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Chimeric antigen receptor (CAR) T cells targeting a pathogenic MHC class II:peptide complex modulate the progression of autoimmune diabetes

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ABSTRACT

A primary initiating epitope in the NOD mouse model of Type 1 Diabetes (T1D) lies between residues 9 and 23 of the insulin B chain. The B:9-23 peptide can bind to the NOD MHC class II molecule (I-A^{g7}) in multiple registers, but only one, (register 3, R3), creates complexes able to stimulate the majority of pathogenic B:9-23-specific $CD4^+$ T cells. Previously we generated a monoclonal antibody (mAb287) that targets this critical I-A^{g7}-B:9-23(R3) complex. When given weekly to pre-diabetic mice at either early or late stages of disease, mAb287 was able to delay or prevent T1D in the treated animals. Although the precise mechanism of action of mAb287 remains unclear, we hypothesized that it may involve deletion of antigen presenting cells (APCs) bearing the pathogenic IA^{g7}-B:9-23(R3) complexes, and that this process might be rendered more efficient by re-directing cytotoxic T cells using a mAb287 chimeric antigen receptor (287-CAR). As anticipated, 287-CAR T cells secreted IFN-γ in response to stimulation by I-A^{g7}-B:9-23(R3) complexes expressed on artificial APCs, but not I-A^{g7} loaded with other peptides, and killed the presenting cells in vitro. A single infusion of 287-CAR $CD8^+$ T cells to young (5 week old) NOD mice significantly delayed the onset of overt hyperglycemia compared to untreated animals ($p = 0.022$). None of the 287-CAR $CD8^+$ T cell treated mice developed diabetes before 18 weeks of age, while 29% of control-CAR T cell treated mice ($p = 0.044$) and 52% of the un-treated mice ($p = 0.0001$) had developed T1D by this time. However, the protection provided by 287-CAR $CD8^+$ T cells declined with time, and no significant difference in overall incidence by 30 weeks between the 3 groups was observed. Mechanistic studies indicated that the adoptively transferred 287-CAR T cells selectively homed to pancreatic lymph nodes, and in some animals could persist for at least 1–2 weeks post-transfer, but were essentially undetectable 10–15 weeks later. Our study demonstrates that CAR T cells specific for a pathogenic MHC class II:peptide complex can be effective *in vivo*, but that a single infusion of the current iteration can only delay, but not prevent, the development of T1D. Future studies should therefore be directed towards optimizing strategies designed to improve the longevity of the transferred cells.

1. Introduction

A primary goal of current type 1 diabetes (T1D) research is to develop an effective antigen specific therapy for the disease. Although in theory any islet antigen might be a suitable target for tolerance induction, there is abundant evidence to suggest that T cells recognizing epitopes within (prepro)insulin may be particularly important to the islet autoimmunity that ultimately leads to T1D [1], making this autoantigen a prime candidate for antigen specific intervention. In the spontaneous diabetic NOD mouse model, amino acids 9 to 23 of the insulin B chain (B:9-23 peptide) contain at least one critical epitope; mice expressing only insulin molecules with a one amino acid mutation, alanine rather than tyrosine at B16, are completely protected from the disease [2]. As peptide binding to MHC class II molecules is relatively promiscuous, and the binding groove is open at both ends, in many instances a single 15mer such as the B:9-23 peptide can bind in multiple registers, with distinct populations of T cells responding to the peptide bound in each register [3]. By trapping the peptide in each potential binding register, Kappler and colleagues demonstrated that most, if not all, islet infiltrating B:9-23 specific $CD4^+$ T cells in NOD mice recognize

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complexes in which the peptide is bound in the energetically unfavorable "register 3," which places the positive charged Arg22 in the basic pocket 9 [4,5]. Accordingly, to target the IA^{g7}-B:9-23(R3) complex we generated a monoclonal antibody, named mAb287, that selectively binds to the complex, but not the free peptide or complexes in other registers or containing other peptides [6]. Administration of mAb287 to pre-diabetic NOD mice significantly reduced the severity of insulitis and delayed or prevented the development of T1D [6]. Moreover, mAb287 treatment inhibited islet infiltration, not only by insulin reactive $CD4^+$ T cells, but also by $CD4^+$ and $CD8^+$ T cells specific to other islet antigens, suggesting that the antibody has a global impact on the function of antigen presenting cells (APCs) co-presenting the B:9-23 peptide and other islet autoantigens.

At present the precise mechanism of action of mAb287 remains unclear, but as the antibody suppresses insulitis in general, it likely involves selective deletion of the target APCs. A potential limitation of the mAb287 reagent is its modest affinity (K_d ~120 nM [6]). This contrasts with other therapeutic antibodies that often have affinities that are 1–2 orders of magnitude greater than that of mAb287. Consequently, it may be necessary to maintain relatively high circulating levels of mAb287 to obtain therapeutic benefit. In contrast, there is evidence to suggest that cytotoxic T cells with receptors having similar or lower affinities to mAb287 are able to delete APCs expressing even a single copy of their cognate ligand [7–9]. This suggests that cytotoxic T cells re-directed with mAb287 may be more efficient in protecting recipients from diabetes than mAb287 itself. Chimeric antigen receptors (CARs, also known as artificial T cell receptors) are used to graft the specificity of a monoclonal antibody onto a T cell. CARs are recombinant receptors typically composed of an antibody-derived targeting domain fused to transmembrane and intracellular signaling domains from molecules involved in T cell activation [10]. CAR T cells have proven highly successful for treating multiple cancers, showing greater efficacy than that afforded by treatment with their parental monoclonal antibodies, and less toxicity than allogeneic cell transplantation [11–13]. Moreover, CAR T cells may also be efficacious in autoimmunity. For example, T1D in the NOD mouse was prevented by adoptive transfer of CAR T cells with peptide-MHC (pMHC) targeting domains specific to pathogenic $CD8^+$ T cells [14]. We now report our initial findings that CAR T cells targeted by mAb287 maintain the specificity of mAb287, and kill antigen presenting cells in vitro. In vivo they can delay the onset of Type 1 Diabetes in a well established preclinical model, although the protection is eventually lost. Nonetheless, this suggests that CAR T cells targeted to APCs expressing pathogenic MHC class II:peptide complexes may be a viable therapy for T1D, and other related autoimmune conditions.

2. Methods

2.1. Animals

Female NOD/LtJ, Thy1.1 NOD, and NOD.SCID mice were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained under specific pathogen-free conditions, with 12 h light/dark cycles and food and water ad libitum, in accordance with protocols approved by the Baylor College of Medicine animal care and use committee.

