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JCI Insight. 2020. https://doi.org/10.1172/jci.insight.136185.

Regulatory T cells (Tregs) play essential roles in maintaining immunological self-tolerance and preventing autoimmunity. The adoptive transfer of antigen-specific Tregs has been expected to be a potent therapeutic method for autoimmune diseases, severe allergy, and rejection in organ transplantation. However, effective Treg therapy has not yet been established because of the difficulty in preparing a limited number of antigen-specific Tregs. Chimeric antigen receptor (CAR) T cells have been shown to be a powerful therapeutic method for treating B cell lymphomas, but application of CAR to Treg-mediated therapy has not yet been established. Here, we generated CD19-targeted CAR (CD19-CAR) Tregs from human peripheral blood mononuclear cells (hPBMCs) and optimized the fraction of the Treg source as CD4^+^CD25^+^CD127^low^CD45RA^+^CD45RO^-^CD19-CAR Tregs could be expanded in vitro while maintaining Treg properties, including a high expression of the latent form of TGF-β. CD19-CAR Tregs suppressed IgG antibody production from primary B cell differentiation in vitro via a TGF-β-dependent mechanism. Unlike conventional CD19-CAR CD8^+^ T cells, CD19-CAR Tregs suppressed antibody production in immunodeficient mice that were reconstituted with hPBMCs with reducing the risk of graft-versus-host disease. Therefore, the adoptive transfer of CD19-CAR Tregs may provide a novel therapeutic method for treating autoantibody-mediated autoimmune diseases.

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CD19-targeted CAR regulatory T cells suppress B cell activities without GvHD

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Running title: CAR-Treg therapy for B cell-mediated autoimmunity
Abstract

Regulatory T cells (Tregs) play essential roles in maintaining immunological self-tolerance and preventing autoimmunity. The adoptive transfer of antigen-specific Tregs has been expected to be a potent therapeutic method for autoimmune diseases, severe allergy, and rejection in organ transplantation. However, effective Treg therapy has not yet been established because of the difficulty in preparing a limited number of antigen-specific Tregs. Chimeric antigen receptor (CAR) T cells have been shown to be a powerful therapeutic method for treating B cell lymphomas, but application of CAR to Treg-mediated therapy has not yet been established. Here, we generated CD19-targeted CAR (CD19-CAR) Tregs from human peripheral blood mononuclear cells (hPBMCs) and optimized the fraction of the Treg source as CD4⁺CD25⁺CD127⁻CD45RA⁺CD45RO⁻. CD19-CAR Tregs could be expanded in vitro while maintaining Treg properties, including a high expression of the latent form of TGF-β. CD19-CAR Tregs suppressed IgG antibody production from primary B cell differentiation in vitro via a TGF-β-dependent mechanism. Unlike conventional CD19-CAR CD8⁺T cells, CD19-CAR Tregs suppressed antibody production in immunodeficient mice that were reconstituted with hPBMCs with reducing the risk of graft-versus-host disease. Therefore, the adoptive transfer of CD19-CAR Tregs may provide a novel therapeutic method for treating autoantibody-mediated autoimmune diseases.
Introduction

Regulatory T cells (Tregs) suppress excess immunity against a variety of antigens, including self-antigens, commensal bacteria-derived antigens, and environmental allergens. Thus, Tregs have been shown to play important roles in suppressing the development of autoimmunity, colitis, and allergies (1). In humans, some clinical trials of ex vivo–expanded polyclonal Tregs adoptive transfer have been performed to suppress graft-versus-host disease (GvHD) after allogeneic hematopoietic stem cell transplantation (2-5) and type 1 diabetes (T1D) (6-8). These phase I studies revealed that the adoptive transfer of polyclonal Tregs is well tolerated and showed promising effects. Although the results of polyclonal Treg adoptive therapy are encouraging, a large number of cells are necessary and the risk of nonspecific immunosuppression should be considered.

The adoptive transfer of antigen-specific Tregs was shown to be a more promising approach because fewer cells are needed to suppress targeted cells, which reduces the risk of nonspecific immunosuppression (9, 10). There are several strategies to generate antigen-specific Tregs, which overexpress a transgenic T cell receptor (TCR) or expand when stimulated with antigen. These antigen-specific Tregs indicate superior suppression of non-specific or polyclonal Tregs for several diseases, such as GvHD (11), hemophilia A (12), and experimental autoimmune encephalomyelitis (EAE) (13). In mice, in vitro generation of induced antigen-specific Tregs (iTregs) in the presence of vitamin C, which stabilizes Foxp3 expression, has been used to suppress GvHD (14), but the methods for generating stabilized human iTregs have not yet been established (15).

Another way to create antigen-specific T cells is chimeric antigen receptor (CAR) expression, which consists of an extracellular single chain antibody (scFv) fused to intracellular signaling domains in primary T cells. These CAR T cells have been successfully used in cancer treatment and are approved to treat patients with certain
types of B cell lymphoma (16, 17). Many clinical trials are ongoing in multiple cancers using the adoptive transfer of CAR T cells that recognize multiple tumor antigens (18). CAR is a way to facilitate Tregs interacting with target cells. CAR Tregs may be safer than conventional CAR-T cells because of the low inflammatory effects and cytotoxicity of Tregs. Some studies have shown that the adoptive transfer of CAR-Tregs suppresses autoimmunity against GvHD (19), hemophilia A (20), EAE (21), and colitis (22) at the preclinical level.

Autoantibodies secreted from B cells are thought to induce various autoimmune diseases (23), such as systemic lupus erythematosus (SLE) (24), Sjögren’s syndrome (25), and IgG4-related diseases (26). B cells targeting therapy such as anti-BAFF neutralizing antibodies “belimumab” or anti-CD20 depleting antibodies “rituximab” are used to treat severe autoantibody-mediated autoimmune diseases (27). Although one report used conventional CD19-targeted CAR (CD19-CAR) CD8+ T cells for the treatment of SLE in murine SLE models (28), there are no reports on the application of CD19-targeted CAR Tregs for autoimmun B cell control. In this study, we demonstrated that CD19-CAR Tregs suppress the antibody production of B cells without harmful side effects such as GvHD. The adoptive transfer of CD19-CAR Tregs might provide a novel therapeutic method to treat autoantibody-mediated autoimmune diseases.

