Defects in IL-2R signaling contribute to diminished maintenance of FOXP3 expression in CD4⁺CD25⁺ regulatory T cells of T1D subjects

Running Title: Low IL-2R signal and FOXP3 persistence in T1D

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Objective: In humans, multiple genes in the IL-2/IL-2R pathway are associated with T1D. However, no link between IL-2 responsiveness and CD4⁺CD25⁺FOXP3⁺ regulatory T cells (Treg) has been demonstrated in T1D subjects despite the role of these IL-2-dependent cells in controlling autoimmunity. Here, we address whether altered IL-2 responsiveness impacts persistence of FOXP3 expression in Treg of T1D subjects.

Research design and methods: Persistence of Treg was assessed by culturing sorted CD4⁺CD25<sup>hi</sup>nTreg with IL-2 and measuring FOXP3 expression over-time by flow cytometry for control and T1D populations. The effects of IL-2 on FOXP3 induction were assessed 48 hours following activation of CD4⁺CD25⁺ T cells with anti-CD3 antibody. Cytokine receptor expression and signaling upon exposure to IL-2, IL-7 and IL-15 was determined by flow cytometry and western blot analysis.

Results: Maintenance of FOXP3 expression in CD4⁺CD25⁺ Treg of T1D was diminished in the presence of IL-2, but not IL-7. Impaired responsiveness was not linked to altered expression of the IL-2R complex. Instead, IL-2R signaling was reduced in Treg and total CD4⁺ T cells of T1D. In some individuals, decreased STAT5 phosphorylation correlated with significantly higher expression of protein tyrosine phosphatase N2 (PTPN2), a negative regulator of IL-2R signaling.

Conclusions: Aberrant IL-2R signaling in CD4⁺ T cells of T1D subjects contributes to decreased persistence of FOXP3 expression that may impact establishment of tolerance. These findings suggest novel targets for treatment of T1D within the IL-2R pathway and suggest that an altered IL-2R signaling signature may be a biomarker for T1D.
There are two major classes of FOXP3+ regulatory T cells (Treg): Natural Treg (nTreg) that develop in the thymus and control peripheral immune responses to self antigens and induced Treg (iTreg) that can be generated from peripheral blood CD4+CD25+ T cells (1,2). While the ontogeny of human peripheral blood FOXP3+ T cells is still debated, it is clear that IL-2 strongly influences the biology of both nTreg and iTreg (3-5). IL-2 regulates FOXP3 expression in a STAT5 dependent manner (6) and both IL-2 and STAT5 are required for the peripheral generation of iTreg (5,7) and maintenance of nTreg (8,9). The dependence of Treg on IL-2 has been clearly demonstrated in knock-out mice where deficiency of IL-2, IL-2Rα, or IL-2Rβ leads to early death due to severe autoimmunity caused by a lack of FOXP3+ T cells (10-12). In rare human cases, deficiency in IL-2Rα results in autoimmunity, lymphadenopathy, and persistent viral infection (13,14) while deficiency of STAT5b results in decreased frequency and function of Treg (15). Together, these data emphasize the essential role of IL-2/IL-2R signaling in peripheral tolerance mediated by Treg.

IL-2 is a T cell growth factor and key cytokine involved in immune regulation that is produced in a transient manner primarily by activated effector T cells (4). Binding of IL-2 to the high affinity IL-2R results in a wide range of biological responses including survival, differentiation and proliferation of multiple cell types including T cells. The IL-2R consists of a heterotramer composed of an alpha chain (CD25), a beta chain (CD122) shared with the IL-15R, and the common gamma chain (CD132) shared with the IL-7R, IL-9R, IL-15R and IL-21R. Engagement of the IL-2R results in a cascade of signaling events initiated by phosphorylation of the tyrosine kinases JAK1 and JAK3 followed by phosphorylation of tyrosine residues on the IL-2Rβ chain which results in phosphorylation of STAT5 and Shc. These proximal activation events lead to downstream signaling cascades resulting in activation of IL-2 dependent genes such as FOXP3 (16). Negative regulators of the IL-2 signaling cascades, including protein tyrosine phosphatases (PTPs), control the intensity and kinetics of these responses (17,18).

