Science Translational Medicine
AAAS

www.sciencetranslationalmedicine.org/cgi/content/full/9/416/eaam7543/DC1

Supplementary Materials for

PD-L1 genetic overexpression or pharmacological restoration in hematopoietic stem and progenitor cells reverses autoimmune diabetes

Moufida Ben Nasr, Sara Tezza, Francesca D'Addio, Chiara Mameli, Vera Usuelli, Anna Maestroni, Domenico Corradi, Silvana Belletti, Luca Albarello, Gabriella Becchi, Gian Paolo Fadini, Christian Schuetz, James Markmann, Clive Wasserfall, Leonard Zon, Gian Vincenzo Zuccotti, Paolo Fiorina*

*Corresponding author. Email: paolo.fiorina@childrens.harvard.edu

Published 15 November 2017, *Sci. Transl. Med.* **9**, eaam7543 (2017)
DOI: 10.1126/scitranslmed.aam7543

The PDF file includes:

Other Supplementary Material for this manuscript includes the following:
(available at
www.sciencetranslationalmedicine.org/cgi/content/full/9/416/eaam7543/DC1)

Table S1. Transcriptomic profiling of murine KLS cells (provided as an Excel file).

Table S2. Genome-wide expression analysis of murine KLS cells (provided as an Excel file).
Table S5. Genome-wide expression analysis of pKL cells: up-regulated genes (provided as an Excel file).
Table S6. Genome-wide expression analysis of pKL cells: down-regulated genes (provided as an Excel file).
Table S9. Transcriptome of $pCD34^{+}$ cells (provided as an Excel file).
Table S10. Primary data (provided as an Excel file).

Materials and Methods

Human studies

Patient characteristics

Blood samples were obtained from newly diagnosed T1D patients (New-onset T1D), long-standing T1D patients (T1D) and healthy controls (CTRL) in accordance with Institutional Review Board committee approval. Baseline characteristics of the study population are summarized in Table S7. New-onset T1D patients were enrolled at their first visit/hospitalization. Peripheral blood mononuclear cell (PBMC) fractions were isolated by Ficoll density gradient centrifugation, and $CD34^-$ cells were isolated with microbeads (Miltenyi Biotec) for cell culture experiments.

Mobilization study with Mozobil

Blood samples were obtained from T1D patients and healthy controls (CTRL) at baseline before treatment and 6 hours after treatment with CXCR4 antagonist (Plerixafor [Mozobil], Sanofi). All patients received a single dose of 0.24mg/kg body weight administered by subcutaneous injection in accordance with the Institution Review Board committee of Padua (2996P), and patients were treated in accordance with the Declaration of Helsinki. T1D patients (n=5, aged 18-65 years) who were enrolled in the study were among those referred to the diabetes outpatient clinic of the University Hospital of Padua. Healthy controls (CTRL, n=8, aged 18-65 years) were enrolled from a group of patients referred to the same outpatient clinic for screening of other metabolic diseases. All patients recruited to the study provided written informed consent. Baseline detailed characteristics of the study population are summarized in Table S8.

Human flow cytometric analysis

To assess PD-L1, PD-L2 and PD-1 expression on human $CD34^+$ cells, fresh blood collected from

healthy patients, T1D and new-onset T1D patients was stained with PE-Cy5.5 anti-human CD34, PE anti-human PD-L1 or PD-L2 or PD-1 (BD Biosciences). Fresh blood was also stained with PE anti-human PD-L1 together with PE-Cy7 anti-human-CD19, APC anti-human CD11c or Pacific Blue anti-human CD16 (all BD Biosciences) to assess PD-L1 expression on B cells, dendritic cells or monocytes, respectively. A BD LSR Fortessa flow cytometer (BD Biosciences) was used to analyze cells with the light scatter properties of stem cells or lymphocytes. Background staining was determined using nonreactive isotype-matched control mAbs with gates positioned to exclude 99% of non-reactive cells. FlowJo software version 8.7.3 (Treestar) was used for analysis. Apoptosis was assessed in isolated $CD34^+$ cells stained with APC Annexin V (BD Biosciences) while dead cells were detected using a Fixable Viability Dye Staining (AmCyan, eBioscience). The following antibodies were used for flow cytometric analysis in human studies: PE-conjugated anti-human PD-L1 (CD274) or APC-labeled anti-human PD-L1 (CD274), PE-conjugated anti-human PD-1 (CD279), PE-conjugated anti-human PD-L2 (CD273), PE-conjugated or PC5-conjugated anti-human CD34, FITC-conjugated anti-human CD45, PE-conjugated anti-human-CD19, PerCP-conjugated anti-human CD11c and Pacific Blue (PB)-conjugated anti-human CD16. Antibodies were purchased from BD Biosciences, Biolegend or Beckman Coulter. The following antibodies corresponded to different isotype controls for the abovementioned human antibodies: PE-conjugated mouse IgG1 κ, mouse PC5-conjugated IgG1, APC-labeled mouse IgG2b κ.

In vitro proliferation assay and glucose challenge of $CD34^+$ cells

$CD34^+$ cells were first isolated using magnetic beads (Miltenyi) from PBMCs obtained from blood samples of enrolled subjects. Next, $CD34^+$ cells were stained with CFSE (Invitrogen) and cultured for 3 days at 37°C in 5% CO_2 in StemSpam SFEM II media (Stemcell Technologies).

Proliferation was analyzed by flow cytometry according to CFSE dilution after 1 and 3 days. To assess whether glucose exposure affects PD-L1 expression on $CD34^+$ cells, we cultured $CD34^+$ cells, previously isolated from PBMCs obtained from CTRL and T1D, in DMEM without serum at different glucose concentrations (5 mM, 20 mM and 35 mM) for 3 days. PD-L1 expression was assessed by FACS as previously described (*19*).

Pharmacological modulation of human CD34$^+$

$1X10^6$ isolated human $CD34^+$ HSCs were cultured in 200µl of StemSpan SFEM II media supplemented with recombinant human SCF (50 ng/ml, Life Technology), recombinant human TPO (50 ng/ml, Life Technology), recombinant human FLT3-L (50 ng/ml, Life Technology), human IFN-β (1000U/ml, R&D Systems Inc.), human IFN-γ (5 ng/ml, R&D Systems) and polyinosinic-polycytidylic acid (Poly[I:C]) (1µg/ml, InvivoGen) in a U-bottomed 96-well plate at 37°C in 5% CO_2. PD-L1 expression was evaluated before and after 1 day of culture by flow cytometry using anti-human CD34 and anti-human PD-L1, with their corresponding isotype controls.

Phenotypic profiling of pCD34$^+$ and CD34$^+$ cells by flow cytometry

Phenotypic characterization of pharmacologically-modulated $pCD34^+$ cells obtained from the peripheral blood of T1D patients as compared to unmodulated-$CD34^+$ cells with regard to costimulatory, pro-inflammatory and anti-inflammatory molecule expression was evaluated by FACS analysis. Staining and analysis were performed as previously described (*19*). The percentage of positive cells was assessed. The following antibodies were used for flow cytometric analysis for assessing phenotypic characterization of $pCD34^+$ cells isolated from the peripheral blood of T1D patients and prepared as previously described (*19*): PE-conjugated anti-human PD-1 (CD279), PE-Cy7-conjugated anti-human CD40, AlexaFluor 700-conjugated anti-

human CD80, APC-conjugated anti-human CD86, and APC-conjugated anti-human CD275 (ICOS ligand) were purchased from BD Biosciences or BioLegend. Cytokines were assessed by using the Fixation/Permeabilization kit (BD Biosciences) and staining with Pacific Blue-conjugated anti-human IFN-γ, PE-conjugated anti-human IL-10, and FITC-conjugated IL-4.

Human ELISPot assay

An ELISPot assay was used to measure the number of IFN-γ-producing cells according to the manufacturer's protocol (BD Biosciences) as previously shown by our group (*2*). $1x10^6$ PBMCs isolated from T1D patients were cultured for 2 days in the presence of IA-2 peptide (100μg/ml) in RPMI media supplemented with 10% FBS. At day one after stimulation, 500μl of media was added to the culture. Cells were collected at day 2 and added to plates coated with anti-IFN-γ antibody with or without $CD34^+$ or pharmacologically-modulated $CD34^+$ cells at a ratio of 1:5 or 1:10 in unsupplemented StemSpan SFEM II media. Spots were counted using an A.EL.VIS Elispot Reader (A.EL.VIS GmbH) or on an Immunospot Reader (C.T.L. Cellular Technology Ltd). Anti-human PD-L1 blocking antibody was purchased from eBioscience and used in *in vitro* functional assays at 1μg/ml.

