THROMBOSIS AND HEMOSTASIS

FVIII-specific human chimeric antigen receptor T-regulatory cells suppress T- and B-cell responses to FVIII

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Key Points

- Generation and functional analysis of FVIII-specific human CAR Tregs.
- Specific regulation of FVIII responses by engineered human CAR Tregs.

Replacement therapy with factor VIII (FVIII) is used in patients with hemophilia A for treatment of bleeding episodes or for prophylaxis. A common and serious problem with this therapy is the patient's immune response to FVIII, because of a lack of tolerance, leading to the formation of inhibitory antibodies. Development of tolerogenic therapies, other than standard immune tolerance induction (ITI), is an unmet goal. We previously generated engineered antigen-specific regulatory T cells (Tregs), created by transduction of a recombinant T-cell receptor (TCR) isolated from a hemophilia A subject's T-cell clone. The resulting engineered T cells suppressed both T- and B-cell effector responses to FVIII. In this study, we have engineered an FVIII-specific chimeric antigen receptor (ANS8 CAR) using a FVIII-specific scFv derived from a synthetic phage display library. Transduced ANS8 CAR T cells specific for the A2 domain proliferated in response to FVIII and ANS8 CAR Tregs were able to suppress the proliferation of FVIII-specific T-effector cells with specificity for a different FVIII domain in vitro. These data suggest that engineered cells are able to promote bystander suppression. Importantly, ANS8 CAR-transduced Tregs also were able to suppress the recall antibody response of murine splenocytes from FVIII knockout mice to FVIII in vitro and in vivo. In conclusion, CAR-transduced Tregs are a promising approach for future tolerogenic treatment of hemophilia A patients with inhibitors. (*Blood*. 2017;129(2):238-245)

Introduction

Hemophilia A is an X-linked disorder, in which mutations in the coagulation Factor VIII (FVIII) gene lead to a loss of FVIII function and can result in serious bleeding episodes. Although these episodes can be treated with recombinant or plasma-derived FVIII protein, unfortunately, ~25% of hemophilia A patients produce inhibitory anti-FVIII antibodies (inhibitors).[1,2] Typically, these patients are treated with repeated high doses of FVIII in a protocol termed immune tolerance induction (ITI), a process that is expensive, time-consuming and not entirely effective. The development of effective tolerogenic therapies to prevent, as well as reverse, inhibitor formation is clearly needed.

Recently, regulatory T cells (Tregs) have been proposed as a potential clinical therapy for a variety of adverse immune disorders, ranging from autoimmune diseases to the development of antidrug antibodies, such as inhibitor formation to FVIII. Treg therapies have already been applied in clinical studies for prevention of graft-versus-host disease (GVHD).[3,4] However, polyclonal Tregs encompass many specificities and could potentially be globally immunosuppressive. For example, results of a GVHD study suggest an increase in viral reactivation up to 30 days after adoptive transfer of umbilical cord–derived polyclonal Tregs.[5] Thus, the use of antigen-specific Tregs seems to be preferable for the next generation of Treg therapeutic approaches. Previously, we developed an approach to render expanded polyclonal Tregs "specific" by transducing them with a T-cell receptor (TCR) containing the variable genes from a T-cell clone derived from a hemophilia A patient.[6] These Tregs were highly effective at suppressing both T- and B-cell responses to FVIII. However, TCR-transduced cells remain MHC-restricted. In contrast, chimeric antigen receptors (CARs) containing a single chain variable fragment (scFv) as the binding domain are not major histocompatibility complex (MHC)-restricted and have been used to specifically direct the lytic function of $CD8^+$ T cells against target cells for more than a decade.[7] Most recently, CARs have also been applied to create alloantigen-specific Tregs that prevented xenogeneic GVHD in a mouse model.[8]

Thus, as an alternative to a FVIII-C2-peptide-specific TCR (called 17195 TCR),[6] we have now used a CAR containing a scFv isolated from a phage library[9] (termed ANS8 CAR) that recognizes the A2 domain of FVIII. In this report, we characterize these FVIII-specific CAR Tregs and demonstrate their ability to inhibit T- and B-cell responses to FVIII in vitro and in vivo.

Methods

Human blood samples and cells

Human blood samples from healthy, anonymous male donors ranging from 20 to 70 years of age were obtained with written consent from the Department of

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Transfusion Medicine, Clinical Center, National Institutes of Health (NIH), and analyzed with the approval of the ethical review committee of the NIH. Peripheral blood cells (PBMCs) were isolated from mononuclear cell buffy coats using Ficoll-Hypaque (GE Healthcare Biosciences, Pittsburgh, PA) by gradient centrifugation. Cells were then frozen in fetal bovine serum (FBS) containing 10% dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO) and kept at −80°C. Cells were thawed and washed in complete media (RPMI 1640 medium with 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, 1 mM nonessential amino acids, 50 μM β-mercaptoethanol, 1 mM sodium pyruvate, and HEPES) immediately before each experiment. T cells were enriched from PBMCs by $CD4^+$ selection using Human CD4 MicroBeads (Miltenyi Biotec Inc., Auburn, CA) according to the manufacturer's instructions. Enriched cells were stained and then sort-purified as T naïve ($CD4^+CD25^-CD127^{hi}CD45RA^+$) and T regulatory (Treg, $CD4^+CD25^{hi}CD127^{lo}CD45RA^+$) cells on a fluorescence-activated cell sorting (FACS) Aria II cell sorter (BD, Franklin Lakes, NJ).

Generation of the FVIII-specific ANS8 CAR

The FVIII-specific single-chain variable fragment (scFv), 1G10, was isolated from the Tomlinson J phage library and cloned into the context of the human IgG1 heavy chain as described previously; this scFv recognizes a FVIII A2 domain epitope.[9] Additional components for the CAR design were derived from the nextprot database (nextprot.org). The TCR α chain leader was put upstream, and the sequence for the CD28 transmembrane and intracellular region and the intracellular domain of the CD3 ζ chain were put downstream of the scFv-IgG1 sequence. The designed ANS8 CAR construct was codon-optimized and synthesized by GenScript USA (Piscataway, NJ) and ligated into the retroviral pRetroX-IRES-ZsGreen1 vector (Clontech, Mountain View, CA). Empty retroviral vector (Mock) was used as control. The vector contains an internal ribosomal entry site (IRES) downstream of the multiple cloning site, followed by the cDNA sequence for green fluorescent protein (ZsGreen) as shown in Figure 1A.

