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Supplementary materials and methods

Members of "the DRI_Biorepository Group"

Members of "the Type 1 Diabetes TrialNet Study Group"

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Fig. S2 Neutrophils infiltrate the pancreas of T1D donors from the Exeter cohort.

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Fig. S4 Peripheral neutrophil transcriptomic signature from autoAb-negative, autoAb-positive and new-onset T1D patients form a single large cluster distinct from that of unrelated non-diabetic controls.

Fig. S5 Interferon gene expression signature found in peripheral neutrophils of at-risk subjects is dysregulated in T1D and at-risk subjects and overlaps with interferon gene signatures previously identified in T1D.

Fig. S6 HLA distribution in subjects of whom neutrophil transcriptomic signature was generated.

SUPPLEMENTARY MATERIALS AND METHODS

Risk of symptomatic T1D at each disease stage. For children screened for genetic risk at birth who seroconvert (i.e., develop 2 or more T1D-autoAb), the 5-year and 10-year risks of symptomatic disease are approximately 44% and 70%, respectively, and the lifetime risk approaches 100%. The risk at this stage is quite similar in genetically at-risk children and in relatives of individuals with type 1 diabetes. For subjects autoAb positive and with impaired glucose tolerance, the 5-year risk of symptomatic disease is approximately 75%, and the lifetime risk approaches 100%. The impact of HLA and non-HLA genetic risk is observed in relatives of individuals with type 1 diabetes, who have a 10-fold to more than 100-fold greater risk than the general population. There is therefore a pre-symptomatic stage defined as the presence of non-HLA single nucleotide polymorphisms and risk allele scores that have been used to stratify risk for both developing islet autoantibodies and progressing from islet autoimmunity to symptomatic T1D (1).

In 2015, the Juvenile Diabetes Research Foundation (JDRF), the Endocrine Society and the American Diabetes Association (ADA) endorsed the adoption of a new staging classification system in T1D (2), which was not however used in our study.

TrialNet studies and data collection. Standard 2-hours oral glucose test tolerance (OGTT) and complete blood counts (CBC) measurements were collected from subjects enrolled in the following TrialNet intervention studies before any treatment administration.

TN10: This 2 arm, multi-center, 1:1 randomized, placebo-controlled phase 2 clinical trial was designed to test the drug teplizumab (Anti-CD3) to determine whether it can delay or prevent stage 2 T1D from progressing to clinical diagnosis (stage 3) (ClinicalTrials.gov identifier #NCT01030861). This study is based on previous clinical trials in which

teplizumab sustained insulin production in subjects with a new T1D clinical diagnosis (stage 3) (3). Data from 71 subjects (out of total 76 enrolled) were included in the current work.

TN18: This 2 arm, multi-center, 1:1 randomized, placebo-controlled phase 2 clinical trial was designed to test the drug abatacept (CTLA-4Ig) to determine whether it can delay or prevent early T1D (stage 1) from progressing to stage 2 and ultimately prevent clinical diagnosis (stage 3) (ClinicalTrials.gov identifier #NCT01773707). This study is based on an earlier clinical trial in which abatacept improved insulin production and delayed insulin loss in subjects with a new T1D clinical diagnosis (stage 3) (4). Data from 139 subjects (out of total 151 enrolled) were included in the current work.

TN20: This 2 arm, multi-center, randomized, open-labeled clinical trial was designed to assess the effects of varying doses and schedules of oral insulin on immunological and metabolic markers in relatives at risk for T1D (ClinicalTrials.gov identifier #NCT02580877). This study is based on an earlier trial in which administration of oral insulin in a subgroup of subjects at risk of developing T1D lead to delayed T1D development (5). Data from 79 subjects (out of total 92 enrolled) were included in the current work.

OGTT measures were performed centrally in a reference lab while CBC were taken locally in each recruiting TrialNet clinical center.

Inclusion criteria for the abovementioned intervention clinical trials are listed in **Table S1**. Characteristics of the subjects enrolled in the studies and of whom OGTT and CBC data were recovered are listed in **Table S2**.

Subjects were enrolled in the Type 1 Diabetes TrialNet Pathway to Prevention Trial (TN01) at the TrialNet Clinical Center of the San Raffaele Hospital. The study was approved by the San Raffaele Hospital Ethics Committee (protocol: NHPROT32803-TN01). The overall objective of this study is to perform baseline and repeated assessments over time of

the immunologic and metabolic status of individuals at risk for T1D (ClinicalTrials.gov identifier #NCT00097292). Briefly, first (age 1 – 45 years) and second degree relatives (age 1 – 20 years) of patients with T1D can be enrolled in TN01, that is divided into screening and monitoring stages. The screening stage involves measurement of GAD65A, IA-2A, and mIAA, (with a positive result for any of these leading to measurement of ICA and ZnT8A) to determine whether the participant is eligible for the monitoring stage. Participants will be eligible for monitoring if they have at least two positive autoantibodies on a screening sample or at least one positive autoantibody on two separate screening samples. The baseline monitoring visit for those with a single confirmed antibody will include an OGTT, HbA1c level, and testing for autoantibodies (autoAb). Further follow-up in the monitoring stage depends on the estimated 5-year risk for diabetes according to results of the baseline monitoring and autoantibody tests during the Screening stage. Annual monitoring by an HbA1c level and autoAb is carried out in participants at lower diabetes risk. Semi-annual monitoring by an OGTT, HbA1c level and autoAb will occur in participants at higher diabetes risk. Participants in the Annual Monitoring Group enter the semi-annual monitoring group if they develop ≥ 2 positive autoantibodies, an HbA1c $\geq 6.0\%$, or an increase in HbA1c level $\geq 0.5\%$ from the last test. All immunological and metabolic tests are performed at a central core laboratory and deposited in a data repository accessible to all TrialNet investigators. In addition to study-related tests, we have a local mechanistic study (approved by the TrialNet Ancillary Studies Subcommittee on January 2012) that allows performance of CBC measurements in all TN01-enrolled subjects. CBC were all performed by the automated haematology analyser Sysmex XE-2100 at the San Raffaele Hospital.

Characteristics of the subjects enrolled in the studies and of whom OGTT and CBC data were recovered are listed in **Table S9**.

Image acquisition and analysis. Images of FFPE pancreatic sections analyzed by immunohistochemistry were acquired using an upright NIKON Eclipse Ni-U microscope (Nikon Instruments Inc, Melville, NY, USA) equipped with DS-Ri2 camera (Nikon) and analyzed with NIS-Elements Analysis D v4.40 software.

Images of FFPE sections analyzed by immunofluorescence were obtained with a HCX PL APO λblue 40X (NA 1.4) Oil on a TCS SP5 Laser Scanning Confocal microscope, equipped with 405nm (Diode) 458nm, 476nm, 488nm, 496nm, 514nm (Ar) 543nm (HeNe) 633nm (HeNe) lasers, and analyzed with Las AF software (Laser Advanced fluorescence 2.6.0.7266, Leica Microsystem).

For all sections analyzed, the number of MPO positive cells was quantified by manually count (by the same operator) and each pancreatic section area was determined by observing slides at low magnification (4X) using NIKON Eclipse Ni-U microscope and NIS-Elements Analysis software (Nikon). Finally, the ratio between the number of MPO positive cells and the section area was calculated.

