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# Regulatory T cells engineered with a novel insulin-specific chimeric antigen receptor as a candidate immunotherapy for type 1 diabetes

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ABSTRACT

Adoptive immunotherapy with *ex vivo* expanded, polyspecific regulatory T cells (Tregs) is a promising treatment for graft-versus-host disease. Animal transplantation models used by us and others have demonstrated that the adoptive transfer of allospecific Tregs offers greater protection from graft rejection than that of polyclonal Tregs. This finding is in contrast to those of autoimmune models, where adoptive transfer of polyspecific Tregs had very limited effects, while antigen-specific Tregs were promising. However, antigen-specific Tregs in autoimmunity cannot be isolated in sufficient numbers.

Chimeric antigen receptors (CARs) can modify T cells and redirect their specificity toward needed antigens and are currently clinically used in leukemia patients. A major benefit of CAR technology is its "off-the-shelf" usability in a translational setting in contrast to major histocompatibility complex (MHC)-restricted T cell receptors. We used CAR technology to redirect T cell specificity toward insulin and redirect T effector cells (Teffs) to Tregs by Foxp3 transduction.

Our data demonstrate that our converted, insulin-specific CAR Tregs (cTregs) were functional stable, suppressive and long-lived *in vivo*. This is a proof of concept for both redirection of T cell specificity and conversion of Teffs to cTregs.

## 1. Introduction

Epidemiological data show a steady increase in the prevalence of autoimmune diseases in Western society over the last decades. The prevalence of type 1 diabetes (T1D) in Western Europe and North America is approximately 0.5% or approximately two million people with a steady upward trend. T1D develops in patients with genetic predisposition under the influence of various environmental factors.

Normally, negative selection prevents the reactivity of T cells to autogenic peripheral antigens. However, even this process can be permeable such that beta cell-reactive T cells can be found in the periphery. These autoreactive T cells are regulated by mechanisms of peripheral tolerance, such as ignorance, anergy, deletion and regulation, e.g., by regulatory T cells (Tregs). It is believed that an imbalance between effector T cells (Teffs) and immune regulation exists in autoimmune diseases. For T1D therapy, the missing insulin is administered by artificial insulin preparations. However, this form of therapy is not a cure and must be continuously administered. Preclinical models and clinical trials have shown that T cell-targeted therapies can influence and improve the course of the disease [1,2]. In these conditions, it has been shown that short-term immune modulation of T cells has a long-lasting effect and that these interventions above all restore the balance between effector T cells and Tregs.

The recovery of tolerance to the autoantigens is evidenced by a nonspecific deletion of individual immune cells, e.g., by the use of humanized anti-CD3 antibodies or the use of systemically effective immunosuppressants [3,4]. However, the clinical trial data show, at best, a persistence of progressive beta cell loss. By contrast, Voltarelli

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and coworkers were able to achieve longer-lasting remission of the disease through autologous stem cell transplantation [5]. This is an indication of the positive effect of immune regulation. However, given its invasiveness, the protocol is not suitable for clinical routine. It has also been shown that Tregs in particular play a central and important role in the protection against T1D. Depleting Tregs in the NOD/Ltj T1D mouse model, preventing costimulation by CD28 deficiency or eliminating IL-2 results in a strong progression of diabetes in the model [6–8]. In humans, information on the Treg content during T1D disease is contradictory. A determination can only occur via the blood and makes the analysis of existing studies difficult to compare. However, the condition proved to be a more functional rather than a numerical defect of Tregs [9]. Antigen-specific T cells that convert to Tregs by retrovirally transduced Foxp3, leading to reversion of established diabetes in NOD mice [10]. However, the experiments were performed with T cell receptor (TCR) transgenic Tregs, whereas polyspecific Tregs have no therapeutic effect in murine models and clinical trials to date. From our transplantation studies, we know that antigen-specific Tregs are very effective in prolonging graft survival [11–13]. However, natural antigen-specific Tregs against autoantigens have a very low frequency, so obtaining a relevant cell number for therapy under GMP conditions is a major challenge. One approach in the T1D model was the use of transgenic TCR (BDC2.5). However, due to MHC restriction, this transgenic model is not transferable to patients given that each patient would require a new TCR. In summary, Tregs are present in autoimmune diseases, such as T1D, but not functional.

In 2017, the US FDA approved the first two chimeric antigen receptor (CAR)-T cell-derived cell products for use in B-cell lymphoma. We were able to show that HLA-specific CAR-Tregs can be used to suppress very strong allospecific immune responses in a humanized transplantation model [12]. The use of beta cell-specific CAR-Treg for remission and treatment of T1D is therefore very promising. Unlike TCR, CARs allow highly specific MHC-independent antigen recognition. To construct a specific CAR, a second-generation CAR construct should be used that is characterized by two intracellular domains: CD3ζ and a costimulatory CD28 domain. However, the centerpiece is the antigen-binding domain, which consists of the immunoglobulin of the antigen-binding domain of an antibody.

Such specific Tregs that are directed against beta cell antigens or diabetes-associated antigens are found only in very small numbers and are even more difficult to clone since the exact epitope is only known in the rarest of cases. The *in vitro* generation of CAR-Treg would therefore be a major step forward. These cells would be highly specific for a defined antigen directly related to diabetes after transduction of the receptor. These cells would regulate autoreactive cells without side effects and enhance the healing effect. The group of Ulrike Protzer showed that CAR-T cells are functional against the soluble hepatitis B antigen (HBsAg) [14]. Although, the antigen is detectable throughout the body, the CAR-T cells can be found preferentially in the liver, where the antigen is present at the highest concentration. We have already generated single-chain fragments (scFvs) against murine beta cells, thus detecting the islets in histology, PET and MRI [15], but these were unable to stimulate as a CAR-construct. Therefore, as an alternative strategy, we generated a CAR against insulin and investigated the possibility to generate large numbers of insulin-specific Tregs by changing the specificity of T cells via transfer of chimeric antigen-receptors.

## 2. Materials and methods

### 2.1. Mice

BALB/cJ, C57BL/6J and nonobese diabetic (NOD/LtJ) mice were bred and maintained under pathogen free conditions at the animal facilities of the Hannover Medical School, Hannover, Germany and University Hospital Essen, Essen, Germany. All animal experiments were approved by the animal use and care committee.