Production of retroviral particles and T-cell transduction

Retrovirus was produced using a Phoenix-Ampho packaging system (Clontech) and used for preparation of T-effector cells or transduced Tregs as described previously.[6] Briefly, to prepare CAR or TCR-transduced T-effector cells or Tregs, either sorted T naïve or Treg cells, were prestimulated with anti-CD3ε (clone 64.3, 5 μg/mL) and CD28 (clone CD28.2, 2 μg/mL) antibodies for 48 hours and transduced with retroviral particles by centrifugation at 32°C for 1.5 hours onto retronectin (10 μg/mL, Clontech)-coated multiwell plates. After transduction, cells were expanded for 3 weeks using restimulation by soluble anti-CD3ε antibody (0.5 μg/mL) and autologous γ-irradiated PBMCs. The experiment was performed with transduced T cells generated from a single donor and repeated >3 times with PBMCs from different donors with similar results.

Flow cytometry analysis and antibodies

Isolated cells were washed (3% FBS in phenol-red free RPMI) and stained with fluorochrome-conjugated antibodies for 20 min at 4°C. Antibodies used for flow cytometry analysis and sorting are listed in supplemental Table 1, available on the *Blood* Web site. FVIII and ovalbumin (OVA, Sigma Aldrich) were biotinylated for binding studies using the EZ-Link Sulfo- NHS-LC-Biotinylation kit (Thermo Scientific, Waltham, MA) following the manufacturer's instructions and detected by the addition of fluorochrome-conjugated streptavidin. For intracellular staining, cells were fixed with Foxp3 Fixation buffer (eBioscience, San Diego, CA) and permeabilized with 1x Permeabilization buffer (eBioscience) followed by staining with respective antibodies. Cell viability dye eFluor780 was obtained from eBioscience. Stained cells were acquired on an LSRII instrument (BD) using BD FACSDiva software and analyzed using FlowJo software (Tree Star, Inc., Ashland, OR). For all analyses, dead cells were excluded by gating singlets with forward-scatter (FSC) and side-scatter (SSC) lymphocyte characteristics followed by exclusion of cells that were positively stained with cell viability dye.

Cell proliferation and in vitro T-cell immunosuppression assay

ANS8 CAR-transduced Tregs and T-effector cells, as well as 17195 TCR (TCR specific for FVIII C2 peptide p2191-2220 and restricted by human HLA-DR1) or Ob2F3 TCR (TCR specific for myelin basic protein [MBP], unpublished data) transduced T-effector cells, were used for the assays. Transduced cells were labeled with 10 μM eFluor 450 cell proliferation dye (CPD, eBioscience) according to the manufacturer's instructions. 2.5×10^5 CPD-labeled transduced ANS8 CAR T-effector cells were incubated with 1 μg/mL soluble recombinant human FVIII (Baxalta, Vienna, Austria), or 1 μg/mL soluble FVIII (or OVA as a negative control) in the presence of 1.25×10^6 autologous PBMCs in 24-well plates. Proliferation of T cells was analyzed by flow cytometric detection of dye dilution after 4 days of incubation.

An in vitro CAR Treg immunosuppression assay was performed as described previously[6] with some modifications. Briefly, 3- to 4-week expanded, sorted GFP^+ ANS8 (A2 domain–specific) CAR Tregs and FVIII-specific 17195 TCR or MBP-specific Ob2F3 TCR T effectors were counted and then mixed at specific ratios of Treg: T effectors as shown on the x-axis in Figure 3. These mixtures were cultured in the presence of γ-irradiated autologous PBMCs and stimulated with FVIII (0.4 μg/mL) or myelin basic protein peptide ($pMBP_{85\text{-}99}$, 0.5 μg/mL, New England Peptide Inc., Gardner, MA) for 4 days. T-effector cells were CPD-labeled before mixing with Tregs. Analysis of immunosuppressive activity was calculated from flow data of the absolute counts of divided GFP^+ T-effector cells.

In vitro secondary antibody response and suppression assay

Human HLA DR1 transgenic mice crossed into the E16-FVIII knockout[10] background (E16-FVIII knockout-HLA-DRB1*01:01, abbreviated hereafter as "E16XDR1")[6] were immunized IV with FVIII (2 μg/mL) and boosted with FVIII (1 μg/mL) intraperitoneally 7 weeks later. Pooled whole splenocytes from 2 immunized mice having a high antibody titer against FVIII were cocultured with human ANS8 CAR Tregs (or 17195 TCR Tregs) at various ratios for 6 days in the presence of FVIII (1 μg/mL). FVIII-specific antibody-secreting cells were enumerated by enzyme-linked immunospot (ELISPOT) assay. Cells were washed, plated on FVIII (2 μg/mL)-coated ELISPOT plates (EMD Millipore, Billerica, MA) in duplicate, and incubated overnight. The captured anti-FVIII antibodies were detected by HRP-conjugated anti-mouse IgG (H+L, Invitrogen), and developed with AEC substrate (BD Biosciences).