Neutrophil collection and processing. Peripheral blood from non-diabetic control subjects was collected from those donors undergoing surgery at the San Raffaele Orthopedic Pediatric Department. Peripheral blood was collected from patients at T1D onset (*i.e.,* within 10 days from first insulin injection) hospitalized at the San Raffaele Pediatric Department. The study was approved by the San Raffaele Hospital Ethic Committee (IRB#DRI-003). At risk subjects were enrolled in the Type 1 Diabetes TrialNet Pathway to Prevention Trial (TN01) at the TrialNet Clinical Center of the San Raffaele Hospital. The study was approved by the San Raffaele Hospital Ethics Committee (IRB# NHPROT32803-TN01) and

peripheral blood was also collected for mechanistic studies (approved by the TrialNet Ancillary Studies Subcommittee on January 2012). Parents of all subjects included in this study signed the informed consent prior to blood donation. Detailed donor characteristics can be found in **Table S15**.

Whole blood was collected in sodium EDTA tubes, and processed for neutrophil purification as previously described (6). Neutrophil purity was assessed by hemocytometer and samples with a purity less than 90% were discarded. Purified neutrophils were lysed in TRIzol reagent (Sigma Aldrich, St Louis, MO) for RNA isolation following manufacturer instructions. RNA samples were treated with DNase I (Sigma Aldrich) to remove genomic DNA contamination. RNA quality was assessed by a 2100 Bioanalyzer (Agilent Technologies). Samples with RNA integrity number (RIN) >7 were processed for library preparation and RNA-sequencing.

RNA sequencing data analysis. Raw Illumina format PE data sets were simultaneously filtered for reads containing base calls with phred scores <20, demultiplexed into constituent sublibraries based on in-line bar codes and converted into FASTQ format using the genome analyzer pipeline software CASAVA version 1.8.1.

FASTQ reads were trimmed in a local Galaxy server in two steps: 1) hard-trimming to remove 1 3'-end base; 2) quality trimming from both ends until minimum base quality for each read ≥ 30 (FASTQ Quality Trimmer tool, v.1.0.0 (7, 8). Reads were aligned in Galaxy using Bowtie and TopHat v.1.4.0 (9). Read counts per Ensembl gene ID were estimated in Galaxy using htseq-count v.0.4.1 (10). Sequencing, alignment, and quantitation metrics were obtained for FASTQ, BAM/SAM, and count files in Galaxy using FastQC, Picard v. 1.128, TopHat, Samtools v. 0.1.18, and htseq-count (7–10).

All RNA-seq samples passed quality controls, based on having greater than 5 million total reads, more than 75% of reads aligned to the reference genome, and median coefficient of variation of read coverage less than 1. We trimmed the dataset to include only protein-coding genes, and normalized counts using the trimmed mean of M values (11) as implemented in the BioConductor package edgeR; this algorithm accounts for sequencing depth while allowing differences in library size based on the assumption that most genes are not differentially expressed. We included genes in analyses if they had greater than 1 count per million in at least 15% of the libraries. Sample identity was verified by comparing patient gender to chromosomal sex determined from the log-transformed ratio of X-chromosome reads to Y-chromosome reads. We excluded two mis-matched samples identified by this step, after confirmation using SNP comparison to another sample from one of the patients. Differential expression of individual genes was assessed using limma and voom (12, 13); these methods account for heterogeneity of variance among genes, incorporate sample-level weights, and moderate test statistics across genes. We used a Benjamin-Hochberg adjusted p-value threshold of 0.1 to determine significance. All models included patient sex as a covariate. Inclusion of patient age and/or sample purity did not substantially alter the results. Hierarchical clustering of samples and genes used the complete linkage method as implemented in R's hclust function.

Principal component analysis (PCA) revealed some samples that were markedly different from the majority of the samples. To ensure that our results were not driven by outliers, we ran analyses with and without these anomalous samples; results were very similar for the two cases.

Neutrophil purity is an important variable to be considered when looking at transcriptomic data (14). To ensure that contamination by eosinophils was not influencing our results, we ran models with eosinophil percentage as a covariate, reran analyses

excluding samples with eosinophil contamination above 6.5%, and tested for correlations between differentially expressed genes and eosinophil percentage. It was indeed shown that low numbers (5%-6%) of contaminating leukocytes in neutrophil preparations contribute very little to the overall gene expression profile of cytokine-stimulated neutrophils (14). In all cases, results indicated that eosinophils were not driving the results.

Functional enrichment analysis was conducted with DAVID v. 6.8 (15), using lists of differentially expressed genes as described above. Interferon-associated genes were determined by membership in interferon-related gene ontology (GO) terms.

To obtain insights on the altered pathway in purified neutrophils, differentially expressed biological processes and molecular functions were investigated through functional annotation analysis with DAVID v. 6.8 and Cytoscape 3.2 (16) along with KEGGscape (0.7.1 version), CytoKegg (1.0.1 version) and ReactomeFIPlugIn (6.1.0 version) plugins. The final pathway was built up by Illustrator CS4 (Adobe) on the basis of the "Influenza A pathway" (KEGG PATHWAY Database) and literature on interferon related genes (17).

We compared our interferon-associated transcriptional signature from purified neutrophils to published interferon signatures in T1D (18, 19), rheumatoid arthritis (RA) (20), and systemic lupus erythematosus (SLE) (21). We used the Jaccard index (22) to quantify overlap between the signatures; this index ranges from 0 (no overlap) to 1 (perfect overlap). We determined significance of overlaps using Fisher's exact test, with the total number of interferon-related genes set at 410 based on all genes associated with interferon-related GO terms. To quantify expression of pre-defined gene sets, we calculated the median expression in each sample across all genes in the gene set.

Custom R codes were used to analyze RNA-seq data and they are available at GitHub (https://github.com/mjdufort/Battaglia_T1D_neutrophils).

HLA genotyping. Genomic DNA was extracted and purified using QIAamp DNA Blood Mini Kit (Qiagen, Germany) and Maxwell 16 Blood DNA Purification kit (Promega Corporation, U.S.A.). HLA-typing was performed in an European Federation for Immunogenetics (EFI) accredited laboratory at the Immuno-hematology and Transfusion Service of the San Raffaele Hospital. Complete HLA-DRB1-DQA1-DQB1 intermediate resolution typing was performed using the HISTO SPOT SSO System (HISTO SPOT typing kits, HISTO SPOT reagent kit, MR.SPOT processor and the HISTO MATCH interpretation software; BAG Health Care GmbH, Germany), according to the manufacturer's protocol. For HLA-DRB1 and for HLA-DQA1/DQB1 the second and the second and third exon were amplified and analyzed, respectively. The intermediate typing results which did not belong to the Common and Well Defined Alleles (CWD) group were excluded. Allele Combinations were assessed in the IPD-IMGT/HLA database version 3.25.0. The HLA-DRB1-DQA1-DQB1 haplotypes were determined by comparison of the phenotype with well-known haplotype frequencies without the family study (23).