In NOD mice, there is a decrease in the frequency and function of Treg at the site of inflammation (19), as well as alterations in the IL-2/IL-2R pathway (20). Recent studies have linked defects in Treg in NOD mice to reduced availability of IL-2. These include reports of the association of the Idd3 susceptibility locus to decreased IL-2 production resulting in impaired Treg function and proliferation at sites of inflammation (21-23). Furthermore, this Treg defect in NOD mice can be rescued by treatment with exogenous IL-2 (22). In humans, the IL-2 gene itself and genes that participate in IL-2R signaling, including CD25 and PTPN2, have been implicated in the pathogenesis of T1D (20,24-28). However, to date, no studies have demonstrated a functional link between IL-2/IL-2R signaling and Treg deficits in humans diagnosed with T1D.

Maintenance of FOXP3 expression is essential for the function of Treg in vivo and has been shown in both mouse and man to be dependent on common gamma chain cytokines, with IL-2 playing a dominant role (7,9). Here, we demonstrate that maintenance of FOXP3 expression in both nTReg and iTReg is diminished in CD4+CD25+ T cells of T1D subjects when cultured in the presence of IL-2. This was associated with diminished IL-2R signaling in response to IL-2 as measured by decreased phosphorylation of STAT5, but not altered IL-2R expression. Decreased STAT5 phosphorylation correlated
with an increase in PTPN2 expression in CD4+ T cells of T1D subjects.

RESEARCH DESIGN AND METHODS

**Human Subjects:** Samples for this study were obtained from subjects diagnosed with T1D and control subjects with no personal or family history of autoimmunity who are participants in the JDRF Center for Translational Research protocol approved by IRBs at both Benaroya Research Institute and Seattle Children's Hospital. Subjects provided written informed consent prior to participation in the study. A total of 66 T1D (mean age 32±13, range 18-58) and 125 controls (mean age 35±12, range 18-67) were used in these studies. The number of samples used for each assay is indicated in figure legends. All experiments were performed in a blinded manner without prior knowledge of disease state.

**Antibodies and reagents:** BD Pharmingen (San Jose, CA) antibodies used include FITC conjugated CD25, AlexaFlour488 STAT5(pY694), PE conjugated CD25, CD122, CD132 and STAT5(pY694), PerCP conjugated CD4, APC conjugated CD4, CD45RO and CD25, and purified anti-CD3 (UCH11) and anti-CD28 (CD28.2). Intracellular AlexaFlour647 conjugated anti-FOXP3 (clone 259D) and matching isotype control were purchased from Biolegend (San Diego, CA). For immunoblots, goat polyclonal anti-PTPN2 antibody was purchased from R&D Systems (Minneapolis, MN), polyclonal rabbit anti-JAK1, polyclonal rabbit anti-JAK3 and monoclonal rabbit anti-STAT5 were purchased from Cell Signaling Technologies. Rabbit polyclonal anti-TFIIB antibody was used as a loading control (Santa Cruz Biotechnology, Santa Cruz, CA) with horseradish peroxidase coupled rabbit anti-goat IgG (Novus Biologicals, Littleton, CO) secondary antibody. IL-2 was purchased from Chiron (Emeryville, CA). IL-7 and IL-15 were purchased from BD Pharmingen. A small molecule inhibitor of PTPN2 (compound 8) was prepared as described (29).

**Flow cytometric analysis for phosphorylated STAT5:** BD Phosphoflow staining was performed as per manufacturer’s instructions. In brief, cells were activated with different concentrations of IL-2, IL-7, or IL-15 for 10 and/or 20 min, fixed with Phosflow Buffer I and permeabilized using BD Phosflow Buffer III prior to staining with anti-pSTAT5(Y694), CD4 and CD25. For some experiments, cells were co-stained with CD122 and CD45RO. Initial experiments were performed using freshly isolated PBMC. Similar results were obtained with thawed PBMC. All pSTAT5 data shown here are from previously frozen PBMC. Data were acquired using a FACS Calibur and analyzed using FloJo or Winlist software. Since CD4+CD25+ T cells are known to be sensitive to the freeze/thaw process and fix/perm staining protocols, samples where less than 1% of CD25+ T cells were detected were not analyzed for pSTAT5. pSTAT5(Y694) mean fluorescence intensity (MFI) data was normalized between experiments by determining the MFI fold increase (geometric MFI of the positive population ÷ geometric MFI of the negative control) as described previously (30).

**Isolation and activation of cells:** Human peripheral blood was obtained from donors and PBMC were prepared by centrifugation over Ficoll-Hypaque gradients. For some experiments, previously processed and frozen PBMC were used. CD4+ T cells and accessory cells were purified as described previously (31). For some freshly isolated samples, CD25hi (top 2-5%) were further isolated from CD4+ T cells by sorting using a FACS Vantage (BD Biosciences).

