Genetic contribution to the divergence in type 1 diabetes risk between children from the general population and children from affected families

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Diabetes Publish Ahead of Print, published online January 23, 2019

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See the Supplemental Acknowledgements for members of the TEDDY Study Group.

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word count main text: 3987

word count abstract: 200

number of tables: 4

number of figures: 4

Non-standard abbreviations

FDR children, children with a first-degree family history of T1D

GP children, children from the general population without a first-degree family history of T1D

TEDDY, The Environmental Determinants of Diabetes in the Young

IAA, insulin autoantibodies

GADA, glutamic acid decarboxylase antibody

BTNL2, butyrophilin like 2

ITGA1, integrin subunit alpha

HR, hazard ratio

LD, linkage disequilibrium

Abstract

The risk for autoimmunity and subsequently type 1 diabetes is ten-fold higher in children with a first-degree family history of type 1 diabetes (FDR) than in the general population. We analyzed children with high-risk HLA genotypes (n=4,573) in the longitudinal TEDDY birth cohort to determine how much of the divergent risk is attributable to genetic enrichment in affected families. Enrichment for susceptible genotypes of multiple type 1 diabetes-associated genes and a novel risk gene, *BTNL2*, were identified in FDR children as compared to general population children. After correcting for genetic enrichment, the risks in the FDR and general population children converged but were not identical for multiple islet autoantibodies (HR, 2.26; 95%CI, 1.6-3.02) and for diabetes (HR, 2.92; 95%CI, 2.05-4.16). Convergence varied depending upon the degree of genetic susceptibility. Risks were similar in the highest genetic susceptibility group for multiple islet autoantibodies (14.3% vs 12.7%) and diabetes (4.8% vs 4.1%), and were up to 5.8-fold divergent for children in the lowest genetic susceptibility group, decreasing incrementally in general population children, but not in FDR children. These findings suggest that additional factors enriched within affected families preferentially increase the risk of autoimmunity and type 1 diabetes in lower genetic susceptibility strata.

ClinicalTrials.gov Identifier: NCT00279318

Introduction

Type 1 diabetes has a pre-symptomatic phase that often starts with the appearance of autoantibodies to pancreatic islet cell antigens (1). The development of islet autoantibodies is strongly influenced by HLA DR and DQ genotypes, with smaller contributions from many other genes (2-4). Children with a first-degree family history of type 1 diabetes (FDR children) have a higher risk of developing islet autoantibodies and diabetes than the risk in children from the general population without a family history of the disease (GP children) (5-7). Enrichment of type 1 diabetes-susceptibility genotypes of HLA and other genes is likely to contribute to the inflated risk in FDR children. Understanding the genetic differences and their contributions to the divergent risks between GP and FDR children could provide paradigms to identify novel genetic and environmental factors that modify risk, and to identify GP children whose *a priori* risk of developing type 1 diabetes is similar to that of FDR children.

The Environmental Determinants of Diabetes in the Young (TEDDY) study has screened over 400,000 newborns to identify and recruit over 8,000 with high-risk HLA genotypes into a follow-up cohort that monitors the development of islet autoantibodies and diabetes (8,9). This cohort includes FDR and GP children, providing a rare opportunity to examine the excess risk of developing islet autoantibodies and diabetes in affected families. The children in the TEDDY study have been extensively genotyped (4). This allowed us to calculate genetic risk scores representing cumulative genetic susceptibility (10-12), and has enabled us to investigate which genes, beyond the known susceptibility regions, may contribute to risk. Here, we address whether the increased risk in FDR children is accounted for by enrichment of genetic susceptibility in TEDDY children with the highest-risk HLA genotypes (*DR3/DR4-DQ8* heterozygotes and *DR4-DQ8/DR4-DQ8*

homozygotes). Using this approach, we could show enrichment of genetic susceptibility for multiple known risk genes and a novel risk gene. Matching FDR and GP children for genetic risk abrogated the excess risk in FDR children in the highest genetic risk stratum, but not in the lower genetic risk strata. These findings provide evidence that additional factors preferentially contribute to type 1 diabetes risk in children without a full complement of genetic susceptibility.

Research Design and Methods

The TEDDY study screened 424,788 newborns for type 1 diabetes-associated HLA genotypes between 2004 and 2010, of which 8,676 children were enrolled and followed in six centers located in the USA, Finland, Germany, and Sweden (Supplemental Figure 1). Detailed information on the study design, eligibility and methods has been published (3,8,9). Here, we used data as of 30 June 2017. Written informed consents were obtained for all participants from a parent or primary caretaker, separately, for genetic screening and for participation in prospective follow-up. The study was approved by local Institutional Review Boards and is monitored by the External Evaluation Committee formed by the National Institutes of Health.

Genotyping and genetic risk score

The HLA genotypes were confirmed by the central HLA Laboratory at Roche Molecular Systems (Oakland, CA) for enrolled subjects. The present report includes 4,572 TEDDY children with the *DR3-DQA1*05:01-DQB1*02:01/DR4-DQA1*03:0X-DQB1*03:02* genotype (HLA *DR3/DR4-DQ8*), or the *DR4-DQA1*03:0X-DQB1*03:02/DR4-DQA1*03:0X-DQB1*03:02* genotype (HLA *DR4-DQ8/DR4-DQ8*), if at least one sample was obtained after birth. SNP analysis was performed using the Illumina ImmunoChip (13). The genetic risk score was calculated from 40 SNPs similar to the previously described merged genetic score (12) except the value of the HLA *DR-DQ* genotype (3.98 for *DR3/DR4-DQ8* or 3.15 for *DR4-DQ8/DR4-DQ8*) was not included in the score.

TEDDY study outcomes

Blood samples were obtained every 3 months until age 4 years and biannually thereafter for the analysis of islet autoantibodies (insulin autoantibodies [IAA], glutamic acid decarboxylase autoantibodies [GADA], insulinoma antigen-2 autoantibodies). All radiobinding assays were performed as previously described (13; 14). A positive outcome was defined as positive in both reference laboratories on two or more consecutive visits. The date of seroconversion (time to first autoantibody) was defined as the date of drawing the first of the two consecutive positive samples. The presence of multiple islet autoantibodies was defined as the presence of at least two islet autoantibodies. Diabetes was diagnosed according to ADA criteria.

Statistics

The risks of developing one or more islet autoantibodies, multiple autoantibodies, and diabetes in FDR and GP children were assessed using Kaplan–Meier plots, and groups were compared using the log-rank test. HRs were computed using Cox's proportional hazards model. Genotype frequencies between GP and FDR children were compared using χ^2 tests with Bonferroni's correction for multiple testing. All analyses were carried out with R 3.4.2 (R Foundation for Statistical Computing, Vienna, Austria), using the GWASTools, haplo.stats, qqman and survminer packages.

Results

Risk of developing islet autoantibodies and diabetes according to HLA genotype and first-degree relative status

Of the TEDDY children with at least one follow-up sample (n=7,894), 3,035 (38.4%) had the HLA *DR3/DR4-DQ8* genotype, and 1,537 (19.5%) had the HLA *DR4-DQ8/DR4-DQ8* genotype. Of these 4,572 children, 423 (9.3%) were FDR children and 4,149 were GP children (Table 1, Supplemental Figure 1). One or more islet autoantibodies developed in 500 (10.9%) children, of which 324 (7.1%) had multiple islet autoantibodies. Diabetes was diagnosed in 192 (4.2%) children.

Matching for HLA *DR-DQ* provided an estimate of the excess risk of developing islet autoantibodies or diabetes in FDR children that was due to factors other than enrichment for these genotypes (Figure 1). Matching for HLA genotypes was sufficient to reduce the >10-fold excess risk usually observed in children from affected families to below 3-fold. The cumulative risk (95%CI) by 6 years of age in HLA *DR3/DR4-DQ8* FDR children was 20.5% (15.4–25.4%) for one or more islet autoantibodies, 17.0% (12.1–21.5%) for multiple islet autoantibodies, and 6.8% (3.8–9.7%) for diabetes compared with 10.0% (8.8–12.2%; $P<0.0001$), 6.4% (5.4–7.4%; $P<0.0001$), and 2.7% (2.0–3.3%; $P<0.0001$), respectively, in GP children with these genotypes (Figure 1A,C,E). Similar differences were observed in the HLA *DR4-DQ8/DR4-DQ8* children (Figure 1B,D,F). A first-degree family history of type 1 diabetes was associated with an increased incidence of islet autoimmunity in the first 3 years of life in children with the high-risk HLA genotypes (Supplemental Figure 2A). This was similar if the outcome was defined as the detection of IAA before other autoantibodies or GADA as the first islet autoantibodies (Supplemental Figure 2B).

*DRB1*04 allele subtype enrichment in children from affected families*

The risk for type 1 diabetes is influenced by the *HLA-DRB1*04* allele (15; 16). We, therefore, searched for FDR enrichment of *DRB1*04* subtypes (Table 2). The high risk *DRB1*04:01* allele was more frequent in the FDR children than in the GP children ($P<0.0001$) for children with either the *DR3/4-DQ8* ($P=0.0067$) or *DR4-DQ8/DR4-DQ8* ($P=0.0005$) genotypes. In contrast, the lower risk *DRB1*04:04* ($P<0.0001$) and *DRB1*04:07* ($P=0.035$) alleles were less frequent in the FDR children than in GP children. There were no differences in the frequencies of the *DRB1*04:02* or the *DRB1*04:05* alleles between the FDR children and the GP children. The remaining *DRB1*04* alleles were infrequent in the study population and were not considered.

Genetic risk scores in FDR and GP children

The additional non-HLA *DR-DQ* gene risk was expressed as a genetic risk score from 40 of the non-HLA *DR-DQ* SNPs (12). Genetic risk scores were higher in the FDR children (median, interquartile range: 10.3, 9.7–11.0) than in the GP children (10.1, 9.4–10.7; $P<0.0001$; Figure 2A). Enrichment of risk genotypes reached significance for six of the 40 SNPs (Supplemental Table 1). Similar genetic risk scores were observed in the multiple islet autoantibody positive FDR (median, interquartile range: 10.4, 10.0–11.3) and GP children (10.5, 10.0–11.1; $P=0.97$; Figure 2B).

Additional genes with allele enrichment in FDR children

HLA *DR-DQ* susceptible genotypes have additional variants in genes that are in linkage disequilibrium (LD) with HLA *DR-DQ* (17). We reasoned that the frequencies of

susceptibility genotypes of such genes may be increased in the FDR children and could account for some of the excess risk in these children. With the rare opportunity to compare a large number of GP and FDR children matched for HLA *DR-DQ* genotype in the TEDDY study, we examined 6,097 SNPs with minor allele frequencies >5% on the short arm of chromosome 6, containing the HLA genes (6p21.32). SNPs rs3763305 (P=7.27×10^{-7}) and rs3817964 (P=8.26×10^{-7}) were enriched in the FDR children (Supplemental Figure 3A). Both SNPs are intronic variants of *BTNL2* and are in complete LD (r^2=1). The *BTNL2* gene is located close to the HLA-*DRA*/HLA-*DRB5*/HLA-*DRB1* cluster and HLA-*DQB1*. Extension of the analysis to all 111,069 ImmunoChip SNPs that passed QC filters identified rs7735139 (intronic SNP in *ITGA1*, Integrin Subunit Alpha 1, 5q11.2) with allelic enrichment in the FDR children (P=4.34×10^{-8}; Supplemental Figure 3B).

Genetic contribution to the additional risk for islet autoantibodies and diabetes in FDR children

Cox proportional hazards models were used to assess the development of one or more islet autoantibodies, multiple islet autoantibodies, and diabetes (Table 3). Model 1 examined the excess risk conferred by a first-degree family history of type 1 diabetes adjusted for HLA genotype (*DR3/4-DQ8* vs *DR4-DQ8/DR4-DQ8*), sex, and country of origin. Hazard ratios (HRs) in the FDR children were 2.12 (95% CI, 1.65–2.72) for one or more islet autoantibodies, 2.77 (95% CI, 2.09–3.68) for multiple islet autoantibodies, and 3.69 (95% CI, 2.60–5.23) for diabetes.

To determine whether the genetic factors that were enriched in FDR children contributed to the excess risk in FDR children, Model 2 added the non-HLA *DR-DQ* genetic risk

score, *DRB1*04* subtype *(enriched: DR3/DRB1*04:01* and *DRB1*04:01/DR*04:xx* where **04:xx* is neither **04:04* nor **04:07* vs non-enriched: other genotypes), the *BTNL2* SNP rs3763305 (enriched: GG vs non-enriched: GA/AA genotypes), and the *ITGA1* SNP rs7735139 (enriched: GG vs non-enriched: GA/AA genotypes). HRs for FDR children were reduced to 1.82 (95% CI, 1.42–2.35) for one or more islet autoantibodies, 2.26 (95% CI, 1.70–3.02) for multiple islet autoantibodies, and 2.92 (95% CI, 2.05–4.16) for diabetes. The enriched *DRB*04:01* subtype (HR, 1.48; 95% CI, 1.08–2.01; P=0.014), and the non-HLA *DR-DQ* genetic risk score (HR, 1.66; 95% CI, 1.47–1.88; P<0.0001) contributed to the risk of multiple islet autoantibodies. Similar HRs for these variables were also observed for the risk of one or more islet autoantibodies and diabetes, some of which reached significance. The *BTNL2* SNP rs3763305 GG genotype conferred additional risk for diabetes (HR, 1.80; 95% CI, 1.11–2.93; P=0.017; Table 3), and this additional risk was also observed when the analysis was restricted to children with a HLA *DR3/4-DQ8* genotype (HR, 1.92; 95% CI, 1.11–3.35; P=0.021; Supplemental Table 2). The *ITGA1* SNP was not associated with risk.

