SUPPLEMENTAL INFORMATION

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

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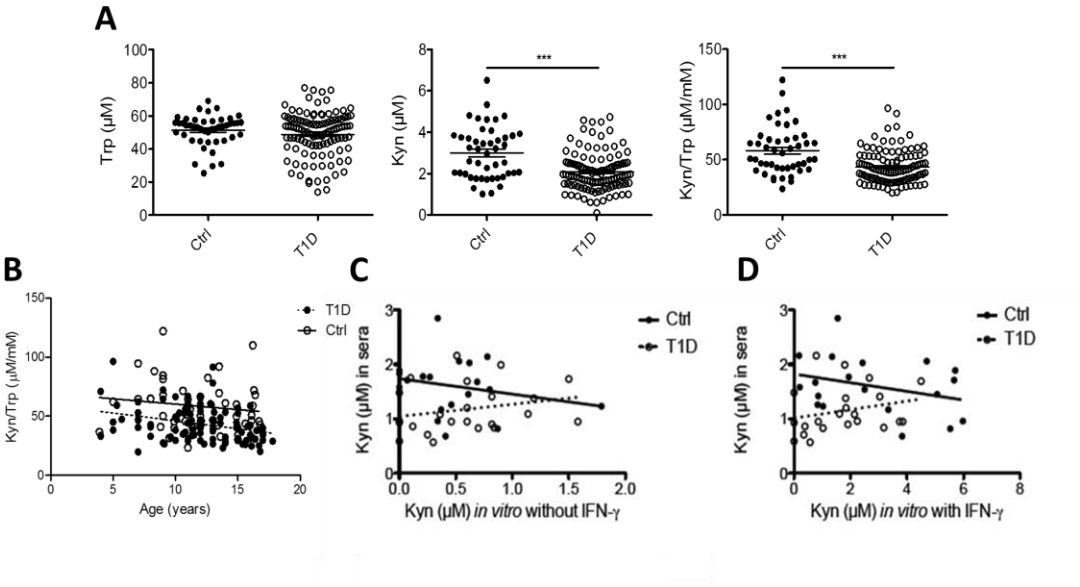

Fig. S1. Trp catabolism is reduced in sera of pediatric patients with T1D. (**A**) Trp and Kyn levels in sera of control subjects (Ctrl; $N = 46$) and T1D patients (T1D; $N = 111$). The Kyn-to-Trp ratio (Kyn/Trp) is also shown. (**B**) Scatterplot showing the relationship between Kyn/Trp and age in both groups ($N = 44$ controls and $N = 105$ T1D). Linear regression analysis of Kyn levels in sera from control subjects ($N = 19$) and T1D patients ($N = 22$) *versus* Kyn concentrations in culture supernatants of PBMCs, either incubated with medium alone (**C**) ($r^2 < 0.06$ and $P > 0.4$ in both groups) or stimulated with 1000 U/ml of IFN-γ shown in (**D**) ($r^2 < 0.1$ and $P > 0.15$ in both groups) for 48 h. Ctrl, control patients. T1D, diabetic patients. Data in **A** were analyzed by two-tailed unpaired Student's t-test. ***, $P < 0.001$.

