Deficiency of immunoregulatory indoleamine 2,3-dioxygenase 1 in juvenile diabetes

Ciriana Orabona, … , Paolo Puccetti, Ursula Grohmann

*JCI Insight.* 2018;3(6):e96244. [https://doi.org/10.1172/jci.insight.96244](https://doi.org/10.1172/jci.insight.96244).

A defect in indoleamine 2,3-dioxygenase 1 (IDO1), which is responsible for immunoregulatory tryptophan catabolism, impairs development of immune tolerance to autoantigens in NOD mice, a model for human autoimmune type 1 diabetes (T1D). Whether IDO1 function is also defective in T1D is still unknown. We investigated IDO1 function in sera and peripheral blood mononuclear cells (PBMCs) from children with T1D and matched controls. These children were further included in a discovery study to identify SNPs in IDO1 that might modify the risk of T1D. T1D in children was characterized by a remarkable defect in IDO1 function. A common haplotype, associated with dysfunctional IDO1, increased the risk of developing T1D in the discovery and also confirmation studies. In T1D patients sharing such a common IDO1 haplotype, incubation of PBMCs in vitro with tocilizumab (TCZ) — an IL-6 receptor blocker — would, however, rescue IDO1 activity. In an experimental setting with diabetic NOD mice, TCZ was found to restore normoglycemia via IDO1-dependent mechanisms. Thus, functional SNPs of IDO1 are associated with defective tryptophan catabolism in human T1D, and maneuvers aimed at restoring IDO1 function would be therapeutically effective in at least a subgroup of T1D pediatric patients.

Find the latest version:

[http://jci.me/96244-pdf](http://jci.me/96244-pdf)
Deficiency of immunoregulatory indoleamine 2,3-dioxygenase 1 in juvenile diabetes

Ciriana Orabona,1 Giada Mondanelli,1 Maria T. Pallotta,1 Agostinho Carvalho,2,3 Elisa Albini,1 Francesca Fallarino,1 Carmine Vaccia,1 Claudia Volpi,1 Maria L. Belladonna,1 Maria G. Berioli,4 Giulia Ceccarini,4,5 Susanna M.R. Esposito,4,5 Raffaella Scattoni,4,5 Alberto Verrotti,4,5 Alessandra Ferretti,1 Giovanni De Giorgi,1 Sonia Toni,6 Marco Cappa,7 Maria C. Matteoli,7 Roberta Bianchi,1 Davide Matino,1 Alberta Iacono,1 Matteo Puccetti,1 Cristina Cunha,2,3 Silvio Bicciato,4 Cinzia Antognelli,1 Vincenzo N. Talesa,1 Lucienne Chatenoud,9 Dietmar Fuchs,10 Luc Pilotte,11,12 Benoît Van den Eynde,11,12 Manuel C. Lemos,13 Luigina Romani,1 Paolo Puccetti,1 and Ursula Grohmann1

1Department of Experimental Medicine, University of Perugia, Perugia, Italy. 2Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, Braga, Portugal. 3ICVS/3B’s - PT Government Associate Laboratory, Braga/Guimarães, Portugal. 4Department of Surgical and Biomedical Sciences, University of Perugia, Perugia, Italy. 5Pediatric Clinic of S. Maria della Misericordia Hospital, Perugia, Italy. 6Juvenile Diabetes Center, Anna Meyer Children’s Hospital, Florence, Italy. 7Unit of Endocrinology and Diabetes, ‘Bambino Gesù’ Children’s Hospital, Rome, Italy. 8Department of Life Sciences, University of Modena and Reggio Emilia, Modena, Italy. 9INSERM U1013, Hôpital Necker-Enfants Malades and Université Paris Descartes, Paris, France. 10Division of Biological Chemistry, Biocenter, Medical University, Innsbruck, Austria. 11Ludwig Institute for Cancer Research, Walloon Excellence in Life Sciences and Biotechnology and 12De Duve Institute, Université Catholique de Louvain, Brussels, Belgium. 13CICS-UBI, Health Sciences Research Centre, University of Beira Interior, Covilhã, Portugal.

Introduction

Type 1 diabetes (T1D), one of the most common chronic and autoimmune diseases in childhood, develops through the interaction of an incompletely defined combination of genetic susceptibilities (1–3) and environmental factors (4–6), which translate into immune destruction of islet β cells and consequent hyperglycemia. Autoimmunity in T1D is often characterized by defect(s) in one or several immune regulatory mechanisms (7). A defect apparently linked to a single pathway may, in turn, represent the result of multiple alterations in physiological mechanisms (8). The functional consequences of genetic variability in the pathogenesis of T1D — and whether those could be targeted pharmacologically — have been unclear (9).
Indoleamine 2,3-dioxygenase 1 (IDO1) — a powerful immunosuppressive enzyme catalyzing the first, rate-limiting step in l-tryptophan (Trp) catabolism — depletes Trp and produces immunoregulatory molecules collectively known as kynurenines (10–14). IDO1 functioning is, in turn, modulated at multiple levels (i.e., transcriptional and/or posttranslational levels; refs. 11, 12, 15). In fact, the expression of IDO1 protein may not be sufficient per se for full enzymatic activity (16, 17) because IDO1 proteasomal degradation occurs in a microenvironment dominated by IL-6 (18, 19). Moreover, genetic variants may likewise influence IDO1 expression and activity (20–23). Therefore, IDO1's defective activity may be the result of alteration(s) in one or multiple mechanisms modulating the enzyme, either positively or negatively.

In NOD mice, a prototypic model for T1D, IDO1 expression — and hence immune tolerance to islet autoantigens — is defective in DCs stimulated with IFN-γ, the main IDO1 inducer (24, 25), owing to a massive production of IL-6 in pancreatic lymph nodes (PLNs) (26). Maneuvers capable of correcting the IDO1 defect in NOD mice will, however, restore autoantigen-specific tolerogenesis by DCs in vivo (25, 27), prolong survival of pancreatic islet grafts (28), and normalize glycemia in diabetic animals (29).

In humans, IDO1 expression and activity are known to exhibit relatively large interindividual variability, particularly under pathological conditions, often as a result of SNPs in the enzyme gene (20–22). However, SNPs in IDO1 and any associated functional defects impacting on human T1D, currently with increasing prevalence in children (30), have not been formally proven (31).