Results

Experimental design to create CD19-targeted CAR Tregs from human peripheral blood T cells

We mainly used the CD19-targeted second generation CAR (CD19-CAR), which consists of the FMC63 extracellular single-chain variable fragment (scFv) domain that is fused to the CD28 hinge, transmembrane and cytoplasmic domains, and the CD3ζ cytoplasmic domain (1928z); the Venus gene is expressed via internal ribosome entry
site-2 (IRES2) to detect gene transduction (Figure 1A) (29). Human Tregs

(CD4^-CD25^-CD127^low) are divided into three main subsets based on Foxp3, CD25, and
CD45RA expression levels, as follows: CD45RA^+Foxp3^low/CD25^low naïve/resting Tregs,
CD45RA^+Foxp3^hi/CD25^hi effector Tregs, and CD45RA^+Foxp3^low/CD25^low, mainly
contain non-Tregs (30, 31). The naïve/resting Treg fraction was first enriched from
healthy donor peripheral blood mononuclear cells (PBMCs) by magnetic negative
selection and then purified by fluorescence activated cell sorting (FACS) based on
marker expression, with CD4^-CD25^-CD127^lowCD45RA^+CD45RO^- cells that are
considered to be naïve/resting Tregs (CD45RA^+Tregs) (Figure 1B). In addition, we also
sorted CD4^-CD25^-CD127^lowCD45RA^-CD45RO^+ T cells, which were considered to be
effector/non-Treg cells (CD45RO^-Treg cells), and
CD4^-CD25^-CD127^-CD45RA^-CD45RO^- T cells, which represented conventional T cells
(Tconvs) (Figure 1B). CD19-CAR retroviruses were transduced into these three
fractions, and then Venus^+ cells were sorted and expanded for 8 days by co-culturing
with human CD19-expressing K562 (hCD19-K562) cells in the presence of IL-2
(Figure 1C), while empty virus-infected (polyclonal) Tregs were expanded by the
stimulation with anti-CD3/CD28 beads with IL-2. The CD19-targeted
CAR-transduction rate in CD45RA^+Tregs (CD19-CAR CD45RA^+Tregs) and Tconvs
(CD19-CAR Tconvs) was approximately 50% and 20%, respectively (Figure 1D). The
CD19-CAR-transduction rate in CD45RO^+Tregs (CD19-CAR CD45RO^+Tregs) was
around 50% (data not shown). The number of CD19-CAR CD45RA^+Tregs increased
approximately 50- to 80-fold 8 days after starting expansion, which was comparable to
that of polyclonal Tregs (Figure 1E), but slightly lower compared with CD19-CAR
Tconvs.

Optimization of CD19-targeted CAR Treg induction

Both CD45RA^- and CD45RO^+ Tregs expressed high levels of Foxp3 and Helios
immediately after isolation (Figure 2A). However, after CAR-transduction and
expansion, CD19-CAR CD45RA⁺Tregs and polyclonal Tregs retained high levels of
Foxp3, Helios, and CTLA4, while CD19-CAR CD45RO⁺Tregs lost most of this
expression (Figure 2B and Supplementary Figure 1A). CD19-CAR CD45RA⁺Tregs
produced high levels of the anti-inflammatory cytokine IL-10, which is comparable to
CD19-CAR CD45RO⁺Tregs, while they expressed much less IFN-γ and IL-2 compared
with CD19-CAR CD45RO⁺Tregs and CD19-CAR Tconvs (Figure 2C and
Supplementary Figure 1B-D; Tconvs data are shown in Supplementary Figure
2A-C). Transforming growth factor-β (TGF-β) has been shown to be involved in Treg
suppression functions (32), which are usually associated with latency-associated peptide
(LAP), and is also known as latent TGF-β. It is activated by glycoprotein-A repetitions
predominant protein (GARP) (33) or other membrane proteins such as integrins or
proteases (32). Surface LAP and GARP were significantly increased in CD19-CAR
CD45RA⁺ Treg cells after co-culture with hCD19-K562 cells but not with parental
K562 cells (Figure 2D), and the induction of LAP⁺GARP⁺ cells was much higher
compared with CD19-CAR CD45RO⁺Tregs or CD19-CAR Tconvs (Figure 2D, Tconvs
data are shown in Supplementary Figure 2D). Next, we examined the cytotoxicity of
CD19-CAR Tregs against CD19⁺ or CD19⁻ K562 cells. To distinguish the cytotoxic
effects and suppression of proliferation, we used irradiated K562 cells because K562
cells doubled within 24 h. CD19-CAR Tconvs killed CD19-expressing K562 cells in an
antigen-specific manner, while CD19-CAR Tregs did not (Figure 2E and
Supplementary Figure 3A). These results were supported by the finding that
CD19-CAR Tregs expressed lower levels of granzyme B and perforin compared with
CD19-CAR Tconvs and CD19-CAR CD8⁺T cells (Supplementary Figure 3B). Thus,
these data suggest that, unlike conventional CAR T cells, CAR Tregs have little
cytotoxic activity.

On the basis of these data, we conclude that CD45RA⁺Tregs are suitable for
CD19-targeted CAR Treg generation.

To optimize the co-stimulatory domain of CD19-CAR, we compared CD28-based CD19-targeted CAR (1928z) and 4-1BB-based CD19-targeted CAR (19BBz). The Foxp3, Helios, and CTLA4 expression levels in 19BBz Tregs were slightly lower than those in 1928z Tregs (Figure 3A). 1928z Tregs proliferated in response to target cells faster than 19BBz Tregs did (Figure 3B). Furthermore, IL-10 expression in 1928z Tregs was higher than that in 19BBz Tregs (Figure 3C and Supplementary Figure 4). In addition, 1928z Tregs expressed higher levels of LAP/GARP than 19BBz Tregs (Figure 3D). Consistently, TGF-β1 was secreted in the supernatants from CD19-CAR Tregs in response to target CD19-K562 cells, and TGF-β1 levels were higher in 1928z Tregs compared with 19BBz Tregs (Figure 3E). Therefore, we considered that CD28-based CD19-CAR is better than 4-1BB-based CD19-CAR for CAR Treg generation; hereafter, we refer to CD28-based CD19-targeted CAR CD45+Treg as CD19-CAR Treg.