*Association between DRB1*04 subtypes and islet autoantibodies or diabetes*

To further assess whether the *DRB1*04:01* allele was associated with increased risk compared with the other *DRB1*04* alleles in the TEDDY children, we removed the confounder of family history, and performed a Kaplan-Meier analysis in the GP children. Among the 1,876 children with the *DRB1*04:01* allele, the cumulative risks (95% CI) at 6 years old were 11.4% (9.8–12.9%) for one or more islet autoantibodies, 7.7% (6.3–9.0%) for multiple islet autoantibodies, and 2.9% (2.1–3.6%) for diabetes compared with 6.8% (5.7–8.0%; P<0.0001), 4.0% (3.1–4.9%; P<0.0001), and 1.4% (0.9–1.9%;

P<0.0001), respectively, among 2,176 children without *DRB1*04:01* (Supplemental Figure 4A,C,E). These differences remained when the analysis was limited to GP children with the HLA *DR3/DR4-DQ8* genotype (Supplemental Figure 4B,D,F).

Association between BTNL2 genotypes and islet autoantibodies or diabetes

Variants in the *BTNL2* gene have not been implicated as independent genetic risk factors for type 1 diabetes previously, likely due to the extensive LD in the MHC region and inadequate sample size. The *BTNL2* rs3763305 GG genotype distribution was increased in children who developed one or more islet autoantibodies (*P*<0.0001), multiple islet autoantibodies (*P*<0.0001), or diabetes (*P*<0.0001), compared with children who remained islet autoantibody negative. These associations were observed separately for children with HLA *DR3/4-DQ8* or *DR4-DQ8/DR4-DQ8* (Table 4). The association between the *BTNL2* rs3763305 GG genotype and type 1 diabetes was also validated in the Type 1 Diabetes Genetics Consortium (T1DGC), using a nested case-control comparison after stratification for HLA *DR3/4-DQ8* (Supplemental Table 3).

The ImmunoChip contained 88 SNPs that were genotyped within *BTNL2*, including 34 that passed all quality-control metrics. The ImmunoChip genotypes were used to define haplotypes using the R package *haplo.stats* (Supplemental Table 4). The risk associated with the four most frequent *BTNL2* genotypes among children with *DR3/DR4-DQ8* and the four most frequent genotypes among children with *DR4-DQ8/DR4-DQ8* was stratified by the presence of haplotype 28, which uniquely contained an A allele at *BTNL2* rs3763305, and a T allele at *BTNL2* rs3817964 (Supplemental Figure 5).

BTNL2 lies close to the HLA-*DRB5*/HLA-*DRB6*/HLA-*DRB1* protein-coding genes in a region of high LD. HLA-*DR3* was in nearly complete LD with the rs3763305 G allele:

the *BTNL2* rs3763305 GG genotype was identified in 1,608 (99.3%) of 1,619 children who had the HLA *DR3/DR3* genotype (Supplemental table 5). We, next examined the second *BTNL2* rs3763305 allele in children with *DR3/DR4-DQ8*. The *BTNL2* rs3763305 G allele was in nearly complete LD with *DRB1*04:01* (allele frequency, 99.5%), *DRB1*04:02* (99.4%), and *DRB1*04:05* (100.0%), whereas the *BTNL2* rs3763305 A allele was associated with *DRB1*0404* (39.2%) and *DRB1*0407* (34.4%) (P<0.0001). These associations were confirmed in a separate cohort of 149 children with type 1 diabetes and the *DR3/DR4-DQ8* genotype from Bavaria, Germany (Supplemental Table 6). The *BTNL2* rs3763305 A allele was also observed together with the protective HLA *DRB1*04:03* allele in five (56%) of nine informative genotypes, and with the protective HLA *DRB1*13:01* allele in 10 (25%) of 40 informative genotypes, but not with other HLA *DRB1* alleles in the German cohort (data not shown).

The *DRB1*04:04* allele together with either the *BTNL2* rs3763305 G or the *BTNL2* rs3763305 A allele was relatively frequent in the TEDDY children and, therefore, provided an opportunity to determine whether the *BTNL2* gene conferred an independent risk to the HLA-DR4 subtype. Risks associated with *BTNL2* genotypes were examined in children with the *DR3/DRB1*04:04-DQ8* or *DRB1*04:04-DQ8/DRB1*04:04-DQ8* genotypes. The cumulative risks (95% CI) at 6 years old in children with the *BTNL2* rs3763305 GG genotype were 9.8% (5.6–13.8%) for one or more islet autoantibodies, 6.3% (2.9–9.6%) for multiple islet autoantibodies, and 3.7% (1.3–6.0%) for diabetes, compared with 8.0% (6.2–9.8%; P=0.46), 4.7% (3.3–6.2%; P=0.096), and 1.6% (0.8–6.0%; P=0.0048) in the children with the GA or AA genotypes (Figure 3). Cox proportional hazards models adjusted for *DR3/DRB1*04:04-DQ8* or *DRB1*04:04-*

*DQ8/DRB1*04:04-DQ8* genotype replicated the additional risk for diabetes conferred by the *BTNL2* rs3763305 GG genotype (*P*=0.0086; Supplemental Table 7).

The additional risk conferred by the *BTNL2* rs3763305 GG genotype may be due to a specific association with specific *DRB1*04:04* subtypes. We, therefore, examined the relationship between the *BTNL2* rs3763305 alleles and *DRB1*04:04* subtypes in the German cohort of patients who had been HLA genotyped by sequencing of HLA *DRB1* exon 2, which harbors variations in all 12 subtypes of *DRB1*04:04*. All subjects with *DRB1*04:04* had the *DRB1*04:04:01* allele regardless of whether the *BTNL2* rs3763305 was A or G, indicating that the *BTNL2* rs3763305 A allele does not appear to mark a subtype of *DRB1*04:04*.

Finally, we examined the effect of BTNL2 knockdown on *in vitro* immune responses (Supplemental Figure 6). As compared to non-targeting siRNA control treated dendritic cells, BTNL2-targeted siRNA treated dendritic cells increased naïve alloreactive $CD4^+$ T cell activation (p=0.031) but not memory antigen-specific $CD4^+$ T cell activation (p=0.43).

Risk excess in FDR children after stratification by genetic risk

We asked whether the observed enrichment of type 1 diabetes genetic susceptibility in the FDR children could account for their excess risk. Four risk strata were defined by HLA *DRB1*04* subtype and genetic risk score. These strata were able to discriminate the risk of developing islet autoantibodies and T1D in the GP children (Figure 4A,C,E). A similar stratification in the GP children was observed if the strata were defined by the children's *BTNL2* genotype and genetic risk score (Supplemental Figure 7). In contrast to the GP children, a discrimination of risk in the FDR children was only achieved in the lowest

risk stratum (Figure 4B,D,F). Comparing FDR children and GP children showed complete convergence of the risks of developing islet autoantibodies and diabetes in the highest-risk stratum, and divergence of risk in the lower-risk strata (Supplemental Table 8). The fold difference in risk for multiple islet autoantibodies between the FDR and GP children was 1.1 in the highest-risk stratum (14.3% vs 12.7%), 1.9 in the second risk stratum (17% vs 9%), 3.3 in the third risk stratum (14.8% vs 4.5%), and 5.8 in the lowest-risk stratum (9.2% vs 1.6%).

Risk in FDR children is modified by maternal, paternal, and sibling type 1 diabetes

Risk divergence at lower genetic susceptibility strata implied that additional factors that influence risk may act differently depending on the *a priori* genetic susceptibility. One factor that is known to affect risk in FDR children is maternal type 1 diabetes (18). The HRs (95% CI) for one or more islet autoantibodies (2.37; 1.71–3.28), multiple islet autoantibodies (2.89; 2.00–4.17), and diabetes (3.06; 1.89–4.94), were increased as compared with GP children if the first-degree relative index case was a father. The HRs were also increased if the first-degree relative was a sibling. By contrast, if the first-degree relative index case was the mother, the HR (95% CI) was not increased for one or more islet autoantibodies (0.85; 0.50–1.44), multiple islet autoantibodies (0.97; 0.52–1.79), and diabetes (1.39; 0.67–2.87; Supplemental Table 9). This relative protection conferred by maternal type 1 diabetes versus paternal or sibling type 1 diabetes was observed in the higher risk strata (Supplemental Figure 8).

Discussion

We found that the excess risk for islet autoantibodies and diabetes in FDR children could be abrogated by accounting for an increased load of type 1 diabetes susceptibility alleles at multiple loci, including a novel susceptibility region marked by SNPs within the *BTLN2* gene. The risk converged when children were matched at the highest genetic susceptibility and became increasingly divergent as genetic susceptibility was attenuated. These data suggest that additional factors shared within families may modify type 1 diabetes risk heterogeneously and are dependent upon *a priori* genetic susceptibility.

The study was performed in a large number of FDR and GP children of mainly European descent who were matched for the two highest-risk HLA class II genotypes. This unique cohort allowed us to assess the contributions of other genetic factors. After selection by HLA genotype, the excess risk for islet autoantibodies and diabetes was around two- to three-fold higher in FDR children, which is markedly less than the >10-fold excess observed without HLA selection. Enrichment of genetic susceptibility was observed for HLA *DR4* subtypes and by an increased genetic risk score for non-HLA loci. The addition of these genetic markers further reduced the excess risk in FDR children, but the adjusted HRs remained above two for the development of multiple islet autoantibodies or diabetes. Remarkably, this excess risk was heterogeneous, and depended on the *a priori* genetic susceptibility. A limitation of our study is that we could not examine children with other HLA genotypes and, therefore, cannot assess whether the divergence continues in children with HLA genotypes associated with moderate or low risk. Although TEDDY is a unique study with unprecedented numbers of FDR and GP children for comparisons, the findings require further validation, especially in different ethnical populations.

The excess risk that remained unaccounted for by susceptibility genes in families is likely due to further genetic enrichment, including rare variants that may be more frequent in familial cases, or other factors, such as a shared environment. The study provided the opportunity to search for additional genetic factors that may contribute to risk by exploring genes with allelic enrichment in FDR children. A limitation of this approach is that, despite the size of the TEDDY study, there was relatively little power to find these genes across the whole genome, particularly for genes with low minor allele frequencies.

We were successful in finding an enrichment of alleles for two additional genes. One of the genes with allelic enrichment in the FDR children, *BTNL2*, lies within the HLA class II region. SNPs within *BNTL2* were previously shown to be associated with other HLA DR-linked diseases, but in almost all cases, including type 1 diabetes, the risk was attributed to LD with HLA DR (19). Our study, which included over 3,000 children with the HLA *DR3/DR4-DQ8* genotype and over 1,500 with the *DR4-DQ8/DR4-DQ8* genotype, had sufficient power to adequately test the independent contribution of *BTNL2*. The G allele of the SNP rs3763305 increased the risk for type 1 diabetes with a HR of around 1.7 in these HLA-selected children. Although our analyses also controlled for the HLA *DRB1*04* subtype, we cannot exclude the possibility that the *BTNL2* SNP marks HLA *DR4* extended haplotypes. However, there was an association between the non-susceptible *BTNL2* allele and *DRB1*04* subtypes that are protective or confer relatively low risk. It is, therefore, equally possible that some of the associations between *DRB1*04* subtype and type 1 diabetes risk are due to variation in *BTNL2* rather than or in addition to HLA DR. *BTNL2* is a negative regulator of immunity that is expressed on antigen-presenting cells and affects the generation, proliferation, and function of regulatory T cells (20-22), and, as shown here, activation of naïve $CD4^+$ T cells. It was

demonstrated that *BTNL2* SNPs confer risk for sarcoidosis (23), a T cell-related inflammatory disease, independently of HLA DR (24) and influence antibody responses to dietary antigens (25). A relationship between the minor allele of the *BTNL2* rs3763305 genotype and *BTNL2* transcriptomic expression has been reported (26). Further studies are required to determine whether there are functional differences between *BTNL2* genotypes that may be relevant to type 1 diabetes susceptibility.

The remaining increased risk for islet autoimmunity and type 1 diabetes in FDR children after accounting for genetic load implies that other factors, which are shared or enriched within affected families, contribute to the child's risk. It is known that a family history of type 1 diabetes is associated with changes in parental practices in an effort to reduce the risk in their unaffected children. It is likely that such practices are more frequent in the children of affected families, and it seems possible that some of these practices may be associated with increased risk. It is also possible that family members more often share infections or diet that increase the risk for islet autoimmunity. One enriched factor in the FDR children is maternal type 1 diabetes, which is known to offer relative protection (18). Indeed, unlike children whose father or sibling had type 1 diabetes, there was no excess risk in children whose mother had type 1 diabetes as compared with GP children in the Cox proportional hazards model. The relative protection conferred by maternal as compared to paternal type 1 diabetes was pronounced in the higher genetic susceptibility strata, suggesting that maternal type 1 diabetes harbors a dominantly protective environment in the presence of enriched genetic susceptibility. These data also suggest that the shared environment of siblings and fathers with type 1 diabetes may be a source from which to identify environmental risk factors.

In summary, we have shown that the increased risk of developing islet autoimmunity in FDR children is largely due to an excess load of genetic susceptibility, we identified a potential novel gene that confers risk for islet autoimmunity, and we have shown that accounting for the excess genetic susceptibility leads to convergence in high-risk strata and divergence in lower-risk strata for the risk of developing islet autoantibodies and diabetes between FDR children and GP children. These findings stress that environmental risk factors of disease will likely exert different effects in a gene-dependent manner, and that searching for these factors may require genetic stratification. Of practical relevance, the study showed that it is possible to identify GP children whose risk for islet autoantibodies and type 1 diabetes is as high as that in the highest-risk FDR children.

