Original article

Maternal type 1 diabetes reduces autoantigen-responsive CD4$^+$ T cells in offspring

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Word Count: 3982

Number of Tables and Figures: 0 tables and 5 figures

Diabetes Publish Ahead of Print, published online January 2, 2020

Abstract

Autoimmunity against pancreatic β-cell autoantigens is a characteristic of childhood type 1 diabetes. Autoimmunity usually appears in genetically susceptible children with the development of autoantibodies against (pro)insulin in early childhood. The offspring of mothers with type 1 diabetes are protected from this process. The aim of this study was to determine whether the protection conferred by maternal type 1 diabetes is associated with improved neonatal tolerance against (pro)insulin. Consistent with improved neonatal tolerance, the offspring of mothers with type 1 diabetes had reduced cord blood $CD4^+$ T cell responses to proinsulin and insulin, a reduction in the inflammatory profile of their proinsulin-responsive $CD4^+$ T cells, and improved regulation of $CD4^+$ T cell responses to proinsulin at 9 months of age, as compared with offspring with a father or sibling with type 1 diabetes. Maternal type 1 diabetes was also associated with a modest reduction in CpG methylation of the *INS* gene in cord blood mononuclear cells from offspring with a susceptible *INS* genotype. Our findings support the concept that a maternal type 1 diabetes environment improves neonatal immune tolerance against the autoantigen (pro)insulin.

Maternal type 1 diabetes confers protection against the development of type 1 diabetes in offspring (1). The likelihood of developing type 1 diabetes in children born to a mother with type 1 diabetes (maternal T1D offspring) is around half that observed in children with a father or sibling with type 1 diabetes and a non-diabetic mother (non-maternal T1D offspring). Understanding the mechanism of this protection may lead to strategies that can prevent type 1 diabetes.

Protection against type 1 diabetes in maternal T1D offspring is observed in the early stages of the disease, with a reduced risk of developing (pro)insulin autoantibodies during the first years of life (2). (Pro)insulin autoantibodies are often the first signs of autoimmunity in children who develop type 1 diabetes (3-5). The susceptibility for developing (pro)insulin autoantibodies is conferred by HLA class II and *INS* genes (6; 7) via mechanisms that hinge on thymic presentation of the self-antigen to T cells. The absence of thymic self-antigen expression can lead to autoimmunity and multiple autoimmune diseases, including type 1 diabetes (8-10). Susceptible *INS* genotypes are associated with decreased expression of (pro)insulin in antigen-presenting cells within the thymus (11; 12), and it is thought that this leads to inadequate deletion of autoreactive T cells and/or impaired generation of self-antigen-specific T regulatory cells (Tregs). Similar mechanisms might also operate in the peripheral lymphoid tissues to augment tolerance (13).

Fetal hyperinsulinemia and increased circulating insulin and proinsulin levels are observed in the first months of life in maternal T1D offspring as a result of increased glucose transfer to the fetus (14-17). The majority of maternal T1D offspring are also exposed to insulin antibodies produced by the mother as part of the mother's disease and in response to insulin replacement therapy (18-20). Although the effects of antibody transfer on diabetes incidence were inconsistent in mice (21) and humans (22), the insulin antibody-insulin immune complexes, which arise from antibody transfer, might extend exposure to the antigen in the offspring. We propose that increased exposure to insulin and proinsulin during

gestation and early infancy in maternal T1D offspring leads to mechanisms that mimic central and peripheral tolerance to (pro)insulin. Here, we sought to corroborate this hypothesis by quantifying and profiling the $CD4^+$ T cell responsiveness to proinsulin and insulin at birth, and examining the regulation of $CD4^+$ T cell responses to proinsulin in the first year of life in maternal T1D offspring. We also examined whether maternal type 1 diabetes is associated with cord blood epigenetic changes in the *INS* gene of offspring.

Research Design and Methods

Study population. Samples for T cell analyses were obtained from the ImmunDiabRisk (IDR) study (23), which included cord blood samples from maternal T1D offspring, non-maternal T1D offspring, and children who did not have a first-degree relative with type 1 diabetes (control offspring; Supplementary Table S1). Cord blood samples were collected from the placental side of the umbilical cord vein and processed within 24 hours. Peripheral blood samples collected in Vacutainer Heparin Tubes (BD Bioscience) at a median age of 9 months were also examined. All samples were collected after obtaining written informant consent from the parents.

Cord blood from newborns who participated in the BABYDIET study (24) was also available for the analysis of DNA methylation, which was performed using the Illumina HumanMethylation 450 BeadChip as previously described (25). Written informed consent was obtained from all participants or their parents.

Frequencies and phenotype of antigen-responsive $CD4^+$ T cells. Cord blood-derived $CD14^+$ monocytes were isolated from fresh cord blood samples by positive isolation using magnetic activated cell sorting (MACS; Miltenyi Biotec). The monocyte-depleted cord blood mononuclear cell (CBMC) component was frozen in human AB serum (PAA) supplemented with 10% DMSO (Sigma-Aldrich) and 0.1 ng/ml IL-7 (R&D Systems), and stored in an Isothermal Liquid Nitrogen Freezer (Custom Biogenic Systems). Dendritic cells (DC) were generated from the isolated $CD14^+$ monocytes in DC medium (CellGenix) supplemented with 3% human AB serum, 2 mM L-glutamine (Lonza) and 100 U/ml penicillin/streptomycin (Lonza) at 37°C, 5% CO_2 and 95% humidity in the presence of 50 ng/ml GM-CSF and 10 ng/ml IL-4 (both R&D Systems) as previously described (26). After 6 days, immature DCs were pulsed with 100 μg/ml insulin (Sigma-Aldrich), proinsulin (Biomm), or KLH (Calbiochem) or the glycated forms of the antigens (27). Monocyte-derived DCs (MO-DCs)

were matured overnight in the presence of 100 units/ml IFN-γ (R&D Systems) and 10 ng/ml lipopolysaccharide (Sigma-Aldrich).

Following MO-DC maturation, cryovials of the same cord bloods were thawed and cell quantity and viability were assessed (Muse; median viability, 79%). $CD25^+$ cells were depleted by MACS using CD25 Microbeads II (Miltenyi Biotec) according to the manufacturer's protocols. The monocyte- and $CD25^+$ cell-depleted CBMCs were washed with $PBS^{-/-}$ (Gibco) and divided into three fractions, which were respectively labeled with 10 μM eFluor450 for 30 min at room temperature, 5 μM eFluor670 for 10 min at 37°C in the dark (both eBioscience), or 1 μM carboxyfluorescein succinimidyl ester (CFSE, BioLegend) for 4 min at 37°C according to the manufacturers' protocols. The investigative protocol used 150,000 cells (50,000 cells labelled with each dye) that were added to 3,000 autologous mature MO-DCs (1:50 final ratio) loaded with or without the antigens of interest in a total of 10 replicates per condition in round-bottom 96-well plates. The co-cultures were incubated in a total of volume of 200 μl RPMI 1640 supplemented with 5% human AB serum, 2 mM L-glutamine and 100 units/ml penicillin/streptomycin at 37°C, 5% CO_2 and 95% humidity in the presence of 0.1 ng/ml IL-7 for 5 days. Human serum AB was previously tested in single proliferation dye assays to exclude non-specific T cell activation. Cells were harvested and stained for 20 min at 4°C using anti-CD4 PerCP (BD Bioscience, clone SK3, catalogue number 347324), anti-CD25 PE (BD Pharmingen, M-A251, 555432), and anti-CD45RO PE-Cy7 (BD Bioscience, UCHL1, 337168) monoclonal antibodies. Cells were washed and stained with Zombie NIR (BioLegend) to assess viability, fixed with 1.5% formalin (Sigma-Aldrich) in $PBS^{-/-}$, and acquired on a Becton Dickinson LSR Fortessa flow cytometer and analyzed with FlowJo software (Version 10; TreeStar Inc.).

For each antigen stimulus, the 10 replicate wells were analyzed for each proliferation dye (30 replicates per antigen). Antigen-responsive $CD4^+$ T cells were identified as dye^{dim} (proliferation) $CD25^+$ (activation) $CD45RO^+$ (conversion to memory) cells (Supplementary

Figure S1). For each sample, the median of the three dyes was calculated for each well and for each antigen, these values for the 10 replicate wells were compared to the 10 wells with unloaded MO-DCs using a Mann-Whitney U test. A positive response was assigned if the two-tailed p value of the comparison was <0.05. Additionally, the responses were expressed as the proportion of total proliferation dye-labeled $CD4^+$ T cells after subtraction of the median value obtained from the 10 wells with unloaded MO-DCs.

For samples with sufficient cell numbers, separate proliferation assays of 1.5×10^5 eFluor450- or eFluor670-labeled, monocyte- and $CD25^+$ cell-depleted CBMCs and 3×10^4 antigen-loaded MO-DCs were performed in four replicates as described for the multi-dye proliferation assays. Antigen-responsive cells were single-cell sorted into 96-well PCR plates (FrameStar) and processed and analyzed by quantitative real-time (RT) PCR using the Fluidigm Biomark HD with an established set of genes as previously described (28; 29).

Regulatory $CD4^+$ T cell immune suppression assays. Frozen PBMCs obtained at follow-up visits were thawed. $CD25^+$ positive cells were enriched by MACS using CD25 Microbeads II and stained for 20 min at 4°C with anti-CD4 Brilliant Violet 510 (BD Bioscience, SK3, 562970), anti-CD25 PE (BD Pharmingen, M-A251, 555432) and anti-CD127 PE-Cy7 (BioLegend, A019D5, 351320) monoclonal antibodies. Live $CD25^+CD127^{low}CD4^+$ cells were sorted on a BD FACS ARIA III. The remaining $CD25^+CD127^{low}$ cell-depleted fraction was separated into $CD8^+$ and $CD8^-$ cells by MACS using CD8 Microbeads II (Miltenyi Biotec) and each fraction was labeled with 10 µM eFluor450 for 30 min at room temperature. 1.5×10^5 eFluor450-labeled CD8-depleted or $CD8^+$ cells were co-cultured with the sorted autologous $CD4^+CD25^+CD127^{low}$ Treg cells at Treg:Tresponder ratios of 1:2 and 0:1 (no Tregs) in X-Vivo15 media (Lonza) supplemented with 10% human AB serum, 2 mM L-glutamine and 100 units/ml penicillin/streptomycin at 37°C, 5% CO_2 and 95% humidity. Each ratio was tested in up to four wells. The autologous Treg/$CD8^+$ cell-depleted co-cultures were

incubated for 5 days in presence of 10 µg/ml proinsulin (Biomm) and the autologous Treg/CD8$^+$ T cell co-cultures were stimulated with anti-CD3/anti-CD28-coupled beads (Dynabeads; Invitrogen) at a bead/cell ratio of 1:50 for 3 days in 96-well plates. Cells were stained with anti-CD4 Brilliant Violet 510 (BD Bioscience, SK3, 562970), anti-CD8 PerCP (BD Bioscience, SK1, 347314), anti-CD25 PE (BD Pharmingen, M-A251, 555432), anti-CD45RO APC (BD Pharmingen, UCHL1, 559865) and anti-CD127 PE-Cy7 (BioLegend, A019D5, 351320). Cells were washed, stained with Zombie NIR to evaluate cell viability and then fixed with 1.5% formalin in PBS$^{-/-}$. Samples were acquired on a Becton Dickinson LSR Fortessa flow cytometer and analyzed with FlowJo software. Responsive T cells were identified as dye^{dim}CD25$^+$CD45RO$^+$. Treg-induced suppression was calculated for each stimulus as the number of responder CD4$^+$ or CD8$^+$ T cells in cultures containing Treg cells divided by the number of responder cells in wells without Treg.

Single cell gene expression. Single cell gene expression profiling was performed by RT-qPCR as previously described (29) with some modifications. After reverse transcription with Quanta qScript TM cDNA Supermix directly on cells, the total cDNA was preamplified for 20 cycles using TATAA GrandMaster Mix (TATAA Biocenter) in the presence of primer pairs for 46 genes (25 nM final concentration for each primer; Supplementary Table S2). Preamplified cDNA (10 µl) was treated with 1.2 U of exonuclease I and expression was quantified by RT-PCR on a BioMark™ HD System (Fluidigm Corporation) using the 96.96 Dynamic Array IFC and the GE 96x96 Fast PCR+ Melt protocol with SsoFast EvaGreen Supermix and Low ROX (BIO RAD) and 5 µM of primers for each assay. Raw data were analyzed using the Fluidigm Real-Time PCR analysis software. Preprocessing and data analysis were conducted using KNIME 3.7.0 and RStudio version 1.1.383 (RStudio). Preprocessing was done using a linear model to correct for confounding sampling effects as previously described (30).

Statistical analysis. Data are provided as mean and 95% confidence intervals. T cell responses were compared among groups using parametric unpaired Student's *t* tests. Two-tailed p-values of < 0.05 were considered significant. Methylation status was compared between groups using the Wilcoxon test. Data were analyzed using GraphPad Prism 7 (GraphPad) or R Studio version 3.2.2 (RStudio) software.

The uniform manifold approximation and projection (UMAP) method was used for dimensional reduction of single cell gene expression data (31). Unsupervised ward clustering was done with hclust in R (version 3.5.1). To model the bi-modal gene expression of antigen-responsive $CD4^+$ T cells, the Hurdle model, a semi-continuous modeling framework, was applied to the pre-processed data using FDR correction and a significance threshold of 0.01 (32). For individual genes that differed between groups, comparisons were also made using the Wilcoxon test.