**CD19-targeted CAR Tregs suppress antibody production from primary B cells in vitro**

Next, we studied the effect of CD19-CAR Tregs on the proliferation and antibody production of primary human B cells in vitro. Primary B cells were isolated by magnetic-activated cell sorting (MACS) and stimulated with anti-IgM and anti-CD40 antibodies in the presence of IL-21, which induced extensive proliferation, class-switch recombination, and plasma cell differentiation (34). CD19-CAR Tregs suppressed the proliferation of B cells more efficiently than empty-vector transduced Tregs (polyclonal Tregs) (Figure 4A). In contrast, CD19-CAR Tregs suppressed the proliferation of conventional T cells as efficiently as polyclonal Tregs, suggesting that CD19-CAR Tregs retain the usual suppression activity as Tregs (Supplementary Figure 5A). CD19-CAR Tregs also strongly suppressed IgG production from primary B cell differentiation (Figure 4B and Supplementary Figure 5B). TGF-β was reported to induce IgA class-switching and reduce IgG production (35, 36). Because CD19-CAR
Tregs expressed high levels of TGF-β (see Figure 2D and Figure 3E), there is a possibility that IgA class-switching is promoted by CD19-CAR Tregs. However, CD19-CAR Tregs suppressed both IgG and IgA production (Figure 4C).

It has been reported that CAR expression may produce tonic signals in the absence of antigen binding (37). To examine whether CAR expression itself affects CAR Treg functions, we generated HER2-targeted CAR Tregs (HER2-CAR Tregs) (38) and compared them with CD19-CAR Tregs. Similar to polyclonal Tregs, HER2-CAR Tregs suppressed neither the proliferation of B cells nor antibody production (Supplementary Figure 5C).

**CD19-CAR Tregs suppress B cells through a TGF-β-dependent mechanism**

Next, to investigate the mechanism of how CD19-CAR Tregs suppressed B cells, we tested various antibodies against known effectors of Tregs including TGF-β, IL-10, CTLA4, PDL-1 and FasL (Figure 5A, left). Among them, anti-TGF-β-neutralizing antibody partly, but significantly reverted the suppressive effect of CD19-CAR Tregs on IgG production in B cells. This effect was confirmed using CA19-CAR Tregs from a different donor (Figure 5A, right). Furthermore, a TGF-β type 1 receptor kinase inhibitor, Repsox, almost completely reverted the suppressive effect of CD19-CAR Tregs at 1 μM (Figure 5B and C).

Since full TGF-β production required stimulation of CD19-CAR Tregs by target cells (Figure 2D and Figure 3E), interaction between CD19-CAR Tregs and B cells may be necessary. As expected, Transwell culture preventing cell–cell contact cancelled the suppression of B cell proliferation by CD19-CAR Tregs (Supplementary Figure 5D).

Since some reports have shown that TGF-β suppresses IgG production in B cells in vitro (39), these data strongly suggest that CD19-CAR-mediated TGF-β signals suppress the proliferation and IgG production in B cells without promoting IgA
CD19-targeted CAR Tregs suppress human B cells in vivo and suppress GvHD effects

Lastly, we investigated the suppressive function of CD19-CAR Tregs on B cells in vivo. Primary human PBMCs were adoptively transferred into immunodeficient NOD.Cg-Prkdc±cidll2rgtm1Wjl/Szj (NSG) mice, which resulted in xenogeneic GvHD and human Ig production (40). When CD19-CAR Tregs were transferred into NSG mice 7 days after human PBMC transfer, both human IgG and IgM levels were reduced on day 14, 21, and 28 (Figure 6A). Polyclonal Tregs (empty-vector transduced Tregs) did not significantly reduce IgG and IgM levels on day 21 and 28 (Figure 6A). CD19-CAR Tregs, but not polyclonal Tregs, significantly suppressed CD20+ B cell expansion in peripheral blood from day 14 to day 28 (Figure 6B and Supplementary Figure 6A). Both transferred CD19-CAR Tregs and polyclonal Tregs were detected on day 14, and maintained similar levels 28 days after transfer (Figure 6C and Supplementary Figure 6B); the number of CD19-CAR Tregs was higher than the number of polyclonal Tregs at all time points. CD19-CAR Tregs also suppressed the expansion of CD20+CD138− plasma cells (Supplementary Figure 6C), indicating that CD19-CAR-Treg could suppress not only Ig production but also B cells differentiation to plasma cells. Similar data were obtained when CD19-CAR Tregs were simultaneously transferred with PBMCs (Supplementary Figure 7). These data indicate that CD19-CAR Tregs specifically suppress Ig production from B cells in vivo.

The transfer of human PBMCs resulted in severe GvHD, leading to weight loss and death whereas the transfer of CD19-CAR Tregs alone did not result in any GvHD symptoms (Supplementary Figure 8A). The co-transfer of CD19-CAR Tregs suppressed GvHD resulting from PBMCs on day 28 (Figure 6D). This GvHD-suppressing effect of CD19-CAR Tregs was greater than that of polyclonal Tregs, probably because CD19-CAR Tregs, but not polyclonal Tregs, expanded in response to
B cells. Since the transfer of human PBMCs in the absence of B cells also resulted in severe GvHD without detectable human IgG antibody (Supplementary Figure 8B and C), B cells were not directly involved in GvHD effects. This suggests that the GVHD-suppressing effect of the CD19-CAR Tregs may be indirect; the exposure to CD19+ B cells in vivo activates CD19-CAR Tregs, which then suppress GVHD through their "usual" Treg TCRs.

A recent study showed that conventional CD19-CAR CD8+ T cells suppressed B-cell-mediated autoimmune diseases by killing B cells (28). However, CD19-CAR CD8+ T cells may cause cytokine release syndrome (41, 42) (characterized by fever, hypotension, and respiratory insufficiency), which is associated with elevated serum cytokines, including interleukin-6 (IL-6). Thus, we compared the effect of CD19-CAR Tregs and CD19-CAR CD8+ T cells on GvHD effects in NSG mice reconstituted with human PBMCs. CD19-CAR Tregs or CD19-CAR CD8+ T cells were transferred 7 days after PBMC injection. Although CD19-CAR Tregs did not affect body weight by day 50 after transfer or reduce the GvHD score, CD19-CAR CD8+ T cells promoted weight loss and did not reduce the GvHD score (Figure 6E). Moreover, CD19-CAR CD8+ T cells, but not CD19-CAR Tregs, increased mouse IL-6 levels and decreased human Tregs 6 days after CD19-CAR CD8+ T cells or CD19-CAR Tregs were simultaneously transferred with PBMCs (Supplementary Figure 9). These data indicate that the transfer of CD19-CAR Tregs may be safer than that of CD19-CAR CD8+ T cells in autoimmune conditions.