A total of $2.5 \times 10^6$ NOD CAR-A6 cTregs or cTregs w/o CAR were injected intravenously into 13-week-old female NOD/Ltj mice. Blood glucose levels were monitored twice a week. After the NOD mice had at least two consecutive blood glucose readings > 200 mg/dl, they were diagnosed as diabetic and sacrificed. Spleen, inguinal lymph nodes (iLN) and mesenteric lymph nodes (mLN) of diabetic mice were analyzed for the persistence of the transferred cTregs via flow cytometry.

### 2.2. Single chain fragment generation by phage display

Antibodies in scFv-format were selected against insulin (Actrapid, Novo Nordisk) from the human naive antibody gene libraries HAL9 and HAL10 [16]. The antibody selection was performed as described previously [17,18]. Briefly, insulin was immobilized in Costar High Binding microtiter plates (Sigma-Aldrich, Germany) and incubated with $5 \times 10^{11}$ scFvs phage from each of the HAL9 and HAL10 libraries. Both libraries are packaged with Hyperphage (PMID: 11135557 + 16996161). The nonbinding antibody phage were removed by one to three washing steps depending on the panning round. The bound scFvs phage were eluted and reamplified by infection of *E. coli* following a coinfection with a M13K07 helper phage and amplification of isolated scFvs phage for further selection rounds. After three rounds of panning, monoclonal soluble scFvs were produced in microtiter plates and screened for insulin binding by antigen-ELISA and immunofluorescent staining (see below). Positive binding scFvs DNA was isolated, sequenced and cloned into the CAR vector system.

### 2.3. ELISA with soluble scFv

To analyze the insulin specificity of selected scFv, Costar High Binding microtiter plates were coated with 100 ng of insulin in 100 µl PBS overnight at 4 °C. BSA-coated (100 ng/100 µl PBS) wells served as control. An anti-lysozyme antibody (DM321-F11) served as positive control. After three washes with PBST (PBS, 1% Tween-20), the wells were blocked with 2% MPBST followed by an additional washing step. The selected and produced scFvs diluted in MPBST were added to the plate, incubated for 1 h and washed thrice. Bound and insulin-specific scFvs were detected using a mouse α-Myc-specific antibody (9E10) in addition to a α-mouse Fc-specific HRP-conjugated antibody (A0168, Sigma-Aldrich). TMB substrate was used for the detection. The reaction was stopped by the addition of 100 µl 1 N $H_2SO_4$ and measured with an ELISA Reader at 450 nm (BioTek Epoch, reference 620 nm).

### 2.4. Isolation/expansion of murine nTregs and generation of murine cTregs

Lymphocytes were pooled from lymph nodes and spleens and stained with anti-CD4-APC (RM4-5, BioLegend) and anti-CD25-PE/Cy7 (PC61, BioLegend, Germany). The nTregs were then sorted based on $CD4^+CD25^{high}$ using a high-speed cell sorter (FACSAria II, BD, Germany). The FACS-based cell sorting was performed at the cell-sorting facility of Hannover Medical School and showed a purity of > 95%.

Converted Tregs (cTregs) were generated by retroviral transduction of $CD4^+$ T cells with a Foxp3 containing plasmid (see below). nTregs and cTregs were cultivated in RPMI media (complete RPMI supplemented with 1% Glutamax, 10% heat-inactivated FCS, 2% HEPES, 1% nonessential amino acids, 1% sodium pyruvate, 0.1% β-mercaptoethanol, 1% penicillin/streptomycin) and expanded by using the Treg Expansion Kit from Miltenyi Biotec (Bergisch-Gladbach, Germany) according to the manufacturer's instructions. For nTreg and cTreg expansion, fresh medium supplemented with IL-2 (2000 U/ml) was added to the cells every 48 h. Before each experiment, Tregs were rested for 3 days in a low IL-2 concentration (50 U/ml). At the end of the culture, anti-CD3 and anti-CD28 beads were removed using the MACSiMAG™ Separator (Miltenyi Biotec).

