

Natural variation in IL-2 sensitivity influences regulatory T cell frequency and function in individuals with long-standing type 1 diabetes

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ABSTRACT

Defective immune homeostasis in the balance between FOXP3⁺ regulatory T cells (Tregs) and effector T cells is a likely contributing factor in the loss of self-tolerance observed in type 1 diabetes (T1D). Given the importance of interleukin-2 (IL-2) signaling in the generation and function of Tregs, observations that polymorphisms in genes in the IL-2 pathway associate with T1D and that some individuals with T1D exhibit reduced IL-2 signaling, indicate that impairment of this pathway may play a role in Treg dysfunction and the pathogenesis of T1D. Here, we have examined IL-2 sensitivity in CD4⁺ T-cell subsets in 70 individuals with long-standing T1D allowing us to investigate the impact of low IL-2 sensitivity on Treg frequency and function. IL-2 responsiveness, measured by STAT5a phosphorylation, was found to be a very stable phenotype within individuals, but exhibited considerable inter-individual variation and was influenced by T1D-associated PTPN2 gene polymorphisms. Tregs from individuals with lower IL-2 signaling were reduced in frequency, were less able to maintain expression of FOXP3 under limiting concentrations of IL-2 and displayed reduced suppressor function. These results suggest that reduced IL-2 signaling may be used to identify patients with highest Treg dysfunction who may benefit most from IL-2 immunotherapy.

INTRODUCTION

Mechanisms leading to type 1 diabetes (T1D) depend on a complex combination of genetics (1-3) and environmental factors resulting in the breakdown of peripheral tolerance. We and others have reported that suppression of autologous conventional CD4⁺ T cells (Tconv) by CD4⁺CD25^{hi}FOXP3⁺ regulatory T cells (Tregs) in individuals with newly-diagnosed T1D (NDT1D) and long-standing T1D (LST1D) is reduced compared to age-matched control subjects (4-8). Although the precise reason for reduced suppressive activity has not yet been fully elucidated, several intrinsic defects in Tregs have been observed in (at least a subgroup of) individuals with T1D, including decreased IL-2 signaling, increased Treg apoptosis and decreased stability of Treg FOXP3 expression (5, 6, 9, 10). However, it is highly significant that, to date, all studies examining functional aspects of Treg biology have observed a large degree of overlap between individuals with and without T1D, with only a subgroup of T1D patients displaying the immune phenotype associated with reduced Treg function. Furthermore, Hughson and colleagues reported in a longitudinal analysis of Treg functions during the first year of T1D diagnosis that not only was there great heterogeneity in patient immunophenotypes but also that the time of sampling and the state of progression of the disease may affect Treg function (11).

IL-2 plays a key role in the generation and maintenance of peripheral fitness and function of Tregs in both mice and humans (12-16). Observations that polymorphisms in genes in the IL-2 signaling pathway associate with T1D (1-3) thus suggest that these genetic variants may alter T1D risk via effects on Treg numbers or function. In support of this, we and others have carried out candidate gene-to-phenotype studies and reported that multiple T1D-associated polymorphisms in the IL-2 receptor alpha

chain (IL-2RA/CD25) and protein tyrosine phosphatase 2 (PTPN2) genes conferred decreased IL-2 signaling in CD4⁺CD25^{hi} Tregs (9, 17-19). We further observed that the presence of the main T1D *IL2RA* susceptibility allele also associated with lower levels of FOXP3 expression in Tregs and a reduction in their ability to suppress proliferation of autologous Tconv (17).

Owing to their constitutively high expression of CD25 (20, 21), Tregs display enhanced sensitivity to IL-2 compared to Tconv and require lower IL-2 levels to support their development, homeostasis and function (17, 21, 22). This key characteristic underlies the use of low-dose IL-2 therapy to enhance Treg frequency and function. IL-2 administration in mouse models of autoimmunity has shown therapeutic effects (23, 24), and has also shown clinical efficacy in humans with chronic graft-versus-host disease (GvHD) (25, 26), hepatitis C virus (HCV)-induced vasculitis (27) and alopecia areata (28). Therefore, there is a strong rationale for investigating IL-2 immunotherapy in human T1D (29-31). Nevertheless, the immune system of a T1D patient is relatively normal (32-35) compared to lymphopenic patients (*e.g.* chronic GvHD) or patients with other severe inflammatory conditions (*e.g.* HCV-induced vasculitis). Furthermore, a recent phase I trial of IL-2/Rapamycin in T1D was terminated because there was a partial decline in beta-cell function (36). The doses and frequency of IL-2 dosing may have been too high in this trial and inadvertent Tconv activation could have accelerated beta-cell damage. These considerations highlight an urgent need to determine dose and frequency of dosing of IL-2 in T1D (31), including identification of baseline characteristics of a patient's immune system that could predict the level of response to IL-2.