Study approval. All clinical investigations were performed in accordance with the principles of the Declaration of Helsinki, with written informed consent from all participants or their parents. The IDR study was approved by the Ethics Committee of the Technical University Munich (No. 5293/12) and the BABYDIET study was approved by ethics committee of the Ludwig-Maximilians University (No. 329/00).

Data and Resource Availability. The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

Results

Maternal type 1 diabetes reduces proinsulin-responsive $CD4^+$ T lymphocytes in offspring cord blood. We first investigated whether maternal T1D offspring showed reduced responses to insulin and proinsulin. We established a cord blood $CD4^+$ T cell antigen assay with 30

replicates for each condition to account for the low expected frequency of antigen-specific cord blood T cells (Supplementary Figure S1). Validating the ability to detect antigen-responsive cord blood $CD4^+$ T cells, responses to the control multi-$CD4^+$ T cell epitope containing stimulus keyhole limpet hemocyanin (KLH) (33) were observed in cord blood from offspring with no first-degree relatives with type 1 diabetes (control offspring), as well as in maternal T1D offspring and non-maternal T1D offspring (Figure 1A).

Cord blood $CD4^+$ T cell responses to proinsulin or insulin were observed in 3 (18.7%) of 16 control offspring, 2 (13.3%) of 15 maternal T1D offspring, and 10 (71.4%) of 14 non-maternal T1D offspring (P=0.0025 vs maternal T1D offspring, P=0.0086 vs control offspring). Responses to proinsulin were observed in 2 (12.5%) of the control offspring, 1 (6.7%) maternal T1D offspring and 7 (50%) non-maternal T1D offspring (P=0.014 vs maternal T1D offspring, P=0.046 vs control offspring). The frequency of responses to insulin were not significantly different between groups. Additionally, the cord blood $CD4^+$ T cell responses to proinsulin (mean 0.14%, range 0–0.31%) and insulin (mean 0.11%, range 0–0.31%) were greater in non-maternal T1D offspring than in control offspring (proinsulin: mean 0.05%, range 0–0.16%, P=0.0032; insulin: mean 0.02%, range 0–0.15%, P=0.0082). By contrast, the cord blood $CD4^+$ T cells responses to proinsulin (mean 0.05%, range 0–0.17%) and insulin (mean 0.04%, range 0–0.14%) in maternal T1D offspring were similar to those of control offspring and lower than those in non-maternal T1D offspring (proinsulin, P=0.0041; insulin, P=0.036). There were no differences in the control stimulus KLH among the groups. The magnitude of responses to KLH were similar to those against proinsulin and insulin in the non-maternal T1D offspring, but higher than those against proinsulin (P=0.039) and insulin (P=0.024) in the maternal T1D offspring and control offspring (P=0.089, P=0.012). The responses were similar and differences were maintained when the cells were stimulated with glycated antigen (Supplementary Figure S2). These findings are consistent with the notion

that maternal type 1 diabetes reduced the number of $CD4^+$ T cells with the capacity to respond to the proinsulin autoantigen.

Gene expression of antigen-responsive $CD4^+$ T cells. We next asked whether there were qualitative differences in the responsive cord blood $CD4^+$ T cells among the antigen and offspring groups. We first examined the expression of 46 genes in 680 single proinsulin- (n=141), insulin- (n=89) and KLH- (n=450) responsive $CD4^+$ T cells from 13 children with positive responses (Figure 2). The cells were projected to nine clusters (Figure 2A). There were differences in the relative proportions of cells responsive to each antigen between clusters ($P<0.0001$). Proinsulin-responsive cells were most frequently found in clusters 1 (15.6% of proinsulin-responsive cells), 4 (24.1%), 6 (15.6%) and 7 (16.3%) and insulin-responsive cells were most frequent in clusters 3 (23.6% of insulin-responsive cells), 4 (32.6%) and 8 (21.3%), and absent in cluster 1 and 2. KLH-responsive cells were rather evenly distributed across the nine clusters. Clusters 1 and 2 were particularly striking and defined by the expression of *CD40*, *IL22*, *CXCR5*, *TBX21*, *IFNG*, and in cluster 1 also *IL9*. These features were mostly absent or infrequent in clusters 3–9 (Figure 2C; Supplementary Figure S3). Strong *CD40* expression is unusual in $CD4^+$ T cells and was previously reported as a feature of aggressive autoreactive T cells in mice (34-37) and in a minority of activated cells in humans (38). None of the proinsulin-responsive cells from both the one maternal T1D offspring and the one control offspring with a positive response was found in clusters 1 and 2, whereas the highest proportion of proinsulin-responsive cells from the non-maternal T1D offspring was observed in cluster 1 (Figure 3).

Clusters 3 and 9 were characterized by abundant expression of chemokine receptors and little or no cytokines (Figure 2, Supplementary Figure S3). Cluster 4 was distinguished by the expression of *ICOS*, *TNFRSF18* (*GITR*) and *BCL6*, along with other features of regulation, and clusters 3, 4 and 5 included 11%, 51% and 7% cells, respectively that expressed *FOXP3* and *IKFZ2* without *IL7R* and *IL2* (Supplementary Figure S4), which is a typical profile of

Treg. The majority of these Treg-like cells also expressed *CCR4,* which is a marker of memory T Helper-like Treg (39). The expression profile of proinsulin- and insulin-responsive Treg-like cells differed to the profile of KLH-responsive Treg-like cells, the former containing more cells from cluster 4 and expressing *TNFRSF18* (GITR), *TNFRSF4*, and the immune checkpoint inhibitor *PDCD1* (Supplementary Figure S4). The proinsulin and insulin-responsive cells with a memory Treg profile included cells from both maternal and non-maternal T1D offspring suggesting that both offspring groups have the capacity to convert naïve T cells to memory (pro)insulin-responsive Treg.

Increased regulation of in vitro CD4$^+$ T cell responses to proinsulin in maternal T1D offspring. Because the peak incidence of (pro)insulin autoantibody development is at 9–12 months of age (3; 5; 40), we investigated whether, in addition to the reduced proinsulin- and insulin-responsive cells at birth, there was evidence of increased regulation of proinsulin-responsive CD4$^+$ T cells in the first year of life (Figure 4). The CD4$^+$ T cell responses to proinsulin were examined in Treg-depleted peripheral blood mononuclear cells (PBMCs) and in Treg-depleted PBMCs co-cultured with Treg (Figure 4A). Responses to proinsulin in the absence of Treg ranged from 0.01 to 0.68% with no difference between the maternal T1D offspring and non-maternal T1D offspring (P=0.36). In non-maternal T1D offspring, the CD4$^+$ T cell response to proinsulin was reduced by 33.5% (range 16.7–51.7%) by co-culture with Tregs. This reduction was significantly greater in maternal T1D offspring (mean 71.0%, range 43.3–91.4%, P=0.0016). This difference remained significant when children with low responses to proinsulin (<0.03%) were excluded from the analysis (P=0.0074). There was no difference in the reduction of CD8$^+$ T cell responses to polyclonal stimulation between maternal T1D offspring (mean 16.1%, range 9.7–23.9%) and non-maternal T1D offspring (mean 13.4%, range, 2.9–19.2%, P=0.48; Figure 4B), suggesting that the maternal T1D

offspring do not show a general increase in Treg fitness or permissiveness of T effectors to regulation.

Maternal type 1 diabetes is associated with altered cord blood INS gene methylation status. The limited cord blood $CD4^+$ T cell response to proinsulin in the maternal T1D offspring was consistent with the notion that there is a more effective immune tolerance, possibly as a result of increased exposure to insulin and/or proinsulin, in this setting. Insulin expression is affected by methylation (41; 42). Although insulin is not expressed in cord blood cells, the methylation status at CpG sites in blood cells can be a useful measure of epigenetic differences between individuals (43), and we previously showed that cord blood methylation of multiple CpG sites within the *INS* gene is increased in children with type 1 diabetes-susceptible *INS* genotypes (25). Therefore, we performed an exploratory investigation as to whether maternal type 1 diabetes was associated with the methylation status of *INS* CpG sites in cord blood mononuclear cells (Figure 5).

Among neonates with the susceptible *INS* genotype AA, methylation in maternal T1D offspring was reduced towards that observed in neonates with protective *INS* genotypes as compared with that in non-maternal T1D offspring at cg25336198 (P=0.04) and cg02749887 (P=0.035). No differences were found among the maternal T1D states in neonates with protective genotypes. We extended the analysis to 40 CpG sites in 33 other T1D susceptibility genes that previously showed marked allele-specific methylation variation (25). Differences between the maternal T1D offspring and non-maternal T1D offspring were found at only three CpG sites (Supplementary Figure S5) located in the *SH2B3* (P=0.047 in offspring with risk genotype), *IFIH1* (P=0.026 in children with a risk genotype), and *ERBB3* (P=0.016 in children with non-risk genotypes) genes.

Discussion

We found that a maternal type 1 diabetes environment is associated with a reduction in newborn $CD4^+$ T cell responsiveness to the type 1 diabetes autoantigen proinsulin, and, in an exploratory analysis, a modest reduction in methylation at CpG sites of the *INS* gene in cord blood mononuclear cells from offspring with a type 1 diabetes susceptible *INS* genotype. These findings are consistent with the notion that maternal type 1 diabetes protects against the development of insulin autoimmunity in offspring potentially by reversing the genetic propensity to release autoreactive (pro)insulin-specific T cells from the thymus.

This study is the first to address the effects of maternal type 1 diabetes on T cell responses to an autoantigen. Antigen-responsive cord blood T cells are infrequent and were previously shown to require competent antigen-presenting cells and T cell growth factors to achieve a response (26). Thus, it was necessary to collect large volumes of cord blood to establish an assay with a considerable number of replicates, a feature that provided a readout with an unprecedented degree of confidence in quantifying these infrequent cells. A limitation of the methodology is its identification of antigen-responsive cells by proliferation. Therefore, it might miss some important antigen-specific cells that do not proliferate following an encounter with antigen-loaded dendritic cells. In the present study, we removed Tregs from the cord blood T cell assays so that we could assess cell response without the confounder of regulation. However, we were unable to determine whether the differences in proinsulin- and insulin-responsive cells were due to fewer precursor numbers or a higher activation threshold in cells from maternal T1D offspring.

We suggest that our findings support a mechanism involving improved immune tolerance to proinsulin and insulin. It was reported that a type 1 diabetes susceptible *INS* genotype is linked to decreased thymic expression of insulin (11; 12), greater risk of developing insulin autoantibodies (7), and increased T cell responses to proinsulin (44). Although modest, it is, therefore, of potential interest that a maternal type 1 diabetes

environment was associated with reduced methylation of CpG sites in *INS* in cord blood DNA of offspring with a type 1 diabetes susceptible *INS* genotype. The magnitude of the observed differences was small and the number of children in our study was too small to further stratify the responses by *INS* genotype or surrogate measures of fetal insulin and glucose. Moreover, cord blood methylation status provides an indirect marker of epigenetic changes, but these may not be relevant to actual expression in the relevant central or peripheral antigen-presenting cells for immune tolerance or may be transient (45). It would, therefore, be important to assess whether maternal type 1 diabetes or hyperglycemia increase insulin expression in the neonatal thymus and whether the effects observed in cord blood persist during childhood.

In our assessment of the *in vitro* cord blood $CD4^+$ T cell responses to antigen, we observed the appearance of an unusual $CD4^+$ T cell profile that was characterized by the co-expression of *CD40*, *CXCR5*, and *IL22*, along with cytokine genes such as *IFNG* and *IL9,* and weak *CD4* expression. A $CD4^{low}CD40^+$ T cell surface phenotype was previously identified and described as an aggressive autoreactive cell type (34; 36-38). We did not examine protein expression, but it is possible that the cells we observed here have a similar phenotype. The *CD40*-positive cell clusters were enriched for proinsulin-responsive cells, reinforcing the potential relevance of proinsulin as an autoantigen (46-49), and no proinsulin-responsive cells from the one positive maternal T1D offspring or control offspring were found in these features. Cells with a memory Treg-like profile were observed in proinsulin- and insulin-responsive cells from both maternal T1D and non-maternal T1D offspring suggesting the ability to convert naïve T cells to Tregs if exposed to antigen and the potential to promote tolerance if exposed to antigen *in vivo*. Suppression assays performed at 9 months indicated that this may have been more efficient in the maternal T1D offspring. These findings require validation since gene expression does not define Treg and suppression assays are crude

measures of Treg activity and are affected by many confounders, including T effector function (50).

Immune tolerance to autoantigens can be achieved by the deletion of autoreactive T cells and the generation of Tregs in the thymus (11) or periphery (13). We provide evidence that maternal type 1 diabetes protects offspring from developing type 1 diabetes by engaging tolerogenic mechanisms. It is also possible that exposure to high glucose affects fetal pancreatic β cell maturation in a manner that provides additional protection against the development of type 1 diabetes.

Acknowledgements

We thank all of the recruiting physicians in pediatric hospitals and private practices for participating in the IDR, BABYDIAB and BABYDIET studies.

Author contributions. JK, AGZ and EB designed the research study and helped conduct the experiments. JK, AG, SD and AL acquired the data. JK, AE, RL, EMS, KW, NH, AGZ, and EB contributed to the analysis and interpretation of the data. JK, AE, AGZ and EB drafted the manuscript. JK, AE, AG, RL, EMS, SD, AL, KW, NH, AGZ and EB critically reviewed and approved the manuscript. EB is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Conflict of interest statement. The authors have declared that no conflicts of interest exist.

Funding. This work was funded by JDRF grant 17-2013-525. EB is supported by the DFG Research Center and Cluster of Excellence - Center for Regenerative Therapies Dresden (FZ 111).