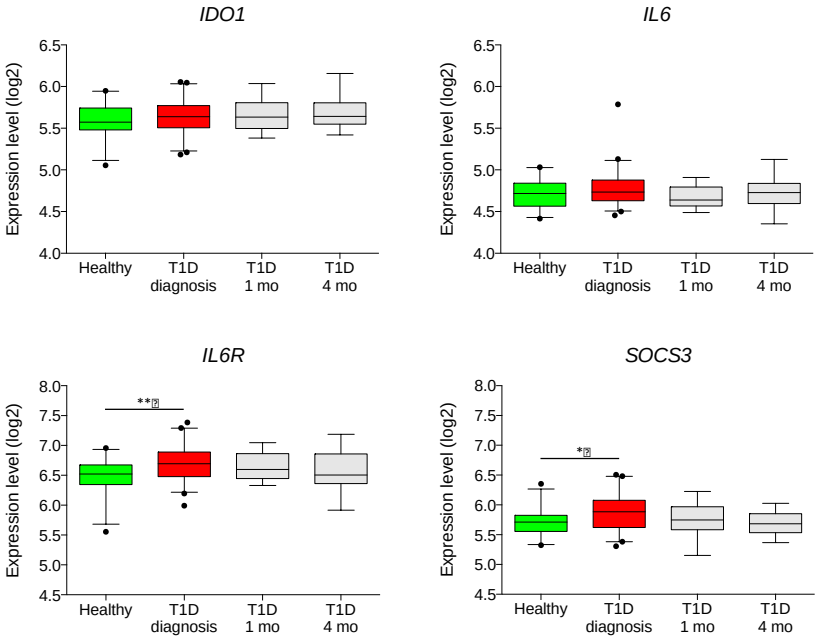

Fig. S2. Expression levels of *IDO1*, *IL6*, *IL6R*, and *SOCS3* in PBMCs of 24 healthy controls (Healthy), 43 patients with newly diagnosed T1D (T1D diagnosis), and 19 samples collected at one (T1D 1 mo) or four (T1D 4 mo) month/s after diagnosis. Raw microarray data (.CEL files) of Affymetrix Human Genome U133A arrays have been downloaded from GEO (GSE9006; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE9006). All data analyses were performed in R (version 3.1.2) using Bioconductor libraries (BioC 3.0) and R statistical packages. Probe level signals were converted to expression values using robust multi-array average procedure RMA *(65)* of Bioconductor *affy* package. Gene expression levels in healthy individuals and T1D patients were compared using two-tailed unpaired *t*-test (*, $P = 0.01 \div 0.05$ and **, $P = 0.001 \div 0.01$).

