg therapy remain, as well as the challenges of the manipulation of Tregs under GMP conditions, and concerns of effector-mediated toxicity due to lack of purity, unstable Treg phenotypes and TCR cross-reactivity. However, alternate approaches are being sought and tested and as the clinical data emerge, these challenges shift to the further evolution of innovative therapeutic approaches.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Early Growth Response Gene 2-Expressing CD4$^+$LAG3$^+$ Regulatory T Cells: The Therapeutic Potential for Treating Autoimmune Diseases

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Regulatory T cells (Tregs) are necessary for the maintenance of immune tolerance. Tregs are divided into two major populations: one is thymus derived and the other develops in the periphery. Among these Tregs, CD4$^+$CD25$^+$ Tregs, which mainly originate in the thymus, have been extensively studied. Transcription factor *Foxp3* is well known as a master regulatory gene for the development and function of CD4$^+$CD25$^+$ Tregs. On the other hand, peripheral Tregs consist of distinct cell subsets including Foxp3-dependent extrathymically developed Tregs and interleukin (IL)-10-producing type I regulatory T (Tr1) cells. Lymphocyte activation gene 3 (LAG3) and CD49b are reliable cell surface markers for Tr1 cells. CD4$^+$CD25$^-$LAG3$^+$ Tregs (LAG3$^+$ Tregs) develop in the periphery and produce a large amount of IL-10. LAG3$^+$ Tregs characteristically express the early growth response gene 2 (*Egr2*), a zinc-finger transcription factor, and exhibit its suppressive activity in a Foxp3-independent manner. Although *Egr2* was known to be essential for hindbrain development and myelination of the peripheral nervous system, recent studies revealed that *Egr2* plays vital roles in the induction of T cell anergy and also the suppressive activities of LAG3$^+$ Tregs. Intriguingly, forced expression of *Egr2* converts naive CD4$^+$ T cells into IL-10-producing Tregs that highly express LAG3. Among the four *Egr* gene family members, *Egr3* is thought to compensate for the function of *Egr2*. Recently, we reported that LAG3$^+$ Tregs suppress humoral immune responses *via* transforming growth factor β3 production in an Egr2- and Egr3-dependent manner. In this review, we focus on the role of *Egr2* in Tregs and also discuss its therapeutic potential for the treatment of autoimmune diseases.

Keywords: Egr2, Egr3, lymphocyte activation gene 3, Foxp3, regulatory T cell, gene therapy, cell therapy

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INTRODUCTION

Autoimmunity can cause a broad range of human diseases. This pathology is observed in at least 5% of the general population (1). To maintain self-tolerance, immune systems evolved mechanisms that discriminate between self and non-self and respond to infection by pathogens such as viruses, bacteria, and parasites, while maintaining unresponsiveness to self-antigens. However, the discrimination between self and non-self is disrupted in some individuals and the immune system is misdirected to attack self-antigens. These conditions can affect one or many different types of organs in the body.

Immune tolerance is maintained by many regulatory cell populations, including CD4$^+$ regulatory T cells (Tregs) (2), CD8$^+$ Tregs (3), regulatory B cells (4), dendritic regulatory cells (5), and

regulatory macrophages (6). Among these regulatory cells, the $CD4^+$ Treg subset, which has a pivotal role in the control of self-tolerance and inflammatory responses, is the most extensively studied in the context of autoimmunity. $CD4^+$ Tregs are divided into two main subsets: one includes thymus-derived, naturally occurring Tregs (nTregs), and the other develops in the periphery. nTregs co-express interleukin (IL)-2 receptor α (CD25) and the transcription factor Forkhead box P3 (Foxp3) protein (7). *Foxp3* is a crucial gene for the development and regulatory function of $CD4^+CD25^+$ Tregs ($CD25^+$ Tregs). The identification of both surface markers and a master regulatory transcription factor has significantly contributed to our understanding of molecular suppressive mechanisms of Tregs. These thymus-derived Tregs (tTregs) can expand in the periphery and exert their antigen-specific suppressive activities to maintain immune tolerance (8, 9).

The majority of the $CD4^+$ Treg subset develops in the periphery, and they likely exert their suppressive activities *via* a Foxp3-independent manner. An experiment of adoptive transfer of $CD4^+Foxp3^-$ cells into non-lymphopenic hosts suggested that peripheral conversion could account for approximately 4–7% of $Foxp3^+$ Tregs (10). Other group reported that $Foxp3^+$ Tregs developed in the periphery comprise ~15% of the peripheral $Foxp3^+$ Tregs (11). These peripherally derived Tregs (pTregs) are thought to play a distinct role in controlling adaptive immunity to restrain allergic inflammation at mucosal surfaces (12).

The lack of specific markers that can reliably distinguish Foxp3-independent Tregs from other T cell populations makes it difficult to assess their suppressive mechanisms. In 2009, we identified a Foxp3-independent IL-10-producing Treg subset, i.e., $CD4^+CD25^-Foxp3^-$ T cells. These cells characteristically express both the lymphocyte activation gene 3 (Lag3) and the transcription factor early growth response gene 2 (Egr2) (13).

In a broad range of autoimmune diseases, these Treg subsets are impaired and decreased in frequency. Therefore, many approaches have been examined to expand functional Treg subsets both *in vitro* and *in vivo*. Gene modification of $CD4^+$ T cells could be used to induce Treg subsets for therapeutic intervention in autoimmune diseases. During the past decade, a numbers of murine and human studies have investigated the therapeutic potential of *Foxp3* gene transduction in $CD4^+$ T cells. The present review focuses on the molecular features of *Egr2* in Tregs and discusses the prospects and obstacles to the clinical development of gene modified Treg cell therapy.

NOMENCLATURE OF $CD4^+$ Tregs

The discovery of the role of Foxp3 is considered the most important finding in Treg biology. Deficiency of the *Foxp3* gene abrogates self-tolerance and causes autoimmune disease (14). Scurfy mice, which have a frame shift mutation in the *Foxp3* gene, fail to generate thymus-derived, nTregs and display extensive severe inflammatory infiltration in multiple organs such as the lung, skin, and liver (15). Immunodysregulation, polyendocrinopathy, enteropathy, and X-linked (IPEX) syndrome, which is caused by mutations in the *FOXP3* gene, is characterized by neonatal autoimmune type 1 diabetes, polyendocrinopathy, autoimmune hemolytic anemia, autoimmune enteropathy, and skin rash (16). A common feature of scurfy mice and IPEX syndrome is a severe deficiency of $CD25^+$ Tregs. Thus, Foxp3 is considered the "master regulator" of $CD25^+$ Tregs. With regard to Foxp3-dependency, Foxp3-dependent Tregs can be divided into three populations (17): first are tTregs, also known as thymus-derived nTreg. Second, $Foxp3^+$ Tregs that differentiate in the periphery from $Foxp3^-$ conventional $CD4^+$ T cells are termed "peripherally derived Tregs." The nomenclature for these two $Foxp3^+$ Tregs populations is clearly based on the anatomical locations of their differentiation. Although it has been widely assumed that freshly isolated $Foxp3^+$ Tregs mainly consist of tTregs, the ratio of tTregs to pTregs has not been completely clarified (18). Third, $Foxp3^+$ Tregs generated *ex vivo* are defined as "*in vitro*-induced Treg (iTreg)." Foxp3 can be upregulated upon T cell receptor (TCR) stimulation of peripheral naive $CD4^+$ T cells in the presence of transforming growth factor (TGF)-β1 (19). Although the term "iTreg" is also widely used to define "extrathymically generated Tregs in the periphery" regardless of Foxp3 dependency, we use the term "iTreg" as "$Foxp3^+$ Treg generated *ex vivo*" in the present review.

On the other hand, Foxp3-independent Treg subsets are thought to develop in the periphery. These Treg subsets consist of heterogeneous subsets, including IL-10-producing $CD4^+$ type I Tregs (Tr1 cells) (20) and iTr35 (21). Tr1 cells are defined by a unique cytokine production profile consisting of high levels of IL-10 and their ability to suppress immune responses through IL-10 production in a Foxp3-independent manner (20). Tr1 cells also produce variable amounts of IL-5, GM-CSF, and IFN-γ and minimal amounts of IL-2, IL-4, and IL-17 (22, 23). Although cytokine profiles of Tr1 cells, such as TGF-β1, IFN-γ, and IL-5, are dependent on experimental conditions, IL-10 production is thought to be the true hallmark of Tr1 cells (24). Production of IL-4 is consistently undetectable, which is distinct from Th2 cells. In this review, in accordance with conventional nomenclature, we mainly use the general term "Tr1 cells" for both "peripherally derived Tr1 cells (pTr1 cells)" and "*in vitro*-induced Tr1 cells (iTr1 cells)."

The Foxp3-dependent Tregs, including tTreg, pTreg, and iTreg, and Foxp3-independent extrathymically developed Tregs such as Tr1 cells are thought to be fundamental for maintaining adequate immune tolerance.

IL-10-PRODUCING $CD4^+CD25^-LAG3^+$ Tregs

Interleukin-10 is an anti-inflammatory cytokine that is produced by a wide range of cell types, including not only $CD4^+$ cells but also $CD8^+$ T cells, B cells, dendritic cells, macrophages, mast cells, natural killer cells, eosinophils, and neutrophils during the course of immune responses (25). As for $CD4^+$ Th cells, IL-10 was first described as a product of Th2 cells that suppressed cytokine secretion from Th1 cells (26). Th1 cells also produce IL-10 *via* ERK1 and ERK2 MAP kinase phosphorylation and IL-12-induced transducer and activator of transcription (STAT) 4 activation (27). IL-10 production from Th17 cells exerts tissue-protective

and immunosuppressive effects (28). During infections, IL-10 production from these Th cell subsets might be an essential mechanism underlying the self-limitation that dampens excessive immune responses and prevents tissue damage (29).