Discussion

In this study, we optimized the conditions for generating stable CD19-targeted CAR Tregs, which are suitable for the adoptive cell therapy of non-tumor, chronic autoimmune diseases. We found that CD19-targeted CAR Tregs efficiently suppressed primary human B cells compared to polyclonal Tregs both in vitro and in vivo. Unlike
conventional CAR CD8⁺T cells, CAR Tregs may be safer for chronic diseases because
CAR Tregs suppressed rather than resulted in GvHD.

Our data is consistent with that of a previous report by Boroughs et al. (43). They
also used CD19-CAR for modifying co-stimulatory domains. They concluded that
CD19-CAR carrying CD28 signaling domains is superior to 4-1BB-based CD19-CAR,
which is consistent with our results. However, there is an important difference, namely,
you showed that CD19-targeted CAR Tregs have mild cytotoxicity, while our
CD19-CAR Tregs did not. This might be caused by the difference in Treg fraction
preparation strategy from PBMCs. They used the CD4⁺CD25hiCD127low fraction as a
source of Tregs whereas we further purified CD45RA⁺ cells from this fraction (Figure
1A). The CD4⁺CD25hiCD127low fraction contains both naïve/resting (CD45RA⁺) Tregs
and CD45RO⁺ effector/non Tregs (30, 31). We showed that Foxp3 expression was
unstable in CD45RO⁺Tregs, and CD45RO⁺Tregs produce a greater amount of effector
cytokines and cytoytic mediators than CD45RA⁺Tregs (Figure 2). Although the
fraction of CD4⁺CD25hiCD127lowCD45RA⁺ cells is small, we found that this fraction
can be expanded vigorously without losing Foxp3 expression and suppression functions,
and the retroviral gene transduction efficiency in this Treg fraction was higher than
Tconv cells, although the exact reason is not clear. Thus, we believe that the source of
Tregs is important in order to generate stable and non-cytolytic Tregs for Treg therapy.

It has been demonstrated that “exTregs” can be pathogenic and that the stability of
Tregs is a bottleneck for Treg cell therapy (15). In this study, we showed that
CD19-CAR CD45RA⁺Tregs did not lose Foxp3 expression after long-term expansion in
vitro (Figure 2B), retained Foxp3 expression and persisted for 1 month in NSG mice,
and did not exhibit any harmful GvHD effects (Figure 6 and Supplementary Figure 7).
Since CD19-CAR CD8⁻T cells persist in the human body in CAR-T therapy, we expect
that CD19-CAR Tregs also persist for a long time and maintain Treg functions.

Another proposed CD19-CAR-based cell therapy for autoimmune diseases uses
conventional CD19-CAR CD8+ T cells that kill B cells (28). Apparently, CD19-CAR
CD8+ T cells have the potential to induce GvHD or cytokine release syndrome. In
contrast, our CD19-CAR CD45RA+Tregs suppress B cell proliferation and IgG
production but do not kill B cells and have little risk of harmful effects. Anti-CD20
antibody therapy is another way to control B cells in autoimmune diseases (27).
Anti-CD20 antibodies also deplete B cells and cause hypogammaglobulinemia, thereby
increasing the risk of infection. CD19-CAR Tregs may also have similar adverse
effects; however, we expect that the recovery of immunoglobulin after CD19-CAR Treg
therapy is faster than anti-CD20 depleting antibodies, because CD19-CAR Tregs might
just suppress B cells rather than deplete them. To improve the safety of CAR Treg
therapy, engineered CARs that are equipped with a suicide gene or work only in the
presence of drugs may be made available (18).

In this study, we propose that TGF-β is a major mechanism for B cell suppression by
CD19-CAR Tregs. Although TGF-β is a secreted cytokine, we showed that Treg-B cell
direct contact is necessary for the suppression of B cells. This is probably because LAP
expression in CD19-CAR Tregs requires the activation of Tregs by target cells. In
addition, molecules expressed on B cells such as integrins and proteases may be
involved in the processing of LAP to release active TGF-β (32). This TGF-β activation
process may require cell-cell contact, and could be the reason why the anti-TGF-β
antibody did not revert the effect of CAR Tregs completely whereas the TGF-β signal
inhibitor completely reverted it. In addition, TGF-β-independent mechanisms may also
play role especially in vivo; the exact interaction between B cells and CD19-CAR Tregs
in in vivo should be clarified in the future study.

Although our data strongly support that TGF-β from CAR Tregs plays a major role in
the suppression of B cells, it has not yet been clarified how TGF-β signals suppress the
proliferation and IgG production of B cells. Although it is well known that TGF-β
signals induce IgA class-switching (35, 36), we have not observed IgA induction by
co-culture with CD19-CAR Tregs. Exogenous TGF-β has been shown to suppress IgG1 or IgG2a production from murine B cells induced by IL-4 and IFN-γ, respectively (44). Exogenous TGF-β has been shown to decrease B lymphocyte Ig secretion by inhibiting the synthesis of Ig mRNA and inhibiting the switch from the membrane form to the secreted forms of Ig mRNAs (45). Further study is necessary to clarify the precise effects of CD19-CAR Tregs on B cells.

CAR-T cells could be therapeutic not only for cancer but also for various diseases in which pathogenic cells are involved. A recent paper showed that the adoptive transfer of cardiac fibroblast-specific CD8+ T cells results in a significant reduction in cardiac fibrosis and restoration of function after injury in mice (46). CAR Tregs could be an alternative way to suppress, but not eliminate, pathogenic cells without harmful side effects. The usefulness of CAR-Tregs should be confirmed by using immunocompetent murine models of autoimmunity. However, it is always important to use human T cells to confirm the therapeutic value of CAR Tregs because methods for isolation and expansion of pure human Tregs have not been completely established (14).

Methods

Human subjects
Human PBMCs (hPBMCs) were obtained from five healthy human subjects by density gradient centrifugation.