2.2. Generation of CAR expression constructs

Total RNA was extracted from hybridoma cells expressing mAb287, and cDNAs encoding the heavy and light chains amplified by 5′ RACE (Clontech). Sequencing indicated that mAb287 is an $IgG_1\kappa$ with parental IGHV5-6/IGHJ1 and IGKV12-44/IGKJ1 gene segments respectively. The basic design of the CARs is summarized in Fig. 1A. They comprise the entire antibody light chain joined via a semi-rigid 33 amino acid linker derived from camel IgG [15] to the mature variable and CH1 domains of the IgG heavy chain, and fused sequentially to the stalk, transmembrane and cytoplasmic domains of mouse CD28 (residues 116-218), and cytoplasmic domain of mouse CD3ζ (CD247; residues 52-164). The 3rd generation CAR also contains the cytoplasmic tail of mouse 4-1BB (CD137; residues 212-256) placed between the other two signaling domains. To create the CAR expression constructs the individual domains were first amplified separately by PCR from appropriate templates, assembled by 2 or 3 rounds of splice overlap PCR, and finally introduced into the retroviral expression vector pMIG-II [16] (generous gift of Dr D. Vignali, St Judes Hospital). To facilitate folding and surface expression the cysteine residue at the C terminus of the κ light chain was mutated to glycine. The control CAR (24.1-CAR) contains the variable regions from the heavy and light chains of mAb24-1 (ATCC HB-11947). This antibody recognizes an intracellular epitope from the human cystic fibrosis transmembrane conductance regulator (residues 1477-1480).

2.3. Primary CAR-T cell generation

Replication defective retroviruses encoding the CARs were generated in Phoenix-ECO cells (ATCC CRL-3214) according to established procedures [17]. In brief, cells were co-transfected with the pMIGII-CAR and pCL-Eco plasmids at a ratio of 4:1 using Lipofectamine 2000 (Life Technologies). After ~48 h the supernatant containing virus was collected, the media replaced, and a second viral supernatant collected 24–36 h later. Supernatants were filtered through a 0.45 μm PES membrane immediately prior to use. To initially validate the CARs, 5 KC TCR^- hybridoma cells [18] were transduced by 2 rounds of spin infection [19]. To generate primary CAR-T cells, mouse $CD8^+$ T cells were isolated from splenocytes of 4-week NOD female mice by negative selection (Miltenyi Biotec) on day 0. Purified cells were activated in vitro using plate bound CD3/CD28 antibodies (pre-coated at concentration of 1 μg/ml) and expanded for 48 h in RPMI containing 10% heat-inactivated fetal bovine serum, 1% ITS (Life Technologies), mouse IL-7 (0.5 ng/ml; R & D Systems) and human IL-2 (100 IU/ml; PeproTech). Cells (0.5×10^6/well) were then transferred to retronectin (Clontech) coated 24-well plates, filtered retrovirus containing supernatant added, and the cells transduced by spinning at 2000g for 90 min at 37 °C. A second transduction was performed on day 3, and the transduced cells then expanded in medium containing IL-2 + IL-7. CAR expressing cells were purified on day 5 or day 6 by FACS based on the expression of GFP from the IRES in the vector (Fig. 1A; Supplemental Fig. 1).

2.4. Flow cytometry analysis of primary CAR-T cells

For phenotypic analysis the sorted CAR- T cells (2×10^5) were stained with APC/Fire™ 750 anti-mouse CD8α, APC-anti-mouse KLRG1, Brilliant Violet 421™ anti-mouse CD152(CTLA-4), Brilliant Violet 711™ anti-mouse CD69, Brilliant Violet 650™ anti-mouse CD25, PE/Cy7 anti-mouse CD11a/CD18 (LFA-1), and APC-anti-mouse CD215 in 3 different panels. Cells were then washed, fixed and permeabilized (BD Bioscience, CytoFix/CytoPerm Buffer), and stained with Brilliant Violet 421™ anti-mouse IFN-γ, and Brilliant Violet 650™ anti-mouse TNF-α. All antibodies were from eBioscience or BioLegend. Samples were run on an LSR Foretessa (BD) and data analyzed using FlowJo software (Tree Star).

2.5. Antigen specific functional assays of primary CAR-T cells

Antigen specificity of CAR T cells was evaluated by measuring their cytokine secretion. Four artificial antigen presenting cells (aAPCs) were used as stimulators. M12.C3.G7 [20] was a generous gift from Dr E Unanue (Washington University). M12.C3(I-A^{g7}-B:WT), M12.C3(I-A^{g7}-B:R3) and M12.C3(TfR-MBP-DTRL) were generated by transduction of M12.C3 cells [21] respectively with retroviruses encoding I-A^{g7} with the native B:9-23 peptide (HLVEALYLVCGERG) or B:R3-mimotope

