Type 1 diabetes linked PTPN22 gene polymorphism is associated with the frequency of circulating regulatory T cells

Valta Milla[1], Gazali Ahmad Mahfuz[2] *, Viisanen Tyyne[2], Ihantola Emmi-Leena[2], Ekman Ilse[2], Toppari Jorma[3,4], Knip Mikael[5,6,7,8], Veijola Riitta[9], Ilonen Jorma[1,10], Lempainen Johanna[1,4,10, **], Kinnunen Tuure[2,11 **]

[1] Immunogenetics Laboratory, Institute of Biomedicine, University of Turku, Turku, Finland

[2] Department of Clinical Microbiology, Institute of Clinical Medicine, University of Eastern Finland, Kuopio, Finland

[3] Institute of Biomedicine, Research Centre for Integrative Physiology and Pharmacology, University of Turku, Turku, Finland

[4] Department of Pediatrics, Turku University Hospital, Turku, Finland

[5] Children's Hospital, University of Helsinki and Helsinki University Hospital, Helsinki, Finland

[6] Research Program for Clinical and Molecular Metabolism, Faculty of Medicine, University of Helsinki, Finland

[7] Folkhälsan Research Center, Helsinki, Finland

[8] Tampere Center for Child Health Research, Tampere University Hospital, Tampere, Finland

[9] Department of Pediatrics, PEDEGO Research Unit, Medical Research Center, University of Oulu, Oulu, Finland and Department of Children and Adolescents, Oulu University Hospital, Oulu, Finland

[10] Clinical Microbiology, Turku University Hospital, Turku, Finland

[11] Eastern Finland Laboratory Centre (ISLAB), Kuopio, Finland

*The current affiliation for Ahmad M Gazali is Faculty of Industrial Sciences and Technology, Universiti Malaysia Pahang, Pekan, Malaysia.

** The senior authors contributed equally to the study

Corresponding author: Milla Valta

Institute of Biomedicine, University of Turku

Medicity, Tykistökatu 6A FI-20521, Turku, Finland

tel: +358 29 450 4387, email: mkmval@utu.fi

Abbreviations:

Treg	regulatory T cell
T1D	type 1 diabetes
DIPP	Diabetes Prediction and Prevention study

ABSTRACT

Dysfunction of FOXP3-positive regulatory T cells (Tregs) likely plays a major role in the pathogenesis of multiple autoimmune diseases, including type 1 diabetes (T1D). Whether genetic polymorphisms associated with the risk of autoimmune diseases affect Treg frequency or function is currently unclear. Here, we analysed the effect of T1D-associated major HLA class II haplotypes and seven single nucleotide polymorphisms in six non-HLA genes [*INS* (rs689), *PTPN22* (rs2476601), *IL2RA* (rs12722495 and rs2104286), *PTPN2* (rs45450798), *CTLA4* (rs3087243) and *ERBB3* (rs2292239)] on peripheral blood Treg frequencies. These were determined by flow cytometry in 65 subjects who had progressed to T1D, 86 islet autoantibody-positive at-risk subjects and 215 islet autoantibody-negative healthy controls. The *PTPN22* rs2476601 risk allele A was associated with an increase in total ($p = 6 \times 10^{-6}$) and naïve ($p = 4 \times 10^{-5}$) CD4+CD25+CD127lowFOXP3+ Treg frequencies. These findings were validated in a separate cohort comprising ten trios of healthy islet autoantibody-negative children carrying each of the three *PTPN22* rs2476601 genotypes AA, AG and GG ($p = 0.005$ for total and $p = 0.03$ for naïve Tregs, respectively). In conclusion, our analysis implicates the autoimmune *PTPN22* rs2476601 risk allele A in controlling the frequency of Tregs in human peripheral blood.

INTRODUCTION

Type 1 diabetes (T1D) is a chronic autoimmune disease that results from an immune-mediated loss of pancreatic β cells and/or their functionality [1]. Both environmental and genetic factors are involved in the disease process [2, 3]. Although the exact immunopathogenesis of T1D remains elusive, T cells appear to be the main mediators of β cell destruction [1].

Regulatory T cells (Tregs) expressing the transcription factor FOXP3 are a subpopulation of helper T cells that play a critical role in the maintenance of immune homeostasis [4]. Dysregulation of Tregs is an obvious potential mechanism for autoimmunity, and multiple previous studies have reported alterations in Treg frequencies and/or functionality in various autoimmune diseases [5]. Moreover, several genetic polymorphisms associated with the risk of autoimmune diseases, including T1D, likely affect molecules associated with Treg function (e.g. *IL2RA, PTPN2, PTPN22, CTLA4*) [6]. For example, multiple *IL2RA* and *PTPN2* variants have been shown to affect IL-2 signalling in Tregs [7-9] and a *CTLA4* variant has been reported to alter CTLA-4 expression in Tregs [10].

Studies addressing peripheral blood Treg frequencies in patients with T1D have produced somewhat conflicting results, with studies reporting increased [11], decreased [12] or similar frequencies [13-15] of Tregs in patients with T1D compared to healthy controls. The highly variable approaches to define Tregs in different studies make direct comparison of these results challenging. Our recent large and well-controlled study demonstrated a subtle increase in the frequency of total and naïve circulating FOXP3+ Tregs in children with newly diagnosed T1D [16].

Here, we set out to explore possible associations between peripheral blood Treg frequencies and major genetic determinants of T1D risk. Utilising the large data set generated in our previous study [16], we analysed the effect on Treg frequencies of major HLA risk haplotypes (HLA‑DR3‑DQ2 and HLA‑DR4‑DQ8) and seven non-HLA polymorphisms selected for strong effect and/or a presumed role in Treg cell functionality out of the more than 50 loci currently associated with T1D risk [1].

RESULTS & DISCUSSION

PTPN22 rs2476601 risk allele A is associated with increased Treg frequencies in a T1D study cohort

The effect of genetic factors associated with T1D risk on circulating Treg frequencies was studied in a cohort of 363 children, comprising 65 subjects with newly diagnosed T1D, 83 islet autoantibody-positive (AAb+) at-risk subjects and 215 autoantibody-negative healthy control subjects from which a flow-cytometric evaluation of Treg frequencies was previously performed [16]. The study subjects were genotyped for seven non-HLA polymorphisms repeatedly associated with T1D risk [*INS* (rs689), *PTPN22* (rs2476601), *IL2RA* (rs2104286 and rs12722495), *PTPN2* (rs45450798), *CTLA4* (rs3087243) and *ERBB3* (rs2292239)] [6] . In addition, the effect of the two major T1D risk haplotypes in the HLA region, DR3-DQ2 and DR4-DQ8, on Treg frequencies was assessed. We used two different definitions for Tregs, CD4+CD25+CD127low and CD4+CD25+CD127lowFOXP3+ [16]. These total Treg populations were additionally divided into naïve (CD45RA+ or CD45RO-) and memory (CD45RA- or CD45RO+) Treg subpopulations, as previously defined [16]. To attain a sufficient level of statistical power to detect effects caused by rare polymorphisms, the data from the entire cohort was pooled in our primary analysis.

We observed that the *PTPN22* rs2476601 T1D risk allele A appeared to be strongly associated with an increased total Treg frequency (Table 1). The association could be observed both with the CD4+CD25+CD127low and the CD4+CD25+CD127lowFOXP3+ Treg definitions (Table 1). Post-hoc comparison between children with different *PTPN22* rs2476601 genotypes revealed a statistically significant increase in Treg frequencies in children carrying the AA genotype compared to GG children with both Treg definitions (Figure 1). Additionally, a similar increase was observed

in AG children compared to GG children with the CD4+CD25+CD127lowFOXP3+ Treg definition (Figure 1). These alterations in Treg frequencies appeared to be explained by an increased frequency of naïve but not memory Tregs (Table 1 and Figure 1). Naïve Treg frequencies were elevated both with the CD4+CD25+CD127low and CD4+CD25+CD127lowFOXP3+ Treg definitions (Table 1). In post-hoc analyses, a statistically significant difference between AA and GG children was observed with both naïve Treg definitions, and with the CD4+CD25+CD127lowFOXP3+ naïve Treg definition also between AG and GG children (Figure 1).

