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T cell-specific PTPN2-deficiency in NOD mice accelerates the development of type 1 diabetes and autoimmune co-morbidities.

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SUMMARY

Genome-wide association studies have identified *PTPN2* as an important non-major histocompatibility complex gene for autoimmunity. Single nucleotide polymorphisms that reduce *PTPN2* expression have been linked with the development of varied autoimmune disorders, including type 1 diabetes. The tyrosine-phosphatase PTPN2 attenuates T cell receptor and cytokine signalling in T cells to maintain peripheral tolerance, but the extent to which PTPN2-deficiency in T cells might influence type 1 diabetes onset remains unclear. Non-Obese Diabetic (NOD) mice develop spontaneous autoimmune type 1 diabetes, similar to that seen in humans. T cell PTPN2-deficiency in NOD mice markedly accelerated the onset and increased the incidence of type 1 diabetes, as well as that of other disorders, including colitis and Sjogren's syndrome. Although PTPN2-deficiency in $CD8^+$ T cells alone was able to drive the destruction of pancreatic β cells and onset of diabetes, T cell-specific PTPN2-deficiency was also accompanied by increased $CD4^+$ T-helper type 1 differentiation and T follicular helper cell polarisation and an increased abundance of B cells in pancreatic islets as seen in human type 1 diabetes. These findings causally link PTPN2-deficiency in T cells with the development of type 1 diabetes and associated autoimmune co-morbidities.

INTRODUCTION

Autoimmune diseases encompass a broad range of heterogenous and complex disorders. They include systemic disorders such as Sjogren's syndrome, systemic lupus erythematosus and rheumatoid arthritis, and organ-specific diseases such as Graves' disease, Hashimoto's thyroiditis, Crohn's disease and type 1 diabetes mellitus (1). Although considerable progress has been made in understanding the cellular basis of immune tolerance and the development of autoimmunity, the molecular mechanisms that underpin most autoimmune disorders remain incompletely understood. Most autoimmune diseases are associated with specific alleles of the major histocompatibility complex (MHC) locus, but genome-wide associations studies (GWAS) have identified more than 200 non-MHC loci for which alleles are associated with one or more autoimmune diseases (1). Most polymorphisms identified by GWAS are non-coding variants that are predicted to modulate gene expression, with several implicated in functional pathways that influence T- and B-cell activation (1).

GWAS have identified *PTPN2*, encoding protein tyrosine phosphatase (PTP) non-receptor type 2 (PTPN2), also known as T cell PTP, as an important non-MHC locus gene for autoimmunity (2-8). Non-coding *PTPN2* single nucleotide polymorphisms (SNPs) have been associated with autoimmune disorders including Crohn's disease, rheumatoid arthritis and type 1 diabetes (2-8). For example, the type 1 diabetes-associated *PTPN2* SNP rs1893217 has been linked with a 40% decrease in *PTPN2* expression in $CD4^+CD25^+$ T_{reg} and $CD4^+CD45RO^+$ memory T cells (9). More recently, coding region *PTPN2* variants that affect mRNA stability or alter protein structure have been associated with early onset type 1 diabetes (10), whereas non-coding *PTPN2* SNPs linked with Crohn's disease, ulcerative colitis, rheumatoid arthritis and type 1 diabetes, have been reported to decrease PTPN2 in colonic lamina

propria fibroblasts from patients with Crohn's disease (11; 12). Thus, *PTPN2* SNPs resulting in decreased PTPN2 expression might determine the course of autoimmunity through influencing both immune and non-immune cell populations.

Studies in mice lacking PTPN2 have identified critical roles for this regulatory enzyme in T-cell driven immunity, inflammation and metabolism (13; 14). Changes in these processes are also frequently seen in autoimmune and inflammatory diseases where *PTPN2* polymorphisms are associated with disruption of T cell tolerance (15). PTPN2 dephosphorylates and inactivates the SRC family kinases (SFKs) LCK and FYN to 'tune' T cell receptor (TCR) signalling (15-17) and influence thymocyte development and peripheral T cell responses in the periphery to low affinity self-antigens to prevent inappropriate T cell activation (15; 18; 19). In addition, PTPN2 dephosphorylates and inactivates Janus-activated kinases (JAKs)-1 and -3 (20) and signal transducers and activators of transcription (STATs)-1, -3 and -5 to influence T cell responsiveness to various cytokines, including interferon (IFN)-γ and interleukins (IL)-2, -6, -7, -15, and -21 (15; 17-19; 21). Beyond its role in T cells, PTPN2 might affect the activation of innate and adaptive immune cells, including B cells, dendritic cells and myeloid cells (20; 22; 23) as well as stromal cells (24) to influence disease progression. Consistent with this, *Ptpn2*$^{-/-}$ mice that develop wasting disease succumb within weeks of birth (13; 14). Similarly, the inducible deletion of PTPN2 in the hematopoietic compartment of adult C57BL/6 mice (*Mx1*-Cre;*Ptpn2*$^{fl/fl}$) promotes systemic inflammation and overt autoimmunity within 4 weeks of PTPN2 deletion, with mice exhibiting dermatitis, glomerulonephritis, pancreatitis and overt liver disease (22). Beyond the effector/memory T cell phenotype, hematopoietic PTPN2-deficiency resulted in the accumulation of inflammatory monocytes and B cells and germinal centre formation linked with increased T follicular helper (T_{FH}) cells, germinal centre

B cells and IgG-secreting B cells (22). Therefore, PTPN2 deficiency might not only impact on T cell function, but also drive the pathogenic accumulation of T_{FH} cells and high-affinity antigen-specific IgG secreting B cells to promote autoimmunity.

Mice lacking PTPN2 in the hematopoietic compartment develop insulitis (infiltration of lymphocytes in pancreatic islets of Langerhans) (22) reminiscent of in early stage disease in humans with type 1 diabetes. However, this phenotype does not result in type 1 diabetes in C57BL/6 mice, even when PTPN2 is additionally deleted in pancreatic β cells (22). Similarly, these mice do not develop inflammatory bowel disease or inflammatory arthritis (22); diseases that are often associated with *PTPN2* loss of function SNPs (3; 5-8). Therefore, although deficiencies in T cells or the hematopoietic system may result in a loss of tolerance and the development of autoimmunity in C57BL/6 mice, the extent to which PTPN2 deficiency in T cells and/or other immune cells contributes to the onset, maintenance and severity of human autoimmunity remains unclear.

To explore the influence of PTPN2-deficiency in T cells on autoimmunity, especially type 1 diabetes, we backcrossed the *Lck*-Cre and *Ptpn2*$^{fl/fl}$ loci, originally generated in C57BL/6 mice, onto the Non-Obese Diabetic (NOD) background. Unlike other mouse models, NOD mice develop spontaneous autoimmune type 1 diabetes, similar to that seen in humans, sharing many of the diabetes susceptibility loci and autoantigens that contribute to the development of type 1 diabetes (25). PTPN2 deficiency in T cells in NOD mice markedly accelerated the onset and increased the cumulative incidence of type 1 diabetes. The development of inflammatory/autoimmune co-morbidities was also accelerated. Disease progression was associated with the infiltration of cytotoxic $CD8^+$ T cells, along with $CD4^+$ T-helper type 1 (T_{H1}) and T_{FH} polarisation, as detected in human type 1 diabetes (26).

These findings suggest that PTPN2-deficiency in T cells might be sufficient to promote the development of type 1 diabetes and autoimmunity in genetically susceptible individuals.

METHODS

Mice

Lck-Cre*;Ptpn2*$^{fl/fl}$.C57BL/6J mice (15) were backcrossed onto the NOD/Lt background for 11 generations. A genome-wide screen was performed by the Australian Genome Research Facility using the iPLEX GOLD chemistry and the Sequenom MassArray spectrometer for SNP genotyping. Data was analyzed using the GeneChip Targeted Genotyping System Software. *Lck*-Cre*;Ptpn2*$^{fl/fl}$;NOD and *Ptpn2*$^{fl/fl}$;NOD littermate mice were maintained on a 12 h light-dark cycle in a temperature-controlled specific-pathogen-free high barrier facility (ARL, Monash University) with free access to food and water. Aged- and sex-matched littermates were fed a standard chow diet (8.5% fat; Barastic, Ridley, AgriProducts, Australia).

Materials

Recombinant mouse IL-2, IL-6, IL-12, IFN-γ and TGFβ were purchased from Peprotech. Hamster α-mouse CD3ε (145-2C11) and hamster α-mouse CD28 (37.51) were purchased from BD Biosciences. Mouse α-tubulin (Ab-5) and mouse α-GAPDH were purchased from Sigma-Aldrich. Rabbit α-phospho-Stat1 (Tyr701, clone 58D6) and polyclonal rabbit α-Stat1 were purchased from Cell Signalling. Polyclonal rabbit α-phospho-Stat4 (Tyr693) was from Thermo Fisher Scientific.

Percoll was purchased from GE Healthcare and Dnase I from Sigma-Aldrich. Complete T cell medium [RPMI-1640 supplemented with 10% (v/v) FBS, L-glutamine (2 mM), penicilin (100 units/ml)/streptomycin (100 μg/ml), non-essential amino acids, Na-pyruvate (1 mM), HEPES (10 mM) and 2- mercaptoethanol (50 μM)] was purchased from Thermo Fisher Scientific. Dulbecco-Phosphate Buffered Saline (D-

PBS), Dulbecco's Modified Eagle's Medium (DMEM) and Hank's Buffered Salt Solution (HBSS) were from Thermo Fisher Scientific and collagenase IV from Worthington.

Flow cytometry

Single cell suspensions from thymus, spleen, inguinal lymph nodes, pancreatic lymph nodes and mesenteric lymph nodes were obtained by gently pressing the tissue through a 40 μM FALCON cell strainer (BD Biosciences). Erythrocytes were removed by incubating cells with Red Blood Cell Lysing Buffer Hybri-Max™ for 7 min at room temperature. Cells counts were determined with the Z2-Coulter Counter (Beckman Coulter). For surface staining, cells ($1x10^6$/10μl) were resuspended in D-PBS supplemented with 2% (v/v) FBS and stained in 96-well microtiter plates (BD Falcon™) for 20 min on ice. Cells were washed twice and resuspended in D-PBS/2% FBS and analysed using a LSRII, Fortessa, Symphony (BD Biosciences) or Cyan™ ADP (Beckman Coulter) or purified using an Influx sorter (BD Biosciences). Data was analysed using FlowJo v8.7, v9.4 or v10.2 (Tree Star Inc.) software.

For detection of intracellular FoxP3, the Foxp3/Transcription Factor Staining Buffer set (eBioscience) was used according to the manufacturer's instructions. For the detection of intracellular cytokines, the BD Cytofix/Cytoperm™ Kit according was used to the manufacturer's instructions. Serum cytokines were quantified with the BD Cytometric Bead Array (CBA) Mouse Inflammation Kit according the to the manufacturer's instructions.

Type I diabetes assessment

Glycosuria in was determined using Diastix Reagent Strips for Urinanalyis (Bayer). Mice were scored as diabetic after two positive readings (urine glucose ≥55 mmol/l) 48 hours apart. This was confirmed by measuring blood glucose levels that were found to be greater that 18 mmol/l in each case. Pancreata were fixed in formalin and processed for histological analysis (hematoxylin and eosin: H&E) and scored for the degree of insulitis (grades 0-4). Grade 0 represents no infiltrate, grade 1 periductal accumulation of mononuclear cells, grade 2 circumferential accumulation of mononuclear cells, grade 3 intra-islet infiltration, and grade 4 severe structural derangement and complete β cell loss.

Isolation of pancreas-infiltrating T cells

Pancreata from 5 week old pre-diabetic *Lck*-Cre*;Ptpn2*$^{fl/fl}$.NOD and *Ptpn2*$^{fl/fl}$.NOD littermate mice were harvested and pancreatic lymph nodes removed. Pancreata were digested in DMEM containing 1 mg/ml collagenase IV (Worthington) at 37°C for 15 min and single cell suspensions processed for flow cytometry.

Adoptive transfer into NOD mice

$CD8^+CD44^{lo}CD62L^{hi}$ were purified from single cell suspension obtained from pooled splenocytes and lymph node cells from 5 week old prediabetic *Lck*-Cre*;Ptpn2*$^{fl/fl}$.NOD and *Ptpn2*$^{fl/fl}$.NOD littermate control mice using the naïve $CD8^+$ T cell Isolation Kit (Miltenyi Biotech) according the manufacturer's instructions with the autoMACS Separator (Miltenyi Biotech). Naïve $CD8^+CD44^{lo}CD62L^{hi}$ ($1x10^7$) were adoptively transferred into 28 day old female NOD mice and diabetes incidence monitored.

