INTRODUCTION

Transplantation is the best therapeutic option for end-stage organ failure: it prolongs life expectancy and provides patients with the best possible quality of life. However, mortality and morbidity rates remain high due to the use of immunosuppressive drugs, which prevent allograft rejection but also increase the risk of infections, cancers, and cardiovascular diseases. Developing strategies to minimize the need for immunosuppressive drugs has been a major objective of transplant medicine since its inception.

Cell therapy with autologous donor-specific regulatory T cells (Tregs) is a promising strategy to minimize immunosuppression in transplant recipients. Chimeric antigen receptor (CAR) technology has recently been used successfully to generate donor-specific Tregs and overcome the limitations of enrichment protocols based on repetitive stimulations with alloantigens. However, the ability of CAR-Treg therapy to control alloreactivity in immunocompetent recipients is unknown. We first analyzed the effect of donor-specific CAR Tregs on alloreactivity in naive, immunocompetent mice receiving skin allografts. Tregs expressing an irrelevant or anti-HLA-A2-specific CAR were administered to Bl/6 mice at the time of transplanting an HLA-A2+ Bl/6 skin graft. Donor-specific CAR-Tregs, but not irrelevant-CAR Tregs, significantly delayed skin rejection and diminished donor-specific antibodies (DSAs) and frequencies of DSA-secreting B cells. Donor-specific CAR-Treg–treated mice also had a weaker recall DSA response, but normal responses to an irrelevant antigen, demonstrating antigen-specific suppression. When donor-specific CAR Tregs were tested in HLA-A2-sensitized mice, they were unable to delay allograft rejection or diminish DSAs. The finding that donor-specific CAR-Tregs restrain de novo but not memory alloreactivity has important implications for their use as an adoptive cell therapy in transplantation.

KEYWORDS
alloantigen, B cell biology, basic (laboratory) research/science, cellular biology, cellular transplantation (non-islet), immunosuppression/immune modulation, T cell biology, tolerance, translational research/science
Cell therapy with ex vivo–expanded autologous CD4+FOXP3+ regulatory T cells (Tregs) is one of the most promising approaches to regulate alloimmunity and reduce immunosuppression. Preclinical studies have shown that the potency of Treg therapy can be markedly enhanced by the use of Tregs specific for donor alloantigens (ie, donor-specific Tregs). Clinical grade donor-specific Tregs have been generated successfully through long-term in vitro stimulation with allogeneic antigen-presenting cells; however, this approach is technically challenging. We and others have recently shown that chimeric antigen receptor (CAR) technology can be used successfully to generate donor-specific Tregs, overcoming the limitations of in vitro antigen-stimulation-based protocols. Human donor-specific CAR-Tregs traffic to antigen-bearing grafts where they expand and suppress human skin allograft rejection.

The desired clinical application of donor-specific CAR-Treg therapy would be as a treatment that enables diminished immunosuppression. However, a major risk of immunosuppression minimization is the development of donor-specific anti-HLA antibodies (DSAs) and antibody-mediated rejection. The latter is one of the main causes of allograft loss and there is no effective treatment. Although the ability of Tregs to control T cell–mediated rejection has been studied extensively in preclinical models, whether or not Treg therapy limits humoral alloimmunity is largely unknown because a majority of experimental studies of Treg therapy used immunocompromised mouse models that did not allow investigation of B cell–mediated alloimmunity. Another potential application of CAR-Treg therapy would be in sensitized recipients, who have a higher risk of allograft rejection; whether or not CAR-Tregs can control memory immune responses is unknown.

Here we used a model in which naive or sensitized immunocompetent mice received skin allografts to analyze the impact of donor-specific CAR-Treg therapy.

2 MATERIAL AND METHODS

2.1 Animals

C57Bl/6, FOXP3+ C57Bl/6, FOXP3+ × Thy1.1 C57Bl/6, and HLA-A2+ C57Bl/6 mice (B6.Cg-Immp2lTg (HLA-A/H2-D)2Enge, AAD) were bred in-house and maintained under specific pathogen-free conditions at the animal facility at British Columbia Children’s Hospital Research Institute. Experiments were approved by the University of British Columbia Animal Care and Use Committee (A16-0300). Skin grafts were cut into small pieces and incubated at 37°C for 40 minutes on a heated shaker (800 rpm) in a digestion buffer containing collagenase XI (60 mg/mL, Sigma), hyaluronidase (15 mg/mL, Sigma), and DNase (3 mg/mL, Sigma) diluted in Hanks’ Balanced Salt solution. Cells were then filtered (40 μm), rinsed with complete RPMI medium (RPMI media [Invitrogen] supplemented with 200 mmol/L GlutaMax, 10% FCS, and 10 units/mL penicillin/streptomycin) and washed before staining for flow cytometry.