Here, we investigated IDO1 expression and activity, as well as the association of SNPs in IDO1 and T1D development in peripheral blood mononuclear cells (PBMCs) from pediatric patients with T1D (3–17 years of age; 0.1- to 15-year disease duration), as compared with age-matched controls (i.e., not affected by metabolic or immune pathologies). Our data indicated that (i) pediatric T1D is associated with impaired IDO1 activity in PBMCs; (ii) IDO1 SNPs influences the risk of developing T1D; (iii) defective IDO1 activity is associated with a specific IDO1 genotype at rs7820268 and increased expression of the IL-6 receptor (IL-6R) in T1D patients; and (iv) an IL-6R blocker, currently in use for juvenile arthritis, restores IDO1 activity in vitro in PBMCs from a subgroup of T1D patients with defective IDO1 and normalizes glycemia in the majority of diabetic NOD mice in an IDO1-dependent fashion.

**Results**

**Defective Trp catabolism in children with T1D.** Significant changes in systemic Trp catabolism have been reported in several human diseases (32, 33). To ascertain whether dysfunctional Trp catabolism could be associated with T1D, sera from pediatric T1D patients and controls (Table 1) were analyzed for levels of...
Trp and 1-kynurenine (Kyn), the respective substrate and product of the IDO1’s enzymic activity. Kyn concentrations in sera from T1D patients were significantly lower (\( P < 0.001 \)) than those from controls, whereas Trp levels did not differ in the 2 groups (\( P = 0.2594 \)) (Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/jci.insight.96244DS1). The Kyn/Trp ratio, considered to be an indicator of systemic IDO1 activity (20), was significantly lower (\( P < 0.001 \)) in sera from T1D patients relative to controls (Supplemental Figure 1A and Supplemental Text).

Although IDO1 is an enzyme recognized as an authentic immune regulator (11, 13, 15), other enzymes such as Trp 2,3-dioxygenase (TDO) and IDO2 catalyze the same reaction along the Kyn pathway (15, 34). In particular, liver TDO, which is responsible for the catabolism of >95% of Trp entered with the diet, may greatly contribute to circulating levels of Kyn (35). We thus examined whether the data obtained from sera were reflected in expression and activity changes of Trp catabolizing enzymes in PBMCs from T1D patients and control subjects that were either left untreated or stimulated with IFN-\( \gamma \) at 100 or 1,000 U/ml for 48 hours. We first evaluated the expression of IDO1, IDO2, and TDO2 (coding for TDO) in PBMCs, either unstimulated or stimulated with IFN-\( \gamma \) at 100 or 1,000 U/ml. By means of real-time PCR, IDO2 and TDO2 (coding for TDO) expressions were found to be negligible in both control and T1D PBMCs under all conditions (data not shown).

In contrast, IDO1 expression was upregulated in PBMCs from both control and T1D subjects in response to IFN-\( \gamma \), but no significant difference could be found in samples from T1D patients as compared with their counterparts (i.e., control PBMCs; Figure 1A). In contrast, IDO1 protein was expressed to a lower extent in response to 1,000 U/ml of IFN-\( \gamma \) in PBMCs from T1D patients as compared with controls (Figure 1, B and C).

Figure 1. Tryptophan catabolism is reduced in PBMCs of pediatric patients with T1D. (A) Real-time PCR analysis of IDO1 transcripts in PBMCs, either unstimulated (0) or stimulated for 48 hours with IFN-\( \gamma \) at 100 or 1,000 U/ml, normalized to the expression of ACTB (encoding \( \beta \)-actin), and presented relative to results in untreated cells (dotted line, 1-fold; \( n = 33\)–16, Ctrl; \( n = 97\)–18, T1D). (B) Immunoblot analysis of IDO1 and \( \beta \)-tubulin in lysates of peripheral blood mononuclear cells (PBMCs), either unstimulated (0) or stimulated for 48 hours with IFN-\( \gamma \) at 100 or 1,000 U/ml, from 3 representative control subjects and T1D patients (indicated at the right side). (C) IDO1/\( \beta \)-tubulin ratios of scanning densitometry data obtained from immunoblot analyses as in B (all groups, \( n = 27 \)). (D) Kyn levels in supernatants of PBMCs treated as in A from control subjects (\( n = 74, 67, \) and 38 for 0, 100, and 1,000 U/ml IFN-\( \gamma \), respectively) or T1D patients (\( n = 169, 145, \) and 55 for 0, 100, and 1,000 U/ml IFN-\( \gamma \), respectively). (E) Linear regression analysis of 1-kynurenine (Kyn; \( \mu M \)) versus IDO1/\( \beta \)-tubulin in T1D PBMCs unstimulated or stimulated as in A (\( r^2 = 0.7099 \) and \( P < 0.0001; n = 27 \)). Ctrl, nondiabetic subjects. T1D, diabetic patients. Data (mean ± SEM) in A and C–E are the result of 3 independent measurements performed in triplicate. Data in A and C were analyzed by 2-way ANOVA followed by post hoc Bonferroni’s test. The Kruskal-Wallis with post hoc Dunn’s test was used for the analysis of E. **\( P < 0.01 \); ***\( P < 0.001 \).
Figure 2. Defective tryptophan catabolism in T1D patients is associated with a specific IDO1 genotype. (A) Human IDO1 gene structure and SNP localization. The tag SNPs are identified in bold. Exons and untranslated regions are depicted in dark and light gray, respectively. Genotype frequencies for the T1D patients and healthy controls were used to phase haplotype configuration. Rare haplotypes (frequency <2%) are not represented. (B) IDO1 mRNA (measured as in Figure 1A) in peripheral blood mononuclear cells (PBMCs) stratified according to rs7820268 genotypes (n = 8–36). (C) IDO1/β-tubulin protein ratios of scanning densitometry data obtained from immunoblot analyses as in Figure 1, B and C, from PBMCs stratified as in B (all groups, n = 5–11). (D) L-kynurenine (Kyn) production by PBMCs according to rs7820268 genotypes (n = 7–42). Ctrl, nondiabetic subjects. T1D, diabetic patients. Data (mean ± SEM) in panels B–D are the results of 3 independent measurements performed in triplicates. Data in panels B–D were analyzed by 2-way ANOVA, followed by post hoc Bonferroni’s test.*P < 0.05; **P < 0.01, ***P < 0.001.
On evaluating IDO1 catalytic activity, no differences were found in Kyn productions by PBMCs from T1D patients as compared with control subjects under basal conditions (i.e., in the absence of any inflammatory stimuli in vitro; Figure 1D). As expected, PBMCs from control subjects released significantly higher amounts of Kyn in response to IFN-γ at the concentration of 100 U/ml and more so at 1,000 U/ml. In contrast, Kyn levels in supernatants of PBMCs from T1D patients treated with IFN-γ at 100 U/ml were not significantly different from those in unstimulated T1D cells, but they were significantly lower than in control counterparts ($P < 0.001$). The highest concentration of the cytokine — 1,000 U/ml — did increase Kyn production by T1D PBMCs, although to a lesser extent than in control PBMCs stimulated with the same amount of IFN-γ ($P < 0.001$) (Figure 1D). Importantly, ratios of IDO1 to β-tubulin protein expressions would significantly and positively correlate with the concentrations of Kyn released in culture supernatants by PBMCs, unstimulated or stimulated with different concentrations of IFN-γ, from both control subjects (data not shown) and T1D patients (Figure 1E). However, no significant relationship was found between Kyn values in sera and in vitro Kyn production by PBMCs, either unstimulated or stimulated with IFN-γ in both T1D patients and controls (Supplemental Figure 1). Multiple regression analysis was used to test if participants’ characteristics (age, sex, BMI, disease duration, HbA1c, glycemia, and presence or absence of comorbidities) would significantly predict the responsiveness of PBMCs from T1D patients to IFN-γ in vitro in terms of transcript and protein expressions or catalytic activity of IDO1, but no effect of any of those parameters was found (data not shown).