Serum immunoglobulins

Serum IgM was quantified with the mouse IgM ELISA Ready-SET-Go™ kit from eBioscience according to the manufacturer's instructions. To detect serum IgG subsets MaxiSorp ELISA plates (Nunc) were coated with goat α-mouse IgG (625 ng/ml) in ELISA coating buffer overnight at 4 ºC. Plates were washed five times and blocked in D-PBS supplemented with 3% (w/v) BSA for 2 h at room temperature. Serum (IgM 1:5 serial dilutions; IgG1, IgG2a, IgG2b and IgG3, 1:10 serial dilutions) was added and plates were incubated for 1 h at room temperature. Plates were washed five times and biotinylated α-IgM, α-IgG1, α-IgG2a (to detect α-IgG2c), α-IgG2b or α-IgG3 (1:1000) diluted in D-PBS/1% (w/v) BSA was added. Samples were incubated for 1 h at room temperature. Plates were washed five times and incubated with Streptavidin-HRP (1:10.000 in D-PBS/1% (w/v) BSA) for 30 min followed by seven washes. 3,3',5,5'-tetramethylbenzidine (TMB) was added and samples were incubated for 15 min at room temperature in the dark. Reaction was stopped with 0.5 M sulphuric acid and absorbance was read at 450 nM.

Serum insulin autoantibodies (IAA)

ELISA plates (Costar) were coated with human insulin (10 µg/ml, Actrapid, Novo Nordisk) overnight at 4°C. Plates were blocked with PBS supplemented with 2% BSA for 2 hours at room temperature and then incubated with sera from *Ptpn2*$^{fl/fl}$.NOD, *Lck*-Cre*;Ptpn2*$^{fl/fl}$.NOD and *Lck*-Cre*;Ptpn2*$^{fl/+}$.NOD or C57BL/6 mice (1:10 dilution) for 2 hours at room temperature. Plates were washed four times and further incubated with biotinylated anti–mouse IgG1 (AbCam, 1:10,000 dilution) for 30 min at room temperature. After four washes, Streptavidin-HRP (BioLegend) was added and plates were incubated for 15 min at room temperature. Plates were washed five times, TMB was added and absorbance was measured at 450 nm using a Polarstar (BMG Labtech)

microplate reader. Each sample was run in duplicates, and absorbance (450 nm) of the negative control sample (C57BL/6 serum) was subtracted from absorbance of test sample to calculate the true absorbance value for each test sample.

Statistical analysis

Statistical analysis was performed using the non-parametric using 2-tailed Mann-Whitney U Test or the Student's T-test. Statistical analyses on Kaplan-Meier estimates were performed using a Log-rank (Mantel-Cox) test with one degree of freedom. For all tests $p<0.05$ was considered as significant.

Animal ethics

All experiments were performed in accordance with the NHMRC Australian Code of Practice for the Care and Use of Animals. All protocols were approved by the Monash University School of Biomedical Sciences Animal Ethics Committee (Ethics number: MARP/2012/124) and the UK Home Office (project licence: PB3E4EE13).

RESULTS

PTPN2 deletion in T cells promotes autoimmune diabetes

To determine the extent to which PTPN2 deficiency in T cells might contribute to the development of type 1 diabetes we introduced the *Lck*-Cre and *Ptpn2*$^{fl/fl}$ loci from C57BL/6 mice (15) onto the NOD/Lt (NOD) genetic background by backcrossing for 11 generations. *Lck*-Cre;*Ptpn2*$^{fl/+}$.NOD mice were thereon bred with NOD wild type mice to produce the *Ptpn2*$^{fl/+}$.NOD and *Lck*-Cre;*Ptpn2*$^{fl/+}$.NOD offspring that were interbred. DNA from the 11th generation was genotyped for 597 single nucleotide polymorphisms (SNPs) by the Australian Genome Research Facility using iPLEX GOLD chemistry and the Sequenom MassArray spectrometer for SNP genotyping and analysed by the GeneChip Targeted Genotyping System Software. *Ptpn2*$^{fl/+}$.NOD mice had a contaminating C57BL/6-derived interval between and including *rs13483413* and *rs3656892* (~2 MB) that encompasses the floxed *Ptpn2* locus on chromosome 18, whereas *Lck*-Cre;*Ptpn2*$^{fl/+}$.NOD mice had an additional contaminating C57BL/6-derived interval on chromosome 15 between and including *rs13482618* and *rs13482719* (~28 MB) that encompasses the *Lck*-Cre transgene locus. These C57BL/6 intervals are unavoidable, but previous NODxC57BL/6 outcrosses have not identified *Idd* loci within these intervals (27).

Diabetes pathogenesis in NOD mice is characterized by severe insulitis followed by the CD4$^+$ and CD8$^+$ T cell-mediated destruction of insulin-producing β cells in the pancreatic islets of Langerhans. Previous studies have reported that female NOD mice first develop insulitis at 5 weeks of age and diabetes at approximately 90 days after birth, with 46% of mice having diabetes by 150 days of age (28), although this can be influenced by diet and the gut microbiome (29). By contrast, male mice are significantly less susceptible, with a much lower frequency of diabetes (28). We monitored for

diabetes in female and male *Ptpn2*$^{fl/fl}$.NOD and *Lck*-Cre*;Ptpn2*$^{fl/fl}$.NOD mice (**Fig. 1a-b**). The onset of diabetes (urine glucose > 55 mM; blood glucose > 18 mM) was markedly accelerated in both female and male *Lck*-Cre;*Ptpn2*$^{fl/fl}$.NOD mice. Female *Lck*-Cre;*Ptpn2*$^{fl/fl}$.NOD mice started to develop diabetes at 36 days of age with all *Ptpn2*-deficient mice succumbing by 87 days (**Fig. 1a**). Similarly, type 1 diabetes onset in male *Lck*-Cre;*Ptpn2*$^{fl/fl}$.NOD mice occurred at 29 days with 100% of mice developing diabetes by 108 days (**Fig. 1b**). By contrast neither male nor female *Ptpn2*$^{fl/fl}$.NOD littermate mice or parental NOD mice developed diabetes by 100 days of age. Histological analysis in 30-36 day old *Lck*-Cre;*Ptpn2*$^{fl/fl}$.NOD mice prior to the development of diabetes revealed advanced invasive insulitis [combined histological grades 3 (intra-islet infiltration) and 4 (structural derangement and complete β cell loss)] in 91% of islets with obliterative lesions and marked immune cell infiltrates (**Fig. 1c-d**). The immune infiltrates not only contained CD3$^+$ T cells, but also B220$^+$ B cells (**Fig. 1e**), that are thought to contribute to invasive insulitis and diabetes (30; 31). By contrast, invasive insulitis was only observed in ~2% of islets from 30-36 day old *Ptpn2*$^{fl/fl}$.NOD littermate controls, and islets exhibited minimal peri-insulitis (grade 1) and immune cell infiltration (grade 0; **Fig. 1c-d**). Thus PTPN2-deficiency in T cells in NOD mice markedly accelerates pancreatic islet destruction and the onset of diabetes in NOD mice.

The type 1 diabetes-associated non-coding *PTPN2* SNP rs1893217 is accompanied by a reduction in *PTPN2* mRNA in human CD4$^+$CD45RO memory T cells (9). Similarly, the Crohn's disease-associated non-coding *PTPN2* SNP rs2542151 that is also associated with early onset type 1 diabetes (4) is accompanied by a reduction in PTPN2 protein in primary human colonic lamina propria fibroblasts (11; 12). Thus, *PTPN2* might be haploinsufficient in the pathogenesis of autoimmune disorders such

as type 1 diabetes and Crohn's disease. We have previously shown that PTPN2-heterozygosity in *Lck*-Cre;*Ptpn2*$^{fl/+}$ mice results in an approximate 50% reduction of PTPN2 protein in T cells (18). We therefore monitored for the incidence of diabetes in *Ptpn2*$^{fl/fl}$.NOD and *Lck*-Cre;*Ptpn2*$^{fl/+}$.NOD mice (**Fig. 1a-b**). Although delayed relative to homozygous mice, the onset of diabetes was accelerated in female *Lck*-Cre;*Ptpn2*$^{fl/+}$.NOD mice when compared to their *Ptpn2*$^{fl/fl}$.NOD littermate controls. Moreover, although male *Ptpn2*$^{fl/fl}$.NOD control mice did not develop diabetes, even at 250 days of age, male *Lck*-Cre;*Ptpn2*$^{fl/+}$.NOD heterozygous mice began to develop diabetes by 113 days of age. Histological analysis in 84-105 day old *Lck*-Cre;*Ptpn2*$^{fl/+}$NOD mice revealed invasive insulitis in 50% of islets (**Fig. 1f-g**) whereas in *Ptpn2*$^{fl/fl}$.NOD mice invasive insulitis was only evident in 8% of islets. These results emphasise the importance of PTPN2 in T cell tolerance and suggest that even partial PTPN2 deficiency in T cells might be sufficient to accelerate the onset of diabetes in autoimmune-prone individuals.

Increased T helper type 1 and cytotoxic T cells in pancreata.

T cell responses to pro-insulin occur early in the pathogenesis of type 1 diabetes in NOD mice and are required for the development of immune responses to other antigens (32). Dendritic cells and insulin-specific CD4$^+$ T cells cross-prime CD8$^+$ T cells to elicit cytotoxic responses and the destruction of β cells (33). We have shown previously that PTPN2-deficiency in CD8$^+$ T cells negates the need for cross-priming and drives the differentiation and activation of effector T cells and overt responses to self-antigen (15; 18; 19). C57BL/6 mice deficient for PTPN2 in T cells progressively develop an effector/memory (CD44^{hi}CD62L^{lo}) T cell phenotype and autoimmunity with age (15). In *Lck*-Cre;*Ptpn2*$^{fl/fl}$.NOD mice, we noted a significant increase in

effector/memory ($CD44^{hi}CD62L^{lo}$) $CD4^+$ and $CD8^+$ T cells in the spleens and lymph nodes of 36 day old *Lck*-Cre*;Ptpn2*$^{fl/fl}$.NOD mice prior to the development of diabetes (**Fig. 2a-b**). We also noted a significant increase in effector/memory $CD4^+$ and $CD8^+$ T cells in the pancreatic draining lymph nodes of pre-diabetic *Lck*-Cre;*Ptpn2*$^{fl/fl}$.NOD mice (**Fig. 2c**). Notably, although hardly any $CD4^+$ or $CD8^+$ T cells were in the pancreata of age-matched *Ptpn2*$^{fl/fl}$.NOD littermates, the early onset diabetes in *Lck*-Cre;*Ptpn2*$^{fl/fl}$.NOD mice was preceded by the marked infiltration of $CD44^{hi}CD62L^{lo}$ $CD4^+$ and $CD8^+$ T cells (**Fig. 2d**). Previous studies have established that the initiation of diabetes in NOD mice is reliant on the presence of activated insulin-specific $CD4^+$ T_{H1} cells that produce IFNγ and allow for the licensing of antigen-specific $CD8^+$ T cells (34; 35). We found that the $CD44^{hi}CD62L^{lo}$ $CD4^+$ T cells infiltrating the pancreata of *Lck*-Cre;*Ptpn2*$^{fl/fl}$.NOD mice were enriched for $CD4^+$ T_{H1} ($IFN\gamma^{hi}$) cells (**Fig. 2e**). Consistent with previous studies (36) we found that PTPN2-deficient naive $CD4^+$ T cells differentiated more efficiently into T_{H1} cells *ex vivo*, even in the absence of IL-12, consistent with this being a cell intrinsic effect (**Fig. S1a**). At least in part the enhanced T_{H1} differentiation may be due to the promotion STAT-1 signalling, which is required for T_{H1} commitment and differentiation (37). STAT-1 is a *bona fide* PTPN2 substrate (38) and PTPN2-deficiency was accompanied by heightened basal and IFN-γ-induced STAT-1 Y701 phosphorylation (**Fig. S1b**). By contrast IL-12-induced STAT-4 Y693 phosphorylation in activated $CD4^+$ T cells was unaltered by PTPN2-deficiency (**Fig. S1c**). In addition to the increased presence of T_{H1} cells we found that infiltrating $CD44^{hi}CD62L^{lo}$ $CD8^+$ T cells exhibited a terminally-differentiated and antigen-experienced $CD49d^{hi}KLRG1^{hi}CD127^-$ effector phenotype and expressed high levels of IFN-γ, a marker of cytotoxic $CD8^+$ T cells (**Fig. 2f**). Taken together, these results are consistent with PTPN2 deficiency increasing the activation and pathogenic conversion

of $CD4^+$ and $CD8^+$ T cells into T_{H1} and cytotoxic $CD8^+$ T cells respectively to mediate of destruction of pancreatic β cells.

To explore directly whether PTPN2 deficiency in $CD8^+$ T cells might subvert T cell tolerance to promote the $CD8^+$ T cell-mediated destruction of β cells, we adoptively transferred $CD8^+$ naïve ($CD44^{lo}CD62L^{hi}$) T cells from pre-diabetic *Ptpn2*$^{fl/fl}$.NOD versus *Lck*-Cre;*Ptpn2*$^{fl/fl}$.NOD mice into immunoreplete NOD hosts and monitored for diabetes (**Fig. 3**). Albeit delayed, diabetes was evident by 44 days in mice receiving PTPN2 deficient T cells with 86% of mice succumbing by 172 days post-adoptive transfer (**Fig. 3a**). By contrast the onset of diabetes was not altered in control mice and diabetes was not evident until 117 days of age (**Fig. 3a**). Histological analysis at 120 days post-adoptive transfer revealed that *Lck*-Cre;*Ptpn2*$^{fl/fl}$ $CD8^+$ T cell recipients exhibited increased invasive insulitis accompanied by marked lymphocytic infiltrates in islets (**Fig. 3b-c**). These results are consistent with the accelerated onset of diabetes in *Lck*-Cre;*Ptpn2*$^{fl/fl}$.NOD mice being largely, albeit not completely, due to perturbations in peripheral $CD8^+$ T cell tolerance to self-antigens.