For persistence assays, sorted CD4+CD25hi T cells (nTreg) were seeded in a 96 well plate at a concentration of 1.5x10^6/ml in a total volume of 200µl. Cells were
cultured with media alone or cytokine without stimulation through the TCR. Cells were stained for CD25, CD4 and FOXP3 expression on different days following the sort. Surface expression of CD25, CD122 and CD132 was quantified using Quantum™ R-PE MESF beads (Bangs Laboratories) to calculate molecules of equivalent soluble fluorochrome (MESF).

For induction assays, CD4⁺CD25⁻ T cells were activated with 5µg/ml soluble anti-CD3 bound to irradiated (5000 rad) accessory cells at a concentration of 1.5x10⁶/ml as described previously (31). No cytokine, IL-2 (200 IU/ml) or IL-7 (10ng/ml) was added at the initiation of culture. FOXP3 content in iTreg was determined by flow cytometry.

**Western blot analysis:** Expression of IL-2 pathway proteins was analyzed in peripheral CD4⁺ T cells isolated from fresh PBMC. Whole cell lysates were separated by denaturing SDS polyacrylamide gel electrophoresis (20 µg protein per lane) using NuPage 10% or 4-12% Bi-Tris gels as indicated by the manufacturer (Invitrogen Inc., Carlsbad, CA) and transferred to Immobilon P membranes (Millipore Inc, Billerica, MA). Immunoblots were probed with primary antibodies followed by horseradish peroxidase coupled secondary antibody. Staining was detected by chemiluminescence (Perkin-Elmer Life Sciences, Wellesley MA). Protein expression was quantified by densitometry of films using ImageQuant software vs. 5.1 (GE Healthcare, Piscataway, NJ).

**Statistics:** For analysis of experiments comparing a single variable, statistical significance was determined using two-tailed independent or paired student’s t-tests as noted in the Figure legends. For analysis of multiple variables, an analysis of covariance (ANCOVA) was performed. All analyses were performed using GraphPad Prism v4.03 and all values with p <0.05 were considered significantly different.

**RESULTS**

**Maintenance of FOXP3 expression in CD4⁺CD25⁺ T cells is diminished in T1D subjects.** We compared persistence of FOXP3 expression in nTreg of T1D subjects and controls to determine whether the maintenance of FOXP3 expression is impaired in T1D subjects. Persistence of FOXP3 was determined by culturing nTreg (FACS sorted CD4⁺CD25⁺ T cells) in the presence of media or IL-2 and then measuring FOXP3 expression over time by flow cytometry, as diagramed in Figure 1A. Consistent with prior studies (7), FOXP3 expression in CD4⁺CD25⁺ T cells of control subjects was maintained in the presence of IL-2, but not media alone. In contrast, FOXP3 persistence was significantly decreased in nTreg of T1D subjects cultured in the presence of IL-2 as illustrated for one subject in Figure 1B and a cohort of 17 control (mean -6.42±8.2 SD, ∆FOXP3(day4-day0)) and 13 T1D (mean -17.98±15.3 SD, ∆FOXP3(day4-day0)) subjects in Figure 1C. This effect was specific to IL-2 as there was no difference between the study populations when nTReg were cultured in the presence of media or IL-7.

The decrease in FOXP3 persistence in T1D subjects did not appear to be due to differences in survival of nTreg as there was no difference in viable cell counts, absolute number of FOXP3⁺ cells or CD95 expression between control and T1D subjects when cells were cultured in the presence of IL-2 (Supplemental Data 1 in the online appendix which is available at http://diabetes.diabetesjournals.org). Moreover, FOXP3 expression in nTreg of controls, but not T1D, cultured in media could be rescued by addition of IL-2 mid-culture (Supplemental Data 2). It is possible that the composition of nTreg in control and T1D subjects differs, thereby contributing to altered persistence of FOXP3 in the presence of IL-2. However, we found no difference in
the frequency of resting CD45RA⁺FOXP3⁺ or activated CD45RA⁺FOXP3hi cells, two recently described subsets of nTreg (32,33), in the cohort of control and T1D subjects assessed in Figure 1 (Supplemental Data 3). In fact, demethylation of the FOXP3 gene, an additional marker of Treg (34,35), was similar in nTreg of controls and T1D subjects (Supplemental Data 4). Moreover, persistence of FOXP3 was comparable between control and T1D subjects in the presence of IL-7. Together, these data indicate that the decrease in FOXP3 persistence was not strongly influenced by differences in cell differentiation among the CD4⁺CD25hi sorted populations.

