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Type 1 Diabetes-Associated IL2RA Variation Lowers IL-2 Signaling and Contributes to Diminished CD4⁺CD25⁺ Regulatory T Cell Function

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Numerous reports have demonstrated that CD4⁺CD25⁺ regulatory T cells (Tregs) from individuals with a range of human autoimmune diseases, including type 1 diabetes, are deficient in their ability to control autologous proinflammatory responses when compared with nondiseased, control individuals. Treg dysfunction could be a primary, causal event or may result from perturbations in the immune system during disease development. Polymorphisms in genes associated with Treg function, such as IL2RA, confer a higher risk of autoimmune disease. Although this suggests a primary role for defective Tregs in autoimmunity, a link between IL2RA gene polymorphisms and Treg function has not been examined. We addressed this by examining the impact of an IL2RA haplotype associated with type 1 diabetes on Treg fitness and suppressive function. Studies were conducted using healthy human subjects to avoid any confounding effects of disease. We demonstrated that the presence of an autoimmune disease-associated IL2RA haplotype correlates with diminished IL-2 responsiveness in Ag-experienced CD4⁺ T cells, as measured by phosphorylation of STAT5α, and is associated with lower levels of FOXP3 expression by Tregs and a reduction in their ability to suppress proliferation of autologous effector T cells. These data offer a rationale that contributes to the molecular and cellular mechanisms through which polymorphisms in the IL-2RA gene affect immune regulation, and consequently upon susceptibility to autoimmune and inflammatory diseases. The Journal of Immunology, 2012, 188: 4644–4653.

Type 1 diabetes (T1D) is characterized by autoimmune destruction of pancreatic β cells, a process in which autoreactive T cells play a pivotal role (1–3). There is now a growing body of evidence to suggest that in T1D, this pathological autoimmunity is the direct result of a failure of immune regulation (4). This includes the defective function of various populations of regulatory T cells (Tregs), especially those characterized as CD4⁺CD25⁺FOXP3⁺. In support of this, we and others have demonstrated that the suppression of autologous responder T cells by CD4⁺CD25⁺ Tregs in individuals with newly diagnosed T1D is reduced significantly compared with that observed in age-matched control subjects (5–8). Importantly, we also demonstrated in an independent cohort of patients that defective suppression is not only present close to diagnosis, but also in individuals who have had T1D for >20 y, suggesting that the functional defect represents a phenotype that is stable over time and most likely under genetic control (9).

A candidate gene study identified an association between T1D and the IL-2RA gene (encoding the IL-2 receptor α-chain, CD25) (10, 11). There are three protective IL2RA haplotypes, one of which is marked by the single nucleotide polymorphism (SNP) rs12722495, where the protective allele confers a relative risk for T1D of 0.65. Recently, the disease-associated IL2RA rs12722495 haplotype was correlated with several distinct cellular immunophenotypes, most notably the higher expression of CD25 on memory CD4⁺ T cells and higher levels of IL-2 secretion from these memory T cells (12). IL-2RA is part of the high-affinity IL-2 receptor complex and is constitutively expressed at high levels on both naturally occurring and peripherally induced FOXP3⁺ Tregs (13, 14). Numerous lines of evidence from both mice and humans have demonstrated that IL-2 plays a key role in both the generation and function of FOXP3⁺ Tregs (15–20). Studies in vitro have demonstrated that activation of CD4⁺CD25⁺ T cell suppressor function requires IL-2 (21) and in vivo that IL-2 is required for peripheral survival and expansion of CD4⁺CD25⁺ Tregs (reviewed

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in Ref. 13). Furthermore, signaling via common γ-chain cytokines, of which IL-2 is a key member, is required for the maintained expression of FOXP3 by Tregs, which is essential for their suppressive function (19, 22).

These findings invoke the hypothesis that gene polymorphisms in the IL-2/IL-2RA pathway exert their influence on T1D risk via effects on the number or functional ability of FOXP3+ Tregs. In support of this, recent studies show that Tregs from individuals with T1D are more prone to apoptosis and more easily lose expression of FOXP3 and that these phenotypes may be linked to the IL2RA with altered Treg function is lacking. To address this knowledge gap, we examined whether the T1D susceptibility allele defined by the IL2RA SNP rs12722495 is associated with a reduction in the functional capacity of Tregs. To avoid any potential confounding effects of disease on Treg phenotype, we conducted these studies in individuals without disease. Our studies show that the T1D-susceptibility IL2RA haplotype identified by rs12722495 is associated with decreased signaling via the IL-2 pathway in both memory T cells and Tregs and that this is linked to diminished Treg function.