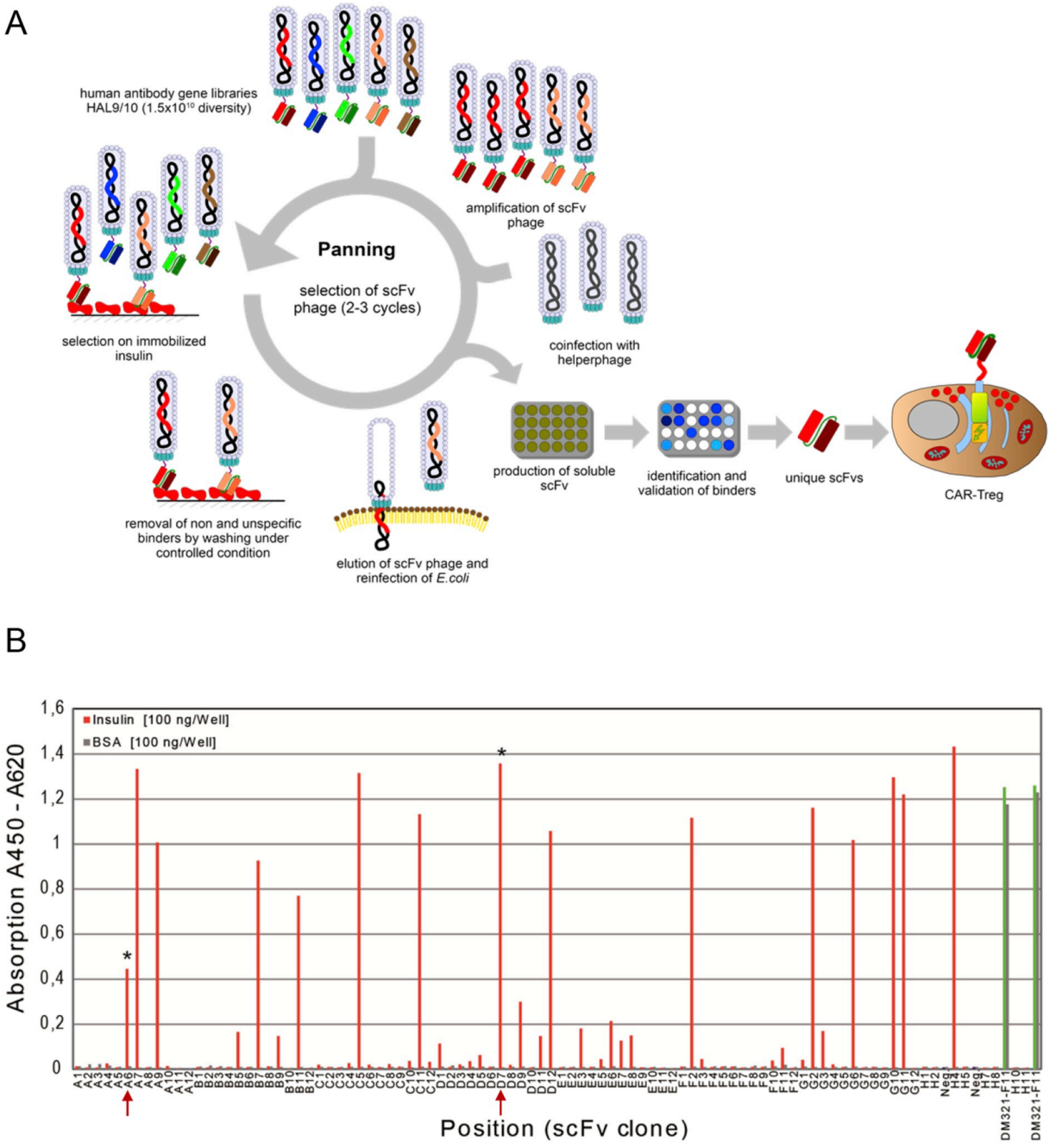


**Fig. 1. Selection and analysis of insulin-specific scFv. (A)** Schematic overview of *in vitro* scFv selection using antibody phage display. The human naive gene libraries (HAL9 and HAL10) were incubated with the immobilized insulin. The bound scFv phage were eluted and infected in *E. coli*. After coinfection with M13 helper phage and amplification, the isolated scFv phage were used for further selection rounds. After three panning rounds, the selected scFvs were produced in *E. coli* and further tested for antigen binding via ELISA or immunohistochemistry to identify insulin-specific scFvs. **(B)** Evaluation of insulin-specific scFvs via monoclonal antigen ELISA. Briefly, 100 ng of insulin were immobilized and incubated with soluble scFv. The bound insulin-specific scFvs were detected by a mouse α-Myc-specific antibody in addition to an α-mouse Fc-specific HRP-conjugated antibody. The negative control (no immobilization of insulin) was indicated as negative. An anti-lysozyme antibody (DM321-F11) served as a positive control (green). Further analyzed clones were depicted with arrows and *.

### 2.5. Retrovirus production and cell transduction

Retroviral vector particles were generated as previously described [19]. Isolated naive CD4$^+$ T cells were stimulated with Treg Expansion beads (Miltenyi Biotec). After 72 h, the cells were transduced with γ-retroviral particles encoding either the CAR-A6 plasmid including an additional Foxp3 sequence, a plasmid with a control CAR including an additional Foxp3 sequence or a control plasmid with Foxp3 but without the CAR. The cells were spin infected in the presence of protamine sulfate. Seventy-two hours after transduction, the transduced cTregs were isolated according to the congenic marker CD90.1 (Thy1.1) expression on their cell surface.

### 2.6. Flow cytometry staining

The following antibodies were used for surface staining: anti-CD4 (GK1.5, RM4-5, BioLegend), anti-CD8 (RM4-5, BioLegend), anti-CD25 (PC61, BioLegend), anti-CD45R/B220 (RA3-6B2, BioLegend), anti-CD62L (MEL-14, BD), anti CD69 (H1.2F3, BD), anti-CD127 (SB/199, BD), anti-GITR (DTA-1, eBioscience, Germany), anti-CD90.1/Thy1.1

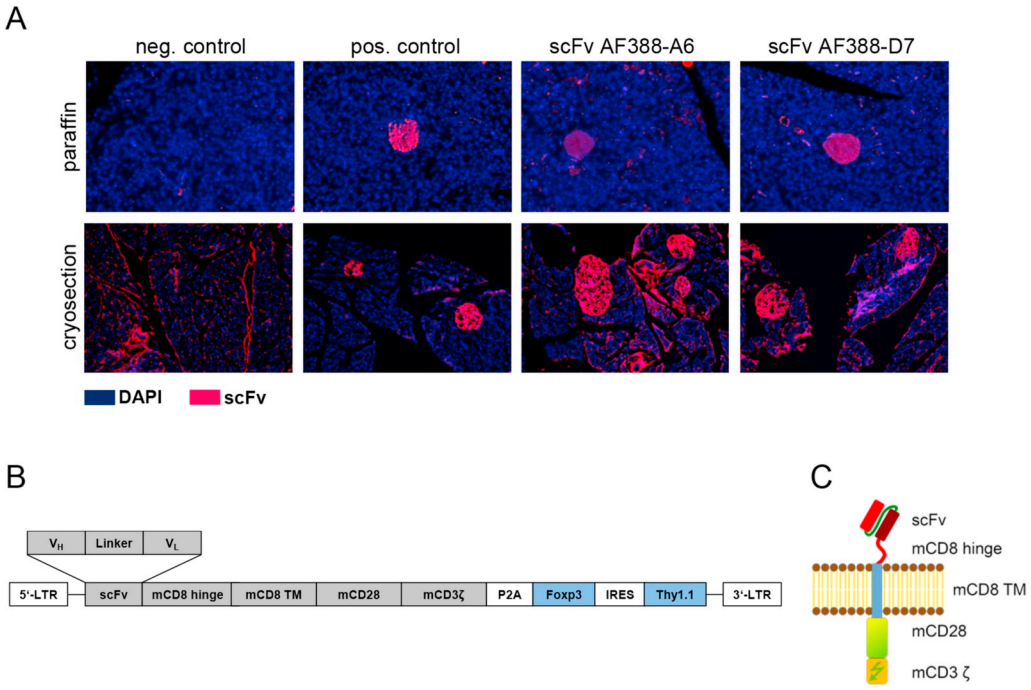


**Fig. 2. Validation of insulin-specific scFvs and generation of insulin-specific CAR. (A)** Immunofluorescence staining of insulin-specific scFvs on mouse pancreas sections. FFPE and cryosections of mouse pancreas were stained with soluble scFv. The detection of specific scFvs was performed with a mouse α-Myc-tag antibody followed by a secondary mouse IgG-Cy3 antibody (red). Cellular DNA was depicted with DAPI staining (blue). Sections only incubated with the secondary antibodies served as a negative control, and sections incubated with a guinea-pig anti-human insulin antibody served as a positive control (40×). **(B)** Modular scheme of a γ-retroviral CAR backbone containing an antigen-specific scFv, a murine CD8-hinge and transmembrane region, a murine CD28 costimulatory domain and a CD3ζ signaling domain followed by a P2A flanked Foxp3 cassette and a surface expression marker Thy1.1 expressed from an internal ribosomal entry site (IRES). **(C)** Structural scheme of the γ-retroviral CAR vector system containing an antigen-specific scFv and the murine T cell activation and costimulation motifs. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(OX-7, BioLegend) and anti-human IgG-F(ab')2 (Jackson). Intracellular staining was performed using the eBioscience Fix/Perm Kit and following antibodies: anti-Foxp3 (FJK-16s, eBioscience), anti-Helios (22F6, BioLegend) and anti-CD152/CTLA-4 (UC10-4F10-11, BD).