Increased FoxP3+ regulatory T cells in PTPN2-deficient mice

$CD4^+FoxP3^+$ regulatory T cells (T_{reg}) prevent autoimmunity by suppressing the activity of autoreactive $CD8^+$ T cells that escape negative selection in the thymus (39). SNPs in genes encoding IL-2 and the IL-2 receptor (CD25) that are required for T_{reg} generation have been linked with various autoimmune diseases, including type 1 diabetes (2). We therefore assessed whether the accelerated onset of type 1 diabetes evident in *Lck*-Cre;*Ptpn2*$^{fl/fl}$.NOD mice might arise through the suppression of T_{reg} development or function (**Fig. 4**). Consistent with our previous studies in C57BL/6 mice (15; 17), we found that PTPN2-deficiency in pre-diabetic 5-week old *Lck*-

Cre;*Ptpn2*$^{fl/fl}$.NOD mice was accompanied by increased thymic (CD25^{-}FoxP3^{+} and CD25^{+}FoxP3^{+}) T_{regs} (**Fig. 4a**). Moreover, PTPN2 deficiency was accompanied by increased proportion of total (**Fig. 4b**), resting (CD44^{hi}CD62L^{hi}) and effector (CD44^{hi}CD62L^{lo}) CD25^{+}FoxP3^{+} T_{regs} in the pancreata and corresponding draining lymph nodes (panLN) (**Fig. 4c-d**) with the increase in T_{regs} in pancreata approximating the increase in effector/memory CD4+ T cells (**Fig. 4e**); total and effector T_{regs} trended higher also in the spleen and inguinal lymphoid tissues (**Fig. S2a**). T_{regs} expand and acquire an activated effector phenotype in response to cognate antigens in the context of infection and autoimmunity (40). PTPN2-deficiency not only increased the proportion of effector T_{regs}, but also increased the number of resting and effector T_{regs} in pancreata and panLNs. By contrast antigen-independent TGFβ/IL-2-induced CD25^{+}FoxP3^{+} T_{reg} generation *ex vivo* was not altered by PTPN2-deficiency (**Fig. S2b**). These findings are consistent with PTPN2 deficiency increasing the antigen-induced expansion and differentiation of T_{regs} *in vivo*. To determine whether PTPN2 deficiency might abrogate T_{reg} function we isolated splenic CD25^{hi} T_{regs} from *Ptpn2*$^{fl/fl}$.NOD versus *Lck*-Cre;*Ptpn2*$^{fl/fl}$.NOD mice and assessed their capacity to repress the TCR-mediated expansion of CD25^{lo} CD4+ T cells *ex vivo* (**Fig. 4f**). We found that *Lck*-Cre;*Ptpn2*$^{fl/fl}$ T_{regs} were just as efficient as *Ptpn2*$^{fl/fl}$ T_{regs} in repressing the α-CD3ε-induced expansion of CD4^{+} T cells. Therefore, the accelerated onset of diabetes in *Lck*-Cre;*Ptpn2*$^{fl/fl}$.NOD mice cannot be attributed to diminished T_{reg} development or function.

Increased CD4^{+} T follicular helper cells and B cells in PTPN2-deficient mice

Our studies indicate that PTPN2 deficiency in T cells not only promotes the infiltration of cytotoxic CD8^{+} and CD4^{+} T cells into the pancreata of NOD mice, but

also the recruitment of $B220^+$ B cells, which promote the expansion and activation of T cells targeting β cell antigens (41). Therefore, another mechanism by which PTPN2 deficiency might influence disease progression in NOD mice is through effects on the B cell compartment. $CD4^+$ T_{FH} cells are required for the promotion of B cell maturation and the production of antigen-specific antibodies (42). Previously, we reported that the inducible deletion of *Ptpn2* in the hematopoietic compartment of adult C57BL/6 mice was accompanied by the marked expansion of T_{FH} cells *ex vivo* (22). To explore the mechanisms by which PTPN2 deficiency in T cells might result in increased B cell pancreatic infiltrates, we investigated the presence of T_{FH} cells and germinal centre B cells in the pancreatic draining lymph nodes of 5 week old *Lck*-Cre;*Ptpn2*$^{fl/fl}$.NOD mice. T cell PTPN2 deficiency in NOD mice resulted in a striking increase in $CD4^+CD44^{hi}CXCR5^{hi}PD\text{-}1^{hi}$ T_{FH} cells in the spleens, inguinal lymph nodes and pancreatic draining lymph nodes (**Fig. 5a)**. In keeping with T_{FH} cell expansion we found that $B220^+GL\text{-}7^{hi}Fas^{hi}$ germinal centre B cells were also markedly increased in spleens, inguinal lymph nodes and pancreatic draining lymph nodes (**Fig. 5b)**. Germinal centre B cell expansion and class switching from IgM to IgG are reliant on interactions with germinal centre T_{FH} cells. Consistent with this we found that circulating IgM and IgG (IgG1, IgG2c, IgG2b, IgG3) were increased in pre-diabetic *Lck*-Cre;*Ptpn2*$^{fl/fl}$.NOD mice (**Fig. 5c)**. Importantly we found that circulating insulin autoantibodies (IAA) were elevated in 5 week old *Lck*-Cre;*Ptpn2*$^{fl/fl}$.NOD mice as well as in 12-15 week old *Lck*-Cre;*Ptpn2*$^{fl/+}$.NOD mice (**Fig. 5d**). These results indicate that T cell PTPN2 deficiency in NOD mice might influence disease progression, not only through effects on $CD8^+$ T cell tolerance, but also through the $CD4^+$ T_{FH}-mediated expansion and maturation of B cells and the production of autoantibodies.

Autoimmune co-morbidities in PTPN2-deficient mice

In addition to type 1 diabetes, NOD mice spontaneously develop autoimmune co-morbidities, including sporadic lymphoid infiltrates in the thyroid gland, mimicking Hashimoto's thyroiditis (43) and sporadic lymphoid infiltrates in the salivary and lacrimal glands, resembling the human autoimmune disorder known as Sjögren's Syndrome (44). Moreover, young NOD mice can develop subclinical colitis after weaning due to defective tolerance to commensal gut bacteria (45). Such autoimmune co-morbidities are also seen in a subset of patients with type 1 diabetes (46). Previous studies have shown that PTPN2-deficiency in T cells or the haematopoietic compartment of C57BL/6 mice promotes systemic inflammation and autoimmunity accompanied by lymphocytic infiltrates in non-lymphoid tissues such as liver, lung, skin and kidney (15; 22). We therefore assessed the impact of PTPN2 deficiency on the development of systemic inflammation and autoimmunity in pre-diabetic 5-6 week old *Lck*-Cre*;Ptpn2*$^{fl/fl}$.NOD mice. PTPN2 deficiency in T cells was accompanied by systemic inflammation as reflected by the increase in circulating pro-inflammatory cytokines TNFα and IL-6 (**Fig. 6a)** and the infiltration of lymphocytes, including B220^{+} B cells and CD3^{+} T cells into the submandibular salivary glands (**Fig. 6b)**. In these young pre-diabetic mice, lymphocytic infiltrates were not evident in liver, lung, kidney or joints and there was no evidence of inflammatory arthritis (**Fig. S3a-b**). Strikingly however, we found marked lymphocytic infiltrates in the colons of pre-diabetic *Lck*-Cre*;Ptpn2*$^{fl/fl}$.NOD mice and the development of overt colitis as reflected by colon shortening (**Fig. 6c)**. The development of colitis was accompanied by an increased number of CD4^{+} and CD8^{+} effector/memory (CD44^{hi}CD62L^{lo}) T cells, T_{FH} cells (CD4^{+}CD44^{hi}CXCR5^{hi}PD-1^{hi}) and germinal centre B cells (B220^{+}GL-7^{hi}Fas^{hi}) in the mesenteric lymph nodes; no alterations were evident in CD4^{+}FoxP3^{+} T_{reg} cells (**Fig.**

7a). Moreover, colitis was accompanied by the accumulation of lamina propria $CD4^+$ and $CD8^+$ effector/memory T cells with T_{H1} and cytotoxic phenotypes respectively (**Fig. 7b-c)**, as assessed by their ability to produce IFNγ *ex vivo* (**Fig. 7c**). No significant differences were evident in the proportion of lamina propria $CD4^+$ T_{H17} cells (**Fig. 7c**) and T_{H17} generation *ex vivo* was not affected by PTPN2-deficiency (**Fig. S4a**). Similarly, intraepithelial and lamina propria $TCR\gamma\delta^+$ T cells in the colons of *Lck*-Cre*;Ptpn2*$^{fl/fl}$.NOD mice were not altered (**Fig. S4b**). These results are consistent with T cell PTPN2 deficiency in NOD mice driving the development of not only type 1 diabetes, but also other autoimmune/inflammatory disorders.

DISCUSSION

Many overlapping tolerance mechanisms exist that normally prevent self-reactive B and T cells from attacking the body during homeostatic processes and antimicrobial defence. Molecules that negatively regulate T cell and B cell signalling are fundamentally important for tuning T cell and B cell responses to prevent overt autoreactivity. PTPN2 negatively regulates TCR signalling to prevent overt autoreactivity to low-affinity self-peptide-MHC complexes (15; 18; 19). Here, we demonstrate that PTPN2 deficiency in T cells alone is sufficient to markedly exacerbate disease onset and severity of diabetes in autoimmune-prone NOD mice.

The development of type 1 diabetes in NOD mice is characterised by the infiltration of antigen-specific T_{H1}-polarised $CD4^+$ and cytotoxic $CD8^+$ T cells into pancreatic islets, resulting in the lymphocyte driven destruction of insulin-producing β-cells (25; 47). In this study we found that the transfer of naive PTPN2-deficient $CD8^+$ T cells alone into immunoreplete NOD hosts accelerated the onset and increased the incidence of type 1 diabetes. These findings are consistent with PTPN2-deficiency in CD8+ T cells enhancing TCR-instigated responses to self to promote autoimmunity. However, as the onset of diabetes in *Lck*-Cre*;Ptpn2*$^{fl/fl}$.NOD mice exceeded that in which PTPN2-deficient $CD8^+$ T cells alone had been transferred, it is probable that perturbations in other T cell subsets might also contribute to disease progression. We have shown previously that PTPN2-deficiency enhances the TCR-induced activation and differentiation of CD4+ T cells into effector/memory T cells (15; 19). Consistent with this, effector/memory $CD4^+$ T cells were increased in the pancreatic draining lymph nodes and the pancreata of *Lck*-Cre*;Ptpn2*$^{fl/fl}$.NOD mice. The recruitment and activation of $CD4^+$ T cells can contribute to the licencing and activation of $CD8^+$ T cells. However, as PTPN2-deficiency in $CD8^+$ T cells negates the need for $CD4^+$ T cell

help during antigen cross-presentation and permits the helper-independent acquisition of cytotoxic activity to self-antigens (19), we surmise that other $CD4^+$ T cell-dependent mechanisms may be more pertinent.

Spalinger et al (36) have reported that PTPN2 deficiency in $CD4^+$ T cells can enhance T_{H1} polarisation, but impair T_{reg} generation in dextran sodium sulfate or T-cell transfer colitis models. Consistent with this we also noted an overt T_{H1} polarisation in pre-diabetic *Lck*-Cre*;Ptpn2*$^{fl/fl}$.NOD mice. Pioneering work by Katz *et al*. (35) has shown that T_{H1} cells, but not T_{H2} cells are required for autoimmune diabetes, whereas a large number of studies have defined the importance of T_{H1} cell signature cytokine in promoting the development of insulitis and type 1 diabetes in NOD mice (48). Therefore, the enhanced T_{H1} polarisation in *Lck*-Cre*;Ptpn2*$^{fl/fl}$.NOD mice is likely to be an important contributor to the accelerated onset of type 1 diabetes. However, in contrast to the findings of Spalinger *et al*. (36) we found that thymic T_{regs} and effector-like T_{regs} in the pancreata and draining lymph nodes in *Lck*-Cre*;Ptpn2*$^{fl/fl}$.NOD mice were increased, rather than decreased. Consistent with this, previous studies have shown that PTPN2 dephosphorylates JAK-1/3 and STAT-5 and attenuates IL-2-induced STAT-5 signalling in thymocytes to suppress the generation of $FoxP3^+$ T_{regs} (49). Moreover we have shown previously that *Ptpn2* deletion in *Lck*-Cre;*Ptpn2*$^{fl/fl}$.C57BL/6 mice or *Mx1*-Cre;*Ptpn2*$^{fl/fl}$.C57BL/6 mice in which *Ptpn2* has been inducibly deleted increases the thymic generation of $CD25^+FoxP3^+$ T_{regs} and does not compromise T_{reg} function *ex vivo* (15; 22). In this study we reaffirmed PTPN2-deficiency does not compromise the suppressor properties of T_{regs} isolated from NOD mice. Therefore, defective T_{reg} generation/function is unlikely to contribute to the loss of tolerance in *Lck*-Cre*;Ptpn2*$^{fl/fl}$.NOD mice.

