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Towards Antigen-specific Tregs for Type 1 Diabetes: Construction and Functional Assessment of Pancreatic Endocrine Marker, HPi2-based Chimeric Antigen Receptor

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Abstract

Type 1 Diabetes (T1D) is an autoimmune disease marked by direct elimination of insulin-producing β cells by autoreactive T effectors. Recent T1D clinical trials utilizing autologous Tregs transfers to restore immune balance and improve disease has prompted us to design a novel Tregs-based antigen-specific T1D immunotherapy. We engineered a Chimeric Antigen Receptor (CAR) expressing a single-chain Fv recognizing the human pancreatic endocrine marker, HPi2. Human T cells, transduced with the resultant HPi2-CAR, proliferated and amplified Granzyme B accumulation when co-cultured with human, but not mouse β cells. Furthermore, following exposure of HPi2-CAR transduced cells to islets, $CD8^+$ lymphocytes demonstrated enhanced CD107a (LAMP-1) expression, while $CD4^+$ cells produced increased levels of IL-2. HPi2-CAR Tregs failed to maintain expansion due to a persistent tonic signaling from the CAR engagement to unexpectantly HPi2 antigen present on Tregs. Overall, we show lack of functionality of HPi2-CAR and highlight the importance of careful selection of CAR recognition driver for the sustainable activity and expandability of engineered T cells.

Introduction

Approximately 1.4 million U.S. adults and children have type 1 diabetes (T1D). Each year, more than 18,000 new diagnoses arise in people < 20 years old and the incidence is

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Credit authorship contribution statement

Ilian Radichev: Methodology, Investigation, Formal analysis, Writing - Original draft preparation. **Jeongheon Yoon**: Methodology, Investigation. **David W. Scott**: Conceptualization, Funding acquisition, Resources, Writing - review & editing. **Kurt Griffin**: Conceptualization, Supervision, Writing - review & editing. **Alexei Savinov**: Conceptualization, Funding acquisition, Resources, Supervision, Project administration, Writing - Original draft preparation.

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increasing, both in the U.S. and worldwide [1, 2]. While a minority within the population who have diabetes, those with T1D incur a disproportionate burden of costs, totaling $14 billion/year in the US [3]. One hundred years after its discovery [4], insulin remains the only approved primary treatment for T1D. While lifesaving, this therapy requires constant vigilance, remains dangerous to administer, and places tremendous burden on young patients and their families. There remains a critical unmet need to address the autoimmunity that is the underlying cause of T1D.

T1D typically presents itself in childhood with profound insulin deficiency due to the autoimmune destruction of β cells in the pancreatic islets of Langerhans. Hyper-activated, self-reactive T effector cells are believed to be at the center of the complex pathogenesis of T1D autoimmunity [5]. Accordingly, multiple attempts to design effective T1D-combating immunotherapies have used generalized immune suppression to fight the underlying cause of the disease. Immunomodulatory drugs such as cyclosporine have been shown to delay T1D progression, but its clinical utility is hindered by the broad immunosuppression it causes [6]. Although antibodies to islet antigens that characterize T1D patients are not pathogenic, B cells can function as professional antigen presenting cells to stimulate T cells. Immunomodulation trials targeting T cells (teplizumab), B cells (rituximab), or co-stimulation (abatacept) have been disappointing, showing similar temporary delays in C-peptide decline for 6–9 months, followed by a return to a rate of decrease parallel to that of the placebo group [7–9].

Insufficient suppression of autoimmune responses by regulatory T cells (Tregs) have been implicated in several autoimmune disorders. Patients with T1D have been demonstrated to carry both numeric and functional deficiencies in Tregs [10–13]. Accordingly, Tregs have attracted increasing interest as a potential T1D therapy. The Bluestone group has established a method to purify and subsequently expand polyclonal Tregs and completed a phase 1 dose escalation study of autologous expanded polyclonal Tregs in adults with T1D [14]. Although insufficiently powered to demonstrate efficacy, most subjects in this study maintained stable C-peptide responses to a mixed meal over one year, and had detectable Tregs, labeled with deuterium during the expansion, at one year. A small study from Trzonkowski group utilized a similar approach and found promising effects on preserving insulin production [15–17].The most recent pediatric phase 2 clinical trial of autologous expanded polyclonal Tregs in new-onset T1D ("T-rex," NCT 02691247) by Sanford Health and Caladrius Biosciences is expected to be completed in fall of 2020.

Despite the promise of efficacy in T1D, only a small subset of polyclonal Tregs recognize antigens relevant to T1D. While Tregs can have significant bystander effects by suppressing local T cells irrespective of antigen specificity [18, 19], concerns remain about the extent to which sufficient numbers of Tregs might localize to islets or the pancreatic lymph node following treatment with unselected polyclonal Tregs. As with current immunomodulatory drugs, there remains at least a theoretical risk that nonspecific, polyclonal Tregs therapies may impede appropriate immune responses against infection or cancer. Indeed, viral reactivation has been observed following treatment with polyclonal Tregs [20].

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Over the last decade, T cells genetically engineered to express Chimeric Antigen Receptors (CARs) have shown efficacy against a variety of cancers, mainly hematologic malignancies [21–23]. In this setting, the typical CAR consists of an extracellular antigen-binding domain, a transmembrane domain, an intracellular signaling domain from CD3ζ and costimulatory domains, such as CD28 or 4–1BB [24–26] expressed on T effectors that hone to the tumor and enhance its killing. Here, we used the CAR approach to design potential antigen-specific T1D immunotherapy. We built a chimeric receptor that recognizes endocrine cells from the islets of Langerhans, introduced this CAR into human peripheral blood Tregs, and tested their islet-recognizing and functional abilities.