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SUPPLEMENTARY TABLES

Table S1. Inclusion criteria TrialNet (TN)-intervention Studies
autoAb: T1D auto antibody
mIAA: anti-insulin autoAb
OGTT: 2-hour oral glucose tolerance test

	TN10 **Teplizumab** **(anti-CD3)**	**TN18** **Abatacept** **(CTLA4-Ig)**	**TN20** **Oral insulin**
Age (yrs)	≥ 8	≥ 6	≥ 3
AutoAb status	≥ 2	≥ 2 excluding mIAA	≥ 2 one of which has to be mIAA
OGTT	Abnormal To confirm in those ≥ 18 yo	Normal	Normal Participants 3-7 yrs at time of randomization can have abnormal OGTT on most recent test
Other	Lymphocytes≥ 1,000/µL Neutrophils ≥ 1,500/µL Platelets ≥ 150,000/µL Hemoglobin ≥ 10g/dL AST or ALT ≤ 1.5xULN Total bilirubin ≤ 1.5xULN		

Table S2. Donor characteristics TrialNet (TN)-intervention Studies.
TN10: Teplizumab (anti-CD3); TN18: Abatacept (CTLA4-Ig); TN20 (oral insulin)
Chi-square and Kruskal-Wallis tests were used to assess differential distributions of categorical and continuous measures, respectively, between studies.

Demographic Characteristic	All subjects N=289	TN10 N=71	TN18 N=139	TN20 N=79	p-value
Age at randomization (yrs)					
Median	12.9	13.4	15.9	8.5	<0.0001
Range	3.0 - 53.1	8.1 - 49.4	6.8 - 53.1	3.0 - 43.6	
< 18 yrs old	215	53	87	75	<0.0001
≥ 18 yrs old	74	18	52	4	
Sex					
Female	140	31	75	34	
Male	145	37	63	45	0.067
not reported	4	3	1	0	
Race					
White	271	69	129	73	
Black/African American	6	0	3	3	0.82
Asian	3	1	1	1	
Multiracial	5	1	3	1	
Unknown or not reported	5	0	3	1	
Ethnicity					
Hispanic or Latino	22	1	15	6	
Not Hispanic or Latino	258	65	122	71	0.026
Unknown or not reported	9	5	2	1	

Table S3. Metabolic and (partial) CBC characteristics of subjects enrolled in TrialNet (TN)-intervention Studies.
TN10: Teplizumab (anti-CD3); TN18: Abatacept (CTLA4-Ig); TN20 (oral insulin).
Chi-square and Kruskal-Wallis tests were used to assess differential distributions of categorical and continuous measures, respectively, between studies.

Metabolic Characteristic	All subjects N=289	TN10 N=71	TN18 N=139	TN20 N=79	p-value
Fasting glucose (mg/dL)					
Median	91	95.4	90	89	<0.0001
Range	71-116	82-116	71-108	71-103	
Missing	1	1	0	0	
Fasting c-peptide (ng/dL)					
Median	1.59	1.59	1.86	1.04	<0.0001
Range	0.41-5.16	0.7-5.16	0.54-4.19	0.41-3.37	
Missing	1	1	0	0	
Early c-peptide (ng/dL) (30-0 min)					
Median	3.63	2.79	4.33	2.63	<0.0001
Range	-0.18 – 16.14	-0.18 – 8.81	0.51 – 12.5	0.71 – 16.14	
Missing	1	1	0	0	
Stimulated c-peptide (Mean AUC)					
Median	5.45	5.26	6.51	4.14	<0.0001
Range	1.85-15.5	1.85-13.75	2.13 -14.6	2.0-15.5	
Missing	1	1	0	0	
Fasting insulin(µU/mL)					
Median	76	81	80	64	
Range	<0.5 – 122	<0.5 – 122	8 –-123	<0.5 – 123	0.009
2-hour glucose (mg/dL)					
Median	115	152	111	109	<0.0001
Range	48 – 213	87 –213	48 –139	53 –199	
HOMA-beta					
Median	86	80.4	95.3	65.7	0.00012
Range	3.2 – 544.5	3.2 – 433.6	32.3 – 544.5	4.5 – 530.2	
Index60					
Median	0.13	1.07	-0.4	0.51	<0.0001
Range	-3.7 – 2.96	-3.4 – 2.96	-3.7 – 1.58	-3.6 – 2.66	
Neutrophils (10^3 cells/µL)					
Median	2.86	2.85	3.16	2.64	0.11
Range	0.64 – 10.7	1.2 – 10.7	0.64 - 8.2	0.86 - 8.1	
Missing	18	1	9	8	
Lymphocytes (10^3 cells/µL)					
Median	2.01	1.80	1.94	2.30	
Range	0.86 – 4.5	1 – 4.05	0.95 – 4.1	0.86 – 4.5	0.0035
Missing	1	0	1	0	

Table S4. Univariate correlation analysis for neutrophils or lymphocytes (log-transformed) and metabolic correlates. Statistically significant correlates are shown in red

	Neutrophils		Lymphocytes	
	r	p	r	p
Fasting glucose	0.02	0.77	-0.18	0.002
Fasting c-peptide*	0.30	<0.0001	-0.12	0.046
Early c-peptide (30-0 min)	0.18	0.002	-0.004	0.94
Stimulated c-peptide (Mean AUC)*	0.27	<0.0001	-0.11	0.062
Fasting insulin	0.046	0.454	-0.15	0.012
2-hour glucose	-0.02	0.72	-0.07	0.23
HOMA-beta*	0.22	0.0003	0.01	0.82
Index-60	0.25	<0.0001	0.039	0.51

* log-transformed

Table S5. Univariate correlation analysis for neutrophils (log-transformed) and clinical correlates. Statistically significant correlates are shown in red.

Characteristic	r	p
Age	0.28	<0.0001
Gender		
Female	3.19	0.025
Male	2.65	
Race		
white *vs* non-white		0.51
hisp. *vs* non-hisp.		0.21
BMI percentile *(only for TN10 and TN18)*	0.24	0.0015
Study (median)		
TN10	2.85	
TN18	3.16	0.11
TN20	2.64	

Table S6. Multivariate correlation analysis for neutrophils (log transformed). BMI was collected only in TN10 and TN18. Statistically significant correlates are shown in red

Factor	Adjusting for age, sex, and protocol (TN10, TN18, TN20) n=289		Adjusting for age, sex, protocol, and BMI% (TN10, TN18) n=210	
	Parameter Estimate (Std. error)	**p-value**	**Parameter Estimate (Std. error)**	**p-value**
Fasting C-peptide*	0.26 (0.07)	0.0006	0.255 (0.09)	0.006
Early C-peptide response *(30-0 minute)*	0.014 (0.008)	0.082	0.01 (0.01)	0.30
Stimulated C-peptide *(Mean AUC c-pep)**	0.20 (0.06)	0.0012	0.18 (0.07)	0.016
HOMA-beta*	0.09 (0.03)	0.003	0.065 (0.04)	0.10
Index60	-0.05 (0.02)	0.008	-0.03 (0.02)	0.11

* log-transformed

Table S7. Multivariable estimated model results to evaluate the impact of fasting C-peptide on neutrophil counts. The final model - obtained with the backward selection procedure - is:

$$\log(neutrophil\ count) \sim 1.134 + 0.195\log\left(Cpeptide_{fasting}\right) + 0.0035\left[\log\left(Cpeptide_{fasting}\right)\ x\ age\ interaction\right]$$

Parameter	Estimate	Std. Error	p-value
Intercept	1.134	0.07	<0.0001
Fasting C-peptide	0.195	0.08	0.018
Fasting C-peptide x age	0.0035	0.0016	0.028

Table S8. Multivariable estimated model results to evaluate the impact of stimulated C-peptide (mean AUC) on neutrophil counts. The final model - obtained with the backward selection procedure - is:

$$\log(neutrophil\ count) \sim 1.305 - 0.006\ BMI\ percentile + 0.0037[\log(Cpeptide_{mean\ AUC})\ x\ BMI\ percentile\ interaction]$$