Prior presentation. Parts of this study were previously presented in poster and oral form at the Immunology of Diabetes Society Congress 2018, London, England, 25 and 26 October 2018.

References

1. Warram JH, Krolewski AS, Gottlieb MS, Kahn CR: Differences in risk of insulin-dependent diabetes in offspring of diabetic mothers and diabetic fathers. N Engl J Med 1984;311:149-152
2. Bonifacio E, Pfluger M, Marienfeld S, Winkler C, Hummel M, Ziegler AG: Maternal type 1 diabetes reduces the risk of islet autoantibodies: relationships with birthweight and maternal HbA(1c). Diabetologia 2008;51:1245-1252
3. Ilonen J, Lempainen J, Hammais A, Laine AP, Harkonen T, Toppari J, Veijola R, Knip M: Primary islet autoantibody at initial seroconversion and autoantibodies at diagnosis of type 1 diabetes as markers of disease heterogeneity. Pediatr Diabetes 2018;19:284-292
4. Krischer JP, Lynch KF, Lernmark A, Hagopian WA, Rewers MJ, She JX, Toppari J, Ziegler AG, Akolkar B: Genetic and Environmental Interactions Modify the Risk of Diabetes-Related Autoimmunity by 6 Years of Age: The TEDDY Study. Diabetes Care 2017;40:1194-1202
5. Ziegler AG, Bonifacio E: Age-related islet autoantibody incidence in offspring of patients with type 1 diabetes. Diabetologia 2012;55:1937-1943
6. Hippich M, Beyerlein A, Hagopian WA, Krischer JP, Vehik K, Knoop J, Winker C, Toppari J, Lernmark A, Rewers MJ, Steck AK, She JX, Akolkar B, Robertson CC, Onengut-Gumuscu S, Rich SS, Bonifacio E, Ziegler AG: Genetic Contribution to the Divergence in Type 1 Diabetes Risk Between Children From the General Population and Children From Affected Families. Diabetes 2019; 68:847-857
7. Walter M, Albert E, Conrad M, Keller E, Hummel M, Ferber K, Barratt BJ, Todd JA, Ziegler AG, Bonifacio E: IDDM2/insulin VNTR modifies risk conferred by IDDM1/HLA for development of Type 1 diabetes and associated autoimmunity. Diabetologia 2003;46:712-720
8. An autoimmune disease, APECED, caused by mutations in a novel gene featuring two PHD-type zinc-finger domains. Nat Genet 1997;17:399-403
9. Anderson MS, Venanzi ES, Klein L, Chen Z, Berzins SP, Turley SJ, von Boehmer H, Bronson R, Dierich A, Benoist C, Mathis D: Projection of an immunological self shadow within the thymus by the aire protein. Science 2002;298:1395-1401
10. Nagamine K, Peterson P, Scott HS, Kudoh J, Minoshima S, Heino M, Krohn KJ, Lalioti MD, Mullis PE, Antonarakis SE, Kawasaki K, Asakawa S, Ito F, Shimizu N: Positional cloning of the APECED gene. Nat Genet 1997;17:393-398
11. Pugliese A, Zeller M, Fernandez A, Jr., Zalcberg LJ, Bartlett RJ, Ricordi C, Pietropaolo M, Eisenbarth GS, Bennett ST, Patel DD: The insulin gene is transcribed in the human thymus and transcription levels correlated with allelic variation at the INS VNTR-IDDM2 susceptibility locus for type 1 diabetes. Nat Genet 1997;15:293-297
12. Vafiadis P, Bennett ST, Todd JA, Nadeau J, Grabs R, Goodyer CG, Wickramasinghe S, Colle E, Polychronakos C: Insulin expression in human thymus is modulated by INS VNTR alleles at the IDDM2 locus. Nat Genet 1997;15:289-292
13. Pugliese A, Brown D, Garza D, Murchison D, Zeller M, Redondo MJ, Diez J, Eisenbarth GS, Patel DD, Ricordi C: Self-antigen-presenting cells expressing diabetes-associated autoantigens exist in both thymus and peripheral lymphoid organs. J Clin Invest 2001;107:555-564
14. Desoye G, Nolan CJ: The fetal glucose steal: an underappreciated phenomenon in diabetic pregnancy. Diabetologia 2016;59:1089-1094
15. Hay WW, Jr.: Placental-fetal glucose exchange and fetal glucose metabolism. Trans Am Clin Climatol Assoc 2006;117:321-339; discussion 339-340
16. Lindsay RS, Walker JD, Halsall I, Hales CN, Calder AA, Hamilton BA, Johnstone FD: Insulin and insulin propeptides at birth in offspring of diabetic mothers. J Clin Endocrinol Metab 2003;88:1664-1671

17. Stumpp C, Beyerlein A, Ziegler AG, Bonifacio E: Neonatal and infant beta cell hormone concentrations in relation to type 1 diabetes risk. Pediatr Diabetes 2014;15:528-533
18. Hamalainen AM, Savola K, Kulmala PK, Koskela P, Akerblom HK, Knip M: Disease-associated autoantibodies during pregnancy and at birth in families affected by type 1 diabetes. Clin Exp Immunol 2001;126:230-235
19. Roll U, Christie MR, Fuchtenbusch M, Payton MA, Hawkes CJ, Ziegler AG: Perinatal autoimmunity in offspring of diabetic parents. The German Multicenter BABY-DIAB study: detection of humoral immune responses to islet antigens in early childhood. Diabetes 1996;45:967-973
20. Ziegler AG, Hillebrand B, Rabl W, Mayrhofer M, Hummel M, Mollenhauer U, Vordemann J, Lenz A, Standl E: On the appearance of islet associated autoimmunity in offspring of diabetic mothers: a prospective study from birth. Diabetologia 1993;36:402-408
21. Greeley SA, Katsumata M, Yu L, Eisenbarth GS, Moore DJ, Goodarzi H, Barker CF, Naji A, Noorchashm H: Elimination of maternally transmitted autoantibodies prevents diabetes in nonobese diabetic mice. Nat Med 2002;8:399-402
22. Naserke HE, Bonifacio E, Ziegler AG: Prevalence, characteristics and diabetes risk associated with transient maternally acquired islet antibodies and persistent islet antibodies in offspring of parents with type 1 diabetes. J Clin Endocrinol Metab 2001;86:4826-4833
23. Warncke K, Lickert R, Eitel S, Gloning KP, Bonifacio E, Sedlmeier EM, Becker P, Knoop J, Beyerlein A, Ziegler AG: Thymus Growth and Fetal Immune Responses in Diabetic Pregnancies. Horm Metab Res 2017;49:892-898
24. Hummel S, Pfluger M, Hummel M, Bonifacio E, Ziegler AG: Primary dietary intervention study to reduce the risk of islet autoimmunity in children at increased risk for type 1 diabetes: the BABYDIET study. Diabetes Care 2011;34:1301-1305
25. Kindt ASD, Fuerst RW, Knoop J, Laimighofer M, Telieps T, Hippich M, Woerheide MA, Wahl S, Wilson R, Sedlmeier EM, Hommel A, Todd JA, Krumsiek J, Ziegler AG, Bonifacio E: Allele-specific methylation of type 1 diabetes susceptibility genes. J Autoimmun 2018;89:63-74
26. Heninger AK, Monti P, Wilhelm C, Schwaiger P, Kuehn D, Ziegler AG, Bonifacio E: Activation of islet autoreactive naive T cells in infants is influenced by homeostatic mechanisms and antigen-presenting capacity. Diabetes 2013;62:2059-2066
27. Monti P, Brigatti C, Krasmann M, Ziegler AG, Bonifacio E: Concentration and activity of the soluble form of the interleukin-7 receptor alpha in type 1 diabetes identifies an interplay between hyperglycemia and immune function. Diabetes 2013;62:2500-2508
28. Eugster A, Lindner A, Heninger AK, Wilhelm C, Dietz S, Catani M, Ziegler AG, Bonifacio E: Measuring T cell receptor and T cell gene expression diversity in antigen-responsive human CD4+ T cells. J Immunol Methods 2013;400-401:13-22
29. Heninger AK, Eugster A, Kuehn D, Buettner F, Kuhn M, Lindner A, Dietz S, Jergens S, Wilhelm C, Beyerlein A, Ziegler AG, Bonifacio E: A divergent population of autoantigen-responsive CD4(+) T cells in infants prior to beta cell autoimmunity. Sci Transl Med 2017;9
30. Bonifacio E, Ziegler AG, Klingensmith G, Schober E, Bingley PJ, Rottenkolber M, Theil A, Eugster A, Puff R, Peplow C, Buettner F, Lange K, Hasford J, Achenbach P: Effects of high-dose oral insulin on immune responses in children at high risk for type 1 diabetes: the Pre-POINT randomized clinical trial. JAMA 2015;313:1541-1549
31. Becht E, McInnes L, Healy J, Dutertre CA, Kwok IWH, Ng LG, Ginhoux F, Newell EW: Dimensionality reduction for visualizing single-cell data using UMAP. Nat Biotechnol 2018
32. McDavid A, Dennis L, Danaher P, Finak G, Krouse M, Wang A, Webster P, Beechem J, Gottardo R: Modeling bi-modality improves characterization of cell cycle on gene expression in single cells. PLoS Comput Biol 2014;10:e1003696

33. Swaminathan A, Lucas RM, Dear K, McMichael AJ: Keyhole limpet haemocyanin - a model antigen for human immunotoxicological studies. Br J Clin Pharmacol 2014;78:1135-1142
34. Baker RL, Mallevaey T, Gapin L, Haskins K: T cells interact with T cells via CD40-CD154 to promote autoimmunity in type 1 diabetes. Eur J Immunol 2012;42:672-680
35. Munroe ME, Bishop GA: A costimulatory function for T cell CD40. J Immunol 2007;178:671-682
36. Vaitaitis GM, Waid DM, Yussman MG, Wagner DH, Jr.: CD40-mediated signalling influences trafficking, T-cell receptor expression, and T-cell pathogenesis, in the NOD model of type 1 diabetes. Immunology 2017;152:243-254
37. Vaitaitis GM, Yussman MG, Waid DM, Wagner DH, Jr.: Th40 cells (CD4+CD40+ Tcells) drive a more severe form of Experimental Autoimmune Encephalomyelitis than conventional CD4 T cells. PLoS One 2017;12:e0172037
38. Vingert B, Tamagne M, Habibi A, Pakdaman S, Ripa J, Elayeb R, Galacteros F, Bierling P, Ansart-Pirenne H, Bartolucci P, Noizat-Pirenne F: Phenotypic differences of CD4(+) T cells in response to red blood cell immunization in transfused sickle cell disease patients. Eur J Immunol 2015;45:1868-1879
39. Halim L, Romano M, McGregor R, Correa I, Pavlidis P, Grageda N, Hoong SJ, Yuksel M, Jassem W, Hannen RF, Ong M, McKinney O, Hayee B, Karagiannis SN, Powell N, Lechler RI, Nova-Lamperti E, Lombardi G: An Atlas of Human Regulatory T Helper-like Cells Reveals Features of Th2-like Tregs that Support a Tumorigenic Environment. Cell Rep 2017;20:757-770
40. Krischer JP, Lynch KF, Schatz DA, Ilonen J, Lernmark A, Hagopian WA, Rewers MJ, She JX, Simell OG, Toppari J, Ziegler AG, Akolkar B, Bonifacio E: The 6 year incidence of diabetes-associated autoantibodies in genetically at-risk children: the TEDDY study. Diabetologia 2015;58:980-987
41. Kuroda A, Rauch TA, Todorov I, Ku HT, Al-Abdullah IH, Kandeel F, Mullen Y, Pfeifer GP, Ferreri K: Insulin gene expression is regulated by DNA methylation. PloS one 2009;4:e6953
42. Yang BT, Dayeh TA, Kirkpatrick CL, Taneera J, Kumar R, Groop L, Wollheim CB, Nitert MD, Ling C: Insulin promoter DNA methylation correlates negatively with insulin gene expression and positively with HbA(1c) levels in human pancreatic islets. Diabetologia 2011;54:360-367
43. Ruchat SM, Houde AA, Voisin G, St-Pierre J, Perron P, Baillargeon JP, Gaudet D, Hivert MF, Brisson D, Bouchard L: Gestational diabetes mellitus epigenetically affects genes predominantly involved in metabolic diseases. Epigenetics 2013;8:935-943
44. Durinovic-Bello I, Wu RP, Gersuk VH, Sanda S, Shilling HG, Nepom GT: Insulin gene VNTR genotype associates with frequency and phenotype of the autoimmune response to proinsulin. Genes Immun 2010;11:188-193
45. Lin X, Teh AL, Chen L, Lim IY, Tan PF, MacIsaac JL, Morin AM, Yap F, Tan KH, Saw SM, Lee YS, Holbrook JD, Godfrey KM, Meaney MJ, Kobor MS, Chong YS, Gluckman PD, Karnani N: Choice of surrogate tissue influences neonatal EWAS findings. BMC Med 2017;15:211
46. Babon JA, DeNicola ME, Blodgett DM, Crevecoeur I, Buttrick TS, Maehr R, Bottino R, Naji A, Kaddis J, Elyaman W, James EA, Haliyur R, Brissova M, Overbergh L, Mathieu C, Delong T, Haskins K, Pugliese A, Campbell-Thompson M, Mathews C, Atkinson MA, Powers AC, Harlan DM, Kent SC: Analysis of self-antigen specificity of islet-infiltrating T cells from human donors with type 1 diabetes. Nat Med 2016;22:1482-1487
47. Pathiraja V, Kuehlich JP, Campbell PD, Krishnamurthy B, Loudovaris T, Coates PT, Brodnicki TC, O'Connell PJ, Kedzierska K, Rodda C, Bergman P, Hill E, Purcell AW, Dudek NL, Thomas HE, Kay TW, Mannering SI: Proinsulin-specific, HLA-DQ8, and HLA-DQ8-

transdimer-restricted CD4+ T cells infiltrate islets in type 1 diabetes. Diabetes 2015;64:172-182
48. Skowera A, Ellis RJ, Varela-Calvino R, Arif S, Huang GC, Van-Krinks C, Zaremba A, Rackham C, Allen JS, Tree TI, Zhao M, Dayan CM, Sewell AK, Unger WW, Drijfhout JW, Ossendorp F, Roep BO, Peakman M: CTLs are targeted to kill beta cells in patients with type 1 diabetes through recognition of a glucose-regulated preproinsulin epitope. J Clin Invest 2008;118:3390-3402
49. So M, Elso CM, Tresoldi E, Pakusch M, Pathiraja V, Wentworth JM, Harrison LC, Krishnamurthy B, Thomas HE, Rodda C, Cameron FJ, McMahon J, Kay TWH, Mannering SI: Proinsulin C-peptide is an autoantigen in people with type 1 diabetes. Proc Natl Acad Sci U S A 2018;115:10732-10737
50. McMurchy AN, Levings MK: Suppression assays with human T regulatory cells: a technical guide. Eur J Immunol 2012;42:27-34