Production of IL-10 is closely related to the function of Treg subsets. IL-10-producing Tregs can be developed *in vivo* in both Foxp3-dependent and Foxp3-independent manners (30). To date, two major subsets of IL-10-producing Tregs have been identified; one subset includes Foxp3$^+$ Tregs and the other is represented by Foxp3-independent Tr1 cells generated extrathymically. However, Foxp3$^+$ Tregs do not produce IL-10 following stimulation after *ex vivo* isolation, unless isolated from the gut. Foxp3$^+$ Tregs inhibit naive T cell proliferation *in vitro* in an IL-10-independent manner. In contrast, Foxp3$^+$ Tregs exert their suppressive activity *in vivo* in an IL-10-dependent manner, suggesting that Foxp3$^+$ Tregs need signals *in vivo* to induce IL-10 (25). On the other hand, the best characterized Foxp3-independent, IL-10-producing Tregs are Tr1 cells. Although other Th subsets also produce IL-10 (see above), Tr1 cells produce greater amounts of IL-10 shortly after activation compared to other Th subsets (31, 32). Andolfi et al. demonstrated that forced expression of IL-10 by human CD4$^+$ T cells confers the phenotype and function of Tr1 cells (2).

Type I Tregs have been a focus of active investigation. Nonetheless, until recently, it has been difficult to assess their *in vivo* physiological function because of the lack of specific cell surface markers and master regulatory genes. However, in 2009, we identified IL-10-producing CD4$^+$CD25$^-$ Tregs that characteristically express cell surface marker LAG3 and transcription factor Egr2 (13). Approximately 2% of the CD4$^+$CD25$^-$ T cell population in the spleen consisted of CD4$^+$CD25$^-$LAG3$^+$ T cells (LAG3$^+$ Tregs) (**Figure 1**). Unlike tTregs, high-affinity interactions with self-peptide/major histocompatibility complex ligands expressed in the thymus are not necessary for the development of LAG3$^+$ Tregs. Those results indicate that LAG3$^+$ Tregs are extrathymic in origin. LAG3$^+$ Tregs, which do not express Foxp3 protein, secrete higher levels of IL-10 than do CD25$^+$ Tregs. In addition, LAG3$^+$ Tregs are hypoproliferative in response to TCR stimulation. Moreover, they suppress the *in vivo* development of colitis induced in RAG-1$^{-/-}$ recipients by the transfer of naive CD4$^+$ T cells in an IL-10-dependent manner. LAG3$^+$ Tregs from Scurfy mice still express *IL10* mRNA and retain regulatory activity *in vitro*. These findings indicate that LAG3$^+$ Tregs are equivalent to pTr1 cells that exist in a steady state.

Groux et al. first reported a unique cytokine production profile of Tr1 cells induced *in vitro*, i.e., IL-10$^+$ IL-4$^-$ IL-5$^+$ TGF-β1$^+$ IL-2$^{low/-}$ IFN-γ$^{low/-}$ (23). Th3 regulatory cell induced *in vivo* is a potent source of TGF-β1, IL-10, and IL-4 protein (33). However, LAG3$^+$ Tregs do not produce TGF-β1, IL-4, and IL-5 protein (13). Thus, although LAG3$^+$ Tregs fulfill the definition of Tr1 cells (24, 34), further studies are required to elucidate the inconsistency between these IL-10-producing Tregs induced *in vitro* or *in vivo* and LAG3$^+$ Tregs, which exist in a steady state, in more detail. Recently, Gagliani et al. reported that concomitant expression of LAG3 and CD49b is specific for Tr1 cells in humans and mice (35). Subsequent findings in antigen-specific immunotherapy based on the administration of cognate peptides in escalating dose immunotherapy (EDI) indicated that the levels of LAG3 and CD49b correlated positively with IL-10 expression in CD4$^+$ T cells, and LAG3 was highly upregulated and was maintained during EDI treatment (36). Although *Lag3* mRNA is expressed on CD4$^+$CD25$^+$Foxp3$^+$ Tregs (37), LAG3 protein was hardly detected on the cell surface of CD4$^+$CD25$^+$ Tregs (38). These observations indicate that LAG3 and CD49b are phenotypic markers of IL-10-producing, Foxp3-independent, extrathymically induced CD4$^+$ Tregs that play a major role in regulating the activity of the immune system (39).

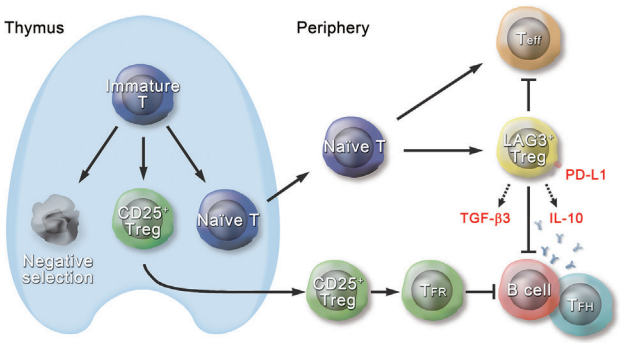

FIGURE 1 | Schematic diagram of the role of CD4$^+$CD25$^-$LAG3$^+$ regulatory T cells (Tregs) in the immune system. Approximately 2% of the CD4$^+$CD25$^-$ T cell population in the spleen consists of CD4$^+$CD25$^-$LAG3$^+$ T cells (LAG3$^+$ Tregs), which are extrathymic in origin. LAG3$^+$ Tregs characteristically express transcription factor early growth response gene 2 (Egr2) and produce large amounts of interleukin (IL)-10 and transforming growth factor (TGF)-β3 in response to antigenic stimulation. Ectopic expression of Egr2 by naive CD4$^+$ T cells confers the phenotype of LAG3$^+$ Tregs, including expression of Prdm1 and IL-10. LAG3$^+$ Tregs suppress effector T cells (Teff) in an IL-10-dependent and Foxp3-independent manner. Like thymus-derived CD4$^+$CD25$^+$Foxp3$^+$CXCR5$^+$ Tregs, called follicular regulatory T (T_{FR}) cells, LAG3$^+$ Tregs control germinal center responses. Although T_{FR} cells express CXCR5 and exist in the germinal center (GC), LAG3$^+$ Tregs do not express CXCR5 protein and are located at the T-B border in the steady state. Suppression of GC responses by LAG3$^+$ Tregs is mediated by TGF-β3, and Egr2 is essential for effective secretion of TGF-β3 from LAG3$^+$ Tregs. TGF-β3-mediated B cell suppression requires programmed cell death 1 (PD-1) expression on B cells, and on the other hand, LAG3$^+$ Tregs highly express PD-1 ligand (PD-L1). Thus, LAG3$^+$ Tregs are equipped with various mechanisms to maintain the immune tolerance.

Interleukin-10 receptor is expressed on a variety of immune cells (40). IL-10-deficient mice develop an inflammatory bowel disease characterized by dysregulated production of pro-inflammatory cytokines (41) and show a prolonged fever in response to lipopolysaccharide (42). Deficiency of IL-10 exacerbates autoimmune pathology in mouse models of systemic lupus erythematosus (SLE) (43), rheumatoid arthritis (RA) (44), and experimental autoimmune encephalomyelitis (EAE) (45), indicating the critical role of IL-10 in the regulation of immune homeostasis. Thus further studies on LAG3$^+$ Tregs could provide insights into the development of new therapeutic targets for autoimmune diseases.

ROLE OF Egr2 IN IL-10-PRODUCING Tregs

Differential gene expression profiles of LAG3$^+$ Tregs, CD25$^+$ Tregs, CD4$^+$CD25$^-$LAG3$^-$ T cells and naive CD4$^+$CD25$^-$CD45RB^{high}

T cells have been constructed by gene array analysis. The data revealed that transcription factor Egr2 were preferentially expressed in LAG3$^+$ Tregs (13). The Egr family consists of four Cys2His2-type zinc-finger transcription factors, Egr-1, -2, -3, and -4. Egr2 plays an essential role in hindbrain development and myelination of the peripheral nervous system. Egr2 deficiency results in perinatal or neonatal death due to respiratory and/or feeding deficits (46). During the last decade, the role of Egr2 in T cells has been extensively elucidated. Egr2 is necessary for the TCR-induced negative regulatory program because it controls the expression of the E3 ligase Cbl-b, which is critical for the regulation of T cell tolerance and anergy (47, 48). Zhu et al. revealed that Egr2 binds directly to the promoter of the cell cycle inhibitor $p21^{cip1}$ in T cells (49).

Our previous study revealed the role of Egr2 in Tregs (13). In the steady state, LAG3$^+$ Tregs, but not Foxp3$^+$ Tregs, specifically expressed not only Egr2 but also IL-10 and Blimp-1, which is a critical regulator for IL-10 production from Th subsets (50–52). To determine whether forced expression of Egr2 in naive CD4$^+$ T cells could convert them to a LAG3$^+$ Treg phenotype, we constructed retroviral vectors that co-expressed green fluorescent protein (GFP) and Egr2. As expected, the Egr2-transduced GFP$^+$CD4$^+$ cells showed significant upregulation of LAG3 and Blimp-1. In addition, these Egr2-transduced GFP$^+$CD4$^+$ cells produced high levels of IL-10 and lower amounts of IL-2, IL-4, and IL-5 proteins, resembling the cytokine profile seen in Tr1 cells (20). We confirmed that the ectopic expression of Egr2 conferred a suppressive function on activated CD4$^+$ T cells (13). These observations clearly demonstrated the critical importance of Egr2 for IL-10 expression in LAG3$^+$ Tregs.