Despite the interest in boosting Treg function in T1D by IL-2 administration, detailed studies directly linking IL-2 signaling with T1D-associated Treg immune phenotypes are lacking. To address this, we examined IL-2 sensitivity in CD4⁺ T-cell subsets in 70 individuals with LST1D and assessed the impact of low IL-2 sensitivity on Treg frequency and function. This study reveals extensive inter-individual variation in IL-2 responsiveness in Tregs that was stable within an individual and influenced by T1D-associated gene polymorphisms. In individuals with low IL-2 signaling, Tregs, especially of the antigen-experienced subset, were reduced in frequency. Furthermore, Tregs from these individuals were less able to maintain expression of FOXP3 under limiting concentrations of IL-2 and displayed reduced suppressor function. Our results indicate that stratification of trial participants by Treg frequency and IL-2 signaling capacity could be a useful strategy in the optimization of IL-2 therapy in T1D.

RESEARCH DESIGN AND METHODS

Subjects.

Blood samples were obtained from 18 non-diabetic controls and 70 individuals with LST1D (>3 years post-diagnosis, <40 years of age) at two time points >3 months apart. Large blood samples from two donors were used as internal biological controls in batch analyses of IL-2 sensitivity. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood samples by density gradient centrifugation (Lymphoprep; Axis-Shield PoC AS, Oslo, Norway) and either cryopreserved in fetal bovine serum (FBS; Gibco) with 10% dimethyl sulfoxide or used immediately for functional studies. In addition, fresh blood samples were obtained from age and sex-matched adult LST1D (n=22), non-diabetic controls (n=20), individuals with NDT1D (n=17; <2 years post-diagnosis) and autoantibody-negative unaffected siblings (UAS; n=15). Details of study participants are shown in Supplementary table 1. Ethical approval for this study was granted by the local ethics committee and informed consent was obtained.

Genotyping.

SNPs in the genes *PTPN2* (rs45450798 and rs478582) and *IL2RA* (rs12722495 and rs2104286) were genotyped using TaqMan 5' nuclease assays (Applied Biosystems) according to the manufacturer's protocol.

Monoclonal antibodies.

Antibodies used in this study are detailed in Supplementary table 2.

Flow cytometric analysis for pSTAT5a.

Phospho-STAT5a analyses for cryopreserved PBMC samples were carried out in a batch manner where each batch consisted of duplicate samples from eight individuals including six with LST1D and two biological controls (Supplementary Fig. 1). The assay was performed using a BD violet fluorescent cell barcoding kit (BD Biosciences). Briefly for the initial IL-2 sensitivity screen, cryopreserved PBMC samples were thawed and rested for 10 minutes at 37°C in X-VIVO-15 media with 1% human pooled AB+ sera (Sigma-aldrich, U.K.). PBMC were then stimulated with 0.1, 0.25 or 10 IU/ml hIL-2 (Proleukin; Novartis) for 30 minutes at 37°C, fixed with BD Lyse/Fix buffer for 10 minutes, washed in PBS and permeabilized with pre-chilled (-20°C) BD Perm buffer III for 30 minutes on ice. Cells were spun and resuspended in pre-chilled 50% BD Perm buffer III (diluted with PBS) and incubated with the barcoding dye mixture at 4°C for 30 minutes. After extensive washes with barcoding wash buffer, samples stimulated with the same IL-2 concentration were combined into a single FACS tube and stained with anti-CD4-FITC, anti-CD25-PE, anti-CD45RA-PerCP-Cy5.5 and anti-pSTAT5a-AlexaFluor647 for 1 hour in the dark at 20°C.

