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Generation of human islet-specific regulatory T cells by TCR gene transfer

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ABSTRACT

Based on the success in animal models of type 1 diabetes (T1D), clinical trials of adoptive regulatory T cell (Treg) therapy are underway using *ex vivo* expanded polyclonal Tregs. However, pre-clinical data also demonstrate that islet-specific Tregs are more potent than polyclonal Tregs at reversing T1D. Translation of this approach into man will require methods to generate large populations of islet-specific Tregs which, to date, has proved to be a major hurdle. Here we demonstrate the feasibility of lentiviral-mediated T cell receptor (TCR) gene transfer to confer antigen specificity on polyclonal human Tregs. Targeting has been achieved using TCRs isolated from human islet-specific and viral-specific $CD4^+$ T cell clones. Engineered T cells demonstrated expression of ectopically-delivered TCRs, resulting in endowment of cognate antigen-specific responses. This enabled antigen-specific suppression at increased potency compared to polyclonal Tregs. However, cells transduced with islet-specific TCRs were less responsive to cognate antigen than viral-specific TCRs, and in some cases, required additional methods to isolate functional antigen-specific Tregs. This study demonstrates the potential of TCR gene transfer to develop islet-specific Treg therapies for effective treatment of T1D, but also highlights that additional optimisation may be required to achieve its full potential.

1. Introduction

Type 1 diabetes (T1D) results from a profound dysregulation of the T-cell immune response. This is manifested by an expansion of pathogenic islet-specific T cells, leading to progressive destruction of pancreatic β-cells [1]. Populations of Tregs found in the periphery, including those expressing the transcription factor Forkhead Box Protein 3 (FOXP3) [2], are crucial in maintaining immunological tolerance. The fundamental role played by Tregs in controlling autoimmunity is clearly demonstrated by the human syndrome, 'Immune dysregulation, polyendocrinopathy, enteropathy, X-linked' (IPEX), where a loss-of-function mutation is present in the FOXP3 gene. This mutation leads to development of a range of autoimmune disorders, including T1D, in >80% of individuals by the age of 2. While it is now established that the frequency of Tregs in individuals with T1D is similar to that seen in control individuals [3,4], several studies have shown that the functional ability of Tregs to suppress autologous effector T cells is significantly reduced in individuals with T1D. This abnormality is evident before clinical diagnosis, at the time of diagnosis and many years following onset of T1D [4–7]. Since defective Treg function appears to be central to the pathogenesis of T1D, it is logical to hypothesise that correction of this imbalance may slow or prevent disease progression. Consequently, strategies aimed at increasing the number or functional potency of Tregs constitute a major focus of clinical trial activity relating T1D.

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One alternative option to therapeutically manipulate Tregs is the adoptive transfer of *ex vivo* cultured cells. This involves the isolation, expansion and/or invigoration of Tregs from an individual, followed by their re-infusion into the patient. This therapeutic approach has shown great success in animal models of transplantation and autoimmunity [8]. Clinical protocols have now been developed to generate large populations of human Tregs by magnetic bead enrichment or fluorescence activated cell sorting (FACS), followed by expansion *in vitro* in the presence of polyclonal stimuli and high concentrations of IL-2. Trials of Treg therapy are underway in several disease indications and have already shown significant clinical benefit in patients with Graft versus host disease [9–11]. In T1D, two clinical trials have been performed demonstrating the feasibility and safety of this approach [12–14].

While polyclonal Treg therapy has now reached the clinic, data from mouse models of T1D have shown that adoptive transfer of antigen-specific Treg populations is more potent at controlling T1D [15–18] and may even reverse disease [15]. To translate antigen-specific Treg therapy into the clinical setting, protocols to generate large populations of antigen-specific Tregs need to be developed. One approach to achieve this is to selectively expand Tregs with the desired specificity by stimulating Tregs with antigen presenting cells (APC) bearing the appropriate epitopes on the cell surface. Whilst this approach has been used to generate populations of alloantigen-specific Tregs for use in the field of transplant tolerance [19], applying these protocols in T1D would prove difficult due to the very low frequency of islet antigen-specific Tregs in the blood. An alternative method for producing antigen-specific T cells is by TCR gene transfer. Feasibility of this approach has been demonstrated in both mouse and human Treg systems [20–22], but only using model antigen-specific TCRs (e.g. virus or tumor specific TCRs). By contrast, this has never previously been achieved using TCRs isolated from *bona fide* autoreactive or islet-specific human T cells.

In the present study we tested the ability of TCRs isolated from two islet-specific T cell clones [23] to re-direct the antigen specificity of polyclonal human Tregs and compared the function of these transgenic Tregs to those generated using TCRs directed against viral antigens. Following lentiviral-mediated transfer of TCR genes, we successfully demonstrated expression of islet-specific TCRs, which resulted in the re-direction of antigen specificity towards cognate islet autoantigens. Islet-specific transgenic Tregs were capable of suppressing $CD4^+$ and $CD8^+$ effector T cell proliferation in a manner that was potentiated by exposure to the appropriate islet autoantigen. Notably, the ability of the islet antigen-specific TCRs to induce Treg activation and mediate antigen-specific suppression was significantly lower when compared to levels of activation or suppression achieved using TCRs with specificity for viral antigens. These findings demonstrate the feasibility of this approach for the development of antigen-specific Treg therapies for the treatment of T1D. They also raise questions regarding the optimal T cell populations to use as sources of autoreactive TCRs.