### 2.7. Hybridoma coculture assay

We used the NFAT-GFP hybridoma cell line (generous gift from Ludger Klein, Munich, Germany), which encodes a GFP under the control of a NFAT-dependent IL-2 promoter [20]. The hybridoma cells were transduced with the retroviral CAR construct as described earlier [12]. Forty-eight hours after transduction, CAR-transduced hybridoma cells were incubated for 20 h with immobilized or soluble insulin. Short-acting Insulin Actrapid (Novo Nordisk) and long-acting Insulin Glargin (Sanofi) were either added fresh to the media (1:100) or coated in PBS (1:100) at 4 °C 14–18 h before initiation of the assay. NFAT-driven GFP expression was analyzed using flow cytometry.

### 2.8. In vitro proliferation and suppression assays

For the proliferation assay, insulin was immobilized by coating a plastic surface with Actrapid or Glargin in PBS (1:100). Soluble insulin was added fresh to the media at a dilution of 1:100. CAR-transduced cTregs were labeled with 5 mM CFSE and incubated for five days. The level of CAR-Treg proliferation was assessed by determining the dilution of CFSE using flow cytometry.

To determine the natural suppression capacity of nontransduced nTregs and transduced CAR-A6 cTregs, we performed an MLR driven by allogeneic stimuli as described previously [21]. In brief, various ratios of BALB/c nTregs or CAR-A6 cTregs were cocultured with naive $CD8^+CD25^-$ Teff BALB/c responder cells, which were CFSE labeled in the presence of allogeneic stimulating cells (irradiated (30Gy) C57BL/6 splenocytes). Teff CFSE dilution was measured five days after the initiation of the culture using flow cytometry. The results are shown as percent inhibition of Teff proliferation relative to Teffs cultured alone.

### 2.9. Histology immunofluorescence staining

For immunofluorescence staining, formalin-fixed, paraffin-embedded (FFPE) and cryosections (5 μm) of murine pancreas were stained by using soluble scFv. The Myc-tag (9B11, Cell Signaling) within the scFvs in addition to an appropriate Cy3-conjugated secondary antibody (mouse IgG-Cy3, Jackson) were used for the detection of positive binding. DAPI was used to stain nuclei. A guinea-pig anti-human insulin antibody (Merck Millipore, Germany) with the appropriate fluorochrome-conjugated secondary antibody served as a positive control. The analysis was performed using an AxioImager M1 microscope (Zeiss, Germany) and Axiovison Rel. 4.8 software.

### 2.10. Statistical analysis

Analysis was performed using Prism 5 software (Graph-Pad). $P < 0.05$ was considered significant. All statistical analyses and p-values are described in the figure legends.

## 3. Results

### 3.1. Generation of insulin-specific scFvs and CAR

We followed the hypothesis that insulin could be a good target for a CAR, as it is constitutively secreted by all beta cells. Therefore, the insulin density within the pancreas is the highest throughout the body. An insulin-targeted CAR should allow cells to migrate into the pancreas and act as regulators at the site of cell destruction. Although insulin is a soluble antigen that is present throughout the body, specific accumulation of CAR-T cells in the liver was detected in the above-described publication on HBsAg, and CAR-T cells were also detected throughout the body [14].

To generate insulin-specific scFv, we used phage-display technology [22](Fig. 1A). Briefly, 2–10 μg insulin (Actrapid®Novo, Nordisk) were immobilized on a plastic surface. On this surface, the phage containing the antibody library were incubated and then washed in several steps. In a subsequent step, only the phage that were able to form a