Western blot

Total protein was extracted from $CD34^+$ cells in Laemmli buffer (Tris–HCl 62.5 mmol/l, pH 6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol), and protein concentration was measured. 35 μg of total protein was electrophoresed on 7% SDS-PAGE gels and blotted onto nitrocellulose (Schleicher & Schuell). Blots were then stained with Ponceau S. Membranes were blocked for 1 h in TBS (Tris [10 mmol/l], NaCl [150mmol/l]), 0.1% Tween-20, 5% non-fat dry milk, pH 7.4 at 25° C, incubated for 12 h with a polyclonal goat anti-human Pdcd-1L1 antibody (Santa Cruz Biotechnology) diluted 1:200 or with a monoclonal mouse anti-β-actin antibody (Santa Cruz

Biotechnology) diluted 1:1000 in TBS–5% milk at 4° C, washed four times with TBS–0.1% Tween-20, then incubated with a peroxidase-labeled mouse anti-goat IgG secondary antibody (or rabbit anti mouse for β-actin) diluted 1:1000 (Santa Cruz Biotechnology) in TBS–5% milk, and finally washed with TBS–0.1% Tween-20. The resulting bands were visualized using enhanced chemiluminescence (SuperSignal). Finally, for the quantification of western blot, images of nitrocellulose membrane filters were analyzed by ImageJ software to quantify size and strength of protein bands.

qRT-PCR

RNA was extracted from isolated $CD34^+$ cells using Trizol Reagent (Invitrogen), and qRT-PCR analysis was performed using TaqMan assays (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. The normalized expression values were determined using the ΔCt method. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) data were normalized for the expression of ACTB, and ΔCt values were calculated as previously performed by our group (*26*). Statistical analysis compared gene expression across cell populations for each patient via one-way ANOVA followed by Bonferroni post-test for multiple comparisons between the population of interest and all other populations (RT^2 profiler PCR Array Data Analysis from Qiagen). For two-group comparisons, Student *t* test was employed. Analysis was performed in triplicate. Reported below are the main characteristics of the primer used:

Gene Symbol	UniGene #	Refseq Accession #	Band Size (bp)	Reference Position
DC274 (PD-L1)	Hs.521989	NM_ 001267706.1	89	614

Confocal microscopy

$pCD34^+$ cells and unmodulated $CD34^+$ cells were collected and washed in 1 ml of BD staining buffer (BD Biosciences). After centrifugation at 400g for 5 min at RT, $pCD34^+$ cells and

unmodulated $CD34^+$ cells were resuspended in 100 ul of BD staining buffer containing 2ul of APC anti-human CD274 (BD Biosciences) and incubated in the dark for 15 min at RT. Cells were washed twice in BD staining buffer and fixed with BD Cytofix/Cytoperm™ (BD Biosciences) for 10 min at RT. After a washing step, cells were stained with DAPI (1:5000) (DAPI 1.5mM solution in H_2O [Molecular Probes, Thermo Fisher) in BD staining buffer and washed, then mounted with FluorSave (FluorSave™ Reagent Calbiochem, Merck KGaA) mounting media into Superfrost™ Plus glass slides and imaged using Leica SP5X system with an upright DM6000 microscope and A1R confocal microscope (Nikon Instruments).

Immunofluorescence and confocal imaging of bone marrow samples

Bone marrow extracted from T1D and healthy control patients were fixed in 4% paraformaldehyde and paraffin-embedded. Sections (7 μm) were prepared using the Microtome (*LEICA* CM3050S) and stained with the corresponding antibodies anti-CD34 (1:100, Novocastra, Newcastel, UK) and anti-PD-L1 (1:50, Thermo Scientific). Photomicrographs (original magnification 63x) were taken using an Olympus BX41 microscope (Center Valley, PA). Histology was evaluated in a blinded fashion, by an expert pathologist. The number of $CD34^+$/PD-$L1^+$ cells was assessed, by counting the number of orange-stained bone marrow elements (the result of green and red signal superimposition) in 10 histological fields at x63, respectively.

Determining PD-L1 DNA methylation

DNA was extracted from HSPCs ($CD34^+$ cells) isolated from PBMCs of T1D patients and healthy controls using a DNeasy Tissue Kit (Qiagen). DNA methylation status of the CpG island 114226 located in Chr9: 5450409 - 5450629 associated with CD274 gene was detected with the

EpiTect Methyl II PCR Assay and DNA Restriction Kit (EPHS114226-1A, Qiagen). The relative amount of methylated and unmethylated DNA fractions were calculated.

qRT-PCR for selected miRNAs

Total RNA was extracted from isolated $CD34^+$ cells using *mir*Vana™ miRNA Isolation Kit (Ambion), according to the manufacturer's instructions. To quantify the expression of selected miRNAs (hsa-miR-7853-5p, hsa-miR-105-5p, hsa-miR-224-3p, hsa-miR-4279, hsa-miR-522-3p, hsa-miR374c-5p) gene-specific reverse transcription was performed using TaqMan MicroRNA Reverse Transcription kit (Applied Biosystem). qRT-PCR was performed using TaqMan MicroRNA Assay containing PCR primers and TaqMan probes (Applied Biosystem) according to manufacturer instructions. Expression values were normalized using U6 small nuclear RNA (U6snRNA). qRT-PCR reactions were performed in triplicate in a 96-well format using an Applied Biosystems 7900HT fast real-time PCR instrument. Relative expression was calculated using the comparative threshold cycle method. For two-group comparisons, Student *t* test was employed.

Transcriptome profiling

Total RNA was isolated from unmodulated-$CD34^+$ and pharmacologically-modulated $pCD34^+$ cells obtained from the peripheral blood of T1D patients (n=5 each group) using the RNeasy Mini Kit (Qiagen) with on-column DNase I digestion. Next, 3 μg total RNA from each sample was reverse-transcribed using the RT2 First Strand kit (C-03; SABiosciences). We used the murine RT² Profiler™ PCR Array Human T-Cell & B-Cell Activation RT2 Profiler PCR Array (PAHS-053Z; Qiagen). The Profiler PCR Arrays quantitatively measure the expression of a panel of genes using SYBR Green-based real-time PCR. Data analysis was performed as previously described above (*26*). Table S9 and Figure S7A-B summarize all data related to

transcriptomic profiling of $CD34^+$ and $pCD34^+$ cells obtained from the peripheral blood of T1D patients.

Murine studies

Mice

Female NOD/ShiLtJ (NOD), C57BL/6, NOD.FVB-Tg (CAG-luc,-GFP)L2G85Chco/FathJ (Luciferase NOD), NOD.CgTg (TcraBDC2.5,TcrbBDC2)1Doi/DoiJ (BDC2.5 NOD) and NOD.Cg-Tg (TcraTcrbNY8.3)1Pesa/DvsJ (8.3 NOD) mice were purchased from The Jackson Laboratory. All mice were used according to institutional guidelines, and animal protocols were approved by the Boston Children's Hospital Institutional Animal Care and Use Committee.

Diabetes Monitoring

Overt diabetes was defined as blood glucose levels above 250 mg/dL for 2 consecutive days. Blood glucose was measured using the Breeze2 (Bayer S.p.A) blood glucose meter.

Reversal studies

Female NOD mice were monitored beginning at 10 weeks of age, and on day 2 of hyperglycemia (>250 mg/dl) were injected with $3x10^6$ PD-L1.Tg KL cells, or unmodulated KL cells, or pharmacologically-modulated KL cells (see description above) administered via the tail vein. Mice were monitored daily by measuring blood glucose until they became terminally hyperglycemic and then were sacrificed, or in some instances were kept alive with insulin administration up to a specific endpoint (40 days). Measurements were performed by tail bleeding according to National Institutes of Health guidelines. We are aware that a recent JDRF-ITN Preclinical T1D consortium showed convincingly that results of immune interventions in the NOD model are highly variable (*27*). Thus, the newly hyperglycemic NOD in this study were treated in a random-like fashion, meaning that each group of treated mice were obtained from

different cages, thus avoiding a selection bias. Furthermore, our NOD colony at Boston Children's Hospital exhibits consistent diabetes penetrance (i.e; 80% at 25 weeks) (*2-3*). Bone marrow cells were obtained from femurs and tibiae of NOD and C57BL/6J mice by flushing with phosphate buffered saline (PBS). Bone marrow cells were lineage depleted using the Lineage Negative Depletion Kit (Miltenyi Biotec). Upon depletion, lineage negative c-kit^{+} cells were isolated using CD117 Microbeads (Miltenyi Biotec), following the manufacturer's instructions.

Anti-PD-1 mAb studies

Anti-PD-1 stimulating mAb (clone PIM-2) was administered in a diabetes prevention study. PIM-2 was injected at a dose of 500 μg on day 0 and then 250 μg on days 2, 4, 6, 8 and 10 via the intraperitoneal route in NOD mice beginning at 10 weeks of age. A group of 10-week-old normoglycemic NOD mice were treated with vehicle only (i.e. PBS) and used as "a control group." For reversal studies, female NOD mice after 2 consecutive hyperglycemic measurements within 24 hours were treated with PIM-2 at a dose of 500 μg on day 0 and then 250 μg on days 2, 4, 6, 8 and 10 via the intraperitoneal route.