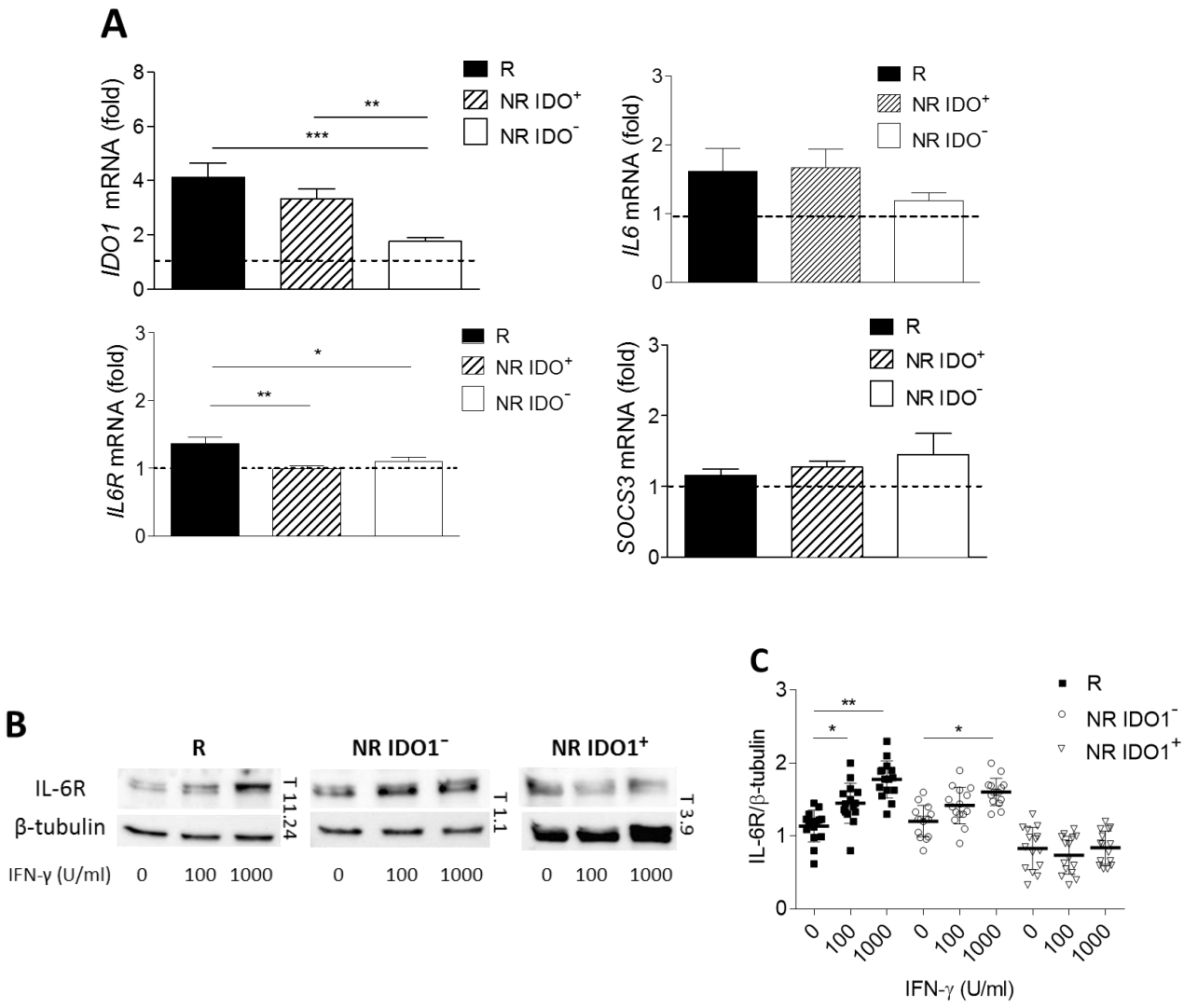

Fig. S3. Expression of IDO1, IL-6, IL-6R, and SOCS3 in PBMCs from T1D subgroups stratified for TCZ responsiveness. (**A**) Real-time PCR analysis of *IDO1*, *IL6*, *IL6R*, and *SOCS3* transcripts in R (N = 23), NR IDO1$^+$ (N = 32), and NR IDO1$^-$ (N = 39) T1D PBMCs stimulated with IFN-γ at 100 U/ml. Data were normalized to expression of *ACTB* (encoding β-actin) and presented relative to results in untreated cells (dotted line, one-fold). (**B**) Immunoblot analysis of IL-6R and β-tubulin in lysates of PBMCs, either unstimulated (0) or stimulated for 48h with IFN-γ at 100 - 1000 U/ml from three representative patients with differential TCZ responsiveness profile (indicated at right side). (**C**) Ratios of IL-6R with β-tubulin obtained from scanning densitometry data of immunoblot analyses of PBMCs, either unstimulated or treated with IFN- γ as in (**A**) (all groups, N = 15). Data in panels **A** and **C** were analyzed by ANOVA followed by post-hoc Bonferroni's test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