Analyses of pSTAT5a in cryopreserved PBMC from selected groups of high (n=12) and low (n=12) IL-2 responders were stimulated with 0.2, 0.4 or 10 IU/ml hIL-2 for 30 minutes at 37°C, barcoded and stained with anti-CD4-APC-eFluor780, anti-CD25-PE, anti-CD45RA-PE-Cy7, anti-FOXP3-AlexaFluor488 and anti-pSTAT5a-AlexaFluor647 using a combination of the BD violet fluorescent cell barcoding kit and BD Pharmingen Transcription Factor PhosphoPlus Buffer set (BD Biosciences, San Diego, CA, USA). Data acquisition was performed on a BD FACSCanto II (BD

Biosciences). Flow cytometry data was analyzed using FlowJo Software (Tree Star Inc., Ashland, OR). Dataset per IL-2 concentration for each cell subset was normalized across batch using the data from the two biological controls as detailed in Supplementary figure 1 to account for day-to-day variation. Phospho-STAT5a analyses for fresh whole blood samples were carried out as previously described (17).

Isolation and analysis of cell populations for functional studies.

Fresh PBMC were stained on ice with anti-CD4-qDot605, anti-CD14-AlexaFluor488, anti-CD19-Pacific Blue, anti-CD25-PE (M-A251) and anti-CD127-PerCP-Cy5.5. Single lymphocytes were identified based on forward and side scatter parameters and populations isolated for functional analyses using a BD FACS Aria II flow cytometer and FACSDiva software (BD Biosciences).

Assessment of maintenance of FOXP3 expression in Tregs in cultured with IL-2.

CD4⁺CD14⁻CD19⁻CD25^{hi}CD127^{lo} Tregs from fresh blood were stained with anti-FOXP3-AlexaFluor647 and anti-Ki67-FITC immediately post-sort and after 48 h cultured with or without limiting concentrations (0.1 or 1 IU/ml) of hIL-2 using FOXP3/Transcription Factor staining buffer set (eBioscience).

***In vitro* co-culture suppression assays.**

Suppression assays were performed in V-bottom 96-well plates using fresh PBMC by co-culturing 500 sorted CD4⁺CD25^{int-lo}CD127⁺ Tconv/well in the presence or absence of CD4⁺CD25^{hi}CD127^{lo} Tregs at various ratios with or without 1×10^3 CD19⁺CD4⁻ B cells as a source of accessory cells in X-VIVO-15 media with 10% human sera. Samples were stimulated either with PHA (4 µg/ml; ALERE) (APC-dependent assay)

or Human T-Activator anti-CD3/CD28 beads (Life Technologies) at a bead:Tconv ratio of 1:1 (APC-independent assay) and incubated at 37°C, 5% CO₂ for 6 days. Proliferation was assessed by the addition of 0.5 µCi/well [³H]thymidine (PerkinElmer, Waltham, MA) for the final 20 h of co-culture. All conditions were run in quintuplicate and proliferation readings (CPM) averaged. Samples with proliferation <3,000 CPM were excluded. In cultures containing stimulated Treg alone, in the absence of Tconv, proliferation was similar to the background of the assay (<500 CPM; mean=166). Percentage suppression was calculated as previously described (17).

Statistical analysis.

The normality of datasets was tested using the D'Agostino and Pearson omnibus normality test and unpaired Student's *t* test, ANOVA or Mann Whitney test was used as appropriate. Correlations were assessed using linear regression. One-tailed tests were performed if there was prior evidence of association otherwise values from two-tailed test were reported (GraphPad Software, Inc., La Jolla, CA, U.S.A.). Case-control matched data was analyzed using a bootstrap analysis (<https://github.com/nicholasjcooper/misc/blob/master/YangBootStrap.R>) accounting for a mixed design of pairs and trios, using the 'boot' package in R (www.r-project.org). Sample size and power calculations were calculated using Stata (www.stata.com) and detailed in Supplementary figure 6).

RESULTS

Assessing IL-2 responsiveness in individuals with LST1D.

In order to assess responsiveness to IL-2, we measured phosphorylation of STAT5a in cryopreserved PBMC from 70 individuals with LST1D following brief *in vitro* exposure to IL-2 (Fig. 1). Barcoding (37) and normalization methods were used to reduce intra- and inter-staining variability of the IL-2 sensitivity assay (Supplementary figure 1). An example of pSTAT5a staining and the gating strategy used to identify CD4⁺ T-cell subsets is shown in Fig. 2. As fixation precluded the use of CD127 as a surface marker, Tregs were identified based on CD4^{lo}CD25⁺ staining as previously described (17) (Fig. 2D). In addition, a more stringent definition was applied to identify Tregs by gating on the top 2% of CD25-staining CD4⁺ T cells (CD25^{hi} Tregs) (5, 6, 17, 38). As previously observed, sensitivity to IL-2 in this assay was lowest for naïve Tconv (nTconv), with memory Tconv (mTconv), CD4^{lo}CD25⁺CD45RA⁺ Tregs, CD4^{lo}CD25⁺CD45RA⁻ Tregs and CD25^{hi} Tregs showing successively higher sensitivities (Fig. 2F), which correlated with their respective CD25 expression levels (17, 21).