Acknowledgements

The TEDDY study is funded by U01 DK63829, U01 DK63861, U01 DK63821, U01 DK63865, U01 DK63863, U01 DK63836, U01 DK63790, UC4 DK63829, UC4 DK63861, UC4 DK63821, UC4 DK63865, UC4 DK63863, UC4 DK63836, UC4 DK95300, UC4 DK100238, UC4 DK106955, and Contract No. HHSN267200700014C from the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), National Institute of Allergy and Infectious Diseases (NIAID), National Institute of Child Health and Human Development (NICHD), National Institute of Environmental Health Sciences (NIEHS), Juvenile Diabetes Research Foundation (JDRF), and Centers for Disease Control and Prevention (CDC). This work was supported in part by the NIH/NCATS Clinical and Translational Science Awards to the University of Florida (UL1 TR000064) and the University of Colorado (UL1 TR001082). The funders had no impact on the design, implementation, analysis and interpretation of the data.

See the Supplemental Acknowledgements for members of the TEDDY Study Group.

The datasets generated and analyzed during the current study are available in the National Institute of Diabetes and Digestive and Kidney Diseases Central Repository at https://www.niddkrepository.org/studies/teddy. TEDDY ImmunoChip (SNP) data that support the findings of this study have been deposited in The National Center for Biotechnology Information Database of Genotypes and Phenotypes (dbGaP) with the primary accession code phs001037.v1.p1.

Author contributions

EB and AGZ designed the study. MH, WAH, JPK, JT, JK, CW, AL, MJR, JS, CCR, SO-G, SSR, and AGZ contributed to data collection. MH, AB, KV, JK, EB, CCR, and AGZ

performed the statistical analyses. MH, AB, WAH, JPK, KV, JK, CW, AL, MJR, AKS, JS, BA, SSR JTEB, and AGZ contributed to the interpretation of the data. MH, AB, CW, EB, and AGZ drafted the manuscript. MH, AB, WAH, JPK, KV, JK, CW, JT, AL, MJR, AKS, JS, BA, CCR, SO-G, SSR, EB, and AGZ critically reviewed the manuscript for important intellectual content. AGZ is the guarantor of this work, as such, had full access to all data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Part of this work has been performed as PhD thesis work (MH) at the Technical University of Munich.

Disclosure: The authors have declared that no conflict of interest exists.

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Table 1. Study characteristics by first-degree relative status

Variable	FDR children (n=423)	GP children (n=4,149)
Males	200 (47.3%)	2,082 (50.2%)
HLA genotype		
DR3/4-DQ8	280 (66.2%)	2,755 (66.4%)
DR4-DQ8/DR4-DQ8	143 (33.8%)	1,394 (33.6%)
Country		
US	194 (45.9%)	1,750 (42.2%)
Finland	51 (12.1%)	792 (19.1%)
Germany	92 (21.7%)	209 (5.0%)
Sweden	86 (20.3%)	1,398 (33.7%)
First-degree relative with T1D		
None	0 (0.0%)	4,149 (100.0%)
Mother	146 (34.5%)	0 (0.0%)
Father	180 (42.6%)	0 (0.0%)
Sibling	79 (18.7%)	0 (0.0%)
Multiplex	18 (4.3%)	0 (0.0%)
Outcome events		
One or more islet autoantibodies	85 (20.1%)	415 (10.0%)
Multiple islet autoantibodies	69 (16.3%)	255 (6.1%)
First-appearing IAA	51 (12.1%)	227 (5.5%)
First-appearing GADA	46 (10.9%)	250 (6.0%)
Diabetes	47 (11.1%)	145 (3.5%)
Genetic risk score available	408 (96.5%)	4,006 (96.6%)

Table 2. Allelic enrichment of *DRB1*04* subtypes in FDR children

DR4 subtype	FDR children (alleles, n=535)	GP children (alleles, n=5,415)	*P*-value*
*DRB1*04:01*	329 (60.15%)	2,788 (51.49%)	<0.0001
*DRB1*04:02*	33 (6.03%)	291 (5.37%)	0.42
*DRB1*04:04*	137 (25.05%)	1,928 (35.60%)	<0.0001
*DRB1*04:05*	23 (4.20%)	226 (4.17%)	0.91
*DRB1*04:06*	1 (0.18%)	1 (0.02%)	0.17
*DRB1*04:07*	7 (1.28%)	153 (2.83%)	0.035
*DRB1*04:08*	4 (0.73%)	25 (0.46%)	0.33
*DRB1*04:10*	0 (0.00%)	1 (0.02%)	1
*DRB1*04:11*	1 (0.18%)	1 (0.02%)	0.17
*DRB1*04:13*	0 (0.00%)	1 (0.02%)	1

Each *DRB*04* allele was counted separately (once for children with the *DR3/DR4-DQ8* genotype and twice for children with the *DR4-DQ8/DR4-DQ8* genotype). *DRB*04* subtype information was missing in 77 *DR3/DR4-DQ8* and 35 *DR4-DQ8/DR4-DQ8* children. Children with the non-risk *DRB1*04:03* allele were excluded *a priori* from the TEDDY study unless they had a first-degree relative with type 1 diabetes, and the 12 occurrences of this allele in FDR children were therefore not considered. * *P*-values were calculated using Fisher's test.

Table 3. Cox proportional hazards models for developing islet autoantibodies and diabetes in FDR children compared with GP children (reference)

	One or more islet autoantibodies				Multiple islet autoantibodies				Diabetes			
	Model 1 *		Model 2 *		Model 1 *		Model 2 *		Model 1 *		Model 2 *	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	
FDR child	2.12 (1.65–2.72)	<0.0001	1.82 (1.42–2.35)	<0.0001	2.77 (2.09–3.68)	<0.0001	2.26 (1.70–3.02)	<0.0001	3.69 (2.60–5.23)	<0.0001	2.92 (2.05–4.16)	
*DRB1*04:01/x* †			1.42 (1.10–1.83)	0.0075			1.48 (1.08–2.01)	0.014			1.38 (0.93–2.04)	
Genetic risk score ‡			1.48 (1.34–1.64)	<0.0001			1.66 (1.47–1.88)	<0.0001			1.67 (1.42–1.96)	
BTNL2 rs3763305 GG §			1.05 (0.79–1.40)	0.73			1.39 (0.96–2.00)	0.080			1.80 (1.11–2.93)	
ITGA1 rs7735139 GG §			1.18 (0.86–1.63)	0.30			1.06 (0.70–1.60)	0.77			1.11 (0.67–1.87)	

* Model 1 and 2 are adjusted for sex, country (reference: US) and HLA genotype (reference: *DR4-DQ8/DR4-DQ8*)

† Reference: *DRB1* without 04:01 or 04:01/04:04 and 04:01/04:07; ‡ per unit increase; § reference: GA/AA genotype

Table 4. *BTNL2* SNP genotype frequencies in relation to the development of islet autoantibodies and diabetes among TEDDY children with HLA *DR3/DR4-DQ8* or *DR4-DQ8/DR4-DQ8* and available genotype information

	Islet autoantibody negative	One or more islet autoantibodies	Multiple islet autoantibodies	Diabetes
HLA *DR3/4-DQ8* (n=3,024)				
BTNL2 SNP rs3763305				
GG	1,839 (69.1%)	272 (75.3%)	190 (79.8%)	120 (82.8%)
GA	823 (30.9%)	89 (24.7%)	48 (20.2%)	25 (17.2%)
AA	1 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
P-value *		0.030	0.0007	0.0007
HLA *DR4-DQ8/ DR4-DQ8* (n=1,532)				
BTNL2 SNP rs3763305				
GG	692 (49.7%)	89 (64.0%)	63 (73.3%)	36 (76.6%)
GA	571 (41.0%)	44 (31.7%)	21 (24.4%)	10 (21.3%)
AA	130 (9.3%)	6 (4.3%)	2 (2.3%)	1 (2.1%)
P-value *		0.0033	<0.0001	0.0010

* Children who remained islet autoantibody negative were compared with children who developed one or more islet autoantibodies, multiple islet autoantibodies and diabetes using Fisher's test.

Figure legends

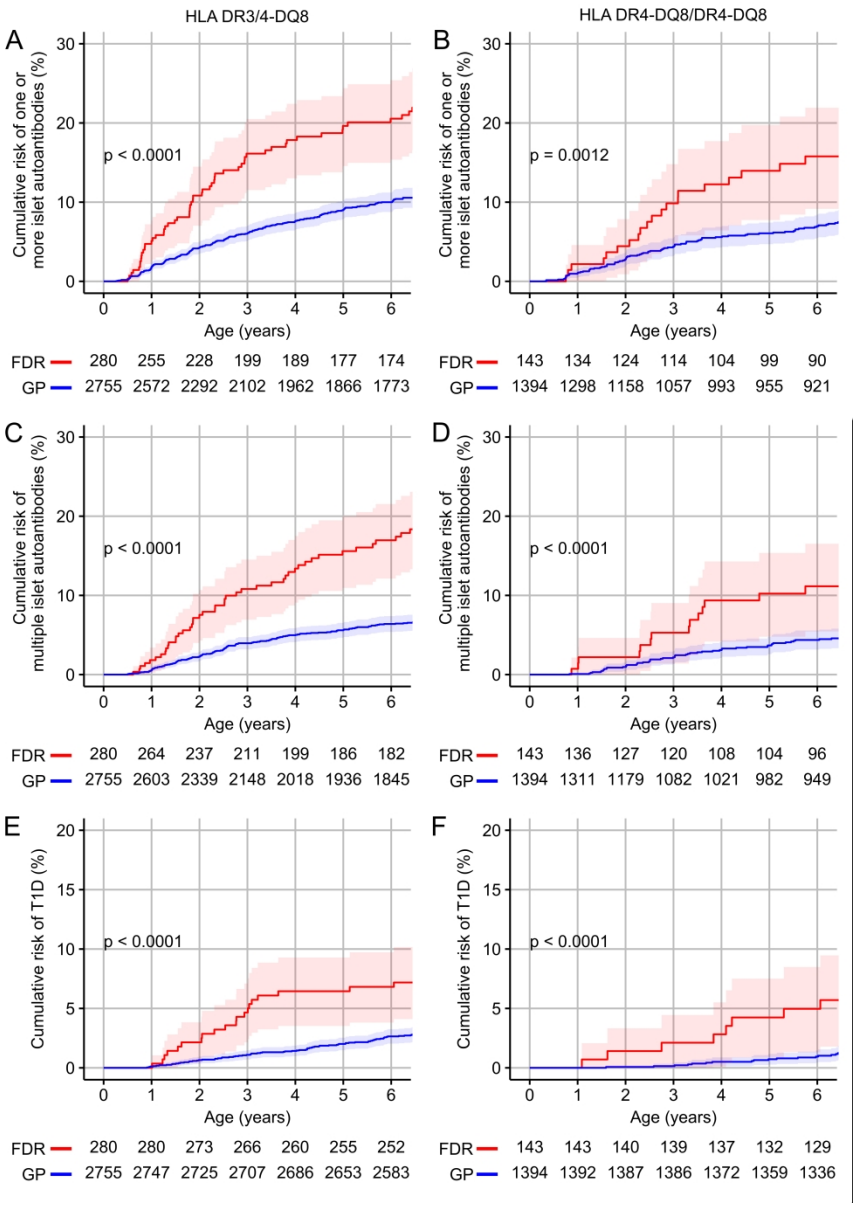
Figure 1. Cumulative risks of islet autoantibodies and diabetes. Kaplan–Meier curves for the risk of one or more islet autoantibodies (A, B), multiple islet autoantibodies (C, D), and diabetes (E, F) in FDR children (red) and in GP children (blue), stratified into children with the HLA *DR3/DR4-DQ8* (A, C, E) or HLA *DR4-DQ8/DR4-DQ8* (B, D, F) genotypes. Shaded areas represent the 95% CI. Numbers represent children at risk. *P*-values were calculated using log-rank tests.

Figure 2. (A) Distribution of non-HLA *DR-DQ* genetic risk scores in all 4,414 *DR3/DR4-DQ8* or *DR4-DQ8/DR4-DQ8* children stratified into FDR children (red) and GP children (blue). (B) Distribution of non-HLA *DR-DQ* genetic risk scores in 317 *DR3/DR4-DQ8* or *DR4-DQ8/DR4-DQ8* children who developed multiple islet autoantibodies (FDR, red; GP, blue). *P*-values were calculated using the two-sided Mann–Whitney U test.

Figure 3. The modification of risk by the *BTNL2* SNP rs3763305 on the development of one or more islet autoantibodies (A), multiple islet autoantibodies (B) and diabetes (C) in children with the *DR3/DRB1*04:04-DQ8* or *DRB1*04:04-DQ8/DRB1*04:04-DQ8* genotypes. Risks are shown for the GG genotype (blue) versus the GA or AA genotypes (red) at rs3763305. *P*-values were calculated using log-rank tests.