Materials and Methods

Subjects and study design

Individuals homozygous for the T1D protective rs12722495 IL2RA haplotype and the fully susceptible IL2RA haplotype (denoted as P1P1 and SS, respectively) were recruited for the present study. The levels of CD25 expression on CD4+ T cell subsets have previously been studied in these individuals, and pairs of P1P1 and SS individuals were preselected who showed typical haplotype-specific patterns of CD25 expression on the conventional memory CD4+ T cell (Tconv) subset (i.e., a relatively higher level of CD25 expression in P1P1 individuals compared with SS).

For PBMC isolation, blood samples were collected in vacutainers containing sodium heparin anti-coagulant, diluted 1:1 with RPMI 1640 supplemented with 100 μg/ml penicillin/streptomycin (Invitrogen, Paisley, U.K.) and stored overnight under constant rotation. The following day, PBMCs were isolated from whole blood by density gradient centrifugation (Lymphoprep;Axis-Shield, Oslo, Norway). For whole blood staining, blood was collected into vacutainers containing sodium heparin anti-coagulant and used on the day of collection. Because of the day-to-day variation inherent in intracellular staining protocols, it was not possible to normalize pSTAT5a and CD25 median fluorescence intensity (MFI) values through time as we were able to do previously using a cell surface staining protocol (12). Therefore, all analyses were performed in a prospective manner with each SS and one SS individual analyzed on a single day. Ethical approval for this study was granted by the local Ethics Committee and informed consent was obtained. All experiments were performed in a blinded manner without knowledge of genotype of the individual.

mAbs and reagents

The following Abs were used in these studies as indicated: PE-conjugated monoclonal anti-CD25 (clones M-A251 and 2A3), FITC-conjugated anti-CD4 (clone SK3), Alexa Fluor 488-labeled anti-STAT5a (pY694) were obtained from BD Biosciences (Oxford, U.K.); eFluor 450-labeled anti-CD4 (clone SK3), anti-CD45RA (clone H100), and PerCP-Cy5.5-conjugated anti-CD127 (clone eBioRDR5) were obtained from eBioscience (Hatfield, U.K.); and Alexa Fluor 700-labeled anti-CD45RA (clone H100),allophycocyanin-Cy7-conjugated anti-CD14 (clone HCD14), FITC-conjugated anti-Helios (clone 22F6), Alexa Fluor 647-labeled anti-FOXP3 (clone 259D), Alexa Fluor 700-labeled anti-CD4 (clone RCA-T4), Pacific Blue-conjugated anti-CD45RA (clone H100), and PE-conjugated anti-FOXP3 (clone 259D) were obtained from BioLegend (Cambridge, U.K.). Ab concentrations used were found to significantly deviate from the normal distribution, either an independent or paired Student t test was used to test for significance as indicated. Where one or more datasets were found to significantly deviate from the normal distribution, statistical significance was determined using Wilcoxon matched-pairs signed rank test for paired data. All statistical analyses were performed using GraphPad Prism (GraphPad Software, La Jolla, CA).

Flow cytometric analysis for pSTAT5a

Analysis of pSTAT5a in PBMCs was performed using BD Phosflow reagents (BD Biosciences) according to the manufacturer’s instructions. Briefly, 1 × 10^6 PBMCs were incubated with various concentrations of IL-2 for 10 min at 37°C, fixed with Phosflow buffer I, permobilized with Fixed Buffer III, stained with anti-CD-FIGT, anti-CD25-PE, anti-CD127-PerCP-Cy5.5, anti-CD45RA-eFluor 450, and anti-STAT5a-pY694-Alexa Fluor 647 and analyzed in a BD FACS Canto II (BD Biosciences). Because of a technical failure in one sample, only nine pairs of PBMC samples were analyzed for expression of pSTAT5a. For simultaneous detection of pSTAT5a and FOXP3, 500 μl fresh blood was incubated with 500 μl X-Vivo media containing various concentrations of IL-2 for 10 min at 37°C, fixed with warm BD Lyse/Fix buffer (BD Biosciences), and permeabilized with 100% methanol for 20 min on ice. After extensive washing with PBS containing 0.2% BSA, cells were stained with anti-CD4-Alexa Fluor 700, anti-CD25-allophycocyanin, anti–CD45RA-Pacific Blue, anti–FOXP3-PE, and anti–STAT5a-pY694-Alexa Fluor 488 and analyzed using a BD Fortessa (BD Biosciences). Results are expressed as the MFI of pSTAT5a staining for all cells within a particular T cell subset.