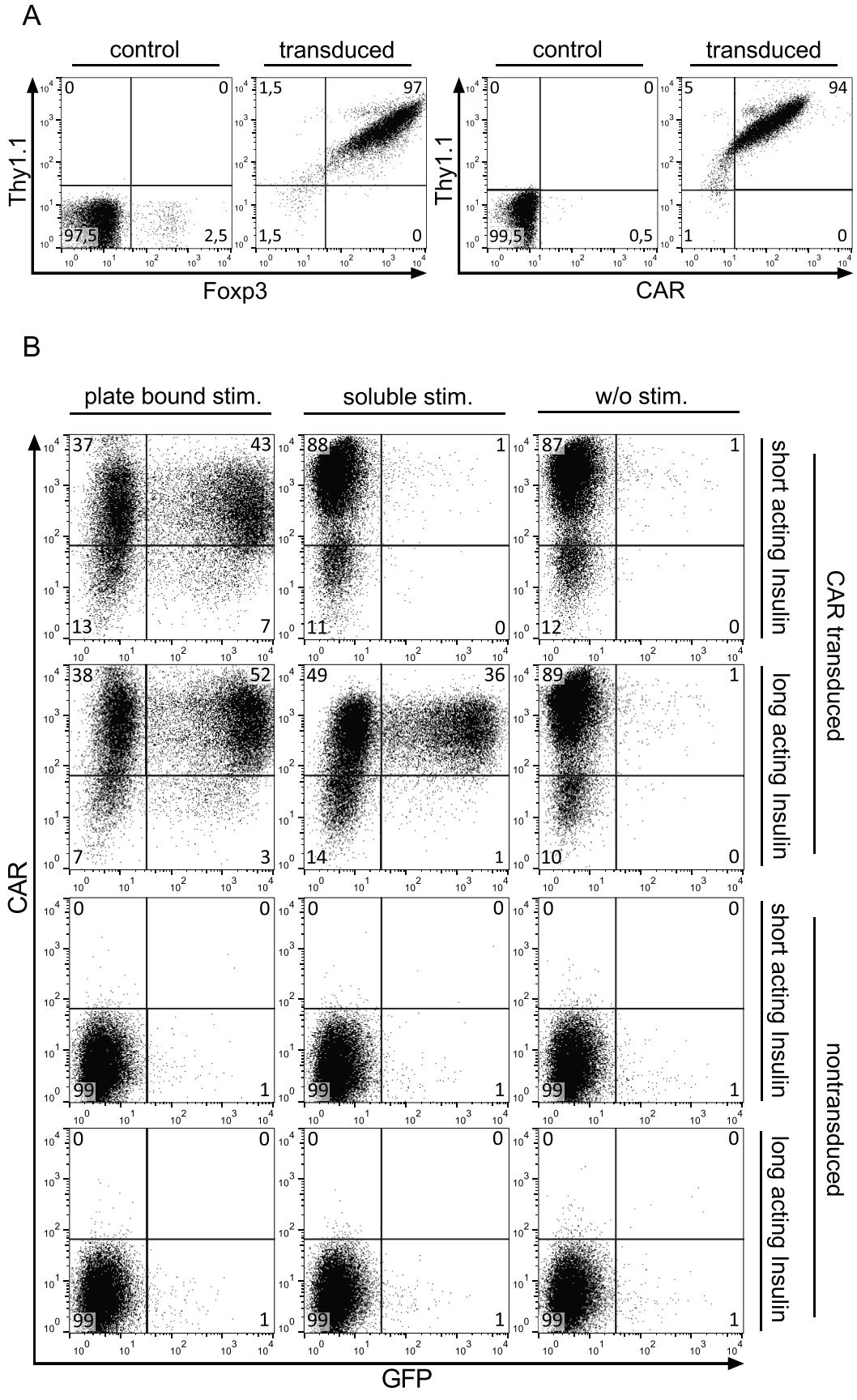


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**Fig. 3. Expression and functionality of insulin-specific chimeric antigen receptor (CAR-A6). (A)** $CD4^+$ T cells were transduced with retroviral particles encoding the CAR plasmid including Foxp3 to convert $CD4^+$ T cells into cTregs and analyzed by flow cytometry. Cells were stained for Thy1.1, Foxp3 and scFvs via anti-IgG-Fab demonstrated surface expression of the CAR-A6. Nontransduced $CD4^+$ T cells were stained as a negative control. **(B)** CAR-A6 transduced hybridoma cells upregulated GFP expression 20 h after antigen-specific NFAT stimulation. Cells were incubated with immobilized or soluble insulin. The following insulin compounds were used for stimulation: short-acting Insulin Actrapid (Novo Nordisk) and long-acting Insulin Glargin (Sanofi). Nontransduced hybridoma cells served as a negative control.

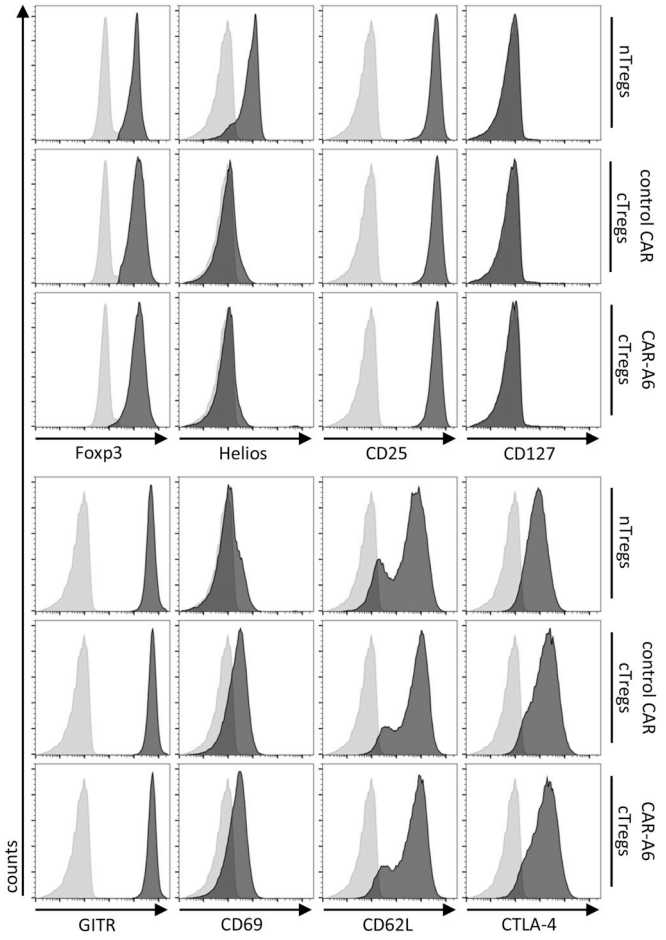


**Fig. 4. Phenotype of nTregs and CAR-cTregs.** For phenotype analysis, $CD4^+$ T cells were transduced either with an insulin-specific or control CAR construct including the Foxp3 sequence. Cells were gated on transduced $Thy1.1^+Foxp3^+$ CAR-cTregs. Expression of Foxp3, Helios, CD25, CD127, GITR, CD69, CD62L and CTLA-4 in CAR-cTregs were compared with that in nTregs. Fluorescence minus one staining served as a control.

sufficiently strong specific binding to the insulin are eluted. To increase the yield and the affinity of specific phage, the elution is used in further "panning" rounds, whereby an increasingly specific suspension and subsequent individual clones are obtained. In the next process step, potential binders are evaluated by an enzymatic-linked immunoassay (ELISA) to detect insulin-specific phage (Fig. 1B). The clones identified as insulin binding were produced as soluble monoclonal scFv. The soluble scFvs have a Myc-tag detection. Insulin was immobilized, and only those clones that were greater than 10 times above background level in the binding ELISA were considered positive.

Subsequently, the ELISA-positive clones were qualitatively analyzed *ex vivo* in histology for their specificity binding properties. For this purpose, paraffin and cryosections of pancreatic tissues were prepared (Fig. 2A). Since which insulin-epitope the scFvs bind is not known, both the formaldehyde-fixed paraffin tissue sections and the natively obtained cryosections were stained. The sections were incubated with the soluble monoclonal antibody fragments, and the bound clones were identified indirectly via the Myc-tag using a second fluorochrome-conjugated anti-Myc antibody. Due to the specificity of the scFvs to insulin, only the insulin-producing beta cells of the pancreas were recognized by the scFv. The two most promising clones (AF388-A6 and AF388-D7) in ELISA (Fig. 1 B with *) and histology were used to generate insulin-specific CARs.