Figure 4. Risk of developing islet autoantibodies and diabetes in FDR children (B, D, F) and in GP children (A, B, C) according to genetic susceptibility strata based on HLA

*DRB1*04* subtype and genetic risk score (GRS). Risks are shown for the development of one or more islet autoantibodies (A, B), multiple islet autoantibodies (C, D), and diabetes (E, F). All of the children had the *DR3/DR4-DQ8* or *DR4-DQ8/DR4-DQ8* genotype. Genetic susceptibility strata were defined as 1. high-risk *DRB1*04* subtype (*DR3/DRB1*04:01* or *DRB1*04:01-DQ8/DR4* without 04:04 or 04:07) AND GRS in the upper quartile (grey); 2. high-risk *DRB1*04* subtype AND GRS in the second quartile, OR lower-risk *DRB1*04* subtype AND GRS in the upper quartile (pink); 3. high-risk *DRB1*04* subtype AND GRS in the lower 50th centile OR lower-risk *DRB1*04* subtype AND GRS in the second quartile (light blue); and 4. lower-risk *DRB1*04* subtype AND GRS in the lower 50th centile (green). The strata appear in this order from top to bottom in the risk tables. *P*-values were calculated across all strata using log-rank tests.



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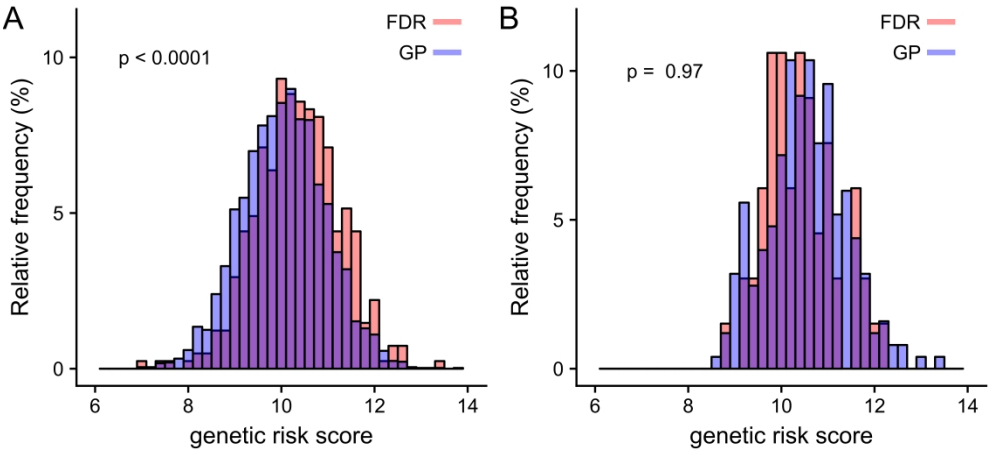

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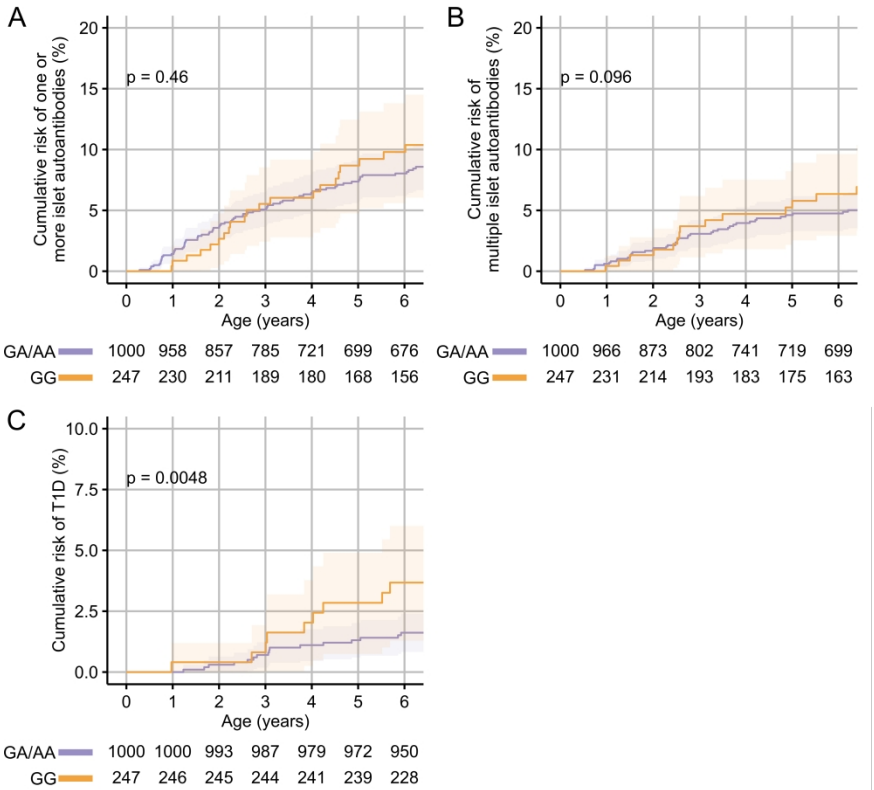

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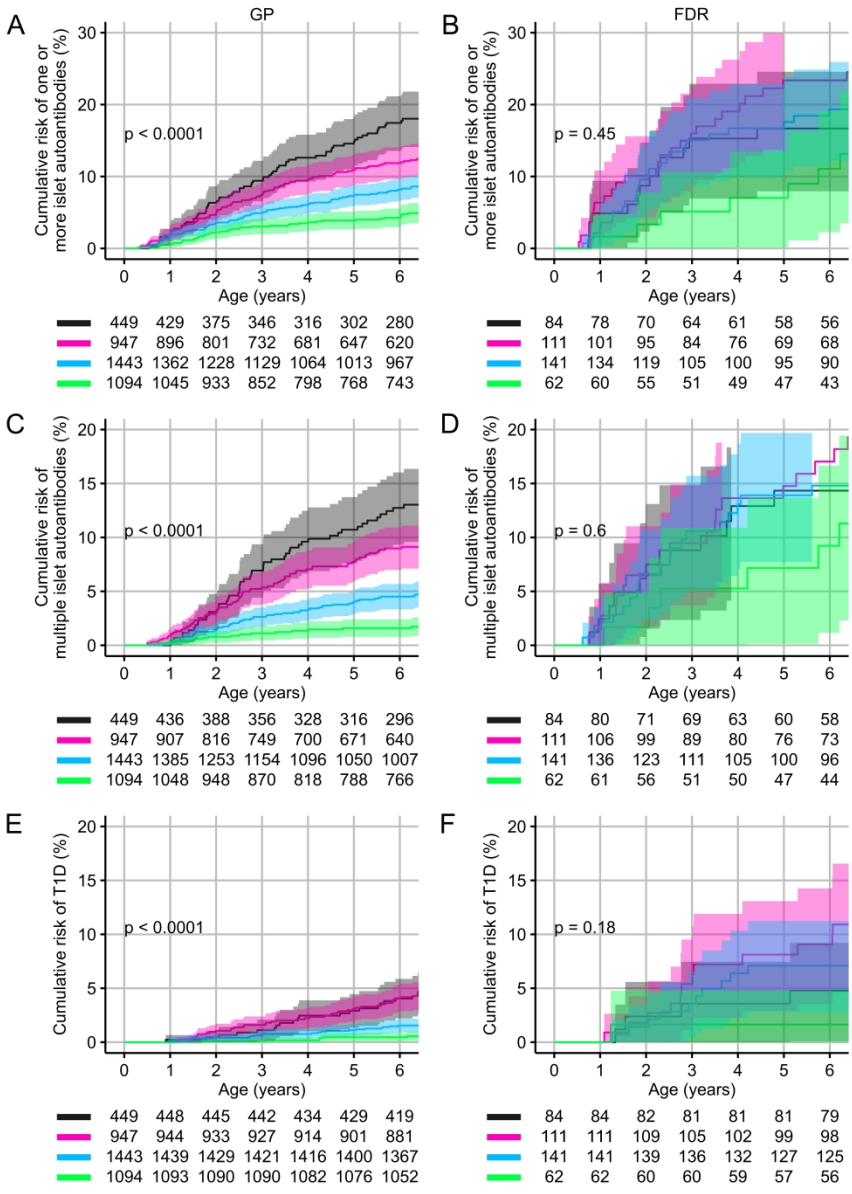

Figure 4: Risk of developing islet autoantibodies and diabetes in FDR children (B, D, F) and in GP children (A, B, C) according to genetic susceptibility strata based on HLA DRB1*04 subtype and genetic risk score (GRS). Risks are shown for the development of one or more islet autoantibodies (A, B), multiple islet autoantibodies (C, D), and diabetes (E, F). All of the children had the DR3/DR4-DQ8 or DR4-DQ8/DR4-DQ8 genotype. Genetic susceptibility strata were defined as 1. high-risk DRB1*04 subtype (DR3/DRB1*04:01 or DRB1*04:01-DQ8/DR4 without 04:04 or 04:07) AND GRS in the upper quartile (grey); 2. high-risk DRB1*04 subtype AND GRS in the second quartile, OR lower-risk DRB1*04 subtype AND GRS in the upper quartile (pink); 3. high-risk DRB1*04 subtype AND GRS in the lower 50th centile OR lower-risk DRB1*04 subtype AND GRS in the second quartile (light blue); and 4. lower-risk DRB1*04 subtype AND GRS in the lower 50th centile (green). The strata appear in this order from top to bottom in the risk tables. P-values were calculated across all strata using log-rank tests.

Genetic contribution to the divergence in type 1 diabetes risk between children from the general population and children from affected families

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Content

Supplemental Figure 7. Risk of developing islet autoantibodies and diabetes in FDR children and in GP children according to genetic susceptibility strata based on *BTNL2* SNP rs3763305 and genetic risk score (GRS).

Supplemental Figure 8. Risk of developing islet autoantibodies and diabetes in children with a mother with type 1 diabetes compared with children with a father or sibling with type 1 diabetes.

Supplemental Tables

Supplemental Table 1. Genotype frequencies for SNPs used in the T1D genetic risk score

Supplemental Table 2. Cox proportional hazards models for the development of islet autoantibodies and diabetes in TEDDY children with the HLA *DR3/4-DQ8* genotype

Supplemental Table 3. Genotype frequencies of *BTNL2* SNP rs3765503 in validation cohort

Supplemental Table 4. Haplotypes of 34 SNPs in *BTNL2*

Supplemental Table 5. Relationship between *BTNL2* SNP rs3765503 genotypes and HLA *DR3* and *DRB1*04* subtype alleles in TEDDY children with the HLA *DR3/DR4-DQ8* genotype

Supplemental Table 6. Relationship between *BTNL2* rs3763305 genotypes with HLA *DRB1*04* subtype alleles in patients from the Bavarian diabetes registry DiMelli

Supplemental Table 7. Cox proportional hazards models for developing islet autoantibodies and diabetes according to *BTNL2* rs3763305 genotype in children with the *DRB1*0404* allele

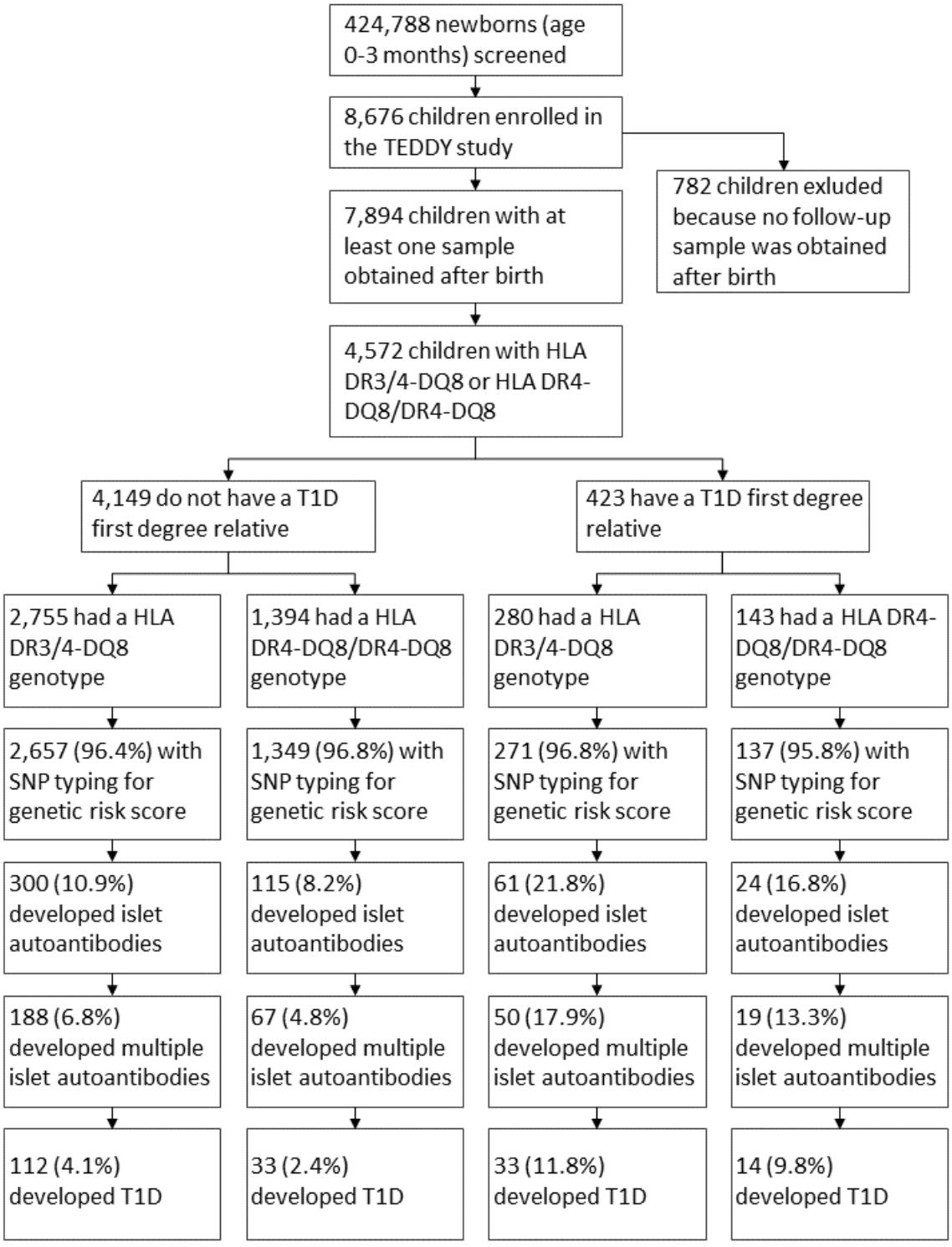
Supplemental Table 8. Cumulative risk of developing islet autoantibodies or diabetes by 6 years old in children stratified by genetic risk score and HLA *DRB1*04* subtype

Supplemental Table 9. Cox proportional hazards models for developing islet autoantibodies and diabetes in FDR children with respect to the affected family member.