Cell culture
K562 cell line (ATCC, Manassas, VA) was cultured in RPMI-1640 (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA, USA), 10 mM HEPES (Invitrogen, Carlsbad, CA, USA), 2 mM L-glutamine (Invitrogen), 1× NEAA (Invitrogen), 1× Penicillin-Streptomycin Mixed Solution (Nacalai Tesque), 0.55 mM β-mercaptoethanol (Thermo Fisher
Scientific), and 1 mM sodium pyruvate (Invitrogen). HEK293T cell line (ATCC) was cultured in DMEM (Nacalai Tesque) supplemented with 10% FBS and 1× Penicillin-Streptomycin Mixed Solution. Primary human T cells were cultured in RPMI-1640 supplemented with 10% normal human AB serum (Innovative Research Inc, Novi, MI, USA), 10 mM HEPES, 2 mM L-glutamine, 1× NEAA, 1× Penicillin-Streptomycin Mixed Solution, 0.55 mM β-mercaptoethanol, and 1 mM sodium pyruvate.

**Mice**

Male/female NOD.Cg-PrkDC cidIl2rgtm1Wjl/Szj (NSG) mice were purchased from Charles River Laboratories Japan Inc. (Kanagawa, Japan). All mice were kept in specific pathogen–free facilities at Keio University.

**Plasmid construction and lentiviral transduction**

Human CD19 gene and human HER2 gene were polymerase chain reaction (PCR)-amplified from the human cDNA library, and cloned into lentiviral vectors CSII-EF1a-MCS-IRES2-Venus. A method for generating CAR genes was previously described (29). Briefly, CD19-targeted CAR genes that consist of signal peptide of human GM-CSFR, anti-human CD19 scFv (FMC63), the extracellular domain, transmembrane region, and cytoplasmic region of human CD28 or human 4-1BB, and the cytoplasmic part of the human CD3ζ molecule, were synthesized and cloned into retroviral vectors pMEI5 containing MCS-IRES2-Venus (pMEI5-MCS-IRES2-Venus). For CD28-based HER2-targeted CAR genes, anti-human HER2 scFv (4D5-7) (38) was used instead of anti-human CD19 scFv (FMC63). The sequences of CARs are described in Supplementary Table.

The virus was prepared as previously described (47). Briefly, the cDNA expression vector was transduced into HEK293T cells along with the VSV-G expression vector and
packaging vector. Eighteen hours after transduction, the vector-containing culture medium was changed into fresh culture medium; 48 h later, the virus-containing medium was collected, passed through a 0.45 μm filter, and concentrated using centrifugation (8,000 g at 4°C for 16 h). The virus pellets were resuspended in culture medium. K562 cells were transduced to express human CD19 (hCD19-K562) or human HER2 (HER2-K562) with lentivirus.

Flow cytometry

Fluorochrome-conjugated monoclonal/polyclonal anti-human CD4 (45-0049-42, RPA-T4), anti-human CD8 (300924 or 12-0087-41, HIT8α or SK1), anti-human CD25 (302606, BC96), anti-human CD127 (351316, A019D5), anti-human CD45RA (304126, HI100), anti-human CD45RO (304228, UCHL1), anti-human CD20 (302310 or 13-0209-82, 2H7), anti-human CD45 (45-0459-42, HI30), anti-human CD19 (13-0199-82, HIB19), anti-human CD38 (12-0388-42, HB7), anti-human CD138 (356516, MI15), anti-human IgD (348210, IA6-2), anti-human LAP (349706, TW4-6H10), anti-human GARP (352504, 7B11), anti-human IFN-γ (506507, B27), anti-human IL-2 (25-7029-42, MQ1-17H12) anti-human IL-10 (506807, JES3-19F1), propidium iodide (421301; PI), anti-human CTLA4 (17-1529-42, 14D3), anti-GFP (A-21311, polyclonal), anti-human Foxp3 (17-4777-42, 561182, 236A/E7), anti-human Helios (12-9883-42, 22F6), and fixable viability Dye eFluor™ 780 (65-0865-14; FVD) were purchased from eBioscience (San Diego, CA, USA), BioLegend (San Diego, CA, USA), BD Biosciences (Franklin Lakes, NJ, USA) or Thermo Fisher Scientific. Intranuclear staining was performed using the fixation/permeabilization buffer solution (eBioscience) or IC fixation buffer (eBioscience), according to the manufacturer’s instructions. Stained cells were analyzed on a FACSCantoII (BD Biosciences) and data were analyzed with Diva software (BD Biosciences) and FlowJo software (Tree Star,
Ashland, OR, USA). Flow-count fluorospheres (Beckman Coulter, Brea, CA, USA) were used to determine the absolute number of cells, according to the manufacturer’s protocol.

**Isolation, transduction, and expansion of primary human T cells**

CD4 T cells and CD8 T cells were separated from hPBMCs using a CD4⁺ T Cell Isolation Kit, human (Miltenyi Biotec, Bergisch Gladbach, Germany) and a CD8⁺ T Cell Isolation Kit, human (Miltenyi Biotec). Naïve/resting Tregs (CD4⁺CD25⁺CD127⁺CD45RA⁺CD45RO⁻) and conventional CD4 T cells (Tconvs; CD4⁺CD25⁻CD127⁻CD45RA⁺CD45RO⁺) were isolated from MACS-purified CD4 T cells by FACS using an Aria II/III cell sorter (BD Biosciences). Naïve/resting Tregs were stimulated with anti-CD3/CD28 dynabeads (Life Technologies, Carlsbad, CA, USA) in the presence of 100 ng/mL human IL-2 (Peprotech, Rocky Hill, NJ, USA). On day 2, CD19-targeted CAR gene or HER2-targeted CAR gene (described above) was transduced with retrovirus by centrifugation at 35°C for 2 h in the presence of 5 μg/mL polybran (Nacalai Tesuque). On day 5 or 6, Venus⁺ cells were isolated by a FACS Aria II/III cell sorter and expanded by co-culture with irradiated (40 Gy) antigen-expressing K562 cells (CD19-K562 cells for CD19-targeted CAR and HER2-K562 cells for HER2-targeted CAR) in the presence of 100 ng/mL human IL-2. Other irradiated antigen-expressing K562 cells were added 3 or 4 days after the first co-culture. Polyclonal Tregs that were transduced with an empty gene or not transduced were cultured with anti-CD3/CD28 dynabeads in the presence of 100 ng/mL human IL-2. Tconvs and CD8 T cells were cultured in the presence of 10 and 30 ng/mL human IL-2, respectively, and gene transduction was carried out on day 1. All T cells were cultured at 4 to 10 x 10⁴ cells/well in 96-well round plates after gene transduction. For purification after expansion, CAR-T cells were stained with biotin conjugated anti-human CD235a antibody (13-9987-82, HIR2, eBioscience), followed by incubation with anti-biotin.
microbeads (Miltenyi Biotec). CAR-T cells were then negatively selected by MACS.