Parameter	Estimate	Std. Error	p-value
Intercept	1.305	0.055	<0.0001
BMI percentile	-0.006	0.002	0.0065
Stimulated C-peptide x BMI percentile	0.0037	0.0009	0.0009

Table S9. Donor characteristics TN01 (Pathway to Prevention – Milan Site)**.**
autoAb: T1D auto-antibody
Stable autoAb/OGTT: subjects who maintain the same autoAb number/OGTT status throughout the study
Change of autoAb/OGTT Status: subjects who changed the autoAb number/ OGTT status throughout the study

Characteristic	
# of subjects	109
Age at the first visit	15.07
median (range)	(2.8 – 45.8)
Age > 18 yrs, n (%)	42 (38.5%)
Gender	
Females, n (%)	53 (48.62%)
Males, n (%)	56 (51.38%)
Race	
White, n (%)	109 (100%)
Ethnicity	
Hispanic or Latino, n (%)	3 (2.78%)
Not Hispanic or Latino, n (%)	105(97.22%)
Unknown or not reported, n	1
autoAb	
Stable NEG, n (%)	5 (4.6%)
Stable POS =1, n (%)	45 (41.3%)
Stable POS ≥ 2, n (%)	39 (35.8%)
Change of autoAb status, n(%)	20 (18.3%)
OGTT	
Stable Normal, n (%)	80 (73,4%)
Stable Impaired, n (%)	8 (7,3%)
Change of OGGT results, n(%)	21 (19,3%)

Table S10. Observation characteristics TN01 (Pathway to Prevention – Milan Site)
n= 303 observations from 109 subjects belonging to 101 families
autoAb: T1D auto-antibody

Characteristic	median (range)
# observations per subject	1 (1 - 11)
autoAb	
NEG, n (%)	36 (11.9%)
POS =1, n (%)	131 (43.2%)
POS ≥ 2, n (%)	136 (44.9%)
Age	16.6 (2.8 - 49.9)
Age > 18 yrs, n (%)	133 (44%)
Gender	
Females, n (%)	165 (54%)
Males, n (%)	138 (46%)
BMI percentile	44.71 (0.36 - 99.86)
Race	
White, n (%)	303 (100%)
Ethnicity	
Hispanic or Latino, n (%)	3 (1%)
Not Hispanic or Latino, n (%)	293 (99%)
Unknown or not reported, n	1
OGTT Results	
Normal, n (%)	226 (76%)
Impaired, n (%)	73 (24%)
Missing time point, n	4
Fasting glucose (mg/dL)	88 (66 - 125)
Fasting C-peptide (ng/mL)	1.495 (0.4 - 4.35)
Early C-peptide (30-0 min)	3.44 (0.18 - 13.85)
Stimulated C-peptide (Mean AUC)	5.89 (1.63 - 6.34)
Fasting insulin (µU/mL)	6.45 (1.1 - 33.35)
2-hr glucose (mg/dL)	112.0 (55.0 – 244.0)
Homa-beta	94.71 (14.9 - 984.52)
Index 60	-0.02 (-4.59 – 3.11)
Neutrophils (10^3 cells/µL)	3 (0.5 - 10.74)
Lymphocytes (10^3 cells/µL)	2 (0.7- 5.4)

Table S11. Results of the longitudinal statistical analysis to evaluate the impact of fasting C-peptide on neutrophil counts. The final model - obtained with the backward selection procedure- is:

$$log(neutrophil\ counts) = 0.611 + 0.002(BMI\ percentile) + 0.264\big(log(Fasting\ C\text{-}peptide + 1)\big) + 0.005(log(Fasting\ C\text{-}peptide + 1) * Age)$$

Parameter	Estimate	Std.Error	p-value
Intercept	0.002	0.093	<0.001
BMI percentile	0.002	0.001	0.023
Fasting C-peptide	0.264	0.117	0.026
Fasting C-peptide x age	0.005	0.002	0.030

Table S12. Results of the longitudinal statistical analysis to evaluate the impact of stimulated C-peptide (mean AUC) on neutrophil counts. The final model, obtained with the backward selection procedure, is:

$$log(neutrophil\ counts) = 0.886 - 0.145(Male) - 0.015(Age) + 0.003(BMI\ percentile) + 0.011(log(Mean\ AUC\ C\text{-}peptide + 1) * Age)$$

Parameter	Estimate	Std.Error	p-value
Intercept	0.886	0.072	<0.001
Gender (Male)	-0.145	0.061	0.049
Age	-0.015	0.007	0.033
BMI percentile	0.003	0.001	0.003
Stimulated c-peptide x age	0.011	0.003	0.001

Table S13. Characteristics of pancreas donors.
AutoAb: T1D auto-antibody

Cohort	Case -ID	Donor	Age (yrs)	Gender	AutoAb	Disease duration
nPOD	6024	non-diabetic	21	M	negative	NA
nPOD	6098	non-diabetic	18	M	negative	NA
nPOD	6153	non-diabetic	15	M	negative	NA
nPOD	6174	non-diabetic	21	M	negative	NA
nPOD	6178	non-diabetic	24	F	negative	NA
nPOD	6179	non-diabetic	22	F	negative	NA
nPOD	6027	AutoAb pos	19	M	$ZnT8A^+$	NA
nPOD	6158	AutoAb pos	40	M	$GADA^+$, IAA^+	NA
nPOD	6167	AutoAb pos	37	M	$IA\text{-}2A^+$, $ZnT8A^+$	NA
nPOD	6197	AutoAb pos	22	M	$GADA^+$, $IA\text{-}2A^+$	NA
nPOD	6052	T1D	12	M	IAA^+, $IA\text{-}2A^+$	1 yrs
nPOD	6087	T1D	17	M	$ZnT8^+$, IAA^+	4 yrs
nPOD	6089	T1D	14	M	IAA^+	8 yrs
nPOD	6113	T1D	13	F	IAA^+	1 yrs
Siena	60217	T1D	39	F	$GADA^+$	from childhood
DiViD	1	T1D	25	F	$GADA^+$,$IA\text{-}2A^+$, $ZnT8A^+$, IAA^+	4 wks
DiViD	2	T1D	24	M	$GADA^+$, $IA\text{-}2A^+$, $ZnT8A^+$	3 wks
DiViD	3	T1D	34	F	$GADA^+$, $IA\text{-}2A^+$, $ZnT8A^+$	9 wks
DiViD	4	T1D	31	M	$GADA^+$, $IA\text{-}2A^+$, IAA^+	5 wks
DiViD	5	T1D	24	F	$GADA^+$, $IA\text{-}2A^+$, IAA^+	5 wks
DiViD	6	T1D	35	M	$GADA^+$	5 wks
Exeter	SC112	T1D	22	M	NA	9 yrs
Exeter	SC76	T1D	20	M	NA	3 wks
Exeter	SC57	T1D	18	F	NA	< 1 wk
Exeter	SC116	T1D	35	unknown	NA	15 yrs
Exeter	E560	T1D	42	F	NA	1.5 yrs