Figure legends

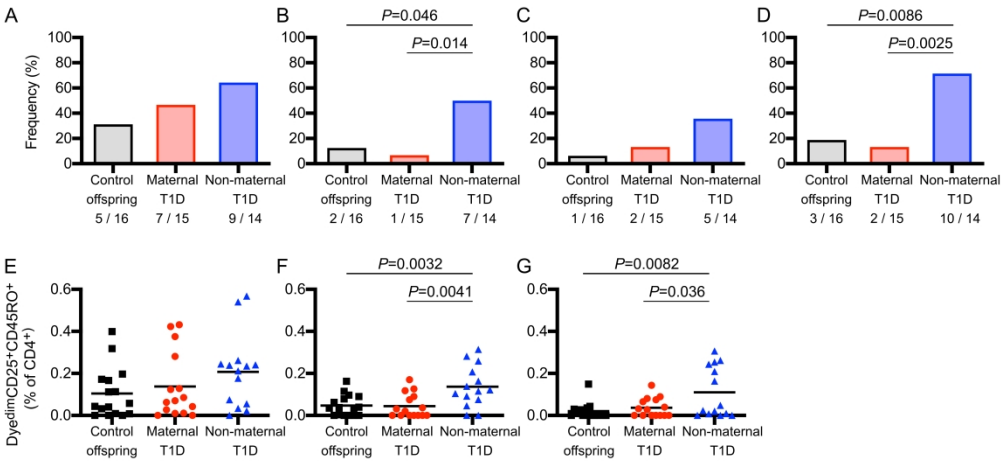
Figure 1. Antigen-responsive cord blood CD4$^+$ T cells. The proportion of cord blood samples with responses (bar graphs, A, B, C, D) and the frequencies of antigen-responsive CD4$^+$ T cells (scatter plots, E, F, G) after 5 days culture with matured MO-DCs loaded with KLH (A, E), proinsulin (B, F), insulin (C, G) and either proinsulin or insulin (D) for all tested cord blood samples are shown. For each antigen, a positive response was defined as a significant increase after comparing the frequency of responding CD4$^+$ T cells in each of 10 wells in the presence of antigen-MO-DCs to the 10 wells with control MO-DCs (see Figure S1). The number with a positive response out of the number tested is indicated. The frequency of antigen-responsive CD4$^+$ T cells for each antigen shown in E-G was determined by calculating the median response in each of 10 wells and subtracting the median of the 10 wells with control MO-DCs. For scatter plots, the mean is indicated as the horizontal line. *P*-values show comparisons made between groups using a two-sided Fischer's exact test for responder status (A, B, C, D) and two-sided, unpaired Student's *t* tests for the frequencies of antigen-responsive cells (E, F, G).

Figure 2. Gene expression in antigen-responsive cord blood CD4$^+$ T cells. A total of 680 antigen-responsive CD4$^+$ T cells were single cell sorted and processed for qPCR. A: UMAP analysis of qPCR data from 46 genes identified nine major clusters (cluster 1, black, 55 cells; cluster 2, brown, 38 cells; cluster 3, blue, 114 cells; cluster 4, light green, 110 cells; cluster 5, yellow, 41 cells; cluster 6, pink, 70 cells; cluster 7, gray, 76 cells; cluster 8, dark green, 123 cells; cluster 9, red, 53 cells). B: Distribution of KLH- (blue, 450 cells), proinsulin- (yellow, 141 cells) and insulin-responsive CD4$^+$ T cells (brown, 89 cells) is shown in the identified clusters. C: Gene expression intensities (white, no expression; brown, highest expression; gene order indicated on the right of the heatmap) were visualized as a heatmap organized by cluster as indicated in the horizontal bar beneath the heatmap. For each cluster, the cells are ordered by antigen as KLH (blue), proinsulin (yellow) or insulin (brown) as shown in the horizontal bar beneath the heatmap.

Figure 3. Gene expression signatures in proinsulin-responsive cord blood CD4$^+$ T cells according to maternal type 1 diabetes status. Expression measured by single cell qPCR of 46 genes in proinsulin-responsive CD4$^+$ T cells. A: UMAP analysis from Figure 2 showing cells from maternal T1D offspring (red, 30 cells), non-maternal T1D offspring (81 cells, blue) and control offspring (gray, 30 cells), in each of the nine clusters described in Figure 2. B: Gene expression intensities (white, no expression; brown, highest expression; gene order indicated on the right of the heatmap) were visualized as a heatmap organized by cluster as indicated in the horizontal bar beneath the heatmap. For each cluster, the cells are presented in the order maternal T1D offspring (red), non-maternal T1D offspring (blue), and control offspring (gray) as indicated below the heatmap.

Figure 4. Regulation of $CD4^+$ T cell responses by maternal T1D offspring and non-maternal T1D offspring $CD4^+CD25^+CD127^{low}$ cells in early childhood. Co-culture experiments of FACS-purified $CD4^+CD25^+CD127^{low}$ Treg with responder dye-labeled $CD4^+$ T cells stimulated with proinsulin (A) or responder dye-labeled $CD8^+$ cells stimulated with anti-CD3/CD28 Dynabeads (B). Upper panels show representative FACS plots of responder dye-dim T cells gated on $CD4^+$ (A) or $CD8^+$ T cells (B) without or in the presence of Tregs at a Treg:Tresponder ratio of 1:2. In the bottom panels, the y axis shows the suppression of proliferating responder T cells calculated in cultures containing Treg cells compared with those without Tregs. Data are shown for samples from maternal T1D offspring (red circles, n=6–7) and non-maternal T1D offspring (blue triangles, n=4–6) obtained at a median age of 9 months. The mean is indicated as the horizontal line in each scatterplot. *P*-values were calculated using two-sided, unpaired Student's *t* tests.

Figure 5. Methylation status of CpG sites within the *INS* gene in maternal and non-maternal T1D offspring. Analysis of cord blood methylation of the CpG sites in *INS* cg25336198 (A) and cg02749887 (B) in maternal T1D offspring (red) and non-maternal T1D offspring (blue) according to susceptible *INS* genotype AA (left) or other *INS* genotypes (right). The median value is indicated as the horizontal line in each scatterplot. *P*-values were calculated using Wilcoxon tests.



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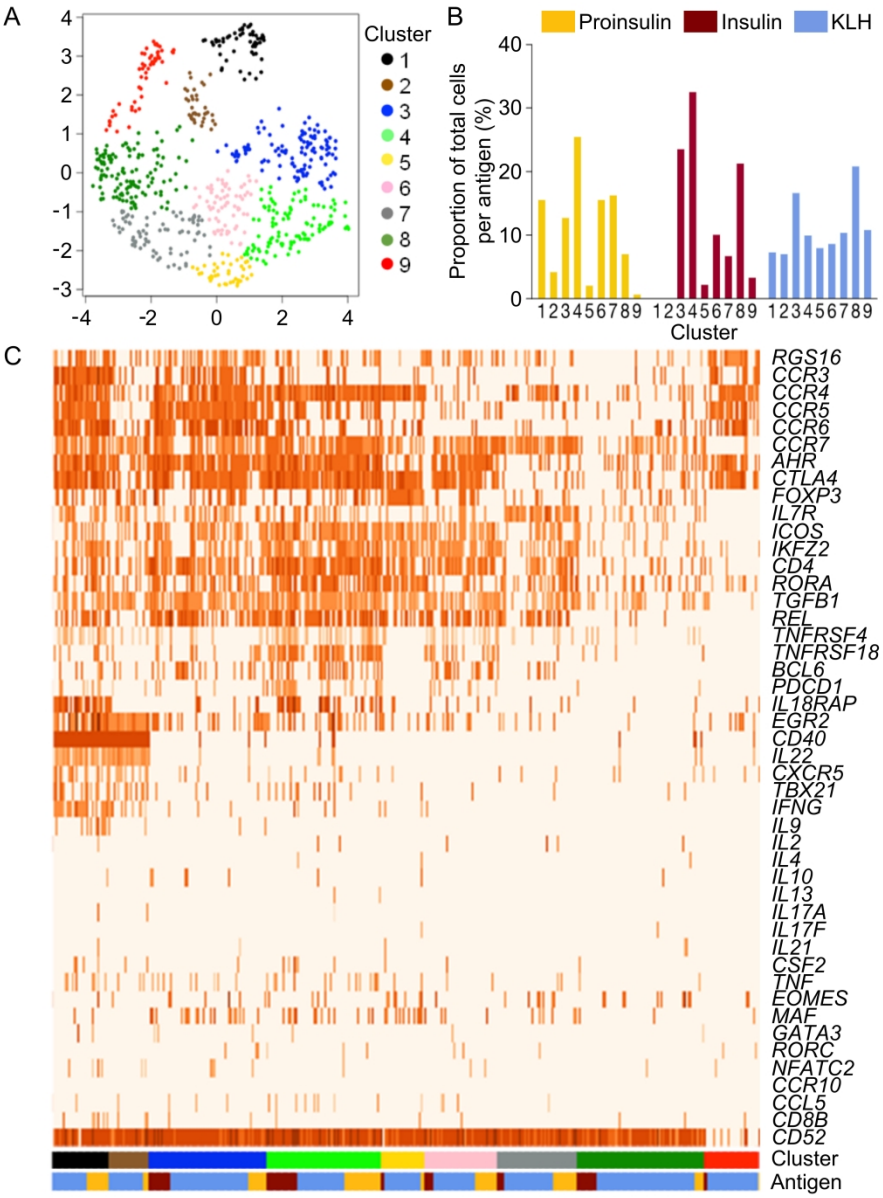

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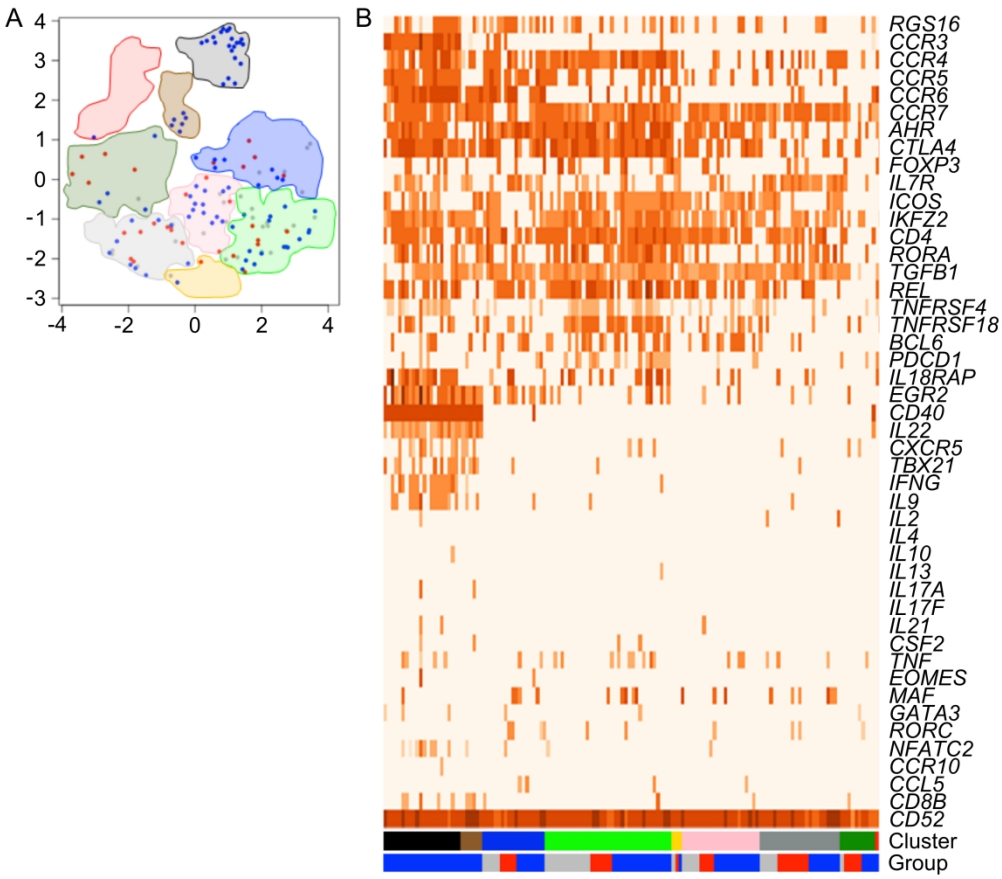