In order to investigate the stability of IL-2 responsiveness and identify individuals who show reproducibly high or low IL-2 responsiveness, we obtained two independent blood draws from each subject, separated by a minimum of three months. We observed considerable inter-donor variation in IL-2 responsiveness in all CD4⁺ T-cell subsets (Fig. 3). Notably, we observed a strong correlation between the two blood draws in all CD4⁺ T-cell subsets ($r^2=0.34-0.87$). Similarly, the frequency of T-cell subsets, including Tregs, was highly correlated between the two bleeds ($r^2=0.68-0.92$;

Supplementary Fig. 2).

Associations of T1D-associated *PTPN2* variants with IL-2 signaling.

Long and colleagues have reported an association between a T1D-associated variant (rs1893217) in *PTPN2* and reduced IL-2 signaling in CD4⁺ T cells in non-diabetic individuals (18). We observed a similar association in all Treg subsets between two independent *PTPN2* risk alleles of SNPs rs45450798 ($r^2=1$ with rs1893217) and rs478582 ($r^2=0.159$ with rs45450798) and reduced IL-2 signaling in our cohort of individuals with T1D ($P=4.4 \times 10^{-3}-0.02$; Fig. 4).

Relationship between IL-2 responsiveness and FOXP3⁺ Treg phenotype and frequency.

Expression of FOXP3 is currently the most reliable marker to identify *bona fide* Tregs by flow cytometry. However to date co-staining of FOXP3 and pSTAT5a in cryopreserved PBMC has been problematic. We, therefore, employed novel staining reagents optimized for this purpose. Using these reagents, we were able to reliably identify and delineate three different populations of CD25⁺FOXP3⁺ T cells as described by Sakaguchi and colleagues (39): resting (rTreg, FOXP3⁺CD45RA⁺, Fr. I), activated (aTreg, FOXP3^{hi}CD45RA⁻, Fr. II) and memory (mTreg, FOXP3⁺CD45RA⁻, Fr. III) Treg (Fig. 5A and B). To confirm the intrinsic differences in IL-2 signaling between individuals were maintained when this more definitive method for identifying Tregs was employed, we selected cryopreserved PBMC from subgroups of individuals with LST1D (n=24) with extremes of IL-2 signaling, identified based on IL-2 responses from their CD4^{lo}CD25⁺CD45RA⁻ and CD25^{hi} Tregs (Supplementary Fig. 3). We observed that low IL-2 responders maintained reduced

number of pSTAT5a⁺ cells in total FOXP3⁺ Tregs compared to high IL-2 responders ($P=3.6 \times 10^{-5}$; Fig. 5C) with the greatest difference being observed in the aTreg subset ($P=4 \times 10^{-4}$ -0.016; Fig. 5D-F). Kinetic analysis of IL-2 induced pSTAT5 induction in selected individuals demonstrated that the difference between high and low responders was maintained at several time points post stimulation (Supplementary Fig. 4A and B). No difference in IL-2 responsiveness was observed between the two groups at higher concentration of IL-2 (10 IU/ml), indicating that optimal/saturating IL-2 concentration could 'recover' deficient response observed in individuals with low IL-2 responsiveness (Supplementary Fig. 4C). Expression of CD25 was also reduced in all Treg subsets from low IL-2 responders, most notably in aTreg ($P=1 \times 10^{-3}$, Supplementary Fig. 5). Furthermore, we observed a reduced frequency of FOXP3⁺ Tregs in individuals with low IL-2 responsiveness ($P=1.8 \times 10^{-3}$; Fig. 6A). The greatest difference in Treg frequency between the two subgroups of individuals with LST1D was observed in aTreg ($P=5 \times 10^{-4}$; Fig. 6B). A similar difference was observed in mTreg ($P=0.011$), but not in antigen-inexperienced rTreg ($P=0.49$) (Fig. 6C and D).

Relationship between Treg fitness and IL-2 signaling.