Murine flow cytometric analysis

For murine KL characterization, KLS or KL cells were extracted from bone marrow were suspended in 200μL of buffer, then stained with the following antibodies and incubated according to the manufacturer's instructions for 30 minutes at 4°C. Cells were washed with buffer, centrifuged at 300 g for 10 minutes and suspended in 300 μl of buffer. The following antibodies were used for the staining: rat anti-mouse CD274 or CD273 or anti-mouse CD279. PD-L1, PD-L2 and PD-1 expression on KL cells were represented as histograms. For non-hematopoietic stem cell characterization, 1x10^{6} cells per sample were stained with anti-mouse

B220-PE to assess B cells, with CD11c-PerCP to assess dendritic cells, anti-mouse F4/80-APC to assess macrophages, CD11b-PE to assess early myeloid cells, CD127-PerCP, Sca-1-FITC and CD25-PE to assess innate lymphoid type 1 cells, CD127-PerCP, CD25-FITC and CD90-PE to assess innate lymphoid type 2 cells, CD127-PerCP, CD117-FITC and CD90-PE to assess innate lymphoid type 3 cells, CD1d-PE, Vβ8.1/Vβ8.2 TCR-FITC and B220-PerCP to assess NKT cells, CD4-PerCP, CD8-PerCP, CD44-PE, CD62L-APC to assess CD4/CD8 effector and CD4/CD8 central memory, and CD4-PerCP, CD25-PE and FoxP3-APC to assess CD4 T regulatory cells. PD-L1 expression in $B220^+$ cells, $CD11c^+$ cells, $CD11b^+$ cells, NKT cells, ILC1 cells, ILC2 cells, ILC3 cells and $F4/80^+$ cells was assessed using anti-mouse CD274-PE and anti-mouse CD274-APC. Briefly, isolated bone marrow cells and splenocytes were washed in flow medium (PBS containing 2% FCS and 0.05% sodium azide) and stained with the appropriate dilution of flow antibodies. Samples were incubated for 30 min in the dark at 4°C, washed again with flow medium and then fixed with 1% formalin. Samples were acquired using a FACS Calibur and results analyzed using Flowjo software (version 8.7.3, Treestar). Anti-mouse CD11c-PerCP was purchased from Biolegend, anti-mouse F4/80 APC from eBioscience, anti-mouse CD11b-PE from BD Biosciences, anti-mouse CD127-PerCP, from BioLegend, anti-mouse Sca-1-FITC from eBioscience, anti-mouse CD25-PE from BD Biosciences, anti-mouse CD25-FITC from BioLegend, anti-mouse CD117-FITC from BioLegend, anti-mouse CD90-PE from eBioscience, anti-mouse CD1d-PE from BioLegend, anti-mouse Vβ8.1/Vβ8.2 TCR-FITC from eBioscience, anti-mouse CD4-PerCP and anti-mouse CD8-PerCP from BioLegend, anti-mouse CD44-PE, anti-mouse CD25-PE and anti-mouse CD62L-APC from BD Biosciences, anti-mouse FoxP3-APC from eBioscience and anti-mouse PD-L1 from BD Biosciences. Bone marrow cells were stained with the following cocktail of anti-mouse antibodies: lineage negative cocktail-APC, c-

kit-PerCP, Sca-1-FITC, CD150-PE, CD41-FITC, CD48-PerCP, CD244-PerCP, PD-L1-PE and PD-L1-APC. (i) *For intracellular cytokine staining:* Anti-mouse IFN-γ PE was purchased from eBioscience, and its corresponding isotype control Rat PE mouse IgG1, κ was also purchased from eBioscience. (ii) *For in vitro functional assays:* Anti-mouse PD-L1 blocking antibody was purchased from Abcam and used for *in vitro* inhibition assays as previously described (*9*). All other antibodies were purchased from eBioscience and from BD Biosciences. Staining and analysis was performed as above. Median fluorescence intensity and percentage of positive cells were assessed. The following antibodies were used for flow cytometric analysis for assessing phenotypic characterization of KL extracted from bone marrow and spleen: phycoerythrin (PE)-conjugated or allophycocyanin (APC)-conjugated Rat anti-mouse PD-L1 (CD274), phycoerythrin (PE)-conjugated Rat anti-mouse PD-1 (CD279), and phycoerythrin (PE)-conjugated Rat anti-mouse PD-L2 (CD273) were purchased from BD Biosciences or Biolegend. The following antibodies corresponded to different isotype controls for the abovementioned murine antibodies: PE Mouse IgG1, κ Isotype Ctrl; APC Mouse IgG2b, κ Isotype Ctrl.

Apoptosis assay

Isolated KL cells were washed twice with cold PBS and then resuspended in 1X Binding Buffer (component no. 51-66121E; BD Biosciences) at a concentration of $1x10^6$ cells/ml. 100 µl of this solution (containing $1x10^5$ HSCs) was transferred into a 5 ml culture tube and was stained with 5µl of PE Annexin V and 5 µl 7-AAD, followed by incubation for 15 min at RT (25°C) in the dark. After incubation, 400 µl of 1X Binding Buffer was added to each tube prior to acquisition on the flow cytometer. The following controls were used to set up compensation and quadrants: unstained cells, cells stained with PE Annexin V (no 7-AAD) and cells stained with 7-AAD (no PE Annexin V). Cells that stained positive for PE Annexin V and negative for 7-AAD were

undergoing apoptosis. Cells that stained positive for both PE Annexin V and 7-AAD were either in the end stage of apoptosis, were undergoing necrosis, or were already dead. Cells that stained negative for both PE Annexin V and 7-AAD were alive and not undergoing measurable apoptosis.

In vitro proliferation assay

Isolated KL cells were washed twice with cold PBS buffer without FCS, then resuspended in half the final volume of buffer at $3x10^7$ cells/ml. Diluted CFSE was added to the cell suspension to a final concentration of 10uM, followed by vortexing and incubation at 37°C for 15 minutes. After incubation, FCS was added to the cell suspension in order to quench any remaining free CFSE, and the tube was filled completely with PBS buffer. After a second wash, cells were resuspended in media and were cultured for 3 days at 37°C in 5% CO_2. After 72h, proliferation of KL cells was visualized by flow cytometric analysis according to CFSE dilution.

Intracellular staining for flow cytometry

Naïve $CD4^+CD25^-$ T cells ($5x10^5$) were isolated from BDC2.5 TCR tg mice using a negative selection strategy with the $CD4^+$ $CD25^+$ Regulatory T cell isolation kit (Miltenyi Biotec) and were stimulated with BDC2.5 peptides and $CD11c^+$ dendritic cells (DCs) ($2.5x10^5$) previously isolated using $CD11c^+$ mAb-coated microbeads. DCs were added in a 1:2 ratio to T cells and were co-cultured with PD-L1.Tg KL cells at ratios of 1:1, 1:5 and 1:10 (1 T cells vs. 1/5 and 1/10 PD-L1.Tg KL/pKL cells) or alone (controls) or with untransduced KL cells or with pKL cells (at ratios of 1:1, 1:5 and 1:10) for 24 hours in RPMI 10% FBS in a humidified incubator 37°C, 5% CO_2. After incubation, cells were collected, washed and plated in RPMI 10% FBS, then stimulated with 50 ng/ml PMA (Sigma Aldrich), 750 ng/ml ionomycin (Sigma Aldrich) and protein transport inhibitor (6 μl per 6 ml of RPMI as recommended by the manufacturer) (BD

GolgiStop) for 5h in a humidified incubator 37°C, 5% CO_2. After incubation, cells were collected, washed, stained for surface markers (i.e CD4 APC), then washed and permeabilized using the BD Cytofix/Cytoperm Kit (BD Biosciences), and then washed and stained with a mAb against IFN-γ (eBioscience). Finally, $CD4^+$ IFN-γ^+ cells were assessed by flow cytometry analysis.

Murine ELISPot assay

An ELISPot assay was used to measure the number of IFN-γ-producing cells according to the manufacturer's protocol (BD Biosciences) as previously shown by our group (*2*). $1x10^6$ of splenocytes isolated from NOD-treated mice (NOD-PD-L1.Tg-treated, NOD-Trifecta-treated, NOD-KL-treated) and NOD-untreated mice were cultured for 24 hours in the presence of the following murine islet peptides (150µg/ml): BDC2.5, IGRP, GAD65 purchased from ANASPEC, while insulin was added at 300µg/ml. Spots were counted using an A.El.VIS Elispot Reader.

Autoreactive T cell apoptosis assay upon co-culture with PD-L1.Tg KL cells

Naïve $CD4^+CD25^-$ T cells ($5x10^5$) isolated from BDC2.5 TCR tg NOD mice or $CD8^+$ T cells ($5x10^5$) from 8.3 TCR tg NOD mice and stimulated with BDC2.5 or IGRP islet peptides and $CD11c^+$ dendritic cells ($2.5x10^5$), were cocultured with KL cells (WT) or PD-L1.Tg KL cells ($5x10^5$) for 24 hours. Cells were collected, washed, stained for surface markers (i.e CD4 PE or CD8 PE), then washed twice with cold PBS and resuspended in 1X Binding Buffer (BD Biosciences) at a concentration of $1x10^6$ cells/ml. 100 µl of this solution (containing $1x10^5$ HSCs) was transferred into a 5 ml culture tube and was stained with 5µl of APC Annexin V and 5 µl 7-AAD, followed by incubation for 15 min at RT (25°C) in the dark. After incubation, 400 µl of 1X Binding Buffer was added to each tube prior to acquisition on the flow cytometer. The

following controls were used to set up compensation and quadrants: unstained cells, cells stained with APC Annexin V (no 7-AAD) and cells stained with 7-AAD (no APC Annexin V). Cells that stained positive for APC Annexin V and negative for 7-AAD were undergoing apoptosis. Cells that stained positive for both APC Annexin V and 7-AAD were either in the end stage of apoptosis, were undergoing necrosis, or were already dead. Cells that stained negative for both APC Annexin V and 7-AAD were alive and not undergoing measurable apoptosis.

Cell lines and cell culture

The Lenti-XTM 293T cell line used in this study was purchased from Clontech as recommended. All procedures involving the HEK293T cell line and lentiviral methodologies were approved by the Institutional Biosafety Committee (IBC) of Boston Children's Hospital Committee, Harvard Medical School.