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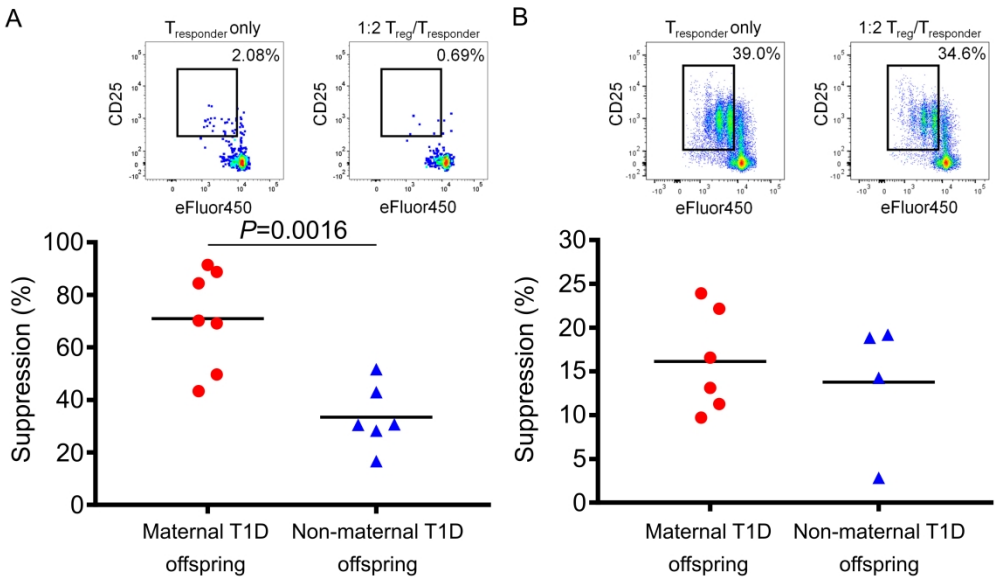

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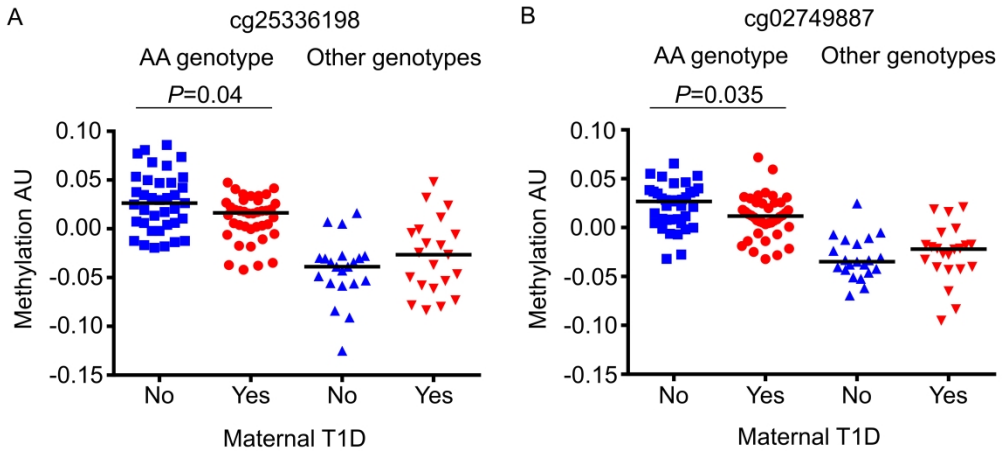

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Original article

Maternal type 1 diabetes reduces autoantigen-responsive CD4$^+$ T cells in offspring

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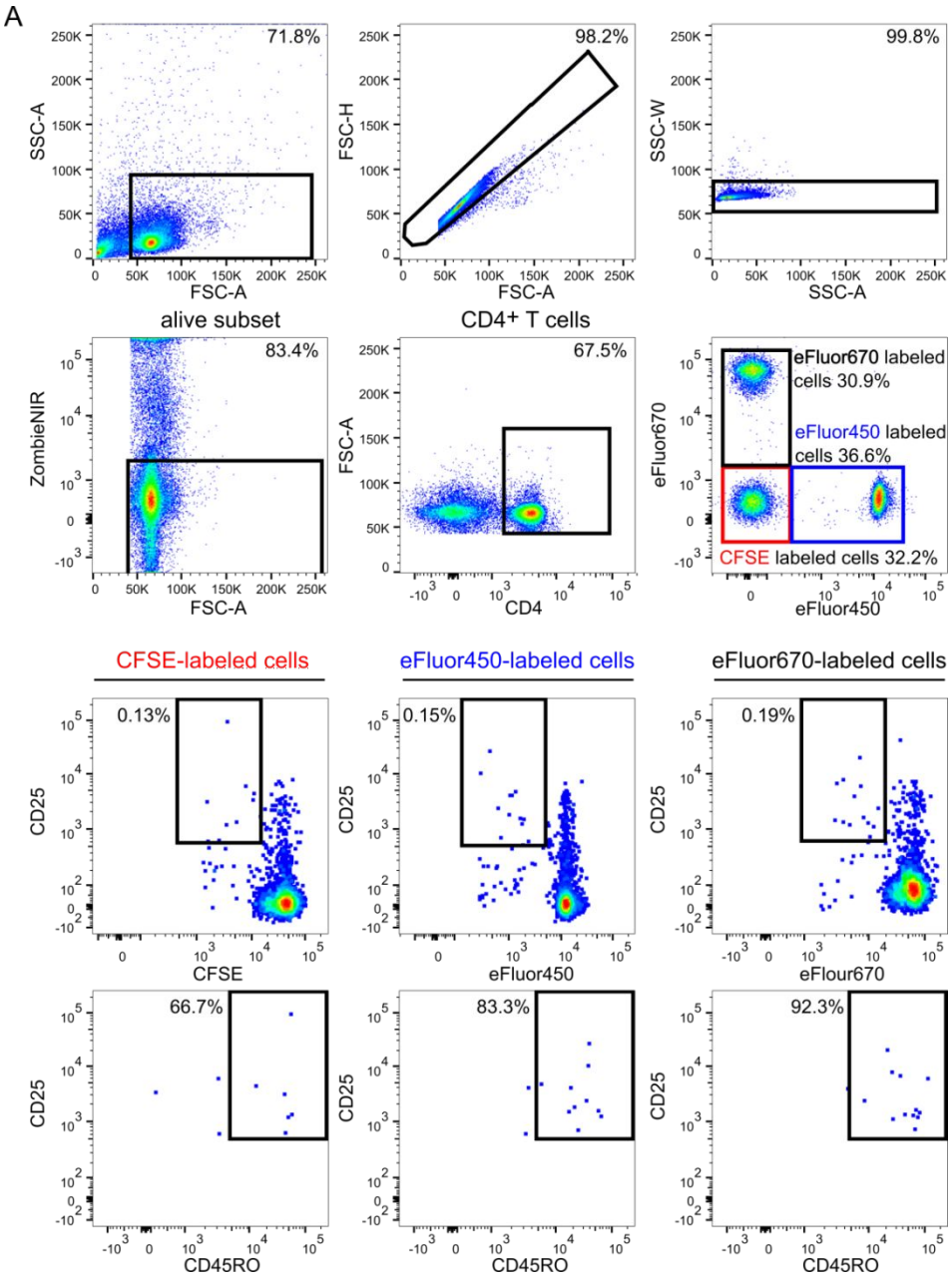
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99.8%
SSC-W
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ZombieNIR
FSC-A
CD4+ T cells
67.5%
FSC-A
CD4
eFluor670 labeled cells 30.9%
eFluor450 labeled cells 36.6%
CFSE labeled cells 32.2%
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0.13%
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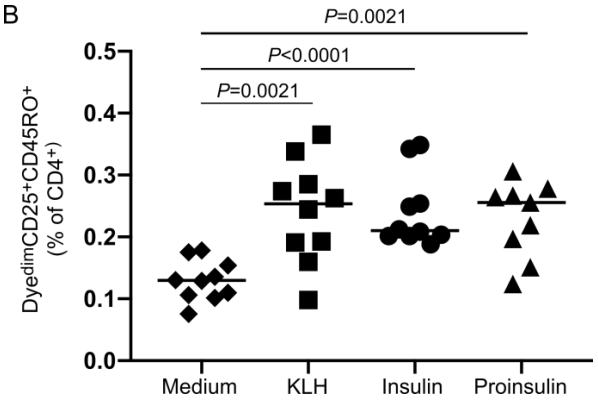
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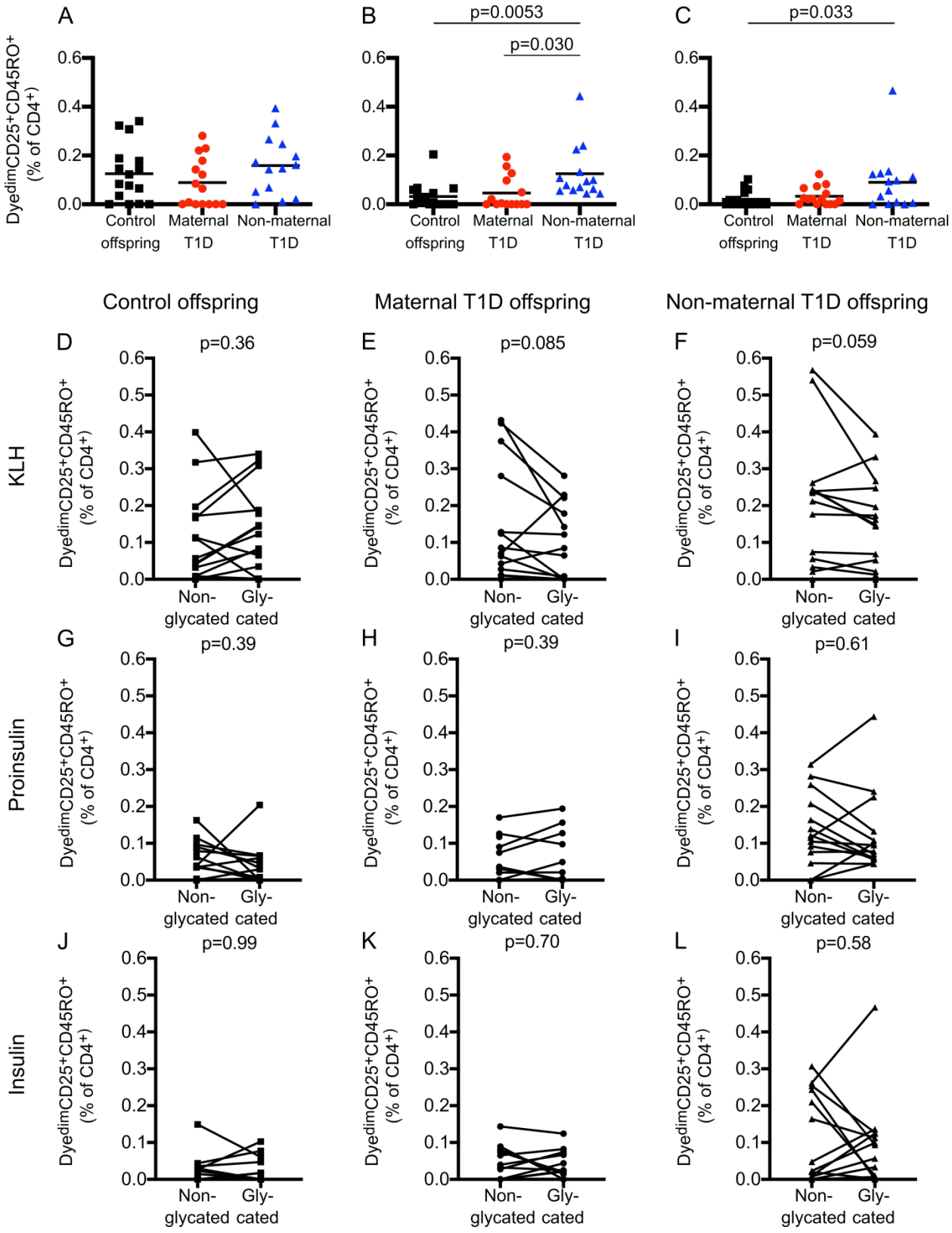
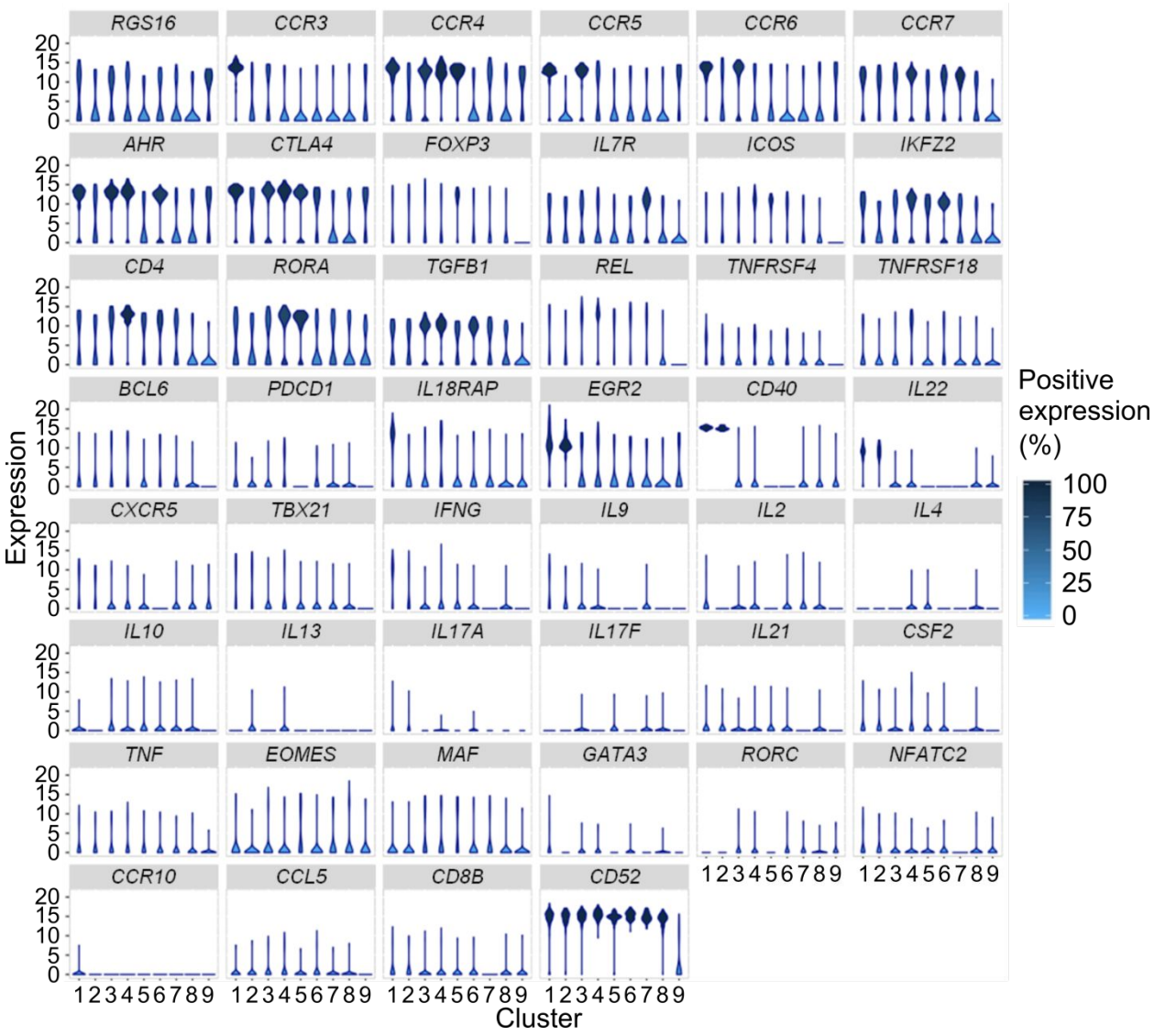
P<0.0001
P=0.0021
DyedimCD25+CD45RO+ (% of CD4+)
0.5
0.4
0.3
0.2
0.1
0.0
Medium
KLH
Insulin
Proinsulin