The scFvs were cloned into a newly generated second generation CAR backbone consisting of a murine CD8 hinge and transmembrane region fused to the intracellular CD28 and $CD3_\zeta$-ITAM motifs for intracellular signaling (Fig. 2B/C) [23]. We decided to use a CD28/CD3 second generation CAR [24] given that Tregs are critically dependent of CD28 costimulation and that mice lacking the CD28 ligands CD80 and CD86 are devoid of Tregs [25]. The human version of the construct was very efficient in a transplant setting [12]. The CD8 region was modified to prevent receptor clustering [12,26]. The insulin-specific CAR was cloned into a gamma-retroviral RSF91 expression vector. For control experiments, we cloned an MHC class I-specific CAR into the RSF91 backbone. To follow CAR-transduced Tregs, CD90.1 (Thy1.1) was expressed by an internal ribosomal entry site (IRES). This molecule can also be used for depleting cells by anti-Thy1.1 administration. In most of the following experiments, $CD4^+$ effector T cells were used to generate Tregs. Therefore, Foxp3 in the construct is cleaved by a proteolytically active P2A motif. This form can migrate in a soluble form posttranslationally into the cell nucleus and provide stable expression of Foxp3, which is essential for cTregs. CAR, Foxp3 and Thy1.1 expression was controlled in transduced murine fibroblasts that lack these molecules before transduction (Supple. Fig. 1). The genetic insertion of Foxp3 has already been shown to improve T1D in Treg therapy [10].

### 3.2. Insulin-specific CAR was functional in vitro

To test the functional specificity of the insulin-specific CAR in further detail, we transduced $CD4^+$ Teffs (Fig. 3A) and a T cell hybridoma cell line with our CAR constructs (Fig. 3B). This hybridoma stably expressed a GFP reporter construct driven by an NFAT-sensitive IL-2 promoter [12]. While insulin-specific CAR-A6 transduced hybridomas did not show any GFP expression after transduction without further stimulation of insulin (Fig. 3B right column), NFAT was strongly upregulated with subsequent GFP expression after stimulation with plate bound, short-acting insulin (Fig. 3B upper/left) but not with soluble short-acting insulin (upper/middle). Similar activation was observed with plate bound and soluble insulin if a long-acting version of the drug was used for the stimulation assay (second row/left and middle). Nontransduced hybridoma cells were not activated by any form of insulin directly (lower rows). The same results could be observed with the other clone CAR-D7 (Supple. Fig. 2). Since the hybridoma does not express any endogenous TCR, the observed signal transduction and activation was dependent on the insulin-specific CAR.

### 3.3. Foxp3-transduced, converted CAR-Tregs (CAR-cTregs) were phenotypically and functionally similar to natural tregs (nTregs)

We compared three different kinds of Tregs (Fig. 4). We analyzed a sorted fraction of nTregs (left panel). On the right-hand side, we sorted $CD4^+CD25^-$ T effector cells (Teffs) and transduced them either with a control CAR including Foxp3 (control CAR-cTregs) or with the insulin-specific CAR including Foxp3 (CAR-A6 cTregs). Based on the sorting strategy, all analyzed Tregs expressed Foxp3. However, it is noteworthy that all Tregs expressed Foxp3 to the same extent as they expressed