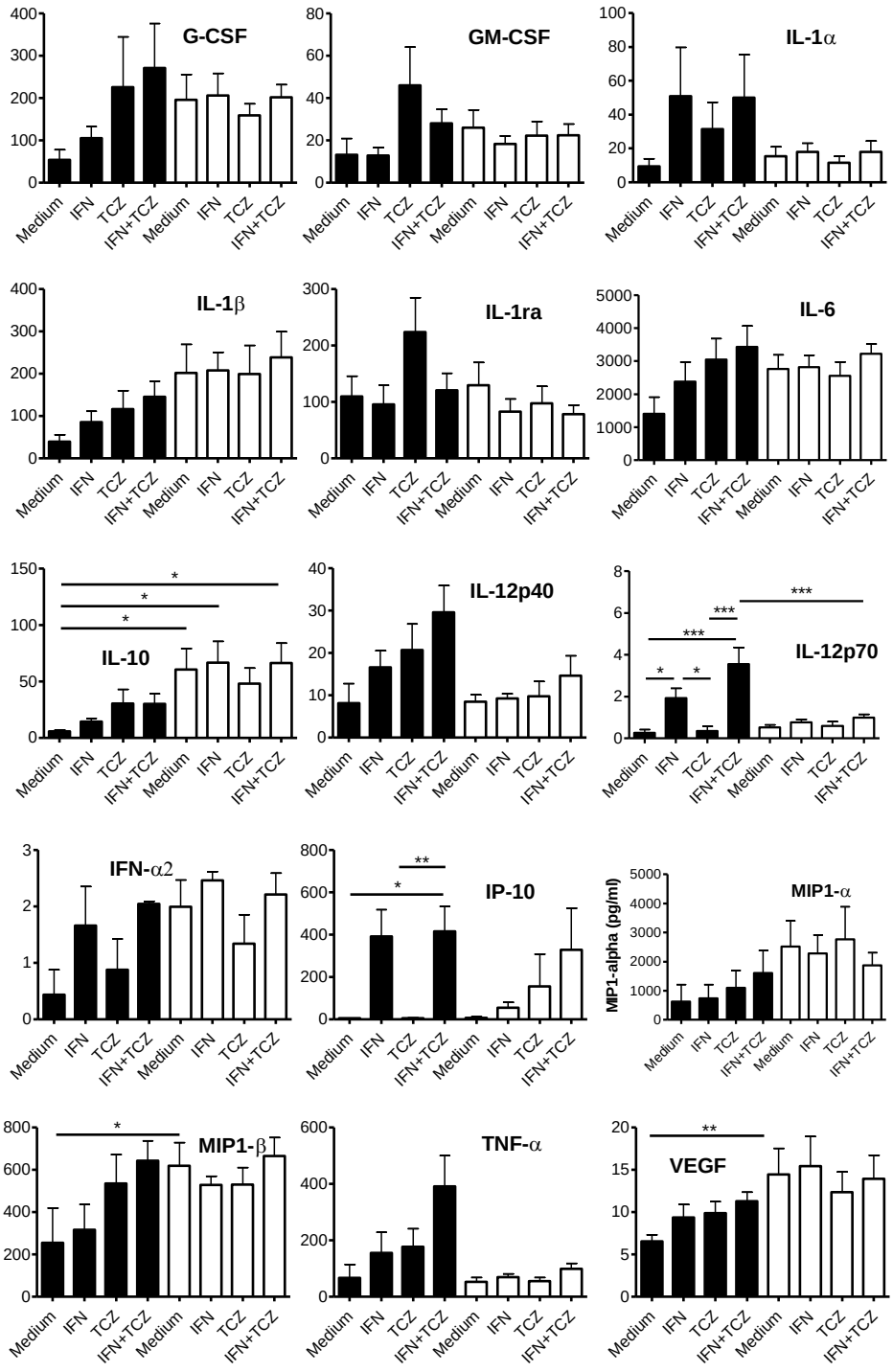

Fig. S4. Cytokine and growth factor profile in control *vs.* T1D PBMCs in the presence or absence of TCZ. Cytokine and growth factors levels were determined in culture supernatants from control or T1D PBMCs, either unstimulated or stimulated with 100 U/ml IFN-γ for 48 h in the presence or absence of TCZ at 10 μg/ml by means of the Human Cytokine/Chemokine magnetic bead panel kit (HCYTMAG-60K) and the Luminex MAGPIX Multiplex instrument (EMD Millipore, Billerica, MA). Black histograms represent control subjects. White histograms indicate T1D patients. Data were analyzed by ANOVA followed by post-hoc Bonferroni's test *, $P < 0.05$; **, $P < 0.01$; ***$P < 0.001$.

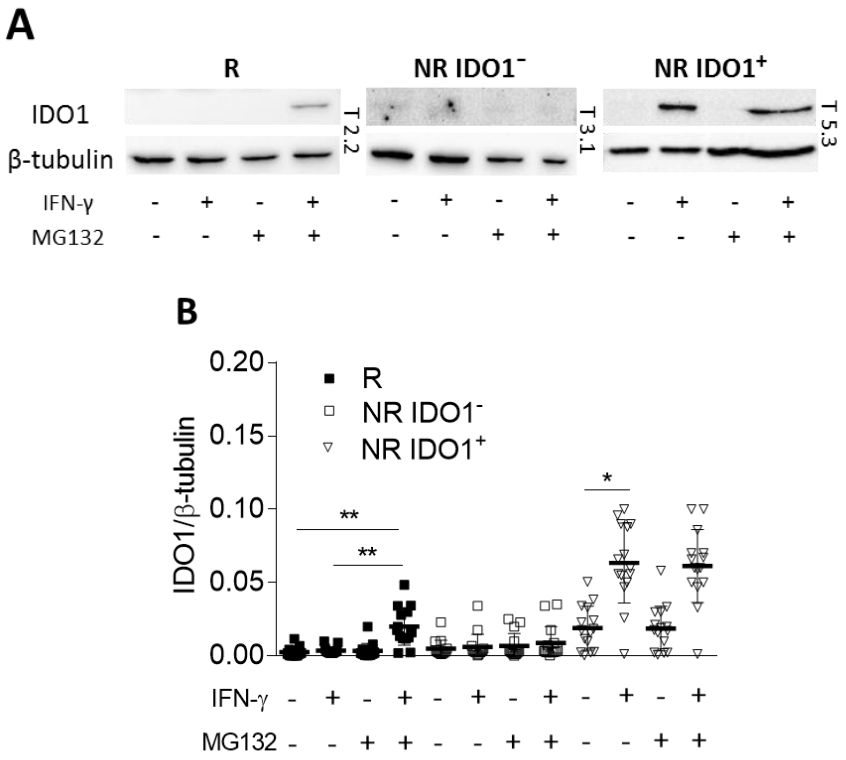

Fig. S5. Effect of MG132, a proteasomal inhibitor, on IDO1 protein expression in PBMCs from T1D subgroups stratified for TCZ responsiveness. (**A**) Immunoblot analysis of IDO1 and β-tubulin in lysates of PBMCs from one R, NR IDO1^{+}, and NR IDO1^{-} representative T1D patient (indicated at the right side), either untreated or treated with IFN-γ at 100 U/ml, MG132 at 20 μM, or a combination of both reagents. (**B**) IDO1/β-tubulin ratios of scanning densitometry data obtained from immunoblot analyses as in (**A**) (all groups, $N = 15$). Data in **B** were analyzed by ANOVA followed by post-hoc Bonferroni's test *, $P < 0.05$; **, $P < 0.01$.