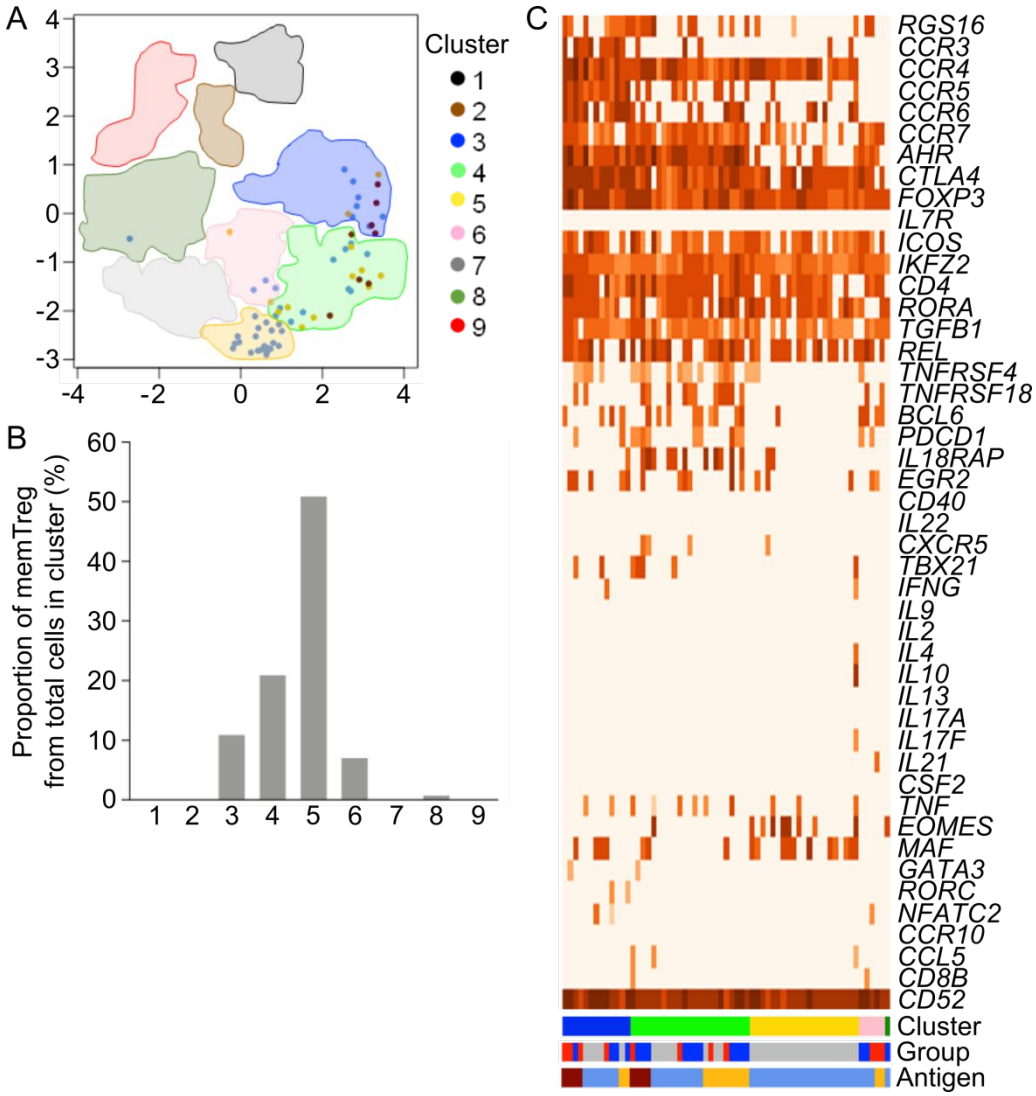
Supplementary Figure S1. Multi-dye, multi-well proliferation assay to identify antigen-responsive cord blood CD4$^+$ T cells. The established multi-dye proliferation assay consists of two parts: (1) maturation of monocyte-derived dendritic cells (MO-DC) from cord blood and (2) multi-parameter detection of antigen-responsive CD4$^+$ T cells. The assay enabled us to assess antigen-responsive frequencies of CD4$^+$ T cells with up to 30 replicates (5×10^4 cells per proliferation dye, resulting in 1.5×10^5 labeled responder cells in each of 10 wells). Ten replicates were analyzed for each antigen stimulus. A: For each dye, the labelled cells were identified as dye positive and negative for the remaining two dyes as indicated in the right hand FACS panel of row 2. Antigen-responsive CD4$^+$ T cells in each of the three dyes were subsequently identified as CD25$^+$CD45RO$^+$Dye^{dim}: each FACS plot in row 3 shows the features for one of the three dyes after selection of the dye positive cells. B: CD25$^+$CD45RO$^+$Dye^{dim} responsive CD4$^+$ T cells expressed as a proportion of the total number of proliferation dye-labelled CD4$^+$ T cells for each of the dyes in each of the 10 replicate wells (median values of three different proliferation dyes per well for each of the conditions shown). For each of the three antigen conditions (KLH, insulin, proinsulin), the values for the 10 replicates were compared to those in wells with medium (unloaded MO-DCs) by the Mann-Whitney U test and a positive response was defined when the two-tailed *P*-value of the comparison was <0.05.


A
B
C
p=0.0053
p=0.030
p=0.033
DyedimCD25+CD45RO+ (% of CD4+)
Control offspring
Maternal T1D
Non-maternal T1D
Control offspring
Maternal T1D
Non-maternal T1D
Control offspring
Maternal T1D
Non-maternal T1D
Control offspring
Maternal T1D offspring
Non-maternal T1D offspring
KLH
Proinsulin
Insulin
D
E
F
G
H
I
J
K
L
p=0.36
p=0.085
p=0.059
p=0.39
p=0.39
p=0.61
p=0.99
p=0.70
p=0.58
Non-glycated
Gly-cated

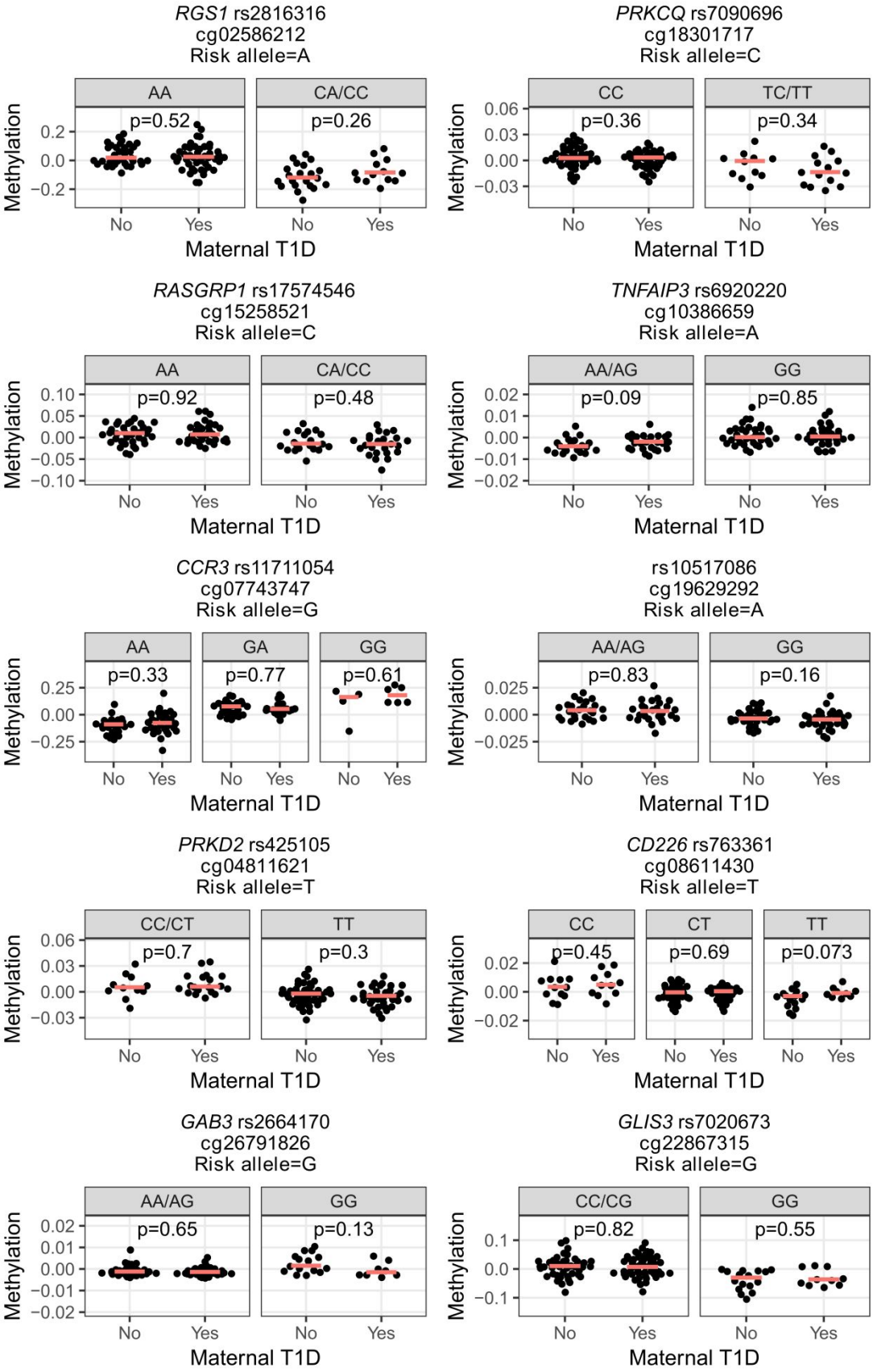
Supplementary Figure S2. Responsiveness of cord blood CD4$^+$ T cells to non-glycated and glycated antigens. Frequencies of antigen-responsive CD4$^+$ T cells after 5 days culture with matured MO-DCs loaded with glycated KLH (A), glycated proinsulin (B) and glycated insulin (C). The mean value is indicated as the horizontal line in each scatterplot. Paired responses to non-glycated and glycated antigens are shown for KLH (D–F), proinsulin (G–I) and insulin (J–L) in control offspring (n=16), maternal T1D (n=15) and non-maternal T1D offspring (n=14). *P*-values were calculated using two-sided, paired Student's *t* tests.



Supplementary Figure S3. Gene expression in antigen-responsive cord blood CD4$^+$ T cells according to cluster. Violin plots of Ct values for 46 genes in 680 antigen-responsive cord blood CD4$^+$ T cells for each of the nine clusters shown in Figure 2.