Early growth response gene 2 is expressed at high levels in LAG3$^+$ Tregs. In contrast, it is barely expressed in other T cell subsets in the steady state (13, 53). Egr2 is induced in T cells after TCR stimulation (54). *In vitro*, Egr2-deficient T cells proliferate normally in response to TCR stimulation. However, Egr2-deficient T cells are hyperresponsive to exogenous IL-2, indicating that Egr2 does not affect TCR signaling, but controls the subsequent expansion of activated T cells (49). Such Egr2-deficient T cells produce higher levels of effector cytokines such as IFN-γ and IL-17. Recently, Du et al. demonstrated that Egr2 plays a pivotal role in T cells' response to influenza virus infection by directly binding to *Tbx21* gene and promoting the expression of T-bet (55). In contrast, another group showed that Egr2 is not required for T cell responses to *Toxoplasma gondii* or lymphocytic choriomeningitis virus (56). On the other hand, Miao et al. reported that Egr2 directly interacts with Batf, a transcription factor that regulates both IL-17 production and Th17 differentiation and blocks its binding to the *IL-17* promoter (57). As for follicular helper T (T_{FH}) cells, during viral infection, Egr2 and Egr3 directly regulate the expression of Bcl6 and differentiation of T_{FH} cells (58). Interestingly, despite increased effector function of Egr2-deficient T cells as described above, IL-2 production and proliferation of T cells from Egr2- and Egr3-deficient T cells are impaired (59). More recently, Miao et al. revealed that Egr2 and Egr3, induced in activated T cells, suppress T cell activation and differentiation but promote clonal expansion of virus-specific T cells (53). These observations indicate that Egr2 and Egr3 have distinct function in homeostatic condition and infection.

IL-27 INDUCES Egr2 EXPRESSION

Interleukin-27, a member of the IL-12 cytokine family, plays a critical role in the development of Tr1 cells and the resolution of inflammation (60). IL-27 is a heterodimeric cytokine composed of subunit proteins IL-27p28 and EBV-induced protein 3 (61). Deficiency of IL-27 signaling results in significant reduction of IL-10-producing T cells during autoimmune disease and infection (60). IL-27 receptor signaling induces expression of cMaf and aryl hydrocarbon receptor, both of which transactivate the *IL10* and *IL21* promoters (62). Recently, Karwacz et al. reported that interferon regulatory factor 1 and BATF were rapidly induced after treatment with IL-27 and were necessary for the differentiation and function of Tr1 cells *in vitro* and *in vivo* (63). Both transcription factors were critical for preparing the chromatin landscape during Tr1 differentiation. We previously demonstrated that Egr2 mediates IL-27-induced IL-10 production in CD4$^+$ T cells (50). Egr2 was induced by IL-27 in a STAT3-dependent manner and directly bound to the promoter region of *Prdm1*, encoding Blimp-1, and enhanced its activity. When cells were deficient for Egr2, IL-27 failed to induce Blimp-1 and IL-10 in CD4$^+$ T cells. These observations support the essential role of Egr2 expression in the induction of IL-10 in CD4$^+$ T cells.

Egr2 AND AUTOIMMUNE DISEASES

In humans, mutations in *Egr2* cause Charcot–Marie–Tooth disease type 1, Dejerine–Sottas syndrome, and congenital hypomyelination neuropathy (64). Recent genome-wide association studies (GWAS) have identified new genetic links between *Egr2* and human autoimmune diseases. Two independent GWAS investigations revealed strong association signals for Crohn's disease (the most common form of chronic inflammatory bowel disease) on chromosome 10q21, within which *Egr2* is located (65, 66). In line with these observations, we previously demonstrated that adoptive transfer of Egr2-expressing LAG3$^+$ Tregs effectively ameliorated intestinal inflammation in a murine T cell transfer model of colitis (13).

A candidate gene analysis revealed that polymorphisms in the *Egr2* gene influenced the susceptibility to SLE (67). SLE is an autoimmune disease characterized by a wide range of anticellular and antinuclear autoantibodies that affect multiple organs. SLE is induced by combinations of environmental and genetic factors (68). Initially, the survival of patients with SLE showed improvements. However, in the last two decades, no substantial improvements in patient survival have been observed (69, 70). Thus, further studies are needed to clarify the precise molecular mechanisms that are involved in the pathogenesis of SLE.

Recent studies have shown an association of the *Egr2* gene with the occurrence of lupus in mice. T cell-specific Egr2 conditional knockout (CKO) mice develop progressive lupus-like autoimmunity with no impact on the development of Foxp3-dependent CD25$^+$ Tregs (49). Moreover, mice deficient for both Egr2 and Egr3 in B and T cells present lethal and early-onset systemic

autoimmunity, suggesting a synergistic role for Egr2 and Egr3 in controlling B cell tolerance (59). The association between Egr2 and autoantibody-mediated systemic autoimmunity suggested a linkage between Egr2-expressing LAG3$^+$ Tregs and the control of lupus activity. To clarify the role of Egr2 in T cells, we generated T-cell-specific Egr2 CKO mice (*Egr2*$^{fl/fl}$ CD4-*Cre*$^+$) (71). Egr2CKO mice showed significant increases in the proportion of T_{FH} cells and germinal center B (GCB) cells and exhibited an enhanced antibody response against T cell-dependent antigens. Transfer of wild-type LAG3$^+$ Tregs significantly suppressed spontaneous T_{FH} and GCB formation and inhibited aberrant antibody responses.

In lupus-prone MRL-*Fas*$^{lpr/lpr}$ (MRL/lpr) mice, adoptive transfer of LAG3$^+$ Tregs from control MRL-*Fas*$^{+/+}$ (MRL/+) mice suppresses the progression of lupus in a TGF-β3-dependent manner. Although the pro-inflammatory role of TGF-β3 was previously demonstrated by the observation that TGF-β3 and IL-6 promote pathogenic Th17 cell differentiation (72, 73), the anti-inflammatory role of TGF-β3 has attracted little attention. As for helper T-cell development, TGF-β3 is autonomously produced by Th17 cells (73). We confirmed that not only Th17 cells but also Th1 cells produced TGF-β3; however, LAG3$^+$ Tregs secreted greater amounts of TGF-β3 compared with Th1 and Th17 cells. TGF-β3 suppressed the phosphorylation of STAT6, Syk, and NF-κB p65 in activated B cells, indicating that TGF-β3 inhibits important pathways for B cell functions (71). TGF-β3 also effectively suppresses human B cells (74).

Moreover, we have demonstrated that LAG3$^+$ Treg-mediated B cell suppression requires programmed cell death 1 (PD-1), which provides negative co-stimulatory signals to both T cells and B cells (75, 76). The *PD1* gene has been identified as an SLE-susceptible gene (68), and its deficiency in mice promotes a lupus-like disease (77). Intriguingly, LAG3$^+$ Tregs highly express PD-1 ligand, and TGF-β3 enhanced PD-1 expression by stimulated B cells. These observations indicate that TGF-β3 produced by LAG3$^+$ Tregs plays a major role in the maintenance of humoral immune tolerance. As IL-10 strongly suppresses development and function of Th17 cells, IL-10 produced by LAG3$^+$ Treg may counteract the pro-inflammatory aspect of TGF-β3 (78). Further studies are necessary to confirm the synergistic effects of TGF-β3 and IL-10 in the immune system.

Recently, we addressed the role of Egr2 in the induction of TGF-β3 in LAG3$^+$ Tregs (79). Among the four Egr family members, Egr3 is able to partially compensate for Egr2 function (49). As expected, the absence of both Egr2 and Egr3 in T cells resulted in a significant reduction of TGF-β3 secretion from LAG3$^+$ Tregs and led to earlier onset of a lupus-like syndrome compared with Egr2CKO mice. Unexpectedly, *Tgfb3* mRNA was observed in LAG3$^+$ Tregs even when Egr2 and/or Egr3 were deficient. TGF-β3 undergoes complex processing steps intracellularly before its secretion from the cell surface (80, 81). After translation, TGF-β3 precursor protein is cut by furin and forms a small latent complex (SLC) that consists of mature TGF-β3 and latency associated peptide (LAP). SLCs are usually associated with latent TGF-β-binding protein (Ltbp) and secreted outside the membrane as a large latent complex. The Ltbp family consists of four members. Among them, the expression of Ltbp1–4 (82) and Ltbp3 is maintained by Egr2, and Egr3 was required for TGF-β3 secretion from LAG3$^+$ Tregs. Thus, Egr2 expressed in CD4$^+$ T cells has an integral role in a broad range of immunological balances.

A THERAPEUTIC PERSPECTIVE

Tregs for Potential Cell Therapy

Therapeutic use of Tregs to treat aberrant immune responses that cause autoimmune diseases is now an important field of investigation. For example, the Scurfy mouse phenotype is ameliorated by adoptive transfer of tTregs (83). Other approaches to adoptive Treg cell therapy using either Foxp3-dependent or Foxp3-independent Tregs have been demonstrated in various autoimmune disease mouse models, including SLE (71, 84), type 1 diabetes (85), EAE (86), inflammatory bowel disease (13, 87), and collagen-induced arthritis (CIA) (88). Adoptive Treg cell transfer is also an effective treatment for allograft rejection (89).