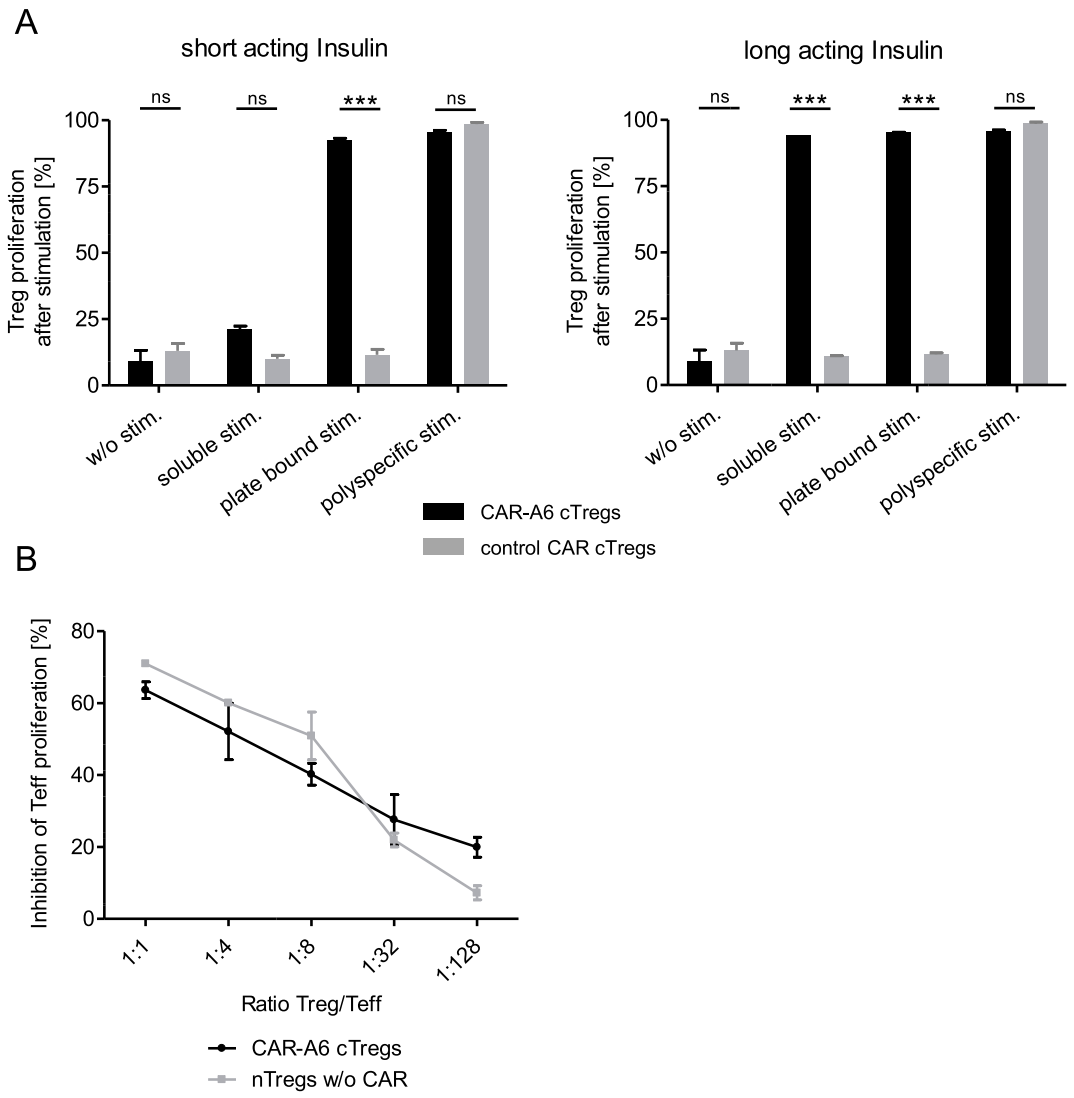


**Fig. 5. cTregs expressing CAR-A6 demonstrate strong proliferation in the presence of insulin and the same suppressive capacity as nTregs. (A)** Proliferation of CFSE-labeled CAR-A6 cTregs and control CAR cTregs five days after stimulation. Cells were stimulated either polyspecific via their T cell receptor with anti-CD3/-CD28 beads or antigen-specific via the CAR with plate bound or soluble insulin (short-acting insulin Actrapid (Novo Nordisk) and long-acting insulin Glargin (Sanofi)). The level of proliferation was assessed by determining the dilution of CFSE. Data represent the mean ± SEM of one experiment performed in duplicate. Significance determined by two-tailed unpaired Student's t-tests. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, ns = not significant. **(B)** Comparison of the suppressive capacity of nTregs and CAR-A6 cTregs in presence of an allogenic stimulus. Mixed lymphocyte reactions (MLR) were performed with C57BL/6 splenocytes as allogeneic stimulator cells, CFSE-labeled BALB/c $CD8^+CD25^-$ responder T cells and BALB/c nTregs or CAR-A6 cTregs. Teff CFSE dilution was measured five days after the initiation of the assay using flow cytometry. Data are presented as the percentage of inhibition of responder Teff proliferation relative to Teffs cultured alone. The Graphs of the MLRs with nTregs and CAR-A6 cTregs were done independently and represent two experiments each. Data represent the mean ± SD of one experiment performed in triplicate.

CD25 and lack CD127 expression. By definition, cTregs also lacked HELIOS that was exclusively expressed by nTregs. Levels of activation markers, such as CD62L and CD69, were as indistinguishable between nTregs and cTregs as GITR and CTLA-4, which are important for Treg function.

The insulin-specific CAR-cTregs have an unaltered proliferative capacity (Fig. 5A and Suppl. Fig. 3). For the *in vitro* antigen-dependent proliferation assay, we used either short-acting (left panel) or long-acting insulin (right panel) as noted in Fig. 3B. Both plate-bound insulins were able to completely activate the specific CAR-A6-cTregs followed by complete proliferation, whereas control cTregs with a noninsulin-specific CAR were not able to induce activation. The long-acting insulin form was even able to induce proliferation of all insulin-specific CAR-cTregs in its soluble form. We then tested whether CAR-cTregs could also inhibit T cell proliferation. Given the lack of insulin-specific T cells in the normal T cell repertoire, we stimulated allospecific effector $CD8^+$ Teffs and added either nTregs (Fig. 5B) or insulin-specific CAR-cTregs in given ratios to the culture. Greater than 50% inhibition was still observed at a 1:4 Treg/Teff ratio, demonstrating the strong suppressive capacity of CAR-cTregs.

### *3.4. Insulin-specific CAR-cTregs were unable to prevent spontaneous diabetes in NOD/Ltj females but were still present even after four months*

Approximately 75% of NOD/Ltj females became spontaneously diabetic after 30 weeks in our colony. In the experimental groups, 4 out of 5 female NOD/Ltj became diabetic without any treatment (Fig. 6A, green line). Neither $2.5 \times 10^6$ cTregs given after week twelve (blue line) nor the insulin-specific CAR-A6-cTregs (red line) was able to prevent diabetes induction to a higher extent. However, even if CAR-A6-cTregs were unable to prevent mice from becoming diabetic (Fig. 6A), CAR-cTregs could be found up to 17 weeks after adoptive