Supplementary Figure S4. Gene expression of antigen-responsive Treg-like CD4$^+$ T cells. A: UMAP analysis showing clusters from Figure 2 and 63 cells with the *FOXP3*$^+$*IKFZ2*$^+$*IL7R*$^-$*IL2*$^-$ Treg-like gene expression profile. Cells are distinguished as proinsulin-responsive (yellow), insulin-responsive (brown) and KLH-responsive (blue). B: Frequency of *FOXP3*$^+$*IKFZ2*$^+$*IL7R*$^-$*IL2*$^-$ Treg-like cells out of total antigen-responsive cells in each of the 9 clusters. C: Gene expression intensities (white, no expression; brown, highest expression; gene order indicated on the right of the heatmap) for the *FOXP3*$^+$*IKFZ2*$^+$*IL7R*$^-$*IL2*$^-$ Treg-like cells were visualized as a heatmap organized by cluster as indicated in the horizontal bar beneath the heatmap. For each cluster, the cells are grouped by control offspring (grey), maternal T1D offspring (red) or non-maternal T1D offspring (blue) and by antigen responsiveness against KLH (blue), proinsulin (yellow) or insulin (brown) as shown in the horizontal bars beneath the heatmap.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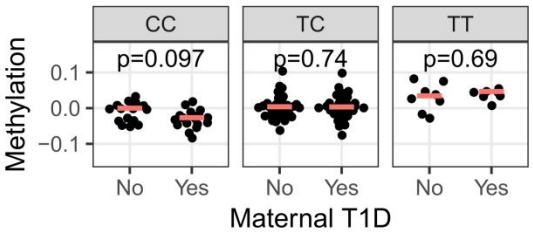
cg02586212
Risk allele=A
AA
CA/CC
p=0.52
p=0.26
PRKCQ rs7090696
cg18301717
Risk allele=C
CC
TC/TT
p=0.36
p=0.34
RASGRP1 rs17574546
cg15258521
Risk allele=C
AA
CA/CC
p=0.92
p=0.48
TNFAIP3 rs6920220
cg10386659
Risk allele=A
AA/AG
GG
p=0.09
p=0.85
CCR3 rs11711054
cg07743747
Risk allele=G
AA
GA
GG
p=0.33
p=0.77
p=0.61
rs10517086
cg19629292
Risk allele=A
AA/AG
GG
p=0.83
p=0.16
PRKD2 rs425105
cg04811621
Risk allele=T
CC/CT
TT
p=0.7
p=0.3
CD226 rs763361
cg08611430
Risk allele=T
CC
CT
TT
p=0.45
p=0.69
p=0.073
GAB3 rs2664170
cg26791826
Risk allele=G
AA/AG
GG
p=0.65
p=0.13
GLIS3 rs7020673
cg22867315
Risk allele=G
CC/CG
GG
p=0.82
p=0.55
Methylation
No
Yes
Maternal T1D

ORMDL3 rs2290400
cg06444195
Risk allele=C



SIRPG rs2281808
cg18612044
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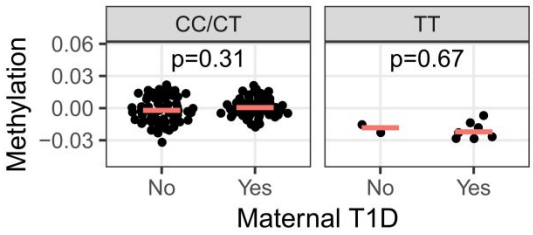

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cg20239604
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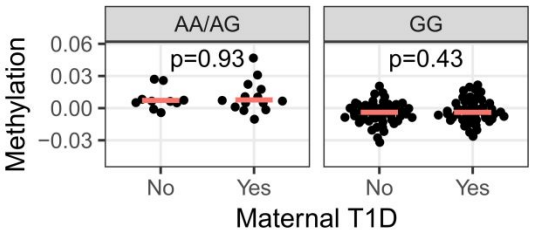

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cg14258366
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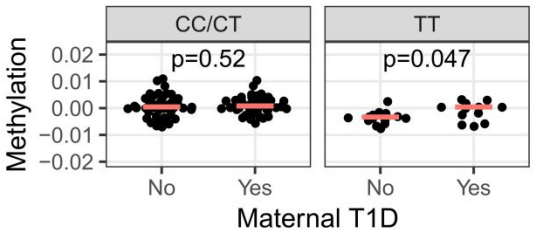

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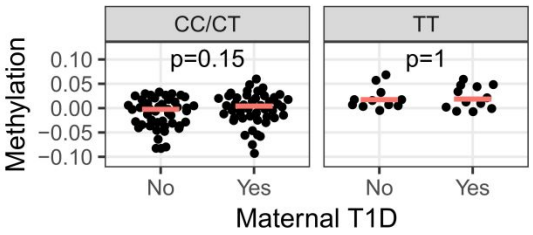

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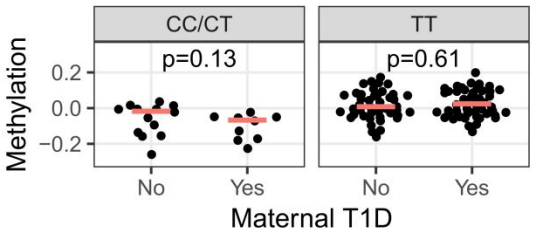
Risk allele=T



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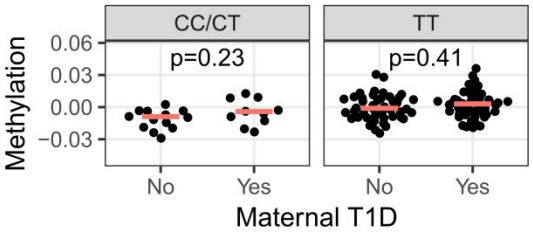
Risk allele=T



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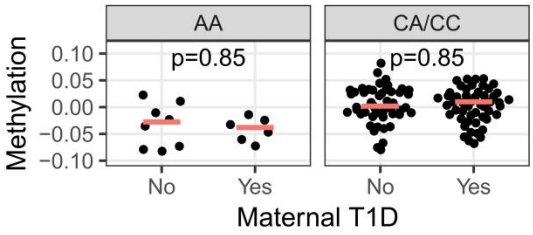
cg16175006
Risk allele=A



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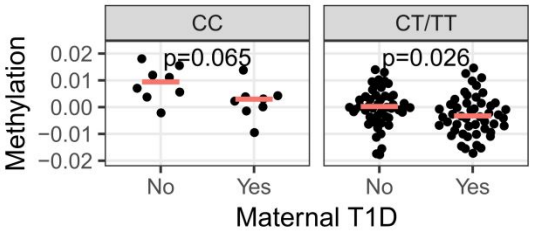

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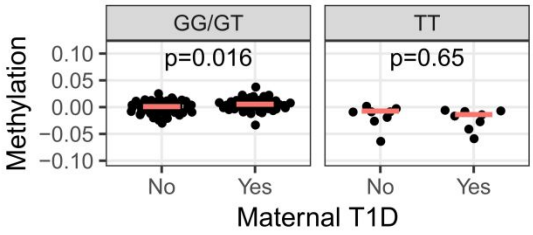

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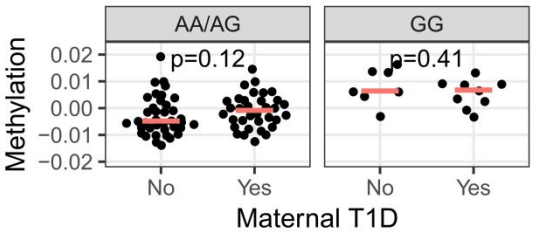

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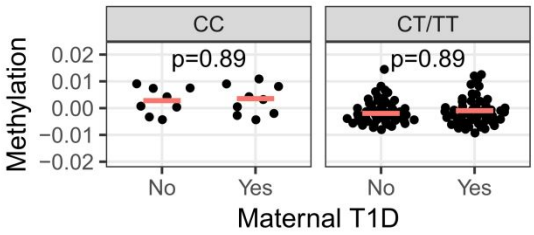

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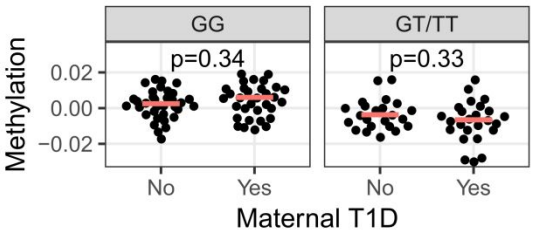
Risk allele=G



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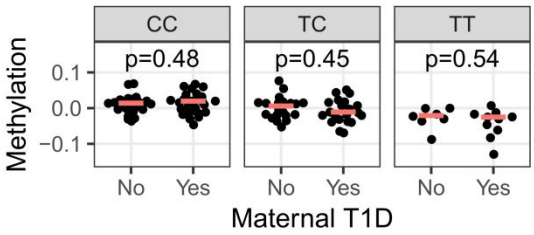
Risk allele=C



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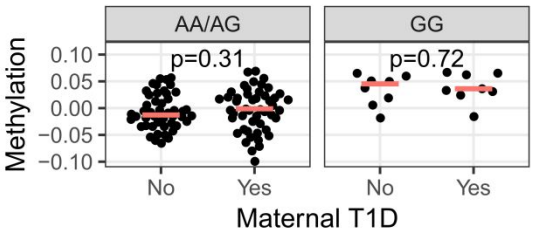
cg05080940
Risk allele=A



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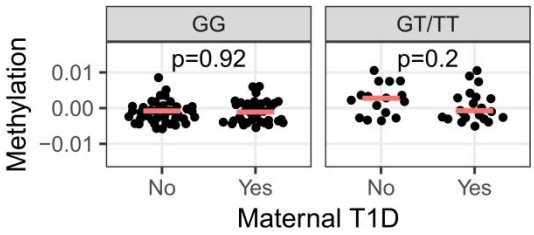

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cg00044050
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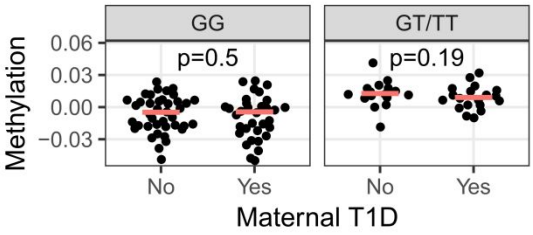

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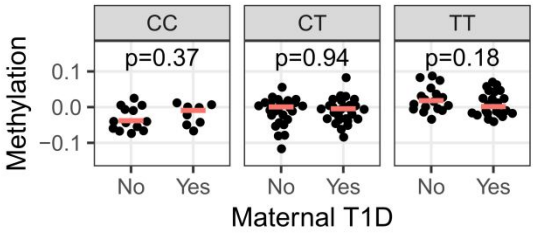

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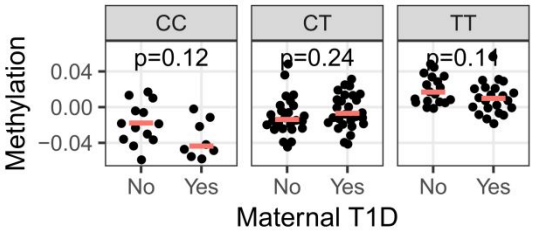

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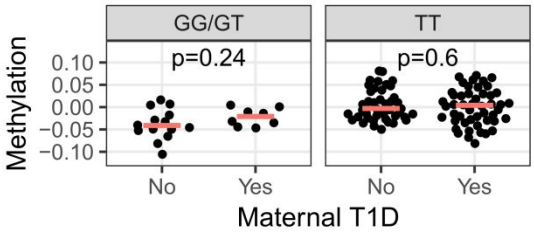