2.4 Skin allograft flow cytometry

Samples were read on an LSRII or Fortessa (BD Biosciences) and results analyzed using FlowJo Software version 10.4.2. Surface staining was performed for anti-CD271 (NGFR; BD Biosciences), HLA-A2 (Biolegend), Myc Tag (clone 9E10, UBC Ablab), mouse CD4 (Biolegend), Thy1.1 (eBioscience), and Thy 1.2 (eBiosciences). Samples were also stained with fixable viability dye (FVD; eBioscience). HLA-A2 tetramers were obtained from the National Institutes of Health (NIH) Tetramer Core facility. For in vivo experiments, 50 μL of blood was collected weekly from a venipuncture. At end points, blood was collected from an intracardiac puncture. Ammonium chloride was used for red blood cell lysis. Cells were then stained for flow cytometry.

2.3 Treg injection and skin transplantation

Eight-week-old to 16-week-old female or male Bl/6 mice were transplanted with skin from HLA-A2+ Bl/6 mice 2 hours after tail vein injection of 1 × 10^6 (equivalent to 30-50 × 10^6/kg) Tregs. HLA-A2+ Bl/6 skin was cut into circular pieces utilizing an 8-mm biopsy punch, placed onto fresh plates with PBS, and kept at 4°C to 8°C until transplanted (~1-4 hours). Previously shaved mice were anesthetized, dorsal skin was cut near the shoulder, and an area of mouse skin of similar size (6-mm) was removed. Grafts from syngeneic Bl/6 and/or HLA-A2+ Bl/6 mice were placed on the exposed area and stabilized with steri-strips (3M, Nexcare). Grafts were covered with a petroleum jelly gauze and wrapped with a 2-cm-wide CoFlex bandage (3M, Nexcare) for up to 7 days. Recipients were inspected daily from the day of bandage removal and monitored for signs of graft rejection. Rejection was defined as skin shrinkage associated with >50% of necrosis including the center of the skin piece. Fifty microliters of blood was collected weekly and at end points. In some experiments, mice received two skin grafts at the same time: one from a syngenic Bl/6 and one from a HLA-A2+ Bl/6 donor.

2.5 Flow cytometry

Skin grafts were cut into small pieces and incubated at 37°C for 40 minutes on a heated shaker (800 rpm) in a digestion buffer containing collagenase XI (60 mg/mL, Sigma), hyaluronidase (15 mg/mL, Sigma), and DNase (3 mg/mL, Sigma) diluted in Hanks’ Balanced Salt solution. Cells were then filtered (40 μm), rinsed with complete RPMI medium (RPMI media [Invitrogen] supplemented with 200 mmol/L GlutaMax, 10% FCS, and 10 units/mL penicillin/streptomycin) and washed before staining for flow cytometry.
resuspended in PBS containing an anti-mouse CD16/32 (Thermo Fisher Scientific) and stained as described in the preceding text.

2.6 | CARs and retrovirus

The extracellular portion of the previously reported anti–HLA-A2 and anti-HER2 human CARs, which encode the relevant single-chain fragment variable Abs (scFv) and a Myc epitope tag, were fused to a stalk region from mouse CD8α, transmembrane and intracellular domains of mouse CD28, and mouse CD3ζ. The resulting A2- and HER2-CARs were cloned into a murine stem cell virus (MSCV)-based retroviral vector that encoded a transduction marker downstream of an internal ribosomal re-entry sequence. Initial experiments were done with vectors encoding surface-expressed truncated human nerve-growth-factor receptor (ΔNGFR) and then switched to vectors encoding the fluorescent intracellular monomeric Kusabira-Orange2 (mKO2) protein as the transduction marker as described by Kurachi et al. Viral particles were produced by using the Platinum-E (Plat-E) Retroviral Packaging Cell Line according to the manufacturer recommendations (Cell Biolabs).