1. Materials and Methods

2.1 Cell lines and human primary T cells

The mouse β cell line, NIT-1 (ATCC CRL-2055), HEK-293 cells (ATCC CRL-1573), and Phoenix-Ampho cells were obtained from ATCC (Manassas, VA). The human pancreatic β cell line, βlox5, was a kind gift from Dr. Clayton E. Mathews (University of Florida, College of Medicine, Gainesville, FL). All cell lines were routinely cultured in DMEM (Corning, Corning, NY) supplemented with 10% FBS (HyClone, Pittsburgh, PA) and 1% Penicillin-Streptomycin solution (Thermo Fisher Scientific, Waltham, MA).

Human T cells were isolated from Leukapheresis Packs (Stemcell Technologies, Cambridge, MA) after centrifugation through Ficoll-Paque PLUS medium (GE Healthcare Bio-Sciences, Pittsburgh, PA) followed by magnetic separation of collected PBMCs. $CD4^+$, $CD8^+$ and Tregs were isolated using EasySep Human $CD4^+$ T cell enrichment kit, EasySep Human $CD8^+$ T cell isolation kit, and Human $CD4^+CD127^{low}CD49d^-$ Treg cell enrichment kit (all from Stemcell Technologies), respectively, following the manufacturer's recommendations. Additionally, for some experiments Tregs were isolated by FACS-based sorting of $CD4^+CD25^{hi}CD127^{lo}$ cells, as previously described [18].

For co-culturing studies, 1×10^5 $CD4^+$ and $CD8^+$ T cells were incubated in 96 well plates (U bottom) in the presence or absence of either 2×10^4 βlox5 or 1×10^5 NIT-1 cells resuspended in complete RPMI. All cells were subsequently collected for FACS analysis as the T cells were distinguished from the co-cultured β cells by their positivity for CD3 or CD8.

2.2 Anti-HPi2 purification

Conditioned medium from mouse anti-HPi2 (clone HIC1–2B4.2B) hybridoma was obtained from Oregon Health & Science University (Portland, OR). The antibody was purified from serum components using Protein L coupled Sepharose (Abcam, Cambridge, United Kingdom). Briefly, 0.2 ml Protein L Sepharose slurry in binding buffer (100 mM sodium phosphate, 150 mM sodium chloride, pH 7.2) was incubated with 2 ml filtered conditioned medium for one hour at room temperature. After extensive washing with binding buffer, anti-HPi2 was eluted in 1 ml Elution Buffer (0.1M Glycine, pH 2.7). The low pH was neutralized with 0.1 M Tris-HCl pH 8.0. The purified antibody was then dialyzed against PBS and concentrated using Amicon Ultra with 10 kDa cutoff (Thermo Fisher Scientific).

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2.3 Anti-HPi2 variable region sequencing, and generation of HPi2-CAR construct

Sequencing of anti-HPi2 variable region was performed by Rapid Novor (Kitchener, Ontario, Canada) using their REmAb with WILD method to distinguish the isomeric residues such as Isoleucine and Leucine. Anti-HPi2 scFv (single-chain variable fragment) was constructed after backtranslation of the variable domains of the heavy and light chains joined by a DNA sequence coding flexible G4S linker. Resultant cDNA was used to replace FVIII-specific scFv in the ANS8 CAR construct [18] as a result introducing anti-HPi2-scFv immediately after a TCRα leader sequence and before IgG1 CH2-CH3 hinge, CD28 transmembrane, CD28 co-signaling, and CD3ζ signaling domains. HPi2-CAR was expressed from a pRetroX-IRES-ZsGreen1 vector (Clontech, Mountain View, CA) allowing easy confirmation transduction efficiency and sorting of CAR-expressing cells.

2.4 Viral production, transfection and transduction

Production of retroviral particles was performed after transfection of Phoenix-Ampho cells using a Pheonix-Ampno packaging system (Clontech) as previously described [27]. Viral supernatants collected at 48 and 72 hours post-transfection with either HPi2-CAR or control plasmid (Mock) were aliquoted and stored at −80°C. Sorted $CD4^+$ or $CD8^+$ T cells (1×10^6 cells) were pre-stimulated for 48 hours with Human CD3/CD28 T Cell Activator (Stemcell Technologies) before transduction in a Retronectin-coated 24-well plate [28]. Transduced T cells were then expanded for up to 3 weeks in the presence of 200 units of IL-2 (Stemcell Technologies) before re-stimulation.

Transfection of HEK-293 cells was performed in 10 cm dish using Lipofectamine LTX with Plus Reagent (Thermo Fisher Scientific) and 7.5 μg DNA following the standard manufacturer's protocol. Cells for immunofluorescence, FACS analysis and dot blot analysis were collected 48 hours post-transfection.

2.5 Antibodies and Flow cytometry

1×10^6 T cells or β cells collected as single-cell suspension in Cell Dissociation buffer (Thermo Fisher Scientific) were stained with anti-HPi2 (1:100) followed by incubation with 1 μg/ml of secondary AlexaFluor488-conjugated anti-mouse (Jackson ImmunoResearch Laboratories, West Grove, PA). Anti-CD3 (1:100), anti-CD8 (1:100), anti-Granzyme B (1:20) and anti-LAMP1 (1:20) were from Biolegend (San Diego, CA). Intracellular staining for IL-2 (1:20; Biolegend), IL-10 (1:20; Biolegend) and TGFβ (1:20; R&D Systems, Minneapolis, MN) was performed after fixation and permeabilization of T cells using Intracellular Fixation & Permeabilization Buffer Set from eBioscience (San Diego, CA). Dead cells were stained with either 7-AAD or LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (both from Thermo Fisher Scientific) for surface and intracellular staining, respectively.