In RA patients, treatment with a humanized anti-IL-6R monoclonal antibody (tocilizumab) reduced circulating Th17 cells and increased pTregs (90). We have confirmed that, similar to murine LAG3$^+$ Tregs, human CD4$^+$CD25$^-$CD45RA$^-$LAG3$^+$ T cells express *Egr2*, *IL10*, and *TGFB3* mRNAs and suppressed antibody production from B cells when co-cultured with T_{FH} cells (71). Recently, we showed that the frequency of LAG3$^+$ Tregs in RA patients was lower, especially those with higher Clinical Disease Activity Index scores, compared to healthy donors. Moreover, LAG3$^+$ Tregs significantly increased after 6 months of treatment with abatacept, a CTLA-4 fusion protein. *In vitro* abatacept treatment conferred LAG3 and Egr2 expression on naive CD4$^+$ T cells, and abatacept-treated CD4$^+$ T cells exhibited suppressive activity (91).

Collectively, these results suggest approaches for the use of Treg subsets in the treatment of human diseases. Recent clinical trials, using either Foxp3$^+$ Tregs or Tr1 cells, proved the safety of Treg cell therapy and suggested possible therapeutic effects (22). However, the frequency of Tregs in the peripheral blood mononuclear cell fraction is very low. *Ex vivo*-expanded Tregs may change their suppressive phenotype posttreatment, because Xu et al. demonstrated that Tregs in the absence of TGF-β can differentiate into Th17 cells (92). Since it is also difficult to ensure the high purity of human Tregs using cell surface markers, these enriched Tregs may contain pathogenic autoreactive Th cells (93).

Gene Transfer Therapy

Gene transfer-based induction of Tregs offers an alternative promising treatment option for autoimmune diseases. Ectopic expression of the *FOXP3* gene in naive human CD4$^+$ T cells from healthy donors or IPEX syndrome patients renders the cells suppressive (94–97). In mice, therapeutic approaches using *Foxp3* gene-transduced CD4$^+$ Tregs have been successful in the induction of tolerance in graft-versus-host disease (98) and some autoimmune diseases (99, 100).

Gene transfer-based therapeutic approaches combining master regulatory genes of Tregs with an antigen-specific TCR could enhance the clinical efficacy of Tregs. This approach could generate large numbers of antigen-specific Tregs and reduce undesirable global immune suppression. The potential advantages are evident, as this treatment option contrasts with traditional drugs such as steroids, other immunosuppressive agents, and

biologic drugs. For example, we isolated a pair of TCR α and β genes from the paw of a mouse with CIA. We co-transduced this clonotype and the *Foxp3* gene into peripheral CD4+ T cells. These antigen-specific, modified Tregs effectively suppressed CIA. We also observed reductions in TNF-α, IL-17A, IL-1β expression and bone destruction even when transfer occurred after the onset of arthritis (101). In contrast, Foxp3-transduced T cells without antigen specificity did not have a therapeutic effect on CIA. Subsequently, another group demonstrated that adoptive transfer of TCRαβ and *Foxp3* gene-transduced CD4+ T cells suppressed T cell cytokine production and the proliferation of allergen-specific effector T cells (102).

Therapeutic Potential of Egr2-Expressing LAG3+ Tregs

Our laboratory demonstrated that forced expression of Egr2, which is preferentially expressed by LAG3+ Tregs, in naive CD4+ T cells could convert them to the phenotype of LAG3+ Tregs (13). We investigated CD4+ T cells from chicken ovalbumin (OVA)-specific TCR transgenic DO11.10 mice transduced with pMIG-Egr2. These cells significantly suppressed delayed type hypersensitivity reactions against OVA compared with BALB/c CD4+ T cells transduced with pMIG-Egr2. Those results indicated the presence of antigen-specific suppressive activity in Egr2-transduced cells. These findings suggest that Egr2-associated Tregs, as well as Foxp3-associated Tregs, can modulate antigen-specific Treg cell therapy. We previously showed that adoptive transfer of IL-10-transduced T cells from chicken OVA-specific TCR transgenic DO11.10 mice ameliorated methylated bovine serum albumin (BSA)-induced arthritis when the arthritic joint was co-injected with OVA in addition to methylated BSA without impairing the systemic immune response to the antigen. Those experiments indicated the feasibility of an optional therapeutic approach using antigen-specific non-Treg cells transduced with regulatory effector molecules specific for Tregs such as IL-10 (103).

Antigen-Specific Chimeric Antigen Receptor (CAR) T Regulatory Cells

There are two major concerns in ectopic expression of antigen-specific TCR. First, there is the potential for mispairing between the ectopically transduced TCR and endogenous TCR (104). Second, there are limitations to the human leukocyte antigen restriction. Elinav et al. engineered hapten-specific CAR-transduced CD4+CD25+ Tregs that effectively ameliorated colitis induced by the same hapten (105). CAR, which redirects specificity for a desired cell surface antigen, consists of antigen-recognizing variable regions (scFVs) from monoclonal antibodies, a hinge/spacer peptide, a transmembrane region, and one or more cytoplasmic signaling domains. Recently, Eyquem et al. demonstrated that a CD19-specific CAR to the TCR alpha constant locus not only resulted in uniform CAR expression in human peripheral blood T cells but also averted accelerated T-cell differentiation and exhaustion. Thus, it increased the therapeutic potency of engineered T cells (106). It appears that CAR T cell therapy provides several advantages compared to gene therapy using a combination of TCR α and β single chains.

TGF-β as a Therapeutic Target

We previously reported that Egr2-expressing LAG3+ Tregs, which suppressed germinal center reactions, were enriched at the T-B border, where B cells interact with T_{FH} cells (71, 79). As described above, LAG3+ Tregs produce large amount of TGF-β3 and Egr2 is necessary for the effective secretion of TGF-β3 from LAG3+ Tregs (79). These findings showed the importance of the induction of Egr2 on T cells to maintain the humoral immune tolerance *via* TGF-β3. Although we confirmed that TGF-β1 produced similar effects as TGF-β3 on B cells, LAG3+ Tregs do not produce TGF-β1 (71). The isotype specific production of TGF-β might be advantage of Egr2-expressing LAG3+ Tregs for therapeutic approach, because TGF-β1, but not TGF-β3, promotes fibrosis (107, 108).

Current Issues of Gene Therapy

Overall, these observations indicate the therapeutic value of genetically modified Tregs in human autoimmune diseases. A previous study suggested that integrating viral vectors do not elicit clinically evident genotoxicity in T cells, unlike hematopoietic stem cells (109). However, many issues such as cell dose, stability, plasticity, epigenetic regulation, antigen specificity, cross-reactivity of engineered TCRs, vector design, and immunological responses to the transferred gene products need to be addressed.

CONCLUSION

Dysregulation of the immune system results in autoimmune diseases or allergies. Although impressive advances in the development of new drugs and optimization of therapeutic protocols are being made in these fields, many pathologies are still resistant to treatment. Many people with autoimmune diseases or organ transplantation require immune-suppressive drugs that are associated with a number of complications, including increased susceptibility to severe lethal infection. Antigen-specific cell therapy is the most physiologic means to manipulate immune responses. Adoptive immunotherapy with Tregs is entering early clinical trials to prove the safety of this novel therapeutic approach (22, 110). However, the difficulty of isolating and cloning stable Tregs involved in antigen-specific inhibition of immune responses has made it impractical. To circumvent these problems, gene therapy using master regulatory molecules and/or regulatory effector molecules of Treg subsets might be a very promising therapeutic approach. For the future development of these Treg-based therapies, further studies that delineate the exact role of each Treg subset, including tTregs, pTregs, and pTr1 cells, in each disease will be required.

AUTHOR CONTRIBUTIONS

TO, KY, and KF designed the outline and wrote the manuscript.

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REVIEW
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Gene Therapy With Regulatory T Cells: A Beneficial Alliance

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Gene therapy aims to replace a defective or a deficient protein at therapeutic or curative levels. Improved vector designs have enhanced safety, efficacy, and delivery, with potential for lasting treatment. However, innate and adaptive immune responses to the viral vector and transgene product remain obstacles to the establishment of therapeutic efficacy. It is widely accepted that endogenous regulatory T cells (Tregs) are critical for tolerance induction to the transgene product and in some cases the viral vector. There are two basic strategies to harness the suppressive ability of Tregs: *in vivo* induction of adaptive Tregs specific to the introduced gene product and concurrent administration of autologous, *ex vivo* expanded Tregs. The latter may be polyclonal or engineered to direct specificity to the therapeutic antigen. Recent clinical trials have advanced adoptive immunotherapy with Tregs for the treatment of autoimmune disease and in patients receiving cell transplants. Here, we highlight the potential benefit of combining gene therapy with Treg adoptive transfer to achieve a sustained transgene expression. Furthermore, techniques to engineer antigen-specific Treg cell populations, either through reprogramming conventional $CD4^+$ T cells or transferring T cell receptors with known specificity into polyclonal Tregs, are promising in preclinical studies. Thus, based upon these observations and the successful use of chimeric (IgG-based) antigen receptors (CARs) in antigen-specific effector T cells, different types of CAR-Tregs could be added to the repertoire of inhibitory modalities to suppress immune responses to therapeutic cargos of gene therapy vectors. The diverse approaches to harness the ability of Tregs to suppress unwanted immune responses to gene therapy and their perspectives are reviewed in this article.