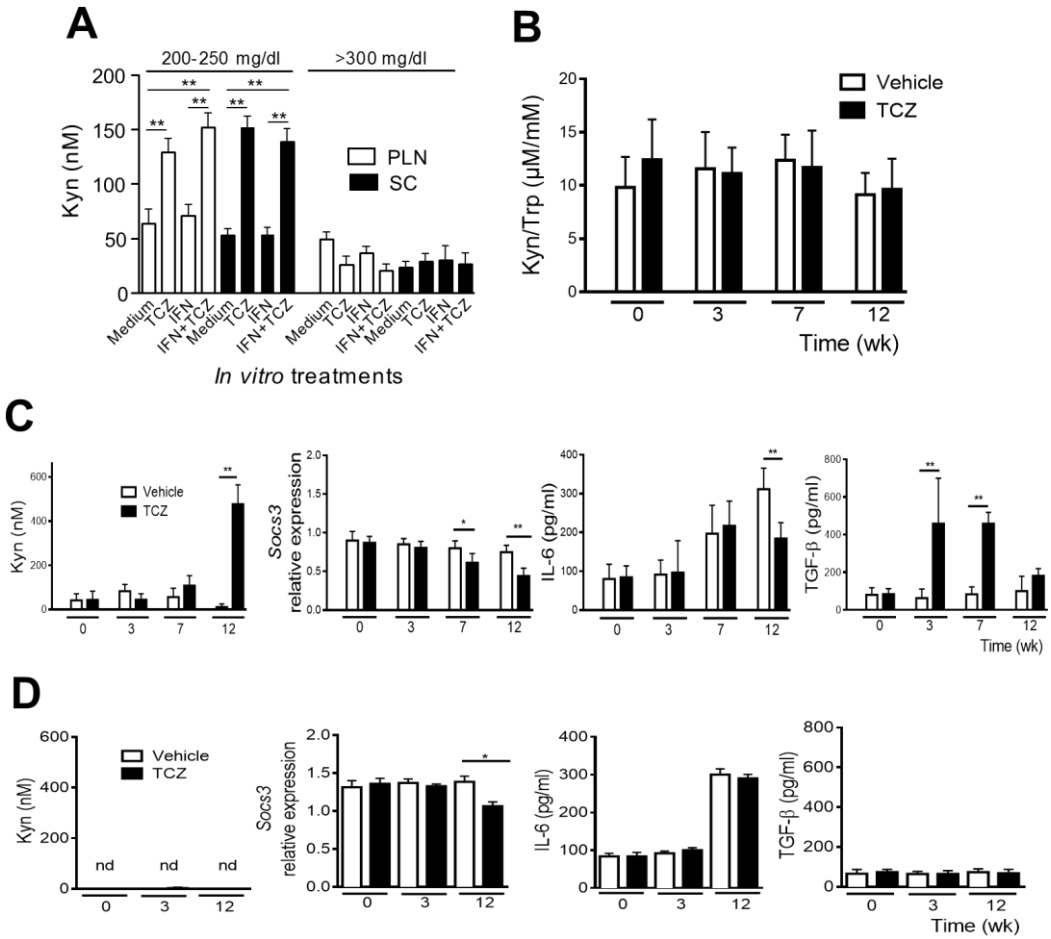

Fig. S6. TCZ exerts therapeutic effects in diabetic NOD mice in an IDO1-dependent fashion. (**A**) Kyn levels released by pancreatic lymph node (PLN) cells or splenocytes (SC; both at 1.5×10^6 cells per well), purified from diabetic NOD mice with lower (200–250 mg/dl) or higher (>300 mg/dl) glycemia levels and incubated in the presence or absence of 200 U/ml IFN-γ, with or without tocilizumab (TCZ; 10 μg/ml) for 48 h. (**B**) Kyn/Trp ratios in sera of mice treated with vehicle alone or TCZ as in Fig. 5A at different times after commencing treatment (time 0). (**C**) and (**D**) Kyn, IL-6, TGF-β, and *Socs3* expressions in supernatants (Kyn, IL-6, and TGF-β) or lysates (*Socs3*) of 24-h cultures of PLN cells from wild-type (**C**) or *Ido1*$^{-/-}$ (**D**) diabetic NOD mice treated with vehicle alone or TCZ as in Fig. 5 and sacrificed at different times (indicated). One experiment representative of three is shown. Data in panel **A** were analyzed by Kruskal-Wallis with post-hoc Dunn's test. The two-tailed unpaired Student's t-test was used for the analysis of panels **B**-**D**. **P* < 0.05; ***P* < 0.01.

Table S1. Allele distributions of SNPs in the *IDO1* gene among patients with T1D (*N* = 265) and control subjects (*N* = 447) and association test results.