Lentivirus production and transduction

Full-length cDNA encoding murine PD-L1 was cloned into the transfer plasmid pHAGE-fullEF1a-TRE-IZsGreen, a third generation non-replicative Lentiviral vector containing internal EF1a promoter, has IRES-ZsGreen (IRES-fluorescent tag); Tet-responsive element (kindly provided by the Rossi Lab at the Harvard Stem Cell institute). performed by co-transfection of the murine PD-L1 transfer plasmid together with the packaging expression plasmids (Gag/Pol, Tat, Rev) and the envelope expressing plasmid encoding for a VSV-G pseudotyped glycoprotein into 293T cells using the Trans-IT 293 transfection reagent (Mirus Bio). 1 or 2 days post transfection, the supernatant containing the viral particles was collected, centrifuged at 1800rpm for 5 minutes to remove dead cells and debris, and concentrated using the Lenti-X concentrator following manufacturer's protocol (Clontech). Viral stocks were stored at -80°C until transduction experiments were performed. Freshly isolated murine KL cells were transduced

with PD-L1 lentiviral particles in Stem SFEMII (Stemcell Technologies) in the presence of 2 μg/mL polybrene, 10 ng/ml of SCF (Miltenyi Biotec) and 100 ng/ml of TPO (Miltenyi Biotec). 24 hours after transduction, cells were collected for FACS analysis and used for reversal studies.

Luciferase assay

KL cells isolated from NOD.FVB-Tg(CAG-luc,-GFP)L2G85Chco/FathJ were transduced with PD-L1 lentivirus and injected intravenously into NOD-hyperglycemic mice. After 24 hours, treated mice were injected with luciferin (ip). Following luciferin injection, luciferase expression was assessed using an IVIS Spectrum (Perkin Elmer).

Pharmacological modulation of murine KL cells

Murine bone marrow KL cells were isolated using magnetic beads and MACS® separation columns (Miltenyi) and ~ $2x10^5$ cells were plated in a U-bottomed 96-well plate with 200 μl of the following medium: Stemspan-SFEMII (Stemcell Technologies) supplemented with 50 ng/ml recombinant human SCF (Stemcell Technologies), 50 ng/ml of mouse TPO (Stemcell Technologies), 50 ng/ml of recombinant mouse IL-3 (R&D Systems), recombinant mouse (1000U/ml) (R&D Systems), mouse IFN-γ (5 ng/ml) (R&D Systems) and 1μg/ml of poly[I:C] (polyinosinic-polycytidylic acid) (InvivoGen). The use of IFN-γ and IFN-β is based on suggestions present in the literature, which connected PD-L1 upregulation with IFN-β and IFN-γ challenge, while the choice of poly(I:C) was based on TLR screening that we performed, and the highest PD-L1 MFI (Fig. 5C) was achieved with TLR3 stimulation (Fig. 5A-E). The color coding shown in in Fig. 5C is as follows: lowest PD-L1 MFI values are shown as orange, median PD-L1 MFI values are yellow and highest PD-L1 MFI values are green. PD-L1 expression was evaluated before culture by FACS using rat anti-mouse PD-L1 **(**BD Biosciences) with the corresponding isotype control Rat IgG2a, λ (BD Biosciences).

Chemokine profiling in pKL, Tg. KL and KL-Veh cells by flow cytometry

Chemokine receptor expression on KL-Veh (unmodulated KL), pKL and Tg.KL was evaluated by FACS analysis. Staining and analysis was performed as previously described above and the percentage of positive cells was assessed. The following antibodies were used for this flow cytometric analysis to assess chemokine phenotypic characterization of pKL, Tg.KL and KL-Veh (unmodulated) extracted from bone marrow of NOD normoglycemic mice: APC-conjugated rat anti-mouse CCR2 (CD192), APC-conjugated rat anti-mouse CCR4 (CD194), APC-conjugated rat anti-mouse CCR8 (CD198), APC-conjugated rat anti-mouse CXCR4 (CD184), PE-conjugated rat anti-mouse CCR5 (CD195) PE-conjugated rat anti-mouse CCR6 (CD196), PE-conjugated rat anti-mouse CCR7 (CD197) and FITC-anti-sphingosine 1-phosphate receptor 1 were purchased from BD Biosciences, BioLegend, or Alomone. The following antibodies corresponded to different isotype controls for the above murine antibodies: PE mouse IgG1, κ isotype ctrl, Armenian hamster IgG; APC mouse IgG2b, κ isotype ctrl, Armenian hamster IgG (Table S4).

Western blot

KL cells were homogenized in RIPA buffer (20mM Tris pH 8.0, 150mM NaCl, 0.1% SDS, 0.5% DOC, 0.5% triton X-100) with protease inhibitor cocktail (Roche). Cell lysates equivalent to 50 μg of total protein were fractionated on 4%-20% SDS-polyacrylamide gradient gels (Bio-Rad) and transferred to nitrocellulose membranes (0.2 μm, Bio-Rad). Membranes were blocked with 5% BSA at room temperature for 1 hour and then incubated overnight with anti-PD-L1 (1:200, Santa Cruz Biotechnology), anti-rabbit GAPDH (1:1000 dilution, Cell Signaling Technology). Detection was performed using anti-rabbit IgG (1:2000) HRP-linked antibodies (Cell Signaling Technology). Finally, for the quantification of western blot bands, images of nitrocellulose

membrane filters were analyzed by ImageJ software to quantify size and strength of protein bands.

qRT-PCR

To measure expression levels of the *PD-L1* gene in KL cells, total RNA was extracted from KL cells and treated at 42℃ for 30 min with 100 μl extraction buffer (Arcturus Picopure, Applied Biosystems), then subjected to different washing steps and eluted in 15 ul of elution buffer according to the manufacturer's instructions. RNA was quantified using a NanoDrop spectrophotometer followed by reverse transcription and pre-amplification using ABI Reverse Transcription and Taqman PreAmp Kit (Applied Biosystems) according to the manufacturer's instructions. TaqMan gene expression assays (Applied Biosystems) were performed on triplicate samples using a StepOnePlus^{TM} Real-Time PCR system (Applied Biosystems). Data were normalized relative to the GAPDH housekeeping gene. Reported below are the main characteristics of the primer used:

Gene Symbol	UniGene #	Refseq Accession #	Band Size (bp)	Reference Position
DC274 (PD-L1)	Mm.245363	NM_021893.3	77	483

Immunofluorescence and confocal imaging of bone marrow samples

Bone marrow extracted from femur and tibiae of 8-week-old NOD and B6 mice were fixed in 4% paraformaldehyde and paraffin-embedded. Sections (7 μm) were prepared using the Microtome (*LEICA* CM3050S) and stained with the corresponding antibodies anti-c-kit (1:20, LSBio) and anti-PD-L1 (1:50, Thermo Scientific). Photomicrographs (original magnification 63x) were taken using an Olympus BX41 microscope. Histology was evaluated in a blinded fashion by an expert pathologist. The number of c-kit^{+}/PD-L1^{+} cells was assessed by counting

the number of orange-stained bone marrow elements (the result of green and red signal super-imposition) in 10 histological fields at x63, respectively.

ZsGreen imaging of the pancreas

Pancreata collected from treated and untreated NOD mice were fixed in PFA 4% for 1hr at 4C, washed 3 times for 20min in PBS, then perfused in 15% sucrose for 1hr followed by another perfusion with 30% sucrose and finally embedded in OCT. Pancreas sections of 7-12um were prepared, then air-dried. Cells were counterstained with blue fluorescent DAPI (1:10000, Biolegend). Cells were photographed under a 63x objective with excitation and emission filters set at 490/20 nm and 528/38 nm for ZsGreen visualization. Images were captured on a Leica SP5X system with an upright DM6000 microscope and A1R confocal microscope (Nikon Instruments).

Immunohistochemistry

Immunohistochemistry was performed as previously described (*3*) and was performed on formalin-fixed, paraffin-embedded pancreas tissue. Pancreas sections (5-μm–thick) were stained with H&E, anti-CD3 and anti-insulin as previously described. The following primary antibodies were used: anti-CD3 (1:100) and anti-insulin (1:500) (Abcam). Photomicrographs (original magnification ×20) were taken using an Olympus BX41 microscope. Histology was evaluated by an expert pathologist.

Insulitis score

Insulitis scoring was performed on 5-μm–thick formalin-fixed, paraffin-embedded, hematoxylin and eosin (H&E)-stained pancreatic sections as previously described (*4*). A score of 0 to 4 was assigned based on islet infiltration by an experienced pathologist, as previously described (*3*). Insulitis scores were graded as follows: grade 0, normal islets; grade 1, mild mononuclear

infiltration (<25%) at the periphery; grade 2, 25–50% of the islets infiltrated; grade 3, >50% of the islets infiltrated; grade 4, islets completely infiltrated with no residual parenchyma remaining. At least 30 islets per group were analyzed and pooled from sections obtained from different mice.

Pancreas digestion and preparation for flow cytometry

Pancreata were collected in ice-cold IMDM medium, cut into small pieces, and digested with Collagenase D for 1h at 37°C, with DNase I added after 30 minutes. Digested pancreata were passed through a 70-μm cell strainer to obtain single cell suspensions and then analyzed by flow cytometry.

Luminex serum cytokine determination

Treated or control NOD mice were bled via tail vein on days 0, 7, 14, and 40, and serum samples were collected. The BeadLyte Mouse Multi-Cytokine Beadmaster Kit (Millipore) was used according to the manufacturer's protocol to determine cytokine levels of IL-2, IL-4, IL-6, IL-10, IL-17A/CTLA8, MIG/CXCL9, IFN-γ, and TNF-α. Briefly, supernatant samples were incubated overnight at room temperature with beads conjugated to the aforementioned cytokines, matched biotinylated reporters were added and incubated for 1.5 h, and streptavidin-phycoerythrin solution was incubated with samples for 30 min. After the addition of stop solution, sample cytokine levels were calculated from a standard curve using a Luminex100 reader (Luminex Corporation).