An additional mechanism by which PTPN2-deficient $CD4^+$ T cells may

facilitate the development of type 1 diabetes is by promoting the role of B cells in progression towards invasive insulitis and diabetes. Although B cells are not essential for the generation or effector function of islet-reactive T cells, they can contribute to the development of autoreactivity (41). Indeed anti-CD20-mediated B cell depletion (50), or the arrest of B cell maturation by disabling IgM production (30), protects against type 1 diabetes in NOD mice, whereas CD19 deletion on NOD B cells diminishes the expansion of β cell antigen-specific T cells (31). In humans, B cell-depletion with anti-CD20 can delay disease progression in patients with newly diagnosed type 1 diabetes (51). In our studies we found a marked infiltration of $B220^+$ B cells into the pancreatic islets of *Lck*-Cre*;Ptpn2*$^{fl/fl}$.NOD mice. This was accompanied by a significant increase in the number of germinal centre $B220^+$ B cells in lymphoid organs, including in the draining lymph nodes of the pancreas. The increase in germinal centre B cells was in turn accompanied by the expansion of $CD4^+$ T_{FH} cells in lymphoid organs. T_{FH} cells are required for the formation of germinal centres, the promotion of B cell proliferation and the production of antigen-specific antibodies (42). In this regard, we identified an increased humoral immunity in *Lck*-Cre*;Ptpn2*$^{fl/fl}$.NOD mice that was reflected by an increase in circulating IgM and IgG and circulating anti-insulin autoantibodies. Recent gene expression profiling in DO11;RIP-mOVA mice (a model of type 1 diabetes) has identified a T_{FH} cell gene signature in islet-specific T cells and has shown that transgenic DO11 T cells with a T_{FH} signature from diabetic animals transfer diabetes to RIP-OVA recipients (26). Importantly, increased T_{FH} cells are linked with the development of autoimmunity and T_{FH} cells are over-represented in patients with type 1 diabetes (26; 52). The production of T_{FH} cells and their homing to germinal centres is dependent on autocrine IL-21 that signals via JAK-1/3 and STAT-3 (53). STAT-3 is required for both T_{FH} and germinal

centre B cell differentiation and STAT-3 gain-of-function mutations that increase T_{FH} cells and autoantibody production may be associated with type 1 diabetes in humans (42; 54). Moreover, along with *Il2*, *Il21* is a candidate gene for the diabetes susceptibility locus *Idd3* and IL-21 signaling is required for diabetes development in NOD mice (55). JAK-1/3 and STAT-3 are *bona fide* substrates of PTPN2 (21) and the inducible hematopoietic deletion of *Ptpn2* in adult C57BL/6 mice is accompanied by heightened IL-21-induced STAT-3 signalling in T_{FH} cells *ex vivo* (22). Therefore, in addition to lowering the threshold for TCR-instigated responses to self-antigen by enhancing LCK signalling, PTPN2-deficiency may drive STAT-3 signalling in $CD4^+$ T cells to enhance the formation of T_{FH} cells and the maturation of B cells to facilitate disease progression in *Lck*-Cre*;Ptpn2*$^{fl/fl}$.NOD mice.

Beyond spontaneously developing type 1 diabetes, NOD mice also exhibit T cell-mediated autoimmunity against other tissues, including the thyroid gland and especially the lacrimal and salivary glands, mimicking Sjogren's syndrome (43; 44). Moreover, young NOD mice also develop low levels of colitis due to disturbed tolerance towards autologous commensal gut antigens (45). This is accompanied by an increase in activated $CD4^+$ T cells and T_{H17} cells in the colonic lamina propria (45). In addition to promoting type 1 diabetes, T cell-specific PTPN2 deficiency in NOD mice was accompanied by marked lymphocytic infiltrates in salivary glands and the formation of what resembled ectopic lymphoid-like structures that included T cells and B cells. Ectopic lymphoid-like structures develop at sites of inflammation, can form functional germinal centres and contribute to disease pathogenesis. Importantly ectopic lymphoid-like structures are seen in the salivary glands of patients with Sjogren's syndrome (56). Similarly, PTPN2 deficiency resulted in widespread immune infiltrates and the formation of lymphoid clusters, probably ectopic lymphoid-like structures that

are a pathologic hallmark of inflammatory bowel disease, including Crohn's disease (57). Consistent with this, lymphocytic infiltrates in mesenteric lymph nodes included not only $CD44^{hi}CD62L^{lo}$ CD4+ T cells, but also T_{FH} cells and germinal centre B cells. In addition, there was a marked infiltration of activated/cytotoxic $CD8^+$ T cells and $CD4^+$ T cells with a predominant T_{H1} phenotype in the lamina propria of *Lck-Cre;Ptpn2*$^{fl/fl}$.NOD mice. Although previous studies have found that in dextran sodium sulfate or T-cell transfer-induced colitis models PTPN2 deficiency resulted in the induction of both T_{H1} and T_{H17} cells and the impaired induction of T_{reg} cells (36), we found that T_{H17} and T_{reg} cells were unaltered in the colons of 5 week-old pre-diabetic mice and the generation of T_{H17} and T_{reg} cells *ex vivo* was not affected by PTPN2-deficiency. Nonetheless, we cannot exclude the possibility that such alterations may occur as disease progresses.

The findings of our study underscore the importance of PTPN2 in tuning T cell responses for the maintenance of T cell tolerance and prevention of autoimmunity. Moreover, as PTPN2 deficiency in T cells alone exacerbated the development of both type 1 diabetes and colitis in NOD mice, our results argue for perturbations in T cell function being causally involved in the early onset type 1 diabetes and Crohn's associated with *PTPN2* loss of function SNPs in humans. Importantly, the findings of this study suggest that beyond driving type 1 diabetes or Crohn's disease, PTPN2 loss of function SNPs in humans might also contribute to the development of associated co-morbidities.

AUTHOR CONTRIBUTIONS

Conceptualization –F.W. and T.T.; Methodology, F.W., T.B., P.K.G., Y.L., G.W.J., D.Y., A.B., S.A.J., T.K., and T.T., ; Investigation – F.W., T.B., P.K.G., Y.L., and G.W.J.. Writing – Original Draft, F.W. T.B., and T.T.; Writing – Review & Editing, F.W., T.B., P.K.G., Y.L., G.W.J., D.Y., A.B., S.A.J., T.K., and T.T.,; Funding Acquisition, T.T.

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“Dr. Tony Tiganis is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis."

CONFLICTS OF INTERTEST

None declared.

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FIGURE LEGENDS

Figure 1. PTPN2-deficiency in T cells promotes the early onset of type I diabetes in Lck-Cre;Ptpn2$^{fl/fl}$.NOD mice. **a-b)** Female and male *Ptpn2$^{fl/fl}$*.NOD, *Lck-Cre;Ptpn2$^{fl/fl}$*.NOD, *Lck-Cre;Ptpn2$^{fl/+}$*.NOD and NOD/Lt mice were monitored for survival and diabetes incidence (urine glucose ≥ 55 mmol/l; blood glucose > 18 mmol/l). **c)** Pancreata from 5 week old female prediabetic *Ptpn2$^{fl/fl}$*.NOD and *Lck-Cre;Ptpn2$^{fl/fl}$*.NOD mice were fixed in formalin and processed for histological assessment (hematoxylin and eosin: H&E) and **d)** the severity of insulitis was determined histologically. **e)** Formalin fixed pancreata were processed for immunohistochemistry staining for B220 and CD3. **f)** Pancreata from 12-15 week old female prediabetic *Ptpn2$^{fl/fl}$*.NOD and *Lck-Cre;Ptpn2$^{fl/+}$*.NOD mice were fixed in formalin and processed for histological assessment and **g)** the severity of insulitis was determined histologically. Results in (a-e) are representative of two independent experiments. The percent of islets graded 0-4 and those with invasive insulitis (grades 3 and 4) in (d) and (g) were determined for the indicated number of mice. Significant differences in invasive insulitis (means ± SEM) were determined using -tailed Mann-Whitney *U* test (non-parametric); **p < 0.01. Statistical analyses on Kaplan-Meier estimates in (a-b**) (*p < 0.05, **p < 0.01, ****p < 0.0001) were performed using a Log-rank (Mantel-Cox) test with one degree of freedom.

Figure 2. Increased effector/memory T cells and T_{H1} cells in Lck-Cre;Ptpn2$^{fl/fl}$.NOD mice. Lymphocytes from 5 week old female prediabetic *Ptpn2$^{fl/fl}$*.NOD and *Lck-Cre;Ptpn2$^{fl/fl}$*.NOD mice were harvested from **a)** spleen, **b)** inguinal lymph nodes (LN), **c)** pancreatic lymph nodes (panLN) and **d-f**) pancreata and **a-d**) stained for CD4, CD8, CD62L and CD44 or **e**) were stimulated with 20 ng/ml PMA/ 1 µg/ml Ionomycin (4 h)

in the presence of Brefeldin A/Monensin, fixed and then permeabilised and stained for CD4, CD8, IL-4, IFN-γ and analysed by flow cytometry. **f)** Pancreatic lymphocytes were stained for CD8, CD62L, CD44, CD49d, KLRG1 and CD127 and analysed by flow cytometry. Representative contour-plots in (a, e, f) are shown. Quantified results are means ± SEM for the indicated number of mice and are representative of two independent experiments. In (a-f) significance was determined using 2-tailed Mann-Whitney U Test; *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 3. Adoptive transfer of PTPN2-deficient naive CD8$^+$ T cells promotes type 1 diabetes in NOD mice. **a)** 1x10^7 naïve CD8$^+$ T cells (CD44^{lo}CD62L^{hi}) from 5 week old female prediabetic *Ptpn2$^{fl/fl}$*.NOD and *Lck*-Cre*;Ptpn2$^{fl/fl}$*.NOD mice were adoptively transferred into replete female NOD hosts, which were monitored for survival and diabetes incidence (urine glucose ≥55 mmol/l). **b)** Pancreata were fixed in formalin and processed for histological assessment and the severity of insulitis determined for the indicated number of mice. **c)** Pancreata from naïve CD8+ T cell recipient NOD mice from (b) were fixed in formalin and processed for histological assessment.

Figure 4. Increased regulatory T cells in Lck-Cre;Ptpn2$^{fl/fl}$.NOD mice. **a)** Thymocytes from 5 week old female prediabetic *Ptpn2$^{fl/fl}$*.NOD and *Lck*-Cre*;Ptpn2$^{fl/fl}$*.NOD mice were stained for CD4, CD25 and intracellular FoxP3 and the numbers of CD4$^+$FoxP3$^+$CD25$^{hi/lo}$ T cells were quantified by flow cytometry. **b)** PanLN cells and pancreatic lymphocytes from 5 week old female prediabetic *Ptpn2$^{fl/fl}$*.NOD and *Lck*-Cre*;Ptpn2$^{fl/fl}$*.NOD mice were stained for CD4 and intracellular FoxP3 and the numbers of CD4$^+$FoxP3$^+$ T cells were quantified by flow cytometry. **c-**

d) PanLN cells and pancreatic lymphocytes from 5 week old female prediabetic *Ptpn2*$^{fl/fl}$.NOD and *Lck*-Cre;*Ptpn2*$^{fl/fl}$.NOD mice were stained for CD4, CD25, CD62L, CD44 and intracellular FoxP3 and the numbers of central-resting (CD44^{hi}CD62L^{hi}) and effector-like (CD44^{hi}CD62L^{lo}) T cells were quantified by flow cytometry. **e)** Ratios of pancreatic CD4^{+}FoxP3^{+} T_{regs} and CD4^{+}CD44^{hi}CD62L^{lo} effector/memory (Eff/mem) T cells were determined by flow cytometry. **f)** FACS-purified naïve CD4^{+}CD25^{lo} lymph node T cells (5x10^{4}) from 5 week old female pre-diabetic NOD mice were stained with CTV (Cell Tracker Violet) and incubated with α-CD3 (1 μg/ml) in the presence of irradiated accessory splenocytes and FACS-purified CD4^{+}CD25^{hi} T_{regs} (5x10^{4}-0.625x10^{4}) from 5 week old female pre-diabetic *Ptpn2*$^{fl/fl}$.NOD and *Lck*-Cre;*Ptpn2*$^{fl/fl}$.NOD mice. After 72 h proliferation was assessed by flow cytometry monitoring for CTV dilution. Representative contour-plots in and representative histograms in are shown. Quantified results are means ± SEM for the indicated number of mice and are representative of two independent experiments. In (a-d) significance was determined using 2-tailed Mann-Whitney U Test; *p < 0.05; **p < 0.01.