SNP rs# number	Alleles[a]	Minor allele frequencies (%)		*P* value[b]
		T1D	Controls	
rs9657182	T>C	0.450	0.428	0.4620
rs3808606	C>T	0.477	0.462	0.6189
rs10089078	G>A	0.347	0.363	0.5839
rs7820268	C>T	0.338	0.414	**0.0083**
rs3739319	G>A	0.485	0.479	0.8485

[a] The major and minor alleles are represented by the first and second nucleotides, respectively.
[b] *P* values were calculated using the Fisher's exact test. Significant associations are reported in bold.

Table S2. Genotype distributions of SNPs in the *IDO1* gene among patients with T1D (*N* = 265) and control subjects (*N* = 447) and association test results.

SNP rs# number	Alleles[a]: status	Genotype, N (%) A/A	A/a	a/a	*P* value[b]
rs9657182	T>C				
	T1D	85 (33.2)	116 (45.3)	55 (21.5)	0.2204
	Controls	140 (31.3)	230 (51.5)	77 (17.2)	
rs3808606	C>T				
	T1D	76 (29.1)	124 (47.5)	61 (23.4)	0.5477
	Controls	127 (28.4)	229 (51.2)	91 (20.4)	
rs10089078	G>A				
	T1D	108 (43.0)	109 (43.4)	34 (13.5)	0.6812
	Controls	179 (40.0)	209 (46.8)	59 (13.2)	
rs7820268	C>T				
	T1D	110 (43.8)	110 (43.8)	31 (12.4)	**0.0382**
	Controls	157 (35.1)	210 (47.0)	80 (17.9)	
rs3739319	G>A				
	T1D	66 (26.5)	122 (49.0)	61 (24.5)	0.9725
	Controls	122 (27.3)	221 (49.4)	104 (23.3)	

SNP, single nucleotide polymorphism; T1D, type 1 diabetes

[a] The major and minor alleles are represented by the first and second nucleotides, respectively.

[b] *P* values were calculated using the Fisher's exact test. Significant associations are reported in bold.

Table S3. Haplotype frequencies in the *IDO1* gene among patients with T1D (*N* = 265) and control subjects (*N* = 447) and association test results.

Haplotype[a]	Alleles[b]: status	Frequency (%)	*P* value[c]
H1	C-T-G-C		
	T1D	0.436	**0.0021**
	Controls	0.348	
H2	T-C-A-T		
	T1D	0.319	0.4565
	Controls	0.298	
H3	T-C-G-C		
	T1D	0.168	0.0943
	Controls	0.133	
H4	T-C-A-C		
	T1D	0.025	**0.0053**
	Controls	0.060	
H5	C-T-G-T		
	T1D	0.009	**2.188E-6**
	Controls	0.066	

[a] Haplotypes with frequencies >0.05 in either T1D patients or controls are shown.
[b] Pairwise linkage disequilibrium (LD) blocks were defined using the confidence intervals method. Genotype frequencies of the selected SNPs were used to phase haplotype configuration.
[c] *P* values were calculated based on approximate chi-square distribution.

Supplementary Text

Systemic tryptophan (Trp) catabolism is defective in children with type 1 diabetes (T1D)

Significant changes in systemic Trp catabolism have been reported in several human diseases (1). Consistent with a dominant role of underlying immune suppression, higher levels of Kyn (the first catabolite in the IDO1 pathway) have repeatedly been observed in sera from neoplastic (2, 3) and HIV-infected patients (4, 5), with effective therapies being characterized by significant reductions in circulating levels (6). Altered systemic Trp catabolism was found in patients with autoimmune, allergic, and chronic inflammatory diseases (7-9), all conditions whereby immunoregulatory mechanisms appear to be deregulated. Administration of 3,4-hydroxyanthranilic acid, a Trp catabolite downstream production, will reverse paralysis in mice with experimental autoimmune encephalomyelitis (10), and IFN-β, one of the most effective agents in human multiple sclerosis, significantly increases blood Kyn levels in that experimental setting (9).

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 Kyn. 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 two groups ($P = 0.2594$) (Supplemental Figure 1A). The Kyn-to-Trp ratio (Kyn/Trp), considered to be an indicator of systemic IDO1 activity (2), was significantly lower ($P < 0.001$) in sera from T1D patients relative to controls (Supplemental Figure 1A). Multiple regression analysis was used to test if participants' characteristics (age, sex, body mass index, and disease status, i.e., T1D versus control) significantly predicted Kyn/Trp values in sera. The results of the regression analysis indicated that two predictors explained 20% of the variance ($R^2 = 0.2$, $F(4, 114)=7.15$, $P < 0.001$). We found that age significantly predicted

Kyn/Trp changes ($\beta = -1.09$, $P < 0.05$), as did disease status ($\beta = -11.3$, $P < 0.01$). Similar results were obtained in a subgroup analysis on data from T1D patients where we included in the model also the following variables: age, sex, body mass index, glycemia, Hb1Ac, disease duration, as well as presence versus absence of comorbidities. Only age was found to significantly predict Kyn/Trp changes ($\beta = -1.28$, $P < 0.05$; and Supplemental Figure 1B).