Thus, our data indicated the existence of a significant defect of Trp catabolism in sera as well as in PBMCs from pediatric T1D patients. Reduced Trp catabolism in PBMCs could be attributed to a defect intrinsically linked to protein but not transcript expressions of IDO1 or other Trp catabolizing enzymes, or to any other tested patients’ variables, including percentages of distinct DC subsets (data not shown). The capacity of IFN-γ to upregulate IDO1 transcripts equally well in PBMCs from healthy controls and T1D patients also excluded any contribution of IFN-γ unresponsiveness to the effect. Lack of a significant correlation of Kyn in sera versus PBMC supernatants indicated that (i) systemic Kyn may

### Table 2. Description of IDO1 SNPs evaluated in T1D patients and control subjects.

<table>
<thead>
<tr>
<th>SNP rs number</th>
<th>Chromosome position</th>
<th>Alleles</th>
<th>Gene location</th>
<th>HapMap MAF</th>
<th>MAF in our study</th>
<th>HWE</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs9657182</td>
<td>39908329</td>
<td>T→C</td>
<td>Near gene 5′</td>
<td>0.483</td>
<td>0.441</td>
<td>0.7199</td>
</tr>
<tr>
<td>rs3808606</td>
<td>39918856</td>
<td>C→T</td>
<td>Near gene 5′</td>
<td>0.481</td>
<td>0.477</td>
<td>0.6630</td>
</tr>
<tr>
<td>rs10089078</td>
<td>39912420</td>
<td>G→A</td>
<td>Near gene 5′</td>
<td>0.336</td>
<td>0.346</td>
<td>1.0000</td>
</tr>
<tr>
<td>rs7820268</td>
<td>39920010</td>
<td>C→T</td>
<td>Intron</td>
<td>0.305</td>
<td>0.378</td>
<td>0.5727</td>
</tr>
<tr>
<td>rs3793919</td>
<td>39927802</td>
<td>G→A</td>
<td>Intron</td>
<td>0.429</td>
<td>0.476</td>
<td>0.6273</td>
</tr>
</tbody>
</table>

Chromosome positions at 8p12–p1 are from NCBI database (www.ncbi.nlm.nih.gov), assembly GRCh 37. MAFs and HWE tests were calculated collectively for T1D patients and healthy controls. SNP, single nucleotide polymorphism; MAF, minor allele frequency; HWE, Hardy-Weinberg equilibrium.

### Table 3. Multivariate analysis of the association of genetic variants in IDO1 with the risk of T1D in the discovery and confirmation studies.

<table>
<thead>
<tr>
<th>Genetic risk factors</th>
<th>Discovery study ($n = 732$)</th>
<th>Confirmation study ($n = 412$)</th>
<th>Combined studies ($n = 1,144$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adjusted OR (95% CI)</td>
<td>$P$ value</td>
<td>Adjusted OR (95% CI)</td>
</tr>
<tr>
<td>SNP model</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs7820268, CC genotype</td>
<td>1.44 (1.05–1.98)</td>
<td>0.028</td>
<td>1.69 (1.13–2.54)</td>
</tr>
<tr>
<td>Haplotype model</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H1/H1 haplotype</td>
<td>1.70 (1.03–2.80)</td>
<td>0.036</td>
<td>1.90 (1.03–3.51)</td>
</tr>
</tbody>
</table>

OR, odds ratio. *ORs were adjusted for patient age and sex. Multivariate analyses were based on conditional logistic regression. In the SNP model, the CT + TT genotype combinations at rs7820268 were the reference categories. Because the CC genotype at rs7820268 conferring risk for T1D was present in the H1/H1 diplotype, the H1/H2 + H2/H2 combinations were the reference categories in the haplotype model.
Figure 3. Peripheral blood mononuclear cells (PBMCs) from T1D patients express abnormal levels of SOCS3 and IL-6R. (A) Absolute expression levels of SOCS3, IL6, and IL6R transcripts in untreated PBMCs normalized to ACTB expression (n = 26–97). (B) Real-time PCR analysis of SOCS3, IL6, and IL6R transcripts in PBMCs treated as in Figure 1A. Data were normalized to expression of ACTB (encoding β-actin) and presented relative to results in untreated
not a reliable measure of IDO1 activity in immune cells, in contrast to previous reports (20, 21, 36), and/or (ii) an immune and metabolic disease such as T1D may be characterized by a significant defect in Trp catabolism involving liver TDO, as well, whose specific contribute cannot, however, be determined in live patients.

**Association of genetic variation in IDO1 with defective Trp catabolic activity in PBMCs.** Considering that genomic heterogeneity (20–23) could have masked the presence of a defect in IDO1 transcript expression in a subgroup of patients (see also Supplemental Text), we examined the possibility of an association between genetic variability in IDO1 and development of T1D.