Figure 5. Enhanced T_{FH} and GC B cells in Lck-Cre;Ptpn2$^{fl/fl}$.NOD mice. **a-b)** Lymphocytes were harvested from spleen, inguinal lymph nodes and panLNs from 5 week old female prediabetic *Ptpn2*$^{fl/fl}$.NOD and *Lck*-Cre;*Ptpn2*$^{fl/fl}$.NOD mice were stained for **a)** CD4, CD44, CXCR5 and CD274 (PD-1) mice to identify CD4^{+}CD44^{hi}CXCR5^{hi}PD-1^{hi} follicular helper (T_{fh}) T cells or **b)** B220, GL-7 and CD95 (Fas) to identify B220^{+}GL-7^{hi}Fas^{hi} germinal centre (GC) B cells and analysed by flow cytometry. **c)** Serum immunoglobulins from 5 week old female prediabetic *Ptpn2*$^{fl/fl}$.NOD and *Lck*-Cre;*Ptpn2*$^{fl/fl}$.NOD mice and **d)** serum IAA (insulin autoantibodies) from 5 week old female prediabetic *Ptpn2*$^{fl/fl}$.NOD and *Lck*-

Cre;*Ptpn2*$^{fl/fl}$.NOD mice and 12 week old female prediabetic *Ptpn2*$^{fl/fl}$.NOD and *Lck*-Cre;*Ptpn2*$^{fl/+}$.NOD mice were determined by ELISA. Representative contour-plots in (a, b) are shown. Quantified results are means ± SEM for the indicated number of mice and are representative of two independent experiments. In (a-d) significance was determined using 2-tailed Mann-Whitney U Test; **p < 0.01, ***p < 0.001.

Figure 6. Type 1 diabetes in Lck-Cre;Ptpn2$^{fl/fl}$.NOD mice is accompanied by systemic inflammation and autoimmunity. **a)** Serum cytokines in 5 week old female prediabetic *Ptpn2*$^{fl/fl}$.NOD and *Lck-Cre;Ptpn2*$^{fl/fl}$.NOD mice were determined by flow cytometry using a BD Cytokine Bead Array (BD Biosciences). **b)** Salivary glands from 5 week old female prediabetic *Ptpn2*$^{fl/fl}$.NOD and *Lck-Cre;Ptpn2*$^{fl/fl}$.NOD mice were fixed in formalin and processed for histological assessment (H&E and immunohistochemistry staining for B220 and CD3). **c)** Colons from 5 week old female prediabetic *Ptpn2*$^{fl/fl}$.NOD and *Lck*-Cre;*Ptpn2*$^{fl/fl}$.NOD mice were assessed for length and processed for histology. Quantified results are means ± SEM for the indicated number of mice and are representative of two independent experiments. In (a, c) significance was determined using 2-tailed Mann-Whitney U Test; **p < 0.01.

Figure 7. Colitis in Lck-Cre;Ptpn2$^{fl/fl}$.NOD mice. Lymphocytes from 5 week old female prediabetic *Ptpn2*$^{fl/fl}$.NOD and *Lck*-Cre;*Ptpn2*$^{fl/fl}$.NOD mice harvested from **a)** mesenteric lymph nodes and **b-c)** lamina propia and were stained for **a, b)** CD4, CD8, CD62L and CD44 or **a)** CD4, CD44, CXCR5 and CD274 (PD-1) and B220, GL-7 and CD95 (Fas) and analysed by flow cytometry. **c**) Lamina propria lymphocytes were stimulated with 20 ng/ml PMA/1 µg/ml Ionomycin (4 h) in the presence of Brefeldin A/Monensin, fixed and then permeabilised and stained for CD4, CD8, IFN-γ and IL-

17A and analysed by flow cytometry. Representative contour-plots or dot-plots in (b, c) are shown. Quantified results are means ± SEM for the indicated number of mice and are representative of two independent experiments. In (a-c) significance was determined using 2-tailed Mann-Whitney U Test; *$p < 0.05$, **$p < 0.01$.

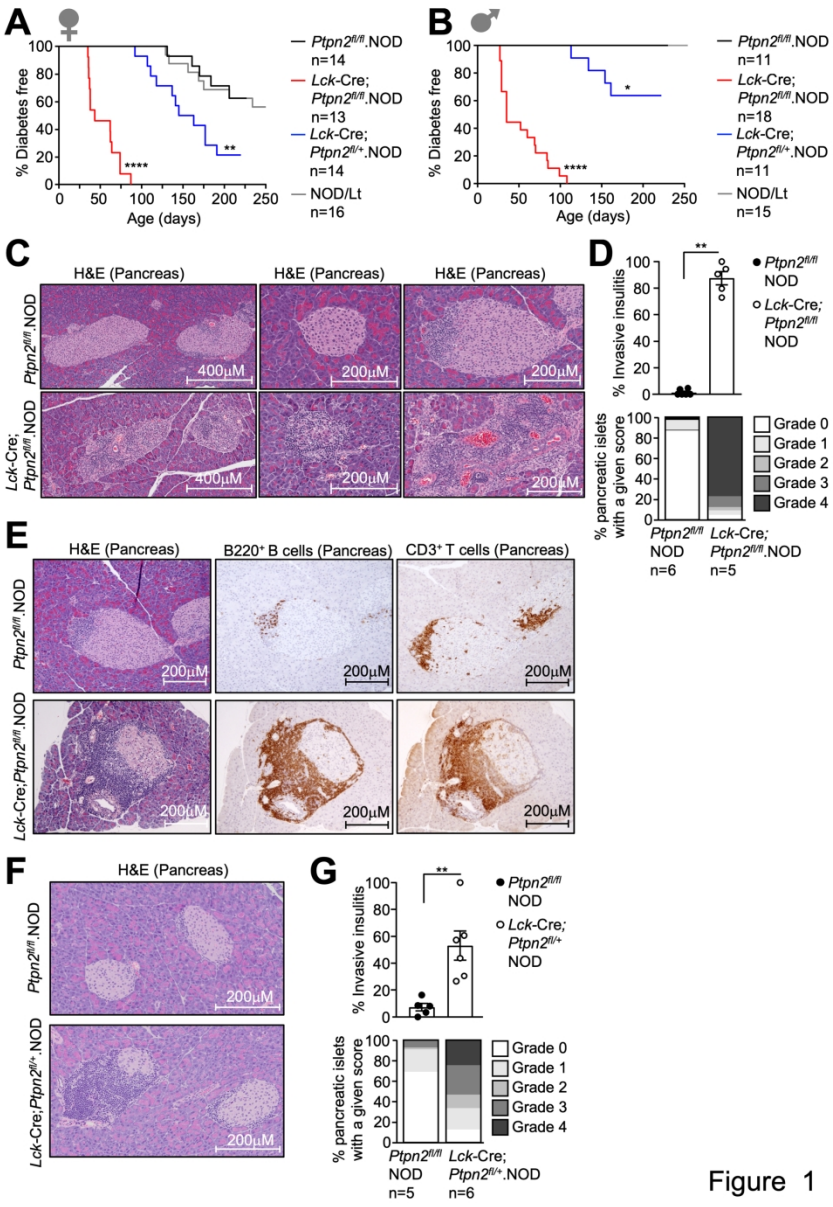

Figure 1

Figure 1

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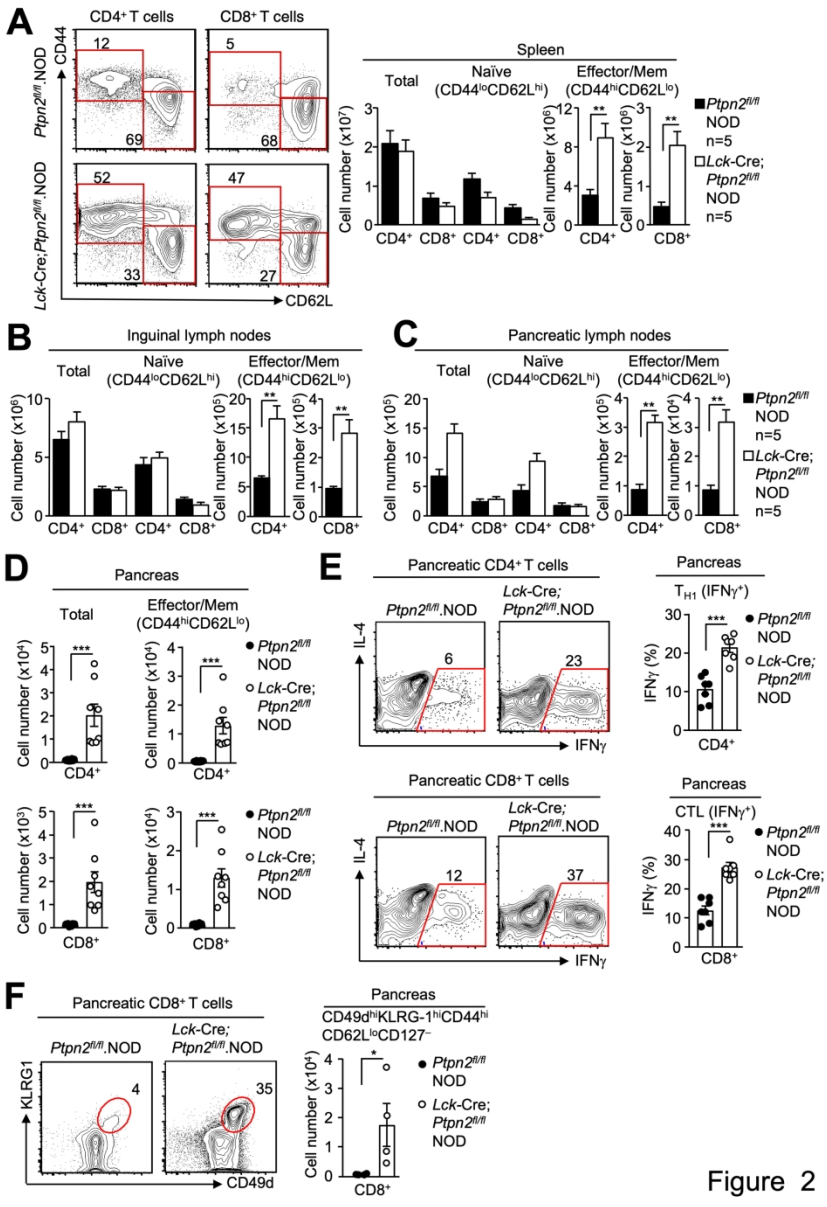

Figure 2

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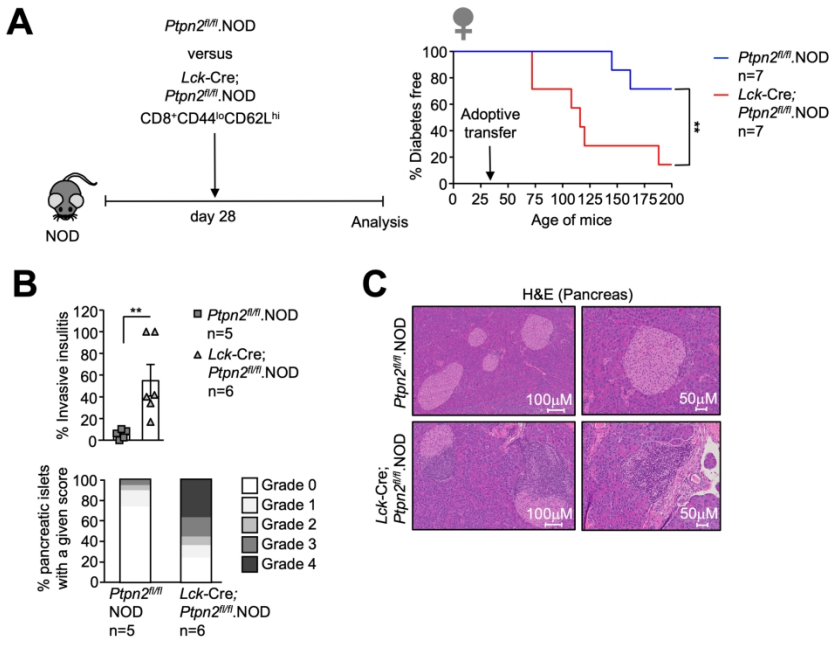

Figure 3

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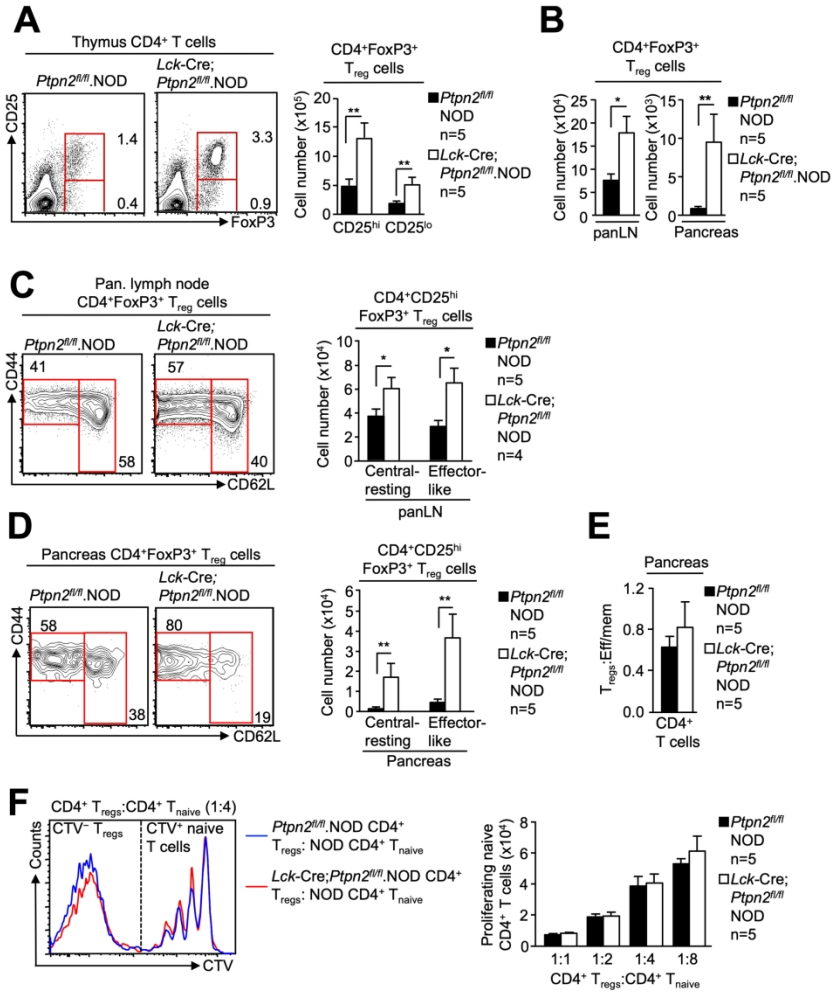