No significant relationship was found between Kyn values in sera and in vitro Kyn production by PBMCs, either unstimulated (Supplemental Figure 1C) or stimulated with IFN-γ (Supplemental Figure 1D) in both T1D patients and controls, suggesting that systemic Trp catabolism may not be entirely attributable to IDO1 activity by PBMCs. In this regard, it is interesting to note that serum levels of neopterin—a pteridine produced by blood immune cells known to correlate with IDO1-mediated, systemic Trp catabolism (8)—did not correlate with Kyn/Trp in either control or T1D patients (data not shown), pointing to the contribution of additional Trp-catabolizing enzymes to circulating Kyn levels.

Association of genetic variation in *IDO1* with defective Trp catabolic activity in PBMCs

IDO1 expression and activity are known to exhibit relatively large interindividual variability. The reasons for this variability have remained unclear, although sparse evidence suggests that genetic factors may be involved. In one study, two rare, naturally occurring single nucleotide polymorphisms (SNPs) in the exons of *IDO1* (frequency < 1%, exclusively in African-Americans) were shown to be responsible for impaired functional activity in vitro (11). In another study, the screening of the *IDO1* promoter in healthy Caucasian subjects led to the identification of a Variable Number of Tandem Repeats (VNTR) polymorphism that significantly correlated with decreased circulating

Trp in healthy female subjects but not with an increase in Kyn or in Kyn/Trp ratios (12). More recently, the genotype carrying the T allelic variant (C/T + T/T) of the rs780268 (C6202T) SNP present in intron 5 of *IDO1* was found to be significantly more frequent in patients with an autoimmune disease such as systemic sclerosis as compared to control subjects. Interestingly, patients with the T allelic variant showed impaired Treg function (13). Finally, the rs9657182 polymorphism in the promoter region of *IDO1* was found to be associated with moderate or severe IFN-α-induced depressive symptoms, thereby further supporting the notion that IDO1 has an important role in cytokine-induced behavioral changes (14).

Restoration of Trp catabolism by tocilizumab in PBMCs from T1D patients

To investigate whether TCZ could modulate the production of soluble mediators, cytokine profile in T1D and/or control PBMCs, supernatants from cells either unstimulated or stimulated with 100 U/ml IFN-γ for 48 h in the presence or absence of 10 μM TCZ were analyzed by multiplex assay (Human Cytokine/Chemokine magnetic bead panel Cat N. HCYTMAG-60K; EMD Millipore Corporation) by means of a MAGPIX system (Luminex Corporation). Levels of eotaxin, IL-2, IL-3, IL-5, IL-7, IL-9, IL-15, IL-17A, and TNF-β were below the detection limit of the assay, whereas those of IL-8 and MCP-1 were over the maximum limit. IFN-γ concentrations were not considered due to the external addition of the cytokine to some groups. IL-13 (range 2.58-4.66 pg/ml) was detected only in PBMCs from one T1D patient affected also by autoimmune thyroiditis. All levels of detectable cytokines are shown in Supplemental Figure 5. As a whole, the results showed that no significant modulatory effect for TCZ could be found in any experimental group. However, some significant differences were found in T1D *versus* control PBMCs, either in basal conditions or in response to IFN-γ. Specifically, the

production of IL-10 and vascular endothelial growth factor (VEGF) was higher in unstimulated T1D PBMCs whereas that of IP-10 and IL-12p70 was lower in IFN-γ−stimulated T1D PBMCs, when compared to their respective control counterpart. In any case, TCZ was not able to restore the control cytokine profile in T1D PBMCs.

As a whole, our data suggested that PBMCs from T1D patients are characterized by a basal inflammatory state that would impede the orchestration of appropriate responses to additional inflammatory signals, possibly confirming the existence of exhausted T cells in autoimmunity (15).