To examine the relationship between IL-2 sensitivity and Treg fitness (FOXP3 maintenance and proliferation), we recalled the same subgroups of individuals with extremes of IL-2 signaling to examine fresh PBMC from a third blood draw. Consistent with the previous data in cryopreserved PBMC (Fig. 6), we observed a reduced frequency of Tregs in low IL-2 responders compared to high IL-2 responders ($P=7.1 \times 10^{-3}$ -0.03; Fig. 7A and B). Tregs were more proliferative (5-16% Ki-67⁺) *in vivo* compared to Tconv (0.8-3% Ki-67⁺), in agreement with previous reports (40).

However, no difference was found for the steady state proliferation of immediately post-sorted CD25^{hi}CD127^{lo} Tregs between high and low IL-2 responders ($P=0.64$; Fig. 7C and D). Previous studies have shown that defects in IL-2 signaling contribute to diminished maintenance of FOXP3 expression in Tregs in a subgroup of individuals with T1D (10). Here, we also observed that Tregs from high IL-2 responders cultured with limiting concentration of IL-2 (1 IU/ml) were better at maintaining FOXP3 expression compared to low IL-2 responders ($P=0.017$; Fig. 7E). Furthermore, the level of FOXP3 maintenance was observed to be positively correlated with the level of IL-2 signaling in FOXP3⁺ Tregs ($r^2=0.22$, $P=0.04$; Fig. 7F).

Relationship between IL-2 signaling and Treg suppressive function.

To determine if *in vitro* Treg suppressive capacity differs between the two subgroups of individuals with extremes of IL-2 signaling, we used an *in vitro* co-culture suppression assay. We observed reduced levels of suppression of Tconv proliferation in low IL-2 responders compared to high IL-2 responders under both APC-dependent and -independent conditions ($P=0.026$ and 0.036 , respectively; Fig. 7G). In cultures without Tregs, low IL-2 responders were observed to have increased Tconv proliferation compared to high IL-2 responders ($P=0.079$ and 0.027 for APC-dependent and -independent assays, respectively; Fig. 7H).

Comparison of IL-2 responsiveness between individuals with and without T1D.

Given the extensive inter-individual variation observed in our LST1D cohort we wanted to test whether IL-2 responsiveness in CD4⁺CD25⁺ T cells from individuals with T1D was different to non-diabetic controls in the light of two previous studies (9,

10). We compared IL-2 signaling in cryopreserved PBMC from 18 non-diabetic individuals recruited contemporaneously with the 70 individuals with LST1D. However, no difference in IL-2 sensitivity was observed in any CD4⁺ T-cell population between these two groups ($P=0.05-0.45$; Fig. 8A-D). In addition, we examined IL-2 sensitivity using fresh whole blood samples from 17 individuals with NDT1D and 22 with LST1D and compared these with 15 matched UAS and 20 non-diabetic controls, respectively. Samples from individuals with T1D were run in parallel with a non-diabetic control in an attempt to minimize inter-day variation. However, despite the paired nature of the study design, and consistent with the studies with cryopreserved PBMC, we observed a similar distribution of IL-2 responsiveness in individuals with and without T1D with no significant difference in IL-2 responsiveness observed between groups in any of the CD4⁺ T-cell subsets analyzed ($P=0.13-0.73$; Fig. 8E-G).

DISCUSSION

A major motivation for our research is to develop stratified or precision medicine for the treatment of T1D. This goal is based on detailed and reproducible knowledge of the mechanisms of disease and identification of accurately-measured phenotypes that not only measure the effects of potential immunotherapies but also might indicate at baseline (before drug administration) which patients might respond more or less than others. To this end, here we have established robust methods and procedures for measuring IL-2 signaling in T cells, including pSTAT5a measurement, in relation to Treg function. We discovered that Tregs from patients with low IL-2 responsiveness were less able to maintain the expression of FOXP3 under limiting concentrations of IL-2 and displayed reduced suppressor function with lower overall frequencies of Tregs in the circulation compared to individuals with higher IL-2 responsiveness. These results suggest that T1D patients with lower IL-2 responsiveness might benefit more, in terms of safely enhancing Treg function, from treatment with physiological, or ultra-low, doses of IL-2.