Isolation and analysis of cell populations for functional studies

PBMCs were stained with anti-CD4-eFluor 450, anti-CD25-PE, anti-CD127-PerCP-Cy5.5, anti-CD45RA-Alexa Fluor 700, and anti–CD14-allophycocyanin-Cy7 and control populations stained with anti–CD4-eFluor 450, IgG1-PE, IgG1-PerCP-Cy5.5, anti–CD45RA-Alexa Fluor 700, and anti–CD14-allophycocyanin-Cy7. Lymphocytes were identified based on forward and side scatter parameters and populations isolated for functional analysis using a BD FACSAria II flow cytometer and FACSDiva software (BD Biosciences). Flow cytometry data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Flow cytometric analysis for FOXP3 and Helios

FOXP3 and Helios staining was performed on cells immediately postsorting and after 48 h culture with various concentrations of IL-2 using the BioLegend FOXP3 Fix/Perm buffer set according to the manufacturer’s instructions. For analysis of FOXP3 and Helios in cultured cells, 10^6 sorted Tregs were incubated in complete media supplemented with IL-2 for 48 h prior to analysis.

In vitro coculture suppression assay

Suppression assays were performed by culturing memory or naive Tconv populations (2.5 × 10^6/well) in the presence or absence of either autologous Tregs or a third party Treg cell line at the ratios indicated. Cells were activated by the addition of Dynabeads Human T-Activator anti-CD3/anti-CD28 beads (Invitrogen) at a bead/conventional cell ratio of 1:1. All conditions were conducted in triplicate. The third party Treg cell line was generated by expanding FACS sorted Tregs from a single donor for 14 d in 600 U/ml IL-2. Expanded Tregs were cryopreserved and a single aliquot was thawed for use with each pair of samples analyzed. After 5 d culture, 100 μl supernatant was removed and stored at −80°C for later cytokine analysis. Proliferation was assessed by the addition of 0.5 μCi/well [H]thymidine (PerkinElmer, Waltham, MA) for the final 18 h coculture. The percentage of suppression was calculated using the following formula: % suppression = 100 – [(cpm in the presence of Tregs × cpm in the absence of Tregs) × 100].

Statistics

The normality of all datasets was tested using the D’Agostino–Pearson omnibus normality test. Where data did not significantly deviate from the normal distribution, either an independent or paired Student t test was used to test for significance as indicated. Where one or more datasets were found to significantly deviate from the normal distribution, statistical significance was determined using Wilcoxon matched-pairs signed rank test for paired data. All statistical analyses were performed using GraphPad Prism (GraphPad Software, La Jolla, CA).

Results

We have previously reported that the IL2RA rs12722495 protective haplotype is associated with significantly higher levels of expression of CD25 on conventional memory CD4+ T cells (mTconv) and we observed a similar trend for FOXP3+ Tregs (12). Therefore, we first sought to investigate whether increased levels of CD25 expression from individuals with the P1P1 IL2RA haplotype resulted in altered responsiveness to IL-2 signaling,
measured via phosphorylation of STAT5a in all T cell populations after brief in vitro exposure to IL-2. Because fixation precluded the use of CD127 as a surface marker of Tregs and FOXP3 costaining was found to be unreproducible using the pSTAT5a staining protocol for PBMC samples, Tregs were initially identified based on a high level of CD25 staining and reduced CD4 staining as previously described by other investigators and as illustrated in Fig. 1A (the frequency of CD4<sup>lo</sup>CD25<sup>+</sup> Tregs defined using this method and via CD25<sup>+</sup>CD127<sup>lo</sup> expression was highly correlated; $R^2 = 0.87$, $p < 0.0001$; Supplemental Fig. 1). Additionally, a more stringent definition was applied to identify Tregs expressing very high levels of CD25 (CD25<sup>hi</sup> Tregs) by gating on the top 1% of CD25-staining CD4<sup>+</sup> cells (CD25<sup>hi</sup> Tregs). Tconv were identified by low/intermediate levels of CD25 staining. (B–D) All populations were analyzed for expression of CD45RA to delineate populations of CD45RA<sup>+</sup> and CD45RA<sup>−</sup> Tconv and Tregs.