Figure 4

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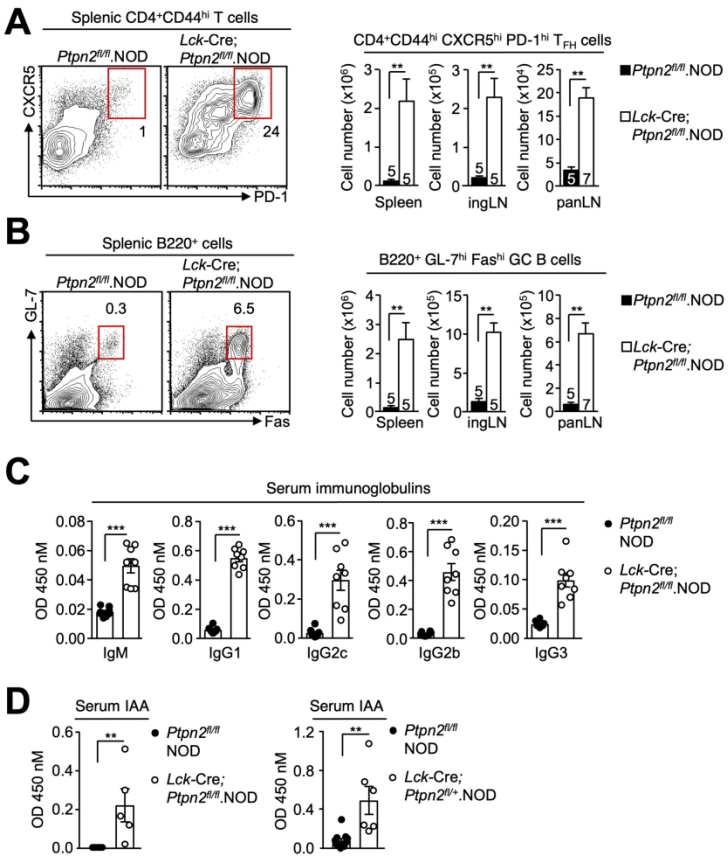

Figure 5

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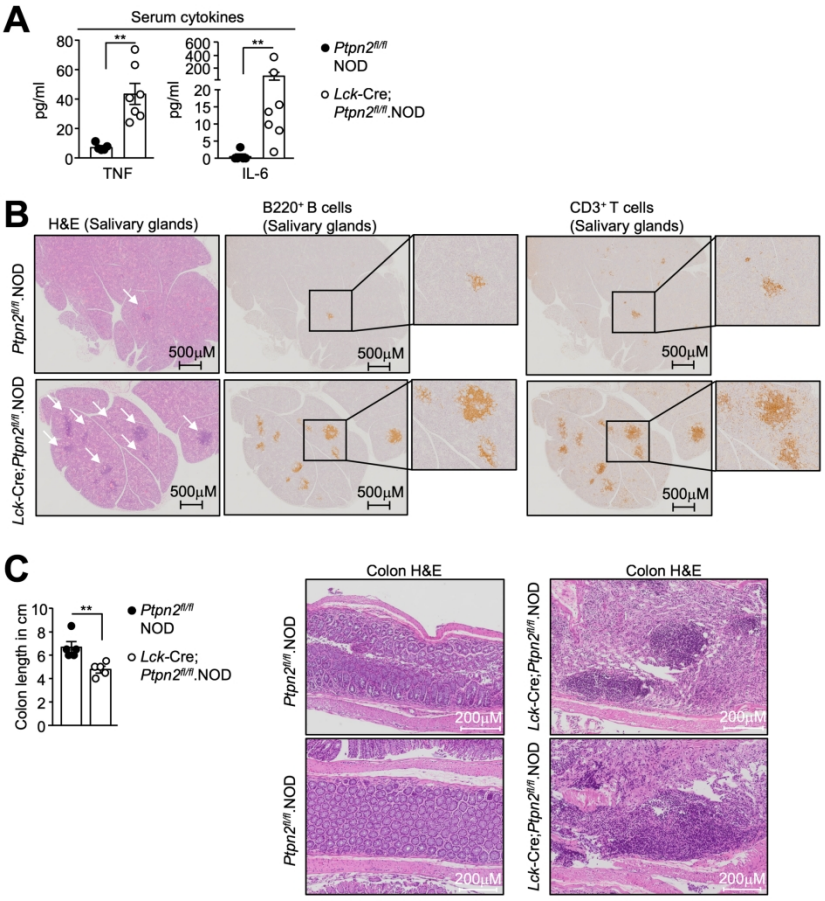

Figure 6

Figure 6

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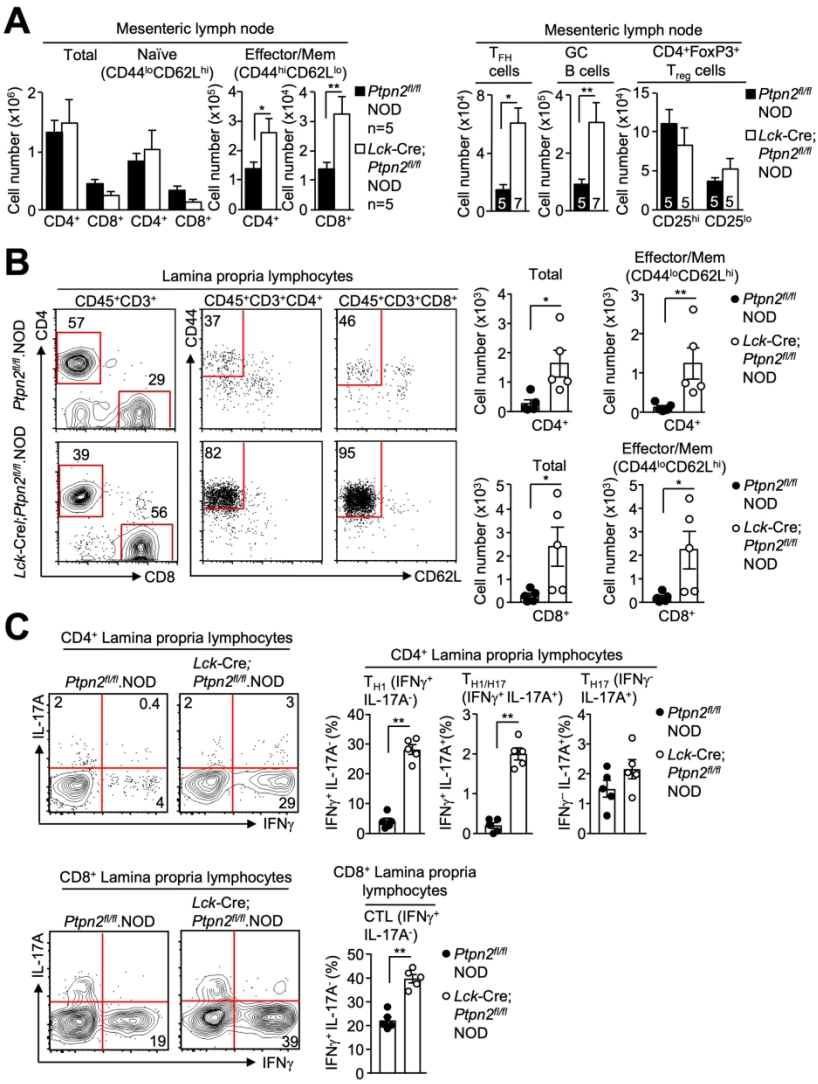

Figure 7

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SUPPLEMENTAL MATERIALS

T cell-specific PTPN2-deficiency in NOD mice accelerates the development of type 1 diabetes and autoimmune co-morbidities.

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Generation of Lck-Cre;Ptpn2$^{fl/fl}$.NOD mice

Lck-Cre*;Ptpn2$^{fl/fl}$*.C57BL/6J mice (1; 2) were backcrossed onto the NOD/Lt genetic background (3) for 11 generations. DNA samples were extracted from tail biopsies and genotyped by standard PCR using oligos specific for the *Ptpn2* floxed allele (*forward primer: 5' GAA TTC CAG GAC AGC CAA GG 3'*; *reverse primer: 5' CTG CTC TTA AAG GGG ATC AGG 3'*) and the Cre transgene (*forward primer: 5' ATG TCC AAT TTA CTG ACC 3'*; *reverse primer: 5' CGC CGC ATA ACC AGT GAA AC 3'*). Amplified products were visualized by gel electrophoresis to distinguish the *Ptpn2* floxed allele (size = 1300 bp) and *wild-type* allele (1100 bp) or the *Lck*-Cre transgene (350 bp). A genome-wide screen was performed by the Australian Genome Research Facility using the iPLEX GOLD chemistry and the Sequenom MassArray spectrometer for SNP genotyping. Data was analyzed using the GeneChip Targeted Genotyping System Software. 11th generation backcrossed NOD mice heterozygous for the *Ptpn2* floxed allele and positive for the *Lck*-Cre transgene were of the NOD genotype across the whole genome except for those markers encompassing the C57BL/6-derived *Ptpn2* floxed allele (chromosome 18) and the C57BL/6-derived *Lck*-Cre transgene (chromosome 15).

Flow cytometry

The following antibodies from BD Biosciences, eBioscience or BioLegend were used for flow cytometry: Phycoerythrin (PE) or peridinin-chlorophyll cyanine 5.5 (PerCP-Cy5.5)-conjugated CD3 (145-2C11); PerCP-Cy5.5 or phycoerythrin-cyanine 7 (PE-Cy7)-conjugated CD4 (RM4-5); Pacific Blue-conjugated (PB) or allophycocyanin-cyanine 7 (APC-Cy7)-conjugated CD8 (53-6.7); PE or APC-Cy7-conjugated CD25 (PC61); Fluorescein isothiocyanate (FITC) or V450-conjugated CD44 (IM7); APC-Cy7-conjugated CD45 (30-F11); APC or APC-Cy7-conjugated CD45R (B220; RA3-6B2); biotin-conjugated CD49d (9C10; MFR4.B); PE–cyanine 5 (PE-Cy5)–conjugated TCR-δ (GL3); PE-Cy7 or APC-

conjugated CD62L (MEL-14); PE-conjugated CD127 (SB/199); biotin-conjugated CD185 (CXCR5; 2G8); PE-Cy7-conjugated CD279 (PD-1, RMP1-14); APC-conjugated KLRG1 (2F1), PE or FITC-conjugated GL-7 (GL-7); PE-Cy7 or PE-conjugated IFNγ (XMG1.2); PE or V421-conjugated IL-17A (TC11-18H10.1) FITC-conjugated IL-4 (11B11), V450-conjugated FoxP3 (clone MF23). APC- or PE-Cy7-conjugated streptavidin were used to detect cells stained with biotinylated antibodies.

Histological assessment of pancreas and salivary glands infiltrating lymphocytes

To detect $CD45R^+$ ($B220^+$) B cells and $CD3^+$ T cells, sections were deparaffinized and rehydrated. Antigen retrieval was performed in citrate acid buffer (pH 6.0) at 95°C for 20 min. Sections were blocked with 20% (v/v) normal goat serum in 0.1 M phosphate buffer and 0.2% (v/v) Triton X-100 for 1 h at room temperature. Sections were incubated overnight at 4°C with α-CD3ε (1:500; RAM 34 clone, 14-0341, eBioscience) or α-CD45R (1:400; RA3-6B2, BD Biosciences). $CD45R^+$ ($B220^+$) B cells and $CD3^+$ T cells were visualized using rabbit or rat IgG VECTORSTAIN ABC Elite and DAB (3,3'-diaminobenzidine) Peroxidase Substrate Kits (Vector Laboratories) and counterstained with hematoxylin. Sections were visualized on a Zeiss Axioskop 2 mot plus microscope (Carl Zeiss) and Aperio imaging software.