Keywords: regulatory T cells, tolerance, gene therapy, chimeric antigen receptor regulatory T cells, adoptive transfer, cell therapy, adeno-associated virus vectors, lentiviral vectors

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INTRODUCTION

Gene therapy has the tremendous potential to completely cure with a single treatment, diseases previously classified as untreatable, or disorders that could be managed but not corrected. Correction is achieved by transferring a functional copy of a gene, which is otherwise mutated in the diseased state, or by editing the defective gene in the patient's body. After a period of major setbacks during the late 1990s and early 2000s, this technique has reemerged as a major breakthrough in regenerative medicine (1, 2). A clear proof of clinical efficacy has mostly been observed in ocular diseases (inherited blindness), primary immune deficiencies, beta-hemoglobinopathies, and more recently hemophilia (2–9). Approaches for gene therapy in the clinic are based on *in vivo* delivery

to post-mitotic cells or tissues, or *ex vivo* delivery into autologous hematopoietic stem cells (HSCs), followed by reinfusion into the patient. Treatment of blindness by *in vivo* gene transfer (NCT00999609 and NCT00516477) is the first representative gene therapy drug approved in the USA by the Food and Drug Administration (Luxturna, Spark Therapeutics). In the meantime, cancer gene therapy drugs have already been approved, which include the virotherapeutic Imlygic (an engineered oncolytic Herpes virus, Amgen), chimeric antigen receptor (CAR) T cell therapy such as tisagenlecleucel-T (Kymriah, Novartis), and most recently, axicabtagene ciloleucel (Yescarta, Kite Pharma). The latter are of particular significance for this review, as they underscore the potential for therapies based on genetically engineered T cells.

IMMUNE RESPONSES TO GENE THERAPY

The aim of successful gene therapy is the safe and effective delivery of the replacement gene at therapeutic levels, potentially for the lifetime of an individual. A key obstacle to successful gene therapy is the host's immune response to both the viral vector and the transgene product. A fatal inflammatory immune response to the adenoviral vector almost brought the field to a stop in 1999 in a gene therapy clinical trial (10), although the safety and efficacy of gene therapy has been clearly established since then.

Gene therapy by vector administration into immune-privileged sites like the brain, eye, and testis has successfully achieved long-term transgene expression (11, 12). However, vector-mediated delivery into immune-competent organs is complicated by prevailing neutralizing antibodies that can limit the efficacy of transduction in patients (13). Although initial trials enrolled patients after a very careful selection process, gene therapy is becoming more common, and patient inclusion criteria are expected to be less exclusive, likely including patients with prevailing neutralizing antibodies or cross-reactive immunologic material- negative mutations.

At present, several viral vectors have been established as vehicles for gene transfer. Common among these are adenoviral vectors, gamma retroviral vectors, adeno-associated virus (AAV) vectors, and lentiviral vectors (LVs). For LV, gene therapy has been clinically approved for *ex vivo* gene transfer (14, 15), and the use of LVs for *in vivo* gene replacement is being evaluated in preclinical models (16, 17). This is facilitated by the low prevalence of neutralizing antibodies to LVs and the capacity to accommodate larger gene inserts. The new generation of replication-deficient vectors is gutted and nonpathogenic. Unlike gamma-retroviruses that favor integration near transcription start sites, LVs have been shown to integrate into active genes, making the chances of insertional mutagenesis and clonal expansion less likely (18). Potential innate and adaptive immune responses, which vary in magnitude, can develop toward the encoded transgene (19), envelope pseudotype or proteins acquired during the packaging process (20). LV-triggered innate immune responses such as type I IFN are primarily mediated by viral genome engagement with TLRs, possibly TLR9 and TLR7 (21–23).

Cytotoxic T lymphocyte (CTL) responses to both viral antigen and transgene have been observed with early-generation adenovirus and in preclinical models of *in vivo* adenoviral gene transfer (24–26). Replication-deficient, first- and second-generation adenovirus vectors are now being used in cancer gene therapy clinical trials, particularly for solid cancers (NCT01811992, NCT02630264, NCT01310179, NCT00870181 and NCT01147965). The high immunogenicity of adenoviral vectors has also made them attractive candidates as vaccine carriers. For example, the recent devastating outbreak of Ebola prompted a rapid phase I clinical trial of the replication-defective, chimpanzee adenovirus type 3-vectored Ebola virus vaccine (cAd3-EBO) (27). There is interest in helper-dependent or gutless third-generation adenoviral vectors, because of reduced *in vivo* immune responses as compared to first- and second-generation adenoviral vectors (28). However, innate immune responses are still elicited (29).

For *in vivo* gene delivery, recombinant AAV is the vector of choice due to its ease of construction, wide tissue tropism, and presumed lack of pathogenicity as it does not efficiently transduce macrophages, mature DC, and other antigen-presenting cells (APCs), although endocytosis of AAV has been associated with innate immune activation (30). It has been shown that the TLR9–MyD88 pathway is crucial for cross priming AAV capsid-specific $CD8^+$ T cells, a process that requires the cooperation of both pDC and cDC subsets of dendritic cells, as well as for activating transgene-specific $CD8^+$ T cell responses (31, 32). Anti-capsid effector T cell responses have been elicited in trials where the vector was administered outside of the retina or CNS. These have been shown to be responsible for deleterious immune responses against transgene-expressing cells, affecting therapeutic efficacy (33–36). Anti-capsid effector T cell responses were not predicted by preclinical studies, highlighting one of the major preclinical challenges when working with AAV (34).

Treg TYPES AND CHARACTERISTICS

The molecular characteristics that enable Tregs to modulate the activation of responder T cells render them uniquely suitable to limit immune responses to a therapeutic gene. Tregs have potent immunosuppressive properties that can be harnessed to confer antigen-specific immunomodulation in a therapeutic setting (37). Treg activity is required to maintain immune homeostasis in the presence of autoreactive T cells. Thus, they have defined roles in diverse clinical conditions including cancer, autoimmune disease, and transplant rejection (38–40). The most commonly studied among them are the $CD4^+CD25^+FoxP3^+$-expressing Treg subset, which are thymus-derived and called thymic, natural, or central Tregs (41). Natural Tregs commonly exhibit specificity to self-antigen and are essential for maintaining tolerance to self-tissues. Treg cells derived from outside the thymus are often referred to as induced, adaptive, or peripheral Tregs. These can be antigen-specific effector T cells induced to express FoxP3, or type 1 Treg (Tr1) cells that are $FoxP3^-$, express surface LAG-3 (CD223) and CD49b, and secrete IL-10 (42, 43). A recent $FoxP3^-$ subset, with a regulatory activity, expressing latency-associated peptide (LAP) on the surface as latent TGF-β complexes has also

been defined, which can be cleaved to release active TGF-β (44). Nonetheless, there is scientific consensus that each Treg subset has suppressive capacity and is integral to maintaining tolerance, as has been observed in treatment for autoimmune diseases and in gene therapy.

Resting natural Tregs are usually $CD25^{hi}$, $CD127^{-}$, L-selectin $(CD62L)^{+}$, CTLA-4 $(CD152)^{+}$, and $ICOS^{\pm}$. Other natural Treg markers such as neuropilin are specific for mice (45, 46). The activation of both natural and peripherally induced Tregs (iTreg) is associated with inducible upregulation of markers, many of which are not Treg exclusive, but are common in activated effector and memory T cells. These include but are not limited to CD69, CD25, CD44 (47, 48), CD39, and CD73 (49), galectin-1 and -10 (50, 51), glycoprotein A repetitions-predominant (GARP) and LAP (52, 53), CTLA-4 (CD152) (54), Ki67, GITR (TNFRSF18), TNFR2, and ICOS (55). In particular, CTLA-4 has been found to regulate many aspects of Treg suppression and can control the progression of autoimmune disease (56–59). In some cases, activated Tregs have been associated with an increase in FoxP3 expression (60–62).

Regulatory T cell suppressive function has been shown to be primarily TCR contact-dependent. TCR signaling is crucial for Treg development, differentiation, and suppressive function (63). Tregs use multiple mechanisms to suppress immune responses, depending on the nature and tissue-specific location of the antigen (lymphoid and non-lymphoid tissues). These include antigen-specific and bystander suppression associated with the release of cytokines such as IL-10, TGF-β, and IL-35 (64), IL-2 deprivation, direct cell killing (65), the production of metabolic intermediates (66, 67), and the modulation of dendritic cell maturation and stimulatory function (68, 69).

EVIDENCE THAT Tregs LIMIT IMMUNE RESPONSES IN MUSCLE GENE TRANSFER

Gene delivery into the muscle is attractive as a potential source for therapeutic protein expression. Muscle gene therapy is ideal for degenerative disorders like the muscular dystrophies, storage disorders leading to metabolic myopathy such as Pompe disease or for the production of enzymes like alpha-1 antitrypsin (AAT) (70). A major barrier to muscle gene transfer is the need to deliver the replacement gene body wide, necessitating multiple injections into various sites throughout the body, thereby increasing the potential for inflammatory immune responses (71, 72). Further, the often-required high vector doses also enhance the risk of provoking an immunological response. Physiologically, delivery into the muscle environment causes inflammation, presumably due to the high frequency of resident macrophages.

Clinical trials using muscle gene transfer have shown promise for many disorders, some of which show very poor prognosis with conventional therapy. For example, gene replacement therapy is a feasible approach for the treatment of the lysosomal storage disorder, Pompe disease, which particularly affects the skeletal and cardiac muscle, and neural tissues. Initial clinical experience in Pompe disease shows that the direct delivery of AAV1–hGAA into the diaphragms of affected children is safe, well tolerated, and efficacious (73, 74). Neutralizing antibody development against the hGAA transgene product and the viral vector prevents therapeutic efficacy and vector readministration, respectively (75, 76). Interestingly, T cell reactivity toward the vector has not been observed to date (73). Preclinical data show that lentiviral correction of HSCs by *ex vivo* transduction was effective in ameliorating Pompe disease in a mouse model (77), which could be a viable alternative for preventing immune responses by facilitating central tolerance.