**FIGURE 1.** Example of the gating used for the identification of T cell populations for STAT5a phosphorylation studies using PBMCs. (A) Lymphocytes identified by their forward and side scatter properties were gated for CD4<sup>+</sup> expression and then examined for expression of CD25. Tregs were identified using two different gating strategies: 1) based on a high level of CD25 staining and reduced CD4 staining (CD4<sup>lo</sup>CD25<sup>+</sup> Tregs), and 2) gating on the top 1% of CD25-staining CD4<sup>+</sup> cells (CD25<sup>hi</sup> Tregs). Tconv were identified by low/intermediate levels of CD25 staining. (B–D) All populations were analyzed for expression of CD45RA to delineate populations of CD45RA<sup>+</sup> and CD45RA<sup>−</sup> Tconv and Tregs.

**FIGURE 2.** Relationship between IL2RA haplotype and CD25 expression on CD4<sup>+</sup> Tconv and Treg populations from PBMCs. PBMCs from donors with the protective rs12722495 IL2RA haplotype (P1P1) and donors with the susceptible IL2RA haplotype (SS) were isolated and analyzed as matched pairs by flow cytometry as shown in Fig. 1. Each symbol type represents a pair of individuals analyzed on the same day and are joined by a horizontal line in paired analysis. MFI is shown of CD25 staining on (A) mTconv, (B) CD4<sup>+</sup>CD25<sup>+</sup> Tregs, (C) CD4<sup>+</sup>CD25<sup>+</sup>CD45RA<sup>−</sup> Tregs, and (D) CD4<sup>+</sup>CD25<sup>+</sup>CD45RA<sup>−</sup> Tregs. Statistical significance was determined using a two-tailed paired Student t test.
expression of CD45RA to delineate memory (CD45RA\(^{-}\), mTconv) and naive (CD45RA\(^{+}\), nTconv) cells (Fig. 1B). Similarly, Tregs were subdivided into CD45RA\(^{-}\)Tregs and CD45RA\(^{+}\) (Fig. 1C). Because CD25\(^{hi}\) Tregs were 90% CD45RA\(^{-}\) (Fig. 1D; i.e., consisted mainly of Ag-experienced Tregs), this population was not subdivided for analysis. In all data presented, a pair of subjects differing at the rs12722495 SNP has been studied in a discrete, simultaneous experiment; each pair is denoted by a distinct symbol that is consistent throughout all graphs in the results section. Details of the gender and age bands of subjects and the symbols used to denote the results are shown in Supplemental Table I.

**Relationship between IL2RA haplotype and expression of CD25 on CD4\(^{+}\) Tconv and Treg populations**

Analysis of isolated PBMCs indicated that individuals with the P1P1 IL2RA haplotype express significantly more CD25 on mTconv \((p = 0.0002)\) and Treg \((p = 0.01)\) compared with individuals with the SS haplotype (Fig. 2A, 2B). The higher levels of CD25 were

**FIGURE 3.** Example of pSTAT5a staining in Tconv and Treg populations from PBMCs. PBMCs were incubated with various concentrations of IL-2 for 10 min, fixed, and stained for CD4, CD25, CD45RA, and pSTAT5a (Y694). (A and B) Representative plot of CD4\(^{+}\) cells from one individual incubated with (A) 0 U/ml and (B) 10 U/ml IL-2. (C) Representative example of a dose response curve in Tconv and Treg populations from one individual: \(\triangle\), nTconv; \(\Delta\), mTconv; ■, CD4\(^{+}\)CD25\(^{-}\)CD45RA\(^{-}\) Tregs; ●, CD4\(^{+}\)CD25\(^{-}\)CD45RA\(^{+}\) Tregs; ◊, represent CD25\(^{hi}\) Tregs.