Assessing cell phenotype and function using fresh blood sample poses several technical challenges, especially when large sample sizes are involved. Using fresh blood, only a relatively few samples can be collected and tested on the same day leading to unavoidable day-to-day variation inherent when measuring the levels of intracellular proteins such as phospho-STAT. Here, we opted to assess IL-2 responsiveness in individuals with LST1D using cryopreserved PBMC from two independent blood draws. In order to reduce day-to-day variation, we carried out batch analyses and exploited barcoding (37) and normalization methods to minimize intra- and inter-assay variability. In accordance with the study by Long and colleagues

(10), we demonstrated that IL-2 responsiveness is a stable phenotype of CD4⁺ T cells within an individual with highly correlated IL-2 signaling between the two blood draws. Extensive inter-individual variation in IL-2 responsiveness was observed not only in cohorts with T1D but also in non-diabetic controls. We and, more recently, Yu and colleagues (21) failed to replicate the finding by others that individuals with T1D have reduced IL-2 responsiveness compared to controls (9, 10). We observed that controls present a similar distribution of IL-2 responsiveness as compared to individuals with T1D with a gradient of response with some controls displaying reduced IL-2 signaling. However, we acknowledge that larger sample sizes are required to rigorously address what may be a subtle phenotype. The level of heterogeneity of this assay between studies precludes us from accurately estimating a combined effect size for the sensitivity to IL-2 signaling. Given the wide range of effect sizes we estimate that sample sizes exceeding 300 individuals would be needed to reveal differences of <5% in IL-2 signaling between cases and controls (Supplementary Fig. 6). It is not surprising that some controls also present reduced IL-2 responsiveness, particularly as the degree of IL-2 responsiveness is influenced by polymorphisms in several genes in the IL-2 signaling pathway, such as T1D-associated variants in *IL2RA* and *PTPN2*, where non-diabetic individuals carrying the risk alleles were observed to have reduced IL-2 signaling in Tregs (17, 18). Here, in a cohort of individuals with LST1D, we replicated the association of T1D-associated *PTPN2* variant(s) with IL-2 signaling that was initially observed in non-diabetic controls (18) further supporting the robustness of our sample quality control and methods.

Definitive identification of *bona fide* Tregs by flow cytometry is problematic

especially as activated CD4⁺ T cells share many phenotypic characteristics with Tregs. For the initial assessment of IL-2 sensitivity in cryopreserved PBMC samples from individuals with LST1D, Tregs were identified based on high levels of CD25 expression, as previously described (5, 6, 17, 38). During the course of the study, we were able to robustly dual-stain for FOXP3 and pSTAT5a in cryopreserved PBMC to allow for more definitive gating of Tregs, thus enabling the confirmation of extremes of IL-2 signaling in FOXP3⁺ Tregs in selected individuals from the screening study.

A study by Long and colleagues observed both a reduced IL-2 responsiveness in CD4⁺CD25⁺ T cells from individuals with T1D and a reduced ability to maintain the expression of FOXP3, although it did not show a direct association of these two phenotypes (10). In the present study we established a direct link between IL-2 sensitivity and expression of FOXP3. One of the pleiotropic roles of IL-2 is its requirement for the maintenance of FOXP3 expression in Tregs (41) to sustain suppressive function since down-regulation of FOXP3 has been associated with a loss of suppressor function (42). Here we observed that Tregs from individuals with T1D with reduced IL-2 responsiveness indeed are inferior at suppressing proliferation by autologous Tconv compared to Tregs from individuals with high IL-2 responsiveness. Interestingly, in cultures without Tregs, proliferation of Tconv was increased in individuals with reduced IL-2 responsiveness upon T-cell receptor stimulation. We, therefore, cannot rule out that Tconv of low IL-2 responders may also have enhanced resistance to Treg suppression, a phenotype that we and others had previously observed in individuals with T1D (7, 43) or that cells in these individuals have an intrinsic proliferative advantage owing to deficient regulation. Recent results suggest that elevated production of IL-21 by Tconv, including T follicular helper cell, could

be part of the intricate balance between Treg and Tconv activities (33, 34, 44, 45).

Intriguingly, we consistently observed a reduced frequency of Tregs in individuals with reduced IL-2 responsiveness using both fresh and cryopreserved PBMC, due to a reduction in numbers of Tregs mainly of the antigen-experienced activated/memory phenotype. There was no difference between the *in vivo* steady state proliferation of Tregs in individuals with extremes of IL-2 responsiveness. Ghosh and colleagues observed a higher level of apoptosis in Tregs using fresh blood samples from individuals with T1D compared to control individuals, which may be partially mediated by IL-2 deprivation resulting in lower expression of anti-apoptotic genes (5, 6). We therefore compared apoptosis in isolated Tregs cultured with low-dose IL-2 and Treg expression of anti-apoptotic Bcl-2 in cryopreserved PBMC by flow cytometry in selected individuals with extremes of IL-2 responsiveness. However, we observed no significant difference between the two groups in any of these analyses (data not shown). The apparent disparity between these results and those obtained by Ghosh are likely due to methodological differences including use of fresh blood versus isolated/cultured or cryopreserved Tregs. Further studies are required to investigate the link between IL-2 responsiveness, Treg frequency and Treg survival.