We compared the frequency of 5 haplotype-tagging SNPs (tagSNPs) in the IDO1 gene (capturing a total of 9 variants based on their ability to tag surrounding variants with an r’ > 0.9) (Figure 2A and Table 2), using genomic DNA samples from both T1D patients (n = 265) and control subjects (n = 447). The rs7820268 SNP displayed a significantly different allele and genotype distribution among T1D patients and control subjects (P = 0.0083 and P = 0.0382, respectively; Supplemental Table 1 and 2). Of note, an association analysis test of rs7820268 genotypes using a genetic dominant model (CC versus T carriers) confirmed a significant association of the CC genotype with an increased risk of T1D (P = 0.0259; data not shown). Haplotypel association analysis further confirmed the role of IDO1 SNPs in T1D (Supplemental Table 3). In particular, the H1 haplotype promoted a significantly higher risk of T1D (P = 0.0021), and the haplotypes H4 and H5 conferred, instead, protection from T1D (P = 0.0053 and P = 2.188 × 10^-4, respectively). In a multivariate model adjusted for age and sex, the odds ratio (OR) for the CC genotype at rs7820268 and the H1/H1 diplotype containing this genotype was 1.4 (P = 0.028) and 1.7 (P = 0.036), respectively (Table 3). The results of both genotype and haplotype association tests were further validated in a confirmation case control study (OR, 1.69 [P = 0.014], and OR, 1.90 [P = 0.042], respectively) and by a meta-analysis including all enrolled patients (OR, 1.51 [P = 0.001], and OR, 1.76 [P = 0.005]).

To determine whether IDO1 SNPs could have an impact in our experimental setting, we compared IDO1 expression and Kyn production among PBMCs, either unstimulated or stimulated with 100 U/ml IFN-γ (i.e., a concentration at which most T1D PBMCs will not respond; Figure 1D) from subgroups of control subjects and T1D patients stratified on the basis of the rs7820268 genotype (Figure 2, B–D). We found no significant difference of IDO1 transcripts among control or T1D groups, either unstimulated or stimulated with the cytokine and harboring distinct genotypes (Figure 2B). In contrast, when assessing T1D toward control samples with the same genotype, a significant reduction in IDO1 protein expression (Figure 2C) and Kyn levels (Figure 2D) was found to occur in association with the CC and CT but not TT genotype at rs7820268 in the presence of IFN-γ. At variance with PBMCs, no significant impact of the rs7820268 SNP could be found on systemic Trp metabolism, further supporting the hypothesis that enzymes other than IDO1 may contribute to Kyn sera levels.

In order to gain insight into the functional meaning (currently unknown) of rs7820268, a SNP residing in the IDO1’s intron between exons 4 and 5, we evaluated whether this could be associated with an alternative splicing of IDO1 transcripts and, thus, loss of function of the IDO1 enzyme. By means of the Human Splicing Finder bioinformatics tool (Universal Mutation Database) (37), the C allele was predicted to create a potential intronic splicing enhancer site. To directly investigate this possibility, IDO1 amplicons were obtained by using primers specific for exons 4 and 5, as well as cDNA from 8 and 6 PBMC samples from T1D patients and healthy subjects (covering all rs7820268 variations), respectively; were sequenced; and were compared with each other. No difference in sequence and length could be found for any amplicon, i.e., from either T1D or controls samples (data not shown). A microRNA has recently been found to interfere with the expression of molecules involved in proteasomal degradation (38). Moreover, in NOD mice as well as in T1D patients, microRNAs downregulate the expression of PD-L1, an immunoregulatory molecule (39). We thus examined the possibility that the rs7820268 SNP could be associated with the production or loss of microRNAs. However, careful analysis performed using the PolymiRTS database...
Based on the available data, the most probable hypothesis could be that the rs7820268 SNP is in linkage disequilibrium (LD) with biologically relevant variability in other genes coding for proteins involved in proteasomal degradation, many of which reside on chromosome 8 (http://www.uniprot.org/docs/humchr08), the same of IDO1.

Therefore, our analyses of IDO1 SNPs demonstrated a significantly different distribution of the rs7820268 SNP in 2 independent association studies, further supporting the importance of IDO1 in autoimmune diabetes. Although the functional meaning of the different rs7820268 genotype distribution between control subjects and T1D patients remains unclear, our data would confirm posttranscriptional...
events as playing a major role in influencing variability of blood IDO1 activity observable within a healthy population of children, as well as between control and T1D subjects.

Occurrence of abnormal expression of IL-6R and SOCS3 in PBMCs from T1D patients. In mouse DCs (18), proinflammatory IL-6 drives regulatory proteolysis of IDO1 via upregulation of SOCS3, a small molecule capable of directly binding the Trp catabolizing enzyme, thus promoting its ubiquitination and subsequent proteasomal degradation (14, 15, 18). Conflicting data on IL-6 serum levels in autoimmune forms of diabetes have been reported. Although some groups reported lower levels of serum IL-6 in children with T1D, others found normal, if not increased, levels of IL-6 (41, 42). Interestingly, Hundhausen et al. observed a significant increase in surface protein expression of IL-6R but not of SOCS3 transcripts in effector T cells purified from PBMCs of adult patients with T1D versus matched controls (42). Analysis of IL6, IL6R, SOCS3, and IDO1 expression in PBMCs from healthy versus T1D children — as conducted with public microarray data obtained by others (43) — demonstrated the existence of significantly higher transcript levels of SOCS3 and more so of IL6R but not IL6 and IDO1 in samples from T1D patients at disease onset (Supplemental Figure 2).

We measured IL-6 serum levels in our cohorts of T1D patients versus controls, but no significant variation was found (data not shown).

On evaluating transcript expression of IL6, IL6R, and SOCS3 genes in PBMCs from our cohort of children with T1D versus matched controls, no significant difference could be found for IL6 expression in cells either untreated (Figure 3A) or stimulated with IFN-γ (Figure 3B). In contrast, IL6R and more so SOCS3 expressions were higher in T1D as compared with control PBMCs under IFN-γ stimulation (Figure 3B) but not basal (Figure 3A) conditions. A linear regression analysis indicated that the in vitro treatment of PBMCs from patients with T1D with 1,000 U/ml IFN-γ increased SOCS3 but decreased IL6 and IL6R expressions over disease duration (Figure 3C).