The subgroups of healthy controls, AAb+ at-risk children and T1D patients were also analysed separately. Although, statistical power was lost with this approach, in most of these separate analyses a tendency mirroring the results of the main analysis could be seen across the groups (Figure 2, Supporting Information Table 1 and Figure 1). Of note, children with T1D carrying the protective *PTPN22* rs2476601 genotype GG had higher frequencies of Tregs compared to healthy controls carrying the genotype GG (Supporting Information Figure 2), demonstrating that in addition to *PTPN22* genotype, other factors are involved in the previously observed increase in Treg frequencies in children with T1D [16]. No associations with Treg frequencies were observed with the other six SNPs and HLA the class II genotypes studied after correction for multiple testing (Table 1, Supporting Information Table 2 and Figure 3).

Healthy children carrying the *PTPN22* rs2476601 risk allele A display elevated Treg frequencies

To confirm our results on the effect of *PTPN22* rs2476601 genotype on Treg frequencies, we analysed a separate validation cohort comprising ten trios of healthy autoantibody-negative children carrying each of the three possible *PTPN22* rs2476601 genotypes, AA, AG and GG. Analyses of

the validation cohort confirmed our initial observations (Figure 3). Both total and naïve

CD4+CD25+CD127lowFOXP3+ Treg frequencies displayed an association with the *PTPN22*

rs2476601 genotype (Supporting Information Table 3). Statistically significant differences were

observed in post-hoc analyses between children with the AA and GG genotypes (Figure 3). In

addition, total Treg frequencies were also increased in AG children compared to GG children

(Figure 3). Finally, a higher Treg frequency was observed in AG and AA children compared to GG

children also by using the definition of CD4+CD25+CD127lowHELIOS+FOXP3+ [16] for Tregs

(Supporting Information Figure 4).

Concluding remarks

 The *PTPN22* gene encodes a protein tyrosine phosphatase involved in T-cell receptor, B-cell

receptor and innate immune signalling [17]. The *PTPN22* rs2476601 autoimmune risk allele A

causes a R620W missense variation that impairs the ability of PTPN22 to bind to c-Src kinase

(CSK) [18] and in addition to T1D, the *PTPN22* rs2476601 polymorphism is associated with the

risk of multiple other autoimmune diseases [17]. The functional consequences of this variation

remain incompletely understood and thus does the exact mechanism how this polymorphism affects

the risk of autoimmunity. Both a gain-of-function [19-22] and loss-of-function [23] effect on

immune cell signalling have been reported. It is also possible that the PTPN22 R620W variant can

have both functions, modulating distinct downstream effects in different immune cell

subpopulations, as recently suggested [17, 24].

PTPN22 is also one of the targets of the transcription factor FOXP3 and its upregulation is inhibited

by FOXP3 expression [25]. It is therefore possible that the *PTPN22* rs2476601 polymorphism

affects Treg development, homeostasis or function. In *Ptpn22*-knockout mice, CD4+CD25+Foxp3+

Treg frequencies in the periphery have been shown to be expanded in multiple different studies [26-

28]. Moreover, mice carrying a *Ptpn22* variant with a mutation analogous to human *PTPN22* R620W also display increasing peripheral Treg frequencies with age [29]. Limited data in humans suggest that Treg cells from individuals homozygous for the *PTPN22* rs2476601 A risk allele (AA genotype) display TCR signalling defects similar to those observed in conventional T cells [30]. No differences in circulating Treg frequencies in association with *PTPN22* genotypes have been reported in two previous studies analysing small cohorts of adult subjects [30, 31], although in line with our observation a tendency for higher Treg frequencies were observed in the group with the AA genotype in the latter study [31]. However, it is important to note that both of these studies have been underpowered compared to our study. Moreover, as the PTPN22 genotype appears to mainly affect naïve Treg frequencies, this difference may be more readily detected in young children analysed in our study compared to adult donors analysed in the previous studies.

The increase in peripheral blood Treg frequencies in individuals carrying the *PTPN22* autoimmune risk allele A may seem counterintuitive at first. However, since PTPN22 signalling alterations affect multiple immune cell subsets, it is possible that the increase in Tregs reflects a compensatory mechanism for autoimmunity-promoting changes in other immune cell subsets.

An important open question is whether the *PTPN22* rs2476601 risk allele A also affects the functionality of Tregs. Tregs isolated from mice expressing the analogous Ptpn22 variant appear to have normal suppressive function [29]. Due to the limited blood sample volumes obtained from paediatric subjects, we could not isolate sufficient numbers of Tregs to study their functionality *in vitro* in this study. However, a previous small study suggested that Tregs isolated from individuals with the *PTPN22* AA genotype were efficient in suppressing the proliferation of effector T cells but defective in suppressing their IFN-γ production *in vitro* [30].

In conclusion, we observed an elevated frequency of circulating Tregs among subjects carrying the *PTPN22* rs2476601 autoimmune risk allele A. This effect was observed both in the total Treg and naïve Treg compartments. Our current findings provide the first evidence that the *PTPN22* rs2476601 risk allele A alters Treg homeostasis in humans, although the exact mechanism behind this phenomenon and its relevance for an increased risk of autoimmunity requires further investigation.

MATERIALS AND METHODS

Study subjects

The first study cohort comprised 65 case subjects diagnosed with T1D (mean age 7.4 years ± SD 3.9) sampled within seven days after diagnosis, as well as 83 islet autoantibody-positive at-risk subjects (mean age 8.6 years ± SD 4.7) and 215 autoantibody-negative control subjects (mean age 8.8 years ± SD 3.9) [16]. Autoantibody-positive and autoantibody-negative subjects were participants in the Finnish Type 1 Diabetes Prediction and Prevention (DIPP) study [32]. All DIPP participants carried an HLA class II genotype associated with an increased risk for the development T1D. Patients with T1D were recruited at the Department of Pediatrics, Turku University Hospital after admission to hospital due to newly diagnosed T1D. Autoantibody-positivity in the DIPP participants was defined as having at least one persistent, biochemical autoantibody either to insulin (IAA), glutamic acid decarboxylase 65 (GADA) or islet antigen-2 antibody (IA-2A). IAA, GADA and IA-2A were analysed as described earlier [33].

The validation cohort comprised ten trios of healthy children from the DIPP study carrying the three possible *PTPN22* rs2476601 genotypes (AA, AG, GG). All of these subjects had remained autoantibody-negative and clinically non-diabetic until sampling for PBMCs analysed here. The

trios were matched for HLA class II genotype, age (mean age 2.3 years ± SD 1.5), sex and date of sampling.

The study was approved by the local ethics committee and written informed consent was provided by the parents of the children participating in this study.

Flow-cytometric analyses

The staining procedure for the primary cohort has been reported previously [16]. For the validation cohort, frozen PBMCs (cryopreserved in 10% DMSO) were thawed and viability staining was performed using Zombie Aqua dye (BioLegend, San Diego, CA) according to the manufacturer's instructions. Immunostaining for surface markers was performed on 10^6 PBMCs per staining by incubating the cells with anti-CD3 APC-F750 (clone SK-7, Biolegend), anti-CD4 PE-Cy7 (RPA-T4, Biolegend), anti-CD25 PE (4E3, Miltenyi Biotec, Bergisch Gladbach, Germany), anti-CD127 PerCP-Cy5.5 (A019D5, Biolegend) and anti-CD45RO BV421 (UCHL1, Biolegend) for 20 to 30 min. Fixation and permeabilization were performed using the Foxp3/Transcription Factor Staining Buffer set (eBioscience, San Diego, CA), followed by staining with anti-FOXP3 A488 (clone 259D) and anti-HELIOS A647 (clone 22F6), both from Biolegend. The samples were analysed with a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA), and the flow cytometry data were examined using FlowJo software (BD Biosciences). The detailed gating strategy for Treg subsets analysed in the primary study cohort has been reported previously [16] and the gating strategy used to analyse Tregs in the validation cohort is shown in Supporting Information Figure 5. Coded samples were used throughout, and the flow-cytometric analyses were performed blinded to the classification of the sample. Flow cytometry experiments were conducted in line with the published guideline [34].

Genotyping

Seven T1D risk associated SNP markers across six loci, *INS* (rs689), *PTPN22* (rs2476601), *IL2RA* (rs2104286 and rs12722495), *PTPN2* (rs45450798), *CTLA4* (rs3087243) and *ERBB3* (rs2292239), were analysed using TaqMan SNP Genotyping Assays (Thermo Fisher Scientific, Pleasanton, CA).