Surface HPi2-CAR on HEK293 cells was analyzed after cell collection in Cell Dissociation Buffer, followed by an incubation with 5 μg/ml biotinylated Protein L (Abcam) and finally stained with APC-conjugated Streptavidin (1:200; Biolegend).

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For the proliferation studies, T cells were labeled with Cell Trace Violet (Thermo Fisher Scientific) before co-culturing with mouse or human β cells.

Staining of human PBMCs with anti-HPi2 was performed using a three step staining protocol. Briefly, PBMCs were treated with Human TruStain FcX (Biolegend) to block the Fc receptors and then stained with anti-HPi2 and AlexaFluor488-conjugated anti-mouse as described above. Cells were then extensively washed and incubated with anti-CD4-APC and anti-CD25-PE (both from Biolegend) before analysis.

FACS was performed on the BD LSR Fortessa (BD Biosciences, San Jose, CA). Data were analyzed using FlowJo (Tree Star, Ashland, OR) software.

2.6 Immunofluorescence

Frozen tissue array, fixed in 100% Acetone, was obtained from US Biomax (Rockville, MD). Mouse and human pancreatic sections (5 μm) were prepared in house from frozen Neg-50 Frozen Section Medium (Thermo Fisher Scientific). Tissue sections were stained with anti-HPi2 (1:50) and secondary donkey anti-mouse conjugated antibody (Jackson ImmunoResearch). Insulin staining with guinea pig anti-insulin (Abcam) was used to locate pancreatic islets in pancreatic sections. Slides were mounted in DAPI-Vectashield medium (Vector Laboratories, Burlingame, CA) and observed under an ECLIPSE Ni-E microscope (Nikon Instruments, Melville, NY).

2.7 Dot blot

βlox5 and HPi2-CAR- or Mock-transfected HEK-293 cells were lysed in modified RIPA buffer (25 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5% Triton-X100, 5 mM EDTA, 0.1 mM PMSF) supplemented with a Protease Inhibitor Cocktail (Thermo Fisher Scientific). Lysates of βlox5 (2 μg protein in 5 μl volume) were applied on a nitrocellulose membrane using a Bio-Dot Apparatus (Bio-Rad, Hercules, CA) and dried. The membrane was then blocked in blocking buffer (1×PBS, 0.1% Tween-20, 1% BSA) and incubated overnight at 4°C with the HEK-293 lysate (25 μg/ml) in blocking buffer. After washing with PBST (1×PBS, 0.1% Tween-20), the membranes were incubated for 1 hour at ambient temperature with 1 μg/ml Protein L-biotin followed by Streptavidin-HRP (1:5,000; Thermo Fisher Scientific). The membranes were then revealed using a chemiluminiscent detection system (Thermo Fisher Scientific). The membranes were scanned on a UVP Biospectrum 500 imaging system.

2. Results

3.1 Anti-HPi2 antibody recognizes human but not mouse islet cells

Despite the unique position as the sole insulin-producing cell type, human β cells are not solely distinguishable from the other cell types based on surface markers expression. In attempt to fill this void, a group from Oregon Health & Science University recently developed a panel of antibodies recognizing alpha, beta, exocrine, or duct cells from human pancreas [29, 30]. Based on the described specificity of those antibodies, we hypothesized that anti-HPi2 (HIC1–2B4) can be used to generate a CAR construct targeting human pancreatic islet cells. Hence, Tregs armored with such a CAR could be retained into the

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pancreatic islets, ultimately protecting them from an autoimmune attack in a T1D setting. In order to confirm the specificity of this antibody, we stained human and mouse frozen pancreatic sections with anti-HPi2 and insulin (Figure 1A). Our results corroborated the published data that anti-HPi2 specifically binds to human but not mouse islet cells without discriminating between insulin-secreting β cells and the other pancreatic endocrine cell subtypes. In order to confirm that this antibody can recognize live islet cells, we also used a FACS analysis of the human and mouse β cell lines βlox5 [31] and NIT-1 [32], respectively (Figure 1B). The FACS results clearly indicated that anti-HPi2 binds only to β cells from human origin.

Next, we proceeded with purification of commercially supplied anti-HPi2 monoclonal mouse antibody for the subsequent sequencing of its heavy (V_H) and light (V_L) chains. In order to avoid a potential contamination from bovine antibodies, which might interfere with the sequencing, we used Protein L-based chromatography to purify the anti-HPi2 from the conditioned media. Protein L binds to the kappa light chain variable domains [33] that are common for mouse and human but not bovine antibodies. After elution from Protein L-Sepharose, we confirmed that anti-HPi2 has high purity without any detectable contaminants by SDS-PAGE and staining with colloidal Coomassie (Figure 1C).

3.2 Generation of HPi2-specific CAR

After obtaining sequences for anti-HPi2's V_H and V_L recognition domains (Figure 2A), we engendered a single-chain variable fragment (scFv), in which the two chains were connected with a 3×(GGGGS) flexible linker. This anti-HPi2 scFv was then introduced in the place of a FVIII-specific single-chain variable fragment, removed from a previously published CAR construct [18] to create a HPi2-CAR (Figure 2B).