In vivo suppression of anti-FVIII antibody response in FVIII knockout mice

Female E16xDR1 mice 5 to 8 months of age were subcutaneously immunized (base of tail) with 2 μg FVIII in incomplete Freund's adjuvant (IFA) at day 0. Within 4 hours, the mice (n = 5 per group, age matched) were adoptively transferred with $1\text{-}2 \times 10^6$ FACS-sorted and in vitro–expanded polyclonal human Tregs expressing either ANS8 CAR, 17195 TCR, or control Ob2F3 TCR. To test the persistence of tolerance, mice were IV boosted with 1 μg FVIII together with unrelated antigen trinitrobenzene sulfonic acid–conjugated sheep red blood cells (TNP-SRBC)[11] in 100 μL volume of phosphate-buffered saline on day 56. The mice were bled weekly starting from 2 weeks after the initial immunization and the anti-FVIII antibody levels were followed using a quantitative enzyme-linked immunosorbent assay (ELISA) as previously described.[12]

Statistics

All statistical analyses were performed using Prism software (v6.0, GraphPad Software, La Jolla, CA). Statistical significance was determined without correction for multiple comparisons using an α value of .05.

Results

FVIII-specific responsiveness of T cells expressing ANS8 CAR

To produce ANS8 CAR–transduced T effectors or Tregs, naïve CD4 T cells ($CD4^+CD25^-CD127^+CD45RA^+$) or Tregs ($CD4^+CD25^{hi}CD127^{lo}$), isolated from healthy donors' PBMCs, were prestimulated with anti-CD3ε and anti-CD28 antibodies, followed by retroviral transduction of empty vector (Mock) or ANS8 CAR (Figure 1A). To

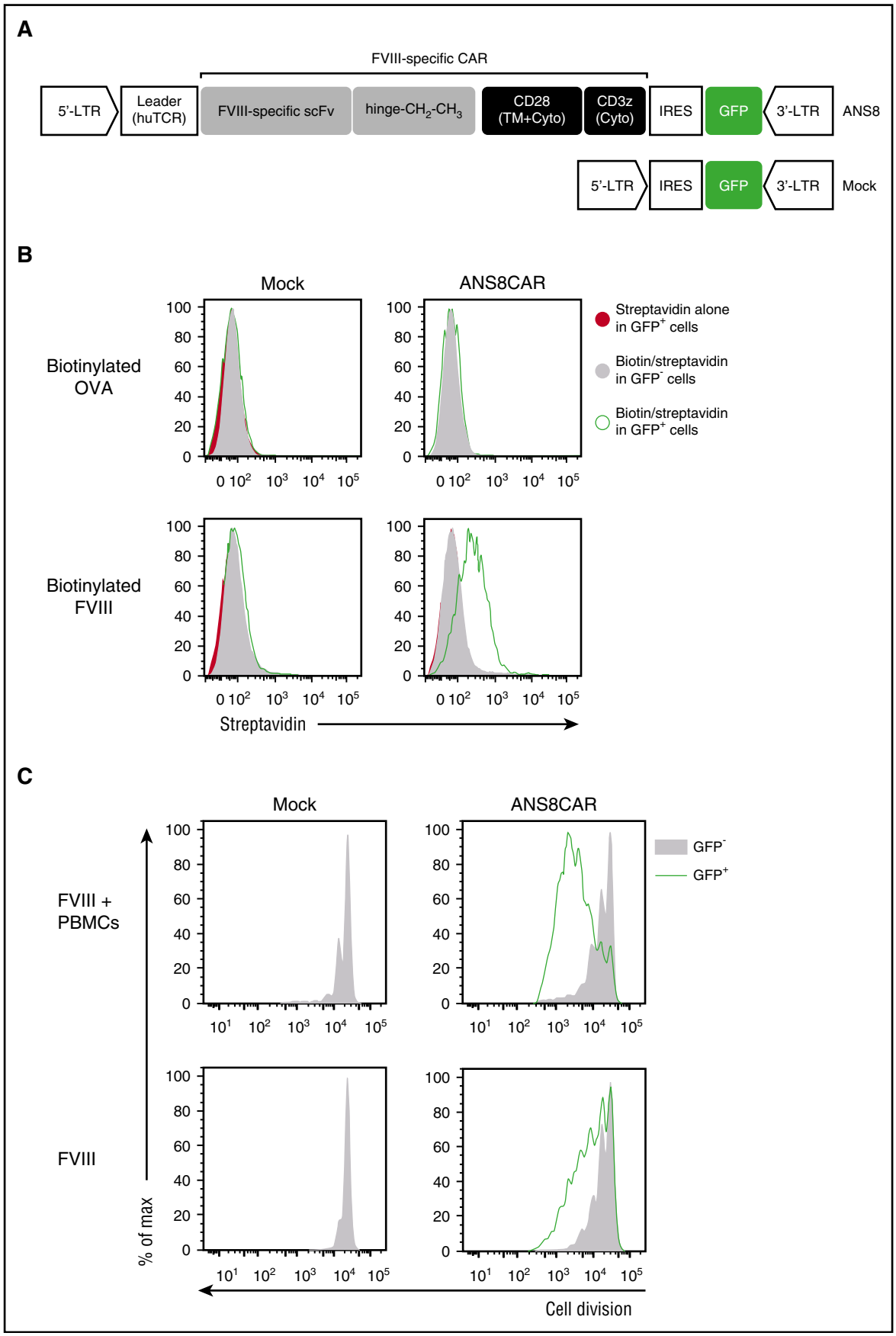

Figure 1. Generation of FVIII-specific CAR T cells. (A) Schematic view of transgenes used. FVIII-specific ANS8 CAR, containing the FVIII-specific scFv 1G10 in comparison with the empty Mock control. (B) Retrovirally transduced Mock or ANS8 CAR Tregs were incubated with biotinylated FVIII (1.8 μg/mL) or OVA (1.8 μg/mL). Cells were stained with PE-conjugated streptavidin and analyzed by FACS. GFP^+ ANS8 CAR Tregs showed specific binding to biotinylated FVIII. (C) Transduced, cell proliferation