Ovalbumin immunization

Ovalbumin peptide (Sigma-Aldrich) emulsified in complete Freund's adjuvant (Sigma-Aldrich) was injected once (100 μg/mouse i.p.) into naïve or treated NOD mice, 14 days after the onset of

diabetes. Splenocytes were then collected after 3 days and used in *in vitro* assays after rechallenge with 1 μmol/l ovalbumin peptide for 24 h (*4*).

Murine Affymetrix microarray

Genome-wide expression analysis was performed following Affymetrix GeneChip WT Pico protocol (Affymetrix). RNA isolation was conducted using Arcturus PicoPure RNA Isolation Kit (Applied Biosystems) and then diluted to approximately 1.0ng. RNA integrity was assessed for all RNA samples, and the final concentration was measured on a Bioanalyzer using RNA Pico Chips (Agilent Technologies). Only RNA with a RIN score of 7 or higher was used. Between 1-2ng was used as template to construct cRNA through a series of reactions involving cDNA synthesis, adaptor synthesis and a 16hr amplification step (Affymetrix). Following cRNA purification and quantitation, ss-cDNA was synthesized, fragmented and labeled (Affymetrix). Each MTA 1.0 or HTA 2.0 Genechip was hybridized for 17hrs at 45C. Arrays were then stained on a FS450 Fluidic station (Affymetrix) and scanned on a Gene Chip 7G Scanner (Affymetrix). Probe intensities were normalized according to a log scale robust multi-array analysis (Expression Console-RMA, Affymetrix) method, and normalized intensities were plotted with Spotfire 6.0 (Perkin Elmer). (i) Table S2 summarizes all data related to GWAS analysis in KLS from NOD and C57BL/6 mice. (ii) Table S2 summarizes all data related to GWAS analysis on Tg.KL cells vs. Mock.KL extracted from NOD mice. (iii) Table S5 and (iv) Table S6 summarize all data related to GWAS analysis on pharmacologically-modulated KL cells (pKL) vs. KL extracted from NOD mice.

PD-L1 DNA methylation

DNA was extracted from KLS isolated from bone marrow of NOD and C57BL/6 mice using a DNeasy Tissue Kit (Qiagen). 500 ng of extracted DNA was bisulfite treated by the Zymo

Research EZ Methylation Kit as recommended by the manufacturer (Zymo Research Corp) and the sequence of interest of the PD-L1 gene was verified by sequencing (Ensembl Gene ID: ENSMUSG00000016496). To study the methylation status of the CpG island localized in the promoter of *PD-L1* gene (CpG loci: -77 to -71) and within the 5'UTR/intron 1 of *PD-L1* gene (CpG loci: -70 to -64), bisulfite modification and pyrosequencing were performed at EpigenDX as recommended by the manufacturer. Bisulfite modification, which converted unmethylated cytosines to uracils, was performed using an EZ DNA Methylation kit (Zymo Research, Irvine, CA) according to the manufacturer's instructions. The pyrosequencing was performed by PSQ96 HS System (Pyrosequencing, Qiagen) following the manufacturer's instructions (Pyrosequencing, Qiagen). The methylation status of each locus was analyzed individually as a T/C SNP using QCpG software (Pyrosequencing, Qiagen). The relative amount of methylated and unmethylated DNA fractions were calculated.

miRNA inhibition

KL cells extracted from NOD mice were cultured in SFEMII (Stemcell Technologies) media in the presence of miRCURY LNA micoRNA inhibitor (Exiqon) at a final concentration of 100 nM per 200000 KL cells and were incubated for 24 h at 37º C. Cells were collected and washed twice with PBS and were subjected to RNA extraction using *mir*Vana™ miRNA Isolation Kit (Ambion), according to the manufacturer's instructions. To quantify the expression of miR-1905, gene-specific reverse transcription was performed using TaqMan MicroRNA Reverse Transcription kit (Applied Biosystem) and qRT-PCR was performed using TaqMan MicroRNA Assay containing PCR primers and TaqMan probes (Applied Biosystem) according to manufacturer instructions. Expression values were normalized using U6 small nuclear RNA (U6snRNA). Another qRT-PCR was performed in order to assess PD-L1 gene expression as

previously described. All qRT-PCR reactions were performed in triplicate in a 96-well format using an Applied Biosystems 7900HT fast real-time PCR instrument. Relative expression was calculated using the comparative threshold cycle method. For two-group comparisons, Student *t* test was employed.

Transcriptome profiling

Total RNA was isolated from KLS cells obtained from bone marrow of C57BL/6 and NOD mice (n=5 each group) using the RNeasy Mini Kit (Qiagen) with on-column DNase I digestion. Next, 3 μg total RNA from each sample was reverse-transcribed using the RT2 First Strand kit (C-03; SABiosciences). We used the murine RT² Profiler™ PCR Array Mouse Cancer Inflammation and Immunity Crosstalk (PAMM-181Z). The Profiler PCR Arrays quantitatively measure the expression of a panel of genes using SYBR Green-based real-time PCR. Data analysis was performed as previously described (*26*). Table S1 summarizes all the data related to transcriptomic profiling of KLS in NOD and C57BL/6 mice.

SUPPLEMENTAL DATA:

Supplemental Figures

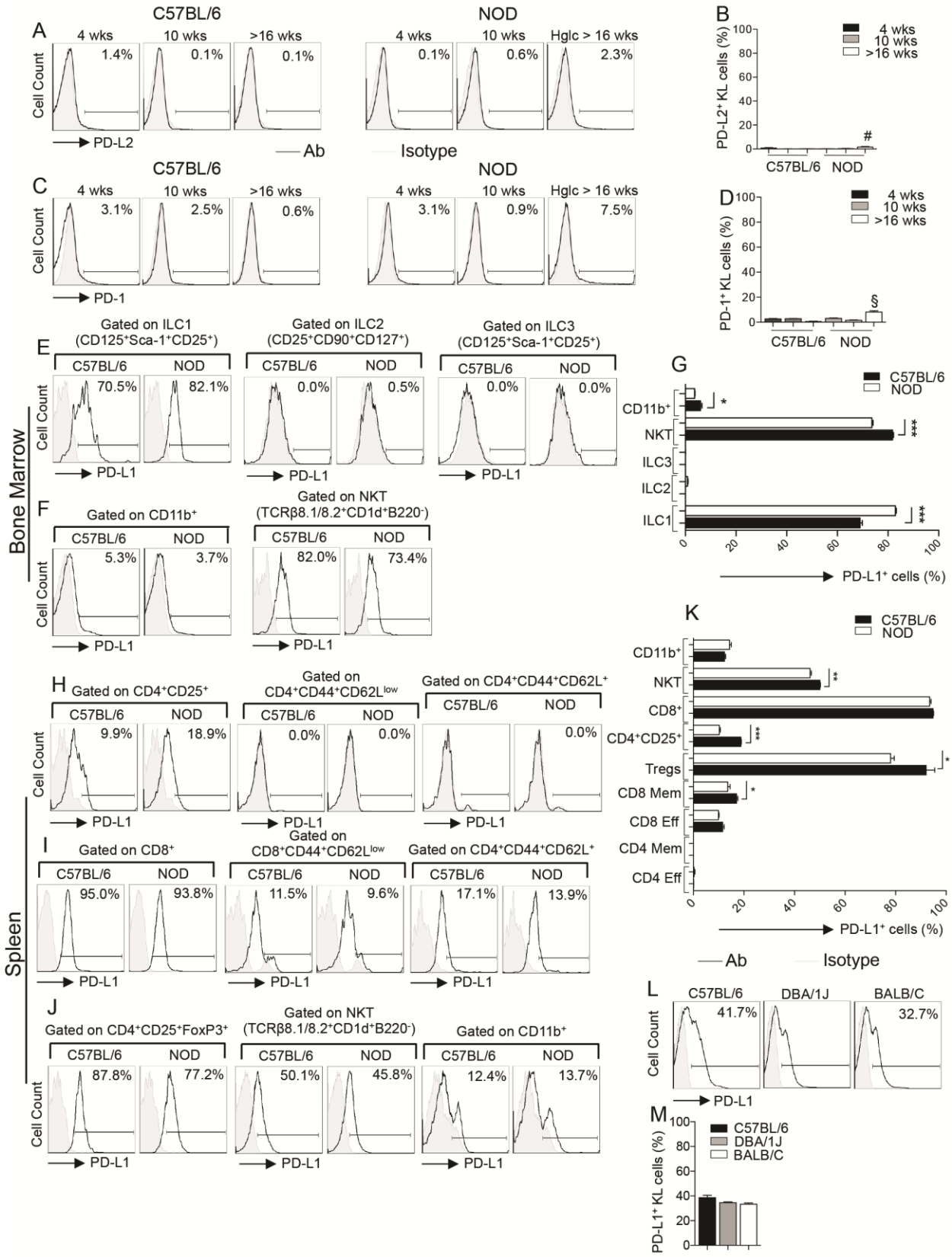

A
C57BL/6
NOD
4 wks
10 wks
>16 wks
Hglc > 16 wks
1.4%
0.1%
0.1%
0.1%
0.6%
2.3%
Cell Count
PD-L2
Ab
Isotype
B
PD-L2⁺ KL cells (%)
4 wks
10 wks
>16 wks
#
C57BL/6
NOD
C
3.1%
2.5%
0.6%
3.1%
0.9%
7.5%
PD-1
D
PD-1⁺ KL cells (%)
§
E
Gated on ILC1 (CD125⁺Sca-1⁺CD25⁺)
Gated on ILC2 (CD25⁺CD90⁺CD127⁺)
Gated on ILC3 (CD125⁺Sca-1⁺CD25⁺)
70.5%
82.1%
0.0%
0.5%
0.0%
0.0%
PD-L1
F
Gated on CD11b⁺
Gated on NKT (TCRβ8.1/8.2⁺CD1d⁺B220⁻)
5.3%
3.7%
82.0%
73.4%
Bone Marrow
G
CD11b⁺
NKT
ILC3
ILC2
ILC1
PD-L1⁺ cells (%)
H
Gated on CD4⁺CD25⁺
Gated on CD4⁺CD44⁺CD62L^low
Gated on CD4⁺CD44⁺CD62L⁺
9.9%
18.9%
0.0%
0.0%
0.0%
I
Gated on CD8⁺
Gated on CD8⁺CD44⁺CD62L^low
Gated on CD4⁺CD44⁺CD62L⁺
95.0%
93.8%
11.5%
9.6%
17.1%
13.9%
Spleen
J
Gated on CD4⁺CD25⁺FoxP3⁺
Gated on NKT (TCRβ8.1/8.2⁺CD1d⁺B220⁻)
Gated on CD11b⁺
87.8%
77.2%
50.1%
45.8%
12.4%
13.7%
K
CD11b⁺
NKT
CD8⁺
CD4⁺CD25⁺
Tregs
CD8 Mem
CD8 Eff
CD4 Mem
CD4 Eff
PD-L1⁺ cells (%)
L
C57BL/6
DBA/1J
BALB/C
41.7%
32.7%
M
PD-L1⁺ KL cells (%)

Fig. S1. PD-L1 expression in non-HSPCs. (**A-D)** Representative flow cytometric analysis and quantitative bar graphs of other costimulatory molecules other than PD-L1 (i.e. PD-L2 and PD-1) in hematopoietic stem progenitor cells isolated from the bone marrow of NOD and C57BL/6 mice. Experiments were run in triplicate and for statistical analysis, one-way ANOVA followed by Bonferroni's Multiple Comparison Test for group comparisons between C57BL/6 and NOD mice; in C: #P<0.05 vs all except NOD-4 wks (P=ns) and in F: §P<0.0001 vs all. (**E-G**) PD-L1 expression on other relevant immune cells (ILC1, ILC2, ILC3, early myeloid $CD11b^+$ cells and NKT cells) obtained from bone marrow of NOD and C57BL/6 mice. (**H-K**) PD-L1 expression on other relevant immune cells ($CD4^+CD25^+$, CD4 effector T cells, CD4 memory T cells, $CD8^+$ T cells, CD8 effector T cells, CD8 memory T cells, $CD4^+CD25^+FoxP3^+$ T regulatory cells, NKT cells and early myeloid $CD11b^+$ cells) obtained from spleen of NOD and C57BL/6 mice. (**L-M**) Representative flow cytometric analysis and quantitative bar graphs of PD-L1 expression on KL isolated from BALB/c and DBA/1 mice as compared to C57BL/6 mice. Experiments were run in triplicate and statistical significance was determined using two-tailed unpaired t-test or with Welch's Correction test if applicable. Data are expressed as mean±standard error of the mean (SEM) unless otherwise specified. *P<0.05; **P<0.01; ***P<0.0001; §P<0.0001 vs. all.

Abbreviations: NKT, natural killer T cells; ILC1, ILC2, ILC3, innate lymphoid cells type 1, 2, 3, respectively.

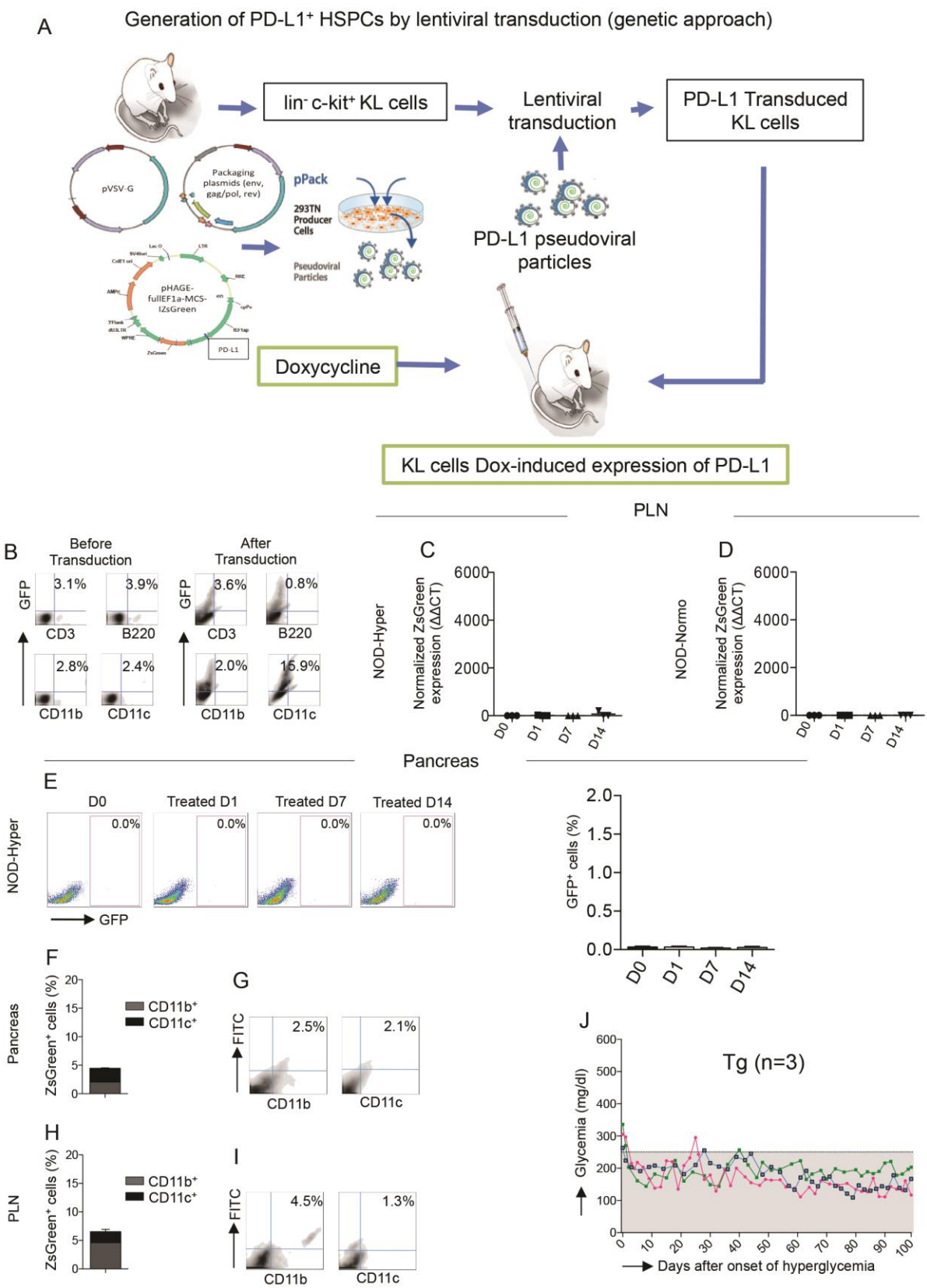

A
Generation of PD-L1+ HSPCs by lentiviral transduction (genetic approach)
lin- c-kit+ KL cells
Lentiviral transduction
PD-L1 Transduced KL cells
pVSV-G
Packaging plasmids (env, gag/pol, rev)
pPack
293TN Producer Cells
Pseudoviral Particles
PD-L1 pseudoviral particles
pHAGE-fullEF1a-MCS-IZsGreen
PD-L1
Doxycycline
KL cells Dox-induced expression of PD-L1
B
Before Transduction
After Transduction
GFP
CD3
B220
CD11b
CD11c
3.1%
3.9%
2.8%
2.4%
3.6%
0.8%
2.0%
15.9%
PLN
C
NOD-Hyper
Normalized ZsGreen expression (ΔΔCT)
D
NOD-Normo
D0
D1
D7
D14
Pancreas
E
D0
Treated D1
Treated D7
Treated D14
0.0%
GFP+ cells (%)
F
Pancreas
ZsGreen+ cells (%)
CD11b+
CD11c+
G
FITC
2.5%
2.1%
H
PLN
I
4.5%
1.3%
J
Tg (n=3)
Glycemia (mg/dl)
Days after onset of hyperglycemia

Fig. S2. Generation of PD-L1.Tg KL cells and their tracking. (**A**) Schematic representation of the genetic approach employed to generate PD-L1.Tg KL cells by lentiviral transduction. (**B**) Representative flow cytometric analysis for lymphoid and myeloid markers of isolated KL cells before and after lentiviral transduction (**C, D**) Quantification of ZsGreen mRNA in the pancreatic lymph nodes of hyperglycemic and normoglycemic NOD mice by qRT-PCR after treatment with PD-L1.Tg KL cells. (**E**) Representative flow cytometric analysis and quantitative bar graphs of GFP^+ KL cells in the pancreas of hyperglycemic NOD mice at 1, 7 and 14 days after treatment with WT KL cells isolated from bone marrow cells of normoglycemic NOD $Luciferase^+$ GFP^+ mice. (**F-I**) Representative flow cytometric analysis and quantitative bar graphs of $CD11c^+ZsGreen^+$ cells and $CD11b^+ZsGreen^+$ cells in the pancreas and PLN of adoptively transferred NOD mice. (**J**) Newly hyperglycemic NOD mice were treated with PD-L1.Tg KL cells and followed for 100 days. All experiments were run in triplicate and for statistical analysis, one-way ANOVA followed by Bonferroni's Multiple Comparison Test for group comparisons. Data are expressed as mean±standard error of the mean (SEM) unless otherwise specified.