**FIGURE 4.** Relationship between IL2RA haplotype and STAT5a phosphorylation in Tconv and Treg populations from PBMCs. PBMCs from donors with the protective rs12722495 IL2RA haplotype (P1P1) and donors with the susceptible IL2RA haplotype (SS) were isolated and analyzed as described in Fig. 3. Populations of conventional and regulatory cells were defined as described in Fig. 1. The percentage of cells positive for pSTAT5a in nTconv (A, B), mTconv (C, D), CD4\(^{hi}\)CD25\(^{+}\) Tregs (E), CD4\(^{hi}\)CD25\(^{-}\)CD45RA\(^{-}\) Tregs (F), CD4\(^{hi}\)CD25\(^{-}\)CD45RA\(^{+}\) Tregs (G), and CD4\(^{+}\)CD25\(^{hi}\) Tregs (H) following exposure to IL-2 as indicated. Each symbol type represents a pair of individuals analyzed on the same day, and individuals are joined by a horizontal line. Results are expressed as the MFI of pSTAT5a staining for all cells within a given population following exposure to IL-2 as indicated. Statistical significance was determined using a two-tailed paired Student t test.
observed both in the CD45RA⁺ (p = 0.005) and CD45RA⁻ CD4⁺ CD25⁺ Tregs (p = 0.008) (Fig. 2C, 2D). We observed no difference in the frequency of mTconv between the P1P1 and SS individuals (mean, 51.5 and 42.7%, respectively), the proportion of CD4⁺ T cells classified as Tregs (mean, 4.28 and 3.8%, respectively), or the percentage of CD45RA⁺ Tregs (mean, 69.2 and 65.2%, respectively). These observations are all in keeping with our previous report (12), even though CD25 levels were quantified using an intracellular staining protocol in the present study.

**mTconv and Tregs from individuals with the protective P1P1 IL2RA haplotype show increased sensitivity to IL-2**

Sensitivity to IL-2 was assessed by measuring phosphorylation of STAT5a in all T cell populations after brief in vitro exposure to IL-2 (Fig. 3A, 3B). As might be predicted from their CD25 levels, sensitivity to IL-2 in this assay was lowest for nTconv, with mTconv, CD4⁺CD25⁺CD45RA⁺ Treg, CD4⁺CD25⁺CD45RA⁻ Treg, and CD25hi Treg showing successively higher sensitivities, especially at very low concentrations of IL-2 (Fig. 3C).

mTconv from individuals with the P1P1 IL2RA haplotype were more responsive to IL-2 at both 10 U/ml (p = 0.01) and 100 U/ml (p = 0.007) compared with individuals with the SS haplotype; however, no such difference was observed in nTconv (Fig. 4A–D). Additionally, we observed that at low concentrations of IL-2, CD4⁺CD25⁺ Tregs from P1P1 individuals were more responsive to IL-2 compared to individuals with the SS haplotype (Fig. 4E; 0.1 U/ml, p = 0.01). This difference was observed in both the CD45RA⁺ and CD45RA⁻ populations (Fig. 4F, 4G; p = 0.005 and p = 0.03, respectively). Interestingly, these significant differences were only observed at the lowest concentration of IL-2 but not at higher concentrations of 1, 10, or 100 U/ml (data not shown). Similarly, we observed that CD25hi Tregs from P1P1 individuals were more responsive to IL-2 compared to individuals with the SS haplotype (Fig. 4H). Again, this was true at low concentrations of IL-2 (0.1 U/ml, p = 0.02) but not at higher concentrations (data not shown).

To confirm and extend these findings, we developed a whole blood assay incorporating additional bona fide markers of Tregs, that is, the transcription factor FOXP3, to examine IL-2 responsiveness (Supplemental Fig. 2). In addition to providing a more definitive marker for Tregs, examination of the expression level of FOXP3 combined with expression of CD45RA also allowed the delineation of three different populations of FOXP3⁺ T cells as described by Mayara and colleagues (26). As an example of the gating strategy used to identify FOXP3⁺ Tregs, the division of this population into resting Tregs (rTregs, FOXP3⁺CD45RA⁻), memory Tregs (mTregs, FOXP3⁺CD45RA⁻), and activated Tregs (aTregs, FOXP3hiCD45RA⁻) and the relative levels of CD25 expression on these populations are shown in Fig. 5A–C. Again, sensitivity to IL-2 in this assay was related to the relative expression levels of CD25 within each T cell population, especially at very low concentrations of IL-2 (Fig. 5D, Supplemental Fig. 2). This assay was then deployed on a fresh cohort of 13 pairs of individuals with the P1P1 or SS IL2RA haplotype consisting of 6 of the original pairs studied above and 7 new pairs. Consistent with the results in isolated PBMCs, staining in whole blood demonstrated that P1P1 individuals express significantly more CD25 on mTconv (p = 0.001) and Ag-experienced FOXP3⁺ mTregs (p = 0.045) and FOXP3hi aTregs (p = 0.0004) compared with individuals with the SS haplotype; however, no such difference was observed in rTregs (Fig. 6A–D). Similarly, results from the whole blood assay again demonstrated that mTconv and FOXP3hi aTregs from individuals with the P1P1 IL2RA haplotype were more responsive to IL-2 at nonsaturating doses (mTconv, 4 U/ml, p = 0.04; aTregs, 0.3 U/ml, p = 0.02) compared with individuals with the SS haplotype (Fig. 6E and 6H, respectively). However, no significant difference was observed in these populations at higher concentrations of IL-2 (mTconv, 10 or 100 U/ml; aTregs, 1–100 U/ml; data not shown). The lack of a significant difference between the P1P1 and SS donors in rTregs and mTregs (Fig. 6F and 6G, respectively) reflects the fact that in three pairs the SS cells were more responsive to IL-2 than were the P1P1
cells. This reflects the fact that in addition to IL2RA, many other genes, including PTPN2 (27), contribute to variation in the signaling pathways that mediate the phosphorylation and dephosphorylation of STAT5a in response to IL-2.