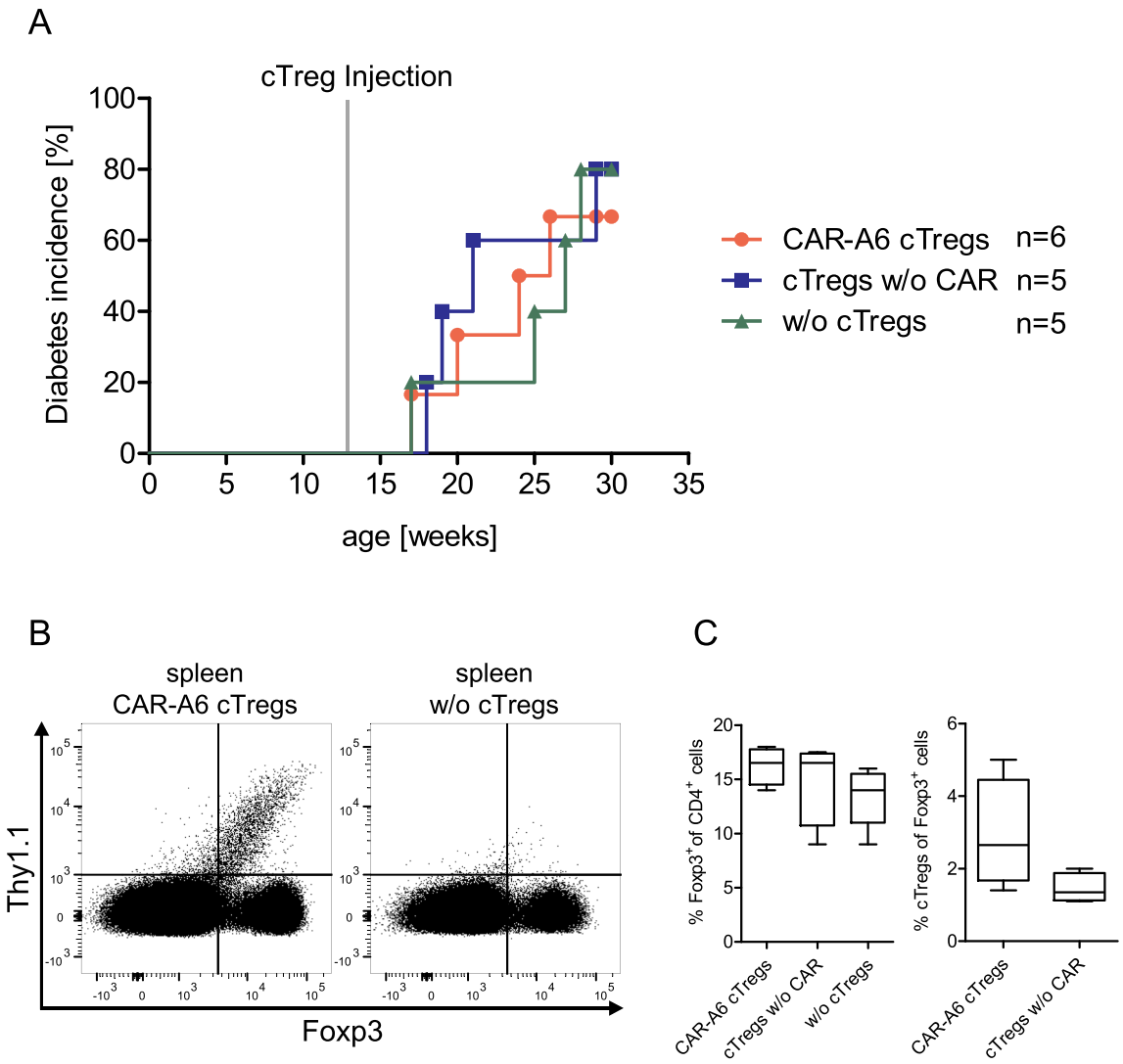


**Fig. 6. Adoptively transferred insulin-specific CAR-cTregs are present in mice after 4 months. (A)** Here, 13-week-old female NOD/Ltj mice were treated with 2,5 × $10^6$ cTregs without CAR (n = 5) or CAR-A6 transduced cTregs (n = 6) or without cTregs (n = 5) as a control. After the NOD/Ltj mice had at least two consecutive blood glucose readings > 200 mg/dL, they were diagnosed as diabetic and sacrificed. **(B)** Spleens were analyzed for persistence of CAR-A6 cTregs using flow cytometry. Dot blots indicate Thy1.1$^+$Foxp3$^+$ CAR-A6 cTregs of the CD4$^+$ population. Representative dot blots are shown. **(C)** Shown is the percentage of Foxp3$^+$ of total CD4$^+$ T cells and transduced cTregs of all Tregs in spleen. Data were collected between weeks 17–30 (4–17 weeks after cTreg injection) when NOD/Ltj mice were diagnosed as diabetic. The results represent the mean ± SEM (cTregs without CAR n = 4, cTreg CAR-A6 n = 4, w/o cTregs n = 5).

transfer, and 2–4% of all splenic Tregs were CAR-cTregs (Fig. 6B/C and Supple. Fig. 4).

## 4. Discussion

We have generated a CAR against human insulin and produced cTregs against it. The resulting insulin-specific CAR-cTregs had a normal Treg phenotype, were suppressive and were long lived in diabetic mice.

These results describe a new, potentially therapeutically useful strategy to generate large amounts of antigen-specific Tregs by changing the specificity of T cells and converting them into Tregs. This protocol can be performed with each donor and a limited *in vitro* culture time and has therefore off-the-shelf capacity. Moreover, cTregs exhibited normal proliferative capacity and were highly responsive to antigen stimulation by proliferation and upregulation of effector molecules.

While CAR-transduced T cells have recently been successfully used to eliminate CD19 $^+$ lymphoma cells [27,28], a major obstacle facing cancer immunology is that many tumor entities do not have specifically identified surface molecules to be targeted by CARs. This situation is in contrast to autoimmunity, e.g., T1D, where well-characterized antigens offer excellent targets that are constitutively expressed on all affected cells of the organ.

However, self-activation by tonic CAR signaling through antigen-independent clustering of CARs has been reported [29]. We recently demonstrated that such antigen-independent signaling did not occur with an alloantigen-specific CAR [12]. The same is true for the insulin-specific CAR in this study given that we detected no NFAT signaling or proliferation in the absence of antigen. This finding also demonstrated the utility of second-generation CARs using CD28 and CD3 signaling domains for cTreg activation as also recently demonstrated [30].

We used an insulin-specific CAR because it could have been used in all patients, but our choice of insulin as the target structure for the CAR-Tregs did not prevent diabetes in mice. We assumed that insulin was accessible not only as a soluble monomer but also in its storage and secreted form as a hexamer for some time [31] because it is known that soluble HBs antigen does not lead to the activation of CAR-T cells [32]. Consistent with these data, we have shown *in vitro* that only the soluble hexamer was able to stimulate the insulin-specific CAR but not the

soluble monomer. This observation is in line with the crosslinking model of antigen receptor activation [33,34]. The monomer had to be immobilized to obtain stimulatory capacity (Figs. 3B and 5A). However, this finding remains a proof of concept, as other target structures of beta cells might be chosen, and CARs can be easily generated. In contrast to transgenic T cell receptor Tregs, CAR-Tregs can be generated as an "off-the-shelf" therapeutic strategy with limited *in vitro* expansion time, as demonstrated in recent trials using CAR-modified T cells in cancer immunology and by us and others in preventing graft rejection [12,35,36].

As the stability of the Treg phenotype in chronically inflamed tissue has recently been questioned [37–40], the transfer of autoantigen-specific Tregs could become problematic, although Bluestone et al. recently demonstrated the stability of adoptively transferred nTregs in clinical trials in patients with T1D [41]. However, to address the stability issues and to increase the yield of Tregs, the CAR construct was complemented by the addition of Foxp3 [10,42,43]. Therefore, we were able to identify CAR-cTregs for four months after injection (Fig. 6 and Supple. Fig. 4).

## 5. Conclusion

This is not the first study that used CAR-Tregs in autoimmunity. In fact, CAR Tregs were used in a mouse model of toxic colitis [44] and EAE [43]. However, this is the first time that CAR-Tregs were generated for T1D.

Thus, CAR-cTregs might offer the possibility to regain immunological tolerance in T1D and in autoimmunity general. This can be achieved without affecting the general immune competence of the recipient. These modified Tregs are available "off-the-shelf" via a short transduction protocol without further need of *in vitro* expansion. Due to the high specificity of their antigens, off-target effects are expected to be minimal.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaut.2019.05.017.

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