IL-2-dependent STAT5a phosphorylation requires over 10-fold lower concentrations of IL-2 in Tregs as compared to Tconv subsets due to expressing higher levels of IL-2RA and common gamma chain and also, as recently observed, an increased activity of endogenous serine/threonine phosphatase 1/2A (17, 21). Well-tolerated ultra-low dose IL-2 has been shown to not only expand the frequency but also augment suppressor function of Tregs, albeit with significant inter-individual variation, in

healthy individuals (46). Most recently, Yu *et al.* demonstrated consistent increase in expression of FOXP3 and CD25 in Tregs, but not mTconv, for all individuals with LST1D upon treatment with low-dose IL-2 (21). Interestingly, their study also showed heterogeneous IL-2-dependent gene expression profile in Tregs in healthy subjects suggesting differential regulation of IL-2-dependent genes in an individual might impact the outcome of low-dose IL-2 therapy. In most, if not all, of these aforementioned IL-2 immunotherapy studies, the ability to enhance frequency of Tregs in response to IL-2 treatment is heterogeneous between individuals (26, 29, 30). As observed in our study and by others, because of the heterogeneity of the responses observed in individuals with autoimmune diseases it may be advantageous to characterize IL-2 responsiveness and/or IL-2-dependent gene expression profile and stratify individuals to be targeted for low-dose IL-2 immunotherapy for T1D and other immune-related diseases.

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FIGURE LEGENDS

FIG. 1. Study design to assess IL-2 sensitivity, fitness and function of Tregs in individuals with LST1D. Cryopreserved PBMC were obtained from 70 individuals with LST1D from two independent blood draws over three months apart. IL-2 sensitivity was assessed in these samples by measuring phosphorylation of STAT5a in response to various concentrations of IL-2. CD4⁺ Tconv and Tregs were distinguished based on the expression for CD25 and CD45RA. Subgroups of high (n=12) and low (n=12) IL-2 responders were then selected for further functional investigation based on the IL-2 responsiveness observed in Tregs. IL-2 sensitivity was assessed again in the selected groups of high and low IL-2 responders where cell populations were distinguished based on the expression of both FOXP3 and CD25 and CD45RA. These two subgroups of individuals were recalled for a third bleed to obtain fresh PBMC to assess fitness and function of Tregs by measuring the expression level of FOXP3 in Tregs in cultured with limited concentrations of IL-2 and quantifying the suppression of CD4⁺ Tconv by Tregs.

FIG. 2. Representative examples of pSTAT5a staining, the gating used to define CD4⁺ T-cell populations and IL-2 dose response. A-C: Examples of pSTAT5a staining in CD4⁺ T cells upon stimulation with 0.1 (A), 0.25 (B) and 10 IU/ml (C) of IL-2 for 30 min, using barcoded cryopreserved PBMC stained with CD4, CD25, CD45RA and pSTAT5a. D: Tregs were identified using two different gating strategies: (i) based on a high level of CD25 staining and reduced CD4 staining (CD4^{lo}CD25⁺ Tregs) and (ii) gated on the top 2% of CD25-staining CD4⁺ cells (CD25^{hi} Tregs). Tconv were identified by low/intermediate levels of CD25 staining. E: All cell populations were analyzed for expression of CD45RA to delineate

populations of CD45RA⁺ and CD45RA⁻ Tconv (pseudo-colour plot) and Tregs (zebra plot). F: An example of dose response curves in Tconv and Treg sub-populations from one individual using percentage pSTAT5a positivity as a read out.

FIG. 3. Relationship between IL-2 responsiveness in CD4⁺ T-cell subsets measured in two independent blood draws. Populations of Tconv and Treg subsets were defined as described in FIG. 2 and stimulated with the indicated IL-2 concentration. A-D: Percentages of pSTAT5a positive CD25^{hi} Treg (A), CD4^{lo}CD25⁺CD45RA⁻ Treg (B), mTconv (C) and nTconv (D) following stimulation of IL-2 were compared between first and second bleeds of patients taken over three months apart.