Previous studies have shown that CD4 co-receptor dimerizes through its D4 domain, a process that maybe critical to enhance signaling through CD4 [34, 35]. In order to check if the dimerization of CAR, including a potential hetero-dimerization with endogenous CD4 molecules, could augment CAR activity, we generated a second CAR construct (HPi2-CAR-2). In this CAR construct, the D4 and transmembrane domains of CD4 molecule were used instead of the CD28 transmembrane region and the CH2-CH3 hinge region of human IgG1 heavy chain (Figure 2B).

Next, we examined if the constructs have expected cell surface localization and if they recognize the HPi2 antigen in βlox5 cells (Figure 2C–F). HEK293 cells were transfected and the surface localization of CAR analyzed by FACS after biotin-Protein L staining instead of a primary antibody and Streptavidin-FITC staining (Figure 2C and D). Surprisingly, Protein L was not able to detect HPi2-CAR-2 on the cell surface, even though there was almost twice more cells expressing HPi2-CAR-2 construct compared to cells with HPi2-CAR (based on GFP expression). Simultaneously, approximately 90% of cells transfected with HPi2-CAR had positive staining on their surface, suggesting that HPi2-CAR-2 is either not folded properly to be recognized by Protein L, or it is not delivered to the cell surface due to folding obstacles and/or degradation.

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To verify that anti-HPi2 scFv portion of the receptor can recognize its antigen, and to address the possibility of HPi2-CAR-2 misfolding, we designed a dot blot experiment where a lysate from βlox5 cells was used as bait against lysate from HEK293 cells transfected with either mock plasmid or one of the CAR constructs (Figure 2D). The results showed a significant increase in Protein L binding when HEK-HPi2-CAR lysate was used (~3 fold over the HEK-mock); while binding of HEK-HPi2-CAR-2 lysate was just slightly elevated (~1.5 fold over the HEK-mock) despite its higher levels of expression. Additionally, in order to rule out any possibility that HEK293 cells have some inherited differences from T cells that might result in inability of a construct containing hinge-transmembrane portions of CD4 to be expressed on the cell surface we also checked the surface expression of our constructs in T cells 5 days post-transduction (Figure 2F). Just like in HEK293, HPi2-CAR-2 was not detected on the cell surface. Therefore, we concluded that the HPi2-CAR-2 construct does not have the potential to be used for T cell therapy.

3.3 HPi2-CAR activates T cells, stimulates their proliferation, and cytokine production

To examine whether HPi2-CAR can successfully activate T cells in presence of islet cells, we isolated $CD3^+$ T cells, transduced them with HPi2-CAR using a viral transduction method, and analyzed their proliferation after co-culturing with human or mouse β cell lines (Figure 3A). In order to have an internal control, we did not sort out the CAR-transduced cells. This allowed us to analyze both sub-populations in the same conditions after gating them in two groups: GFP-positive (CAR-expressing) and GFP-negative (non-expressing CAR). Our results showed that while both CAR-transduced ($CD3^+CAR^+$) and non-transduced cells ($CD3^+CAR^−$) were proliferating similarly when activated via antigen non-specific mechanism (with anti-CD3 and anti-CD28 antibodies), only the human βlox5 cells activated proliferation on $CD3^+CAR^+$ T cells. Moreover, mouse NIT-1 cells did not activate proliferation of neither CAR^+ nor $CAR^−$ $CD3^+$ T cells.

Using similar culture conditions as described above, we also assessed the activation levels of cytotoxic T cells and the degranulation of $CD8^+$ T cells that are present in the HPi2-CAR-transduced $CD3^+$ cells population (Figure 3B). We found that the percentage of cells with high Granzyme B staining in total $CD3^+$ population was slightly increased when T cells were co-cultured with βlox5 (~30%) in comparison to those cultured alone (~25%) or in the presence of NIT-1 (~24%). Similarly, we observed a strong increase of degranulation (based on LAMP-1 staining) in HPi2-CAR-expressing $CD8^+$ T cells co-cultured with βlox5 (76%) in comparison to the control conditions (18% in NIT-1 co-cultures, and 30% for T cells alone). Notably, despite being in non-activating conditions, the percentage of both Granzyme B-positive $CD3^+$ and degranulated $CD8^+$ T cells appeared to be relatively high in HPi2-CAR-transduced cells in comparison to non-transduced cells, showing a potential presence of tonic signaling in those cells.

Next, we analyzed expression of classical regulatory cytokines: IL-2, IL-10 and TGFβ in isolated human $CD4^+$ T cells transduced with HPi2-CAR to check if it could also activate anti-inflammatory responses in the presence of human β cells (Figure 3C and Supplemental Figure 1. Strikingly, we observed approximately 3-fold increase in the percentage of cytokine-producing GFP^+CD4^+ (HPi2-CAR-transduced) T cells when compared to mock-

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transduced $GFP^{+}CD4^{+}$ T cells, suggesting that HPi2-CAR is also functionally active in $CD4^{+}$ T cells with a regulatory phenotype.