**Cytotoxicity assays and cytokine production**

T cells were co-cultured with irradiated (40 Gy) CD19-K562 cells (labeled with CellTrace Violet (Thermo Fisher Scientific) in PBS at 37°C for 8 min) at a ratio of 4:1 or 1:1 (T cells:Targeted cells) for 16 to 20 h. The culture supernatants were then collected and the percentage of dead cells in the targeted cells (PI+ in CellTrace Violet+) or surface TGF-β expression (LAP+GARP+) in CD4 T cells was estimated using flow cytometry analysis. Cytokine concentrations (TGF-β1, IFN-γ, and IL-2) in the culture supernatants were measured using enzyme-linked immunosorbent assay (ELISA) (eBioscience). For intracellular cytokine staining, CAR-T cells were stimulated with irradiated (40 Gy) CD19-K562 cells and K562 cells at a 1:1 ratio, or with PMA (50 ng/mL, Nacalai Tesque)/ionomycin (1 μg/mL, Nacalai Tesque) for 4 to 5 h in the presence of Brefeldin A (1:1000 dilution, eBioscience). Then, intracellular IFN-γ, IL-2, and IL-10 were stained and analyzed by flow cytometry, as previously described (48).

**B cell suppression assay and antibody production assay in vitro**

Primary human B cells were isolated from hPBMC using a B Cell Isolation Kit II, human (Miltenyi Biotec) and stimulated with anti-IgM (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) and anti-CD40 antibodies (R&D Systems, Minneapolis, Minnesota, USA) in the presence of 100 ng/mL IL-21 (peprotech) for 3 days (suppression assay) and 7 days (antibody production assay) (2–5 × 10^4 cells/well in 96-well round plates) (34). In some experiments, various neutralizing antibodies (anti-TGF-β antibody (16-9243-85, 1D11.16.8, eBioscience), anti-IL-10 antibody (16-7108-85, JES3-9D7, eBioscience), anti-CTLA4 antibody (16-1529-82, 14D3, eBioscience), aPD-L1 antibody (16-5983-82, MIH1, eBioscience), and anti-FasL antibody (16-9919-81, NOK-1, eBioscience), 10 μg/mL) or Repsox (Sigma-Aldrich, St.
Louis, MO, USA) (1 or 10 μM) were added. In the suppression assay, B cells were labeled with CellTrace Violet in PBS at 37°C for 8 min. CD19-targeted CAR Tregs (CD19-CAR Tregs) or empty/polyclonal Tregs (polyclonal Tregs) were co-cultured with B cells in a ratio of 1:1/10, 1:3/10, or 1:1 (B cells: Tregs). In a suppression assay, proliferated B cells (CD4 CellTrace Violet low) were analyzed by flow cytometry. In an antibody production assay, differentiated B cells (FVD CD4 IgD CD38+) were estimated by flow cytometry, and the concentration of human total IgG antibody and IgA antibody in the supernatant was measured by ELISA (Bethyl Laboratories, Montgomery, TX). In some experiments, Transwell plates (0.4 μm; Corning, NY, USA) were used to investigate the contact-dependent mechanism. “Proliferation index” was calculated as the percentage of divided B cells (CellTrace Violet low fraction).

CD19-targeted CAR Tconvs suppression assay

CD19-targeted CAR Tconvs (CD19-CAR Tconvs) at day 13 were labeled with CellTrace Violet in PBS at 37°C for 8 min and stimulated with anti-CD3/CD28 dynabeads (cells : beads = 10 : 1) for 2 days. CD19-targeted CAR Tregs (CD19-CAR Tregs) or empty/polyclonal Tregs (polyclonal Tregs) were co-cultured with CD19-CAR Tconvs in a ratio of 1:1 (Tconvs: Tregs). The number of CD19-CAR Tconvs (CD4 CellTrace Violet+) was analyzed by flow cytometry.

Xenograft mouse model for antibody production in vivo

The 8- to 11-week-old male and female NSG mice were intravenously injected with 2 × 10⁶ CD19-targeted CAR Tregs or empty Tregs 4 to 6 h or 7 days after intravenous injection of 5 × 10⁶ hPBMCs. Saline-injected mice served as controls. On days 14, 21, and 28, peripheral blood and spleens were collected, and then erythrocytes were lysed. B cells (FVD hCD45 hCD20+), injected Tregs (FVD hCD45 hCD4+Venus+), and plasma cells (FVD hCD45 hCD4 hCD8 hCD20 CD138+) were measured by flow
cytometry. The serum concentrations of human total IgG antibody and IgM antibody were measured by ELISA (Bethyl Laboratories). To estimate GvHD, body weight, GvHD score, and survival were measured from day 0 to day >100. GvHD score was calculated based on body weight, posture, activity level, fur texture, and skin integrity, with 0 to 2 points per category as previously described (49). Mice were euthanized if they experienced >8 GvHD score. In some experiments, hPBMCs were stained with biotin conjugated anti-human CD19/CD20 antibody, followed by incubation with anti-biotin microbeads, and then B cells–deficient hPBMCs were negatively selected by MACS. B cells–sufficient or B cells–deficient hPBMCs (5 × 10^6) were intravenously injected into NSG mice.

**Statistical analysis**

All data are presented as the mean ± standard error of the mean (SEM). Statistical analyses were performed using the Student’s unpaired t-test (two-tailed) or one-/two-way analysis of variance (ANOVA) with Tukey’s tests, using GraphPad Prism version 7 software (GraphPad Software, San Diego, CA, USA). p < 0.05 was considered to be statistically significant, and the p values were represented as follows: **p < 0.01** and *p < 0.05.

**Study approval**

Animal experiments were performed in strict accordance with the recommendations in the Guidelines for Proper Conduct of Animal Experiments of the Science Council of Japan. All experiments using mice were approved by the Animal Ethics Committee of Keio University (Approval number: 08004) and were performed according to the Animal Ethics Committee’s guidelines. All human study was approved by the Institutional Review Board of Keio University School of Medicine (Approval number: 20120039), and written informed consent was obtained from all five subjects.
Author contributions
Y.I., M.I., T.K., and M.A. performed the experiments; Y.I. analyzed the data; A.Y.
supervised the research; and Y.I., M.A., and A.Y. wrote the manuscript.