Collectively, our results show that Ag-experienced CD4+ Tconv and Tregs from individuals with the protective P1P1 IL2RA haplotype showed increased sensitivity to IL-2.

Tregs from individuals with the protective P1P1 IL2RA haplotype maintained higher levels of FOXP3 in the presence of IL-2

Lymphocytes were gated for CD4+CD14- expression, examined for expression of CD25 and CD127, and sorted into regulatory (CD4+CD14-CD25+CD127hi) and Tconv populations (non-Treg gate) as previously described (28) and shown in Fig. 7A. We then measured the expression of FOXP3 in isolated Tregs both immediately after flow cytometric sorting and after culture for 48 h in various concentrations of IL-2. Tregs were also stained for Helios, a member of the Ikaros family of zinc finger transcription factors that is expressed at high levels in thymic-derived Treg cells but not peripherally induced Tregs (iTregs) or activated Tconv (29). An example of the staining is shown in Fig. 7B. Experimental conditions were selected that represent nonsaturating (2 U/ml) and saturating concentrations (20 U/ml) of IL-2 as determined in preliminary experiments (Fig. 8A–D). Immediately postsort

FIGURE 6. Relationship between IL2RA haplotype, CD25 expression, and STAT5a phosphorylation in response to IL-2 in T cell subsets identified by FOXP3-pSTAT5a staining in whole blood. Whole blood from donors with the protective rs12722495 IL2RA haplotype (P1P1) and donors with the susceptible IL2RA haplotype (SS) was stimulated and analyzed as described in Fig. 5. (A–D) Relationship between IL2RA haplotype and CD25 expression. MFI of CD25 staining on (A) mTconv, (B) FOXP3+ rTregs, (C) FOXP3+ mTregs, and (D) FOXP3hi aTregs. (E–H) Relationship between IL2RA haplotype and pSTAT5a expression. MFI of pSTAT5a on (E) mTconv, (F) FOXP3+ rTregs, (G) FOXP3+ mTregs, and (H) FOXP3hi aTregs following exposure to IL-2 as indicated. Each symbol type represents a pair of individuals analyzed on the same day, and individuals are joined by a horizontal line. Results are expressed as the MFI of pSTAT5a staining for all cells within a given population. Statistical significance was determined using a two-tailed paired Student t test.

FIGURE 7. Example of the gating used for the isolation of CD4+ T cell populations for functional studies and staining of isolated Treg populations to investigate maintenance of expression of FOXP3 and Helios. (A) Lymphocytes identified by their forward and side scatter properties were gated for CD4+CD14- expression and then examined for expression of CD25 and CD127 (inset plot shows isotype control staining). Tregs were isolated based on CD4+CD14-CD25+CD127hi. (B) Isolated Tregs were fixed, permeabilized, and stained for expression of the transcription factors FOXP3 and Helios (dot plot) or relevant isotype control (density plot).
there was no significant difference in the proportion of Tregs that expressed FOXP3 (mean, 80.1 ± 7.8% SD and 79.6 ± 7.9% SD, respectively) or those that expressed Helios (mean, 73.3 ± 7.4% SD and 77.6 ± 6.1% SD, respectively) between individuals with the P1P1 or SS IL2RA haplotype (data not shown). As previously reported, culture of Tregs in the absence of IL-2 resulted in a decrease in both the percentage of cells expressing FOXP3 and the level of expression. FOXP3 expression can be “rescued” by addition of IL-2 (22, 24). Our results indicated that FOXP3 maintenance under these conditions is significantly dependent on IL2RA haplotype. We show that Helios+ Tregs from individuals with the protective P1P1 IL2RA haplotype expressed significantly higher levels of FOXP3 under conditions of limiting IL-2 (0 U/ml, \( p = 0.03 \) and 2 U/ml IL-2 (G), or 20 U/ml IL-2 (H) as described above. Each symbol type represents a pair of individuals analyzed on the same day, and individuals are joined by a horizontal line. Statistical significance was determined using a one-tailed Wilcoxon matched-pairs signed rank test.