FIG. 4. Relationship between IL-2 sensitivity and T1D-associated *PTPN2* SNPs, rs45450798 and rs478582. Treg populations were defined as described in FIG. 2 and stimulated with the indicated IL-2 concentration. A-D: Percentages of pSTAT5a positive CD25^{hi} Treg (A and C) and CD4^{lo}CD25⁺CD45RA⁻ Treg (B and D) were compared between individuals with LST1D stratified by genotypes at rs45450798 (A and B) or rs478582 (C and D) in the *PTPN2* gene. Lines indicate group mean with standard error. Statistical significance was determined using a one-tailed one-way ANOVA.

FIG. 5. Example of FOXP3⁺ Treg and subset gating and the comparisons between IL-2 sensitivity in FOXP3⁺ Tregs of low and high IL-2 responders. A-B: Cryopreserved PBMC were stained for CD4, CD25, FOXP3, CD45RA and pSTAT5a. Tregs were gated on CD25^{hi} and FOXP3⁺ cells (A) and subdivided into three populations, FOXP3^{hi}CD45RA⁻ activated (Fr. II; aTreg), FOXP3⁺CD45RA⁻ memory (Fr. III;

mTreg) and FOXP3⁺CD45RA⁺ resting (Fr. I; rTreg) Tregs (B). C-F: Comparisons of percentage of pSTAT5a positive total FOXP3⁺ Tregs (C) as well as the three sub-populations (D-F) between low and high IL-2 responders when cells were stimulated with 0.4 IU/ml IL-2 for 30 min. Lines indicate group mean with standard error. Statistical significance was determined using a one-tailed Student's *t* test.

FIG. 6. Comparison of FOXP3⁺ Treg frequency between low and high IL-2 responders. A-D: Frequencies of total FOXP3⁺ (A), FOXP3^{hi}CD45RA⁻ activated (Fr. II; aTreg) (B), FOXP3⁺CD45RA⁻ memory (Fr. III; mTreg) (C) and FOXP3⁺CD45RA⁺ resting (Fr. I; rTreg) (D) Tregs out of total CD4⁺ T cells stained from cryopreserved PBMC were compared between low and high IL-2 responders. Lines indicate group mean with standard error. Statistical significance was determined using a two-tailed Student's *t* test.

FIG. 7. Comparison of Treg frequency and function between low and high IL-2 responders using fresh PBMC. A-B: Percentages of FOXP3⁺ (A) and CD25^{hi}CD127^{lo} (B) Tregs out of total CD4⁺ T cells were measured in low and high IL-2 responders. C: Tregs were cell sorted based on being CD4⁺, CD25^{hi} and CD127^{lo} for further functional studies. D: Steady state turnover of immediately post-sorted CD25^{hi}CD127^{lo} Tregs was measured based on Ki67 staining. E: Maintenance of FOXP3 expression was measured in Tregs in cultured with or without limiting concentrations of IL-2 for 48 h. F: Relationship between the level of FOXP3 maintenance and the level of IL-2 signaling. G: The suppression of proliferation of Tconv by Tregs was measured using tritium by *in vitro* co-culture at the indicated Treg:Tconv ratio, either stimulated with PHA using B cells as APCs or stimulated

with anti-CD3/CD28 beads for 6 days. H: Proliferation of Tconv cultured alone was measured under stimulation conditions as indicated. Filled squares = low IL-2 responders; open circles = high IL-2 responders. Lines indicate group mean with standard error. Statistical significance was determined using a two-tailed Student's *t* test.

FIG. 8. Comparisons between IL-2 sensitivity in age-matched individuals with and without LST1D. A-D: Comparisons of percentage of pSTAT5a positive CD4^{lo}CD25⁺CD45RA⁻ Treg (A), CD4^{lo}CD25⁺CD45RA⁺ Treg (B), mTconv (C) and nTconv (D) between 18 non-diabetic controls and 70 individuals with LST1D using cryopreserved PBMC, stimulated with the indicated IL-2 concentration for 30 min. Lines indicate group mean with standard error. Statistical significance was determined using a one-tailed Student's *t* test. E-G: Comparisons of percentage of pSTAT5a positive total FOXP3⁺ Tregs (E), mTconv (F) and nTconv (G) between 15 unaffected siblings (UAS) and 17 individuals with newly diagnosed T1D (NDT1D) and between 20 non-diabetic controls (Control) and 22 individuals with long-standing T1D (LST1D) using fresh whole blood, stimulated with the indicated IL-2 concentration for 30 min. Matched pairs of T1D and controls are joined by horizontal lines. Statistical significance was determined using an average matched-pair test.