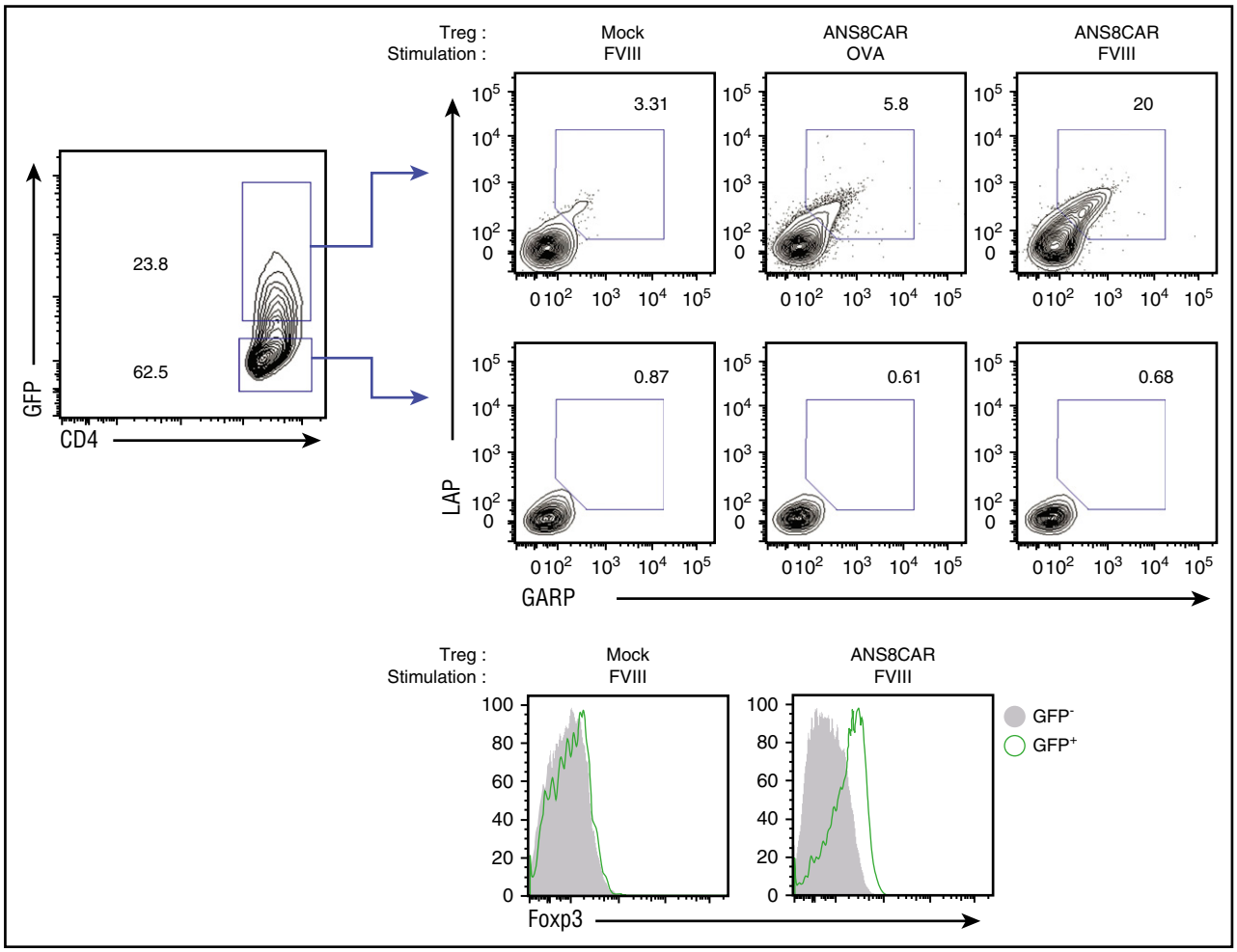

Figure 2. CAR-mediated stimulation of FVIII-specific CAR Tregs. Sorted human Tregs were transduced with ANS8 CAR or Mock, expanded in the presence of IL-2, rested, and then stimulated with FVIII. Gating strategy for GFP^+ and GFP^- cells is shown on the left. Intracellular staining for LAP and GARP in GFP^+ cells was increased in the presence of FVIII but not in the presence of OVA. Foxp3 expression is increased in the ANS8 CAR GFP^+ population in the presence of FVIII. Data are representative of 3 independent experiments.

confirm the extracellular expression of ANS8 CAR in Tregs, biotinylated FVIII (Biotin-FVIII) or biotinylated OVA (Biotin-OVA) were prepared for staining of Mock Tregs or ANS8 CAR transduced Tregs. In flow cytometric analysis, GFP^+ ANS8 CAR Tregs, as well as ANS8 CAR T effectors (not shown), were positively stained only with Biotin-FVIII, but not with Biotin-OVA. There was no specific staining of Mock Tregs by Biotin-FVIII or Biotin-OVA (Figure 1B). The expression of ANS8 CAR (~75 kDa) was also verified by western blot analysis using an antibody specific for human IgG (supplemental Figure 1).

Next, we evaluated FVIII-specific stimulation of ANS8 CAR-transduced cells. For this, FVIII-specific responsiveness of ANS8 CAR–transduced T-effector cells (ANS8 T effectors) was addressed in various restimulating conditions by measuring cell division after cell proliferation dye (CPD) labeling. Although GFP^+ Mock T cells showed very weak cell division after the addition of FVIII, GFP^+ ANS8 CAR T effectors showed FVIII-specific proliferation compared with untransduced GFP^- T effectors (Figure 1C). Of note, FVIII-specific proliferation of ANS8 T effectors was promoted by co-addition of autologous PBMCs with FVIII to the culture. In the presence of FVIII and PBMCs, the GFP^+ population increased from 25.6% to 43.7% during 4 days of culture (data not shown). When only FVIII was added to CAR-transduced cells, no significant increase in GFP^+ cells was measurable during that time (27.6% GFP^+ compared with 25.6%). Thus, reactivity to fluid phase FVIII was weak, which may be important in vivo.