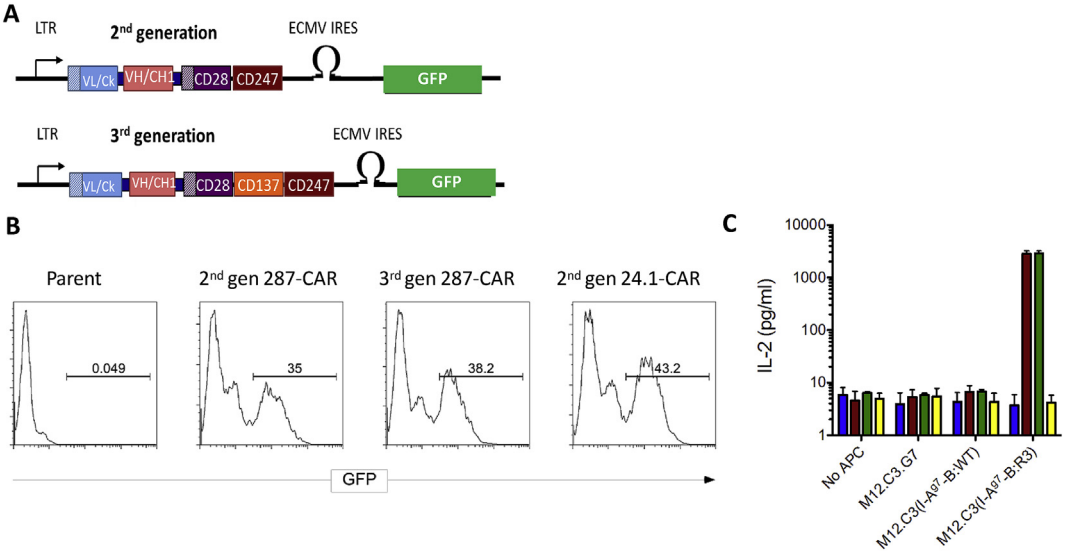

Fig. 1. Expression of CARs in 5KC thymomas. **A**: Cartoon illustrating the main features of the retroviral CAR constructs. They encode the antibody light chains (blue boxes) including their native leader peptides (shaded box) joined by a flexible linker (black bar) to the VH and CH1 domains (red boxes) and fused to the transmembrane (shaded) and signaling domains of CD28 (purple boxes) and cytosolic domain of CD247 [CD3ζ] (brown boxes). The 3rd generation CAR has an additional CD137 [4-1BB] signaling domain (orange box). The construct also contains an IRES driven GFP to allow detection of transduced cells. Expression is driven by the viral LTR. **B**: 5 KC cells were transduced with replication defective retroviruses as described in methods. Expanded cells were analyzed by flow cytometry. **C**: Expanded cells (10^5) were co-cultured in triplicate with an equal number of the indicated aAPCs for 20 h and secreted IL-2 quantified by ELISA. Means and SD of co-cultures containing parental 5 KC cells (blue bars), or cells expressing the 2nd generation 287-CAR (brown bars), 3rd generation 287-CAR (green bars), or 2nd generation 24.1-CAR (yellow bars) from one representative experiment are shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(HLVE**R**LYLVCGE**E**G [5]) fused to the N-terminus of the beta chain [22], and a fusion protein comprising the transmembrane and endocytosis deficient truncated cytoplasmic domain of the human transferrin receptor [23] and maltose binding protein (excised from pMal-c2x; NEB) linked at the C terminus to a peptide containing 3 copies of the 24.1 epitope separated by Gly-Ser linkers (**DTRL**GS**DTRL**GS**DTRL**). 5 KC transductants (10^5) were co-cultured with an equal number of aAPCs for 20 h and secreted mouse IL-2 quantified by ELISA [24]. For primary CAR-T cells, 4×10^4 cells were cultured with increasing numbers of aAPCs. After 16 h the secreted IFNγ was quantified by ELISA (eBioscience). In vitro cytotoxicity was assessed by co-culturing primary CAR T cells (4×10^4) with aAPCs at an effector:target (E:T) ratio of 2:1 for 4 h. The cells were then harvested and co-stained with PE anti-mouse CD19 and propidium iodide (PI). Killing was calculated as the percentage of $CD19^+$ cells that co-stained with PI.

2.6. Analysis of CAR T cells following adoptive transfer

Sorted GFP^+CD8^+ T cells were expanded in complete medium containing IL-2 as described above. Without additional re-stimulation cells continued to proliferate in vitro until day 8–10 post-transduction. Accordingly, for *in vivo* studies, cells were adoptively transferred cells to hosts on day 6 post transduction. To assess survival/proliferation *in vivo,* lymphoid tissues (spleen, pancreatic lymph nodes, and inguinal lymph nodes) were collected 3, 5, 9, and 14 days post transfer. GFP^+CD8^+ T cells were quantified by flow cytometry analysis. To further analyze the distribution of the transferred cells, 5×10^6 CAR-T cells from congenic Thy1.1 NOD donors were transferred to 8-week old female NOD (Thy1.2) mice. On day 5 post-transfer, pancreata and pancreatic lymph nodes (PLNs) were collected and fixed with 10% formalin, then CAR-T cells and endogenous B cells were identified by immunohistochemistry. Briefly, paraffin sections were prepared and stained as described previously [25]. Sections were incubated with primary antisera including mouse anti-Thy1.1 (#202508; Biolegend, San Diego, CA), monoclonal mouse anti-CD3 (#ab17143; Abcam, Cambridge, MA), rat anti-mouse B220 (#557390; BD Biosciences, San Jose, CA), followed by secondary antisera conjugated to Cy3 or Cy5 (Jackson ImmunoResearch, West Grove, PA) and DAPI (Molecular Probes, Eugene, OR, USA) as previously [26]. Sequential staining was performed with T cell specific antibodies followed by B cell specific antibodies to avoid cross reactivity. Images were captured with a Zeiss AxioImager M1 (Carl Zeiss, Thornwood, NY) and Volocity 6.1.1 software (PerkinElmer, Waltham, MA, USA).

2.7. Diabetes protection by 287-CAR T cells

Sorted and expanded CAR T cells ($3–5 \times 10^6$/mouse) were transferred via the tail vein to 5 week old female NOD mice. Beginning at 10 weeks of age blood glucose levels were monitored weekly with an OneTouch Ultra2 monitor (LifeScan Inc.). Animals with values of ≥250 mg/dL were re-tested the following day. Diabetes was diagnosed after two consecutive blood glucose values ≥ 250 mg/dL[6]. Mice were euthanized either following diagnosis of diabetes, or at 25 weeks of age, and the spleens, pancreatic lymph nodes (PLNs) and pancreata were harvested for flow cytometric analysis or for insulitis evaluation. Serum was collected at 4 weeks (prior to the treatment), and at 10, 12 and 14 weeks of age for analysis of serum insulin, TNF α and IFN γ by ELISA and insulin auto-antibodies (IAA) by radioimmunoassay [27]. In a second experiment, 5×10^6 diabetic splenocytes were transferred alone or together with 5×10^6 CAR T cells into groups of 5–6 week old NOD.SCID mice. Animals were monitored daily for signs of diabetes and followed for up to 7 weeks post-transfer.

2.8. Statistical analysis

Survival curves were analyzed using PRISM7 software (Graphpad, San Diego, CA), and *p*-values ≤0.05 considered statistically significant. The Generalized Gehan-Breslow-Wilcoxon test was used for analysis of time to diabetes data because it tends to be more powerful than the log rank test when the assumption of proportional hazards is violated, as is often the case in mouse models. To analyze the effect of treatment on the onset of diabetes at a certain time point, a global significance test

(Pearson's Chi-squared test) was performed initially; then pairwise comparisons between groups analyzed by Fisher's Exact Test. The frequencies of IAAs were analyzed by Fisher's exact test, and titers by 2 tailed Mann Whitney *U* test.