nTReg of T1D subjects display impaired IL-2R signaling. A decreased responsiveness to IL-2 in nTreg may be due to altered expression of components of the IL-2R or altered function of the IL-2R signaling cascade. Flow cytometric analysis was performed to examine the level of CD25, CD122 and CD132 expression on CD4⁺CD25⁺ T cells. In these studies, expression of IL-2R components was comparable between control and T1D subjects (Figure 2A). Additionally, no differences in the level of CD25 expression in the study populations were observed following four days of culture (data not shown).

To address function of the IL-2R signaling pathway, we measured phosphorylation of STAT5 (pSTAT5) in the CD4⁺CD25⁺ population by flow cytometry following exposure to IL-2 as shown in Figure 2B for a representative control and T1D sample. When analyzing a cohort of subjects, we found that the majority (85.83%±12.4 SD) of CD4⁺CD25⁺ T cells isolated from control subjects responded to IL-2 stimulation as measured by pSTAT5. In comparison, significantly fewer (72.44%±15.2 SD) CD4⁺CD25⁺ T cells of T1D subjects responded to IL-2 (p=0.0307)(Figure 2C). Similar differences in pSTAT5 between Treg of control and T1D subjects were observed at different time-points and with different doses of IL-2 (Supplemental Data 5). Moreover, the level of total STAT5 protein expression did not significantly contribute to the frequency of pSTAT5 as the level of total STAT5 protein did not differ between control and T1D subjects and the relative expression of STAT5 did not correlate with pSTAT5 frequencies for either population (Supplemental Data 6). Together, these data suggest that decreased IL-2 responsiveness in nTreg of T1D is linked to defects in IL-2R signaling.

To determine whether the effects of IL-2 on FOXP3 expression altered other IL-2 dependent Treg populations, we compared induction of FOXP3 expression in control and T1D subjects by stimulating CD4⁺CD25⁺ T cells with soluble anti-CD3 and irradiated APC in the presence of media alone, IL-2 or IL-7 and measured FOXP3 expression after 48 hours. Expression of FOXP3 upon activation in the presence of IL-2 was significantly lower in T1D than control CD4⁺ T cells while no difference was seen between the two populations when CD4⁺ T cells were activated in the presence of media alone or IL-7 (Figure 3A). To rule out the possibility that the decrease in FOXP3 expression in CD4⁺ T cells of T1D subjects stimulated in the presence of IL-2 was due to differences in total activation between control and T1D populations, we analyzed CD69 expression levels. We found that CD69 expression levels were comparable between the control and T1D study populations while FOXP3 expression in T1D subjects was diminished as compared to controls in the presence of IL-2 when analyzed with respect to CD69 (Figure 3B and C). Comparable results were obtained using CD25 as an activation marker (Supplemental Data 7). Thus, modulation of FOXP3 expression by IL-2 is diminished in both nTReg and iTReg of T1D subjects.
**Diminished IL-2 responsiveness in CD4^+ T cells of T1D subjects is a stable phenotype associated with impaired IL-2R signaling.**

To better define the mechanism of decreased IL-2 responsiveness in T cells of T1D subjects, we measured STAT5 activation in total CD4^+ T cells, as diagramed in Figure 4A following exposure to IL-2. Experimental conditions included exposure to media alone, IL-2, IL-15 or IL-7 all of which share the common gamma chain receptor component. IL-2 and IL-15 also share the IL-2R β chain. Consistent with other reports (30), the majority of CD25^+ T cells phosphorylate STAT5 in response to IL-2 while a subset of CD4^+CD25^+ T cells also respond to IL-2. When analyzing a cohort of control and T1D subjects, we found a significant decrease in the frequency of total CD4^+ T cells that phosphorylated STAT5 in response to higher (100 IU/ml) and lower (25 IU/ml) doses of IL-2 (Figure 4B). A similar decrease in responsiveness of CD4^+ T cells from T1D subjects was observed upon IL-15 exposure, but not upon exposure to IL-7 suggesting a defect in signaling associated with the IL-2Rβ chain which is shared between IL-2 and IL-15, but not IL-7 (Figure 4C). Similarly, the levels of pSTAT5 in response to IL-2 and IL-15 as measured by MFI were significantly decreased in T1D as compared to controls while responses to IL-7 were similar in these study populations (Figure 4D). The decrease in pSTAT5 observed here was not due to obvious differences in the composition of the T cell compartments of control and T1D subjects as no difference was observed between the frequency of control and T1D CD45RO^+ and CD122^+ CD4^+ T cells in this study (data not shown). Thus, CD4^+ T cells of T1D subjects display diminished responsiveness to IL-2 that may be associated with signaling through the β chain of the IL-2R.