Induction of activated Treg markers in FVIII-treated ANS8 CAR Tregs

Next, to verify ANS8 CAR-mediated Treg activation, mock and ANS8 CAR Tregs were restimulated with OVA or FVIII, followed by the analysis of the Treg activation markers surface latency–associated peptide (LAP), glycoprotein A repetitions predominant (GARP), and forkhead box P3 (Foxp3). It has been reported that human $Foxp3^+$ CD4 Tregs express GARP-anchored LAP after activation.[13] The expression of LAP and GARP was increased only in GFP^+ ANS8 CAR–transduced Tregs after FVIII and not OVA stimulation, indicating a FVIII-specific activation of ANS8 CAR Tregs (Figure 2). In addition,

Figure 1 (continued) dye-labeled ANS8 CAR T-effector cells were incubated with FVIII or FVIII in combination with irradiated autologous PBMCs for 6 days and analyzed for cell proliferation. ANS8 CAR–transduced cells strongly proliferated in the presence of FVIII and PBMCs. The histogram shows viable $CD4^+$ cells. Similar results were obtained in independent experiments from at least 3 different healthy T-cell donors.

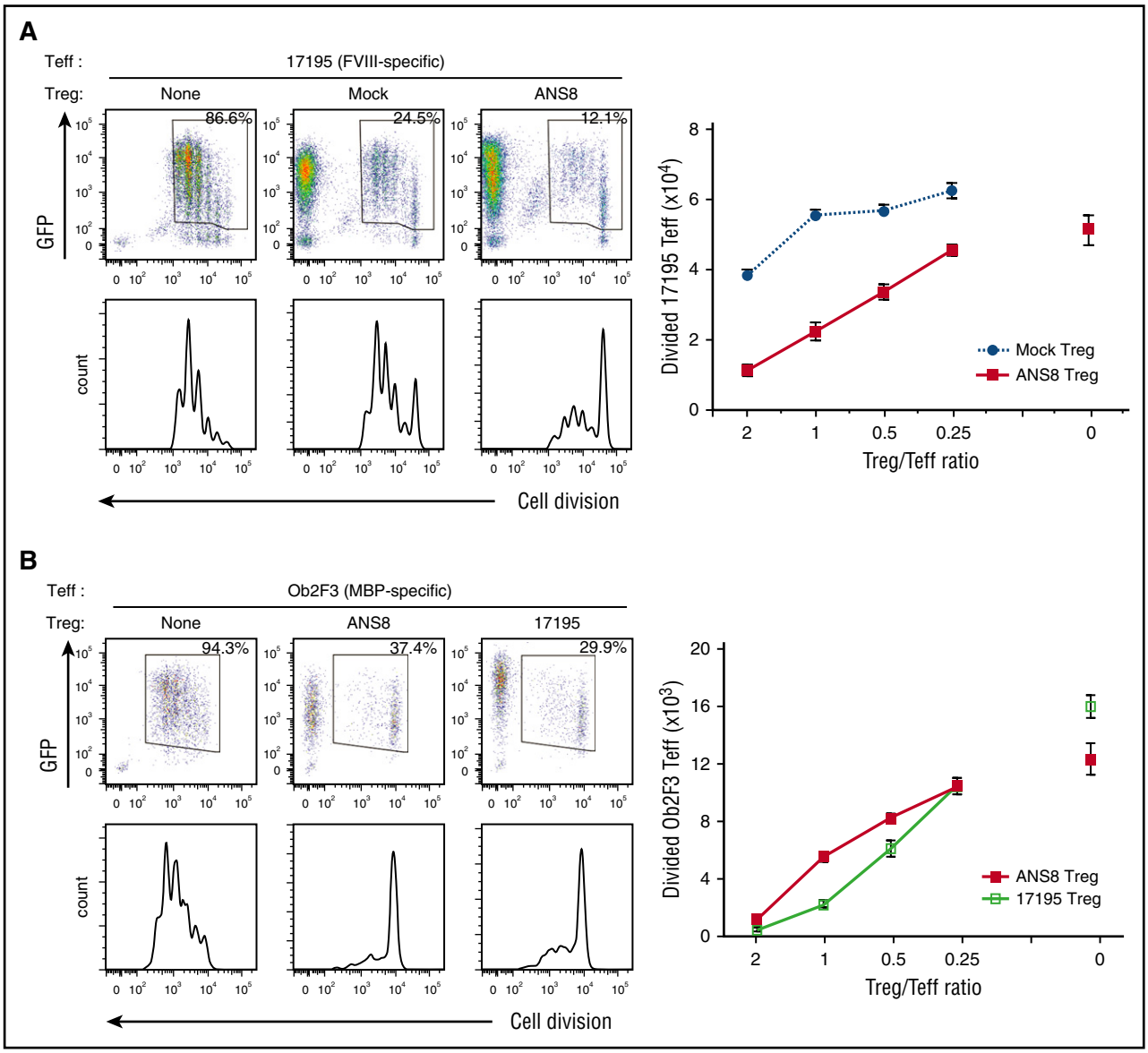

Figure 3. FVIII-specific and bystander immunosuppression by ANS8 CAR Tregs. Sorted human Treg and T-effector cells were transduced with Mock, ANS8 CAR, 17195 TCR, or Ob2F3 TCR, expanded, and rested. (A) Cell proliferation dye (CPD)-labeled 17195 T-effector cells were cocultured with different numbers of Mock or ANS8 CAR Tregs in the presence of FVIII protein (0.4 μg/mL) for 4 days. ANS8 CAR Tregs suppressed FVIII-specific effector T-cell proliferation. (B) CPD-labeled Ob2F3 T effectors were cocultured with different numbers of either ANS8 CAR or 17195 TCR Tregs in the presence of FVIII protein and pMBP for 4 days. ANS8 CAR and 19175 TCR Tregs suppressed MBP-specific effector T-cell proliferation but only when both pMBP and FVIII were present. This figure shows representative cell proliferation data of 17195 TCR (A) or Ob2F3 TCR (B) T-effector cells in the presence of the different Treg populations (left). Graphs show absolute counts of divided GFP^+ T-effector cells after coculture with differently engineered Tregs (right, dotted line and closed circle, mock Tregs; closed square, ANS8 CAR8 Tregs; open square, 17195 TCR Tregs). Data indicate mean values for triplicates ± standard error of the mean (SEM). The experiment was repeated with separately transduced T cells from different donors with similar results.

a FVIII-specific increase in Foxp3 expression was observed only in FVIII-stimulated GFP^+ ANS8 CAR Tregs.