The detection of T cell responses to the capsid in peripheral blood mononuclear cells is not always associated with a deleterious immune response, as seen during gene transfer trials with AAT. Despite the detection of T cell reactivity against the vector and infiltrates into the treated muscle, the transgene was still expressed in subjects who received an AAV1 vector encoding for AAT (78–81). Interestingly, $CD4^{+}CD25^{+}FoxP3^{+}$ Tregs were found within the infiltrating cells (~10%) in vector-injected muscle and were associated with a time-dependent decrease in muscle inflammation, which may have prevented the destruction of transduced myofibers (82).

Similarly, a population of Tregs was shown to accumulate in muscles of dystrophic mice and in muscle biopsies from Duchene muscular dystrophy (DMD) patients (83, 84). These IL-10-secreting Tregs improved the dystrophic phenotype by decreasing inflammation associated with the disease, and their depletion resulted in worsening of the disease phenotype. Therapeutic targeting of Tregs with IL-2/anti-IL-2 complexes had a beneficial effect of reducing muscle inflammation and injury in dystrophic mice. Thus, these observations demonstrate the potential of Treg-modulating agents to induce a local Treg population in muscle at the time of gene transfer to reduce muscle inflammation and favor the maintenance of transgene expression in DMD. Another feasible alternative is adoptive immunotherapy with polyclonally expanded or antigen-specific Tregs at or during the time of gene therapy. In an earlier pivotal study, the administration of exogenous transgene-specific Tregs concomitantly with AAV gene transfer was shown to lower anti-transgene immune reactivity and allow stable transgene expression in normal muscle (85). This established that adoptively transferred $CD4^{+}CD25^{+}$ regulatory T cells can induce a sustained transgene engraftment in solid tissues. Combinatorial treatments using adoptive Treg transfer as adjunct therapy may thus enhance the therapeutic effect of gene delivery by developing tolerance toward the gene delivery vehicle or transgene product.

THE LIVER AS AN IDEAL SITE FOR IMMUNE REGULATION

The administration of gene therapy systemically leads to rapid accumulation of high levels of vector particles within the liver. Specialized liver-resident cells mediate the "liver tolerance effect," which establishes local and systemic tolerance to self and foreign antigens. This has been attributed to the expression of inhibitory

cell surface ligands for T cell activation and the production of anti-inflammatory mediators (86).

The utilization of liver tropic viruses, engineered vector serotypes, and liver-specific promoter and enhancer elements have improved liver gene delivery and increased gene expression to clinically therapeutic levels (87). Much of the present interest in the development of liver-directed gene therapy stems from recent clinical success in treating the X-linked coagulation disorder hemophilia B, with restoration of clinical levels of factor IX (FIX) to hemophilia B patients for sustained periods greater than 5 years (7, 36). A transient increase in liver enzymes, presumably due to the reactivation of a memory CTL response to the vector, was earlier observed, although intervention with corticosteroid administration at the first sign of hepatocellular injury could halt the increases in liver enzymes and sustain FIX expression (34). Similarly, high endogenous levels of clotting factor have been reported in recent clinical trials for both hemophilia A and B (88, 89).

The development of inhibitory antibodies that neutralize factor VIII (FVIII) or FIX is a major complication of protein replacement therapy as well as gene therapy for patients with hemophilia (90). Preclinical studies in small and large animal models of hemophilia have demonstrated that gene therapy strategies and the continuous exposure to clotting factor can promote tolerance and eradicate preexisting antibodies (91–94). Nonetheless, there is still a risk of developing neutralizing antibodies to the coagulation factor product following hepatic gene transfer (95). There is strong evidence that Tregs are an important element of the mechanism by which self-tolerance is maintained and inhibitor development, a T helper-dependent response, is prevented (96–99). In many cases, immune tolerance to hepatic gene transfer of hFIX has also been associated with the induction of Tregs (100–102). We propose that the adoptive transfer of Tregs in the setting of liver gene therapy has the potential to avoid the general immunosuppression that many corticosteroid drugs pose, instead favoring tolerance to the transgene in an antigen-specific, safe, and transient manner.

Another field where liver gene therapy has garnered interest is in the treatment of autoimmune disorders like rheumatoid arthritis, multiple sclerosis, and type 1 diabetes (T1D). Replacement gene delivery in these cases is complicated by the development of an immune response to the therapeutic gene. Studies have demonstrated that gene therapy into the tolerogenic liver microenvironment can abrogate the development of experimental autoimmune encephalomyelitis (EAE) even if the target antigen for the inflammatory T cell response is in a distant organ, such as the central nervous system (103, 104). Protection from EAE was dependent on the induction of antigen-specific $CD4^+CD25^+Foxp3^+$ Tregs (103, 104). Using the same principle in a preclinical mouse model T1D, Akbarpour et al. showed that targeting LV-mediated insulin gene expression to hepatocytes induced regulatory T cells specific for insulin, which halted immune cell infiltration into the pancreatic islet and protected from T1D (105). Thus, it appears that targeting gene transfer to hepatocytes can favor the induction of antigen-specific Tregs systemically, making the liver an attractive target for achieving transgene tolerance.

IN VIVO TOLERANCE INDUCTION WITH Treg

Given the critical role of Treg in maintaining immune regulation of transgene-specific responses, an obvious treatment of choice is the *in vivo* induction of antigen-specific Treg by a specific or a combination drug treatment. Global immune suppression by steroid or chemotherapeutic drugs, while beneficial when given transiently, does not have the advantage that a more targeted and a lasting transgene product-specific Treg response can offer. One method of inducing Treg is to coadminister the antigen with the macrolide immunosuppressant rapamycin (sirolimus), which inhibits cell cycle progression of activated T cells by mTOR pathway blockade, leading to T cell anergy or deletion (106). At the same time, the inhibition of the T cell stimulatory activity of dendritic cells (107) and mTOR-independent signaling by Tregs (108) result in the enrichment of antigen-specific $CD4^+CD25^+FoxP3^+$ Treg (109–111). This effect can be enhanced by the addition of cytokines such as IL-10 or Flt3L, which have been shown to promote tolerance in protein replacement therapy (96, 99). Prophylactic therapy of IL-10 in combination with rapamycin and antigen has also been successful in the prevention and reversal of inhibitory antibody responses in muscle gene transfer of therapeutic FIX in hemophilia B mice (96, 99, 112). Likewise, the introduction of rapamycin with liver gene therapy resulted in a markedly enhanced expression of human acid-α-glucosidase in nonhuman primates, likely due to the induction of hepatic autophagy and is being evaluated for readministration of the AAV vector (113).

Tolerance to antigens administered by the oral route is another approach to inhibit antigen-specific immune responses by targeting the gut-associated lymphoid tissue (114). Multiple immune cell types have been shown to be involved in mediating this state of non-responsiveness, including gut resident dendritic cells, $FoxP3^+$ Tregs and $CD4^+CD25^-LAP^+$-expressing Tregs (115–117). Gut homing receptors and cytokines such as TGF-β and IL-10 have been shown to be responsible for the infiltration/differentiation/local expansion of these Treg subtypes and the induction of tolerance (118). Significantly, oral tolerization improved long-term transgene persistence and expression as shown in a recent study using AAV-mediated gene transfer of the model antigen OVA (119).

CELLULAR THERAPY WITH Treg

Extensive preclinical studies have demonstrated that Tregs play a key role in both the induction and maintenance of tolerance. Adoptive immunotherapy with autologous or donor Tregs has shown promise in several clinical trials for autoimmune disorders and in transplant conditions (120, 121). With new GMP protocols in place, $FoxP3^+$ Tregs can undergo polyclonal or antigen-specific expansion with high purities (122, 123). Protocols to generate donor-specific Tr1 cells are also well established (124–126). Clinical trials with freshly isolated or *ex vivo* expanded $FoxP3^+$ (127–130) or Tr1 cells (ALT-TEN trial) (131) (from umbilical cord blood or peripheral blood) as a cellular therapy given at

or shortly before/after transplantation have been carried out for tolerance to graft-versus-host disease (GvHD) in patients undergoing allo-HSCT for hematological malignancies. Alternatively, ultra-low-dose (ULD) IL-2 has been suggested to selectively expand nTreg *in vivo*, suppressing alloreactive responses in GvHD prophylaxis and treatment (132–134). Supplementing Treg infusion with ULD IL-2 to promote Treg persistence and survival for the treatment of onco-hematological diseases is being tested (NCT02991898).

Studies testing the safety and feasibility of autologous polyclonal or alloantigen-specific Treg infusion for conferring tolerance in solid organ transplantation are currently ongoing [NCT02145325 (TRACT), NCT02088931, NCT02711826 (TASK), UMIN-000015789] (135). The ThRIL study (NCT02166177) has been initiated to evaluate the efficacy of Treg cell therapy, in combination with immune-suppressive drugs, in liver-transplant recipients.

The ONE Study (www.onestudy.org) is a phase I/IIa clinical trial aimed at testing the safety and feasibility of seven different regulatory T cell populations in living donor kidney transplants. This multicenter study compares autologous *ex vivo* expanded polyclonal $CD4^+CD25^+$ nTregs from peripheral blood, Tr1 cells, donor alloantigen-driven Tregs (darTreg), and alloantigen-driven T cells anergized by costimulation blockade, tolerogenic dendritic cells, and regulatory macrophages (Mregs). Comparisons will be made between patients receiving standard immunosuppressive treatment (basiliximab followed by tacrolimus, mycophenolate mofetil, and prednisolone) and those receiving immunotherapy (136).