Genotyping for the major risk HLA-DR-DQ haplotypes was performed using sequence-specific oligonucleotide probes, as described earlier [35]. The subjects were divided into four groups according to their HLA-DR-DQ genotypes: children with the DR3-DQ2 (DQA1*05-DQB1*02) haplotype, children with the DR4-DQ8 (DRB1*04:01/02/04/05-DQA1*03-DQB1*03:02) haplotype, children with both and children with neither of these haplotypes.

Statistical analyses

Statistical analyses were performed using IBM SPSS Statistics 24.0 (Armonk, NY). The flow-cytometric and genotyping data were combined in ANCOVA with the age of the child at the time of sample collection as a covariant to account for the maturation of the immune system. Primary analyses were conducted with pooled data from the entire cohort (T1D, AAb+ and control groups) to gain sufficient level of statistical power to detect differences caused by all studied polymorphisms (minor allele frequencies ranging from 0.06 to 0.33) [36]. Additionally, post-hoc analyses were conducted using the Bonferroni method to explore the differences between different genotypes. $p < 0.05$ was considered statistically significant. The p-values of the primary analysis were adjusted for multiple testing using Bonferroni correction with a factor of 8 (seven SNPs and HLA class II genotype; $p < 0.006$), as the frequencies of the investigated cell populations are dependent on one another.

CONFLICT OF INTEREST DISCLOSURE

The authors have no competing interests.

REFERENCES

1 **Katsarou, A., Gudbjörnsdottir, S., Rawshani, A., Dabelea, D., Bonifacio, E., Anderson, B. J., Jacobsen, L. M., et al,** Type 1 diabetes mellitus. *Nat. Rev. Dis. Primers* 2017. **3:** 17016. 10.1038/nrdp.2017.16.

2 **Pociot, F. and Lernmark, Å,** Genetic risk factors for type 1 diabetes. *Lancet* 2016. **387:** 2331-2339. 10.1016/S0140-6736(16)30582-7.

3 **Rewers, M. and Ludvigsson, J.,** Environmental risk factors for type 1 diabetes. *Lancet* 2016. **387:** 2340-2348. 10.1016/S0140-6736(16)30507-4.

4 **Wing, J. B., Tanaka, A. and Sakaguchi, S.,** Human FOXP3+ regulatory T cell heterogeneity and function in autoimmunity and cancer. *Immunity* 2019. **50:** 302-316. //doi.org/10.1016/j.immuni.2019.01.020.

5 **Grant, C. R., Liberal, R., Mieli-Vergani, G., Vergani, D. and Longhi, M. S.,** Regulatory T-cells in autoimmune diseases: Challenges, controversies and—yet—unanswered questions. *Autoimmun. Rev.* 2015. **14:** 105-116. //doi.org/10.1016/j.autrev.2014.10.012.

6 **Todd, J. A.,** Etiology of type 1 diabetes. *Immunity* 2010. **32:** 457-467. //doi.org/10.1016/j.immuni.2010.04.001.

7 **Cerosaletti, K., Schneider, A., Schwedhelm, K., Frank, I., Tatum, M., Wei, S., Whalen, E., et al,** Multiple autoimmune-associated variants confer decreased IL-2R signaling in CD4+ CD25(hi) T cells of type 1 diabetic and multiple sclerosis patients. *PloS One* 2013. **8:** e83811. 10.1371/journal.pone.0083811.

8 **Garg, G., Tyler, J. R., Yang, J. H. M., Cutler, A. J., Downes, K., Pekalski, M., Bell, G. L., et al,** Type 1 diabetes-associated IL2RA variation lowers IL-2 signaling and contributes to diminished CD4+CD25+ regulatory T cell function. *J. Immunol.* 2012. **188:** 4644-4653. 10.4049/jimmunol.1100272.

9 **Yang, J. H. M., Cutler, A. J., Ferreira, R. C., Reading, J. L., Cooper, N. J., Wallace, C., Clarke, P., et al,** Natural variation in interleukin-2 sensitivity influences regulatory T-cell frequency and function in individuals with long-standing type 1 diabetes. *Diabetes* 2015. **64:** 3891-3902. 10.2337/db15-0516.

10 **Chen, Y., Chen, S., Gu, Y., Feng, Y., Shi, Y., Fu, Q., Wang, Z., et al,** CTLA-4 +49 G/A, a functional T1D risk SNP, affects CTLA-4 level in treg subsets and IA-2A positivity, but not beta-cell function. *Sci. Rep.* 2018. **8:** 10074. 10.1038/s41598-018-28423-9.

11 **Ferreira, R. C., Simons, H. Z., Thompson, W. S., Rainbow, D. B., Yang, X., Cutler, A. J., Oliveira, J., et al,** Cells with treg-specific FOXP3 demethylation but low CD25 are prevalent in autoimmunity. *J. Autoimmun.* 2017. **84:** 75-86. 10.1016/j.jaut.2017.07.009.

12 **Kukreja, A., Cost, G., Marker, J., Zhang, C., Sun, Z., Lin-Su, K., Ten, S., et al,** Multiple immuno-regulatory defects in type-1 diabetes. *J. Clin. Invest.* 2001. **109:** 131-140. 10.1172/JCI13605.

13 **Brusko, T. M., Wasserfall, C. H., Clare-Salzler, M., Schatz, D. A. and Atkinson, M. A.,** Functional defects and the influence of age on the frequency of $CD4^+CD25^+$ T-cells in type 1 diabetes. *Diabetes* 2005. **54:** 1407-14. 10.2337/diabetes.54.5.1407.

14 **Lindley, S., Dayan, C. M., Bishop, A., Roep, B. O., Peakman, M. and Tree, T. I. M.,** Defective suppressor function in $CD4^+CD25^+$ T-cells from patients with type 1 diabetes. *Diabetes* 2005. **54:** 92-9. 10.2337/diabetes.54.1.92.

15 **Hamari, S., Kirveskoski, T., Glumoff, V., Kulmala, P., Simell, O., Knip, M. and Veijola, R.,** Analyses of regulatory CD4(+) CD25(+) FOXP3(+) T cells and observations from peripheral T cell subpopulation markers during the development of type 1 diabetes in children. *Scand. J. Immunol.* 2016. **83:** 279-287. 10.1111/sji.12418.

16 **Viisanen, T., Gazali, A. M., Ihantola, E., Ekman, I., Näntö-Salonen, K., Veijola, R., Toppari, J., et al,** FOXP3+ regulatory T cell compartment is altered in children with newly diagnosed type 1 diabetes but not in autoantibody-positive at-risk children. *Front. Immunol.* 2019. **10:** 19. 10.3389/fimmu.2019.00019.

17 **Bottini, N. and Peterson, E. J.,** Tyrosine phosphatase PTPN22: Multifunctional regulator of immune signaling, development, and disease. *Annu. Rev. Immunol.* 2014. **32:** 83-119. 10.1146/annurev-immunol-032713-120249.

18 **Bottini, N., Musumeci, L., Alonso, A., Rahmouni, S., Nika, K., Rostamkhani, M., MacMurray, J., et al,** A functional variant of lymphoid tyrosine phosphatase is associated with type I diabetes. *Nat. Genet.* 2004. **36:** 337-338. 10.1038/ng1323.

19 **Vang, T., Congia, M., Macis, M. D., Musumeci, L., Orrú, V., Zavattari, P., Nika, K., et al,** Autoimmune-associated lymphoid tyrosine phosphatase is a gain-of-function variant. *Nat. Genet.* 2005. **37:** 1317-1319. 10.1038/ng1673.

20 **Rieck, M., Arechiga, A., Onengut-Gumuscu, S., Greenbaum, C., Concannon, P. and Buckner, J. H.,** Genetic variation in PTPN22 corresponds to altered function of T and B lymphocytes. *J. Immunol.* 2007. **179:** 4704-4710. 10.4049/jimmunol.179.7.4704.

21 **Aarnisalo, J., Treszl, A., Svec, P., Marttila, J., Öling, V., Simell, O., Knip, M., et al,** Reduced CD4 +T cell activation in children with type 1 diabetes carrying the PTPN22/lyp 620Trp variant. *J. Autoimmun.* 2008. **31:** 13-21. 10.1016/j.jaut.2008.01.001.