2.7 | Treg sorting, transduction, and expansion

CD4+ T cells were isolated from FOXP3GFP Bl/6 (for in vitro experiments) or Thy1.1+ FOXP3GFP Bl/6 (for in vivo experiments) reporter mice. After spleen and lymph node cells were harvested, CD4+ T cells were enriched to >90% purity by negative selection using magnetic enrichment kits (STEMCELL Technologies). Live CD4+CD8α− T cells were sorted using a MoFlo Astrios cell sorter (Beckman Coulter). Sorted Tregs were resuspended in complete RPMI medium (RPMI media [Invitrogen] supplemented with 200 mmol/L GlutaMax, 10% FCS, 50 mmol/L β-mercaptoethanol, 25 mmol/L Hespeps, and 10 units/mL penicillin/streptomycin) and stimulated with mouse CD3/CD28 dynabeads (ThermoFisher Scientific) in the presence of 1000 U/mL of IL-2 (Proluken) and rapamycin (50 nmol/L, Sigma-Aldrich). Two days later, cells were transduced with retrovirus. Retrovirus was added to the cell culture with Lipofectamine 2000 (2 µg/mL, ThermoFisher Scientific) and hexadimethrine bromide (Polybrene, 1.6 µg/mL, Sigma) and cells were centrifugated for 1.5 hours at 32°C. The next day, serial dilutions were incubated with K562-HLA-A2 cells and complement (Cedarlane Labs). Cells were washed with PBS after 45 minutes and then switched to vectors encoding the fluorescent intracellular ΔNGFR protein as the transduction marker downstream of an internal ribosomal re-entry sequence. Initial experiments were done with vectors encoding surface-expressed truncated human nerve-growth-factor receptor (ΔNGFR) and then switched to vectors encoding the fluorescent intracellular monomeric Kusabira-Orange2 (mKO2) protein as the transduction marker as described by Kurachi et al. Viral particles were produced by using the Platinum-E (Plat-E) Retroviral Packaging Cell Line according to the manufacturer recommendations (Cell Biolabs).

2.8 | Proliferation assays

To test the effects of HLA-A2-mediated stimulation, Tregs were labeled with a cell proliferation dye (CPD, eBiosciences) at day 8 and restimulated with irradiated (10 Gy) K562 cells or K562-HLA-A2 cells (K562 cells transduced with a lentivirus encoding HLA-A2) at a 1:2 (K562/Tregs) ratio for 3 days in the presence of 100 U/mL IL-2. Treg proliferation was assessed by flow cytometry on day 3.

2.9 | Suppression assays

CD4+ T cells were isolated from the spleen of OTII mice and B cells were isolated from the spleen of either Bl/6 wild-type mice or HLA-A2 transgenic mice (B6.Cg-Immp2Tg(HLA-A/H2-D)2Enge, AAD) by negative selection using magnetic enrichment kits (STEMCELL Technologies). T cells (5 × 10^6 responder OTII CD4+) were labeled with CPD eF450 (eBiosciences) and stimulated with 2 × 10^2 HLA-A2-positive or HLA-A2-negative B cells in the presence of an ovalbumin peptide 323-339 (200 ng/mL). Increasing ratios of anti-HLA-A2-CAR or anti-HER2-CAR Tregs were added to the culture as indicated. After 4 days, division of OTII CD4+ T cells was measured by flow cytometry.

2.10 | ELISAs

The concentration of anti-HLA-A2 IgG antibodies in the serum of recipients was determined by enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well ELISA plates (ThermoFisher Scientific) were coated with HLA A2-monomers (2.5 µg/mL, Biolegend) and washed before recipient sera were added and incubated for 1 hour at RT. After washing, anti-mouse IgG-alkaline phosphatase antibody (Mabtech) was added as detection reagents for 1 hour at RT. After extensive washing, BCIP/NBT substrate was added. Absorbance was measured at 415 nm using the Spectramax M5 plate reader. To determine the concentration of anti-HEL IgG antibodies, plates were first coated overnight with HEL peptide (1 µg/mL in coating buffer). Then, after several washes, they were incubated in blocking buffer for 1 hour before sera dilutions or an anti-HEL IgG1 isotype control (standard, LifeTein) was added. After washing, an anti-IgG conjugated with HRP was added (1:10 000, Sigma). The signal was revealed with TMB Substrate (BD Biosciences) and absorbance measured at 450 nm using a Spectramax M5 plate reader.

2.11 | Cytotoxicity assay

Sera were collected at the peak of the humoral response (day 21) and serial dilutions were incubated with K562-HLA-A2 cells and complement (Cedarlane Labs). Cells were washed with PBS after 45 minutes and cell viability was assessed by flow cytometry after staining with 7-AAD (R&D systems). The cytotoxicity index was defined as cell mortality in the presence of immune sera divided by mortality in the presence of nonimmune sera.

2.12 | ELISPOT assays

For the IFNγ ELISPOT assay, PVDF plates (Mabtech) were humidified with 70% ethanol for 1 minute and then coated overnight with an anti-IFNγ antibody (1 µg/mL, eBioscience) in coating buffer (0.5 mol/L carbonate-bicarbonate buffer, pH 9.6). The next day, serial dilutions of recipient spleen cells were stimulated with mitomycin C–treated HLA-A2

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or third-party NOD spleen cells for 18 to 24 hours at 37°C. Then, a biotinylated anti-IFNγ antibody was added to cell cultures for 2 hours. Spots were revealed using a streptavidin-alkaline phosphatase for 1 hour at RT, followed by BCIP/NBT substrate (Mabtech). The resulting spots were analyzed using an ImmunoSpot Analyzer.