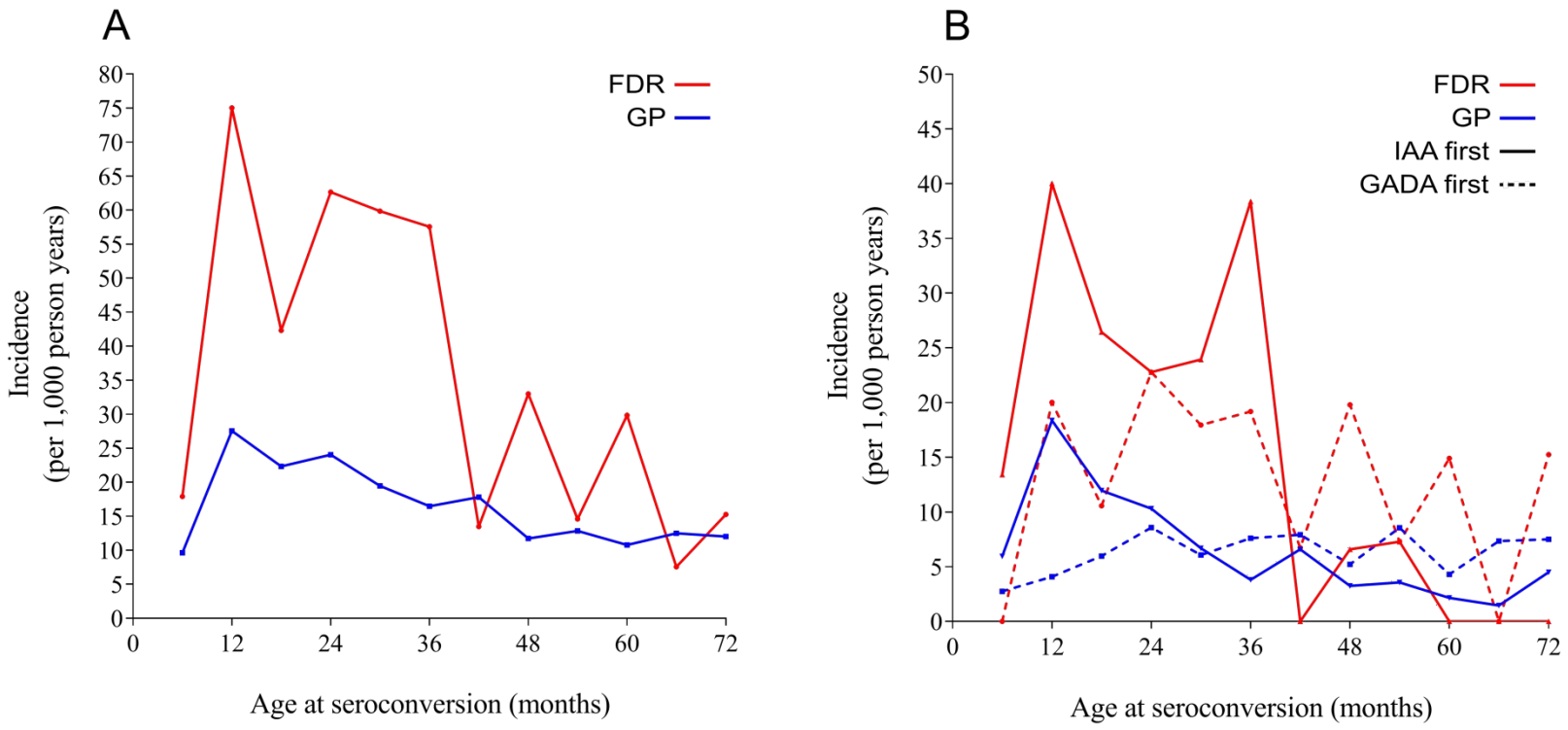
Supplemental Acknowledgements

Supplemental References

Supplemental Figure 1. Flow chart of study population

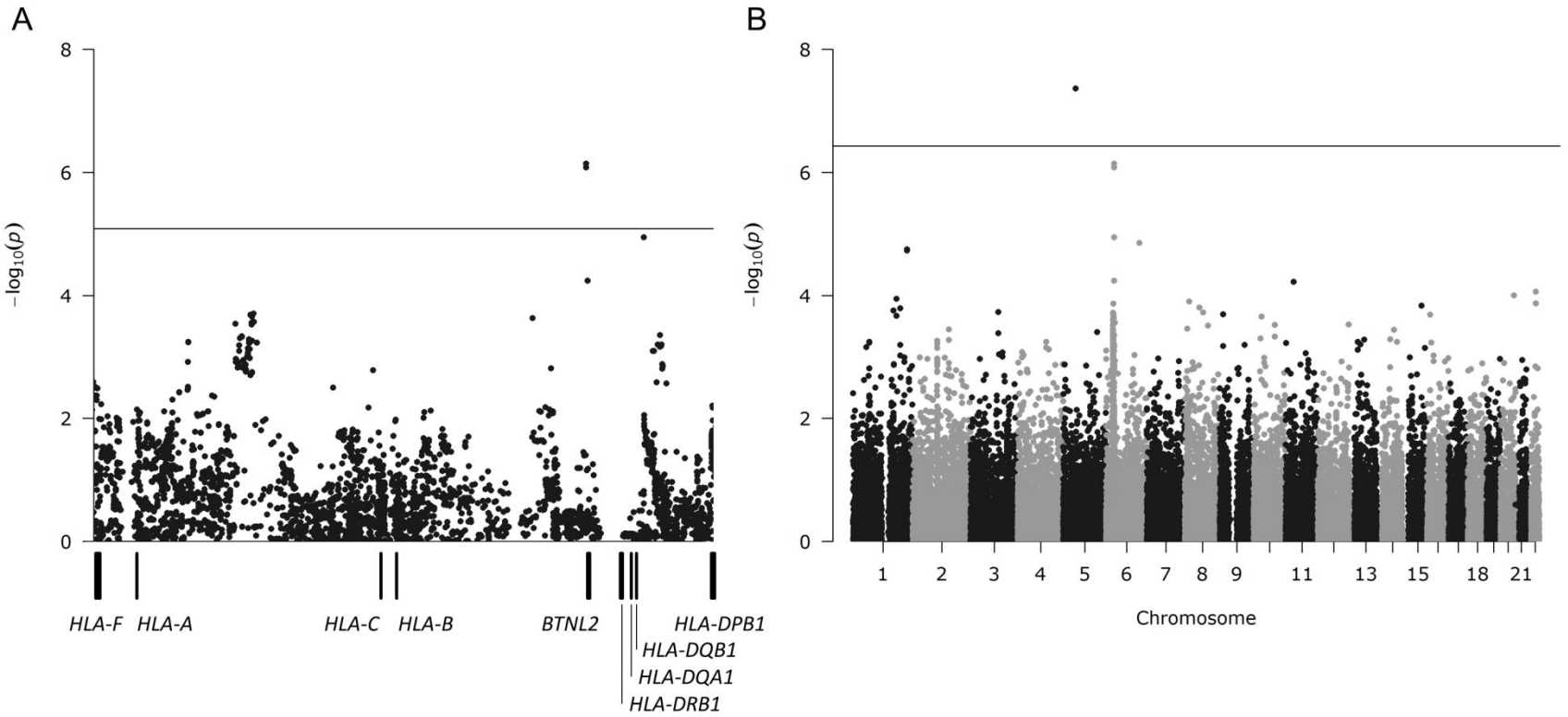


Supplemental Figure 2. (A) Incidence of one or more islet autoantibodies among *DR3/4-DQ8* and *DR4-DQ8/DR4-DQ8* FDR children (red) compared with GP children (blue) by the age of seroconversion. (B) Incidence of first-appearing IAA (solid lines) and first-appearing GADA (broken lines) at seroconversion in *DR3/4-DQ8* and *DR4-DQ8/DR4-DQ8 FDR* children (red) compared with GP children (blue) by the age of seroconversion.

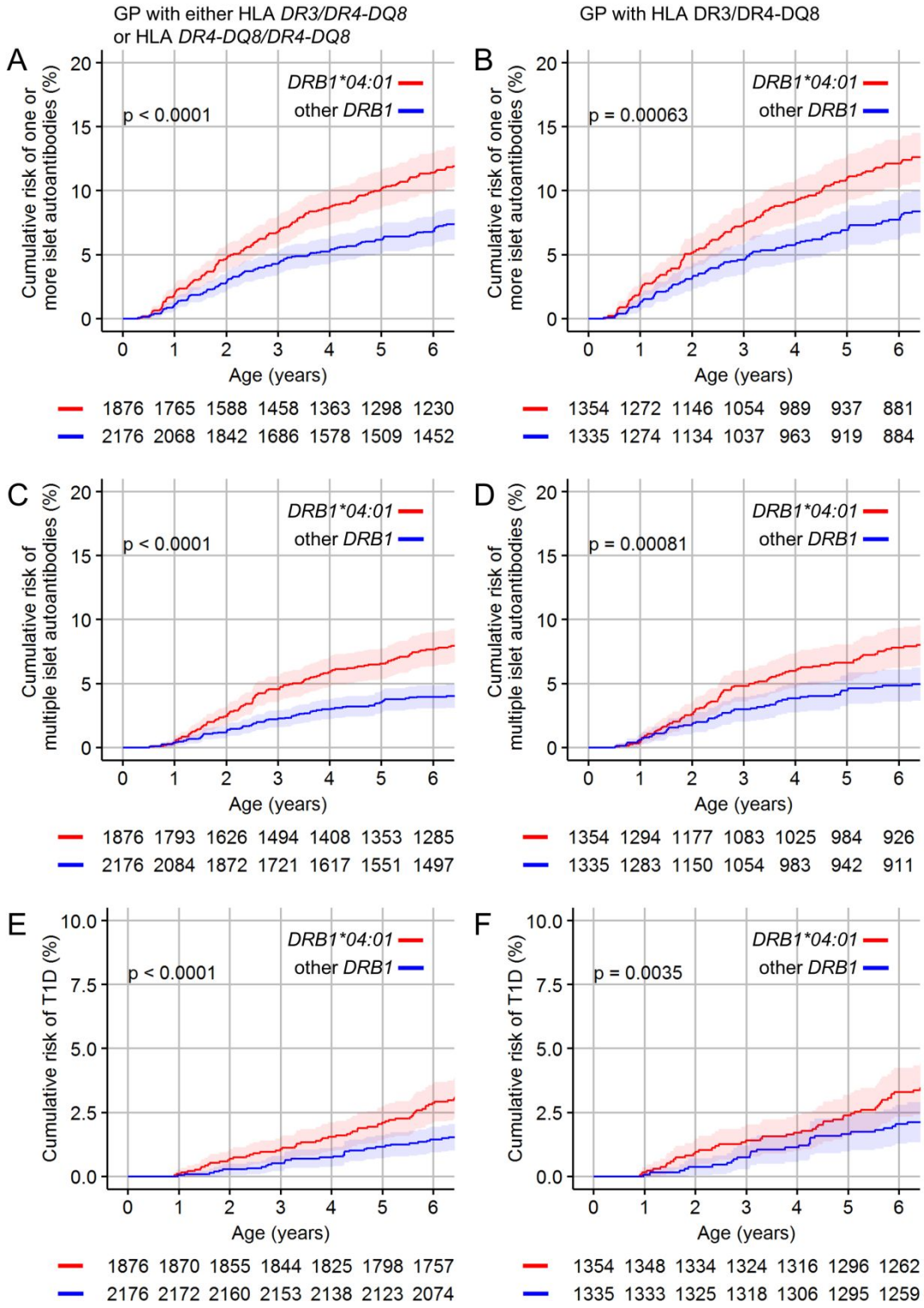


Supplemental Figure 3. Manhattan plot of allele enrichment in FDR children. SNPs were analyzed across the HLA region on chromosome 6 (A) and across all Immunochip data (B).