3. Results

3.1. Single chain Fab CARs maintain the binding specificity of the parent antibodies

The design of the 2nd and 3rd generation CARs is shown in Fig. 1A. The interaction between pMHCs and T cell receptors (TCRs) takes place in areas of close contact in which the adjacent membranes are spaced ~13 nm apart [28]. This spacing is critical, particularly for low affinity ligands [29]. Consequently, to approximate the dimensions of a TCR, we fused single chain Fab fragments (scFabs) directly to the CD28 stalk. To validate the binding specificity of the CARs, we initially expressed them in 5 KC cells; a murine thymoma that lacks expression of endogenous TCRα or β chains [18]. As shown in Fig. 1B, after transduction, 35–45% of the 5 KC cells express GFP from the IRES in the retroviral construct. Staining with a polyclonal anti-mouse IgG confirmed that GFP and CAR surface expression were directly proportional (data not shown). As anticipated, co-culture of 287-CAR expressing 5 KC cells with aAPCs expressing I-A^{g7}-B:R3, but not I-A^{g7} loaded with peptides from endogenous mouse proteins, resulted in a robust IL-2 response (Fig. 1C). In contrast, 287-CAR expressing 5 KC cells responded very weakly to I-A^{g7}-B:WT, consistent with the majority of the native peptide being bound in registers 1 or 2. Similarly, 5 KC cells expressing the 24.1-CAR did not respond to any of the I-A^{g7} aAPCs, but responded robustly to M12-C3 cells expressing the TfR-MBP-DTRL fusion protein (data now shown). These data confirm that the scFabs maintain the binding specificity of the parent antibodies.

3.2. Primary CAR T cells secrete IFN-γ in response to their cognate ligands, and lyse the antigen presenting cells

In NOD mice, insulin autoantibodies (the first sign of islet autoimmunity) rarely appear before 6 weeks of age. Thus, to ensure a predominantly naïve starting population we isolated $CD8^+$ T cells from the splenocytes of 4-week old female NOD mice. After in vitro activation 150–240 fold expansion of the CD8 T cells was observed by day 8 of culture. After two rounds of transduction, 60%–90% of the $CD8^+$ cells co-expressed GFP analyzed at the time of cell sorting (Supplementary Fig. 1).

To begin to characterize the sorted and expanded primary CAR T cells, we first analyzed the expression of selected activation markers by flow cytometry on days 8 or 9. These results are summarized in Table 1. Approximately 98% of the viable GFP^+ cells retained expression of CD8, and as expected, robust expression of activation markers such as LFA-1, CD25, and CD69, but not CD152 (CTLA-4), was also observed. Approximately 29% of the cells expressed KLRG1, a marker of memory cells, whereas more than 90% co-expressed the pro-inflammatory cytokines IFN-γ and TNF-α.

Table 1
Characterization of expanded CAR T cells.

Marker	% positive cells (mean ± SD)[a]
CD8	97 ± 1.5
KLRG1(MAFA)	29 ± 1.7
CD152(CTLA-4)	2.5 ± 1.5
CD69	91 ± 1.8
CD25	65 ± 9.8
CD11a/CD18(LFA-1)	94 ± 0.8
IFNγ	91 ± 3.1
TNFα	94 ± 2.2

Expanded CAR T cells were stained with antibodies to the indicated markers on day 8 or 9 and analyzed by flow cytometry. Results are expressed as the percentage of viable GFP^+ cells.

[a] Mean values of 4 CAR T cell lines from repeated experiments.

We next analyzed the ability of primary CAR-T cells to respond to their cognate antigens. Day 8 CAR T cells were co-cultured for 16 h with increasing numbers of aAPCs cells. As expected, 287-CAR T cells secreted IFN-γ in response to I-A^{g7}-B:R3 stimulation in a dose dependent manner, but did not respond to aAPCs expressing native I-A^{g7} or the 24.1 ligand (Fig. 2A). In contrast, 24.1-CAR T cells only responded to the M12 cells displaying the CFTR peptide (Fig. 2B). To determine if the CAR T cells were able to kill the aAPCs, in a second experiment we stained the cells with propidium iodide (PI) and anti-CD19 after co-culture for 4 h at an effector/target ratio of 2:1. Dead M12 cells will stain with both markers while viable cells will exclude the nuclear dye (Supplementary Fig. 2A). As shown in Fig. 2C (blue bars), after 4 h co-culture with 287-CAR T cells, less than 50% of the M12.C3 (I-A^{g7}-B:R3) cells remained viable, while > 85% excluded PI after co-culture with 24.1-CAR T cells (Fig. 2C, green bars). The ~10% increase in PI positivity from baseline observed in the co-cultures of M12.C3(I-A^{g7}-B:R3) and 24.1-CAR T cells was also seen in 4 h cultures of aAPCs without T cells (not shown) and co-cultures of M12.C3.G7 cells with either 287 or 24.1 CAR T cells (Fig. 2C, purple and brown bars) suggesting that this rate of cell death is intrinsic to the lymphoma cell lines under the conditions used.

3.3. 287-CAR T cells home to pancreatic lymph nodes

To deplete APCs that express I-A^{g7}-B:R3 complexes 287-CAR T cells must enter PLNs where the insulin peptides are presented. To examine their homing capacity we measured the accumulation of GFP^+ T cells in PLNs by flow cytometry on days 3, 5, 9, and 14 post-transfer (Supplementary Fig. 2B). These results are summarized in Fig. 3A. At early time points (days 3 and 5) with one exception the highest levels of transferred cells were observed in the spleen, with similar proportions of antigen specific (red symbols) and control (blue symbols) CAR T cells evident in this tissue. However, the CARs did not appear to proliferate or survive in this location, and few GFP positive cells could be detected in spleens harvested from either test or control animals 9 or 14 days post-transfer. A similar result was observed with control (inguinal) lymph nodes. In contrast, in 50% of animals given 287-CAR T cells GFP^+ cells were clearly evident in the PLNs 9 and 14 days post-transfer, comprising > 5% of the total $CD8^+$ cells present in those animals (Fig. 3A, red symbols). This was not observed in mice given the control 24.1-CAR (Fig. 3, blue symbols), suggesting preferential homing/expansion of the 287-CAR T cells in PLNs containing APCs expressing their cognate antigens. The difference in expansion/survival of the 287-CAR T cells at later time points likely reflects the beneficial role of the CD137 signaling domain, with high levels of GFP^+ cells seen only in animals given the 3rd generation reagent. The transferred cells retained an activated phenotype in the PLNs, with > 90% expressing CD69 at day 5 post transfer.