The frequencies of IgG-secreting donor-reactive antibody-secreting cells were determined using ELISpot for mouse IgG kit (Mabtech). Brieﬂy, serial dilutions of recipient cells isolated from the spleen were cultured for 20 hours in RPMI media supplemented with 10% of fetal bovine serum in 96-well plates coated with anti-mouse IgG antibody (4 × 10^6-0.06 × 10^6 cells/well). Then, biotinylated HLA-A2 molecules (Flex-TTM HLA-A*02:01 Monomers UVX [Biolegend]) were added as detection reagents for 2 hours at 37°C. After extensive washes, the plates were incubated with streptavidin-alkaline phosphatase for 1 hour at RT, followed by BCIP/NBT substrate.

2.13 | Trogocytosis assay

HLA-A2- or -A24-expressing K562s were labeled with CPD and then 1 × 10^5 were co-cultured with 1 × 10^5 A2-CAR-Tregs, HER2-CAR-Tregs or untransduced Tregs. After 4 hours, cells were washed. In some experiments, sera from mice that were or were not sensitized to HLA-A2 were added for 30 minutes. Then, after additional washes, cells were stained with a viability dye, CD4, HLA-A2, and for the transduction marker as above.

2.14 | Statistics

Analysis was performed using Prism 7.0 software (GraphPad). P < .05 was considered signiﬁcant. Signiﬁcance of survival was determined by log-rank (Mantel-Cox) test. Other tests for signiﬁcance were determined by 2-way analysis of variance (ANOVA) test with Sidak’s multiple comparisons test as indicated in the text.

### FIGURE 1

Generation and in vitro testing of dsCAR-Tregs from Bl/6 mice. A, Schematic diagram of retroviral vectors encoding anti-HLA-A2- or HER2-speciﬁc CARs to generate donor-speciﬁc (dsCAR-Tregs) or irrelevant CAR Tregs (irrCAR-Tregs), respectively. B, Work ﬂow of mouse CAR-Treg manufacturing. CD4+Thy1.1+FOXP3^{GFP} Tregs were sorted and stimulated with anti-CD3 and CD28-coated beads, IL-2, and rapamycin. After 2 days, cells were left untransduced (UT) or transduced with retrovirus encoding the indicated CAR, and then expanded for an additional 5 days and rested overnight before testing in vitro or in vivo. C, Expression of FOXP3^{GFP}. CAR (based on c-myc epitope tag expression), and the transduction marker were assessed by ﬂow cytometry on day 7. Shown are representative data, numbers represent the mean ± SD of three independent experiments. D, UT, irrCAR-Tregs, or dsCAR-Tregs were stained with HLA-A2 tetramers and binding was assessed by ﬂow cytometry. Data are representative of three independent experiments. E, UT, irrCAR-, or dsCAR-Tregs were co-cultured with cell-proliferation dye (CPD)-labeled HLA-A2- or HLA-A24-expressing K562 cells. After 4-hour co-cultures, co-cultures were stained with anti-HLA-A2 mAbs and Tregs were gated as live CPD^{GFP} cells, and irrCAR- and dsCAR-Tregs as transduced live CPD^{GFP} cells. Shown is a histogram depicting HLA-A2 expression on the indicated type of Treg. Data are representative of n = 2 independent experiments. F, Cell-proliferation dye (CPD)-labeled irrCAR-Tregs or dsCAR-Tregs were co-cultured for 3 days without (gray histogram) or with (open histogram) blank or HLA-A2+ K562 cells and proliferation was assessed by ﬂow cytometry. Representative and average (mean ± SD) of two independent experiments. G, H, B cells from wild-type or HLA-A2+ Bl/6 mice were co-cultured with CPD-labeled OTII CD4+ T cells in the presence of an ovalbumin peptide and the absence or presence of increasing numbers of irrCAR-Tregs or dsCAR-Tregs. After 3 days, OTII CD4+ T cell proliferation was analyzed by ﬂow cytometry. Representative (G) and (H) mean ± SD of two independent experiments, significance determined by 2-way ANOVA and *P < .05 was considered significant
To assess the suppressive effect of dsCAR-Tregs, HLA-A2+ B cells were co-cultured with CPD-labeled ovalbumin-specific OTII CD4+ T cells in the presence of ovalbumin peptide, and the absence or presence of irrCAR- or dsCAR-Tregs (Figure 1G). Parallel cultures with HLA-A2-negative B cells were negative controls. In the presence of HLA-A2+ B cells, the proliferation of OTII CD4+ T cells was significantly lower in the presence of dsCAR-Tregs compared to co-cultures with irrCAR-Tregs. In contrast, in the presence of HLA-A2-negative APCs (ie, wild-type Bl/6), irrCAR- and dsCAR-Tregs had similar inhibitory effects (Figure 1H). These data demonstrate that dsCAR-Tregs have an antigen-specific and dose-dependent suppressive effect in vitro.