22 **Arechiga, A. F., Habib, T., He, Y., Zhang, X., Zhang, Z., Funk, A. and Buckner, J. H.,** Cutting edge: The PTPN22 allelic variant associated with autoimmunity impairs B cell signaling. *J. Immunol.* 2009. **182:** 3343-3347. 10.4049/jimmunol.0713370.

23 **Zhang, J., Zahir, N., Jiang, Q., Miliotis, H., Heyraud, S., Meng, X., Dong, B., et al,** The autoimmune disease–associated PTPN22 variant promotes calpain-mediated lyp/pep degradation associated with lymphocyte and dendritic cell hyperresponsiveness. *Nat. Genet.* 2011. **43:** 902-7. 10.1038/ng.904.

24 **Vang, T., Nielsen, J. and Burn, G. L.,** A switch-variant model integrates the functions of an autoimmune variant of the phosphatase PTPN22. *Sci. Signal.* 2018. **11:** eaat0936. 10.1126/scisignal.aat0936.

25 **Marson, A., Kretschmer, K., Frampton, G. M., Jacobsen, E. S., Polansky, J. K., MacIsaac, K. D., Levine, S. S., et al,** Foxp3 occupancy and regulation of key target genes during T-cell stimulation. *Nature* 2007. **445:** 931-935. 10.1038/nature05478.

26 **Brownlie, R. J., Miosge, L. A., Vassilakos, D., Svensson, L. M., Cope, A. and Zamoyska, R.,** Lack of the phosphatase PTPN22 increases adhesion of murine regulatory T cells to improve their immunosuppressive function. *Sci. Signal.* 2012. **5:** ra87. 10.1126/scisignal.2003365.

27 **Maine, C. J., Hamilton-Williams, E., Cheung, J., Stanford, S. M., Bottini, N., Wicker, L. S. and Sherman, L. A.,** PTPN22 alters the development of T regulatory cells in the thymus. *J. Immunol.* 2012. **188:** 5267-5275. 10.4049/jimmunol.1200150.

28 **Zheng, P. and Kissler, S.,** *PTPN22* silencing in the NOD model indicates the type 1 Diabetes–Associated allele is not a loss-of-function variant. *Diabetes* 2013. **62:** 896-904. 10.2337/db12-0929.

29 **Dai, X., James, R. G., Habib, T., Singh, S., Jackson, S., Khim, S., Moon, R. T., et al,** A disease-associated PTPN22 variant promotes systemic autoimmunity in murine models. *J. Clin. Invest.* 2013. **123:** 2024-2036. 10.1172/JCI66963.

30 **Vang, T., Landskron, J., Viken, M. K., Oberprieler, N., Torgersen, K. M., Mustelin, T., Tasken, K., et al,** The autoimmune-predisposing variant of lymphoid tyrosine phosphatase favors T helper 1 responses. *Hum. Immunol.* 2013. **74:** 574-585. 10.1016/j.humimm.2012.12.017.

31 **Chemin, K., Ramsköld, D., Diaz-Gallo, L., Herrath, J., Houtman, M., Tandre, K., Rönnblom, L., et al,** EOMES-positive CD4+ t cells are increased in PTPN22 (1858T) risk allele carriers. *Eur. J. Immunol.* 2018. **48:** 655-669. 10.1002/eji.201747296.

32 **Ilonen, J., Hammais, A., Laine, A., Lempainen, J., Vaarala, O., Veijola, R., Simell, O. and Knip, M.,** Patterns of β-cell autoantibody appearance and genetic associations during the first years of life. *Diabetes* 2013. **62:** 3636-3640. 10.1080/08916930701619169.

33 **Siljander, H. T. A., Simell, S., Hekkala, A., Lähde, J., Simell, T., Vähäsalo, P., Veijola, R., et al,** Predictive characteristics of diabetes-associated autoantibodies among children with HLA-conferred disease susceptibility in the general population. *Diabetes* 2009. **58:** 2835-42. 10.2337/db08-1305.

34 **Cossarizza, A., Chang, H., Radbruch, A., Akdis, M., Andrä, I., Annunziato, F., Bacher, P., et al,** Guidelines for the use of flow cytometry and cell sorting in immunological studies. *Eur. J. Immunol.* 2017. **47:** 1584-1797. 10.1002/eji.201646632.

35 **Ilonen, J., Kiviniemi, M., Lempainen, J., Simell, O., Toppari, J., Veijola, R. and Knip, M.,** Genetic susceptibility to type 1 diabetes in childhood – estimation of HLA class II associated disease risk and class II effect in various phases of islet autoimmunity. *Pediatr. Diabetes* 2016. **17:** 8-16. 10.1111/pedi.12327.

36 **Karczewski, K. J., Francioli, L. C., Tiao, G., Cummings, B. B., Alföldi, J., Wang, Q., Collins, R. L., et al,** Variation across 141,456 human exomes and genomes reveals the spectrum of loss-of-function intolerance across human protein-coding genes. *bioRxiv.* 2019. 531210. 10.1101/531210.

FIGURE LEGENDS

Figure 1. Increased frequency of total and naïve Tregs in children carrying the *PTPN22* rs2476601 T1D risk allele A. Differences in frequencies of total (A), naïve (B) and memory (C) CD4+CD25+CD127low Tregs, and total (D), naïve (E) and memory (F) CD4+CD25+CD127lowFOXP3+ Tregs in children with the *PTPN22* rs2476601 genotypes GG, AG and AA. The analysis was conducted by using pooled Treg frequency data from flow cytometry analysis of PBMCs [16] from 363 children (65 T1D patients, 83 autoantibody-positive (AAb+) subjects and 215 healthy (AAb-) controls). ANCOVA with Bonferroni post-hoc comparisons were used to analyse differences between the genotypes. Horizontal lines indicate mean values.

Figure 2. Treg frequencies in children with the PTPN22 rs2476601 genotypes GG, AG and AA analysed separately in groups of healthy control subjects, autoantibody-positive (AAb+) at-risk children and children with newly-diagnosed T1D. Total, naïve and memory CD4+CD25+CD127lowFOXP3+ Treg frequencies in PBMCs are displayed for 104 healthy autoantibody-negative (AAb-) controls (A-C), 44 AAb+ subjects (D-F) and 33 T1D patients (G-I) analysed as separate cohorts. Horizontal lines indicate mean values.

Figure 3. ***PTPN22* rs2476601 type 1 diabetes risk allele A is associated with increased Treg frequencies in the validation cohort. .** Representative examples of CD3+CD4+CD25+ CD127lowFOXP3+ flow-cytometric Treg stainings in PBMCs from one trio of children carrying the different *PTPN22* genotypes GG, AG and AA (A). The antibodies used are listed in Materials and Methods, and the detailed gating strategy is shown in Supporting Information Fig. 5. The

frequencies of total (B), naïve (C) and memory (D) CD4+CD25+CD127low FOXP3+ Tregs in ten trios of healthy children carrying the different *PTPN22* rs2476601 genotypes. ANCOVA with Bonferroni post-hoc comparisons were used to analysed differences between the genotypes. Horizontal lines indicate mean values.

Table 1. Associations of selected non-HLA single nucleotide polymorphisms with regulatory T cell frequencies.