2. Materials and methods

2.1. Construction of TCR lentiviral plasmids

To identify the nucleotide sequences of each TCR, RNA was extracted from T cell clones using the RNeasy total RNA extraction kit (Qiagen). First strand cDNA was produced by 5′RACE (SMARTer 5′RACE kit, Clontech) and cDNA for TCR α and β chains were amplified in separate reactions using a TCR− chain specific and 5′RACE primers (TRBC primer: 5′-GCTGACCCCACTGTGCACCTCC TTCCC-3′ TRAC primer: 5′-CCAGGCCACAGCACTGTTGCTCTTGA AGTCC-3′), using Phusion polymerase (New England Biolabs).

DNA products were ligated into the pGEM-T easy vector (Promega) and three independently isolated plasmids were sequenced for each TCR chain. Sequences were analyzed using the International Immunogenetics information system (IMGT)/V-QUEST [24,25] to identify the allele usage of the TCR chain. Following identification of TCR gene sequences, a porcine teschovirus-1 (P2A) ribosomal skip sequence was constructed to link TCR α and β coding sequences using overlap extension PCR. *Avr*II and *Sal*I restriction sites were introduced to the 5′ and 3′ of the insert for ligation into the pELNSxm lentiviral backbone, which also encodes for mCherry as a fluorescent reporter.

2.2. Production of lentivirus and transduction of Jurkat cells

To produce lentivirus (LV), 293T cells were co-transfected with the following plasmids: TCR expression plasmid, and pCRV-1 [26] encoding viral *gag/pol* and vesicular stomatitis virus G (VSV-G) viral envelope protein. Vector was collected 72 h later, titrated and stored at −80 °C. Wild-type Jurkat cells (containing endogenous TCR) or J76 Jurkat cells (lacking an endogenous TCR) were transduced with LV by spin inoculation and transgene expression assessed by flow cytometry after 72 h.

2.3. Production and expansion of transgenic Treg populations

Peripheral blood mononuclear cells (PBMCs) were isolated from 100 ml fresh blood from HLA-compatible donors. PBMCs were stained with anti-human CD4-APC (BioLegend), anti-human CD25-PE (BD Biosciences) and anti-human CD127-Brilliant Violet 421 (BioLegend). Single lymphocytes were identified based on forward and side scatter parameters and $CD4^+CD25^{hi}CD127^{lo}$ cells were isolated as the Treg population using a BD FACS Aria III flow sorter and FACSDiva software (BD Biosciences). Isolated Tregs were activated with a 1:1 ratio of anti-CD3/CD28 coated microbeads (Dynal, Thermo Fisher) in round-bottomed 96 well plates at 5×10^4 cells/well in 200 μl Treg media (X-VIVO 15 media (Lonza) supplemented with 5% human serum (Sigma), 1% pen/strep/fungizone, 1200 IU/ml IL-2 (Proleukin Novartis) and 125 ng/ml Rapamycin (Rapamune, Pfizer)). Rapamycin was included in cultures to promote the selective expansion of FOXP3+ Tregs [27,28]. After 48 h, Tregs were harvested and spin inoculated at a multiplicity of infection of 4 TU/cell, washed and cultured in flat-bottomed 96 well plates, splitting cells as required. After 8–12 days, transduced Tregs were identified by expression of mCherry red fluorescent protein and isolated by flow sorting before being re-expanded as described above for 1–2 cycles. Following the final expansion cycle, Tregs were incubated in media lacking IL-2 and Rapamycin for 48 h prior to phenotypic and functional assessment. The purity of expanded Tregs was assessed by flow cytometry after staining with anti-CD4-APC, CD25-PE, and FOXP3-V450 using the eBioscience FOXP3 staining kit.

2.4. Assessment of antigen specificity of transgenic T cell populations

Initial testing of TCR function measured upregulation of the early activation marker CD69 on Jurkat cells. Briefly, Jurkat cells were incubated with HLA-compatible Epstein Barr virus (EBV) transformed B cells labeled with CellTrace violet (Life Technologies) at a ratio of 1:2 in round-bottomed 96 well plates at 37 °C, in the presence or absence of cognate peptide (listed in Table 1) or CytoStim (Miltenyi) as a positive control. After 16–18 h, cells were harvested, stained with anti-CD69-APC (BioLegend) and analyzed by flow cytometry. Assessment of TCR function in Tregs used a similar protocol, except CellTrace Violet labeled PBMCs were used as the source of APC at a ratio of 10:1 with transgenic Tregs.