ANS8 CAR Tregs suppress FVIII-specific and MBP-specific T-effector responses

To address whether ANS8 CAR Tregs suppress the FVIII-specific immune response of T-effector cells, we cocultured A2 domain-specific ANS8 CAR Tregs and CPD-labeled C2-peptide–specific 17195 TCR T-effector cells in the presence of HLA DR1 PBMCs and FVIII. The quality of transduced Tregs was verified by measuring intracellular Foxp3 and Helios expression (supplemental Figure 2). Proliferation of GFP^+ 17195 T-effector cells in the presence of FVIII was suppressed by ANS8 CAR Tregs, whereas suppression of proliferation in the absence of Tregs or the presence of Mock Tregs was minimal even at high Treg/Teff ratios. The suppressive effect of ANS8 CAR on the T-effector cell population increased in a Treg/Teff ratio–dependent manner (Figure 3A). Thus, ANS8 CAR-transduced Tregs, specific for an A2 epitope on FVIII, were capable of suppressing the response of C2-specific T effectors locally. This suggests that Tregs specific for one epitope may be able to suppress the antibody response to multiple epitopes on FVIII.

Considering that the FVIII recognition by ANS8 CAR Tregs is not MHC-restricted but requires recognition of conformational determinants, we hypothesized that FVIII CAR Tregs would exert a suppressive function once activated by FVIII regardless of the T effector cell's antigen

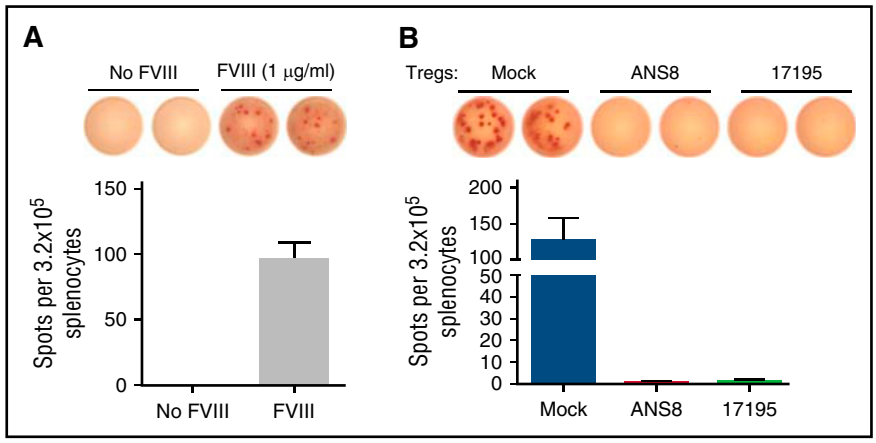

Figure 4. In vitro suppression of antibody production by FVIII-specific CAR Tregs. Whole splenocytes isolated from E16×DR1 mice with high antibody titers (measured by ELISA) were cultured in the presence of FVIII for 6 days and antibody-secreting cells were enumerated by ELISPOT assay. Bars indicate mean ± SEM. (A) FVIII-specific antibody-secreting cells were detected in splenocyte cultures only in the presence of FVIII. (B) Coculture with either ANS8 CAR or 17195 TCR Tregs (but not Mock Tregs) inhibited formation of FVIII-specific antibody-secreting cells. The experiment was repeated with separately transduced Tregs from different donors with similar results.

specificity. To address this, we tested whether FVIII-specific CAR Tregs can suppress the proliferation of T-effector cells with specificity toward MBP. Cells were prepared by retroviral transduction of the MBP-specific Ob2F3 TCR, and then labeled with CPD. Coculture of ANS8 CAR Tregs with Ob2F3 TCR T effectors was performed in the presence of HLA DR1/DR2 PBMCs plus FVIII and MBP peptide for 4 days. The Ob2F3 TCR T-effector cells alone proliferated well after the addition of both FVIII and MBP peptide (Figure 3B). This MBP-specific T-effector cell proliferation was completely blocked by the coculture of FVIII-specific 17195 TCR bystander Tregs. Importantly, ANS8 CAR Tregs also suppressed MBP-specific proliferation of Ob2F3 TCR T effectors effectively. Both ANS8 CAR Tregs and 17195 TCR Tregs were able to similarly suppress MBP-specific T effectors in "bystander assays" (Figure 3B, right). Indeed, we independently showed that ANS8 CAR Tregs activated by FVIII could suppress T-effector cell responses to MBP but only in the presence of both specific activating ligands, in the local milieu of the immune response.

FVIII-activated ANS8 CAR Tregs suppress the production of FVIII-specific antibodies in vitro

Previously, we demonstrated that FVIII-specific TCR-engineered Tregs inhibited the production of FVIII-specific antibodies using an ex vivo restimulation of murine FVIII- specific memory B cells.[6] As mentioned before, FVIII-activated CAR Tregs suppress the TCR-specific activation of T-effector cells without the limits of MHC restriction. Therefore, we also addressed whether ANS8 CAR Tregs could control the recall FVIII-specific antibody response from murine FVIII-specific memory B cells (obtained from hyperimmunized mice). Splenocytes were isolated from FVIII-immunized DR1×E16 mice with a high anti-FVIII antibody titer, and cocultured with Mock Tregs, ANS8 CAR Tregs, or 17195 Tregs in the presence of FVIII for 6 days. In the absence of Tregs, FVIII-specific antibody-producing B cells were detected by day 6 of in vitro culture in response to FVIII (Figure 4A). Antibody formation was significantly diminished with 17195 TCR Tregs as described in our previous report.[6] Importantly, ANS8 CAR Tregs suppressed FVIII-specific antibody formation as much as 17195 TCR Tregs (Figure 4B). Moreover, these data clearly verify the bystander effect of A2 domain-specific ANS8 CAR Tregs because FVIII knockout mice immunized with human FVIII show a diverse antibody response to multiple FVIII domains.[14]

Suppression of anti-FVIII antibody response in vivo by ANS8 Tregs

To further confirm the functional utility of ANS8 CAR Tregs in suppressing an anti-FVIII immune response, we conducted a xenogeneic suppression assay in DR1×E16 mice. Mice were immunized to initiate an anti-FVIII antibody response. Within 4 hours, the mice were treated with expanded human Tregs expressing ANS8 CAR, 17195 TCR, or Ob2F3 TCR (Figure 5A).