	INS/rs689	PTPN22/rs2476601	IL2RA/rs2104286	IL2RA/rs12722495	PTPN2/rs45450798	CTLA4/rs3087243	ERBB3/rs2292239
Total Treg (CD4+CD25+CD127- of CD4+)	0.537	**0.002**	0.804	0.271	0.583	0.11	0.35
Naive Treg (CD4+CD25+CD127-CD45RA+ of CD4+)	0.836	**0.0002**	0.426	0.431	0.71	0.068	0.477
Memory Treg (CD4+CD25+CD127-CD45RA- of CD4+)	0.231	0.19	0.898	0.314	0.635	0.811	0.572
FOXP3+ Treg (CD4+CD25+CD127-FOXP3+ of CD4+)	0.511	**0.000006**	0.68	0.034	0.566	0.033	0.925
FOXP3+ Naive Treg (CD4+CD25+CD127-CD45RO-FOXP3+ of CD4+)	0.596	**0.00004**	0.978	0.085	0.632	0.042	0.642
FOXP3+ Memory Treg (CD4+CD25+CD127-CD45RO+FOXP3+ of CD4+)	0.945	0.23	0.461	0.193	0.868	0.551	0.653

Seven single nucleotide polymorphisms associated with T1D risk (INS (rs689), PTPN22 (rs2476601), IL2RA (rs2104286 and rs12722495), PTPN2 (rs45450798), CTLA4 (rs3087243) and ERBB3 (rs2292239)) were analysed. ANCOVA was used to detect differences in the Treg frequencies between the three genotypes for each marker. Pooled Treg frequency data [16] from 363 children (65 T1D patients, 83 autoantibody-positive

(AAb+) subjects and 215 healthy (AAb-) controls) was used for the analysis. The age of the children at the time of sampling was used as covariant. p-values for each comparison are shown. After Bonferroni correction, a p-value of < 0.006 was considered statistically significant (bolded).

FIGURES
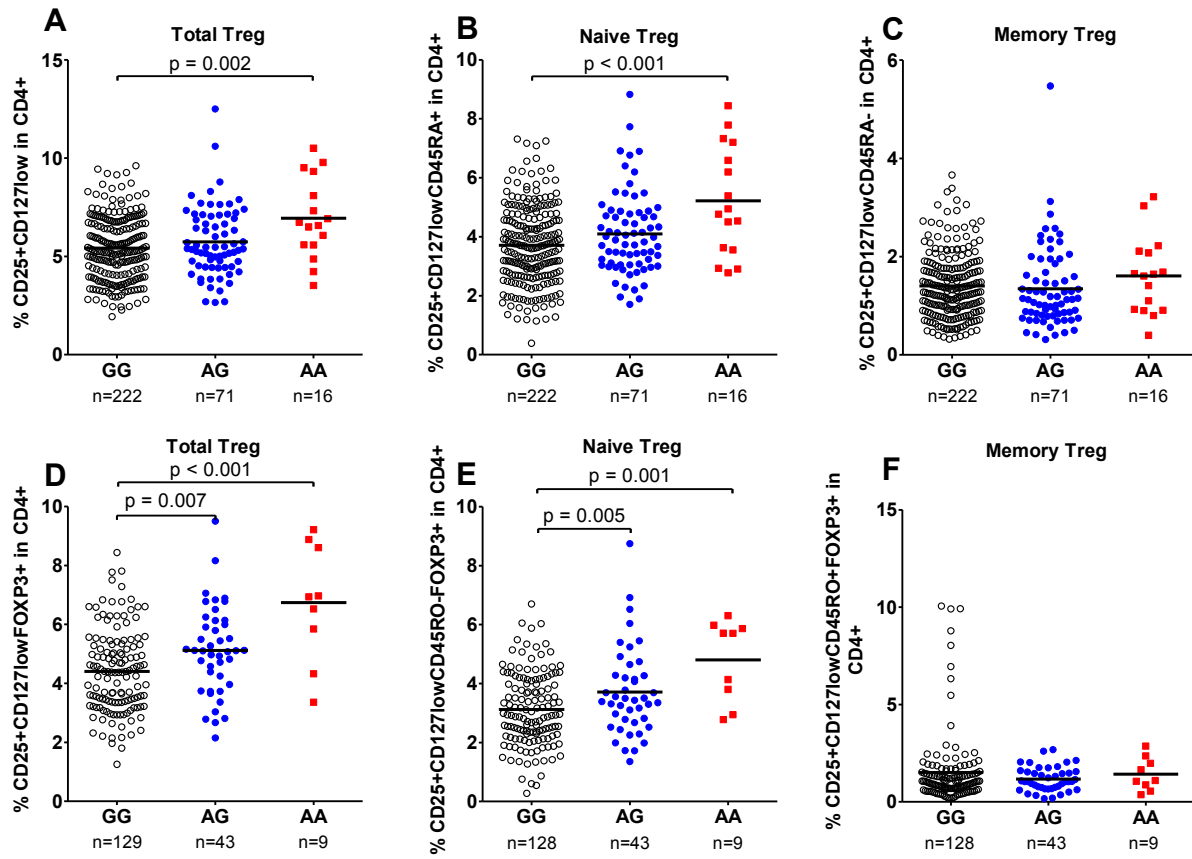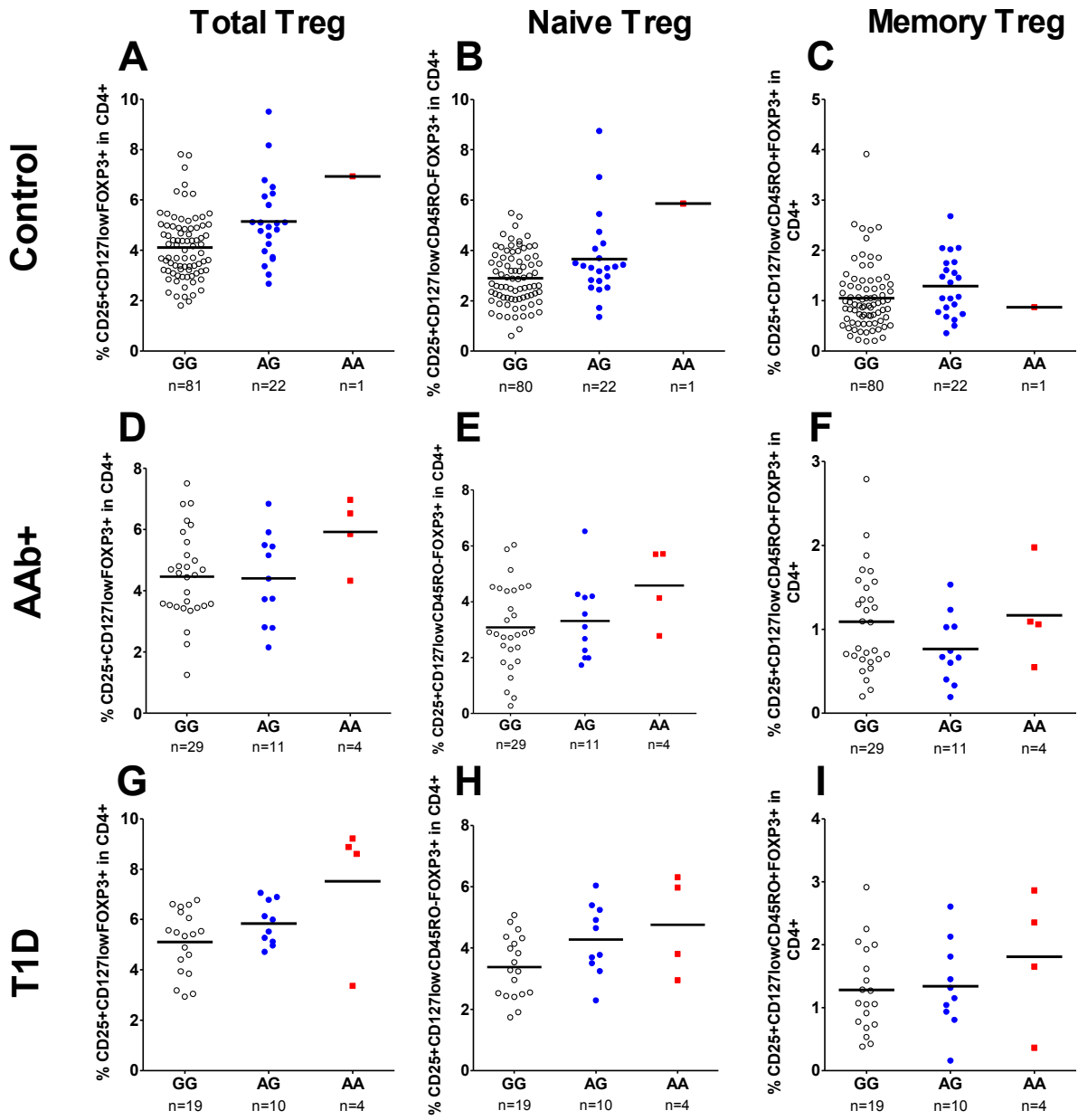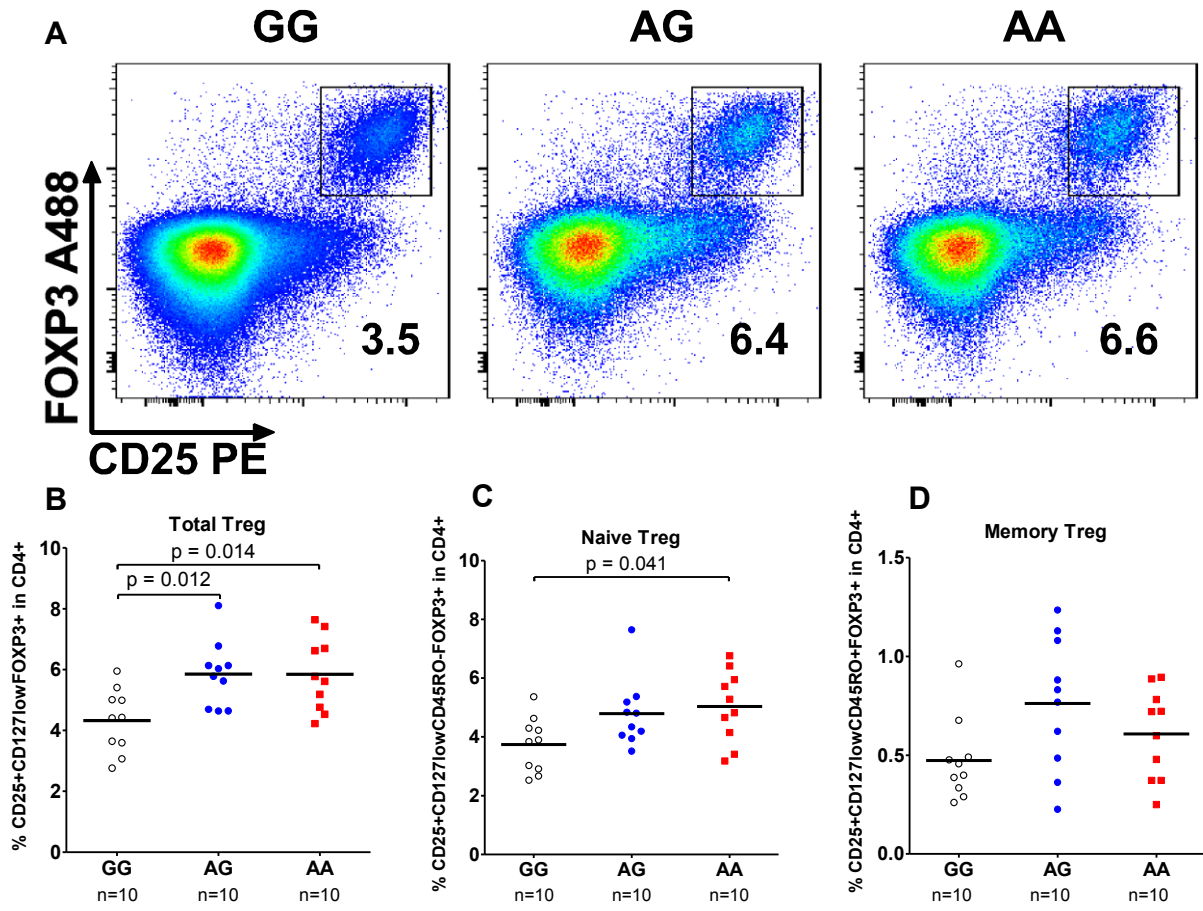