**Discussion**

In the present study we addressed the impact of an IL2RA haplotype associated with autoimmune T1D on Treg fitness and suppressive function. We showed that the presence of a disease-associated (SS) IL2RA haplotype leads to diminished IL-2 responsiveness, resulting in lower levels of FOXP3 expression by Tregs and a reduction in their ability to suppress proliferation of autologous mTconv cells. These data offer a rationale that potentially accounts for the molecular and cellular mechanisms through which polymorphisms in the IL-2RA gene affect immune regulation, and consequently affect susceptibility to autoimmune and inflammatory diseases.

Numerous reports have demonstrated that CD4+CD25+ Tregs from individuals with a range of human autoimmune diseases, including T1D, are deficient either in their frequency or in their ability to control autologous proinflammatory responses when...
compared with nondiseased, control individuals (5, 6, 30–36). However, there is a major gap in present knowledge as to how CD4+CD25+ Treg function relates to the development of human autoimmune disease in terms of causality. Namely, is Treg dysfunction a primary, causal event or is it a result of alterations in the immune system due to the disease process? We hypothesized that if a diabetes susceptibility haplotype could be linked to altered (decreased) Treg function in individuals with no history of autoimmune disease, this would support the proposal that Treg dysfunction is causal in T1D. Several genes within the IL-2/IL-2RA pathway have been identified that influence susceptibility to human autoimmune diseases (e.g., \textit{IL2}, \textit{IL2RA}, \textit{IL2RB}, and \textit{PTPN2}) (11, 37–42), and given the vital importance of IL-2 and IL-2 signaling to the generation and function of CD4+CD25+FOXP3+ Tregs, it is likely that these genes exert their effects by altering Treg frequency or functional ability. In this and our previous report (12), the diabetes-susceptible \textit{IL2RA} haplotype (rs12722495) is shown to confer reduced CD25 expression and IL-2 secretion by mTconv cells. Although the frequency of Tregs detectable in the peripheral blood is not influenced by rs12722495, the ability of Tregs to signal via IL-2 is altered by this polymorphism and function is thereby impaired. This constellation of findings is resonant with observations made in patients with T1D: the frequency of Treg populations is normal, but Treg function is reduced (5, 6, 9). Additionally, a number of recent studies show that patients with T1D have impaired IL-2 signaling and increased Treg apoptosis (7, 24). This implies that impairment of the IL-2 pathway, through its effect on Treg fitness and immune regulation, is a key pathway in autoimmune disease pathogenesis.

Binding of IL-2 to its high-affinity receptor complex leads to a cascade of signaling events, including activation of the Ras/MAPK, JAK/STAT, and PI3K/Akt pathways. In Tregs, a major cellular consequence of IL-2 signaling is the phosphorylation and activation of STAT5a, which binds to the \textit{FOXP3} promoter, leading to sustained \textit{FOXP3} expression and enhanced suppressive capacity (19, 21, 43). IL-2 signaling and STAT5a phosphorylation are also vital for the generation of induced \textit{FOXP3}+ Tregs (22). In our studies, mTconv from individuals with the susceptible \textit{IL2RA}

FIGURE 9. Percentage suppression of mTconv proliferation by autologous or third party Tregs. Tregs and Tconv were isolated from donors with the protective rs12722495 \textit{IL2RA} haplotype (P1P1) and donors with the susceptible \textit{IL2RA} haplotype (SS). The suppression of proliferation of Tconv by Tregs was measured by in vitro coculture. Plots show (A) suppression of mTconv by autologous Tregs, (B) suppression of mTconv by standard third party Tregs, and (C) suppression of mTconv by autologous Tregs. Each symbol type represents a pair of individuals analyzed on the same day, and individuals are joined by a horizontal line. Statistical significance was determined using a one-tailed Wilcoxon matched-pairs signed rank test.