Isolation of intraepithelial and lamina propria lymphocytes

For the isolation of intraepithelial (IEL) and lamina propria (4) lymphocytes colons excised from 5 week old prediabetic *Lck*-Cre*;Ptpn2*$^{fl/fl}$.NOD and *Ptpn2*$^{fl/fl}$.NOD littermate control mice was cut into 0.5–1 cm pieces and incubated twice with Ca^{2+} and Mg^{2+}-free HBSS supplemented with 5% (v/v) FBS, 2 mM EDTA, 0.15 mg/ml DTT (dithiothreitol) and 10mM HEPES shaking at 250 rpm for 15 min at 37°C to isolate intraepithelial lymphocytes. Intestine pieces were further digested in Ca^{2+} and Mg^{2+}-containing HBSS supplemented with 5% (v/v)

FBS, 1.5 mg/ml Collagenase D, 0.02 mg/ml Dnase I shaking at 250 rpm for 1h at 37 °C. Lymphocytes from digested colon pieces were enriched using a two-layer Percoll gradient at 40 and 80% (v/v) in DMEM supplemented with 10% (v/v) FBS.

Antigen-induced arthritis (AIA)

8-12 week-old C57BL/6J male mice were immunized subcutaneously with 100 µl mBSA (1 mg/ml; Sigma-Aldrich) emulsified in an equal volume of Complete Freund's Adjuvant (CFA). Mice also received 160 ng of heat-inactivated *Bordetella pertussis* toxin as a single intraperitoneal injection (Sigma-Aldrich). One week later, mice received an identical subcutaneous booster immunization of methylated BSA (mBSA) in CFA. 21 days after the initial immunization, inflammatory arthritis was induced by intraarticular administration of 10 µl mBSA (10 mg/ml) into the right knee joint. Arthritis development by measuring knee joint diameters using a POCO 2T micrometer (Kroeplin).

Histological assessment of joint inflammation

Knee joints were fixed in 10% (v/v) neutral buffered formal saline (Sigma-Aldrich) and decalcified in 10% (v/v) formic acid at 4°C before embedding in paraffin. Parasagittal serial sections (7 µm) were stained with haematoxylin (VWR International), fast green and safranin O (both from Sigma-Aldrich) for histological evaluation of joint pathology. Two observers blinded to the experimental groups scored the sections for sub-synovial inflammation (0 = normal to 5 = ablation of adipose tissue due to leukocyte infiltrate), synovial exudate (0 = normal to 3 = substantial number of cells with large fibrin deposits), synovial hyperplasia (0 = normal with 1-3 cells thick to 3 = over 3 layers thick with overgrowth onto joint surfaces with evidence of cartilage/bone erosion), and cartilage/bone erosion (0 = normal to 3 = destruction of a significant part of the bone). Joint histopathology in pre-diabetic 5 week old *Lck*-

Cre*;Ptpn2*$^{fl/fl}$.NOD and *Ptpn2*$^{fl/fl}$.NOD littermate control mice was compared to C57BL/6J mice with antigen-induced arthritis.

In vitro generation of T_{H1}, T_{reg} and T_{H17} cells

FACS-purified naïve CD4^{+}CD44^{lo}CD62L^{hi}CD25^{lo} lymph node T cells (5x10^{4}) isolated from 6-8 week old *Lck*-Cre*;Ptpn2*$^{fl/fl}$ and *Ptpn2*$^{fl/fl}$ littermate control mice were stimulated with plate-bound α-CD3ε (1 μg/ml) in the presence of soluble α-CD28 (10 μg/ml) in complete T cell medium overnight in 96-well round bottom plates. For the generation of T_{H1} cells 10 μg/ml soluble α-IL-4 (clone BVD4-1D11, WEHI) was added and for the generation of T_{H17} cells 10 μg/ml soluble α-IL-4 (clone BVD4-1D11, WEHI) and 10 μg/ml soluble α-IFNγ (clone XMG1.2, WEHI) were added. Cells were harvested and transferred to new 96-well round bottom plates and incubated with IL-2 (10 ng/ml) alone or various concentrations of IL-12 to generate T_{H1} cells or TGFβ (5 ng/ml) to generate T_{reg} cells. To generate T_{H17} cells, cells were incubated in IL-6 (50 ng/ml) and TGFβ (1 ng/ml) in the absence of IL-2. Cells were harvested at day 4 and 5 and processed for flow cytometry.

Treg suppression assay

FACS-purified naïve CD4^{+}CD25^{lo} responder T cells (5x10^{4}) were labelled with CTV (Cell Tracker Violet, Molecular Probes) and cultured for 72 h with X-ray irradiated (4,000 rad) splenocytes (5x10^{4}) and 1 μg/ml α-CD3ε (145-2C11) in the presence of the indicated ratio of FACS-purified CD4^{+}CD25^{hi} suppressor T cells.

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SUPPLEMENTARY FIGURE LEGENDS

Supplementary figure 1. PTPN2 deficiency enhances the in vitro generation of T_{H1} cells. **a)** FACS-purified naïve $CD4^+CD44^{lo}CD62L^{hi}CD25^{lo}$ lymph node T cells from *Ptpn2*$^{fl/fl}$ and *Lck*-Cre;*Ptpn2*$^{fl/fl}$ mice were cultured under T_{H1} polarising conditions in the presence of IL-2 (10 ng/ml) and various concentrations of IL-12. At day 4 cells were harvested and stained for intracellular IFNγ and the percentage of $IFN\gamma^+$ T_{H1} cells was determined by flow cytometry. **b-c)** FACS-purified naïve $CD4^+$ $CD44^{lo}CD62L^{hi}CD25^{lo}$ lymph node T cells from *Ptpn2*$^{fl/fl}$ and *Lck*-Cre;*Ptpn2*$^{fl/fl}$ mice were incubated with plate-bound α-CD3ε (5 μg/ml)/CD28 (5 μg/ml) for 48 h and then stimulated with **b)** 50 U/ml IFNγ or **c)** 10 ng/ml IL-12 for the indicated times and processed for immunoblotting. Quantified results are means ± SEM for the indicated number of mice and are representative of two independent experiments. Significance was determined using Student's T-test; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

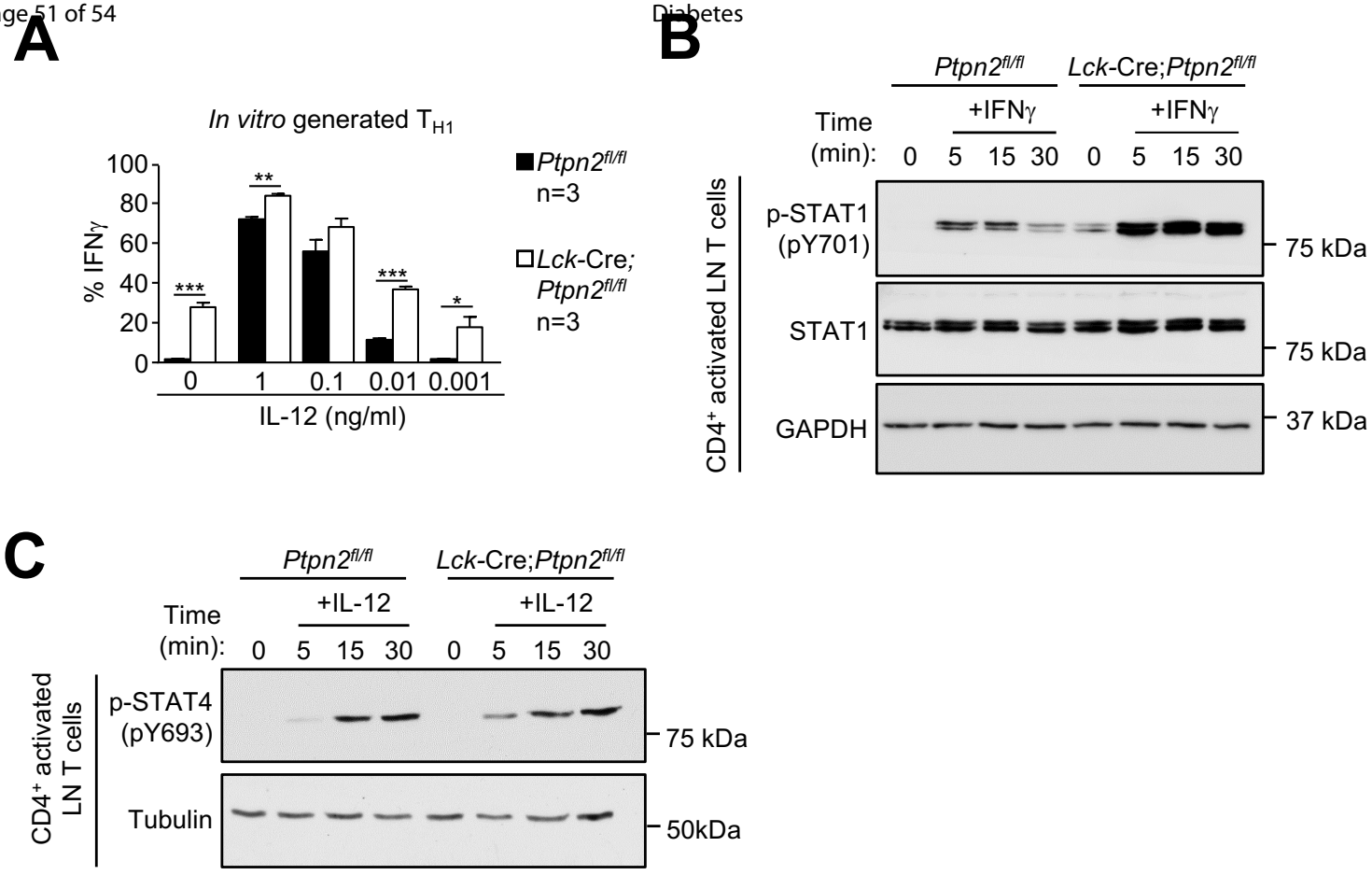
Supplementary figure 2. Regulatory T cell development in vivo and ex vivo. **a)** Inguinal lymph node cells and splenocytes from 5 week old female prediabetic *Ptpn2*$^{fl/fl}$.NOD and *Lck*-Cre;*Ptpn2*$^{fl/fl}$.NOD mice were stained for CD4, CD25, CD62L, CD44 and intracellular FoxP3

and the numbers of central-resting ($CD44^{hi}CD62L^{hi}$), and effector-like ($CD44^{hi}CD62L^{lo}$) T_{reg} cells were quantified by flow cytometry. **b)** FACS-purified naïve $CD4^{+}CD44^{lo}CD62L^{hi}CD25^{lo}$ lymph node T cells were cultured under T_{reg} polarising conditions in the presence of IL-2 (10 ng/ml) and TGFβ (5 ng/ml). At day 4 and 5 cells were harvested and stained for CD25 and intracellular FoxP3 and the percentage of $FoxP3^{+}CD25^{hi}$ T_{regs} were determined by flow cytometry. Quantified results are means ± SEM for the indicated number of mice and are representative of two independent experiments.

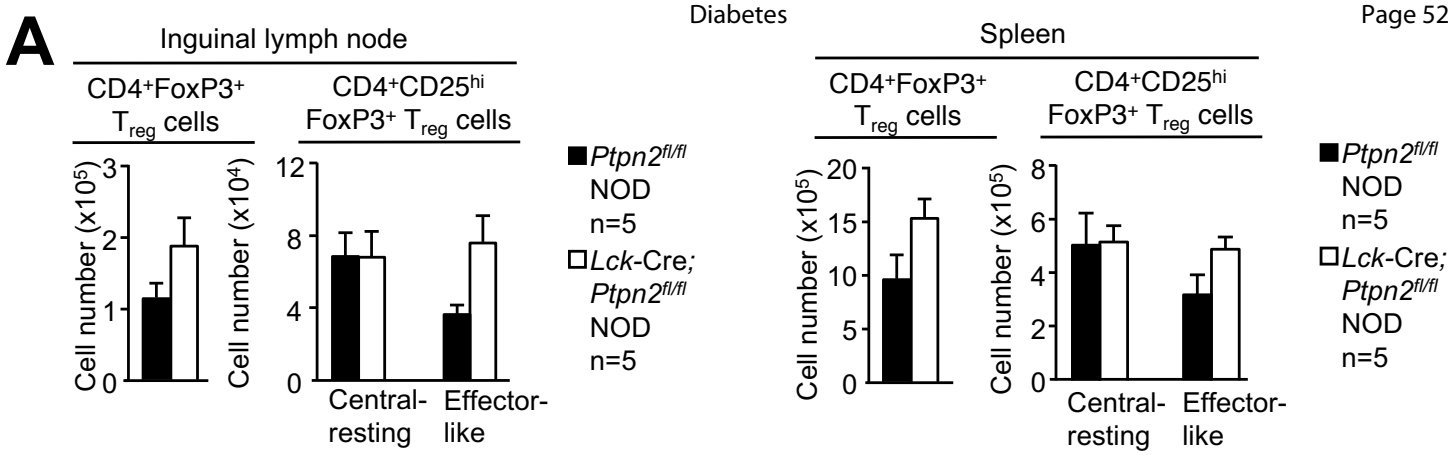
Supplementary figure 3. Histological assessment of liver, lung, kidney and knee-joints. **a)** Liver, lung and kidneys from 5 week old female prediabetic *Ptpn2*$^{fl/fl}$.NOD and *Lck*-Cre*;Ptpn2*$^{fl/fl}$.NOD mice were fixed in formalin and processed for histological assessment (hematoxylin and eosin: H&E). **b)** Knee joints from 5 week old female prediabetic *Ptpn2*$^{fl/fl}$.NOD and *Lck*-Cre*;Ptpn2*$^{fl/fl}$.NOD mice fixed in formalin and decalcified in formic acid. Parasagittal serial sections were processed for histological assessment (H&E, fast green and safranin O). Joint histopathology in *Lck-Cre;Ptpn2*$^{fl/fl}$ and *Ptpn2*$^{fl/fl}$ mice was compared to C57Bl/6 mice with antigen-induced arthritis (AIA).