Table S14. Sections used from each pancreas donors

Cohort	Case -ID	Sections
nPOD	6024	(head-01 sec 03) (head-08 sec 01)
nPOD	6098	(body-01 sec 01) (tail-02 sec 04) (body-01 sec 04)
nPOD	6153	(head-04 sec 01)
nPOD	6174	(body-04 sec 03) (body-04 sec 01)
nPOD	6178	(body-04 sec 01) (tail-02 sec 01) (body-04 sec 01)
nPOD	6179	(body-04A sec 01) (tail-02A sec 02) (tail-02A sec 02)
nPOD	6027	(head-06 sec 01) (tail-04 sec 02) (head-06 sec 03)
nPOD	6158	(body-10 sec 03) (tail-04 sec 01)
nPOD	6167	(body-10 sec 01) (tail-08 sec 04) (tail-08 sec 03)
nPOD	6197	(body-06 sec 03) (head-03 sec 04)
nPOD	6052	(body-04 sec 03) (body-04 sec 01)
nPOD	6087	(body-01 sec 01) (tail-02 sec 02) (body-01 sec 01)
nPOD	6089	(body-04 sec 03) (tail-02 sec 04)
nPOD	6113	(body-01 sec 01) (head-01 sec 06) (body-01 sec 02)
Siena	60217	(body sec 30) (head sec 20) (tail sec 20)
DiViD	1	(block 2102-2) (block 2102-04)
DiViD	2	(block 6079-4) (block 1618)
DiViD	3	(block 1754B) (block 4838-4)
DiViD	4	(block 1759) (block 5189-2)
DiViD	5	(block 5191-1) (block 5191-2)
DiViD	6	(block 1822) (block 16036)
Exeter	SC112	(slide #10)
Exeter	SC76	(slide #1)
Exeter	SC57	(slide #10)
Exeter	SC116	(slide #13)
Exeter	E560-mid	(slide #21)

Table S15. Transcriptomic Blood Neutrophil Signature: donor characteristics. Significance evaluated for age, and neutrophil purity by Kruskal–Wallis test, for # autoAb by Mann–Whitney test and for gender by Fisher's test.

	unrelated non-diabetic controls n=16	**AutoAb NEGATIVE n= 13**	**AutoAb POSITIVE n=8**	**T1D new onset n=5**	p-value
Age (yrs)					
Median	13	12	13	6	0.15
Range	12-18	5-16	5-17	5-15	
Gender					
Females (%)	8 (50%)	6 (46%)	5 (62%)	3 (60%)	0.92
Males (%)	8 (50%)	7 (54%)	3 (38%)	2 (40%)	
# autoAb					
Median	NA	0	2	3	0.54
Range			1-4	2-3	
Days from diagnosis					
Median	NA	NA	NA	5	
Range				3-7	
Neutrophil purity (%)					
Median	95.5	95.6	97.8	95.7	0.65
Range	90.1-98.9	90.6 - 100	93 - 99.7	90.4-98.4	

SUPPLEMENTARY VIDEOs

Supplementary Video 1. Immunofluorescence analysis of a OCT pancreatic section from an autoAb positive nPOD donor (#6027) stained with anti-MPO antibody (green) and Hoechst 33342 for DNA detection (white). All images were acquired by confocal microscopy and assimilated as Z-stacked. The video shows a single plane per frame. Scale bar 25 μm.

Supplementary Video 2. Immunofluorescence analysis of a OCT pancreatic section from T1D nPOD donor (#6052) stained with anti-MPO antibody (green) and Hoechst 33342 for DNA detection (white). All images were acquired by confocal microscopy and assimilated as Z-stacked. The video shows a single plane per frame. Scale bar 25 μm.

Supplementary Video 3. Immunofluorescence analysis of a FFPE pancreatic section from T1D DiViD donor (#5) stained with anti-MPO antibody (green), citrullinated Histone H3 (red) and Hoechst 33342 for DNA detection (white). A sample field was acquired by confocal microscopy and assimilated as Z-stacked. The video shows a single plane per frame. Scale bar 7.5 μm.

Supplementary Video 4. Immunofluorescence analysis of a FFPE pancreatic section from T1D DiViD donor (#1) stained with anti-MPO antibody (green), citrullinated Histone H3 (red) and Hoechst 33342 for DNA detection (white). A sample field was acquired by confocal microscopy and assimilated as Z-stacked. The video shows a single plane per frame. Scale bar 7.5 μm.