Acknowledgements
We thank Mari Ikeda, Yoshiko Noguchi, Yasuko Hirata, and Yukiko Tokifuji (Keio
University) for their technical assistance; Mika Inoue (Keio University) and Kasane
Imura-Kishi (The University of Tokyo) for manuscript preparation; and Yuzo Koda
(Keio University) for discussions. This work was supported by JSPS KAKENHI (S)
JP17H06175, Challenging Research (P) JP18H05376, and AMED-CREST
JP19gm1110009 grants for A.Y.; and JSPS KAKENHI 17K15667, 19H04817, and
19K16618, AMED-PRIME 19gm6210012, JSPS KAKENHI 19H04817 and 19K16618
for M.I.; and by the Tomizawa Jun-ichi & Keiko Fund of the Molecular Biology
Society of Japan for Young Scientists, Research grant for young investigators by the
Mitsubishi Foundation, Mochida Memorial Foundation for Medical and Pharmaceutical
Research, Takeda Science Foundation, Uehara Memorial Foundation, Naito Memorial
Foundation, Kanae Foundation, SENSHIN Medical Research Foundation, Astellas
Foundation for Research on Metabolic Disorders, Inoue Research Award, Life Science
Research Award, and Keio Gijuku Academic Developmental Funds.

Conflict of interest statement
The authors have declared that no conflicts of interest exist.
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Figures and Figure legends
A

| Signal peptide | FMC63 scFv (anti-hCD19) | CD28 (EC-TM-CP) | CD3ζ (CP) |
|----------------|-------------------------|-----------------|-----------|
|                | IRES2 Venus             | Empty           |           |
| CD19-targeted  |                         |                 |           |
| CAR (1928z)    |                         |                 |           |

B

(Casted on CD4+)

CD4 Tcells

CD45RA+Tregs

CD25

CD127

CD26

CD45RA

CD45RO

C

TCR Stimulation → CAR gene transfer → Sorting (CAR+ cells) → CAR stimulation with hCD19-K562 cells → Assays

D

% Venus+ (CAR)-T cells

E

Proliferation (Fold change)

Venus

CD4

CD45RA+ Tregs

CD19 CAR Tconvs

CD19 CAR CD45RA+ Tregs

CD19 CAR CD45RA- Tregs

Polyclonal CD45RA+ Tregs

D0-D10

D10-D20

D20-D30

D30-D40

D40-D50

D50-D60

D60-D70

D70-D80

D80-D90

D90-D100

D100-D110

D110-D120

D120-D130

D130-D140

D140-D150

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D900-D910

D910-D920

D920-D930

D930-D940

D940-D950

D950-D960

D960-D970

D970-D980

D980-D990

D990-D1000

NS

*
**Figure 1.**

**Generation of CD19-targeted CAR-Tregs**

(A) Schematic representation of constructs carrying CD19-targeted CAR and empty vector. scFv, single-chain variable fragment; EC, extracellular domain; TM, transmembrane domain; CP, cytoplasmic domain. (B) Sorting strategy to isolate naïve/resting CD45RA⁺ regulatory T cells (CD45RA⁺ Tregs), CD45RO⁺ Tregs, and conventional CD4 T cells (Tconvs). (C) Scheme for the generation and expansion of CD19-targeted CAR (CD19-CAR) Tregs and CD19-CAR Tconvs. (D) Flow cytometric analysis of CAR (Venus) when transduced cells were sorted at day 4 to 6. The left panels show representative FACS profiles and the right panel shows quantification of the transduction rate (n = 23 or 26). Data are representative of twenty six independent experiments using human samples that were provided by five healthy donors. (E) Fold expansion at day 12 to 14, 8 days after CAR-T cells were sorted (n = 17, 14, or 12). Data are representative of seventeen independent experiments using human samples provided by five healthy donors. *p values were determined using (D) a two-tailed Student’s *t*-test or (E) a one-way analysis of variance (ANOVA) (*p < 0.05, **p < 0.01). Data are presented as the mean ± SEM.
**Figure 2.**

CD19-targeted CAR Tregs maintain the phenotype and suppressive abilities of Tregs after expansion

(A, B) Flow cytometric analysis of Foxp3, Helios, and CTLA4 at day 0 after sorting (A) and at day 13 after expansion (B). (C) Flow cytometric analysis of IL-10, IFN-γ, and IL-2 in the indicated cells 4 h after stimulation with hCD19-K562 cells, K562 cells, or PMA/ionomycin (PMA/iono) (n = 3). (D) Flow cytometric analysis of LAP and GARP in the indicated cells 1 day after co-culture with hCD19-K562 cells or K562 cells (n = 3). (E) The cytotoxicity of CD19-targeted CAR T cells 1 day after co-culture with hCD19-K562 or K562 cells. The graph shows residual live targeted cells after co-culture (n = 3). Data are collected using human samples that were provided by one healthy donor. *p values were determined using (C and D) a two-tailed Student’s *t*-test or (E) a one-way analysis of variance (ANOVA) (**p < 0.01 and NS, not significant). Data are presented as the mean ± SEM.
**A** % of positive cells

- **Foxp3**
  - 1928z Tregs: 92.6%
  - 19BBz Tregs: 84.3%
  - Isotype control:...

- **Helios**
  - 1928z Tregs: 72.5%
  - 19BBz Tregs: 66.6%
  - Isotype control:...

- **CTLA4**
  - 1928z Tregs: 96.6%
  - 19BBz Tregs: 80.3%
  - Isotype control:...