3.4 Rapid collapse of HPi2-CAR T cells due to a broad expression of HPi2 antigen by multiple cell types

During the expansion step following transduction, we observed a rapid proliferation and formation of big colonies in T cells transduced with HPi2-CAR (Figure 4A). In contrast, mock-transduced T cells grew in smaller, but increased number of colonies. This observation suggested to us a possibility of strong activating tonic signaling in HPi2-CAR-transduced cells. Confirming such hypothesis was our growing curves during the expansion step. Generally, CAR-T cells are expanded for 2 to 3 weeks before their use for functional studies [36–38]; however, the expansion of our HPi2-CAR T cells not only stopped, but the numbers of cells in culture started to decline in less than two weeks post activation (Figure 4B). This effect was even more drastic for isolated $CD4^{+}CD25^{+}CD127^{low}CD49d$- Tregs, which pointed towards a substantial problem with the expansion of HPi2-CAR Tregs and prevented their complete functional testing. This observation was detected multiple times using either positively sorted by Flow Cytometry Tregs ($CD4^{+}CD25^{+}CD127^{low}CD49d^{-}$) as well as negatively sorted *via* magnetic beads ($CD4^{+}CD25^{+}$) Tregs with similar results (data not shown).

A recent study suggested that the HIC1–2B4 mAb, which we used to create the HPi2-CAR, may be binding to CD98 antigen [39]. CD98 is a heterodimeric transporter for neutral amino acids with multiple functions including cell activation and signaling, it can be found in multiple cell types. Moreover, CD98 expression is upregulated during lymphocyte activation [40–42]; CD98 was also reported to be expressed in regulatory T cells [43]. Therefore, we analyzed the specificity of anti-HPi2 antibody by immunofluorescence using a tissue array (Figure 4C). Our results showed that anti-HPi2 binds to majority of tested tissues with the strongest signal observed for cerebrum, kidneys and placenta. Finally, the analysis of PBMCs by FACS determined that all $CD4^{+}$ T cells are positive for HPi2 (Figure 4D), indicating that HPi2-CAR cannot be used for therapeutic purposes in Tregs as it provides constant tonic signaling which could be resulting in their exhaustion.

3. Discussion

T1D is a complex autoimmune disease in which activation of autoreactive T cells specific for β cell antigens results in diminishing insulin production, and ultimately, in impaired glycemic control. A major mechanism governing maintenance of peripheral tolerance is the suppression of autoreactive T effector cells by Tregs. Thus, multiple groups have hypothesized that increased Tregs' numbers along with improved retention in the vicinity of pancreatic islets would delay or even stop T1D-underlying autoimmunity (reviewed in [44]).

Tregs devotes a majority of its suppressive influence modulating the local microenvironment of inflamed tissue by producing anti-inflammatory soluble factors such as IL-10, TGFp and IL-35, funneling pro-inflammatory IL-2, and stimulating recruitment of other immunosuppressive cell populations such as T regulatory 1 (Tr1) cells and Tregs of different specificities [45–48]. Therefore, in order to raise Tregs' numbers in the islets and/or

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pancreasdraining lymph node(s), we need to generate Treg clones recognizing β cells antigens. Unfortunately, despite extensive research, insulin remains to be the only reliable marker for β cells. Insulin is a rapidly metabolized soluble peptide; it is delivered throughout the bloodstream to all parts of the body, and its concentration, while the highest in the islets, varies greatly during the day. Moreover, in T1D patients, exogenous insulin is injected into subcutaneous sites distant from the pancreas. All these features make insulin a questionable target for the CAR-based Treg therapy. Despite having a longer half-life, the C-peptide is also not an appropriate CAR target because, just like insulin, it is a soluble peptide and therefore not a dependable driver of islet-specific Tregs accumulation. Hence, finding cell surface molecules that are either β cell specific or at least explicitly present on pancreatic endocrine cells is crucial for a successful CAR-based T1D therapy.

The proof of concept that Tregs could protect pancreatic islets from an immune attack in mouse models of T1D was reported in recent studies where either insulin-specific CAR constructs [49] or CAR directed against MHC-mismatched pancreatic islet allografts [50] were used. However, both of these strategies have major limitations that make them hardly translatable into human T1D patients. Thus, we decided to assess the ability of anti-HPi2 monoclonal antibody (clone HIC1–2B4), specific for the human pancreatic endocrine cells, ([29] and Figure 1), to activate human CAR Treg cells when co-cultured with human islet cells (Figure 3). We hypothesized that equipping our proposed CAR with a recognition marker that is not limited to β cells, but is also present on all endocrine cell subtypes comprising islets of Langerhans would likely enhance retention of HPi2-CAR Tregs in islets surroundings, where the ongoing in T1D patients' autoimmune process is already wiping out vast majority of endogenous β cells.

Previously, it was shown that the hinge region connecting the scFv-recognition portion of CAR to its transmembrane domain might significantly affect CAR activation [51–54]. Therefore, along with the classical CAR hinge domain (CH2-CH3 portion of hIgG1), we also created a construct (HPi2-CAR-2) where as a hinge we used the D4 domain of CD4 molecule (Figure 2B). The poor CAR expression/function is an undesirable, but quite common feature described for multiple CARs constructed to target tumor antigens [53, 55, 56]. Similarly, as evident by the two factors, our HPi2-CAR-2 proved to be nonfunctional. First, HPi2-CAR-2 was not detected by FACS on the cells' surface (Figure 2D). This is either due to a disturbed trafficking, or to a folding issue of the anti-HPi2-scFv portion that prevented Protein L binding. Second, the lysates from the cells expressing HPi2-CAR-2 were not very efficient in recognizing their antigen from βlox5 cells (Figure 2E), despite the fact that HPi2-CAR-2 expression levels (judged from the ZsGreen intensity) were much more prominent (Figure 2D). All these data additionally highlight the importance of the hinge region for the CAR functionality. Further optimization of this domain might be important for improved CAR activity since studies from different groups showed that CH2-CH3 hinge might provide a low tonic signaling that could affect long-term survival of CAR-expressing cells [53].