Finally, Treg therapy has also been applied to autoimmune and inflammatory disorders (e.g., TRIBUTE trial for Crohn's). An autologous antigen-specific Tr1 therapy for refractory Crohn's disease is in development (Ovasave, Txcell). In a study of pediatric patients with T1D, single and repeat infusions of polyclonal expanded Tregs were found to be safe and effective in patients (137). As with GvHD, the effect of low-dose IL-2 on *in vivo* induction of Tregs for 12 autoimmune and inflammatory diseases is being tested in a multicentric trial (TRANSREG, NCT01988506). Moreover, studies to assess the safety of Treg immunotherapy supplemented with IL-2 and the persistence of infused autologous Tregs in patients with recent onset T1DM are being undertaken (TILT study, NCT02772679) (138). In an earlier report by the same group, a study of 14 adult subjects with T1D who received *ex vivo* expanded polyclonal Tregs saw a subset of Tregs remaining in circulation at 1 year after transfer (139).

Taken together, these studies reveal that infusions of *ex vivo* expanded $FoxP3^+$ or $FoxP3^-$ (Tr1) cells are safe, well tolerated, and can aid in tolerance in many inflammatory and autoimmune conditions.

SUPPLEMENTING GENE THERAPY WITH Treg ADOPTIVE TRANSFER

The immune suppressive properties of Tregs have generated interest in utilizing this cell population for tolerance toward the transgene product. Not only are Tregs critical for establishing central tolerance during development and in preventing autoimmunity, they are also involved in inducing tolerance toward exogenous antigens, such as therapeutic proteins. Ideally, immune modulation to suppress vector or transgene-specific responses should eliminate undesired immune cells while sparing protective immunity.

There is ample evidence that adoptive immunotherapy with polyclonal or engineered Tregs can improve protein replacement therapy in inherited protein deficiencies (98). On the other hand, very few studies on infusing Tregs to improve tolerance to gene therapy have been carried out. So far, gene therapy into immune privileged sites like the eye has not been associated with a deleterious immune response. Likewise, gene delivery into tolerogenic organs, particularly the liver, has in fact been shown to induce Tregs *in vivo*. However, liver-directed gene therapy, while successfully diminishing immune responses toward the transgene product, does not completely eliminate the development of cytotoxic T cells that can subsequently lead to the potential immune-mediated deletion of transgene-expressing cells (140). Similarly, although the development of neutralizing antibodies to the transgene product has so far not been observed in the small number of liver gene therapy clinical trials in humans, the possibility remains a concern as observed in preclinical studies for the immunogenic FVIII molecule (140, 141) (unpublished observations). Strategies such as using microRNA target sequences (miR-142-3p) in the LV to de-target transgene expression from professional APCs, coupled with restricted expression in either hepatocytes or liver endothelial cells, have led to improved transgene expression. This has been shown to correlate with the emergence of transgene-specific Tregs, which induced tolerance in preclinical models of hemophilia A and B (17, 142, 143).

In an earlier study on complementing gene therapy with Treg adoptive transfer, Gross et al. established that the injection of influenza hemagglutinin (HA)-specific $CD4^+CD25^+$ Tregs, concomitant with gene transfer, enabled persistent HA transgene expression in the muscles of mice (85). Cytotoxic T cell responses, as well as circulating anti-IgG antibodies to HA, were impaired in HA-Treg recipients. These findings were applied to a disease setting for hemophilia A, where nonviral gene transfer of the therapeutic FVIII plasmid resulted in supraphysiological levels of FVIII, but triggered inhibitory antibody development and loss of functional FVIII activity. Adoptive transfer of cells enriched for FVIII-specific Tregs into naïve hemophilic mice, followed by plasmid challenge, led to a significantly diminished inhibitory antibody formation for a prolonged period, as compared to control animals (144). These studies establish the potential of Tregs to modulate immune responses to the transgene product in an antigen-specific manner (**Figure 1**). Our group added to these initial studies by demonstrating that adoptively transferred *ex vivo* expanded Treg could be used to improve gene therapy of FIX in a mouse model of hemophilia B (98). In the study, polyclonal *ex vivo* expanded autologous $CD4^+CD25^+FoxP3^+$ Treg administered at doses similar to those currently used in clinical trials (~5×10^7 cells/kg) was able to prevent the formation of an adaptive immune response in hemophilia B mice receiving AAV1 hFIX muscle directed gene transfer. Despite limited *in vivo* persistence of the adoptively transferred cells, a sustained

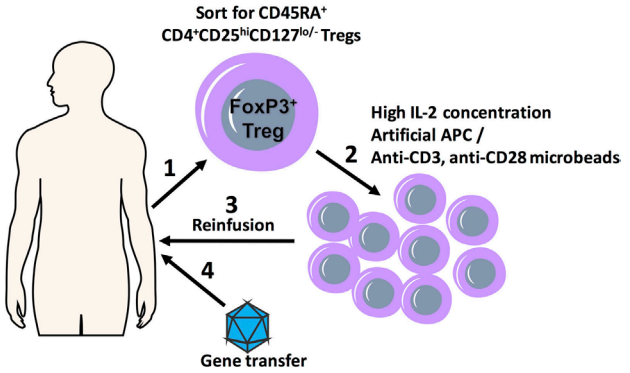

FIGURE 1 | A scheme detailing combination regulatory T cell (Treg) adoptive therapy with gene transfer for tolerization of immune responses. FoxP3+ Treg cells with polyclonal specificity are harvested from the patient (1) and *ex vivo* expanded in the presence of high IL-2 concentrations and artificial APC (aAPC) or anti-CD3, anti-CD28 microbeads using GMP protocols (2); expanded Tregs are transplanted back into the patient (3), which is followed shortly by gene transfer (4).

suppression lasting 10 weeks was observed. This was attributed to the emergence of antigen-specific suppression *via* the induction of endogenous Treg, which was facilitated by the transplanted Treg (**Figure 2A**). It has been shown that *ex vivo* expansion improves the suppressive properties of polyclonal Tregs, rendering them functionally superior to freshly isolated Tregs (145). Expanded Tregs highly upregulate CTLA-4 expression, which competes with the costimulatory molecule CD28 for binding to CD80/86 on APCs. Suboptimally activated APCs facilitate the induction of iTreg cells (57, 146).

Although cell therapy with expanded polyclonal Tregs has many advantages, it has been demonstrated that antigen-specific Tregs are more potent at 10- to 100-fold lower frequencies (147). One way to recover a sufficient number of antigen-specific Tregs is to expand them in the presence of alloantigens through a process of indirect allospecificity. This has been successfully used to promote transplantation tolerance, by expanding the recipient's Treg pool toward donor antigens (148–151). In some cases, Treg expansion and therapeutic potential were improved by the addition of IL-2 and IL-12 (152). However, it is unclear whether it would be possible to isolate rare antigen-specific Tregs to supplement gene therapy, especially in the case of inherited protein deficiencies, where the antigen is not expressed and central tolerance may not be achieved.

THERAPY WITH GENETICALLY MODIFIED CELLS

More recently, the applicability of gene therapy has moved beyond gene correction to a wider spectrum of diseases. Gene-modified cells, such as CAR-modified T cells for the eradication of hematologic cancers, have achieved breakthrough success in clinical trials (153–156). Glaxo Smithkline has introduced the first *ex vivo* stem cell gene therapy to treat patients with ADA-SCID, Strimvelis, which received approval from the European Medicines Agency in 2016 (157). Zalmoxis, a donor cell-derived T cell therapy used for H-SCT, is also poised for the market. These novel and successful trials are making way for other cutting-edge technology, such as the development of gene-editing techniques using CRISPR-Cas to increase the stability of CAR-T cells (NCT03166878) or for treating hematological malignancies in patients with HIV (NCT03164135).

Gene modification to increase antigen specificity has been recently applied to Tregs. The difficulty of isolating cells with a rare antigen specificity from the natural polyclonal T cell repertoire has hampered the clinical translation of targeted therapy with antigen-specific Tregs. On the other hand, treatment with polyclonally expanded Tregs requires the infusion of large numbers of clinical-grade autologous cells, with a possibility for general immunosuppression. Using clinical-grade LVs to genetically reprogram cells represents an attractive strategy to fine-tune Treg populations for a particular specificity (**Figure 2B**). One example is the ectopic overexpression of FoxP3 in conventional CD4+ T cells from healthy donors, with the aim of generating a large number of homogeneous and functional Treg cell populations. This technique has been applied successfully to conventional CD4+ T cells of patients with immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome (dysfunction in FoxP3 gene), and in other preclinical animal models of autoimmunity (158–161). The possibility of reversion to an effector T cell phenotype is a concern, given the plastic nature of many Treg populations. However, adoptively transferred, FoxP3 overexpressing Tregs were shown to be stable in steady-state and inflammatory conditions and continued to be suppressive *in vivo* (161). The requirement for antigen specificity of FoxP3 gene-transferred cells and the dose of cells required for suppression, as well as persistence *in vivo*, are questions that still need to be addressed.

Another similar approach for engineering Treg specificity using TCR gene transfer has been shown to improve Treg potency, as observed in preclinical models for diabetes, transplantation tolerance, arthritis, and hemophilia A (61, 162–165). Engineered TCRs provide a viable alternative to redirect Treg specificity to a single antigenic epitope with a potentially high TCR affinity. However, this approach is HLA restricted and thus limits the number of patients to those with common HLA alleles.