As expected, nonspecific Ob2F3 Tregs could not effectively control the development of anti-FVIII antibodies. In contrast, the development of anti-FVIII antibody response was effectively suppressed both by ANS8 and 17195 Tregs in vivo because only minimal levels of anti-FVIII antibodies were detected in recipients of ANS8 CAR or 17195 TCR Tregs over 8 weeks (Figure 5B). This was especially impressive considering that human T cells were undetectable in immunocompetent recipient mice

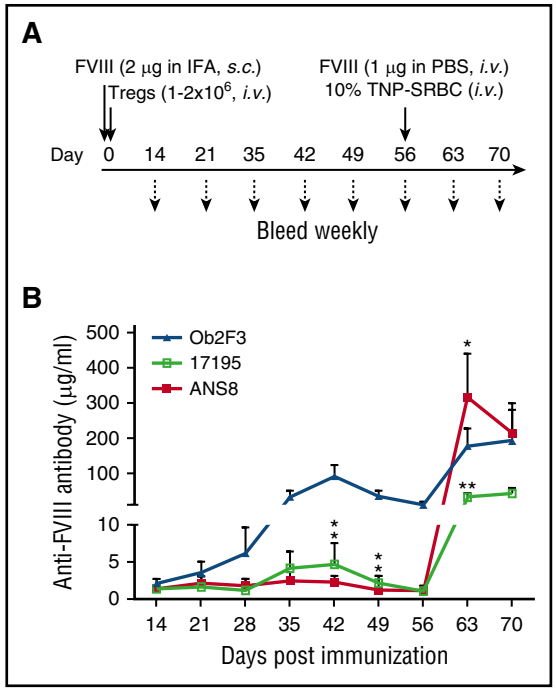

Figure 5. Xenogeneic suppression of anti-FVIII antibody response in vivo by human ANS8 CAR Tregs. (A) Experimental schema. Female E16×DR1 mice (n = 5 per group) were subcutaneously immunized with FVIII in incomplete Freund's adjuvant on day 0. Four hours after immunization, the mice were adoptively transferred with GFP$^+$ Tregs expressing ANS8 CAR, 17195 TCR, or control Ob2F3 TCR. The anti-FVIII antibody levels were followed weekly after the immunization (dotted arrows). Mice received one additional challenge with FVIII, together with 10% TNP-SRBC to test antigen-specificity of tolerance on day 56 (solid arrows). (B) Time course of anti-FVIII antibody response. Data indicate mean ± SEM. Multiple Student *t* tests were performed to determine statistical significance. $^*P < .05$, $^{**}P < .01$.

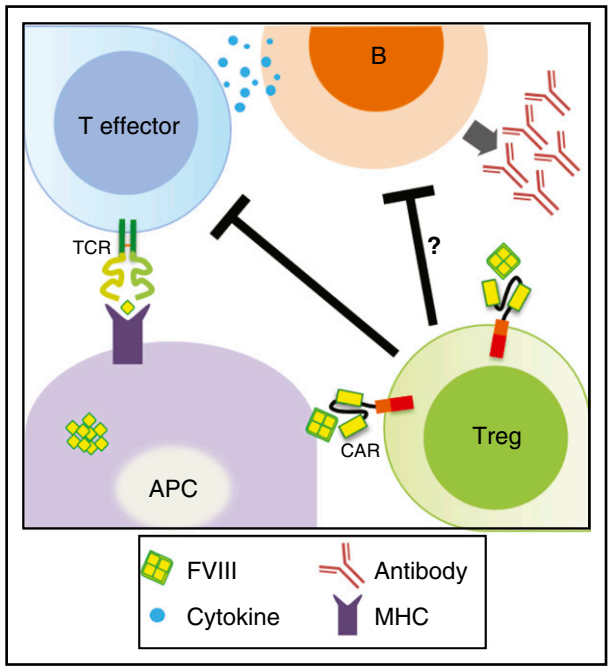

Figure 6. Model of FVIII-specific human CAR Treg function. Cartoon depicting suppressive function of FVIII-specific CAR-engineered human Tregs to FVIII-specific TCR-engineered T-effector cells or antibody-producing B cells in response to FVIII. FVIII-loaded APCs might bring T-effector cells and Tregs into close proximity. Thus, activated Tregs can inhibit T-effector cell activation in this local milieu (eg, by IL-2 consumption). Inhibition of T-effector cells also leads to prevention of FVIII inhibitor formation as costimulatory signals of T-effector cells are essential for B-cell activation. In addition, activated Tregs might influence B cells directly by still unknown mechanisms.

2 weeks after adoptive transfer (data not shown). The results of this in vivo experiment are in agreement with our previous[6] and current studies, which demonstrated effective suppression of FVIII-specific memory responses by both types of antigen-specific of Tregs in vitro. However, rechallenge with FVIII at day 56 resulted in a loss of tolerance in the ANS8 CAR group as the anti-FVIII antibody development reached the levels of the control Ob2F3 TCR mice. Anti-FVIII antibody levels in the 17195 TCR group increased as well. However, they remained significantly lower 1 week after the rechallenge, suggesting a different mechanism of action between ANS8 CAR and 17195 TCR Tregs (Figure 5B). Interestingly, all groups developed comparable amounts of anti-TNP antibody levels when the mice were rechallenged at day 56 with TNP-SRBC, suggesting that Treg-treated mice were not nonspecifically suppressed (supplemental Figure 3).