3.2 Donor-specific CAR-Tregs delay skin allograft rejection in immunocompetent mice

We next assessed the ability of irrCAR- vs dsCAR-Tregs to suppress allograft rejection in vivo. IrrCAR- or dsCAR-Tregs were injected intravenously into wild-type Thy1.2+ Bl/6 mice on the same day they were transplanted with a skin graft from an HLA-A2-transgenic Bl/6 donor (Figure 2A). To assess cell quality prior to injection, cell viability, GFP (FOXP3), and CAR expression were measured, revealing a consistently high level of GFP expression and that approximately 65% or 75% of cells were transduced with the irrCAR or dsCAR, respectively (Figure 2B). Because the survival of therapeutic cells generally correlates with cell therapy success,26 we assessed if injected Thy1.1+ Tregs could be detected in the blood, spleen, and graft-draining lymph nodes of recipients. We found that Thy1.1+ cells were present in all these locations 7 days after skin transplantation/cell injection (Figure 2B). It is notable that more than 90% of the cells remained GFP+, demonstrating the stability of their regulatory phenotype in vivo (Figure 2C).

Homing of therapeutic Tregs to allografts is required for Treg-mediated suppression of alloimmunity,27 and we recently demonstrated that donor-specific human CAR-Tregs migrated to skin allografts in NSG mice reconstituted with human PBMCs.11 To test if dsCAR-Tregs homed to the allograft in immunocompetent recipients, we transplanted recipients with side-by-side HLA-A2-negative or HLA-A2-positive skin grafts and injected either dsCAR- or irrCAR-Tregs. Seven days later, both grafts were collected and digested, and the presence of Thy1.1+ Tregs was assessed by flow cytometry (Figure 2D). Although Thy1.1+ irrCAR- and dsCAR-Tregs were detected in both grafts, there was a significantly higher proportion of Thy1.1+ cells in the HLA-A2-positive skin in mice injected with dsCAR-Tregs (Figure 2D). Moreover, only in this condition did the cell population retain a proportion of CAR-expressing cells, which was similar to the proportion injected (Figure 2E). These data show that in immunocompetent recipients, dsCAR Tregs home to alloantigen-expressing grafts and that antigen exposure is an important factor in preserving survival of dsCAR-Tregs.

To assess the allosuppressive effect of irrCAR- vs dsCAR-Treg therapy, allograft survival was monitored from the day of bandage removal on day 7. Figure 2F shows representative photos of skin grafts 10 days after transplantation in 5 different recipients, with graft survival quantified macroscopically in Figure 2G. We found that dsCAR- but not irrCAR-Tregs significantly prolonged skin allograft survival (median survival time: 8.0, 8.5, and 14.0 days in the PBS, irrCAR-, and dsCAR-Tregs groups respectively; P < .0001 by log rank). These results are consistent with those of other studies that tested antigen-specific Treg therapy in irradiation-free, drug-free, immunocompetent skin allograft recipients.28

3.3 Donor-specific CAR-Tregs inhibit humoral alloreactivity

We next studied the effect of therapeutic Tregs on the cellular and humoral arms of the anti-donor adaptive response. We first performed an IFN-γ ELISPOT assay to assess the impact of irrCAR- and dsCAR-Tregs on the anti-donor cellular response. Cells from graft draining lymph nodes were isolated 21 days posttransplant, stimulated with mitomycin-treated HLA-A2+ splenocytes, and after 20 hours the number of IFN-γ-secreting cells was enumerated. The number of IFN-γ-secreting cells was lowest in the dsCAR-Treg group, although the difference did not reach statistical significance (Figure 3A).