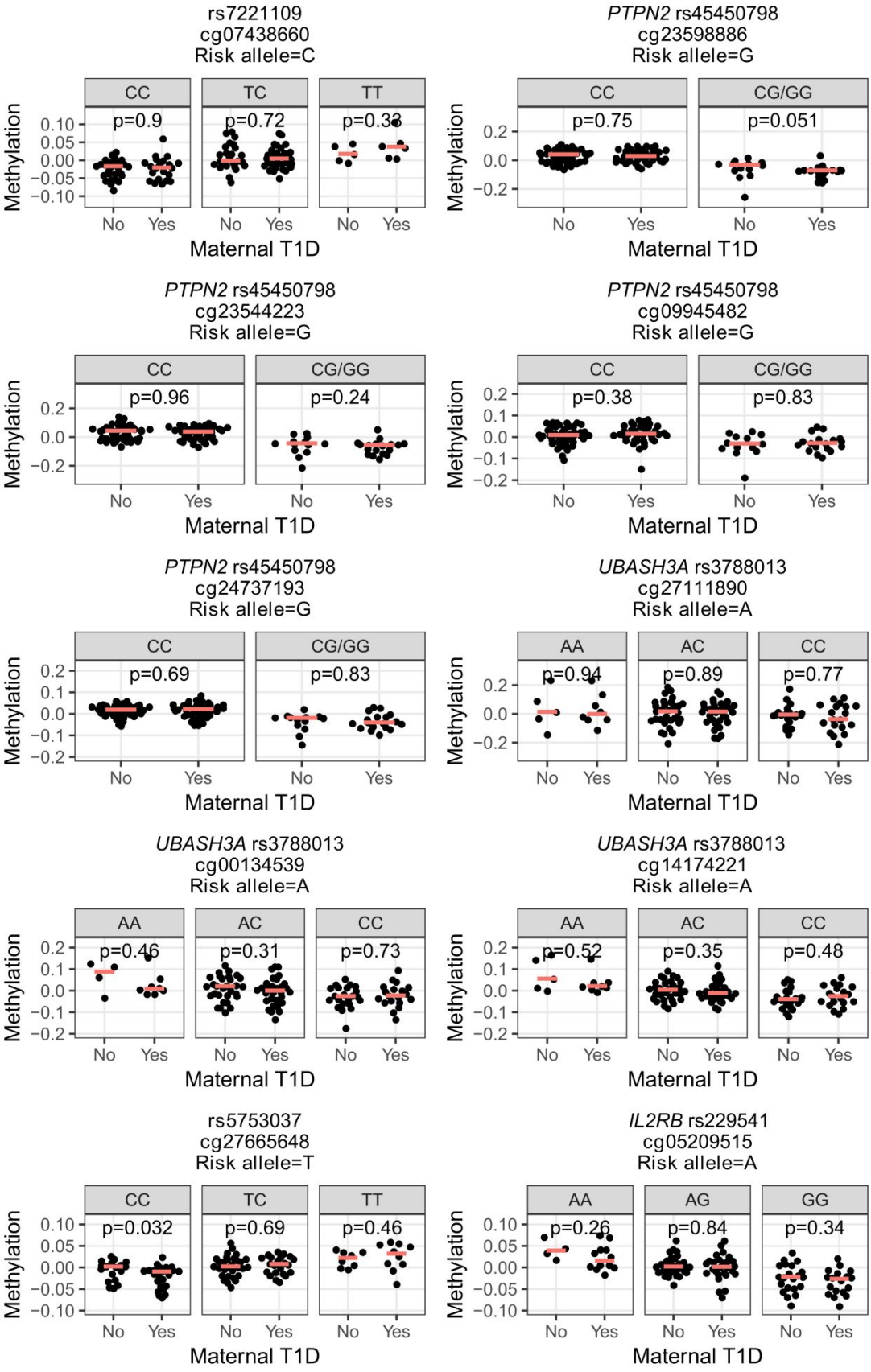
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cg07438660
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CC
TC
TT
p=0.9
p=0.72
p=0.33
PTPN2 rs45450798
cg23598886
Risk allele=G
CC
CG/GG
p=0.75
p=0.051
PTPN2 rs45450798
cg23544223
Risk allele=G
CC
CG/GG
p=0.96
p=0.24
PTPN2 rs45450798
cg09945482
Risk allele=G
CC
CG/GG
p=0.38
p=0.83
PTPN2 rs45450798
cg24737193
Risk allele=G
CC
CG/GG
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p=0.83
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cg27111890
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AA
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rs5753037
cg27665648
Risk allele=T
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p=0.032
p=0.69
p=0.46
IL2RB rs229541
cg05209515
Risk allele=A
AA
AG
GG
p=0.26
p=0.84
p=0.34
Methylation
Maternal T1D
No
Yes

Supplementary Figure S5. Methylation status of CpG sites within type 1 diabetes susceptibility genes. Cord blood methylation status of the CpG sites of T1D susceptibility genes that showed marked allele-specific methylation changes (25). The offspring are grouped according to SNP genotype and by maternal T1D status (no, non-maternal T1D offspring; yes, maternal T1D offspring). The gene, SNP, and CpG site are indicated above each graph. The median value is indicated as a horizontal line in each scatterplot. *P*-values were calculated using Wilcoxon tests.

Supplementary Table S1. Characteristics of samples used for T cell studies

	Control offspring	Maternal T1D	Non-maternal T1D
	n	n	n
Precursor frequencies of antigen-responsive, naive $CD4^+$ T cells (n=45)			
n	16	15	14
Sex (m/f)	8/8	10/5	7/7
HLA DR3	0	6	4
HLA DR4	1	10	5
INS genotype (AA/AT/TT)*	-	(6/7/2)	(4/9/1)
Gene expression profile of antigen-responsive, naive $CD4^+$ T cells (n=13)			
n	4	5	4
Sex (m/f)	2/2	4/1	3/1
HLA DR3	0	3	1
HLA DR4	0	3	3
INS genotype (AA/AT/TT)*	-	(2/3/-)	(-/3/1)
Suppressive capacity of antigen-specific, regulatory $CD4^+$ T cells (n=13)			
n	-	7	6
Sex (m/f)	-	5/2	6/0
Median age, years	-	0.76	0.76
HLA DR3	-	2	2
HLA DR4	-	2	3

*A, susceptible allele; T, protective allele

Supplementary Table S2. Primers used in single cell gene expression profiling of antigen-responsive CD4$^+$ T cells at birth

Gene	Preamplification 5'	Preamplification 3'	qPCR 5'	qPCR 3'
AHR	TAAAGCCAATCCCAGCTGAA	GACGCTGAGCCTAAGAACTGA	TAAAGCCAATCCCAGCTGAA	GACGCTGAGCCTAAGAACTGA
BCL6	AGCCGTGAGCAGTTTAGAGC	AAGTCCAGGAGGATGCAGAA	AGCCGTGAGCAGTTTAGAGC	AAGTCCAGGAGGATGCAGAA
CCR10	GCTGCTCTTCAGCCAGGAT	GCGTAGCAGGCTACCATGAC	GCTGCTCTTCAGCCAGGAT	GCGTAGCAGGCTACCATGAC
CCR3	TGTCTCGTTCTCCCTCTGCT	AGCCACATTGTAGGGTGTCC	TGTCTCGTTCTCCCTCTGCT	AGCCACATTGTAGGGTGTCC
CCR4	CAAATACAAGCGGCTCAGGT	AGCCCACCAAGTACATCCAG	CAAATACAAGCGGCTCAGGT	AGCCCACCAAGTACATCCAG
CCR5	GGCCATCTCTGACCTGTTTTT	AAACACAGCATGGACGACAG	GTCCCCTTCTGGGCTCACTA	AAACACAGCATGGACGACAG
CCR6	TCAGCGATGTTTTCGACTCC	CACCAGAATATTCCCCAGGA	TCAGCGATGTTTTCGACTCC	CACCAGAATATTCCCCAGGA
CCR7	CAATGAAAAGCGTGCTGGT	ATAGGGAGGAACCAGGCTTT	GTGGTGGCTCTCCTTGTCAT	ATAGGGAGGAACCAGGCTTT
IL7R	CTGAGGCTCCTTTTGACCTG	CTGCAGGAGTGTCAGCTTTG	CTGAGGCTCCTTTTGACCTG	CTGCAGGAGTGTCAGCTTTG
TNFRSF4	AAGCCTGGAGTTGACTGTGC	GGTCCCTGTCCTCACAGATT	CCACACAGGACACAGTCTGC	GGTCCCTGTCCTCACAGATT
CD4	ACCGGGGAGTCCCTTTTAG	CATTCAGCTTGGATGGACCT	ACCGGGGAGTCCCTTTTAG	CATTCAGCTTGGATGGACCT
CD40	GTGAGAGCTGTGTCCTGCAC	GCTTGTCCAAGGGTGACATT	GTGAGAGCTGTGTCCTGCAC	GCTTGTCCAAGGGTGACATT
CD52	GCGCTTCCTCTTCCTCCTAC	CTGAAGCAGAAGAGGTGGATT	GCGCTTCCTCTTCCTCCTAC	CTGAAGCAGAAGAGGTGGATT
CD8B	GCTGGACTTCGCCTGTGATAT	TTGTCTCCCGATTTGACCAC	GCTGGACTTCGCCTGTGATAT	TTGTCTCCCGATTTGACCAC
MAF	GGACGCGTACAAGGAGAAAT	GCTTCCAAAATGTGGCGTAT	GGACGCGTACAAGGAGAAAT	GCTTCCAAAATGTGGCGTAT
CTLA4	TGACAGCCAGGTGACTGAAG	GTTGCCTATGCCCAGGTAGT	TGGGGAATGAGTTGACCTTC	GTTGCCTATGCCCAGGTAGT
CXCR5	AAATGGACCTCGAGAACCTG	CTTGAAGGAGGCCATGAGG	AAATGGACCTCGAGAACCTG	CTTGAAGGAGGCCATGAGG
EGR2	TGGAGAGAAGAGGTCGTTGG	GTTGAAGCTGGGGAAGTGAC	TGGAGAGAAGAGGTCGTTGG	GTTGAAGCTGGGGAAGTGAC
EOMES	CACAAATACCAACCCCGACT	GGGACAATCTGATGGGATGA	CACAAATACCAACCCCGACT	GGGACAATCTGATGGGATGA
FOXP3	GTAGCCATGGAAACAGCACAT	GCGTGTGAACCAGTGGTAGAT	ACATTCCCAGAGTTCCTCCAC	GCGTGTGAACCAGTGGTAGAT
GATA3	CCGCCCTACTACGGAAACTC	TTGGAGAAGGGGCTGAGAT	CCGCCCTACTACGGAAACTC	TTGGAGAAGGGGCTGAGAT
TNFRSF18	GAGTGGGACTGCATGTGTGT	TGCAGTCTGTCCAAGGTTTG	GAGTGGGACTGCATGTGTGT	TGCAGTCTGTCCAAGGTTTG
CSF2	CACTGCTGCTGAGATGAATGA	AGGGCAGTGCTGCTTGTAGT	CACTGCTGCTGAGATGAATGA	AGGGCAGTGCTGCTTGTAGT
IKFZ2	CGAAAGGGAGCACTCCAATA	ATGGCCCCTGATCTCATCT	CGAAAGGGAGCACTCCAATA	ATGGCCCCTGATCTCATCT
ICOS	GGACCATTCTCATGCCAACT	TCGTGCACACTGGATGAATA	GGTTACCCATAGGATGTGCAG	TCGTGCACACTGGATGAATA
IL18RAP	TTGCAGGAGAGCGAATTAAA	GGTGAGAGTCGATTTCTGTGG	TTTAAGGGTTACCTGGGTTGC	GCCTTGCTCTTGTTTTCACAG
IL10	TGCTGGAGGACTTTAAGGGTTA	GCCTTGCTCTTGTTTTCACAG	GTACTGTGCAGCCCTGGAAT	TTTACAAACTGGGCCACCTC
IL13	GGTCAACATCACCCAGAACC	TTTACAAACTGGGCCACCTC	GCCAAATTCTGAGGACAAG	GGGGACAGAGTTCATGTGGT

IL17A	TGGGAAGACCTCATTGGTGT	CCGGTTATGGATGTTCAGGT	GCCTGTGCCAGGAGGTAGTA	ATGCAGCCCAAGTTCCTACA
IL17F	TCCAAAAGCCTGAGAGTTGC	ATGCAGCCCAAGTTCCTACA	TTGCAGGAGAGCGAATTAAA	GGTGAGAGTCGATTTCTGTGG
IL2	TGGAGCATTTACTGCTGGATT	GCACTTCCTCCAGAGGTTTG	TGGAGCATTTACTGCTGGATT	GCACTTCCTCCAGAGGTTTG
IL21	TCGCCACATGATTAGAATGC	AAGCAGGAAAAAGCTGACCA	TCGCCACATGATTAGAATGC	AAGCAGGAAAAAGCTGACCA
IL22	TCCAGCAGCCCTATATCACC	GTTCAGCACCTGCTTCATCA	TCCAGCAGCCCTATATCACC	GTTCAGCACCTGCTTCATCA
IL4	TGCCTCCAAGAACACAACTG	CTCTGGTTGGCTTCCTTCAC	GGCAGTTCTACAGCCACCAT	CTCTGGTTGGCTTCCTTCAC
IL9	CTCATCAACAAGATGCAGGAAG	TGTTTGCATGGTGGTATTGG	CTCATCAACAAGATGCAGGAAG	TGTTTGCATGGTGGTATTGG
IFNG	CTGTTACTGCCAGGACCCAT	TGGATGCTCTGGTCATCTTT	GGTCATTCAGATGTAGCGGA	TGGATGCTCTGGTCATCTTT
NFATC2	AACTCGTCTTTGGCGAGGA	AGACGGTAATCCATGATGTGG	AAGAAGAGCCGAATGCACATA	AGAAACTTCTGCGGCCCTAC
PDCD1	ACCTGCAGCTTCTCCAACAC	GCAGTTGTGTGACACGGAAG	GCTTCCGTGTCACACAACTG	GCACTTCTGCCCTTCTCTCT
CCL5	CGCTGTCATCCTCATTGCTA	ACACACTTGGCGGTTCTTTC	ATCTGCCTCCCCATATTCCT	ACACACTTGGCGGTTCTTTC
REL	ACAAATGTGAAGGGCGATCA	CCGTCTCTGCAGTCTTTTCC	GGAGCACAGCACAGACAACA	CCGTCTCTGCAGTCTTTTCC
RGS16	CACGCTTTCCTGAAGACAGA	GACCTCTTTAGGGGCCTCAC	CACGCTTTCCTGAAGACAGA	GACCTCTTTAGGGGCCTCAC
RORA	CACCAGCATCAGGCTTCTTT	GGTCTGCCACGTTATCTGCT	CACCAGCATCAGGCTTCTTT	GGTCTGCCACGTTATCTGCT
RORC	TCCCGAGATGCTGTCAAGTT	TCCCTCTGCTTCTTGGACAT	TCCCGAGATGCTGTCAAGTT	TCCCTCTGCTTCTTGGACAT
TBX21	CCGTGACTGCCTACCAGAAT	ATCTCCCCCAAGGAATTGAC	CCGTGACTGCCTACCAGAAT	ATCTCCCCCAAGGAATTGAC
TGFB1	TACCTGAACCCGTGTTGCT	CACAACTCCGGTGACATCAAA	TACCTGAACCCGTGTTGCTT	CAACTCCGGTGACATCAAAA
TNF	CCCCAGGGACCTCTCTCTAA	TGAGGTACAGGCCCTCTGAT	CCCGAGTGACAAGCCTGTAG	TGAGGTACAGGCCCTCTGAT