To measure whether impaired IL-2R signaling is a property of CD4^+ T cells of T1D subjects, we compared pSTAT5 responses of CD4^+ T cells from the same individual stimulated with either IL-2 or IL-15. Overall, responsiveness to IL-2 correlated with responsiveness to IL-15 (Figure 5A). This was a stable phenotype, as CD4^+ T cells isolated from the same individual at different times responded similarly to IL-2 stimulation (Figure 5B). Thus, CD4^+ T cells of individual T1D subjects consistently display impaired responsiveness to both IL-2 and IL-15 suggesting an intrinsic property of T1D CD4^+ T cells linked to the β chain of the IL-2R.

**Increased expression of PTPN2 in T1D subjects correlates with decreased STAT5 activation.** Decreased pSTAT5 in T1D subjects may be due to alterations in the level of expression of molecules that participate in the IL-2R signaling pathway. To address this question, we measured protein expression levels of STAT5, JAK1, JAK3 and the negative regulator, PTPN2, in CD4^+ T cells of control and T1D subjects (Figure 6 and Supplemental Data 6 and 8). We then compared these expression levels to pSTAT5 responses following exposure to IL-2. No differences were found in STAT5, JAK3 or JAK1 protein expression comparing controls and T1D subjects. By contrast, PTPN2 expression was uniform in controls but was increased overall in T1D subjects with some T1D subjects showing a 4-fold greater level of expression as compared to controls (Figure 6C). This increased expression of PTPN2 correlated with decreased pSTAT5 following IL-2 exposure (Figure 6D), consistent with a down-modulatory role of PTPN2 (18). In T1D subjects with high PTPN2 expression, decreased IL-2 response could be rescued by pre-treatment with a PTPN2 inhibitor (29) (Figure 6E and F). In fact, where PTPN2, pSTAT5 and FOXP3 persistence were measured on the same individual, we found that a T1D subject with elevated PTPN2 expression also showed reduced pSTAT5
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(4.84% of CD4, 100 IU/ml for 10min) and diminished persistence of FOXP3 expression (-18.92, ∆ FOXP3(day4-day0)). Together, these data suggest that increased expression of PTPN2 in CD4+ T cells of some T1D subjects, may contribute to reduced levels of pSTAT5 in response to IL-2.

DISCUSSION

While the precise mechanisms by which IL-2 influences CD4+CD25+FOXP3+ Treg control of IDDM are currently being dissected in mouse models, many basic questions remain to be answered regarding FOXP3+ Treg in humans diagnosed with T1D. Previous studies in T1D subjects have focused on the number and function of Treg (36-41). Given the established role of IL-2 and IL-2R signaling in Treg maintenance (42) and the association of genes within the IL-2/IL-2R pathway with T1D (20,24-28), we investigated whether IL-2 dependent persistence of Treg was impaired in T1D subjects. We found that maintenance of FOXP3 expression in the presence of IL-2 was decreased in both nTReg and iTReg of T1D subjects. This correlated with alterations in the IL-2Rβ chain signaling pathway, as opposed to expression levels of components of the IL-2R, suggesting that this diminished responsiveness to IL-2 may contribute to loss of tolerance in T1D subjects.

The decrease in FOXP3 persistence observed here in nTreg and iTReg of T1D subjects suggests that resistance to IL-2 may result in a decrease in the persistence of Treg in vivo. However, the frequency of FOXP3+ T cells in the peripheral blood does not differ between control and T1D subjects (36). IL-2 is primarily produced by effector T cells upon activation at the site of inflammation and, in this manner, IL-2 production controls local Treg frequency and function (22,43,44) while IL-7 is produced primarily by stromal cells and can also support homeostasis of lymphocyte populations including FOXP3+ Treg (45). Thus, persistence of Treg in T1D subjects may be diminished at the sites of inflammation due to decreased responsiveness to IL-2 produced during inflammation while peripheral homeostasis of the FOXP3+ Treg population may not be affected. This is consistent with the observation that the frequency of Treg in NOD mice is impaired in the beta islets and pancreatic lymph nodes but not in peripheral sites (22). Alternatively, currently measures of Treg frequency detected ex vivo may not be sensitive enough to measure alterations in the biology of Treg, however, when challenged in vitro, deficiencies in the maintenance of Treg of T1D subjects may become more obvious. Here we show a deficit in Treg persistence in T1D subjects, however, immune regulation by Treg is also diminished in T1D subjects through an additional mechanism. Effector T cells of T1D subjects are more resistant to regulation than effector T cells of control subjects (40). Taken together, we suggest a model in which IL-2-dependent FOXP3 persistence may be decreased at the site of inflammation due to impaired responsiveness to IL-2 and effector T cells may be more resistant to regulation by these less stable Treg, together resulting in unregulated organ-specific inflammation.