In fact, tonic signaling, which usually stems from the antigen-independent CAR activation, is extensively studied and is considered to be one of the major problems hampering the use of CAR T cells as therapeutics in multiple cancers settings [55–60]. Such constant tonic

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conditioning, even at the low levels, has been demonstrated to initiate and enhance development of exhaustion [61]. Therefore, for the targets that in addition to the tissue of interest are also expressed in the normal tissues, fine-tuning of CAR signaling should be of a critical importance to minimize tonic signaling. Unfortunately, for the HIC1–2B4 mAb-based anti-HPi2 CAR we constructed, such fine-tuning seems to be impossible due to the high levels and broad spectrum of CD98 antigen (target of HIC1–2B4 mAb) expression (Figure 4). Moreover, the environment of constant tonic signaling enhances the possibility of some Tregs to convert into a cytotoxic T cells [62, 63].

CAR Tregs have great potential to be used as a treatment against autoimmunity including T1D [64], therefore, exploring target-specific CARs is crucial for developing new therapeutics. Regrettably, our attempt to use anti-HPi2-driven CAR was unsuccessful because the anti-HPi2 antibody appeared not to be exclusively specific for human pancreatic islets (Figure 4). Moreover, HPi2 antigen was highly expressed on all $CD4^+$ T cells, which resulted in quick proliferation collapse. Thus, HPi2-based CAR fell short of being a viable therapeutic approach; the future studies using alternative islet-specific targets should be explored. With the recent advancement of 10x single cell sequencing technology into β cell biology field, different reports suggested that NTPDase3 [65], DPP6 [66] and (FXYD2)Ya [67] could be such potential pancreatic β cell markers. Additional studies though are needed to prove their specificity as well as their ability to activate CAR-specific regulatory T cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- HPi2 antibody raised against human islet cells is not an exclusive islet marker.
- All $CD4^+$ T cells are also HPi2 positive.
- Rapid decline in expansion of T cells expressing CAR construct.
- Tregs are more sensitive to CAR-driven proliferation blockade than pan-$CD4^+$cells.

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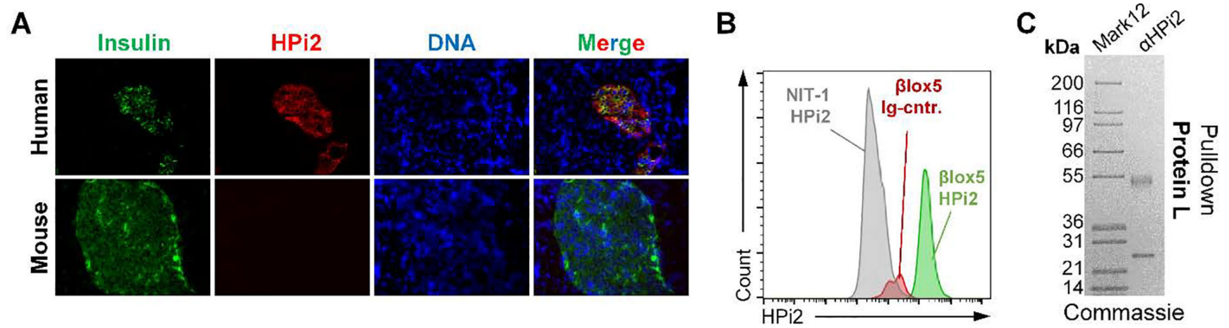
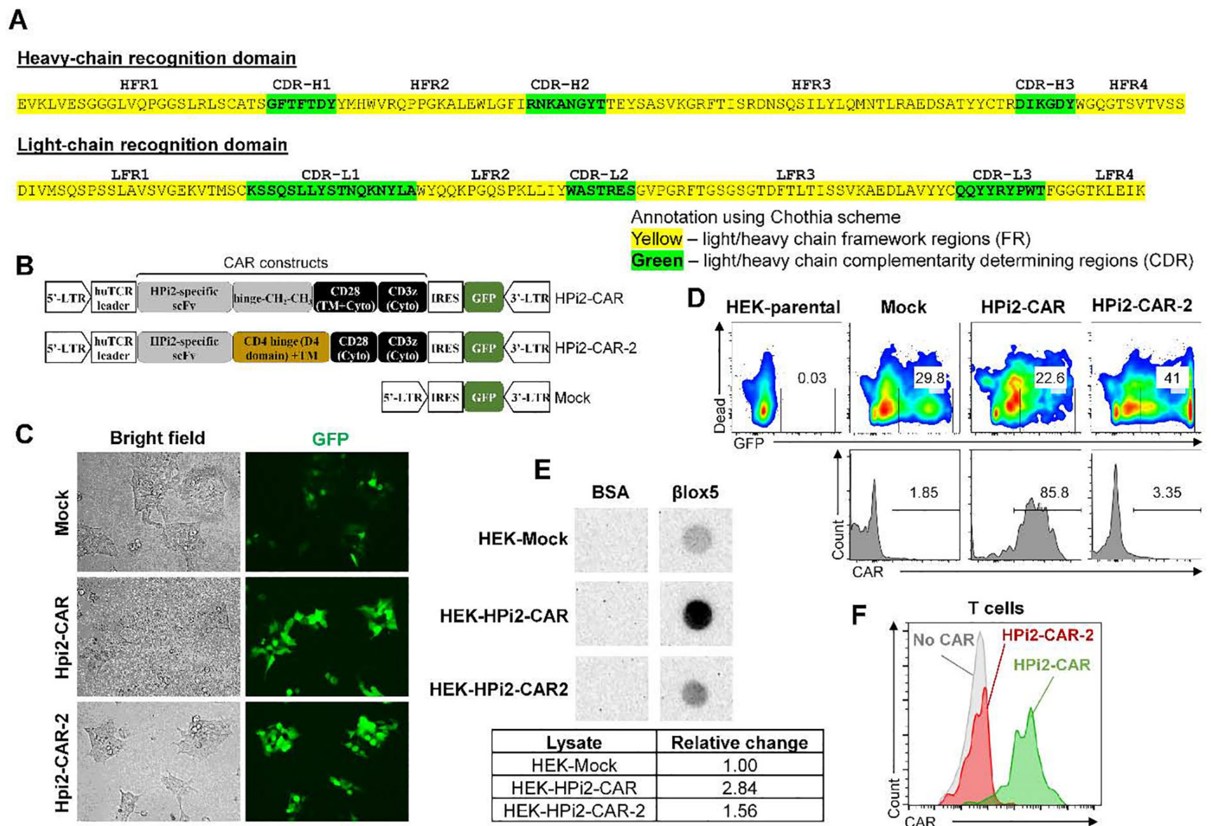