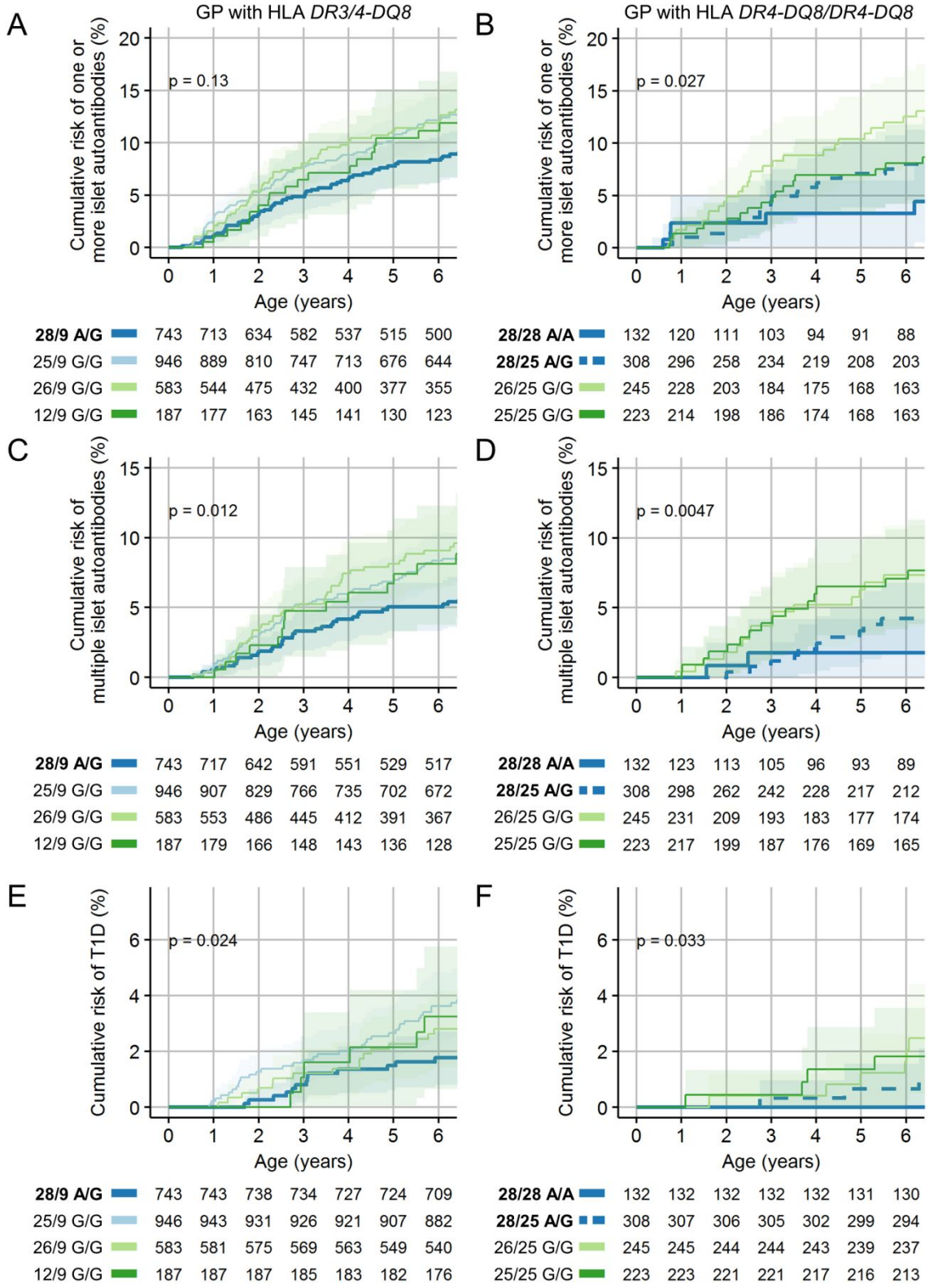
P-values were calculated using χ^2 tests. The thresholds for *P*-values after Bonferroni correction (8.2×10^{-6} and 4.5×10^{-7}) are indicated using sold lines.



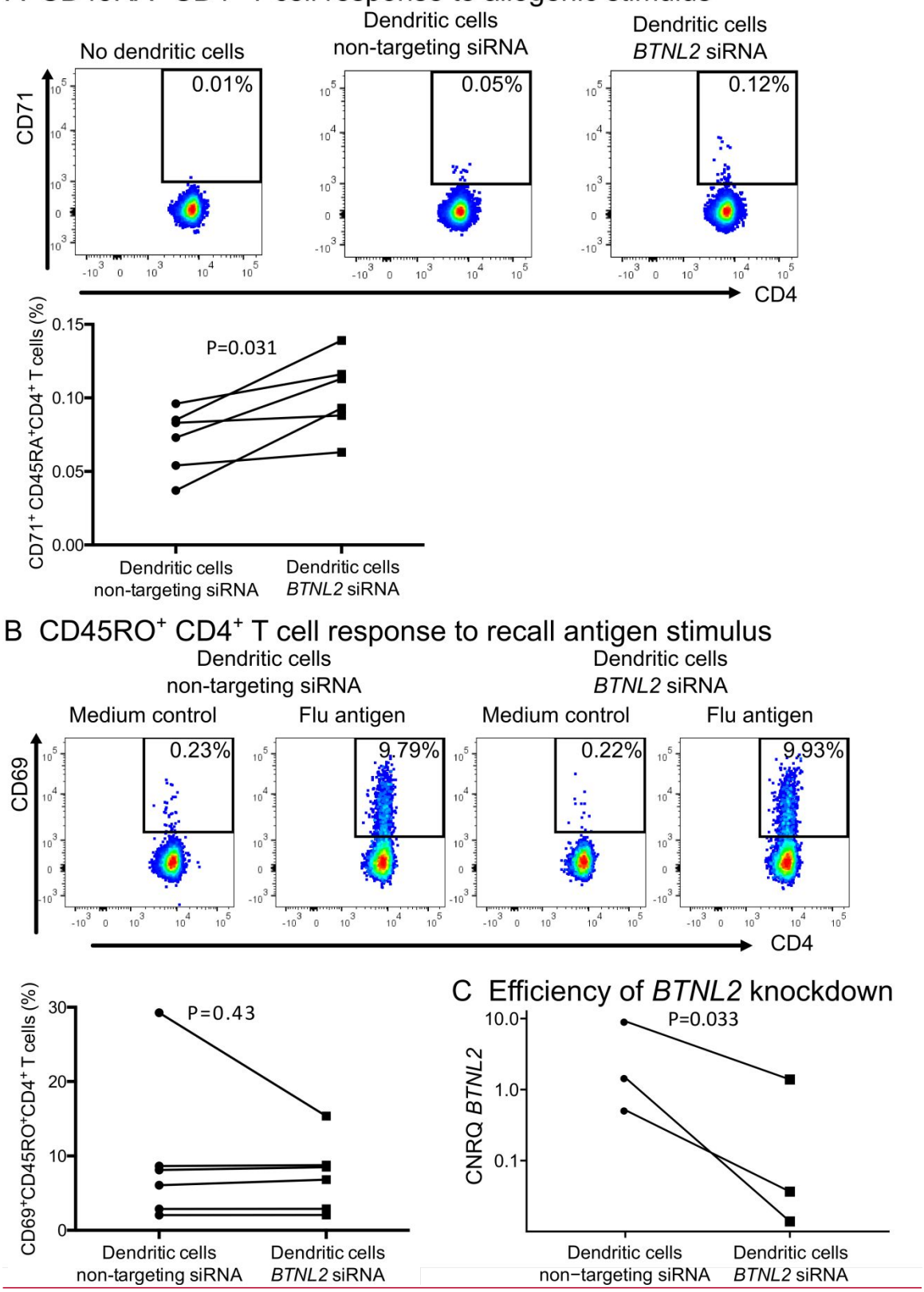
Supplemental Figure 4. Risk of developing one or more islet autoantibodies (A, B), multiple islet autoantibodies (C, D) and diabetes (E, F) in GP children with HLA *DR3/DRB1*04:01-DQ8* or *DRB1*04:01-DQ8/DRB1*04:xx-DQ8*, where **04:xx* was any allele other than *DRB1*04:04* or *DRB1*04:07* (red) vs children without *DRB1*04:01* (blue). The risks are also shown separately for GP children with HLA *DR3/DR4*04:01-DQ8* (B, D, F). *P*-values were calculated using log-rank tests.



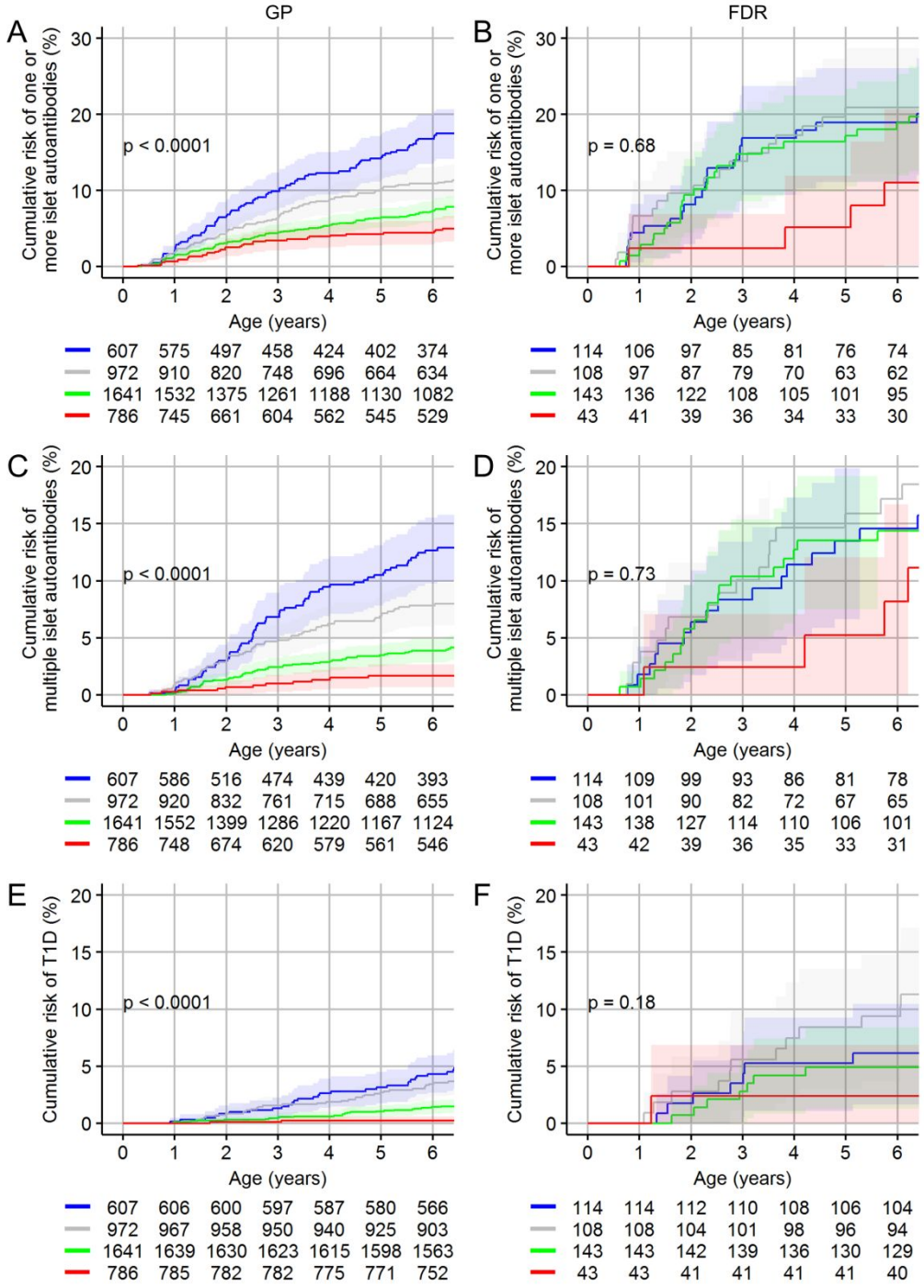
Supplemental Figure 5. Kaplan–Meier curves for the risk of one or more islet autoantibodies (A, B), multiple islet autoantibodies (C, D), and diabetes (E, F) in GP children stratified into children with the *HLA DR3/DR4-DQ8* (A, C, E) or *HLA DR4-DQ8/DR4-DQ8* (B, D, F) genotypes and according to *BTNL2* haplotypes. For both HLA genotypes, the 4 major *BTNL2* genotypes are shown. The genotypes that include haplotype 28, which is the only *BTNL2* haplotype that has the SNP rs3763305 A allele, are indicated as thick blue lines. Shaded areas represent the 95% CI. Numbers represent children at risk. P-values were calculated using log-rank tests.