Abbreviations: WT, wild type; KL, $lineage^-c\text{-}kit^+$ cells; Tg, transgenic; PLN, pancreatic lymph nodes; GFP, green fluorescent protein.

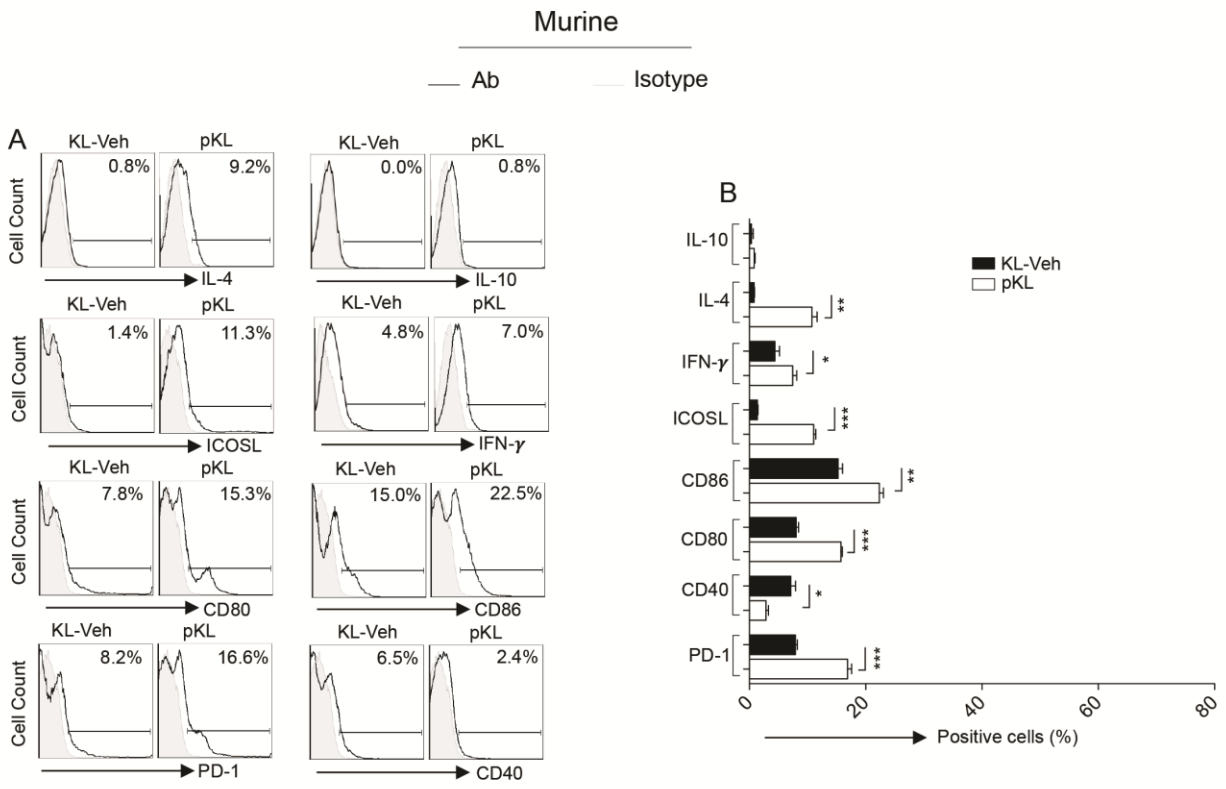


Fig. S3. Immunophenotype of pKL cells. (**A-B**) Representative flow cytometric analysis and quantitative bar graph of positive and negative costimulatory molecules, (CD40, CD80, CD86, ICOSL, PD-1) and of select pro-inflammatory and anti-inflammatory cytokines (IFN-γ, IL-10, IL-4) in pharmacologically-modulated KL cells (pKL) from NOD mice as compared to unmodulated KL-Veh cells isolated from the bone marrow of normoglycemic NOD mice. Experiments were run in triplicate and for statistical significance was determined using two-tailed unpaired t-test or with Welch's Correction test if applicable. Experiments were performed in triplicate and for statistical analysis, one-way ANOVA followed by Bonferroni's Multiple Comparison Test for group comparisons between C57BL/6 BALB/c and DBA/1 mice. Data are expressed as mean±standard error of the mean (SEM) unless otherwise specified. **P*<0.05; ***P*<0.01; ****P*<0.0001.

Abbreviations: pKL, pharmacologically-modulated lineage$^-$c-kit$^+$ cells; KL-Veh, lineage$^-$c-kit$^+$-vehicle-treated cells.

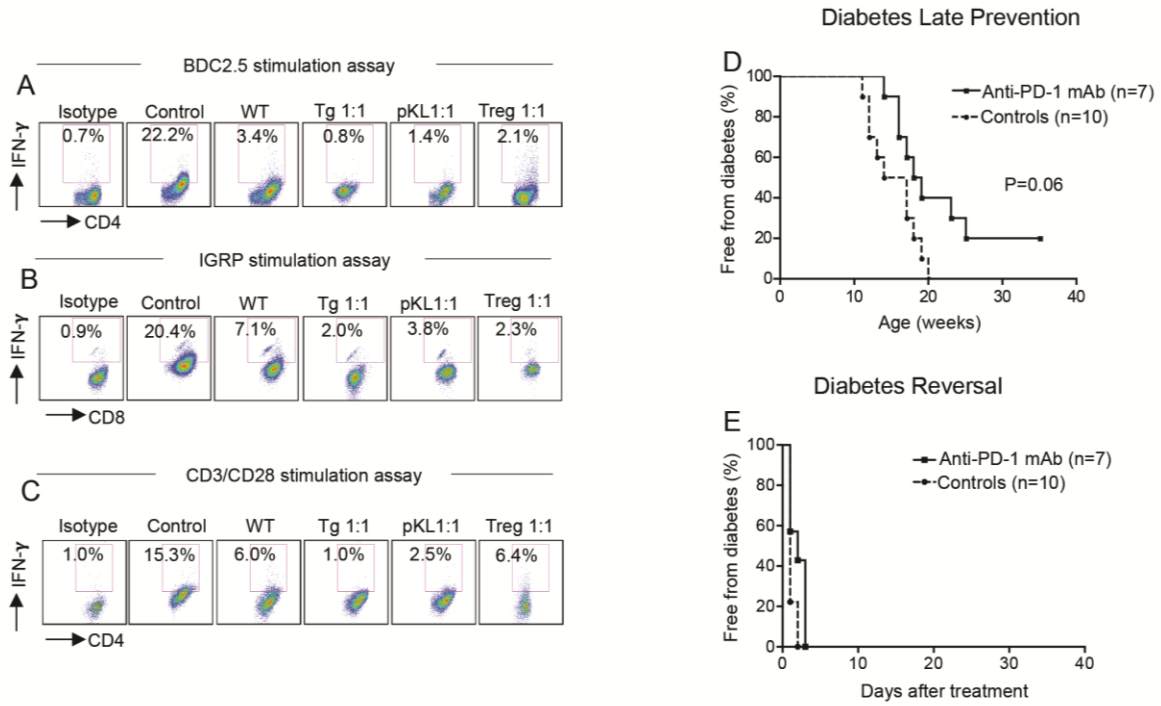


Fig. S4. Anti–PD-1 studies. (**A**) Representative flow cytometric analysis of IFN-γ^+CD4$^+$ T cells isolated from NOD-BDC2.5 TCR Tg mice stimulated with BDC2.5 peptide in the presence of DCs (Control) or upon co-culture with unmodulated KL cells (WT), with pharmacologically-modulated KL cells (pKL), with PD-L1.Tg KL cells or with CD4$^+$CD25$^+$ T regulatory cells. (**B**) Representative flow cytometric analysis of IFN-γ^+CD8$^+$ T cells isolated from NOD-8.3 TCR Tg mice stimulated with IGRP peptide in the presence of DCs (Control) or upon co-culture with unmodulated KL cells (WT), with pKL cells, with PD-L1.Tg KL cells, or with CD4$^+$CD25$^+$ T regulatory cells. (**C**) Representative flow cytometric analysis of IFN-γ^+CD4$^+$ T cells isolated from normoglycemic NOD mice and stimulated with soluble anti-CD3/anti-CD28 (Control) or upon co-culture with unmodulated KL cells (WT), with pKL, with PD-L1.Tg KL cells or with CD4$^+$CD25$^+$ T regulatory cells. (**D, E**) Incidence of diabetes in pre-diabetic 10-week-old NOD mice and newly hyperglycemic NOD mice treated with anti-PD-1 stimulating mAb (clone

PIM2). Incidence of diabetes in all groups of NOD mice (untreated, anti-PD-1-treated mice) was compared using the Log-rank (Mantel-cox) test.

Abbreviations: WT, wild type; pKL, pharmacologically-modulated lineage$^-$c-kit$^+$ cells; Tg, transgenic; Treg, CD4$^+$CD25$^+$ T regulatory cells.