To determine how dsCAR-Tregs influenced DSA generation, we enumerated DSA-secreting cells day 21 posttransplant with an ELISPOT assay, finding that the number of DSA-producing cells

FIGURE 2 Donor-specific CAR-Tregs prolong skin allograft survival in immunocompetent recipients. A, Schematic diagram of experimental setup. 1 × 10^6 Thy1.1+ irrCAR-Tregs or dsCAR-Tregs were injected into Thy1.2+ Bl/6 mice on the same day they were transplanted with an HLA-A2+ Bl/6 donor skin graft. B, Quality control data of CAR-Tregs injected in vivo. Cell viability, FOXP3^GFP^, CAR expression, and transduction efficiency; mean ± SD; irrCAR n = 3, dsCAR n = 6 from three experiments. C, Proportion of transferred Thy1.1+ cells day 21 posttransplant (CD4+ and FOXP3^GFP^ population). Mean ± SD; significance was determined by log-rank test and *P < .05 was considered significant.
FIGURE 3  Impact of CAR-Tregs on anti-donor cellular and humoral immunity. A, Draining lymph node cells were isolated day 21 after transplantation and restimulated with HLA-A2* Bl/6 splenocytes overnight. An ELISPOT assay was used to quantify the number of IFN-γ-secreting cells. Mean ± SD for PBS n = 5, irrCAR-Tregs n = 5, and dsCAR-Tregs n = 5 from one independent experiment. B, The frequency of anti-HLA-A2 IgG secreting cells in the spleen of recipients as determined by ELISPOT day 21 after transplantation. Mean ± SEM, PBS n = 5, irrCAR-Tregs n = 5, and dsCAR-Tregs n = 5 from one independent experiment. Significance was determined by 2-way ANOVA. C, Levels of circulating anti-HLA-A2 IgG donor-specific antibodies (in serum) were measured weekly by ELISA. Mean ± SEM, PBS n = 19, irrCAR-Tregs n = 15, and dsCAR-Tregs n = 20 from four independent experiments. Significance was determined by 2-way ANOVA. D, Serial dilutions of day 21 sera were incubated with A2* K562 cells and complement. Cell viability was assessed by flow cytometry after 45 minutes. Cytotoxicity index = cell mortality in presence of immune sera/cell mortality in presence of nonimmune sera. Mean ± SEM, PBS n = 5, irrCAR-Tregs n = 5, and dsCAR-Tregs n = 5, from one independent experiment. Significance was determined by 2-way ANOVA. E, Schematic diagram of experiments in G and H; 1 × 10⁶ dsCAR-Tregs were injected into Bl/6 to mice on the day of an HLA-A2* skin graft. Forty-two days later mice were immunized with a subcutaneous (SC) injection of HEL protein + HLA-A2* splenocytes and hen egg lysozyme (HEL) in incomplete Freund’s adjuvant (IFA). Levels of circulating (F) anti-HLA-A2 and (G) anti-HEL IgG antibodies were measured after rechallenge by ELISA. Mean ± SEM, PBS n = 5, and dsCAR-Tregs n = 6 from one independent experiment. Significance was determined by 2-way ANOVA. *P < .05 was considered significant.
was significantly lower in dsCAR- vs irrCAR-Treg or PBS recipients (Figure 3B). To confirm these data, we measured the serum concentration of anti-HLA-A2 IgG (ie, DSAs) weekly after transplantation. The levels of DSAs were similar in the PBS and irrCAR-Treg groups, whereas treatment with dsCAR-Tregs significantly decreased the concentration of anti-HLA-A2 IgG DSAs (Figure 3C).

To evaluate antibody-dependent cytotoxicity toward HLA-A2+ cells, sera were collected at the peak of the humoral response (day 21) and serial dilutions were incubated with A2-K562 cells and complement. Cell viability was assessed after 45 minutes and a cytotoxicity index was calculated. The serum cytotoxicity against HLA-A2+ cells was significantly lower in the dsCAR-Tregs group compared to both the PBS and irrCAR-Treg groups (Figure 3D), providing further evidence that dsCAR-Tregs suppress humoral alloreactivity.

We next aimed to determine if the inhibitory effect of dsCAR-Tregs on the anti-donor humoral response persisted after a second encounter with the alloantigen. Mice that received an HLA-A2+ Bl/6 skin graft without or with dsCAR-Tregs were rechallenged 6 weeks after transplantation by injection with HLA-A2+ Bl/6 splenocytes in IFA. As a control-irrelevant antigen, they also received HEL proteins (Figure 3E). Even upon re-challenge of the recall B cell response, the levels of anti-HLA-A2 IgG DSAs remained significantly lower in mice previously treated with dsCAR-Tregs (Figure 3F). It is notable that the levels of anti-HEL antibodies were comparable between treated and nontreated mice (Figure 3G), indicating that dsCAR-Tregs did not cause nonspecific suppression. At the time of rechallenge, dsCAR-Tregs were not detectable in blood (not shown), suggesting that attenuation of the recall response was likely due to the inhibited generation of donor-reactive memory T cells and B cells in the peri-transplant period.