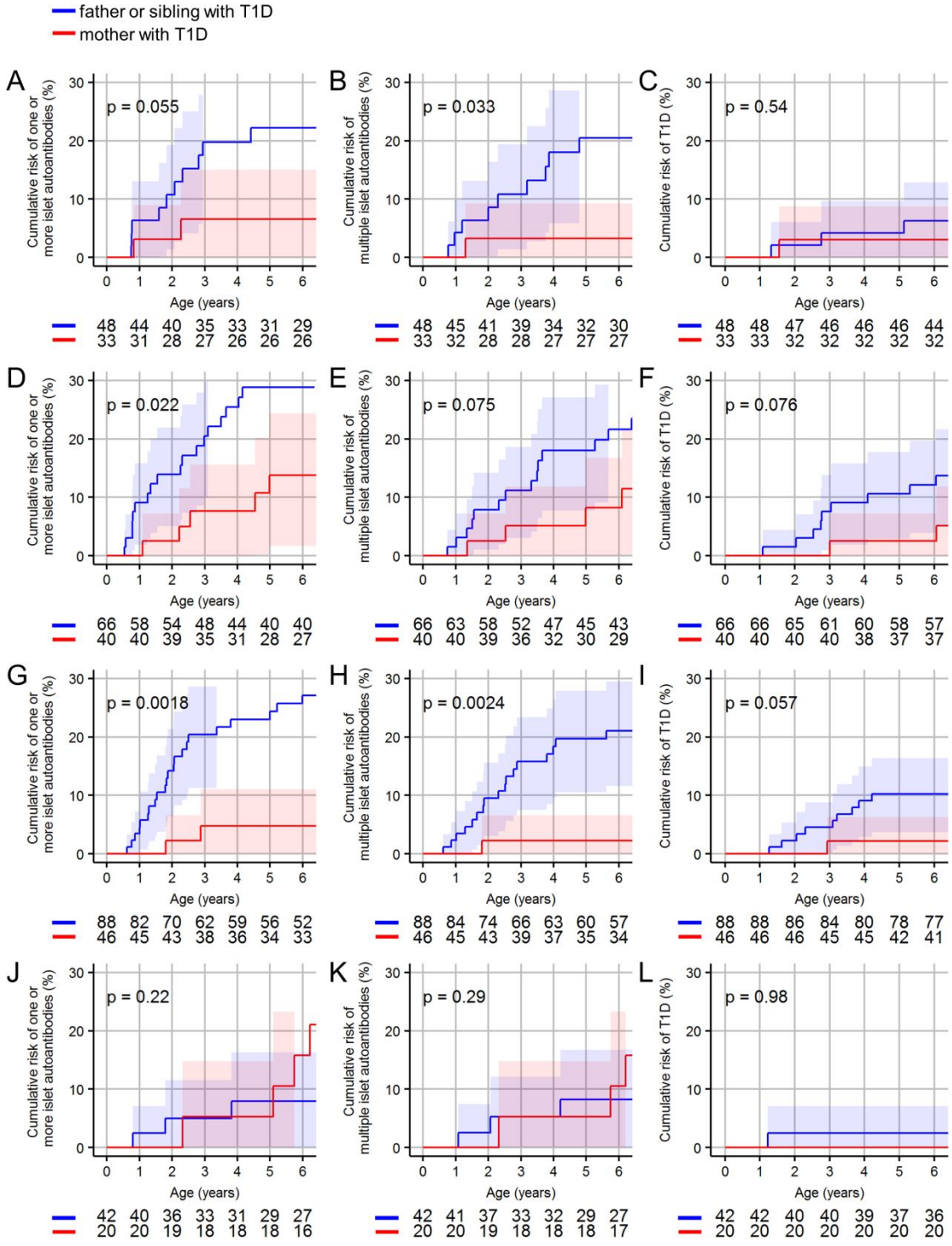
Supplemental Figure 6. Effect of BTNL2 knockdown in monocyte-derived dendritic cells on $CD4^+$ T cell activation. A. Activation of isolated $CD4^+$ $CD25^-$ T cells by allogeneic monocyte-derived dendritic cells transfected with non-targeting siRNA or BTNL2 targeting siRNA in mixed lymphocyte cultures. CD71 expression, previously reported as a marker of allo-reactive T cell activation, was used to measure the activation of the $CD4^+$ T cells. Dendritic cells were transfected as previously described (1). The upper panels are exemplary FACS plots for 42 hour cultures of $CD4^+$ T cells only and in the presence of allo-reactive transfected dendritic cells. The lower graph indicates the mean frequency $CD71^+$ $CD45RA^+$ $CD4^+$ T cells at the end of the 42 hour culture (4 replicates) after subtraction of the mean frequency of $CD71^+$ $CD45RA^+$ $CD4^+$ T cells in quadruplicate cultures without dendritic cells. Each of three dendritic cell samples was tested against two different allogeneic $CD4^+$ T cell preparation yielding 6 data sets. Activation was increased when $CD4^+$ T cells were activated with dendritic cells transfected with *BTNL2*-targeting siRNA as compared to non-targeting siRNA ($p=0.031$, Wilcoxon matched pair sign test). B. Activation of isolated $CD4^+$ $CD25^-$ T cells by autologous monocyte-derived dendritic cells transfected with non-targeting siRNA or *BTNL2*-targeting siRNA in the presence of flu or tetanus toxoid antigen. CD69 expression was used to measure the activation of the $CD4^+$ T cells. The upper panels are exemplary FACS plots for 42 hour cultures of $CD4^+$ T cells plus dendritic cells in the presence and absence of flu antigen. The lower graph indicates the mean frequency $CD69^+$ $CD4^+$ T cells at the end of the 42 hour culture (triplicates) after subtraction of the mean frequency of $CD69^+$ $CD4^+$ T cells in triplicates cultures without antigen. Each of three dendritic cell samples was tested against flu and tetanus toxoid yielding 6 data sets. Activation was not different when $CD4^+$ T cells were activated with dendritic cells transfected with BTNL2-targeting siRNA as compared to non-targeting siRNA ($p=0.43$, Wilcoxon matched pair sign test). C. Efficiency of knockdown with BTNL2 vs non-targeting siRNA in dendritic cells used for A and B. 200ng of RNA from siRNA transfected cells was subjected to cDNA synthesis using a mix of oligo-dT-primer and random primer with the iScript cDNA synthesis Kit (Bio-Rad) followed by pre-amplification with gene specific primers and the SsoAdvanced PreAmp Supermix (Bio-Rad). qbase+-software (Biogazelle) was used for analysis of qPCR experiments. BTNL2 expression was normalized to reference genes TELO2 and TRMT61A and the Calibrated Normalized Relative Quantities (CNRQ) relative to the treatment with non-targeting siRNA is shown ($p=0.033$).


A CD45RA+ CD4+ T cell response to allogenic stimulus
No dendritic cells
Dendritic cells non-targeting siRNA
Dendritic cells BTNL2 siRNA
0.01%
0.05%
0.12%
CD71
CD4
CD71+ CD45RA+CD4+ T cells (%)
P=0.031
Dendritic cells non-targeting siRNA
Dendritic cells BTNL2 siRNA
B CD45RO+ CD4+ T cell response to recall antigen stimulus
Dendritic cells non-targeting siRNA
Dendritic cells BTNL2 siRNA
Medium control
Flu antigen
Medium control
Flu antigen
0.23%
9.79%
0.22%
9.93%
CD69
CD4
CD69+CD45RO+CD4+ T cells (%)
P=0.43
Dendritic cells non-targeting siRNA
Dendritic cells BTNL2 siRNA
C Efficiency of BTNL2 knockdown
P=0.033
CNRQ BTNL2
Dendritic cells non-targeting siRNA
Dendritic cells BTNL2 siRNA

Supplemental Figure 7. Risk of developing islet autoantibodies and diabetes in FDR children (B, D, F) and in GP children (A, B, C) according to genetic susceptibility strata based on *BTNL2* SNP rs3763305 and genetic risk score (GRS). Risks are shown for the development of one or more islet autoantibodies (A, B), multiple islet autoantibodies (C, D), and diabetes (E, F). All of the children had the *DR3/DR4-DQ8* or *DR4-DQ8/DR4-DQ8* genotype. Genetic susceptibility strata were defined as follows: 1. rs3763305 GG AND GRS in the upper quartile (red); 2. rs3763305 GG AND GRS in the second quartile, OR rs3763305 GA or AA AND GRS in the upper quartile (grey); 3. rs3763305 GG AND GRS in the lower 50th centile OR rs3763305 GA or AA AND GRS in the second quartile (green); and 4. rs3763305 GA or AA AND GRS in the lower 50th centile (blue). *P*-values were calculated across all strata using log-rank tests.



Supplemental Figure 8. Risk of developing one or more islet autoantibodies (A, D, G, J), multiple islet autoantibodies (B, E, H, K) and diabetes (C, F, I, L) in children with a mother with T1D (red) compared with children with a father or sibling with T1D (blue). Children have been stratified by genetic risk score and HLA *DRB1*04* subtype into four risk strata from highest genetic susceptibility (A, B, C), to the lowest genetic susceptibility (J, K, L). *P*-values were calculated using log-rank tests.



Supplemental Table 1. Genotype frequencies for SNPs used in the genetic risk score in FDR children and in GP children

		Genotype frequency [%] *						
		FDR children			GP children			
SNP	**Gene**	**PP**	**SP**	**SS**	**PP**	**SP**	**SS**	***P*-value**
rs6897932	*IL7R*	11.8	34.5	53.7	7.7	41.0	51.3	0.0020
rs1004446	*INS*	9.0	44.7	46.3	14.4	45.6	40.0	0.0025
rs3825932	*CTSH*	10.9	40.0	49.2	13.9	44.1	42.1	0.014
rs3024505	*IL10*	2.4	20.9	76.8	2.2	27.0	70.8	0.025
rs3184504	*SH2B3*	27.4	46.8	25.8	30.9	48.8	20.3	0.027
rs2292239	*ERBB3*	42.8	44.0	13.2	46.4	44.1	9.6	0.043
rs1990760	*IFIH1*	13.5	50.1	36.3	18.3	47.4	34.3	0.053
rs10517086	*Unknown*	46.8	46.6	6.6	51.4	40.7	7.9	0.058
rs229541	*IL2B*	29.8	49.4	20.8	34.5	48.3	17.2	0.064
rs7804356	*SCAP2*	3.3	34.0	62.6	5.7	35.2	59.1	0.079
rs2069763	*IL2*	35.5	51.1	13.5	39.4	45.5	15.1	0.088
rs3757247	*BACH2*	28.9	48.6	22.5	33.2	47.7	19.1	0.10
rs4948088	*COBL*	0.2	5.9	93.9	0.3	8.8	90.9	0.12
rs7020673	*GLIS3*	21.7	47.3	31.0	24.4	49.0	26.5	0.12
rs2476601	*PTPN22*	76.1	22.5	1.4	80.3	18.6	1.1	0.12
rs2816316	*RGS1*	5.0	29.6	65.5	3.3	30.4	66.3	0.20
rs9388489	*C6ORF*	26.7	51.8	21.5	29.7	47.6	22.7	0.25
rs425105	*PRKD2*	1.4	28.6	70.0	2.7	27.1	70.2	0.26
rs45450798	*PTPN2*	66.7	29.3	4.0	69.6	27.5	2.9	0.26
rs5753037	*Unknown*	42.8	43.3	13.9	40.6	47.2	12.2	0.27
rs3087243	*CTLA4*	14.7	47.8	37.6	17.5	47.0	35.5	0.31
rs2395029	*HLA_B_5701*	0.0	1.2	98.8	0.0	2.2	97.7	0.32
rs17574546	*Unknown*	61.6	33.4	5.0	64.3	31.8	3.9	0.41
rs2281808	*SIRPG*	9.7	46.0	44.3	11.8	44.5	43.7	0.43
rs1465788	*ZFP36L1*	7.1	44.4	48.5	7.9	41.2	50.9	0.43
rs3788013	*UBASH3A*	35.9	50.1	13.9	35.0	48.6	16.4	0.44
rs4788084	*IL27*	17.5	47.8	34.8	19.4	48.3	32.3	0.50
rs763361	*CD226*	26.0	48.7	25.3	27.1	49.8	23.0	0.57
rs6920220	*TNFAIP3*	64.5	30.5	5.0	63.6	32.0	4.4	0.73
rs1738074	*TAGAP*	19.9	46.3	33.8	18.6	48.0	33.4	0.76
rs12708716	*CLEC16A*	12.6	42.2	45.3	12.2	44.0	43.8	0.76
rs7221109	*unknown*	13.9	44.2	41.8	13.6	45.9	40.4	0.79
rs2664170	*GAB3*	56.5	22.7	20.8	57.6	21.4	20.9	0.83
rs4763879	*CD69*	37.7	49.1	13.3	38.9	47.7	13.4	0.86
rs947474	*PRKCQ*	3.5	30.5	66.0	3.2	30.1	66.8	0.89
rs5979785	*TLR8*	17.5	18.4	64.1	18.2	18.5	63.3	0.92

rs7202877	*unknown*	79.7	19.1	1.2	79.1	19.5	1.3	0.95
rs1264813	*HLA_A_24*	82.7	16.6	0.7	82.3	17.0	0.7	0.98
rs10509540	*C10orf59*	7.1	38.3	54.6	7.3	38.3	54.4	0.98
rs12722495	*IL2R*	0.7	16.5	82.7	0.7	16.6	82.7	1.00

* P refers to the protective allele and S refers to the susceptible allele

Supplemental Table 2. Cox proportional hazards models for the development of islet autoantibodies and diabetes in FDR children compared with GP children in TEDDY children with the HLA *DR3/4-DQ8* genotype

Variable	One or more islet autoantibodies				Multiple islet autoantibodies				Diabetes			
	Model 1 *		Model 2 *		Model 1 *		Model 2 *		Model 1 *		Model 2 *	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
First-degree relative	2.24 (1.67–3.01)	<0.0001	1.95 (1.45–2.63)	<0.0001	2.92 (2.09–4.07)	<0.0001	2.47 (1.76–3.46)	<0.0001	3.48 (2.30–5.28)	<0.0001	2.86 (1.88–4.36)	<0.0001
*DRB1*0401/x* †			1.26 (0.95–1.68)	0.11			1.24 (0.88–1.74)	0.22			1.17 (0.76–1.79)	0.47
Genetic risk score ‡			1.50 (1.33–1.68)	<0.0001			1.63 (1.41–1.88)	<0.0001			1.56 (1.29–1.87)	<0.0001
BTNL2 rs3763305 GG §			1.09 (0.79–1.51)	0.60			1.43 (0.95–2.17)	0.090			1.92 (1.11–3.35)	0.021
ITGA1 rs7735139 GG §			1.26 (0.88–1.82)	0.20			1.14 (0.73–1.80)	0.56			1.25 (0.72–2.18)	0.43

* Model 1 and 2 are adjusted for sex, country (reference: US) and HLA genotype; † reference: *DRB1* without 0401 or 0401/0404 and 0401/0407; ‡ per unit increase; § reference: GA/AA genotype

Supplemental Table 3. Genotype frequencies of *BTNL2* SNP rs3765503 in validation cohort

	BTNL2 rs3765503 genotype			
	GG	AG	AA	*P*-value*
Control	487 (71%)	200 (29%)	0 (0%)	<0.0001
Diabetes	3496 (81%)	842 (19%)	0 (0%)	

* *P*-value was calculated using Fisher's exact test.