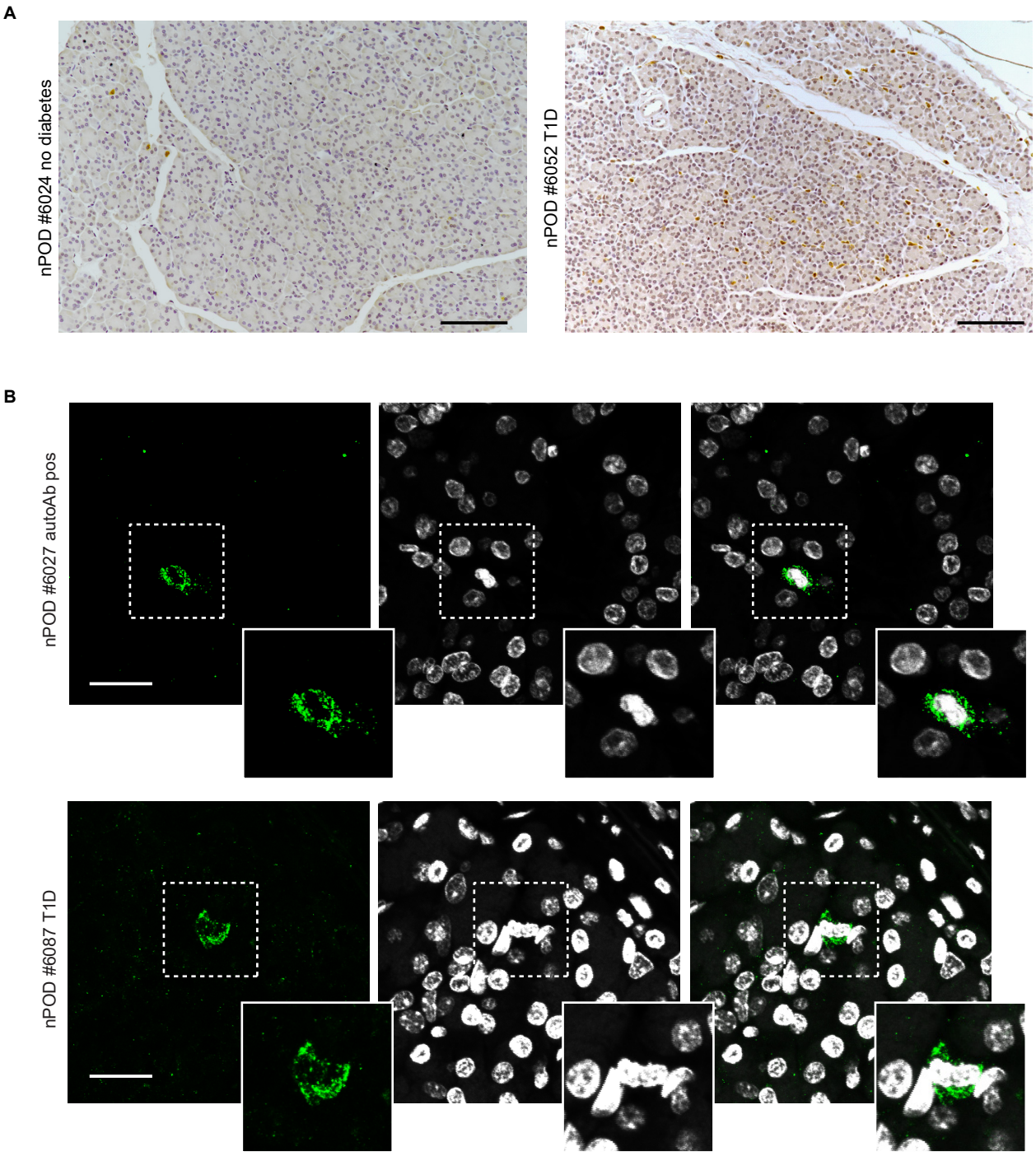

Figure S1. Intact- and NETting-neutrophils infiltrate the pancreas of pre-symptomatic and symptomatic T1D donors. (**A**) One representative image of an immunohistochemical (IHC) analysis of a formalin fixed paraffin embedded (FFPE) section from a non-diabetic control (#6024, left panel, out of 6 total subjects/sections analyzed) and with T1D (#6052, out of 4 total subjects/sections analyzed) nPOD donors stained with hematoxylin and anti-MPO (neutrophil-specific) antibody (brown staining). Scale bar 100 µm. (**B**) Representative images of immunofluorescence (IF) analyses of FFPE nPOD sections stained with anti-MPO antibody (green, left column) and Hoechst 33342 for DNA detection (white, middle column). Expression signals was then merged (right column). Donor ID and characteristics are shown on the left (n= 14 donors/sections). Images are represented as Z-stacked following projection. Insets were cut (dotted square) and magnified 1.5X at the bottom of each panel and they highlight the occurrence of decondensed DNA co-localized with MPO suggestive of the presence of pancreas-residing NETting neutrophils. Scale bar 20 µm.

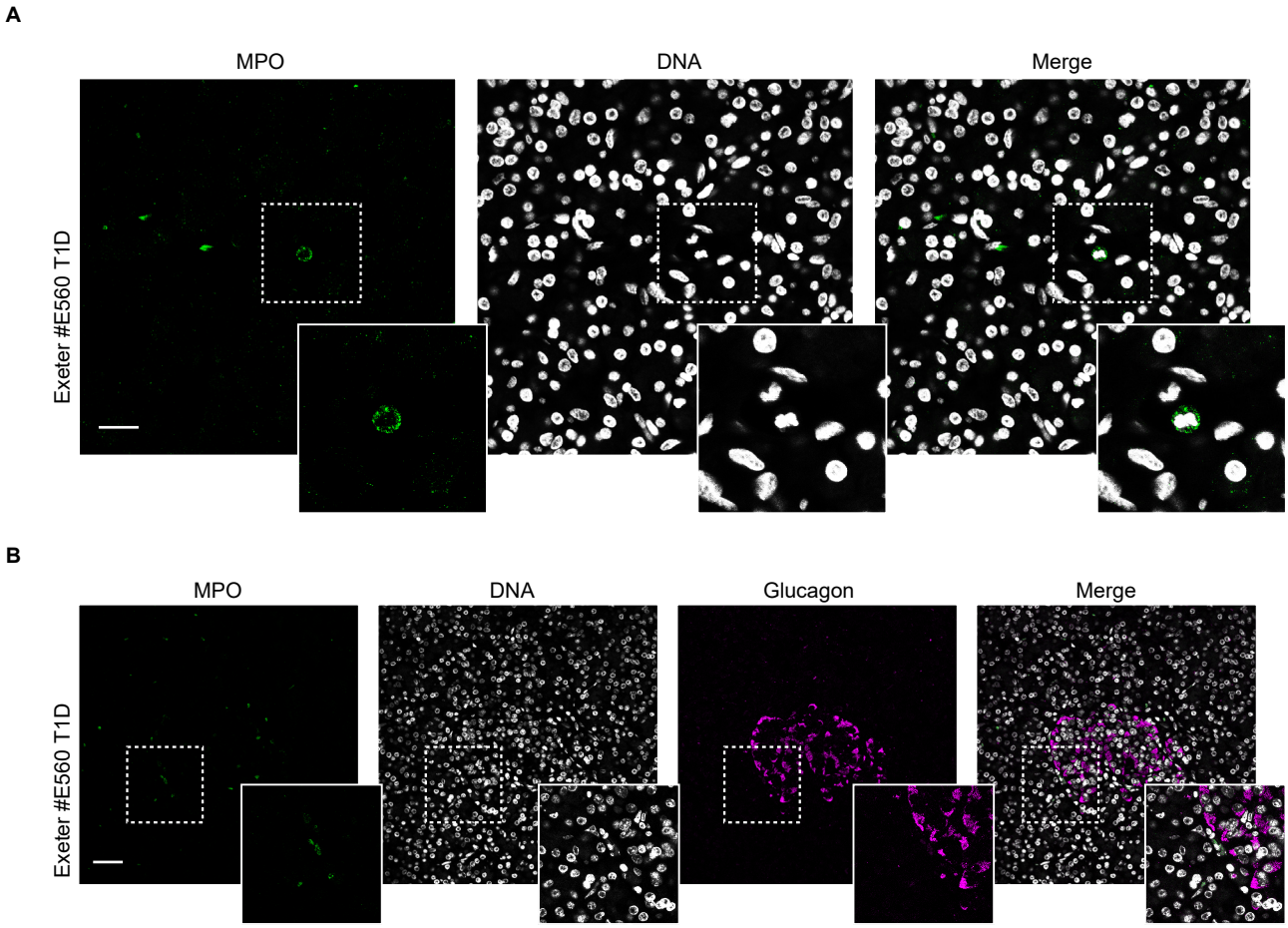

Figure S2. Neutrophils infiltrate the pancreas of T1D donors from the Exeter cohort. (**A**) One representative image of IF analysis of a formalin fixed paraffin embedded (FFPE) section from an Exeter donor with T1D (#E560, out of 5 total subjects/sections analyzed) stained with anti-MPO antibody (green, left column) and Hoechst 33342 for DNA detection (white, middle column). Expression signals was then merged (right column). Donor ID and characteristics are shown on the left. Scale bar 25 µm. (**B**) One representative image of IF analysis of a FFPE section from an Exeter donor with T1D (#E560) stained with anti-MPO antibody (green), Hoechst 33342 for DNA detection (white) and anti-glucagon antibody (pink). Expression signals was then merged (right column). Donor ID and characteristics are shown on the left. Scale bar 20 µm.