Reduced IL-2 responsiveness is likely a result of impaired activation of STAT5 through the IL-2Rβ chain as CD4+ T cells of T1D subjects displayed diminished pSTAT5 in response to both IL-2 and IL-15, cytokines with receptors containing the IL-2Rβ chain, but not IL-7. Several mouse models shed light on the role of IL-2R β chain on FOXP3+ Treg biology (reviewed in (4,20)) and, when taken into consideration with data shown here, reveal possible mechanisms of immune dysregulation in T1D subjects. Mice lacking both IL-2 and IL-15 have reduced numbers of Treg which can be rescued by forced expression of constitutively active STAT5 (3,8). When the active form of STAT5 was
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constitutively expressed (46,47) or specific tyrosine residues within the cytoplasmic domain of the IL-2Rβ chain were mutated (48), a clear link was shown between IL-2 responsiveness and Treg development and homeostasis. Here, we find that FOXP3 expression in the presence of IL-2 is reduced as compared to controls but can be rescued in the presence of IL-7. This is consistent with a requirement for STAT5 activation through the IL-2Rβ chain. Together, these data suggest that impaired persistence of FOXP3 in Treg of T1D may be due to defects in IL-2Rβ chain signaling and offer novel options for targeted therapy.

Decreased pSTAT5 in response to IL-2 was not only observed in nTreg, but also in total CD4+ T cells suggesting an intrinsic property of CD4+ T cells of T1D subjects. In fact, a similar decrease in pSTAT5 upon exposure to IL-2 was observed with CD8+ T cells (data not shown). Whether this intrinsic property of CD4+ T cells of T1D is due to genetic and/or environmental factors is not clear. Multiple genes in the IL-2/IL-2R pathway are associated with T1D (20,24-28) but none to date have been found to associate with impaired Treg biology in T1D. More studies with other autoimmune diseases, type 2 diabetics and first degree relatives are required to better determine whether this phenotype is specific to T1D. More broadly, this defect may have additional implications for other biological processes that require IL-2.

Increased expression of PTPN2 in CD4+ T cells of some T1D subjects suggests a molecular mechanism for the reduced responsiveness to IL-2 in T1D subjects. Consistent with the down-modulatory role of PTPN2 in signal transduction (17), others have shown a role for PTPN2 in decreasing STAT1 phosphorylation in response to IFNγ stimulation of human beta islet cells (49). Here, we show for the first time that in some T1D subjects PTPN2 protein is significantly increased in CD4+ T cells, a cell subset of the immune system known to express high amounts of PTPN2 (50). We further demonstrate a mechanistic link between increased expression of PTPN2 in T1D subjects and decreased pSTAT5 by rescuing IL-2 responsiveness with a PTPN2 specific inhibitor. However, some T1D subjects have diminished pSTAT5 in response to IL-2 but the level of PTPN2 expression is comparable to controls. This suggests that in some T1D subjects increased expression of PTPN2 may contribute to altered IL-2 responsiveness, while in others, different molecular mechanisms may be involved, both resulting in a decreased pSTAT5 phenotype. Two independent variants within introns of the PTPN2 gene are associated with T1D (27) and may impact PTPN2 expression. This is the focus of current research in our laboratory. Interestingly, when we analyze control and T1D subjects that do not carry risk-alleles of PTPN2, we still find a significant difference in PTPN2 expression and pSTAT5 (unpublished data).

Cytokine signaling signatures are associated with autoimmune diseases. Here, we demonstrate that two phenotypes of CD4+ T cells of T1D subjects involve molecules in the IL-2R signaling pathway. Whether altered PTPN2 and pSTAT5 phenotypes indicate a comprehensive defect in the IL-2R signaling pathway of T1D is not yet known. However, in conjunction with further studies, these data may suggest that CD4+ T cells of T1D are marked by altered IL-2R signaling. Gaining a better understanding of the mechanisms leading to these phenotypes may guide development of diagnostic assays and targeted therapies.