Figure 1. Anti-HPi2 recognizes an epitope on the surface of human but not mouse islet cells.
(**A**) Immunostaining of human and mouse pancreatic sections with anti-HPi2 (red). Islets were visualized after counterstaining with anti-insulin (green), and DNA – with DAPI (blue). (**B**) FACS analysis of human (βlox5) or mouse (NIT-1) β cells using anti-HPi2. (**C**) Coomassie staining of anti-HPi2 after its purification from conditioned medium using Protein L affinity chromatography.



Lysate	Relative change
HEK-Mock	1.00
HEK-HPi2-CAR	2.84
HEK-HPi2-CAR-2	1.56

Figure 2. Generation of and validation of HPi2-CAR construct.

(**A**) Amino-acid sequence of anti-HPi2's V_H and V_L recognition domains. (**B**) Design of the HPi2-specific CAR construct. (**C**) Validation of expression. HEK293 cells were transiently transfected with an empty plasmid (Mock) or a plasmid carrying HPi2-CAR and then analyzed by fluorescent microscopy for GFP expression 48 hrs post-transfection. (**D**) Flow cytometry–based detection of surface expression of CAR constructs in HEK293 cells after staining with biotinylated Protein L (recognizes the kappa chain of CARs' anti-HPi2 domain) and Streptavidin-APC. (**E**) Confirmation of HPi2-CAR specificity against human β cells by dot blot analysis. Cell lysates from βlox5 were blotted on a membrane (2 μg protein per spot) and then incubated with a lysate from HEK293 cells transfected with CAR or CAR-2 (HEK-HPi2-CAR or HEK-HPi2-CAR-2, respectively) or empty plasmid (HEK-Mock). After washing, the scFv portion of CARs was revealed using biotinylated Protein L and Streptavidin-HRP. The intensity of the signal was evaluated by densitometry (shown under the dot blot). (**F**) Flow cytometry-based detection of surface expression of CAR constructs in T cells. Human T cells were transduced with HPi2-CAR or HPi-CAR-2 and 5 days post-transduction the surface presence of the CAR constructs was analyzed as described in (**D**) in the ZsGreen$^+$ population.

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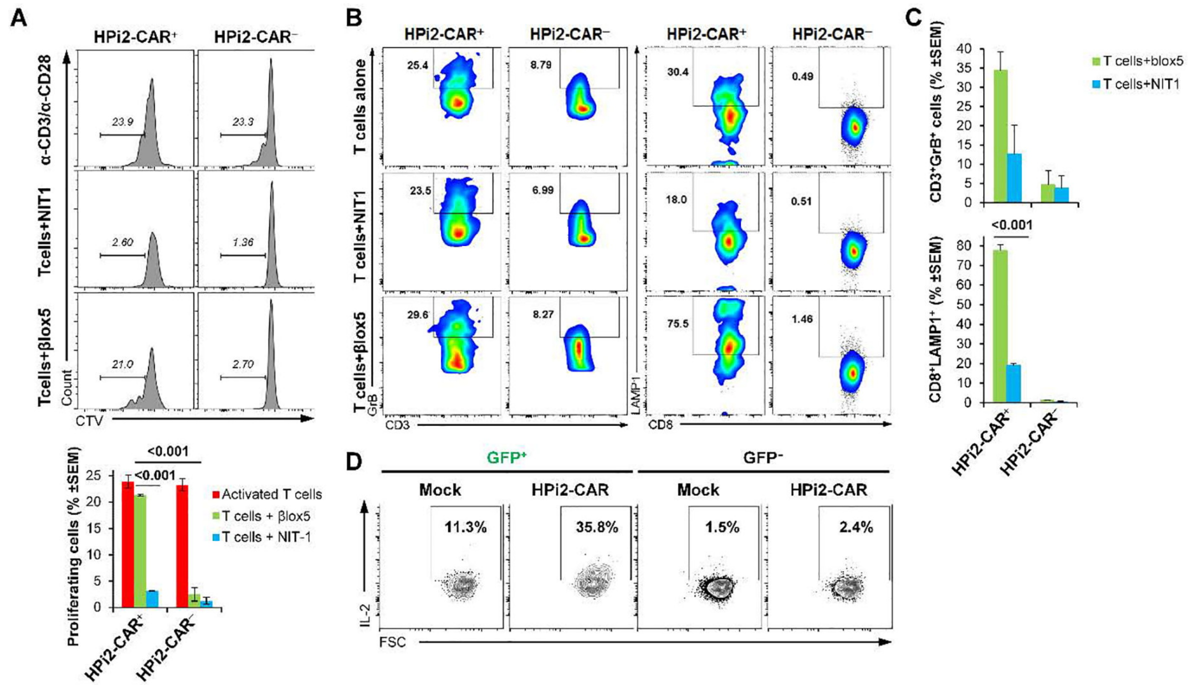

Figure 3. T cell-activation is stimulated by HPi2-CAR following co-culturing with human insulin-producing cells.