To determine if the transferred cells could home to inflamed islets, in a second experiment we adoptively transferred CAR-T cells generated from congenic Thy1.1 NOD donors to 8 week old female NOD (Thy1.2) recipients. As shown in Fig. 3B, the transferred cells (red) were readily detectable in islets that had significant levels of B lymphocyte infiltration (green), confirming that they can home to the target tissue. Consistent with the previous flow cytometric analyses (Fig. 3A), the transferred cells could also be detected in PLNs by histological staining (data not shown).

3.4. A single CAR-T cell infusion can delay the onset of T1D in NOD mice, but the protection is lost with time

To determine whether 287-CAR T cells can mimic the protection

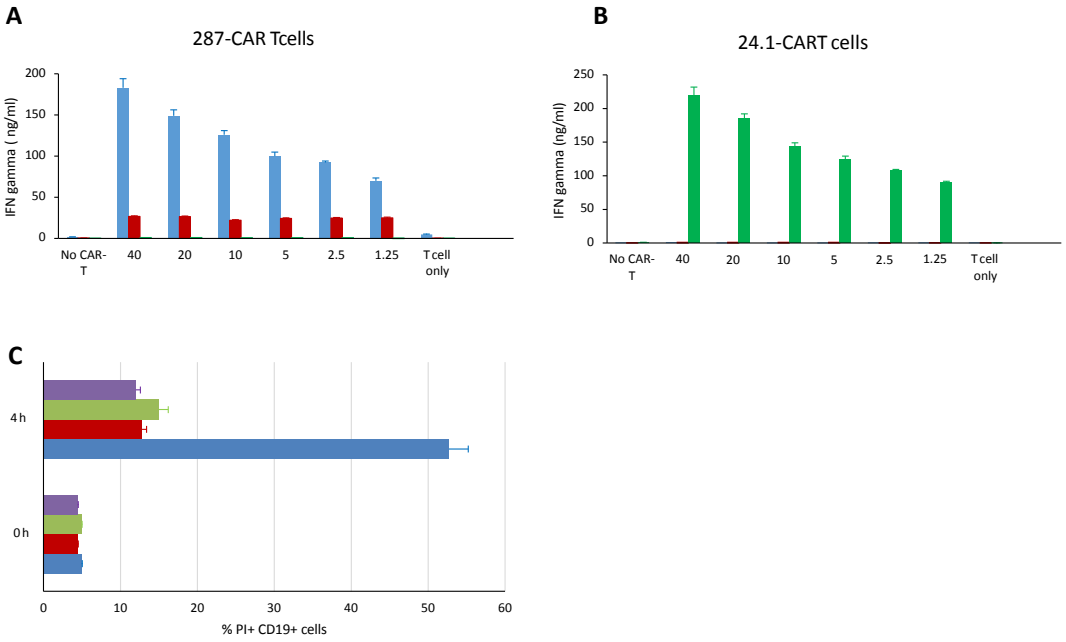

Fig. 2. Functional analysis of primary CD8$^+$ CAR T cells. **A, B**: FACS purified CAR T cells (4×10^4) were co-cultured with increasing numbers of M12.C3(IA^{g7}-B:R3) cells (blue bars), M12.C3 (IA^{g7}) cells (red bars), or M12.C3(TFR-MBP-DTRL) cells (green bars) for 16 h and secreted IFNγ quantified by ELISA. **C**: 287-CAR T cells (blue and brown bars) or 24.1-CAR T cells (green and purple bars) were co-cultured with M12.C3 (IA^{g7}-B:R3) cells (blue and green bars) or M12.C3 (IA^{g7}) cells (brown and purple bars) at an effector to target ratio of 2:1 for 0 or 4 h. Cells were then co-stained with Allophycocyanin conjugated anti-B220 and propidium iodide and immediately analyzed by flow cytometry. The percentages of PI positive B220$^+$ cells are shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

provided by the parent antibody, we infused expanded cells into 5 week old female NOD mice and monitored the development of T1D in the treated animals. Three batches of mice were treated using CAR T cells from three separate transductions. The pooled results from this experiment are shown in Fig. 4. Overall, treatment with 287-CAR T cells showed modest protection from disease compared to untreated mice ($p = 0.022$ by Gehan-Breslow-Wilcoxon test) but no significant difference when compared to the 24.1-CAR T cell treatment ($p = 0.27$).

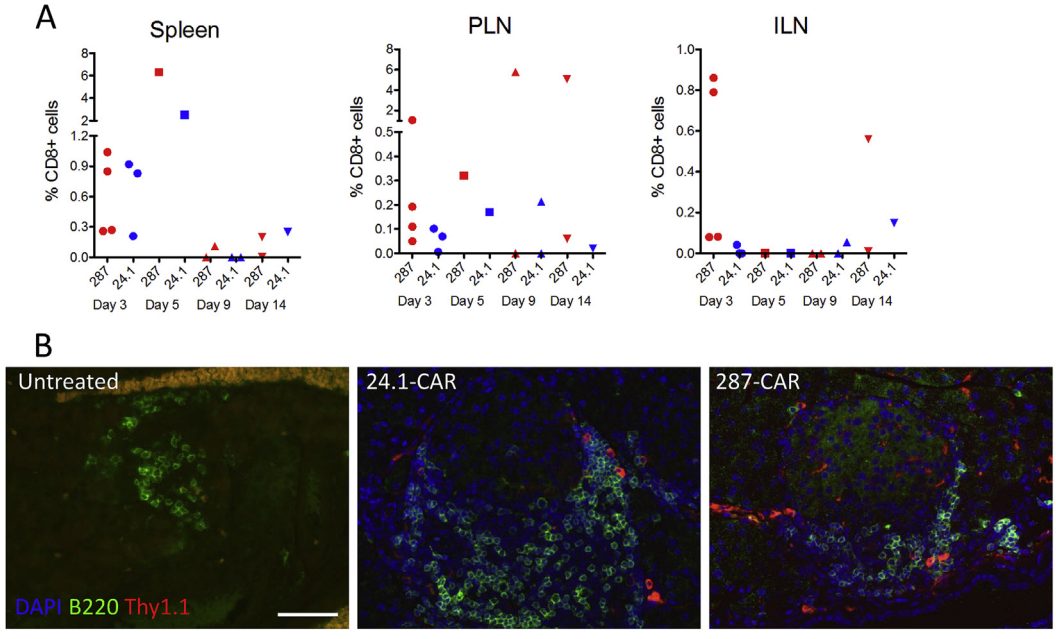

Fig. 3. Distribution of CAR T cells in secondary lymphoid tissues and pancreas after adoptive transfer. Expanded CAR T cells (3–5 x 10^6) were transferred to 5-week old (A) or 8 week old (B) NOD mice. **A**. At the indicated times post transfer individual mice were euthanized and spleens, PLNs and ILNs harvested for flow cytometric analysis. Data represents the percentage of viable CD8$^+$ cells that co-express GFP for individual animals from 4 separate experiments. Animals given 287-CARs are indicated with red symbols and those given 24.1-CARs with blue symbols. Background signals determined using the equivalent gating strategy for tissues harvested from un-manipulated littermates have been subtracted. **B**. On day 5 post transfer pancreata were harvested, fixed with 10% formalin, and embedded in paraffin. Slides were stained with anti-Thy1.1 (red), B220 (green), and DAPI (blue). Representative immunofluorescence images are shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