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Figure Legends

Figure 1: Maintenance of FOXP3 expression in CD4⁺CD25⁺ nTreg of T1D subjects is impaired in the presence of IL-2. As diagramed in (A), fresh CD4⁺CD25⁺ T cells were sorted from PBMC isolated from control and T1D subjects, placed in culture with media alone or 200 IU/ml IL-2 and FOXP3 and CD25 expression were assessed over-time by flow cytometry. (B) One control and T1D sample is shown. (C) CD4⁺CD25⁺ T cells from multiple control (n=17) and T1D (n=13) subjects were assayed as in (A) in the presence of media alone, 200 IU/ml IL-2 or 10ng/ml IL-7. Bars show means and symbols represent individual subjects. Analysis was performed by gating on live CD4⁺CD25⁺ T cells. Statistical significance was determined using an independent student’s t-test. Cohorts of control and T1D subjects had mean ages of 38 (range 18-61) and 34 (range 21-46), respectively.

Figure 2: nTReg of T1D display impaired IL-2R signaling. (A) Level of expression of CD25, CD122 and CD132 in the CD4⁺CD25⁺ population of control (n=17) and T1D (n=11) subjects from Figure 1C was determined using Quantum™ R-PE MESF beads. (B) Thawed PBMC from a representative control and T1D subject were stimulated with 100 IU/ml IL-2 for 20min prior to fixation and staining with CD4, CD25 and pSTAT5(Y694). Dashed lines are treatment with media alone and solid lines are treatment with IL-2. (C) Multiple control (n=15) and T1D (n=17) subjects were stimulated as in (B). Bars show means and symbols represent individual subjects. Analysis of pSTAT5 was performed by gating on live CD4⁺CD25⁺ T cells. Statistical significance was determined using an independent student’s t-test.

Figure 3: FOXP3 expression in iTreg of T1D subjects is impaired in the presence of IL-2 but not IL-7. (A) CD4⁺CD25⁺ T cells were isolated from previously frozen control (n=15) and T1D (n=13) subjects and activated with 5µg/ml anti-CD3 antibody and irradiated accessory cells in the presence of media alone, 100 IU/ml IL-2 or 10ng/ml IL-7. FOXP3 expression 48hrs following activation was determined by flow cytometry by gating on live, total CD4⁺ T cells. Asterix denotes significant difference from media alone using a paired student’s t-test. Error bars represent means ± SEM. (B) CD69 expression 48 hours after activation was assessed by flow cytometry for a subset of controls(n=9) and T1D(n=10) shown in (A). (C) Linear regression was performed for samples in (B) activated in the presence of 100 IU/ml IL-2 to determine the association between CD69 and FOXP3 expression for control (R²=0.8496, p=0.0004) and T1D (R²=0.1702, p=ns) subjects. The difference between the slopes of the lines was measured using an analysis of covariance with the p value noted in the graph.

Figure 4: CD4⁺ T cells of T1D subjects display diminished responsiveness to IL-2. Thawed PBMC from control subjects were stimulated with 100 IU/ml IL-2 for 10 minutes prior to fixation and staining for CD4, CD25, and pSTAT5(Y694). Analysis was performed by gating on total live CD4⁺ T cells and comparing response to media alone versus cytokine stimulation. (A) Staining for one representative sample is shown. (B) The frequency of CD4⁺ T cells that were pSTAT5(Y694)⁺ in response to IL-2 was determined for control (n=59) and T1D (n=33) subjects. Bars represent means while symbols represent individual subjects. (C) Control (n=12)
and T1D (n=13) subjects were assayed for pSTAT5(Y694) in response to stimulation for 10 minutes with 200pg/ml IL-15 or 40pg/ml IL-7. Bars represent means±SEM. (D) MFI Fold Increase of pSTAT5(Y694) in STAT5+ CD4+ T cells of thawed PBMC from control (n=12) and T1D (n=14) subjects was determined by comparing pSTAT5(Y694) MFI following stimulation with IL-2, IL-7 and IL-15 or media alone. Bars show means and symbols represent individual subjects. All p values were determined using an independent student’s t-test.