We next investigated whether the differences observed in the expression of IL6R and SOCS3 transcripts could translate into significant protein variations. IL-6 levels were measured in supernatants from T1D or control PBMCs, cultured with or without IFN-γ at 100 U/ml, in the presence or absence of TCZ (10 μM). In vitro incubation with TCZ did not increase IDO1 activity significantly in PBMCs from control subjects under any conditions (Figure 4A). In contrast, TCZ made IFN-γ capable of upregulating Kyn release to a significant, though moderate, extent in T1D

<table>
<thead>
<tr>
<th>TCZ R NR IDO1+ NR IDO1–</th>
<th>TCZ R NR IDO1+ NR IDO1–</th>
</tr>
</thead>
<tbody>
<tr>
<td>IDO1 mRNA</td>
<td>–        ++        ++          ±</td>
</tr>
<tr>
<td>IDO1 protein</td>
<td>–        –         ++          –</td>
</tr>
<tr>
<td>IL-6R protein</td>
<td>–        ++        +          ++</td>
</tr>
<tr>
<td>Kyn</td>
<td>–        –         ++          –</td>
</tr>
<tr>
<td>Kyn</td>
<td>+        ++        +          –</td>
</tr>
<tr>
<td>TGF-β</td>
<td>–        ++        +          +</td>
</tr>
<tr>
<td>TGF-β</td>
<td>+        ++        +          +</td>
</tr>
</tbody>
</table>

Table 4. Comparative profile of IDO1 expression (IDO1 mRNA and IDO1 protein) and activity (Kyn), IL-6R protein expression, and TGF-β production in subgroups of T1D PBMCs either untreated or incubated with TCZ.
PBMCs as compared with the same cells incubated with medium or TCZ alone. No TCZ effect could be observed in T1D PBMCs not stimulated with the cytokine.

To evaluate whether a variability in TCZ responsiveness could have masked the effect in PBMCs of individual patients, we searched for the presence of effective TCZ responders (i.e., PBMCs in which the drug did significantly increase Kyn production in at least 2 experiments; hereafter referred to as R) and nonresponders (NR) in the tested T1D population. The subgroup of TCZ NR (65.8% of the whole population) was capable

Figure 5. Tocilizumab (TCZ) restores normoglycemia in diabetic NOD mice via an IDO1-dependent mechanism. NOD mice proficient (A–D) or deficient (E–H) in Ido1 expression were treated i.p. with saline (control) or TCZ at the dose of 5 mg/kg every other day for 3 weeks and once a week for the next 3 weeks. (A and E) Percentages of diabetic animals (i.e., treated with TCZ or saline when showing a glycemia of 200–250 mg/dl) whose course of glycemia at the individual level is represented over time in B and F (n = 8 per group; 1 experiment representative of 3). (C and G) Histology of 1 representative mouse per group. Scale bars: 50 μM. (D and H) Degree of insulitis in the various groups. Data of diabetes incidence (A and E) were analyzed by Kaplan-Meier and survival curves were compared by log-rank test. ***P < 0.001.
of significantly upregulating Trp catabolism in response to IFN-γ (but not to IFN-γ plus TCZ). In contrast, TCZ R PBMCs (34.2%) increased their Kyn production exclusively in the copresence of IFN-γ and TCZ (Figure 4B). Moreover, incubation with TCZ and IFN-γ rendered cells from the TCZ R group capable of producing Kyn to an extent comparable with healthy subjects (i.e., approximately 1 μM; Figure 4, A and B).

A further analysis of the TCZ NR population led to the identification of 2 distinct TCZ NR subpopulations (Figure 4C), in which TCZ either did not exert any modulatory effect on Kyn production (NR IDO1+) or significantly impaired Kyn release as induced by IFN-γ alone (NR IDO1−). To investigate other possible functional effects of TCZ on PBMCs that might corroborate the differences in the 3 T1D subgroups, we measured the levels of TGF-β — an immunosuppressive cytokine that promotes IDO1 upregulation and acts as a functional antagonist of IL-6 on Trp catabolism (44–46) — in culture supernatants. Data showed that R and NR IDO1− samples released lower levels of the cytokine as compared with the NR IDO1+ group, under both basal conditions and in the presence of IFN-γ alone (Figure 4D). However, R — but not NR IDO1− or NR IDO1+ — cells produced significantly higher levels of TGF-β in the presence of TCZ, in an IFN-γ–independent fashion. Besides an overrepresentation of the CT genotype in R samples, analysis of the rs7820268 genotype in the 3 subgroups revealed a prevalence of the TT (i.e., T1D protective; Supplemental Table 2 and 3) or CC (T1D pathogenic) genotype in NR IDO1+ and NR IDO1− patients, respectively (Figure 4E). Interestingly, all comorbidities (i.e., 13 cases out of overall 114 T1D patients) were found to segregate in the NR groups (4 in IDO1+ and 9 in IDO1−; data not shown).

Therefore, although the majority of PBMCs from T1D patients may exhibit defective IDO1 activity (Figure 1) and a deregulated IL-6R–SOCS3 axis (Figure 3), together with a few alterations in the cytokine profile (Supplemental Figure 4 and Supplemental Text), the use of an IL-6R blocker allowed us to identify 3 subgroups of patients characterized by distinct profiles of Trp catabolism, TCZ responsiveness, and production of TGF-β, as well as distinct frequencies of IDO1 rs7820268 genotypes.

Differential distribution of a functional SNP of the IL6R gene in the 3 T1D subgroups. Similarly to IDO1, the genes coding for IL-6 and IL-6R are also characterized by the presence of SNPs. Several SNPs have been described in the IL6 gene, namely rs1800795, a promoter SNP that is known to influence the expression of the cytokine and has been correlated with many distinct inflammatory diseases, including type 2 diabetes (47). Moreover, the rs2228145 AC and CC genotype variants of IL6R have been shown to confer protection from several inflammatory diseases, including T1D (48). In fact, the AC and CC genotypes are functional — i.e., they determine a lower expression of membrane IL-6R and, thus, a reduced IL-6R signaling activity as compared with the rs2228145 AA genotype.

In order to strengthen the link between IL-6R signaling and IDO1 in T1D and evaluate additional features of the 3 T1D subgroups, we performed a genetic polymorphism analysis of IL6 rs1800795 and IL6R rs22281145 SNPs in parallel with measurements of IL-6 and IL-6R transcript and protein expressions in PBMCs, either unstimulated or stimulated with IFN-γ, from the 3 T1D subgroups (TCZ R, NR IDO1+ and NR IDO1−). No differential IL-6 transcript and protein expressions, as well as no distinct distribution of the IL6 rs1800795 SNP, could be observed in the 3 subgroups (data not shown). The analysis of IL6R, along with that of IDO1 transcript expressions (Supplemental Figure 3A), revealed that both the R and NR IDO1+ subgroups upregulated IDO1 expression in response to the cytokine to a similar extent, which was significantly greater than that found in NR IDO1− samples. In contrast, samples from R — but not NR IDO1− — patients expressed higher levels of IL6R transcripts in response to IFN-γ as compared with the other groups. Western blotting analyses indicated that, in the presence of IFN-γ at 100 U/ml, IL-6R protein expression was significantly higher in R and NR IDO1− samples (Supplemental Figure 3, B and C). However, as expected from the data of IDO1 activity (Figure 4, B and C), no upregulation of the IDO1 protein by IFN-γ alone could be observed in NR IDO1− and R cells, at variance with NR IDO1+ cells.