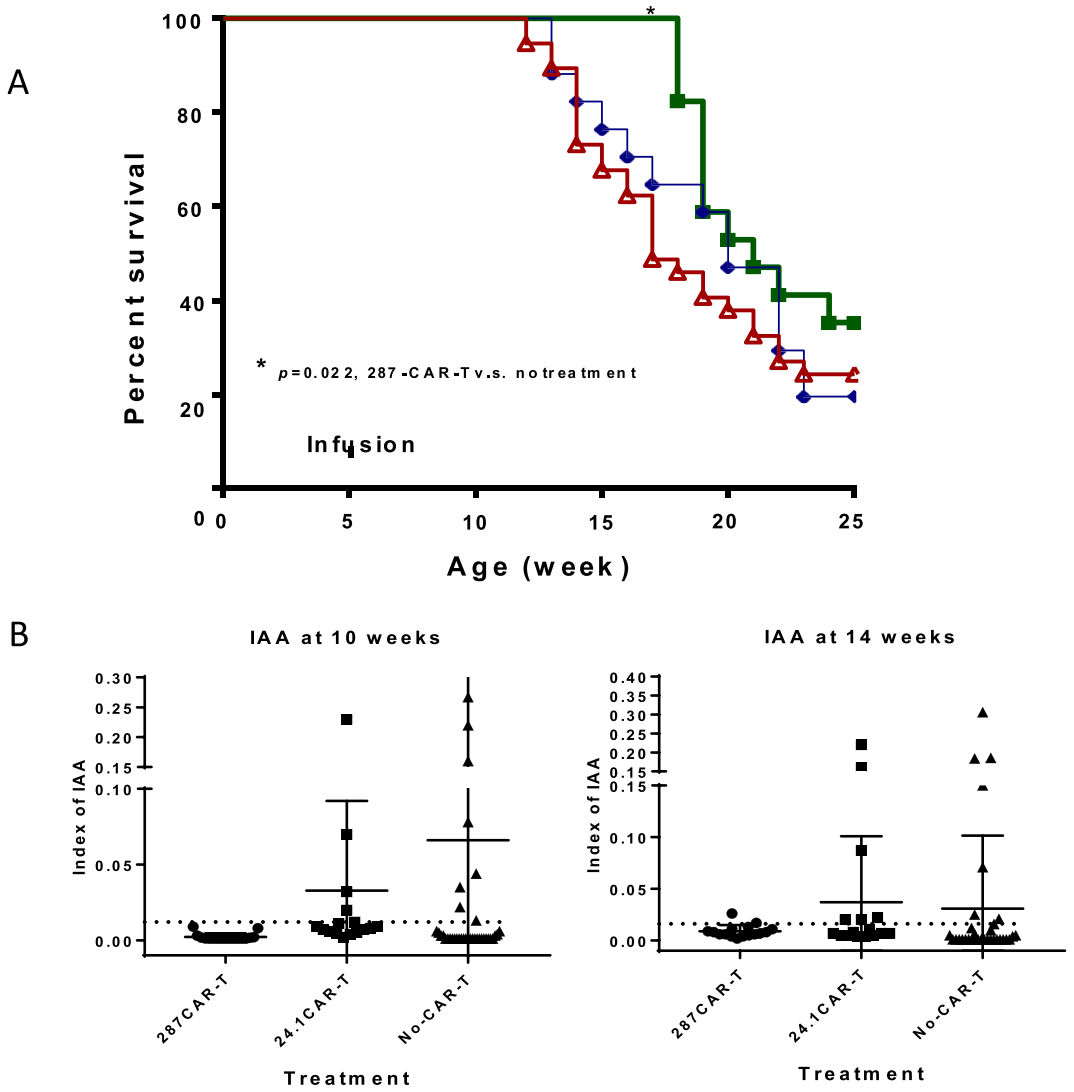

Fig. 4. Development of spontaneous T1D following adoptive transfer of CAR T cells. Groups of 5 week old female NOD mice were infused with 3–5x10^6 CAR T cells and monitored for persistent hyperglycemia for up to 20 additional weeks. Animals diagnosed with T1D were euthanized. **A**. Survival curves of un-manipulated animals (n = 38; red triangles), and those given 287-CAR T cells (n = 17; green squares), or 24.1-CAR T cells (n = 17; blue diamonds), are shown. Data is pooled from 3 separate control- and 287- CAR T cell preparations. *$p < 0.05$. Right table: the contingency table shows the number of diabetic and non-diabetic mice at 18 weeks. B. Insulin autoantibodies were measured in serum from 10 week old (left panel) and 14 week old (right panel) animals. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2
T1D development in 18 week old treated and control mice.

Treatment	T1D mice number	Non-T1D number	Total number
287-CAR-T	0	17	17
24.1-CAR-T	5	12	17
No-CAR-T	20	18	38

The proportions of animals who had developed T1D by 18 weeks are shown. Data was analyzed by Fisher's exact test. 287-CAR-T v.s. No-CAR-T, $p = 0.0001$; 287-CAR-T v.s. 24.1-CAR-T, $p = 0.044$; 24.1-CAR-T v.s. No-CAR-T, $p = 0.147$.