Inspired by the clinical success of using CAR-T cells to treat certain types of cancers, a similar approach has been applied that engineers Tregs to express extracellular single-chain antigen-binding domains (scFv) fused to intracellular signaling molecules (**Figure 2C**). CARs can directly recognize their corresponding antigen irrespective of HLA. Further, issues such as TCR chain mispairing, which is a potential concern with TCR gene transfer, do not arise. At present, it is unclear how CAR-Tregs exert their suppressive effect and which cell populations they interact with. It has been postulated that the optimal activation of CAR-Tregs requires the presence of APCs (62). It is possible that CAR-Tregs recognize antigen that is immobilized on the surface of the APC, although molecular interactions or receptors that may be involved remain to be defined. The ability of CAR-Tregs to respond directly to soluble antigen or to recognize antigen bound to a B-cell receptor (BCR) is also still an open question (**Figure 2C**).

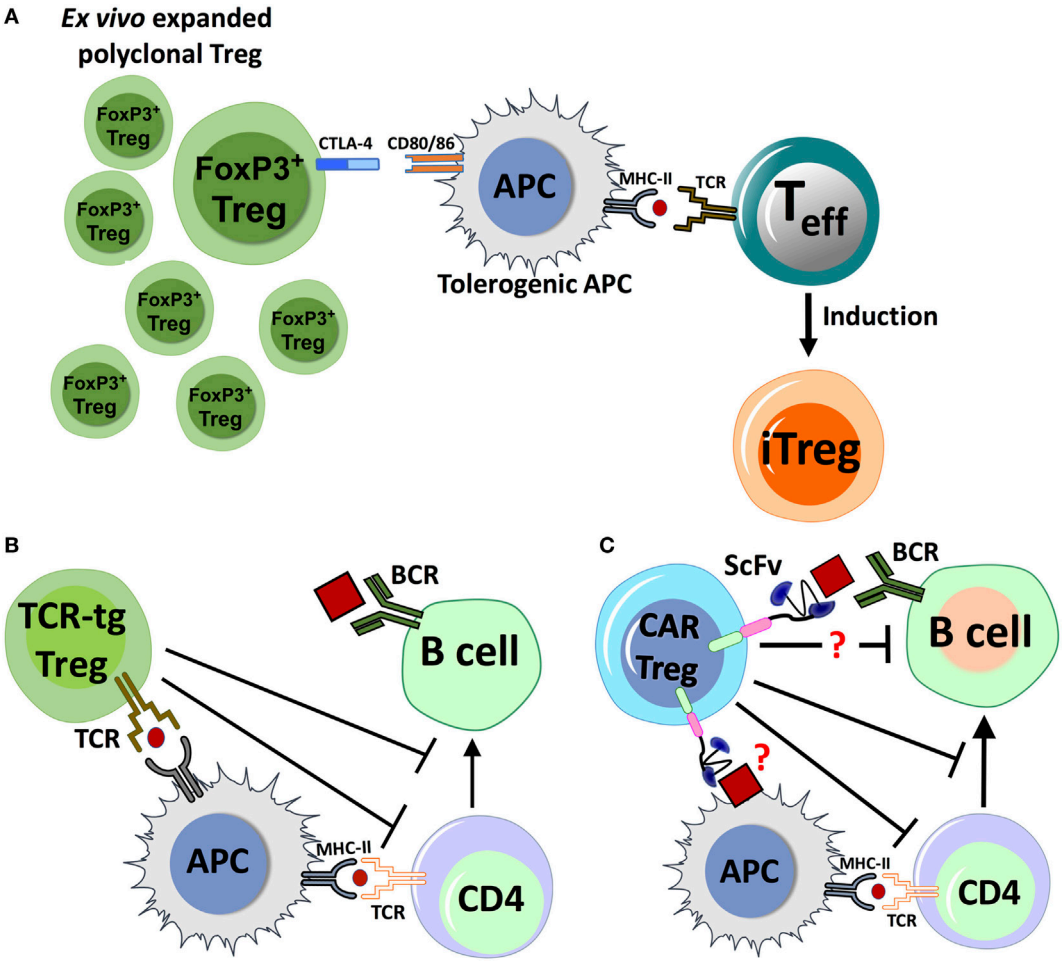

FIGURE 2 | Proposed mechanisms for immune suppression by adoptive transfer of polyclonal FoxP3+ regulatory T cell (Treg), chimeric antigen receptor (CAR)-Treg or TCR-transgenic (TCR-tg) Treg. **(A)** Adoptively transferred *ex vivo* expanded Treg with polyclonal specificity can interact with antigen-presenting cell (APC). Inhibitory receptors like CTLA-4 can compete with the costimulatory molecule CD28 to bind to CD80/86 receptors, and combined with other factors, it can lead to APC tolerization. Tolerogenic APCs interact with activated antigen-specific T effector (T_{eff}) cells, which leads to conversion of T_{eff} to induced Treg (iTreg). **(B)** Natural Treg engineered with TCR specificity for antigen (TCR-tg Treg) can recognize antigen presented by APCs, directly suppressing the APC's capacity to costimulate T_{eff} cells. TCR-tg Treg can also directly inhibit CD4+ T helper cells, which in turn affects T cell help to antigen-specific B-cells. **(C)** Putative mechanisms for antigen recognition and suppression by CAR-Treg. CAR-Tregs may recognize either a B-cell bound antigenic epitope or antigen on the surface of APC, which can trigger the activation and proliferation of the CAR-Treg through transmembrane and intracellular-signaling domains. The mechanisms by which CAR-Tregs exert their suppressive effects are not clearly defined, but may include interactions with key cell types.

Chimeric antigen receptor regulatory T cells are being tested in preclinical models of EAE, allograft rejection, colitis, rheumatoid arthritis, and hemophilia A (62, 166–174). The first CAR-Treg trial, by the French company TxCell, for the prevention of transplant rejection is expected to commence in 2018. Such clinical trials will be able to address questions such as immunogenicity of the novel CAR molecule (175), or the possibility of cytokine release syndrome, which is a serious side effect of CAR-T cell treatments for cancer (176). Meanwhile, new CAR strategies are being developed to improve the specificity and function of CAR-modified T cells/Tregs. For example, the transient expression of a CAR construct that recognizes the FITC molecule can be used to target Treg function to transplanted organs by binding to FITC-conjugated monoclonal antibodies against donor MHC antigens (174). Alternatively, the surface expression of the antigenic domain, rather than the scFv, conjugated to primary and secondary signaling molecules, can bind the BCR of the corresponding antigen-specific B-cell, thus promoting B-cell depletion or suppression, as demonstrated in a model for autoimmunity and hemophilia A (177, 178).

CHALLENGES AND FUTURE DIRECTIONS

Beginning with the discovery in 1990 and 1995 that adoptively transferred CD4+CD25+ Tregs can maintain tolerance in an autoimmune animal model (179), the clinical prospects of Tregs have expanded in the past decade (36). It is apparent from studies with disease models and clinical trials that Treg-suppressive mechanisms can counter immune activation caused by gene replacement therapy.

Although clinical trials using adoptively transferred Tregs to supplement gene therapy have not been attempted thus far, this review highlights several benefits for combining these two approaches. For example, existing obstacles faced in recent clinical trials such as unwanted immune responses to gene therapy and the inability to readminister vector could be mitigated by the codelivery of Treg with the vector. Adoptive immunotherapy with Tregs has shown clinical efficacy in autoimmune diseases such as T1D (which is characterized by a detrimental inflammatory response) and can tolerize against inflammatory reactions to a transplanted organ. We therefore propose that augmenting gene transfer applications, either by promoting the *in vivo* induction and expansion or Tregs or by immunomodulation with adoptively transferred Tregs can work synergistically and lead to successful gene transfer.

It is crucial, however, to emphasize the importance of good manufacturing practice-compliant cell therapy procedures, especially for the generation of polyclonal Tregs, which require dosing at larger cell numbers to reach therapeutic efficacy (180). A current challenge with using Tregs in the clinic is the need for the isolation and expansion of a pure population of functional and stable cells in sufficient numbers. FoxP3 is an intracellular marker and can be transiently expressed by activated $CD4^+$ and $CD8^+$ T cells. Optimization of cell sorting, such as employing double sorting of $CD4^+CD25^{hi}CD127^{lo}$ cells can ensure increased purity of the starting population to help control for the outgrowth of effector T cells, which expand exponentially faster than Tregs in culture.

Regulatory T cell infusion may be beneficial not only in gene replacement settings to suppress capsid and transgene-specific immune responses but may also have the potential as adjunct therapy to prevent immune responses toward vector readministration. The formation of neutralizing antibodies constitutes a major obstacle to vector readministration, as they are elicited at high titers following gene transfer and can persist for years. Repeat administration of vector may be required when the gene product does not reach therapeutic levels, or when administered to pediatric patients, where an increasing organ turnover may limit the therapeutic dose over time. Successful gene therapy would expose the patient's immune system to the newly delivered vector and/or transgene, generating B and T cell responses that would limit the ability to readminister vector. Plasmapheresis and transient immunosuppression (anti-CD40 Ab, CTLA-4 Ig, high-dose corticosteroids, rapamycin, rituximab, or combinations of these treatments) are currently being tested to allow for repeat injections (NCT02240407) (76, 113, 181). The use of adoptive Treg therapy in these scenarios has not been tested, and thus it remains a possible combinatorial therapy product for blocking potential immune responses.

AUTHOR CONTRIBUTIONS

MB, SK, CT, and RH made substantial contributions to the concept of this work, drafted, and made critical revisions and final approval. MB, SK, CT, and RH agreed to be accountable for all aspects of the work.

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