Figure 1

Figure 2

A

GG   AG   AA

FOXP3 A488 (vertical axis)

CD25 PE (horizontal axis)

3.5   6.4   6.6

B Total Treg

p = 0.014
p = 0.012

% CD25+CD127lowFOXP3+ in CD4+

GG   AG   AA
n=10   n=10   n=10

C Naive Treg

p = 0.041

% CD25+CD127lowCD45RO-FOXP3+ in CD4+

GG   AG   AA
n=10   n=10   n=10

D Memory Treg

% CD25+CD127lowCD45RO+FOXP3+ in CD4+

GG   AG   AA
n=10   n=10   n=10

Figure 3

Supporting Information

Supporting Information Table 1. Association of PTPN22 rs2476601 with Treg frequencies analysed separately for healthy control subjects (n=210), autoantibody-positive (AAb+) at-risk children (n=83) and children with newly-diagnosed T1D (n=65).

Controls

	PTPN22 rs2476601
Total Treg (CD4+CD25+CD127- of CD4+)	0.806
Naive Treg (CD4+CD25+CD127-CD45RA+ of CD4+)	0.437
Memory Treg (CD4+CD25+CD127-CD45RA- of CD4+)	0.297
FOXP3+ Treg (CD4+CD25+CD127-FOXP3+ of CD4+)	**0.004**
FOXP3+ Naive Treg (CD4+CD25+CD127-CD45RO-FOXP3+ of CD4+)	**0.003**
FOXP3+ Memory Treg (CD4+CD25+CD127-CD45RO+FOXP3+ of CD4+)	0.205

AAb+

	PTPN22 rs2476601
Total Treg (CD4+CD25+CD127- of CD4+)	0.68
Naive Treg (CD4+CD25+CD127-CD45RA+ of CD4+)	**0.023**
Memory Treg (CD4+CD25+CD127-CD45RA- of CD4+)	0.549
FOXP3+ Treg (CD4+CD25+CD127-FOXP3+ of CD4+)	0.084
FOXP3+ Naive Treg (CD4+CD25+CD127-CD45RO-FOXP3+ of CD4+)	0.083
FOXP3+ Memory Treg (CD4+CD25+CD127-CD45RO+FOXP3+ of CD4+)	0.366

T1D

	PTPN22 rs2476601
Total Treg (CD4+CD25+CD127- of CD4+)	0.067
Naive Treg (CD4+CD25+CD127-CD45RA+ of CD4+)	0.126
Memory Treg (CD4+CD25+CD127-CD45RA- of CD4+)	0.421
FOXP3+ Treg (CD4+CD25+CD127-FOXP3+ of CD4+)	**0.017**
FOXP3+ Naive Treg (CD4+CD25+CD127-CD45RO-FOXP3+ of CD4+)	0.058
FOXP3+ Memory Treg (CD4+CD25+CD127-CD45RO+FOXP3+ of CD4+)	0.232

ANCOVA was used to analyse differences between the three PTPN22 genotypes.

Supporting Information Table 2. The association of HLA-DR/DQ genotypes on Treg frequencies.

	HLA
Total Treg **(CD4+CD25+CD127- of CD4+)**	0.166
Naive Treg **(CD4+CD25+CD127-CD45RA+ of CD4+)**	0.14
Memory Treg **(CD4+CD25+CD127-CD45RA- of CD4+)**	0.531
FOXP3+ Treg **(CD4+CD25+CD127-FOXP3+ of CD4+)**	0.111
FOXP3+ Naive Treg **(CD4+CD25+CD127-CD45RO-FOXP3+ of CD4+)**	0.049
FOXP3+ Memory Treg **(CD4+CD25+CD127-CD45RO+FOXP3+ of CD4+)**	0.859

The study subjects were divided into four groups based on their HLA-DR/DQ genotypes: children carrying DR3-DQ2, children carrying DR4-DQ8, children carrying both and children carrying neither of these haplotypes. ANCOVA was used to analyse differences between the HLA class II genotypes. After Bonferroni correction, a p-value of < 0.006 was considered statistically significant.