Figure 5: Diminished IL-2 responsiveness is a stable phenotype and intrinsic property of CD4+ T cells of T1D. Thawed PBMC from control (n=18) and T1D (n=20) subjects were stimulated with 100 IU/ml IL-2 or 200pg/ml IL-15 for 10 minutes prior to fixation and staining for CD4, CD25, and pSTAT5(Y694). Linear regression was performed to determine the relationship between pSTAT5 responses in the same cells following stimulation with either IL-2 or IL-15. Trend lines represent linear regression of control (R^2=0.3713, p=0.0073) and T1D (R^2=0.2003, p=0.0478) data. (B) Cells isolated from the same individual but at different dates were assayed as described in (A) for response to IL-2. Sample collection dates varied from 5 months to 6 years. The number of months between sample collection did not correlate with SD or coefficient of variation for the controls, T1D subjects or all samples combined as analyzed by linear regression.

Figure 6: Altered expression of molecules in the IL-2R signaling cascade in CD4+ T cells of T1D subjects. CD4+ T cells were isolated from fresh PBMC of control and T1D subjects and whole cell protein lysates were analyzed by western blot. Immunoblots were probed with JAK1, JAK3 and PTPN2 specific antibodies and an anti-TFIIB antibody as a loading control. Protein expression was determined by densitometry, normalizing each sample to TFIIB and expressing total protein levels relative to a Jurkat control present on each blot. Total JAK1 (A) and JAK3 (B) protein expression were compared between control (n=20) and T1D (n=18 and 20 for JAK1 and JAK3, respectively) subjects. (C) PTPN2 protein expression was compared between control (n= 21) and T1D (n= 18) subjects. Significance was determined using an independent student’s t-test. (D) Thawed PBMC from these same samples were assayed for pSTAT5 upon exposure with 100IU/ml IL-2 for 10min as in Figure 4. Using linear regression, protein expression was compared to pSTAT5 for control(n=13) and T1D(n=15) subjects. Correlation between PTPN2 protein expression in the total population (control and T1D combined) and pSTAT5 is noted in the graph. Solid trend line (R^2=0.02, p=0.6) and squares denote controls and dashed trend line (R^2=0.234, p=0.067) and open circles denote T1D subjects. (E) Thawed PBMC from the T1D subject with the highest PTPN2 expression or (F) T1D with PTPN2 expression above the mean 0.8 (n=6) were incubated with a PTPN2 inhibitor (compound 8 in reference (29)) for 30 minutes prior to stimulation with IL-2 for 10 minutes as in Figure 4.
REFERENCES


27. Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. *Nature* 447:661-678, 2007


Figure 1

A

Sort CD4+CD25hi T cells (top 2-5%)

Fresh PBMC

Assess FOXP3 content and CD25 expression over time

B

%FOXP3 of CD4+CD25

Days

C

Δ% FOXP3 of CD4+CD25

Cntrl T1D media

Cntrl T1D IL-2

Cntrl T1D IL-7

p=0.0125

ns
Figure 2

A

B

CD4

CD25

C

Low IL-2R signal and FOXP3 persistence in T1D

20min IL-2

Cntrl

T1D

pSTAT5(pY694)

p=0.0307

% pSTAT5(pY694)

of CD4+CD25+

Cntrl

T1D
Figure 3

A  

48hrs  
% FOXP3 of CD4  
Cntrl  T1D  
media  IL-2  IL-7 

ns  
p=0.0147  

B  
% CD69 of CD4  
Cntrl  T1D 

ns 

C  
% FOXP3 of CD4  
% CD69 of CD4 

p=0.0032
Figure 4

A

B

C

D
Figure 5

A

% pSTAT5 of CD4
(IL-1.5)

% pSTAT5 of CD4
(IL-2)

Cntrl
T1D

B

% pSTAT5(pY694) of CD4

Sample (# mo between collection)

Cntrl
T1D

1 (11)
2 (7)
3 (9)
4 (11, 24)
5 (39)
6 (23)
A (26)
B (72)
C (27)
D (24)
Figure 6

A

JAK1

(relative expression)

Ctrl T1D

p=ns

B

JAK3

(relative expression)

Ctrl T1D

p=ns

C

PTPN2

(Relative expression)

Ctrl T1D

p=0.0062

D

%pSTAT5(pY694) of CD4

PTPN2

(relative protein)

R²=0.1748

p=0.0268

E

PTPN2 inhibitor

DMSO 10nM 50nM

25 IU/ml IL-2

100 IU/ml IL-2

F

% pSTAT5 of CD4

25 100

DMSO 10nM 50nM