**B**

- **Proliferation (Fold change)**
  - 1928z Tregs
  - 19BBz Tregs

**C**

- **% IL-10+ cells**
  - K562
  - hCD19-K562
  - PMA/iono

**D**

- **% LAP-GARP+ cells**
  - none
  - k562
  - hCD19-K562

**E**

- **TGF-β1 (pg/mL)**
  - K562
  - hCD19-K562
Figure 3.
CD28-based CD19-CAR is better than 4-1BB-based CD19-CAR for CAR Treg generation

(A) Flow cytometric analysis of Foxp3, Helios, and CTLA4 in 1928z Tregs and 19BBz Tregs at day 13. (B) Fold expansion at day 13, 8 days after CAR Tregs were sorted (n = 3). (C) Flow cytometric analysis of IL-10 in the indicated cells 4 h after stimulation with hCD19-K562 cells, K562 cells, or PMA/ionomycin (PMA/iono) (n = 2). (D) Flow cytometric analysis of LAP and GARP in the indicated cells 1 day after co-culture with hCD19-K562 or K562 cells (n = 2) (E) The amount of TGF-β1 produced by CD19-CAR Tregs (1928z) and 19BBz Tregs 1 day after co-culture with hCD19-K562 cells or K562 cells (n = 3). Data are collected using human samples that were provided by one healthy donor. p values were determined using (B) a two-tailed Student’s t-test or (E) a one-way analysis of variance (ANOVA) (*p < 0.05 and **p < 0.01). Data are presented as the mean ± SEM.
**A** Frequency of proliferated B cells

| Condition   | Frequency |
|-------------|-----------|
| NC          | 19.3%     |
| IL-21       | 51.8%     |
| CD19-CAR Tregs (0.1) | 30.0%     |
| CD19-CAR Tregs (1)   | 26.9%     |
| Polyclonal Tregs (0.1) | 46.7%     |
| Polyclonal Tregs (1)   | 30.0%     |

**B** HD #1

![Graphs showing total IgG (mg/mL) and plasma cells (x10^3) for different conditions and dilutions of IL-21.](image)

**C** HD #2

![Graphs showing total IgA (mg/mL) for different conditions and dilutions of IL-21.](image)
Figure 4.

CD19-targeted CAR Tregs efficiently suppress B cells and antibody production

Primary human B cells were stimulated with anti-IgM and anti-CD40 antibodies in the presence of IL-21. (A) Flow cytometric analysis of CellTrace violet dilution of CellTrace violet-labeled primary human B cells 3 days after co-culture with CD19-CAR Tregs or polyclonal Tregs at ratios of 1:0.1 and 1:1 (B cells: Tregs) (n = 3). The fraction of CellTrace violet\textsuperscript{low} B cells in the absence of drug and CD19-CAR Tregs is shown as 100% in the right panel. (B) Total IgG antibody levels produced by B cells and the flow cytometric analysis of differentiated B cells (CD4\textsuperscript{+}FVD\textsuperscript{+}IgD\textsuperscript{+}CD38\textsuperscript{+}) 7 days after co-culture with CD19-CAR Tregs or polyclonal Tregs at ratios of 1:0.1, 1:0.3, and 1:1 (B cells: Tregs) (n = 3-4). (C) Total IgA antibody levels after co-culture (n = 3). (A and B) Data are representative of independent experiments using two different healthy donors. (C) Data are collected using human samples that were provided by one healthy donor. (A-C) \( p \) values were determined using a one-way analysis of variance (ANOVA) (*\( p < 0.05 \), **\( p < 0.01 \) compared with the indicated two columns; NS, not significant, \#\( p < 0.05 \) and \##\( p < 0.01 \) compared with each black circle). Data are presented as the mean ± SEM.
TGF-β from CAR-Treg cells play a major role in the suppression of B cell proliferation and IgG production

Primary human B cells were stimulated with anti-IgM and anti-CD40 antibodies in the presence of IL-21. (A) Total IgG antibody levels produced by primary human B cells 7 days after co-culture with CD19-CAR Tregs in the presence of various neutralizing antibodies (10 μg/mL) (n = 4-5). (B) Flow cytometric analysis of CellTrace violet dilution of CellTrace violet-labeled primary human B cells after co-culture with CD19-CAR Tregs in the presence of TGF-β type 1 receptor inhibitor Repsox (0.1 and 1 μM). The fraction of CellTrace violet\textsuperscript{low} B cells in the absence of drug and CD19-CAR Tregs is shown as 100% in the right panel (n = 3). (C) Total IgG antibody levels produced by primary human B cells 7 days after co-culture with CD19-CAR Tregs in the presence of Resox (1 μM) (n = 3). (A) Data are representative of independent experiments using two different healthy donors. (B and C) Data are collected using human samples that were provided by one healthy donor. (A-C) \( p \) values were determined using a one-way analysis of variance (ANOVA) (*\( p < 0.05 \), **\( p < 0.01 \) compared with the indicated two columns; NS, not significant, ##\( p < 0.01 \) compared with each black circle). Data are presented as the mean ± SEM.
A

hPBMCs
5 x 10^6 cells
CD19-CAR Tregs or Polyclonal Tregs
2 x 10^6 cells
NSG mice

Analysis

Days

% of D0 weight

GvHD score

B

CD20+ B cells
(x10^3 cells/mL)

C

Injected Tregs
(x10^3 cells/mL)

D

Human total IgG (mg/mL)

E

Human IgM (mg/mL)

GvHD score

Days

% of D0 weight

CD19-CAR Tregs
Polyclonal Tregs
hPBMCs
Figure 6.

Therapeutic adoptive therapy of CD19-targeted CAR Tregs efficiently suppresses B cells and antibody production in vivo

Severely immunodeficient (NOD.Cg-PrkDC cidIl2rgtm1Wjl/Szj, NSG) mice were intravenously (IV) injected with $5 \times 10^6$ human PBMCs. Autologous CD19-CAR Tregs and polyclonal Tregs ($2 \times 10^6$) were adoptively transferred 7 days after PBMC injection. (A) Total IgG antibody and IgM antibody levels in serum on day 14, 21, and 28 (n = 4-7). (B, C) The number of B cells (FVD’hCD45’hCD20+) (B) and injected Tregs (FVD’hCD45’hCD4’Venus+) (C) in the peripheral blood on day 14, 21, and 28 measured by flow cytometric analysis (n = 4-7). $p$ values were determined using (C) a two-tailed Student’s $t$-test or (A, B and D) a one-way analysis of variance (ANOVA) (*$p < 0.05$, **$p < 0.01$; NS, not significant, compared red or blue symbols with each black symbol, # $p < 0.05$ and ## $p < 0.01$ compared black symbols with each open symbol). Data are presented as the mean ± SEM. (D) GvHD score was measured on day 28 (n = 5).

(E) NSG mice were IV injected with $5 \times 10^6$ human PBMCs. Autologous CD19-CAR Tregs and CD19-CAR CD8 T cells ($2 \times 10^6$) were adoptively transferred 7 days after PBMCs injection. GvHD score and body weight were measured from day 0 to day 50 (n = 5). (E) $p$ values were determined using a two-way ANOVA (*$p < 0.05$, **$p < 0.01$; NS, not significant compared with black circle). Data are presented as the mean ± SEM.