Supporting Information Table 3. Association of PTPN22 rs2476601 with regulatory T cell frequencies in the validation cohort comprising ten trios of healthy children carrying each of the three possible genotypes, AA, AG and GG.

|	PTPN22 rs2476601
Total Treg **(CD4+CD25+CD127low FOXP3+ of CD4+)**	**0.005**
Naive Treg **(CD4+CD25+CD127low FOXP3+CD45RO- of CD4+)**	**0.03**
Memory Treg **(CD4+CD25+CD127low FOXP3+CD45RO+ of CD4+)**	**0.038**

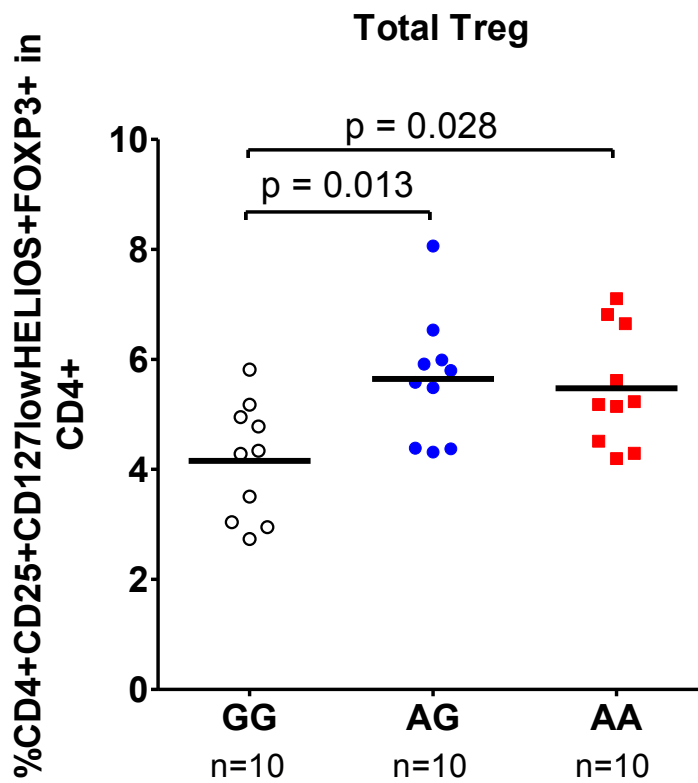ANCOVA was used to analyse differences between the three PTPN22 genotypes.
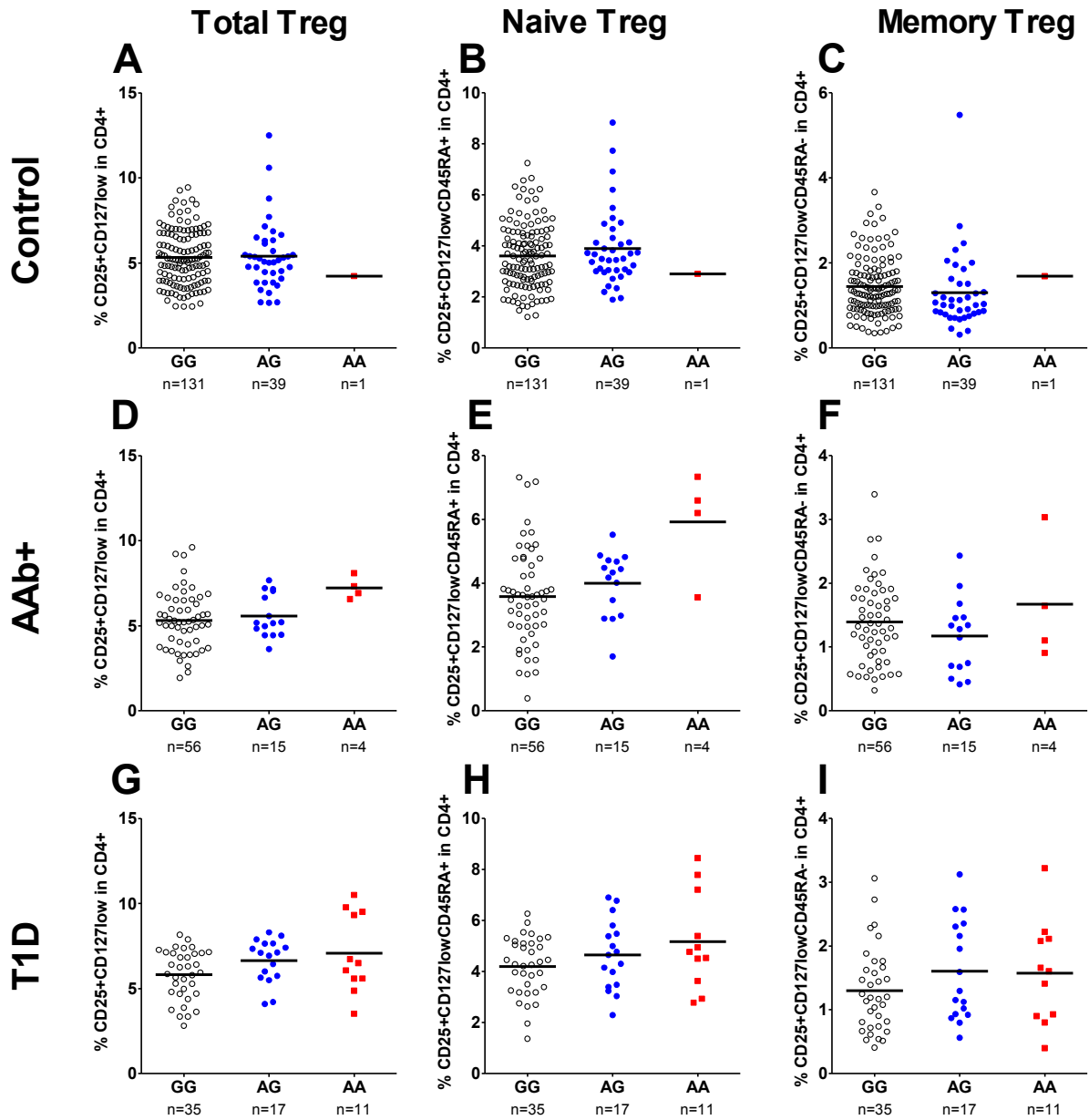
Supporting Information Figure 1. CD4+CD25+CD127low Treg frequencies in children with the *PTPN22* rs2476601 genotypes GG, AG and AA analysed separately in groups of healthy control subjects (A-C), autoantibody-positive (AAb+) at-risk children (D-F) and children with newly-diagnosed T1D (G-I). Horizontal lines indicate mean values.
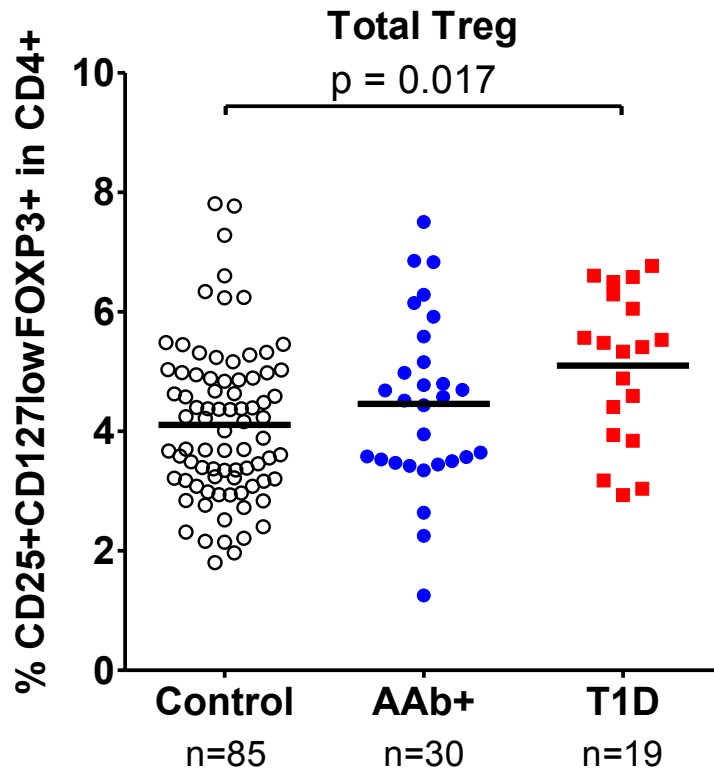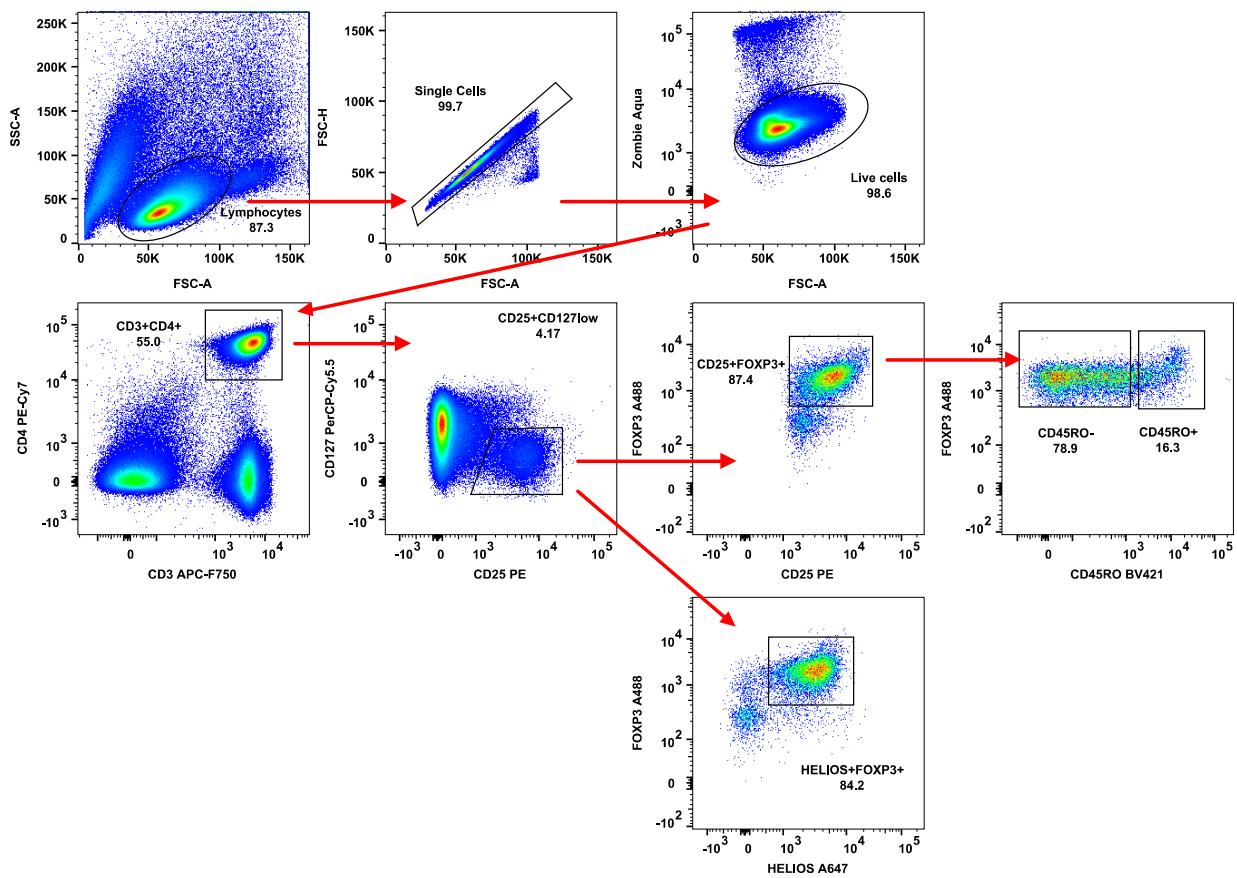
Supporting Information Figure 2. Increased frequency of CD4+CD25+CD127lowFOXP3+ Tregs in children with newly-diagnosed type 1 diabetes carrying the T1D-protective *PTPN22* rs2476601 genotype GG compared to healthy controls carrying the same genotype. ANCOVA with Bonferroni post-hoc comparisons were used to analyse differences between the genotypes. Horizontal lines indicate mean values.