In mice given the control 24-1 CAR T cells (diamonds) T1D first appeared after 13 weeks, 1 week later than in the untreated cohort (triangles). In contrast, no animal given 287-CAR T cells developed diabetes before 18 weeks of age (squares). Thus, while 5/17 (29%) 24.1-CAR T cell treated mice and 20/38 (52%) of untreated mice had developed T1D by 18 weeks of age, 17/17 287-CAR T cell treated mice remained normoglycemic at this time point ($p = 0.044$ v 24-1 CAR; $p = 0.0001$ v untreated; Table 2). However, protection was not maintained as animals started developing T1D after 18 weeks of age, and only 6/17 (35%) remained diabetes free by 25 weeks when the experiment was terminated (compared to 4/17 (23%) 24.1-CAR T cell treated mice and 9/38 (24%) untreated animals). Histological analysis of pancreata from non-diabetic 287-CAR treated mice at 25 weeks (n = 3) revealed both profound peri-insulitis and intact islets (Supplementary Fig. 3). In contrast, all of the diabetic mice, whether they received CAR-T cells or not, had severe insulitis, and very few intact islets could be found.

Like humans, the first evidence of islet autoimmunity in NOD mice is the appearance of autoantibodies to insulin (IAA), which in mice typically peak at around 8–10 weeks of age. To investigate if CAR-T cell treatment inhibits the development of autoantibodies, we measured serum IAA levels at 4, 10 and 14 weeks. As expected, no IAAs were detected at 4 weeks (1 week prior to cell transfer). In contrast, at 10 weeks IAA could be detected in both control groups, but remarkably, not in the mice treated with 287-CAR T cells ($p < 0.01$ vs 24.1-CAR T; $p < 0.05$ vs non-treated mice) (Fig. 4B). By 14 weeks, 5 of the 287-CAR T cell treated mice had developed IAA, and at this time point no

significant difference was detected between the frequencies of IAA positivity in the surviving members of the three groups. However, whilst there was no difference in overall frequency, there was an apparent difference in the titers of the positive sera. The median index of the test group was 0.013 (range 0.011–0.026). This contrasted with 0.071 (range 0.012–0.306) in the untreated animals ($p = 0.0453$) and 0.022 (range 0.011–0.220) in the 24.1-CAR treated animals ($p = 0.1013$). Together this data suggests that a single infusion of 287-CAR T cells can transiently suppress islet autoimmunity, but that the control-CAR T cells cannot.

As the development of IAAs were suppressed in the test animals we also investigated whether serum insulin levels were affected. As shown in Supplementary Fig. 4A, no significant difference in pancreatic tissue insulin levels was observed in any of the treated or nice treaded mice on day 5 post transfer, and no significant difference in circulating insulin levels was observed either at 12 weeks of age in the euglycemic mice either. Thus, CAR-T cell treatment did not appear to have any adverse metabolic side effects. Similarly, at the same time point no significant differences in pancreatic IFN-γ or TNF-α concentrations were seen (Supplementary Fig. 4B).

To determine the long term survival of the transferred cells PLNs were analyzed by flow cytometry, either upon diagnosis of T1D, or in normoglycemic animals sacrificed at 25 weeks of age (n = 3 for 287-CAR-T treated mice, n = 2 for 24.1 CAR-T treated mice, n = 3 for non-treated mice); no detectable GFP^+ T cells could be found at these time points (data not shown).

Adoptive transfer of “diabetic” splenocytes to NOD.SCID mice induces disease in the recipients. To determine if CAR T cells can also impact this model we transferred 5×10^6 splenocytes from recently diabetic NOD mice to a small group of NOD.SCID recipients either alone, or together with an equal number of 287-CAR or 24.1-CAR T cells. As shown in Supplementary Fig. 5, 287-CAR T cells could not suppress disease in this model, with all mice developing T1D by 7 weeks post-transfer, irrespective of treatment group. However, like the previous experiment, T1D developed first in the control animals with 2/5 animals given splenocytes alone (circles) and 1/5 animals given splenocytes plus 24.1-CAR T cells (triangles) becoming hyperglycemic by 28d post transfer, a time point when 4/4 animals given splenocytes plus 287-CAR T cells (squares) remained normo-glycemic. Similarly, 10/10 control animals, but only 3/4 test animals, developed T1D by 40d post transfer. Thus, although the small group sizes preclude any firm conclusions from being drawn, these data are also suggestive that the antigen specific therapy may have provided some marginal benefit.

3.5. CAR-T cell transfer does not grossly change the immune system

To determine if the infusion of CAR-T cells had systemic side effects on the whole immune system, we evaluated the splenic populations of $CD4^+$, $CD8^+$, B and NK cells by flow cytometry on day-6 post transfer to. As shown in Supplementary Fig. 6, there were no significant difference in the percentages of CD4, CD8, B cells or NK cells among the three groups. Immune cells of the mice in the disease prevention study were evaluated at the age of 25 weeks, treatment did not cause significant change of immune cells (data not shown).

4. Discussion

There is an urgent need to develop safe and effective therapies for T1D. Although several agents that cause global immunomodulation have shown promise [1,30] none have so far provided durable protection from disease, suggesting that more sophisticated approaches will likely be required. Recent advances in *ex vivo* cell culture and cellular engineering have led to the development of innovative adoptive cell therapies (ACTs) that are transforming immunotherapy for many human cancers [10,31]. For example, redirection of T cells with CARs specific for CD19 has achieved impressive results in cases of refractory pre-B cell acute lymphoblastic leukemia and diffuse large B cell lymphoma, and is now FDA approved [10,31]. However, application of this technology to autoimmunity is currently lagging behind. One potential reason for this delay is a lack of suitable reagents to selectively target only those immune cells that are involved in the pathogenic process, while sparing those that are required for protective immunity. Our data described above provide a potential solution to this critical problem.

Research by our group and others has shown that the presentation of the insulin B:9-23 peptide in register 3 by $I\text{-}A^{g7}$ is critical for the activation of a key population of pathogenic T cells, and the concurrent initiation of islet autoimmunity, in NOD mice [4–6]. Moreover, T cells recognizing HLA-DQ8:B:11-23(R3) have also been isolated from the peripheral blood of humans with T1D [32], suggesting that there may be shared mechanism between mice and humans. Previously, we demonstrated that immunization with an antibody specific for the pathogenic $I\text{-}A^{g7}$:B:R3 complex (mAb287) could delay or prevent T1D in half of the treated mice [6]. We now report that a scFab variant of this antibody can also function in the context of a chimeric antigen receptor, both in vitro and *in vivo*, creating an alternative method to selectively re-engineer the immune system that may ultimately overcome some of the limitations of our previous protocol.