Isolated CD3+ (**A, B, C**) or CD4+ (**D**) T cells were pre-activated, transduced with HPi2-CAR and then expanded for 2 weeks before analysis of their ability to activate in the presence of human β cells by FACS. (**A**) FACS analysis of proliferating HPi2-transduced, CFSE-labeled CD3+ cells after 4 days of non-specific activation (α-CD3/α-CD28), or after co-culturing with either mouse (NIT-1) or human (βlox5) β cells; HPi2+CAR+, cell population gated for live GFP-positive CD3+ T cells; HPi2+CAR−, cell population gated for live GFP-negative CD3+ T cells; CTV, CellTrace Violet dye. (**B**) Representative plots of FACS analysis of CD3+ T cells' Granzyme B levels *(left two rows)* and a degranulation assay (measured by LAMP1) of CD8+ T cells *(right two rows),* performed 4 hrs post-incubation with or without β cells. (**C**) Comparison of Granzyme B (n=2) and LAMP1 (n=3) levels in HPi2-CAR+ T cells post co-culturing with NIT1 or βlox5 cells. (**D**) FACS analysis of IL-2 levels in HPi2-CAR or mock-transduced CD4+ T cells after incubation for 16 hrs with or without β cells.

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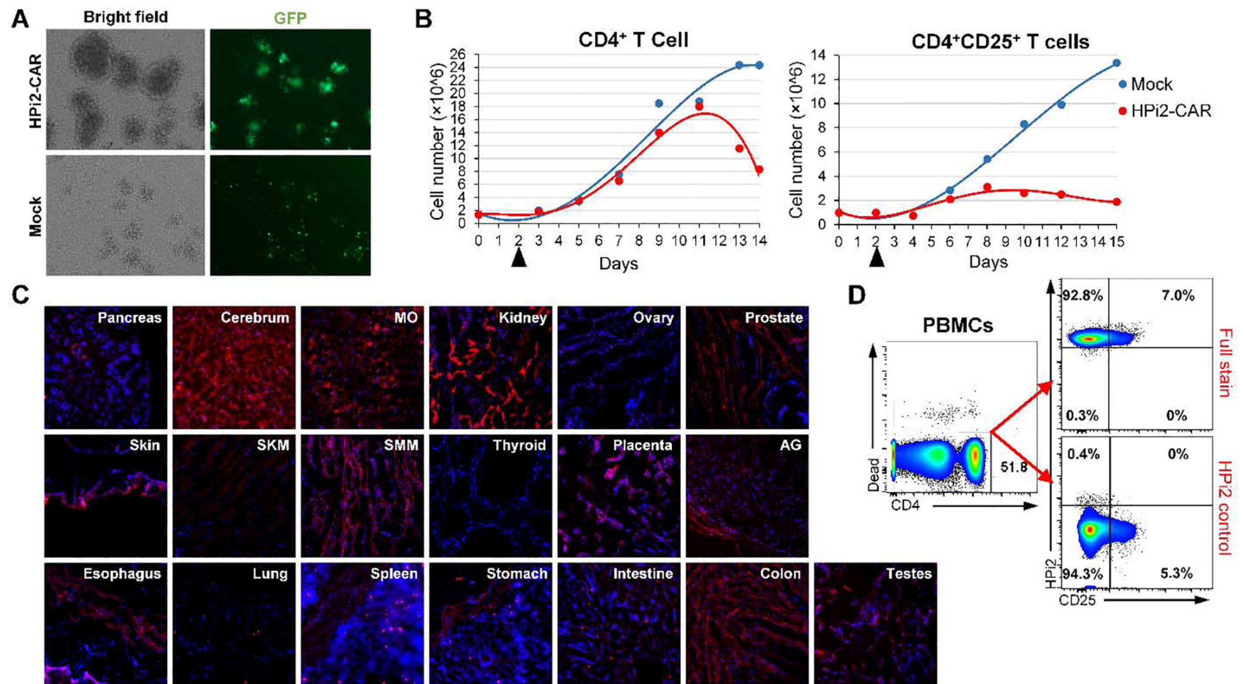

Figure 4. The HPi2 antigen is widely expressed in multiple tissues, including activated T cells, providing a constant tonic signaling.

(**A**) Microscopy of T cells transduced with HPi2-CAR or empty vector (Mock). (**B**) Growing curve of CD4$^+$ and CD4$^+$CD25$^+$ T cells after transduction with HPi2-CAR or Mock plasmid. 1×10^6 isolated T cells were stimulated with anti-CD3 for 48 hrs days before the transduction (arrowhead). (**C**) Immunofluorescence of tissue array with anti-HPi2 (red); *Blue* – DAPI. MO – *medulla oblongata;* SKM – skeletal muscle; SMM – smooth muscle; AG – adrenal gland. (**D**) FACS analysis of CD4$^+$CD25$^+$HPi2$^+$ cells within the isolated peripheral blood mononuclear cells (PBMCs)in live *lymphocytes gate. HPi2 control – the secondary antibody only*