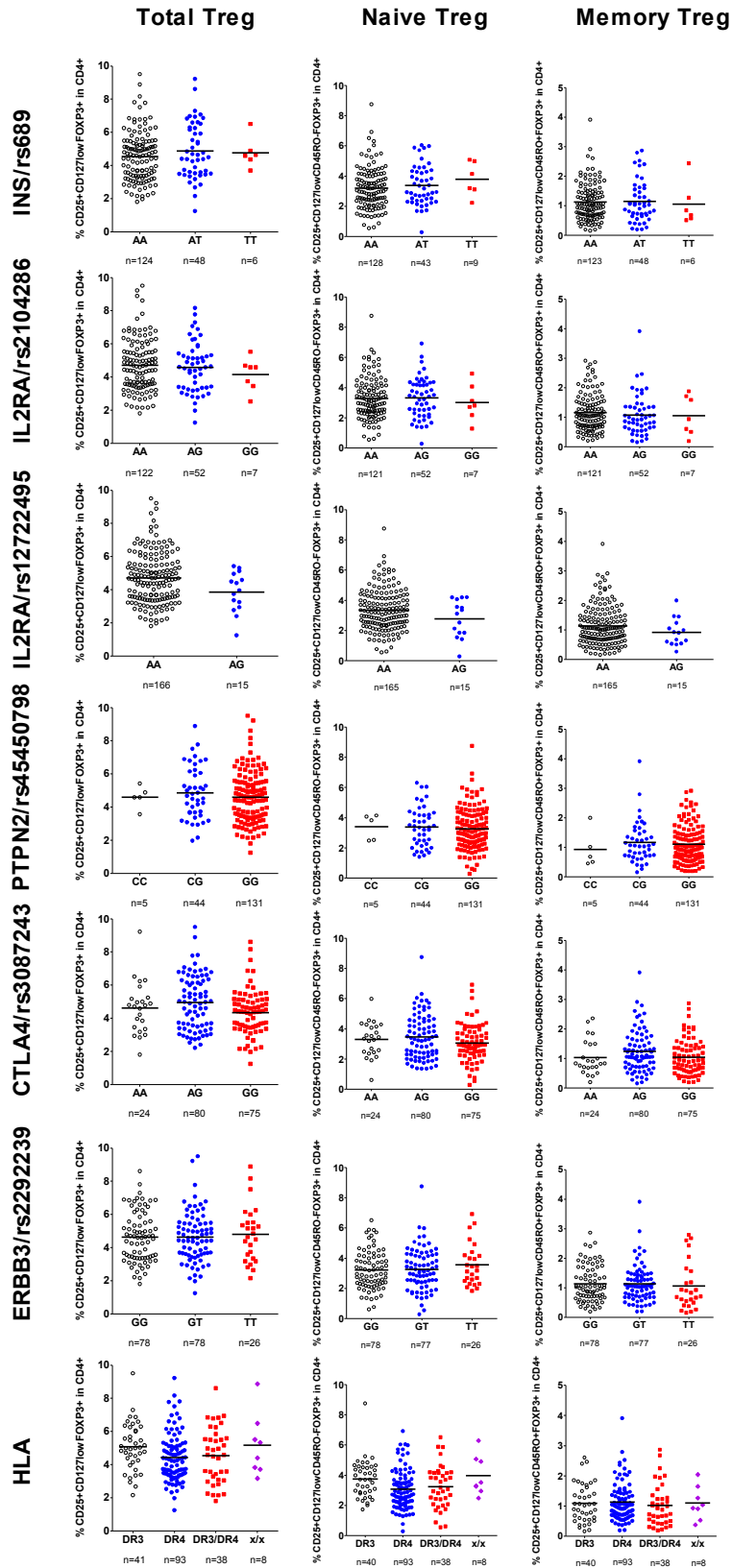
Supporting Information Figure 3. CD4+CD25+CD127lowFOXP3+ Treg frequencies in children carrying different genotypes of *INS (rs689), IL2RA* (rs2104286 and rs12722495), *PTPN2* (rs45450798), *CTLA4* (rs3087243) and ERBB3 (rs2292239) and different HLA class II genotypes. Horizontal lines indicate mean values.

Supporting Information Figure 4: The frequencies of CD4+CD25+CD127lowHELIOS+FOXP3+ Treg cells in trios of healthy children with the *PTPN22* rs2476601 genotypes GG, AG and AA. ANCOVA with Bonferroni post-hoc comparisons were used to analyse differences between the genotypes. Horizontal lines indicate mean values.

Supporting Information Figure 5. Demonstration of the gating strategy for the flow cytometric analysis of peripheral blood Treg cells from PBMC samples. Lymphocytes were first gated based on FSC and SSC properties, followed by elimination of cell doublets through FSC-A and FSC-H gating, and exclusion of dead cells through Zombie Aqua staining. Treg cells were subsequently defined as CD3+CD4+CD25+CD127lowFOXP3+ and further separated into CD45RO- naive, CD45RO+ memory or HELIOS+ Treg subsets.