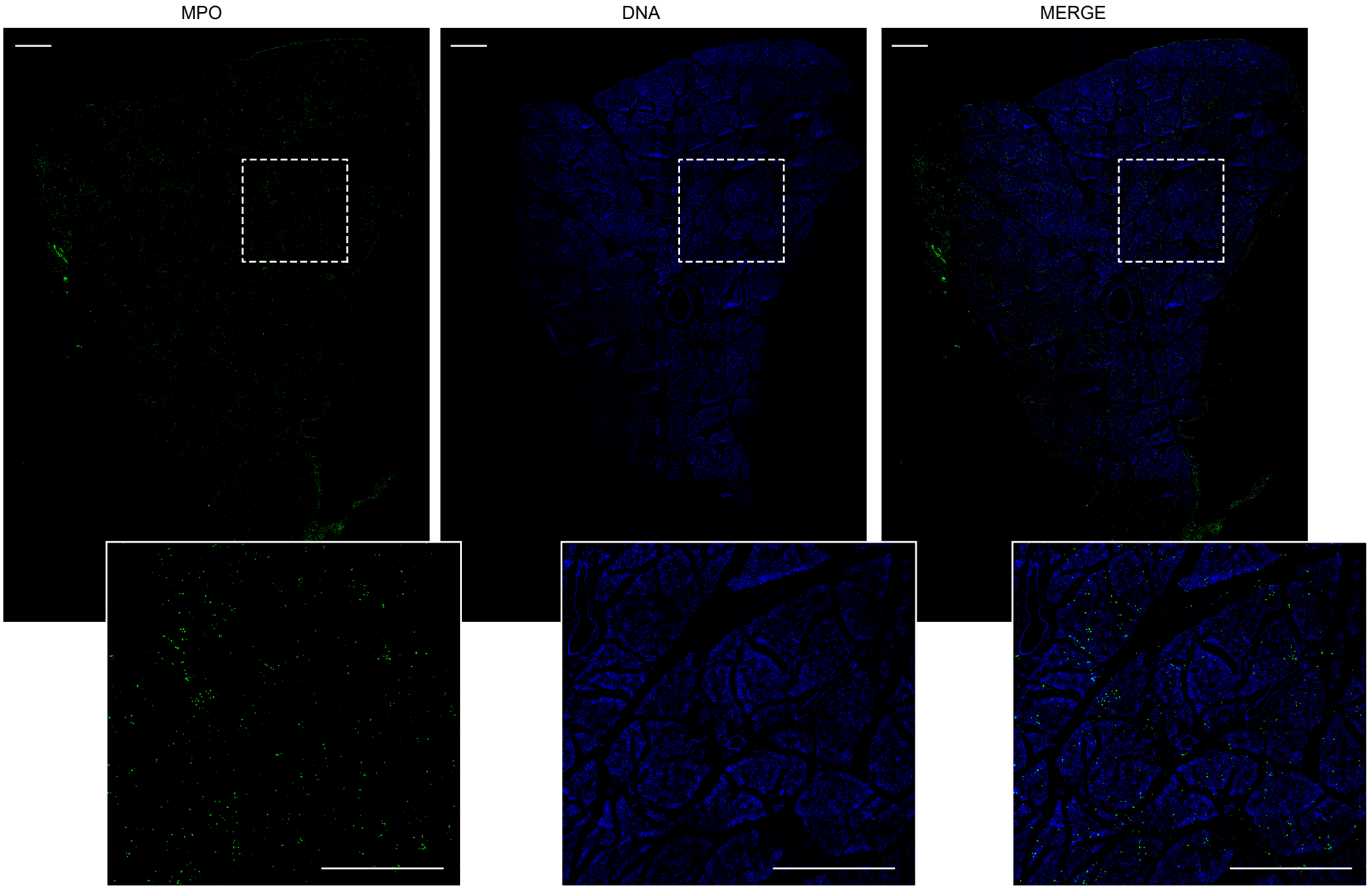

Figure S3. Neutrophils infiltrate the whole pancreas. One representative image of an immunofluorescence (IF) analyses of a frozen OCTembedded section from a nPOD donor with T1D (#6052) stained with anti-MPO antibody (green, left column) and Hoechst 33342 for DNA detection (white, middle column). Expression signals was then merged (right column). All images were acquired by confocal microscopy and represented as stitched images (204 images) of the optimal autofocus on a single plan. Insets were cut (dotted square) and magnified at the bottom of each panel. Scale bar 1 mm.

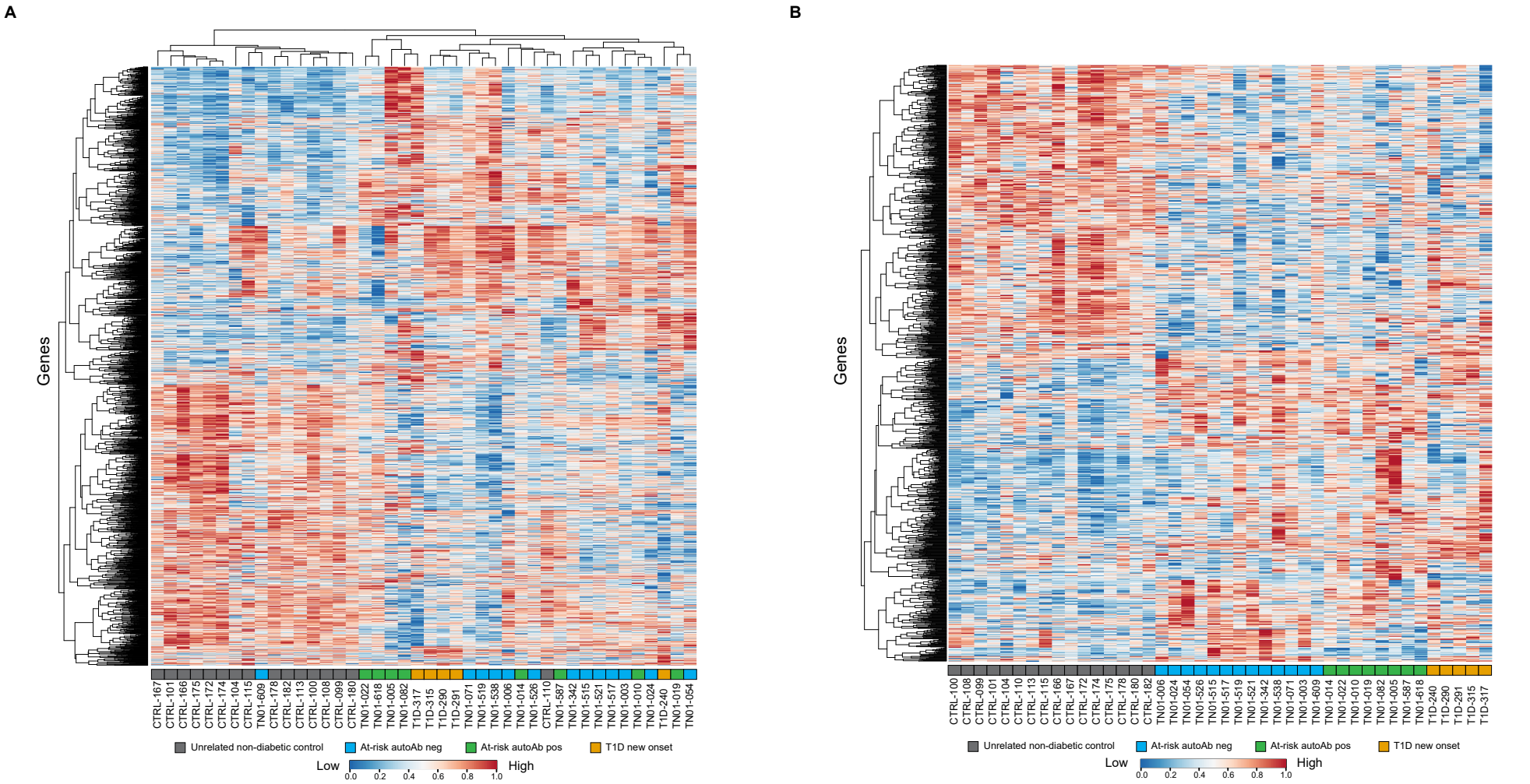

Figure S4. Peripheral neutrophil transcriptomic signature from autoAb negative, autoAb positive and new-onset T1D patients form a single large cluster distinct from that of unrelated non-diabetic controls. (**A**) Heat map showing 2058 genes differentially expressed in neutrophils, with patients arranged by hierarchical clustering of their transcriptional profiles. (**B**) Heat map showing 1350 genes differentially expressed in freshly isolated neutrophils after adjustments for age and neutrophil purity.- Scale bar represents the relative gene expression and ranges from the minimum log expression (0.0) to the maximum log expression (1.0) for both panel **A** and **B**. (**C**) Venn diagram displaying the overlap in genes differentially expressed between each at-risk or T1D donor group and unrelated non-diabetic controls (total of 2751 genes) is shown.