A potential drawback of our previous studies with the parental antibody was the need for weekly injections throughout the pre-diabetic period to achieve efficacy [6]. Such a dosing regimen is unlikely to be suitable for direct translation to the clinic, especially given the long prodromal phase between stage 1 T1D and the development of overt hyperglycemia in many individuals [30]. At present the precise mechanism of action of mAb287 is uncertain. Given that treatment causes a global decrease in insulitis, simple blockade of T cells specific for the target pMHC complex seems unlikely to be the major factor. One possible mechanism for the observed protection is that binding of mAb287 may induce antibody dependent cellular cytotoxicity resulting in selective deletion of APCs expressing $I\text{-}A^{g7}$:B:R3 complexes. This led us to the hypothesis that reprogramming cytotoxic T cells with a CAR based on mAb287 might achieve the same result, and if correct, provide proof of concept that this form of ACT could represent a viable treatment modality for T1D. The results described above support this idea. Specifically, in this study we demonstrate that a single dose of the 287-CAR T cells was sufficient to delay the onset of T1D in otherwise un-manipulated animals for approximately 6 weeks. This data suggests that CAR-T cells are likely a superior therapeutic modality to the parent mAb287, as a one-time treatment with mAb287 could neither prevent nor delay the onset of T1D (our unpublished data). Consistent with the observed delayed onset, 287-CAR T cells could home to, and survive in, PLNs, a primary site for activation of diabetogenic T cells. It is highly likely that the same APCs that present insulin B:R3 will also present other insulin peptides, as well as peptides from other β-cell components, and consequently that their selective deletion will lead to a significant reduction in islet autoimmunity to multiple autoantigenic targets by a process analogous to linked suppression. As discussed elsewhere [4,5], binding of the B:9-23 peptide in register 3 is highly unfavorable, and it is likely that the formation of such complexes is essentially restricted to APCs in the pancreatic draining lymph nodes and islet infiltrates where the high levels of antigen can compensate for the low affinity of binding. Like all other CAR-T cells, the 287 CAR-T cells retain their endogenous T cell receptors (TCRs), which may allow them to have specificities in addition to $I\text{-}A^{g7}$-B:R3 complexes. Research from other laboratories suggests that concomitant activation of the CAR and TCR can diminish the *in vivo* efficacy of CAR-CD8 T cells but not CAR-CD4 T cells [33]. To attempt to minimize interference from endogenous TCRs we used naïve T cells to generate the CAR-T cells. This will likely ensure that the majority of the transferred CAR T cells will express functionally irrelevant TCRs, although we cannot eliminate the possibility that a few may target islet antigens, and contribute to autoimmunity. Nonetheless, given that disease was not accelerated by either the control or test CAR T cells, we do not believe that the

potential presence of autoreactive TCRs within the transferred population represents a major concern for this form of ACT. Moreover, we believe that improved CAR-CD8 T cells with longer survival time *in vivo*, or CAR-CD4 regulatory T cells, which we are actively working on, will increase the efficacy of T1D prevention using our antigen specific immune therapy.

Unfortunately, although disease onset was delayed by 287-CAR T cell infusion, the effect was not durable. At present we cannot be certain of the reason for this, but our inability to recover any GFP positive cells from the diabetic animals suggests that the most likely explanation is that the transferred cells had only a limited lifespan. Indeed, the high rate of progression between 18 and 20 weeks observed in the animals treated with 287-CAR T cells is suggestive of the acute loss of an agent that had previously been keeping the disease in check. Such results are reminiscent of those obtained in early iterations of CAR T cell therapy for cancer, and could indicate that our current CAR design and/or dosing strategy is sub-optimal. As reviewed by Schubert and colleagues [31], the design of the intracellular signaling domain is a critical parameter for CAR T cell functionality *in vivo*. For these studies we selected two well studied variants, a “second generation” CAR having CD28 and CD3ζ domains, and a “third generation” CAR with an additional domain from CD137 (4-1BB). Both variants were well expressed (although primary cells transduced with the 2nd generation reagents tended to expand more efficiently in vitro than those transduced with the 3rd generation reagents), and all responded robustly and selectively to their cognate antigens. However, while both 2nd and 3rd generation 287-CAR T cells could be recovered from PLNs within 5 days of transfer, only cells transduced with the 3rd generation variant persisted *in vivo* beyond 2 weeks. Recently, June and colleagues reported that CAR T cell persistence can be enhanced through ICOS and 4-1BB co-stimulation [34], raising the possibility that replacement of the CD28 transmembrane and signaling domains with those from ICOS could improve the longevity of the 287-CAR T cells.

Beyond CAR design, another critical variable that remains to be optimized is the dosing strategy. Since our long term goal is to develop a therapy that can be translated to the clinic, we believed it important to attempt to minimize the number of infusions, and so for this proof of concept study focused only on a single ACT dose given early in the disease process. Although encouraging, the results obtained clearly indicate that with our current one-time CAR-T treatment strategy was not successful. At present we are uncertain why the protection waned with time but one possibility is that the amount of target antigen present at the time of treatment was insufficient to achieve robust proliferation, with those transferred cells that did expand eventually becoming exhausted. If this is the case, additional infusions at later time points may significantly improve efficacy. Alternatively, since our previous studies with the parental antibody demonstrated that it could also be effective in animals at the stage II/stage III transition [6], it may also be possible to change the initial infusion to the late pre-diabetic period [35] when the increased level of insulitis will mean that the density of IA^{g7}/B:R3 complexes on the target APCs will likely be much higher. Finally, we are also aware that our scFab might also be used to redirect other subsets of T cells, such as $CD4^+$ T regulatory cells (Tregs). For this proof of concept study our primary goal was to mimic the likely mode of action of the parent antibody, and hence we elected to redirect cytotoxic cells. However, a possible limitation of this approach is that by targeting their cognate APCs we may have negatively impacted the generation of memory T cells necessary to sustain the response for extended periods, or the expansion of induced or “natural Tregs. Since this is less likely to occur if the target APCs are modulated, rather than killed, we are actively exploring the feasibility of utilizing other T cell subsets, possibly using a 4th generation construct to boost or stabilize the regulatory function of the transduced cells.

In summary, in this pilot study we provide the first demonstration that CAR T cells can be used to selectively target APCs presenting pathogenic T cell epitopes relevant to autoimmunity. Although many variables remain to be optimized, we believe that our study provides significant support for the hypothesis that an effective antigen-specific adoptive cell therapies for T1D that is based on targeting disease relevant epitope presentation can be developed. Such a therapy is likely to have far fewer side-effects that other treatments currently in clinical trials.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jaut.2018.08.004.

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