The genetic polymorphism analysis of the IL6R rs22281145 SNP in our cohort of patients revealed that the AA, AC, and CC genotypes were differentially distributed among the 3 subgroups of T1D patients (TCZ R, NR-IDO1+, and NR-IDO1−) (Figure 4F). Moreover, the results showed that the ratio of AC + CC/AA (i.e., protective/nonprotective genotype in T1D) was higher in T1D patients with no detectable IDO1 defect (NR-IDO1+) than in IDO1-defective patients (TCZ R and NR-IDO1−) (Figure 4G).

Therefore, because defective IDO1 patients were characterized by a prevalence of the nonprotective IL6R rs22281145 genotype and, thus, higher IL-6R expression, these data confirm the importance of a link between IL-6R signaling and IDO1 defect in T1D and further underline the distinctive immune features identified in the 3 subgroups of pediatric patients with autoimmune diabetes (Table 4).
IDO1-mediated, immunotherapeutic effects of TCZ in diabetic NOD mice. In order to understand whether the TCZ effects in vitro on human PBMCs from pediatric patients with T1D could be of therapeutic importance, we performed parallel experiments with NOD mice. In fact, in NOD mice, IDO1 expression and activity are almost negligible under inflammatory conditions (24, 25, 29), possibly due to an overproduction of IL-6, particularly evident in pancreata (26). A previous study reported that the systemic administration of IL-6-neutralizing antibodies results in a marked suppression of the diabetic disease in those animals (49). We have recently demonstrated that a proteasomal inhibitor approved for multiple myeloma therapy, besides protecting NOD mice from autoimmune diabetes in an IDO1-dependent fashion, significantly upregulates IDO1 protein expression and activity in NOD splenocytes and PLN cells in vitro (27).

In order to corroborate our data suggesting an aberrant IL-6R signaling leading to an accelerated proteasomal degradation of IDO1 in a selected group of T1D patients (i.e., TCZ R), we performed Western blot analysis of IDO1 protein expression and Kyn measurements using PBMCs from T1D patients of the TCZ R, NR IDO1+, and NR IDO1− phenotype, either untreated or treated in vitro with IFN-γ, a proteasomal inhibitor (i.e., MG132), or a combination of the 2 reagents. Representative blots and densitometric analyses of 15 such blots (Supplemental Figure 5, A and B) suggested that MG132, in combination with IFN-γ, significantly increases IDO1 protein expression and activity in TCZ R but not NR IDO1+ and NR IDO1− PBMCs.

Because of the proven pathogenetic role of IL-6 in NOD mice and the similar effects of BTZ in mouse and human settings, we performed experiments with TCZ in NOD animals. Based on the ability of TCZ to rectify IDO1 activity in PLN and spleen cells from NOD mice in vitro (Supplemental Figure 6), and because preliminary experiments indicated that the therapeutic efficacy of TCZ is inversely related to glycemia (data not shown), NOD animals with a glycemia of 200–250 mg/dl were administered TCZ or vehicle alone. At 3 months of drug treatment, all control mice (i.e., on vehicle alone) were markedly hyperglycemic (Figure 5, A and B). In contrast, greater than 70% of TCZ-treated animals had glycemia levels below 170 mg/dl at about 1–3 weeks of TCZ treatment. Glycemia remained in the range of 120–170 mg/dl for the entire duration of the experiment. Histological analyses of pancreata at 3 months of treatment revealed the presence of several islets without invasive insulitis or with minimal or no peri-insulitis in mice administered TCZ (Figure 5, C and D, and Supplemental Figure 6). In order to evaluate the role of IDO1 in TCZ therapeutic effects, we treated diabetic NOD mice lacking IDO1 expression, recently generated in our laboratory (Methods and F. Fallarino, unpublished observations), with the same regimen of TCZ used for WT NOD animals. The results indicated that TCZ’s efficacy in terms of normalization of glycemia, after a transient period of 3 weeks, was lost in the vast majority (>80%) of NOD Ido1−/− mice (Figure 5, E and F). Similarly, lack of IDO1 expression also abrogated TCZ effects in terms of histopathology (Figure 5, G and H).

The apparently incomplete reversal of TCZ effectiveness in Ido1−/− NOD animals may indicate that IL-6R blockade also acts, at least in part, via IDO1-independent mechanisms in autoimmune diabetes.

Thus, our data indicated that — similarly to IL-6 neutralization — IL-6R blockade is therapeutically effective in an experimental model of mouse autoimmune diabetes, and in addition, TCZ effects do require IDO1 expression. Furthermore, our data suggest that the NOD strain may represent a predictive experimental model for some (i.e., those connoted here as TCZ R and characterized by an excessive proteasomal degradation of IDO1) but not all patients with T1D.

Discussion

Current treatment of T1D is substitutive and — despite major constraints of hypoglycemia and multiple insulin injections, as well as incomplete control of cardiovascular complications over the long term — guarantees a good quality of life in the majority of patients (50). Although encouraging results have been obtained with anti-CD3 (51–53) and -CD20 (54) antibodies, no immunotherapy currently exists that can effectively halt the immune-mediated destruction of β cells in human T1D (55). An important caveat of past clinical trials might lie in a substantial disease heterogeneity of T1D patients (56). A recent study indeed indicated that T1D is characterized by the existence of distinct endotypes, reflecting different pathophysiological states (57). Thus, a personalized immunotherapy may be required for effective T1D immunotherapy.