REFERENCES

1. Chen, Y., and Guillemin, G.J. 2009. Kynurenine pathway metabolites in humans: disease and healthy States. *Int J Tryptophan Res* 2:1-19.
2. Suzuki, Y., Suda, T., Furuhashi, K., Suzuki, M., Fujie, M., Hahimoto, D., Nakamura, Y., Inui, N., Nakamura, H., and Chida, K. 2010. Increased serum kynurenine/tryptophan ratio correlates with disease progression in lung cancer. *Lung Cancer* 67:361-365.
3. de Jong, R.A., Nijman, H.W., Boezen, H.M., Volmer, M., Ten Hoor, K.A., Krijnen, J., van der Zee, A.G., Hollema, H., and Kema, I.P. 2011. Serum tryptophan and kynurenine concentrations as parameters for indoleamine 2,3-dioxygenase activity in patients with endometrial, ovarian, and vulvar cancer. *Int J Gynecol Cancer* 21:1320-1327.
4. Fuchs, D., Forsman, A., Hagberg, L., Larsson, M., Norkrans, G., Reibnegger, G., Werner, E.R., and Wachter, H. 1990. Immune activation and decreased tryptophan in patients with HIV-1 infection. *J Interferon Res* 10:599-603.
5. Favre, D., Mold, J., Hunt, P.W., Kanwar, B., Loke, P., Seu, L., Barbour, J.D., Lowe, M.M., Jayawardene, A., Aweeka, F., et al. 2010. Tryptophan catabolism by indoleamine 2,3-dioxygenase 1 alters the balance of TH17 to regulatory T cells in HIV disease. *Sci Transl Med* 2:32ra36.
6. Zangerle, R., Widner, B., Quirchmair, G., Neurauter, G., Sarcletti, M., and Fuchs, D. 2002. Effective antiretroviral therapy reduces degradation of tryptophan in patients with HIV-1 infection. *Clin Immunol* 104:242-247.
7. Raitala, A., Karjalainen, J., Oja, S.S., Kosunen, T.U., and Hurme, M. 2006. Indoleamine 2,3-dioxygenase (IDO) activity is lower in atopic than in non-atopic individuals and is enhanced by environmental factors protecting from atopy. *Mol Immunol* 43:1054-1056.
8. Schroecksnadel, K., Zangerle, R., Bellmann-Weiler, R., Garimorth, K., Weiss, G., and Fuchs, D. 2007. Indoleamine-2, 3-dioxygenase and other interferon-gamma-mediated pathways in patients with human immunodeficiency virus infection. *Curr Drug Metab* 8:225-236.
9. Amirkhani, A., Rajda, C., Arvidsson, B., Bencsik, K., Boda, K., Seres, E., Markides, K.E., Vecsei, L., and Bergquist, J. 2005. Interferon-beta affects the tryptophan metabolism in multiple sclerosis patients. *Eur J Neurol* 12:625-631.
10. Platten, M., Ho, P.P., Youssef, S., Fontoura, P., Garren, H., Hur, E.M., Gupta, R., Lee, L.Y., Kidd, B.A., Robinson, W.H., et al. 2005. Treatment of autoimmune neuroinflammation with a synthetic tryptophan metabolite. *Science* 310:850-855.
11. Arefayene, M., Philips, S., Cao, D., Mamidipalli, S., Desta, Z., Flockhart, D.A., Wilkes, D.S., and Skaar, T.C. 2009. Identification of genetic variants in the human indoleamine 2,3-dioxygenase (IDO1) gene, which have altered enzyme activity. *Pharmacogenet Genomics* 19:464-476.
12. Soichot, M., Hennart, B., Al Saabi, A., Leloire, A., Froguel, P., Levy-Marchal, C., Poulain-Godefroy, O., and Allorge, D. 2011. Identification of a variable number of tandem repeats polymorphism and characterization of LEF-1 response elements in the promoter of the IDO1 gene. *PLoS One* 6:e25470.
13. Tardito, S., Negrini, S., Conteduca, G., Ferrera, F., Parodi, A., Battaglia, F., Kalli, F., Fenoglio, D., Cutolo, M., and Filaci, G. 2013. Indoleamine 2,3 dioxygenase gene polymorphisms correlate with CD8+ Treg impairment in systemic sclerosis. *Hum Immunol* 74:166-169.

14. Smith, A.K., Simon, J.S., Gustafson, E.L., Noviello, S., Cubells, J.F., Epstein, M.P., Devlin, D.J., Qiu, P., Albrecht, J.K., Brass, C.A., et al. 2012. Association of a polymorphism in the indoleamine- 2,3-dioxygenase gene and interferon-alpha-induced depression in patients with chronic hepatitis C. *Mol Psychiatry* 17:781-789.
15. McKinney, E.F., Lee, J.C., Jayne, D.R., Lyons, P.A., and Smith, K.G. 2015. T-cell exhaustion, co-stimulation and clinical outcome in autoimmunity and infection. *Nature*.