The validation cohort consists of 5,025 Caucasian subjects with European decent and HLA *DR3/4-DQ8* genotype according to the algorithm defined by Barker et al. (2), which is based on the tag SNPs rs7454108 and rs2040410. Samples were genotyped on the Illumina Immunochip array and imputed to the TOPMed Reference Panel. rs3763305 was directly genotyped on the Immunochip. rs2040410, and rs7454108 were imputed with high confidence (R2>0.99). Principal components were generated by calculating PC axes in unrelated controls using a set of 83,458 LD-pruned variants and projecting the remaining samples onto this PC space. A set of 33,249 European ancestry unrelated case-control subjects were identified for analysis by comparing PCs to 1000 Genomes Phase 3 subjects (3). We ensured samples were unrelated (less than second degree relationship) using KING version 2.13 (http://people.virginia.edu/~wc9c/KING/). 5,025 of these samples have the HLA *DR3/4-DQ8* genotype.

Supplemental Table 4. Haplotypes of 34 SNPs in *BTNL2* and their frequencies in HLA *DR3/4-DQ8* and *DR4-DQ8/DR4-DQ8*. SNPs in bold are those that were identified as enriched within FDR children. Haplotype ID 'X' indicates that they are not among the 30 most frequent haplotypes.

frequency [%] in																																			
DR3/4-DQ8	*DR4-DQ8/ DR4-DQ8*	Haplotype ID	rs3817969	rs3129953	rs28362678	rs2076530	rs9268480	rs2076529	rs9268481	rs3129954	rs3129955	rs4248166	rs2294884	rs2294883	rs2294882	rs2294881	rs2294880	rs9268482	rs2294878	rs3817966	**rs3817964**	rs3817963	rs3817962	**rs3763305**	rs2076525	rs2076523	rs2076522	rs3793127	rs10947260	rs10947261	rs10947262	rs3806155	rs3806156	rs3806157	rs2395158	rs3763307
42.82	0.26	9	G	A	G	A	G	A	A	G	G	A	A	T	A	A	A	A	C	A	A	A	C	G	A	A	C	G	A	C	G	T	C	A	G	T
18.12	18.70	25	G	G	G	G	A	G	G	G	G	A	A	T	A	A	G	T	A	G	A	G	A	G	G	G	G	A	A	C	G	A	A	C	A	A
15.14	14.62	28	G	G	G	G	A	G	G	G	G	A	A	T	A	A	G	T	A	G	**T**	G	A	**A**	G	G	G	A	A	C	G	T	A	C	A	A
12.48	12.29	26	G	G	G	G	A	G	G	G	G	A	A	T	A	A	G	T	A	G	A	G	A	G	G	G	G	A	A	C	G	T	A	C	A	A
9.86	3.56	12	G	G	G	A	G	A	A	A	A	A	A	T	A	A	A	A	C	A	A	A	C	G	A	A	C	G	A	C	G	T	C	A	A	T
0.60	0.09	27	G	G	G	G	A	G	G	G	G	A	A	T	A	A	G	T	A	G	A	G	A	G	G	G	G	G	A	C	G	T	A	C	A	A
0.31	0.27	16	G	G	G	A	G	A	A	G	G	A	A	T	A	A	A	A	C	A	A	A	C	G	A	A	C	G	A	C	G	T	C	A	A	T
0.26	0.07	5	A	G	A	G	G	G	A	G	G	G	C	A	G	G	A	A	A	A	A	A	C	G	A	G	C	G	G	A	A	T	A	C	A	T
0.15	0.10	21	G	G	G	A	G	A	A	G	G	G	C	A	G	G	A	A	A	A	A	A	C	G	A	G	C	G	G	A	A	T	A	C	A	T
0.09	0.03	4	A	G	A	G	G	G	A	G	G	G	C	A	G	G	A	A	A	A	A	A	C	G	A	A	C	G	A	C	G	T	C	A	A	T
0.05	0.03	23	G	G	G	G	A	G	G	G	G	A	A	T	A	A	A	T	A	G	A	G	A	G	G	G	G	A	A	C	G	T	A	C	A	A
0.00	0.07	3	A	G	A	G	G	G	A	G	G	A	A	A	G	G	G	A	C	G	A	G	C	G	A	G	C	G	A	A	A	T	A	A	A	T
0.03	0.00	8	G	A	G	A	G	A	A	G	G	A	A	T	A	A	A	A	C	A	A	A	C	G	A	A	C	G	A	C	G	T	C	A	A	T
0.03	0.00	13	G	G	G	A	G	A	A	A	A	A	A	T	G	G	A	A	A	A	A	A	C	G	A	G	C	G	G	A	A	T	A	C	A	T
0.02	0.00	17	G	G	G	A	G	A	A	G	G	A	A	T	A	A	A	A	C	A	A	A	C	G	A	A	C	G	A	C	G	T	C	A	G	T
0.00	0.00	1	A	A	A	G	G	G	A	G	G	G	C	A	G	G	A	A	A	A	A	A	C	G	A	G	C	G	G	A	A	T	A	C	A	T
0.02	0.00	6	A	G	G	G	G	G	A	G	G	G	A	A	G	G	G	A	C	G	A	A	A	G	A	G	C	G	G	C	A	T	A	A	A	T
0.00	0.02	10	G	A	G	A	G	A	A	G	G	A	A	T	G	G	A	A	A	A	**T**	A	C	**A**	A	G	C	G	G	A	A	T	A	C	A	T
0.02	0.00	11	G	A	G	G	A	G	G	G	G	A	A	T	A	A	G	T	A	G	**T**	G	A	**A**	G	G	G	A	A	C	G	T	A	C	A	A
0.00	0.00	14	G	G	G	A	G	A	A	A	G	A	A	T	A	A	A	A	C	A	A	A	C	G	A	A	C	G	A	C	G	T	C	A	G	T
0.00	0.00	15	G	G	G	A	G	A	A	G	A	A	A	T	A	A	A	A	C	A	A	A	C	G	A	A	C	G	A	C	G	T	C	A	A	T
0.00	0.02	24	G	G	G	G	A	G	G	G	G	A	A	T	A	A	G	T	A	G	A	G	A	G	G	G	G	A	A	C	A	T	A	C	A	A
0.00	0.00	x	G	A	G	A	G	A	A	G	G	A	A	T	G	G	A	A	A	A	A	A	C	G	A	G	C	G	G	A	A	T	A	C	A	T
0.00	0.00	x	G	G	G	A	G	A	A	G	G	G	C	A	G	G	A	A	A	A	**T**	A	C	**A**	A	G	C	A	G	A	A	T	A	C	A	T

Supplemental Table 5. Relationship between *BTNL2* SNP rs3765503 genotypes and HLA *DR3* and *DRB1*04* subtypes in children with the HLA *DR3/DR4-DQ8* genotype

HLA *DR* genotype	*BTNL2* rs3763305 genotype			Allele frequency (%)	
	AA	GA	GG	A	G
DR3/DR3	2 (0.1%)	9 (0.6%)	1608 (99.3%)	0.4	99.6
*DR3/DRB1*04:01*	0 (0.0%)	15 (1.0%)	1496 (99.0%)	0.5	99.5
*DR3/DRB1*04:02*	0 (0.0%)	2 (1.2%)	165 (98.8%)	0.6	99.4
*DR3/DRB1*04:04*	1 (0.1%)	837 (78.1%)	233 (21.8%)	39.2	60.8
*DR3/DRB1*04:05*	0 (0.0%)	0 (0.0%)	134 (100.0%)	0.0	100.0
*DR3/DRB1*04:06*	0 (0.0%)	1 (100.0%)	0 (0.0%)	50.0	50.0
*DR3/DRB1*04:07*	0 (0.0%)	33 (68.8%)	15 (31.2%)	34.4	65.6
*DR3/DRB1*04:08*	0 (0.0%)	1 (4.8%)	20 (95.2%)	2.4	97.6
*DR3/DRB1*04:10*	0 (0.0%)	0 (0.0%)	1 (100.0%)	0.0	100.0

Supplemental Table 6. Association of the *BTNL2* rs3763305 genotype with HLA *DRB1*04* subtype alleles in children with the HLA *DR3/DR4-DQ8* genotype from the Bavarian diabetes registry DiMelli

	BTNL2 rs3763305 genotype		
*DRB1*04* subtype	AA	AG	GG
*DR3/DRB1*04:01*	0 (0.0%)	0 (0.0%)	89 (100.0%)
*DR3/DRB1*04:02*	0 (0.0%)	0 (0.0%)	21 (100.0%)
*DR3/DRB1*04:04*	0 (0.0%)	18 (75.0%)	6 (25.0%)
*DR3/DRB1*04:05*	0 (0.0%)	0 (0.0%)	7 (100.0%)

Supplemental Table 7. Cox proportional hazards models for developing one or more islet autoantibodies, multiple islet autoantibodies and diabetes according to *BTNL2* rs3763305 in children with the HLA *DR3/DRB1*0404-DQ8* or HLA *DRB1*0404-DQ8/DRB1*0404-DQ8* genotypes adjusted for the HLA *DR3/DR4-DQ8* genotype

	One or more islet autoantibodies		Multiple islet autoantibodies		Diabetes	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
HLA *DR3/DR4-DQ8* *	1.48 (0.81–2.70)	0.20	1.64 (0.70–3.82)	0.25	1.26 (0.44–3.59)	0.66
BTNL2 rs3763305 GG †	1.13 (0.74–1.74)	0.57	1.49 (0.88–2.54)	0.14	2.38 (1.25–4.55)	0.0086

* Reference: *DR4-DQ8/DR4-DQ8*; † reference: GA/AA genotype

Supplemental Table 8. Cumulative risk of developing islet autoantibodies or diabetes by 6 years old in children stratified by genetic risk score and HLA *DRB1*04* subtype

Risk strata	One or more islet autoantibodies		Multiple islet autoantibodies		Diabetes	
	GP children	FDR children	GP children	FDR children	GP children	FDR children
1: High-risk *DR4* * AND 1st quartile GRS	17.4% (13.6–21.2%)	16.7% (7.9–24.6%)	12.7% (9.3–16.0%)	14.3% (6.1–21.9%)	4.1% (2.2–5.9%)	4.8% (0.1–9.2%)
2: High-risk *DR4* AND 2nd quartile GRS OR low-risk *DR4* * AND 1st quartile GRS	11.9% (9.6–14.1%)	23.4% (14.7–31.2%)	9.0% (7.0–10.9%)	17.0% (9.3–24.1%)	4.1% (2.8–5.3%)	9.1% (3.5–14.3%)
3: High-risk *DR4* AND GRS <50th centile OR low-risk *DR4* AND 2nd quartile GRS	8.2% (6.7–9.7%)	19.3% (12.2–25.9%)	4.5% (3.3–5.7%)	14.8% (8.4–20.7%)	1.5% (0.9–2.2%)	7.1% (2.8–11.2%)
4: Low-risk *DR4* AND GRS <50th centile	4.3% (2.9–5.5%)	11.1% (2.3–19.1%)	1.6% (0.8–2.4%)	9.2% (1.2–16.6%)	0.5% (0.1–0.9%)	1.6% (0.0–4.8%)
P-value †	<0.0001	0.45	<0.0001	0.60	<0.0001	0.18

* High-risk *DR4* was defined as *DR3/DRB1*0401* or *DRB1*0401-DQ8/DR4* without 0404 or 0407, and low risk were all other genotypes; † *P*-values were calculated as log-rank tests per column over the four strata. GRS, genetic risk score

Supplemental Table 9. Hazard ratios (HRs) and 95% CIs for developing one or more islet autoantibodies, multiple islet autoantibodies and diabetes in FDR children (FDR mother vs FDR father vs FDR sibling vs FDR multiplex), adjusted for sex, genetic factors (reference: *DR4-DQ8/DR4-DQ8*), and country (reference US)

	One or more islet autoantibodies		Multiple islet autoantibodies		Diabetes	
	HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value
FDR multiplex	1.94 (0.62–6.12)	0.26	3.37 (1.06–10.66)	0.039	5.90 (1.84–18.94)	0.0029
FDR mother	0.85 (0.50–1.44)	0.54	0.97 (0.52–1.79)	0.91	1.39 (0.67–2.87)	0.38
FDR father	2.37 (1.71–3.28)	<0.0001	2.89 (2.00–4.17)	<0.0001	3.06 (1.89–4.94)	<0.0001
FDR sibling	2.68 (1.70–4.22)	<0.0001	3.41 (2.07–5.62)	<0.0001	5.15 (2.94–9.03)	<0.0001
*DRB1*04:01/x* *	1.44 (1.11–1.86)	0.0057	1.49 (1.09–2.04)	0.012	1.40 (0.94–2.07)	0.096
Genetic risk score †	1.49 (1.35–1.65)	<0.0001	1.68 (1.48–1.90)	<0.0001	1.68 (1.43–1.98)	<0.0001
BTNL2 rs3763305 GG ‡	1.02 (0.77–1.36)	0.88	1.34 (0.93–1.94)	0.12	1.74 (1.07–2.84)	0.026
ITGA1 rs7735139 "GG" ‡	1.25 (0.91–1.73)	0.17	1.14 (0.76–1.72)	0.52	1.21 (0.72–2.03)	0.47

* Reference: *DRB1* without 0401 or 0401/0404 and 0401/0407; † per unit increase; ‡ reference: GA/AA genotype

Supplemental Acknowledgements

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