Prompted by our previous observations in NOD mice (24–27, 29, 58), we here investigated whether an IDO1 defect could also be demonstrated in the blood of children with T1D and, if so, which mechanism/s could be possibly involved and whether the defect could be corrected. Determinations of Kyn in both sera and supernatants of PBMCs stimulated with IFN-γ, the main IDO1 inducer, clearly indicated that the...
majority of patients with T1D is characterized by defective Trp catabolism, although sera but not in vitro measurements were found to have a linear relationship with age. Our data also indicated that defective IDO1 activity in PBMCs from children with T1D is mainly imputable to a deregulated IL-6 signaling—associated with a prevalence of the AA rs2228145 IL6R genotype causing increased expression of membrane IL-6R (42) and risk for T1D (48)—that would favor IDO1 proteasomal degradation in inflammatory environments, i.e., dominated by IFN-γ. Cell incubation with TCZ, an IL-6R blocker, restored normal levels of IDO1 catalytic activity in response to IFN-γ in PBMCs from approximately one third—but not all—of the examined T1D population. PBMCs from TCZ Rs showed peculiar characteristics upon stimulation with IFN-γ—namely, they could be categorized as IDO1hiIL6RhiKynhi and carried the CT or CC allele at rs7820268 IDO1. In vitro treatment of PBMCs from TCZ Rs with the IL-6 blocker also increased production of TGF-β, an immunosuppressive cytokine that generally opposes IL-6 effects (59) and has been recognized as a positive modulator of IDO1 expression and activity (45, 46, 60). TCZ NRs could be differentiated in 2 additional T1D subgroups, those upregulating and not upregulating IDO1 expression and activity. Although the occurrence of T1D patients with physiological IDO1 expression may detract from the importance of IDO1 as a restraint mechanism of juvenile diabetes, our current data emphasized the fact that human T1D is heterogeneous in nature, and they suggest that personalized approaches may be mandatory for an effective immunotherapy of all T1D patients. As a matter of fact, PBMCs from IDO1hiIL6RhiKynhi TCZ NRs, while upregulating IDO1 activity in response to IFN-γ, significantly downregulated Trp catabolism in the presence of the drug, possibly indicating that cautions should be taken in treating uncharacterized T1D patients, at least in pediatric diabetology.

Although we cannot predict the exact individual response of PBMCs to a specific cytokine milieu in vivo, our data would point to 2 specific cytokines, IL-6 and TGF-β, playing a major role in affecting IDO1 expression in PBMCs from T1D patients. Thus, maneuvers capable of downregulating IL-6 signaling and/or upregulating TGF-β activity may be therapeutically effective in promoting IDO1-mediated tolerogenic mechanisms in T1D.

In conclusion, our study demonstrated that the majority of T1D patients are characterized by a substantial defect in Trp catabolism detectable in whole PBMCs and that maneuvers aimed at restoring IDO1 function would be therapeutically effective in at least a portion of T1D patients. Because the IDO1 defect was found to be mainly associated with dysregulated IL-6 responsiveness, IDO1 and IL-6R may represent reliable biomarkers for stratified immunotherapeutic approaches in T1D patients.

Methods

Subject recruitment. Peripheral venous blood (3−5 ml) was obtained from T1D patients of European descent and age-matched children (i.e., those without T1D or any autoimmune or allergic disease) attending the Day Service of S. Maria della Misericordia Hospital (Perugia, Italy); the Juvenile Diabetes Center, Anna Meyer Children’s Hospital (Florence, Italy); and the Unit of Endocrinology and Diabetes, ‘Bambino Gesù’ Children’s Hospital (Rome, Italy) during the years 2009−2017. A total of 165 patients with T1D and 87 control subjects were recruited to this study (University of Perugia Ethical Committee). Main subject demographic and disease characteristics are summarized in Table 1. Typically, blood from T1D patients and control subjects was drawn at the same time of the day to avoid any potential confounding effects of diurnal rhythm and season, as well as experimental variability. To limit the effects of diet on circulating Trp, serum samples were collected after an overnight fasting. Individuals included in our studies were free of known infections at the time of blood collection. Due to the small blood volume that can be obtained from pediatric subjects, all assays were not performed on each sample. Metabolic control was assessed by measurement of HbA1c and glycemia at the time of blood sampling. In the genetic discovery study, genomic DNA samples from the children recruited within the functional study, and from additional 100 children with T1D (Hôpital Necker-Enfants Malades) and 361 controls, were screened for IDO1 SNPs. A replication set of 206 adults with T1D and matched controls (CICS-UBI) was used to validate the results from the discovery set.

Cell purification. PBMCs were isolated on a Ficoll-Hypaque gradient and left untreated or exposed for 48 hours at 37°C to IFN-γ (100−1,000 U/ml; Novus Biologicals LLC) in the presence or absence of 10 μg/ml TCZ (Chugai Pharmaceutical Co.) or 10 μM MG132 (MilliporeSigma) in complete medium (RPMI with 10% FCS, 10 mM HEPES, and 50 μM 2-ME). All experiments were performed with 5 × 104/well PBMCs in 24-well plates. Sera were obtained by blood centrifugation at 450 g for 5 minutes and frozen at −80°C in criovials until analyses.
Kyn assay, real-time PCR, immunoblot analysis, and cytokine determination. IDO1 activity was measured in terms of the ability to metabolize Trp to Kyn, whose concentration was measured by HPLC in supernatants of PBMCs at 48 hours of culture after the addition of 100 μM Trp for the final 8 hours. Kyn and Trp were also measured in sera by HPLC as described (61). Kyn/Trp ratios were calculated dividing Kyn (μmol per liter) by Trp concentrations (mmol per liter) concentrations (62). Real-time PCR analysis was done as described (45, 60) with primers specific for human IDO1, IL6, IL6R, and SOCS3. Data were presented as the ratio of gene expression to ACTB (coding for human β-actin) expression determined by the relative quantification method (ΔCT). IDO1 expression was investigated by immunoblot with an affinity purified, mouse mAb (clone 4.16H1) highly specific for human IDO1 (63) in lysates from PBMCs cultured for 48 hours with complete medium either alone or in the presence of IFN-γ and/or TCZ. Rabbit polyclonal Abs with specificity for human SOCS3 (catalog 2923) and IL-6R (catalog 128008) were from Cell Signaling Technology and Abcam, respectively. Anti-human β-tubulin Ab (clone AA2, MilliporeSigma) was used as a normalizer. Relative protein expression was quantified by using an ImageQuant TL LAS4000 mini densitometer and the Analysis Toolbox software (GE Healthcare). Densitometric analysis of the specific signals was performed within a linear range of exposure of the blots, selecting in each experiment the 2 lowest exposure times useful to detect the signals, as described (64). Human IL-6 and TGF-β1 concentrations were determined in PBMC supernatants by using specific ELISA kits (eBioscience Inc. and Promega Corporation, respectively).