Discussion

Although polyclonal regulatory T-cell populations have been used in clinical trials to prevent GVHD and treat type I diabetes,[5,15-17] these polyclonal Tregs could potentially be immunosuppressive. Instead, we previously proposed that the use of engineered antigen-specific Tregs would be preferable for future therapeutic strategies in hemophilia A patients with inhibitors because they would be more efficacious, bearing a lower risk of global immunosuppression. We previously demonstrated that human Tregs could be engineered to be specific for FVIII by transducing them with a TCR recognizing a FVIII C2 domain peptide in the context of HLA DR1.[6] These Tregs (17195 TCR) inhibited the proliferation and cytokine production by C2-specific effector cells, as well as the production of FVIII-specific antibodies of murine FVIII-specific memory B cells in vitro. Herein, we demonstrate that human Tregs can be rendered specific for FVIII as well as by transducing them with a chimeric antigen receptor (CAR), called ANS8 CAR, containing a FVIII A2 domain–specific single-chain variable fragment (scFv) isolated from a phage display library. As opposed to TCR-transduced Tregs, the ANS8 CAR Tregs are not MHC-restricted and thus could be used to treat any patient regardless of the MHC background.

ANS8 CAR T-effector cells proliferated in response to a FVIII-specific stimulus, and FVIII-specific stimulation of ANS8 CAR Tregs led to the induction of the Treg activation markers surface LAP and glycoprotein A repetitions predominant (GARP). In addition, Foxp3 expression was increased in FVIII-stimulated ANS8 CAR-expressing Tregs. Thus, as already shown for Tregs transduced with a FVIII-specific TCR,[6] the regulatory phenotype of CAR-transduced Tregs seems to be stabilized by the antigen-specific stimulation, which is important for in vitro generation of Treg numbers sufficient for therapeutic approaches.

ANS8 CAR Tregs recognizing an epitope in the FVIII A2 domain suppressed C2-specific T-effector cell proliferation in the presence of FVIII as well as proliferation of T-effector cells specific for the MBP in the presence of MBP and FVIII. In addition, ANS8 CAR Tregs prevented antibody formation of restimulated FVIII-specific splenocytes against the whole FVIII molecule in vitro. This demonstrates bystander suppression and suggests that one could use a single CAR Treg population to completely suppress FVIII inhibitor formation. Bystander suppression has already been reported for Tregs.[18] Thus, ANS8 CAR Tregs share this suppressive function with regular Tregs expressing antigen-specific TCRs. As stated before, this bystander suppression is essential for tolerance induction against whole proteins. Conversely, bystander suppression might be one reason for global immunosuppressive side effects of Treg therapies. Therefore, reducing the number of transfused Tregs by the use of more efficacious antigen-specific cell populations is of high interest for the generation of safer Treg therapeutic strategies.

A postulated scheme of ANS8 CAR Treg interactions is given in Figure 6. After activation, ANS8 CAR Tregs suppress T-effector cells in the local milieu (eg, by IL-2 consumption and possibly by secretion of inhibitory cytokines like transforming growth factor-β). Interestingly, ANS8 CAR Tregs were optimally activated when FVIII was presented on PBMCs, suggesting that either signals provided by the loaded PBMCs (acting as antigen-presenting cells) or immobilization of FVIII are important to effectively deliver CAR-mediated proliferation signals, as opposed to minimal activation by FVIII in solution. This is an important feature because it suggests that CAR Tregs function best in the local milieu where an immune response is occurring, rather than in the bloodstream where their effect would be diluted.

A recent study has shown that the anti-FVIII antibody response could be effectively mitigated with in vitro expanded, autologous polyclonal mouse Tregs.[19] Our in vivo data clearly show that both the FVIII-specific CAR and TCR-engineered Tregs were suppressive compared with the nonspecific Ob2F3 TCR Tregs, in terms of suppression of anti-FVIII antibody development. Direct dose response comparisons between polyclonal Tregs and engineered FVIII-specific Tregs have not been done in vivo, although the latter should be more effective based on in vitro data. Adoptively transferred human Tregs were not detectable in any group of the mice as analyzed by FACS at day 14 (data not shown), because they were probably rejected by the immunocompetent animals. However, their suppressive effect lasted for at least 8 weeks, suggesting that the tolerance to FVIII was induced rather early after adoptive transfer of FVIII-specific CAR or TCR-engineered human Tregs. The in vivo suppression by engineered TCR Tregs was specific, because no significant difference was seen in the level of anti-TNP antibody after the challenge with unrelated antigen at day 56. In addition, the transferred Tregs might activate endogenous

host Tregs as well. Interestingly, the in vivo mechanism of action of 17195 TCR and ANS8 CAR Tregs is likely to be different because the ANS8 CAR Tregs need to "see" FVIII B-cell epitopes for continued activation or function, whereas the 17195 TCR Tregs may recognize persisting MHC peptide complexes. This might explain the loss in tolerance after FVIII rechallenge in ANS8 CAR Treg mice while 17195 TCR Treg mice still had some protection in this experimental setup.

A recent study reported direct killing of antigen-specific B-cell hybridoma cells in vitro and in vivo by using a modified form of CAR CD8 T cells.[20] However, we have not been able to demonstrate direct cytotoxicity of ANS8 CAR Tregs. Although further studies of the mechanism of suppression by these engineered FVIII-specific Tregs need to be done, our results support the hypothesis that CAR-transduced Tregs might be used to efficiently control FVIII inhibitor formation and may serve as a platform to regulate other adverse immune responses.

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Authorship

Contribution: J.Y., A.S., A.-H.Z., and Y.C.K. performed the experiments and analyzed the data; Y.C.K. and D.W.S. designed the experiments; C.K. and D.W.S. conceived the project; J.Y., A.S., and D.W.S. wrote the paper; and all authors read and approved the final manuscript.

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FVIII-specific human chimeric antigen receptor T-regulatory cells suppress T- and B-cell responses to FVIII

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