### 3.4 Donor-specific CAR-Tregs are ineffective in presensitized recipients

Finally, we determined if dsCAR Tregs could have a suppressive effect, even in previously sensitized recipients. Mice were sensitized against HLA-A2 by immunizing with HLA-A2+ Bl/6 splenocytes in CFA and then boosting in IFA. Six weeks later, irrCAR- or dsCAR-Tregs were injected intravenously to the sensitized mice on the same day that they were transplanted with a skin graft from an HLA-A2-transgenic Bl/6 donor (Figure 4A). Cell quality prior to injection, was assessed, confirming a high level of viability and GFP (FOXP3)-expression and that the majority of the cells were CAR+ (Figure 4B). IrrCAR- and dsCAR-Tregs were detected in the blood of sensitized recipients, with an average of 89 ± 10% of cells remaining GFP (FOXP3)-positive 7 days after injection (Figure 4C).

However, in contrast to the experiments in nonsensitized animals, graft survival was not improved by the injection of dsCAR-Tregs (Figure 4D). We investigated if the absence of effect of dsCAR-Tregs on allograft survival in sensitized recipients was due to insufficient control of cellular and/or humoral rejection. Graft-draining lymph node cells were collected 21 days post-transplantation and IFN-γ ELISPOT assays revealed that dsCAR-Treg recipients did not have less HLA-A2-stimulated IFN-γ-secreting cells (Figure 4E). Moreover, the levels of circulating DSAs (Figure 4F) and the frequency of DSA-producing cells in the spleen of sensitized recipients (Figure 4G) were not different between the PBS, irrCAR-, and dsCAR-Tregs groups.

### 4 DISCUSSION

To date, the majority of preclinical studies of Treg therapy have focused on studying T cell–mediated rejection in immunodeficient recipients that had not been previously sensitized to the relevant antigen(s). Using CAR-Treg technology as an efficient and clinically relevant method to generate dsCAR-Tregs, we show that these cells can suppress humoral immunity, and delay allograft rejection in unsensitized, immunocompetent recipients. On the other hand, dsCAR-Tregs were unable to limit memory T cell or B cell responses, or delay graft rejection in sensitized recipients. These data have important implications for how dsCAR-Treg therapy can be used in the clinic and indicate that additional therapies would be necessary to utilize this approach in DSA-positive recipients.

Our data, combined with those of other studies of antigen-specific Tregs5-7,28-32 suggest that antigen-specificity is an efficient way to increase the potency of Treg therapy. Consistent with our studies of human dsCAR-Tregs,11 antigen specificity appears to be inextricably linked with tissue localization, as only dsCAR-Tregs expanded within HLA-A2+ grafts. In addition, persistence of the dsCAR-Treg appeared to require antigen as there was a clear survival advantage of untransduced Tregs unless both CAR and antigen were present. This finding is in line with findings from the study of CARs in oncology where antigen loss is related directly to poor CAR-T cell persistence.33

Although we found significant effects of dsCAR-Tregs in a stringent model of skin transplantation, the effect was not long lasting and the grafts were eventually rejected. Because lymphodepletion, administration of pro-tolerogenic immunosuppression, and/or IL-2 therapy can be used in combination with Tregs,7,30,34 exploration of how such strategies could be combined with CAR-Tregs to further improve their effect is an area for future investigation. It is also important to test if repeat dosing of dsCAR-Tregs and/or infusion of higher cell numbers may further improve their therapeutic effect.