Genetic screening. Genomic DNA from whole blood samples was isolated using the QIAamp DNA Blood Mini kit (QIAGEN) following the manufacturer’s instructions and stored at ~20°C. SNPs in IDO1 were selected either from the literature (rs9657182) (22) or based on their ability to tag surrounding variants in the HapMap-CEU population of the International HapMap project, NCBI build B36 assembly HapMap phase III (http://www.hapmap.org). The Haploview version 4.2 software was used to select haplotype-based tagSNPs by assessing LD blocks with a pairwise correlation coefficient \( r^2 \) of at least 0.80 and minor allele frequency of >0.5 in the HapMap-CEU population using the confidence intervals method (65) and the LD-plot function of this software. Genotype frequencies were used to derive haplotype configurations and calculate Hardy-Weinberg equilibrium using the accelerated expectation-maximization algorithm similar to the partition/ligation method (66). Evaluated SNPs and haplotypes are indicated in Figure 2A and Table 2. For the IL6R gene, the functional SNP rs2228145 was selected for analysis (67). Genotyping was performed using the KASPar chemistry (LGC Genomics) according to the manufacturer’s instructions in an Applied Biosystems 7500HT Fast qPCR system (Invitrogen). Each genotyping set comprised randomly selected replicates of sequenced samples and negative controls. Concordant genotyping was obtained for >99% of the assays. Laboratory personnel were blind to the sample status.

Drug administration, histopathology, cell purification, and cytokine determinations in mice. NOD/MrkTac female mice, 12 weeks old, were purchased from Taconic. Ido1−/− NOD mice were derived by means of backcrossing of Ido1−/− C57/BL6 animals into the NOD/MrkTac strain. After 10 backcrossings, analysis of DNA microsatellite markers demonstrated the complete transfer of KO Ido1 into NOD mice (F. Fallarino, unpublished observations). For TCZ treatment, NOD mice, either proficient or deficient for Ido1 expression, were recruited when their glycemia was in the range of 200–250 mg/dl. TCZ or saline was administered i.p. at the dose of 5 mg/kg every other day for 3 weeks and once a week until the end of the experiment. Animals were sacrificed for ex vivo analyses at different times from the initiation of drug treatment. For histopathology, 3–4 μm of paraffin-embedded sections of pancreata (5 per organ) were stained with H&E and analyzed by light microscopy. Insulitis scoring was according to standard criteria as described (26, 29). Pancreatic leukocytes and splenocytes were purified and their cytokine production measured as described (26, 27).

Statistics. All analyses were performed using Prism version 6.0 (GraphPad Software). Data usually met normality and were analyzed by 2-tailed unpaired Student’s \( t \) test or 2-way ANOVA followed by post hoc Bonferroni’s test, when 3 or more samples were under comparison, respectively. Alternatively, the nonparametric Mann-Whitney test or Kruskal-Wallis with post hoc Dunn’s test was used. A \( P \) value less than 0.05 was considered significant. Overall results, obtained by at least 3 replicates per experimental parameter, were shown as mean (± SEM). Association between 2 variables was analyzed by the least square regression analysis. Multiple regression analysis was used to test whether clinical characteristics significantly predicted Trp catabolism in sera and supernatants. IDO1 allele and genotype distributions were compared using Fisher’s exact test. Significance tests for the haplotype analyses were calculated based on approximate \( \chi^2 \) distribution.
In mice, data of diabetes incidence were analyzed by Kaplan-Meier. Survival curves were compared by log-rank test. Ex vivo determinations, shown as means ± SD, were from at least 3 independent experiments and compared by 2-tailed unpaired Student’s t test. Mice were considered nondiabetic when glycemia measurement gave values <200 mg/ml on at least 3 consecutive measurements (3 per week).

Study approval. All parents provided informed written consent for the collection of samples and subsequent analysis. Ethical approvals were as follows: University of Perugia Ethical Committee, 19.1.2009, 2013-50, and 2016-05; Hôpital Necker-Enfants Malades, ID-RCB 2008-A01538-47; and CICS-UBI, CE-FCS-2011-003 and 2013-017. Each child in scholar age was informed orally about the study by a nurse immediately before blood drawing during routine analyses. In vivo studies were in compliance with national (Italian Approved Animal Welfare Assurance A-3143-01) and Perugia University Animal Care and Use Committee guidelines.

Author contributions
CO introduced the use of TCZ and performed most of the experiments with the drug. GM and MTP performed the largest part of experiments. AC analyzed IDO1 genotypic polymorphisms and statistical analysis thereof and wrote the related parts. EA, C. Volpi, FF, MLB, C. Vacca, RB, AI, MP, CC, CA, and VNT contributed in some experiments. SB performed the bioinformatic analysis shown in Supplemental Figure 2. LP and BVDE provided anti–human IDO1 monoclonal antibody. LC provided genomic samples from the Hôpital Necker-Enfants Malades and gave crucial input for the manuscript. MCL provided the Portuguese confirmation cohorts. DF measured Trp and Kyn in sera. LR and PP contributed to revision of the manuscript. MGB, GC, SMRE, RS, AF, GDG, AV, ST, MC, and MCM supervised patient recruitment. UG conceived and directed the project, performed statistical analyses together with DM, and wrote the manuscript.

Acknowledgments
The authors wish to thank patients and subjects who participated in this study, as well as nurses and staff of the Pediatric Clinic of S. Maria della Misericordia Hospital (Perugia), Juvenile Diabetes Center-Anna Meyer Children’s Hospital (Florence), Unit of Endocrinology and Diabetes-‘Bambino Gesù’ Children’s Hospital (Rome), Hôpital Necker-Enfants Malades (Paris), and Diabetes and Metabolism Service-University Hospital Centre of Coimbra (Coimbra). The authors wish also to thank Roberto Gerli for the gift of TCZ, Giovanni Ricci for histologies, and Francisco Carrilho and Eduarda Coutinho for providing and processing, respectively, DNA samples from the Portuguese cohorts. This work was supported by the European Research Council (338954-DIDO to UG) and, in part, by Associazione per l’Aiuto ai Giovani con Diabete Italia e dell’Umbria (to UG) and the Northern Portugal Regional Operational Programme (NORTE 2020), under the Portugal 2020 Partnership Agreement, through the European Regional Development Fund (FEDER) (NORTE-01-0145-FEDER-000013 to AC) and the Fundação para a Ciência e Tecnologia (contracts IF/00735/2014 to AC, and SFRH/BPD/96176/2013 to CC).

Address correspondence to: Ursula Grohmann, Department of Experimental Medicine, University of Perugia, Piazzale Gambuli n.1, 06132 Perugia, Italy. Phone: 39.075.5858240; Email: ursula.grohmann@unipg.it

AV’s present address is: Department of Pediatrics, University of L’Aquila, L’Aquila, Italy.

AF’s present address is: Hospital of Gualdo Tadino, Gualdo Tadino, Italy.