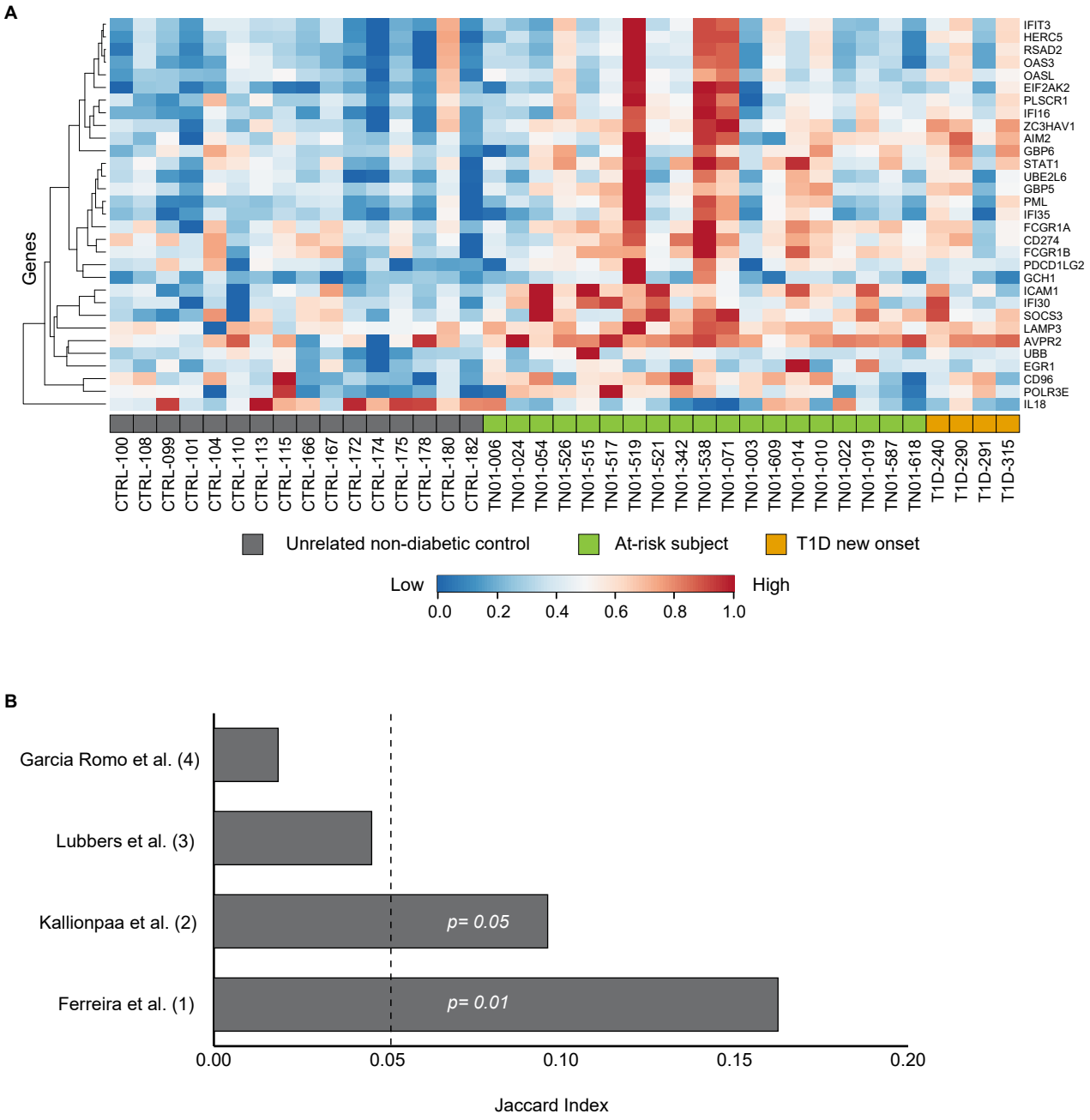

Figure S5. Interferon gene expression signature found in peripheral neutrophils of at-risk subjects is dysregulated in T1D and at-risk subjects and overlaps with interferon gene signatures previously identified in T1D. (**A**) Heat map showing 31 interferon-associated genes differentially expressed between at-risk and unrelated non-diabetic controls, after excluding samples with anomalous gene expression. Scale bar represents the relative gene expression and ranges from the minimum log expression (0.0) to the maximum log expression (1.0). (**B**) Overlap of the interferon gene signature identified in purified neutrophils of at-risk subjects with interferon gene signatures previously identified in T1D (ref 1, 2) and other autoimmune diseases (ref 3,4). Jaccard Index estimation was used to measure the strength of the similarity (see online methods) with a cutoff of 0.05 (dotted line based on hypergeometric p-value). Significant p-values are shown.

1) Ferreira et al. "A type I interferon transcriptional signature precedes autoimmunity in childrengenetically at risk for type 1 diabetes" Diabetes; 2014: 63(7) 2538-50

2) Kallionpaa et al. "Innate immune activity is detected prior to seroconversion in children with HLAconferred type 1 diabetes susceptibility" Diabetes; 2014: 63(7) 2402-14

3) Lubbers et al. "The type I IFN signature as a biomarker of preclinical rheumatoid arthritis" Annals of the rheumatic diseases; 2013: 72(5) 776-80

4) Garcia-Romo et al. "Netting neutrophils are major inducers of type I IFN production in pediatric systemic lupus erythematosus" Science Translational Medicine; 2011: 3 (73) 73ra20

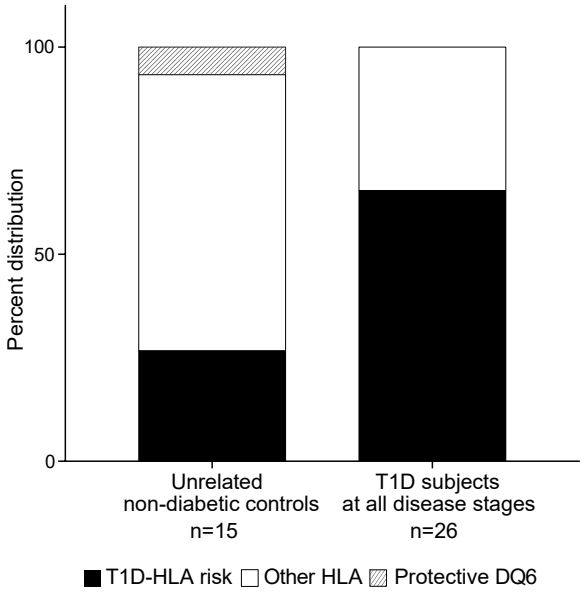

Figure S6. HLA distribution in subjects of whom neutrophil transcriptomic signature was generated. T1D-HLA risk (DR3/4-DQ2/8; DR3/X; DR4/X), T1D-protective HLA-DQ6 and other HLA alleles distribution in unrelated non-diabetic controls and in T1D subjects at all disease stages (i.e., from autoAb negative to T1D new onset subjects) are shown. The Fisher's exact test was applied to test differences between groups (the T1D-protective HLA-DQ6 and other HLA alleles were grouped) (p=0.025).