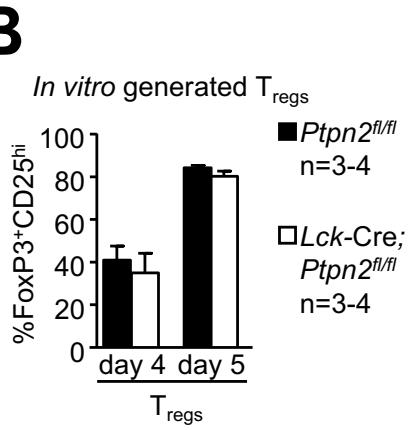
Supplementary figure 4. Colon resident intraepithelial and lamina propria γδ-T cells in Lck-Cre;Ptpn2*$^{fl/fl}$*.NOD mice. **a)** FACS-purified naïve $CD4^{+}CD44^{lo}CD62L^{hi}CD25^{lo}$ lymph node T cells from *Ptpn2*$^{fl/fl}$ and *Lck*-Cre*;Ptpn2*$^{fl/fl}$ mice were cultured under T_{H1} polarising conditions in the presence of IL-6 (50 ng/ml) and TGFβ (1 ng/ml). At day 4 cells were harvested and stained for intracellular IL-17A and the percentage of $IL\text{-}17A^{+}$ T_{H17} cells was determined by flow cytometry. **b)** Lymphocytes from were isolated from the colon of 5 week old female prediabetic *Ptpn2*$^{fl/fl}$.NOD and *Lck*-Cre*;Ptpn2*$^{fl/fl}$.NOD mice and intraepithelial versus lamina propria lymphocytes were stained for CD3 and TCR-δ and analysed by flow cytometry.

Representative contour-plots are shown. Quantified results are means ± SEM for the indicated number of mice and are representative of two independent experiments.

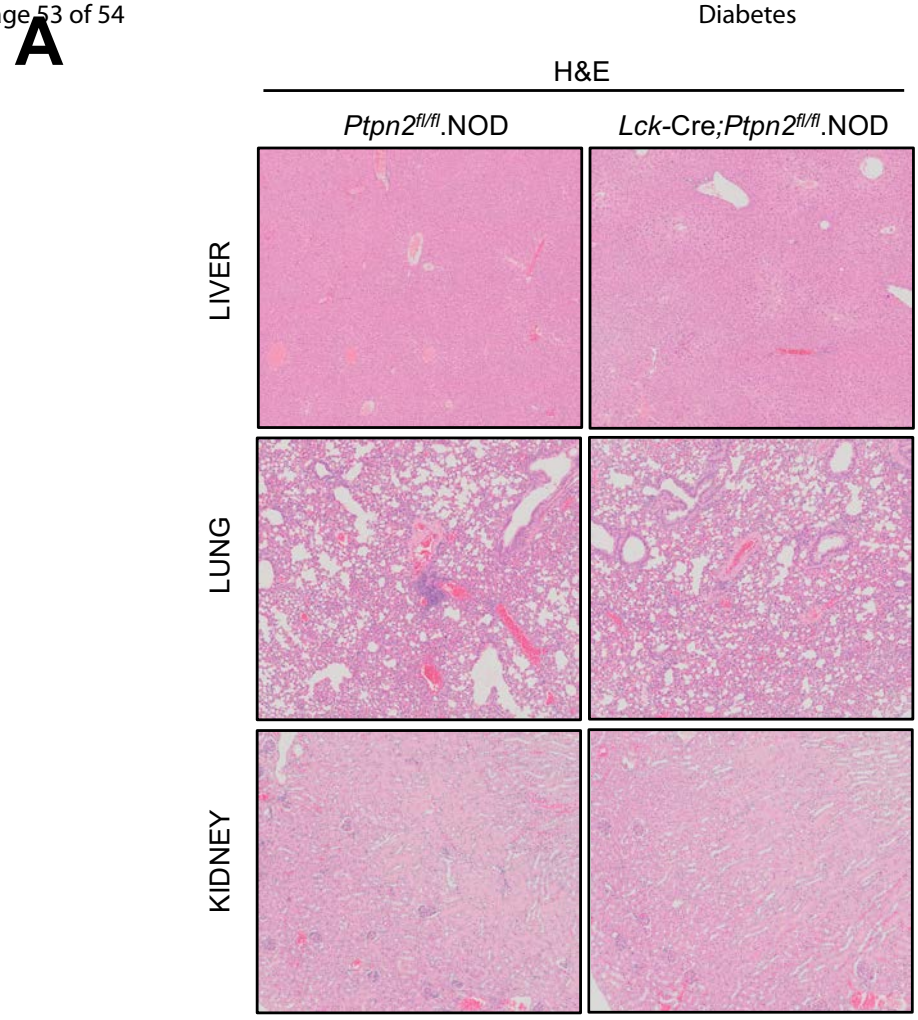


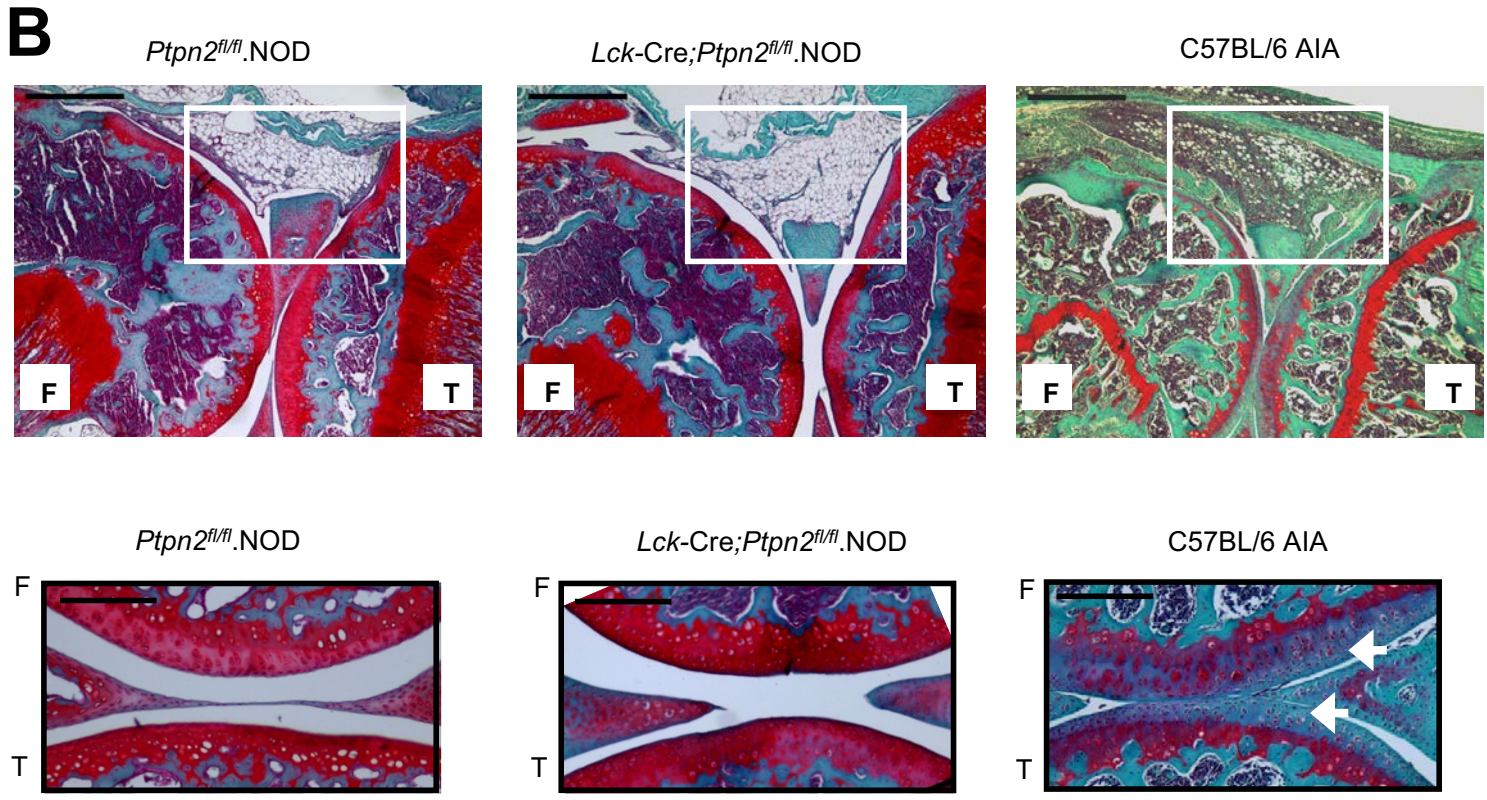
Supplementary figure 1



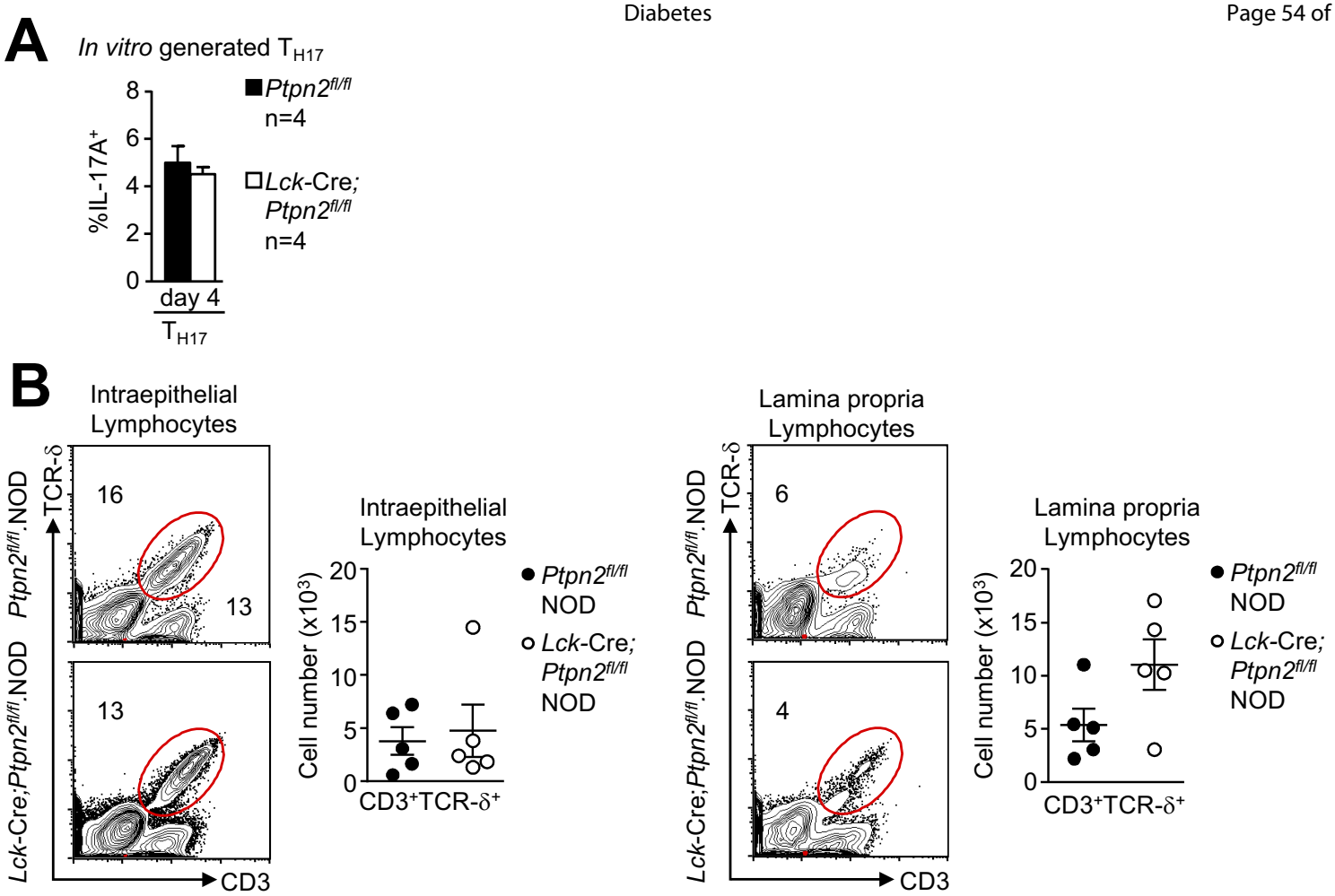


Supplementary figure 2





Supplementary figure 3



Supplementary figure 4