Our findings provide the first evidence that Tregs with direct specificity for donor antigens can suppress not only T cell– but also B cell-mediated alloreactivity. Tregs suppress humoral responses either indirectly by reducing CD4+ T cell help, or directly by cell-to-cell interactions with B cells.35 Because a significant proportion of recipient-derived APCs can acquire intact donor MHC molecules through the uptake of donor exosomes and/or trogocytosis,36-38 this may be a mechanism by which dsCAR-Tregs could directly suppress recipient-derived B cells. Moreover, our data suggest that the dsCAR-Tregs themselves can also acquire donor MHC molecules. The ability of CAR-Tregs to trogocytose their cognate antigen is consistent with the...
FIGURE 4 Impact of CAR-Tregs on alloimmune responses in sensitized recipients. A, Schematic diagram of experimental design. Mice were sensitized to HLA-A2 and hen egg lysozyme (HEL) by injection with HLA-A2⁺ Bl/6 splenocytes and HEL protein in complete Freund's adjuvant (CFA) and 14 days later with incomplete Freund's adjuvant (IFA). On day 42, irrCAR-Tregs and dsCAR-Tregs were injected into the sensitized mice on the same day they were transplanted with an HLA-A2⁺ Bl/6 skin graft. B, Quality control data of CAR-Tregs injected in vivo. Cell viability, FOXP3<sup>GFP</sup>, CAR expression, and transduction efficiency. Mean ± SD, n = 5 for dsCAR- and irrCAR-Tregs, respectively, from 2 experiments. C, Proportion of FOXP3<sup>GFP</sup> cells (within live, CD4<sup>+</sup>Thy1.1<sup>+</sup> cells) in the blood on day 7 after adoptive transfer and skin transplantation. Mean ± SD, n = 7 for dsCAR-, and n = 10 irrCAR-Tregs, from 2 experiments. D, Kaplan-Meier curves for skin allograft survival determined daily from the day of bandage removal (days 6-7). PBS n = 7, irrCAR Tregs n = 9, and dsCAR-Tregs n = 8 from 2 independent experiments. E, Day 21 after transplantation an IFN-γ ELISPOT assay was performed on draining lymph node cells restimulated with HLA-A2⁺ splenocytes. Mean ± SEM, PBS n = 6, irrCAR-Tregs n = 11, and dsCAR-Tregs n = 7 from 2 independent experiments. F, Circulating levels of anti-HLA-A2 IgG (shown as the ratio anti-HLA-A2 IgG/anti-HEL IgG) after transplantation were determined by ELISA. Mean ± SEM, PBS n = 7, irrCAR-Tregs n = 11, and dsCAR-Tregs n = 8 from 2 independent experiments. G, The frequency of anti-HLA-A2 IgG-secreting cells in the spleen of recipients was determined by ELISPOT assay on day 21 after transplantation. Mean ± SEM, PBS n = 4, irrCAR-Tregs n = 5, and dsCAR-Tregs n = 4 from one independent experiment.
fact that antigen-specific Tregs also trogocytose specific peptide-MHC complexes. This phenomenon has been observed previously with double-negative Tregs and for FOXP3+ Tregs could represent a previously unknown mechanism of action in transplantation as removal of allogenic MHC molecules from donor cells could decrease direct and semi-direct presentation. The consequence of interactions between donor-antigen-positive dsCAR-Tregs and donor-specific B cells remains to be investigated.

The ability of Treg therapy to suppress alloreactive memory T cells has been investigated previously using a variety of non-CAR-based methods to generate dsTregs, but effects on humoral alloreactivity were unknown. Consistent with Yang et al, we found that dsCAR Tregs did not suppress the formation of IFN-γ-producing memory T cells or delay memory T cell–mediated skin allograft rejection. On the other hand, Dai et al reported that dsTregs could suppress CD8+ memory T cells via a CD30-dependent mechanism. These contrasting results are likely due to differences in the models: Yang et al used TCR transgenic CD4+ memory T cells and immunodeficient mice, whereas Dai et al used immunocompetent mice and wild-type or TCR transgenic CD8+ memory T cells to mediate rejection. In our model, which better reflects the clinical situation, dsCAR-Tregs were unable to suppress memory T or B cell–mediated responses.

The inability of dsCAR-Tregs to suppress alloreactive memory in our model could be due to one or more possible mechanisms. One mechanism could be via a previously unknown ability of dsCAR-Tregs to trogocytose the CAR antigen (ie, HLA-A2) making the resulting HLA-A2-positive dsCAR-Tregs susceptible to DSA-dependent, cell-mediated, and/or complement-mediated cytotoxicity. dsCAR-Tregs may be unable to inhibit DSA-mediated cytotoxicity, for example, toward graft endothelial cells, and moreover preexisting DSA may mask the HLA-A2 epitope recognized by dsCAR-Tregs, thereby decreasing their activation. Furthermore, because we did not enrich for CXCR5+ expressing cells, the dsCAR-Tregs may not have effectively trafficked to B cell follicles where follicular regulatory cells are known to control B cells. Finally, antibody-secreting plasma cells are unlikely to be targets of direct allosuppression. Further research into how T cell– and/or B cell–depleting therapies could be combined with dsCAR-Treg therapy and/or alternate Treg-dosing regimens will be an important step toward applying this approach in presensitized individuals.

In conclusion, the use of CAR technology is a clinically relevant approach to enhance the potency of Treg therapy in transplantation. dsCAR-Tregs remained FOXP3+ in vivo and there was no evidence of cytotoxicity toward the graft, demonstrating the safety of the approach. The ability of dsCAR-Tregs to suppress humoral alloreactivity is a key feature that supports their use in recipients undergoing immunosuppression minimization.

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DISCLOSURE
The authors of this manuscript have conflicts of interest to disclose as described by the American Journal of Transplantation. MKL and CL have patents (PCT/CA2018/051167 and PCT/CA2018/051174) pending on alloantigen-specific chimeric antigen receptors and have licensed this technology to Sangamo Therapeutics, Inc. MKL has received research funding from TxCell, Pfizer, Bristol Myers Squibb, Takeda, and CRIPSR Therapeutics. The other authors have no conflicts of interest to disclose.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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