FIGURE 10. A model to explain the link between IL-2 pathway gene polymorphisms and the development of autoimmunity. Data from the present study and from Dendrou et al. (12) suggest that the rs12722495 \textit{IL2RA} susceptibility genotype may influence two distinct immunophenotypes: less IL-2 production from mTconv and a reduced sensitivity to IL-2 signaling in Tregs resulting in lower levels of pSTAT5a and reduced expression levels of \textit{FOXP3}. We propose that these two immunophenotypes could act synergistically to reduce Treg function.
IL2RA haplotype had a lower level of pSTAT5a in response to IL-2 stimulation. A key question is the extent to which this might also affect the ability of an individual to respond to immune regulatory cues and generate iTregs. nTconv (which express lower levels of CD25 and in whom most IL-2–induced STAT5a phosphorylation will occur in a CD25-independent manner) from IL2RA-susceptible individuals did not show a reduced pSTAT5a response. However, our in vitro studies did not take into account the fact that priming of naive T cells in vivo requires TCR ligation, a process that alters CD25 expression and hence IL-2 responsiveness. It remains to be established, therefore, whether lower levels of IL-2 sensitivity can result in reduced ability to generate iTregs from naive T cells.

The definitive identification of Tregs by flow cytometry is challenging, as activated T cells share many of the phenotypic characteristics of Tregs. Because fixation precluded the use of CD127 when measuring pSTAT5a in PBMC samples, we adopted a highly conservative approach to identify CD25hi Tregs by gating on the top 1% of CD25-staining CD4+ T cells, an approach adopted by other researchers (7, 25). Gating on an analogous population in the unfixed samples demonstrated that this population consisted of almost exclusively CD127+ cells, and we were therefore confident that the observed difference in STAT5a phosphorylation is due to a difference in sensitivity to IL-2–mediated signaling in Tregs rather than in any contaminating Tconv. These findings were then confirmed in a separate cohort of individuals using a whole blood assay that facilitated the simultaneous detection of FOXP3 and pSTAT5a to allow for more definitive gating of bona fide Tregs based on expression of FOXP3. Importantly, in both groups of individuals tested, this difference was only observed under nonsaturating concentrations of IL-2, suggesting that there was not an inherent difference in the ability of STAT5a to be phosphorylated in individuals with the diabetes-susceptible IL2RA haplotype, but rather a reduced sensitivity to IL-2 signaling that is only apparent under conditions of limiting IL-2.

The decreased STAT5a phosphorylation observed in individuals with the susceptible IL2RA haplotype also translated to a relative inability to maintain high levels of FOXP3 expression under limiting conditions of IL-2. Maintained expression of high levels of FOXP3 are a requirement for sustained suppressive function, and downregulation of FOXP3 has been associated with a rapid loss of regulatory function (44, 45). This difference was only observed in Tregs coexpressing Helios, recently identified as a transcription factor that is proposed to identify thymic-derived FOXP3+ Tregs (nTregs) and distinguish them from those that are generated in the periphery (iTregs) (29). It is possible that nTregs and iTregs have different requirements for sustained IL-2 signaling to maintain high levels of FOXP3 and that, at the concentrations of IL-2 chosen for this study, a difference could only be observed in the nTregs.

In the light of these and other studies, a model can be elaborated to explain the link between the IL-2 pathway gene polymorphisms and the development of human autoimmunity. This proposes that diminished Treg function is influenced by the IL-2–IL-2RA signaling pathway via two distinct immunophenotypes: lower levels of IL-2 production by Tconv and a lower level of responsiveness to signaling via IL-2 in Tregs, the combined result of which is impaired immune regulation (Fig. 10). This dual effect may not grossly affect Treg populations under steady-state conditions (e.g., as measured in peripheral blood), but it may only be revealed under conditions of inflammation, such as those present in the pancreas and pancreatic lymph nodes during development of T1D (46). The net effect would be an inability to sustain the suppressive action of activated Tregs and a subsequent failure to suppress pathogenic autoimmunity.

In summary, we have demonstrated that a disease-susceptible IL2RA haplotype is associated with reduced Treg fitness and suppressive function in vitro. To our knowledge, this represents the first demonstration that a gene polymorphism that increases susceptibility to autoimmune diseases such as T1D is associated with altered Treg function. Further studies will be required to determine whether other susceptibility genes also contribute to the defective Treg function that characterizes individuals with autoimmunity.

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Disclosures

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References