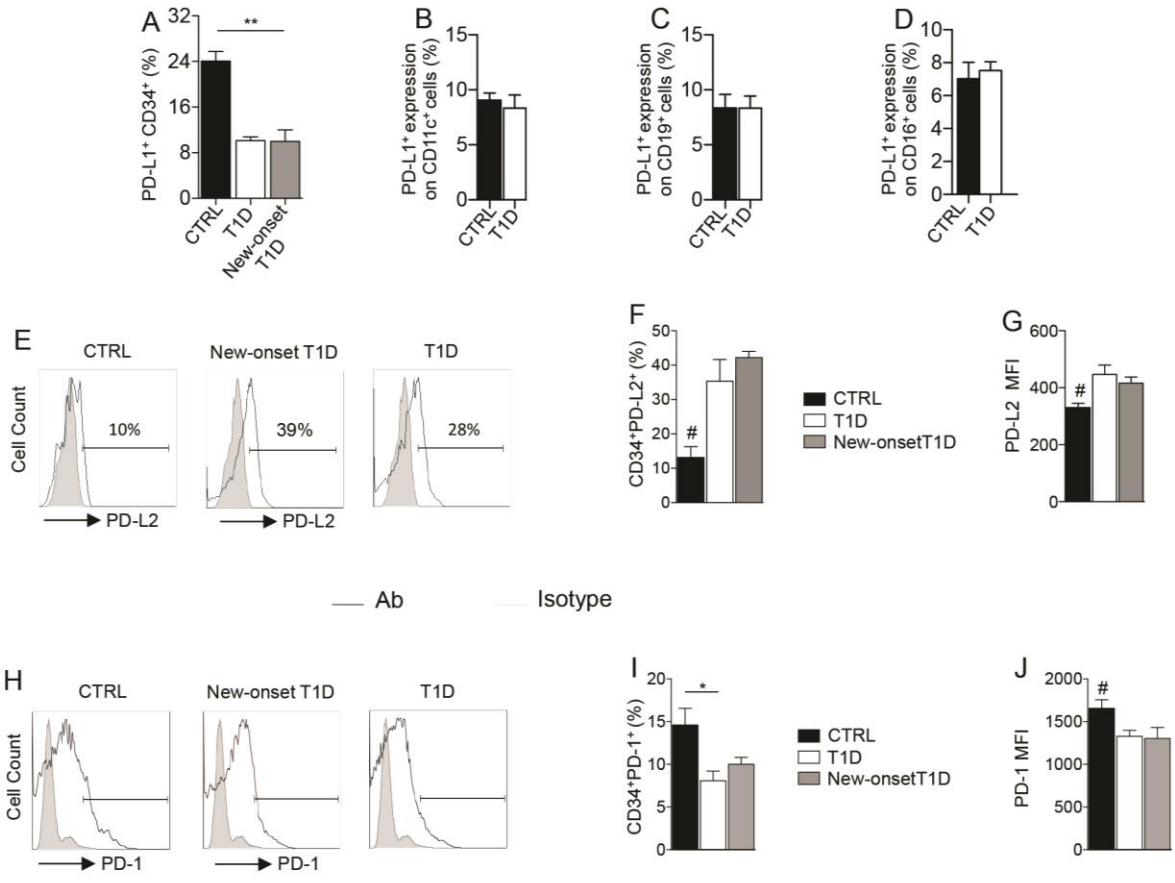


Fig. S5. CD34$^+$ cells characterization in T1D patients and in healthy controls. (**A**) Quantification of PD-L1$^+$CD34$^+$ cells by flow cytometry in long-standing or new-onset T1D patients and in healthy controls (CTRL). (**B, C, D**) PD-L1 expression on other relevant immune cells in patients with T1D and in healthy controls. (**E to J**) Representative flow cytometric analysis and quantitative bar graph of PD-L2$^+$CD34$^+$ cells and PD-1$^+$CD34$^+$ in long-standing or new-onset T1D patients and in healthy controls (CTRL) in CD34$^+$ cells isolated from peripheral blood of healthy controls or from long-standing or new-onset T1D patients, MFI was also shown. Experiments were run in quadruplicate (n=5 samples for CTRL) and statistical significance were

performed using One-way ANOVA with Dunnett's Multiple Comparison Test. *P<0.05; **P<0.01; ***P<0.001. #P<0.05 vs. all.

Abbreviations: CTRL, healthy controls; T1D, type 1 diabetes; MFI, mean fluorescence intensity.

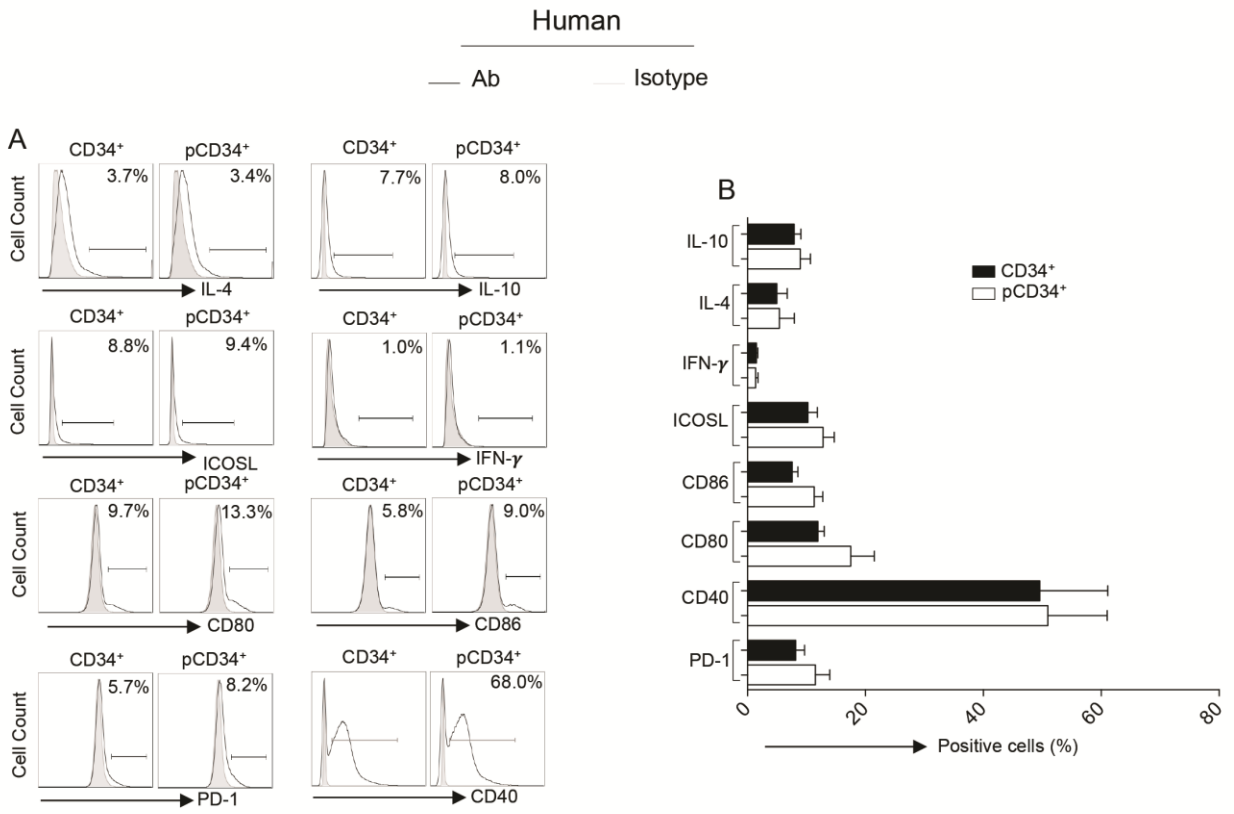
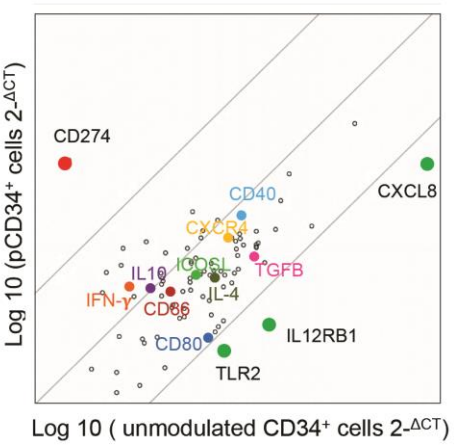


Fig. S6. Immunophenotype of pCD34$^+$. (**A-B**) Representative flow cytometric analysis and quantitative bar graph of positive and negative costimulatory molecules, (CD40, CD80, CD86, ICOSL, PD-1) and of select pro-inflammatory and anti-inflammatory cytokines (IFN-γ, IL-10, IL-4) in human pharmacologically-modulated CD34$^+$ cells (pCD34$^+$) as compared to unmodulated CD34$^+$ cells isolated from peripheral blood of T1D patients (n=3). Statistical analysis was performed using two-tailed unpaired t-test. Data are expressed as mean±standard error of the mean (SEM) unless otherwise specified.

Abbreviations: pKL, pharmacologically-modulated lineage$^-$c-kit$^+$ cells; KL-Veh, lineage$^-$c-kit$^+$-vehicle-treated cells; pCD34$^+$, human pharmacologically-modulated CD34$^+$cells.

A



B

Gene Symbol	P-value	Fold change
CD274 (PD-L1)	0.028999	13.9304

Fig. S7. Transcriptome of pCD34^{+}. (**A-B**) Scattered dot plot and fold change representing transcriptomic profiling of immune-related molecules in pCD34^{+}cells as compared to unmodulated CD34^{+} cells isolated from peripheral blood of T1D patients (n=3). statistical analysis was performed by using the software available RT2 profiler PCR Array Data Analysis (Qiagen). Data are expressed as mean±standard error of the mean (SEM) unless otherwise specified.

Abbreviations: pCD34^{+}, human pharmacologically-modulated CD34^{+}cells.

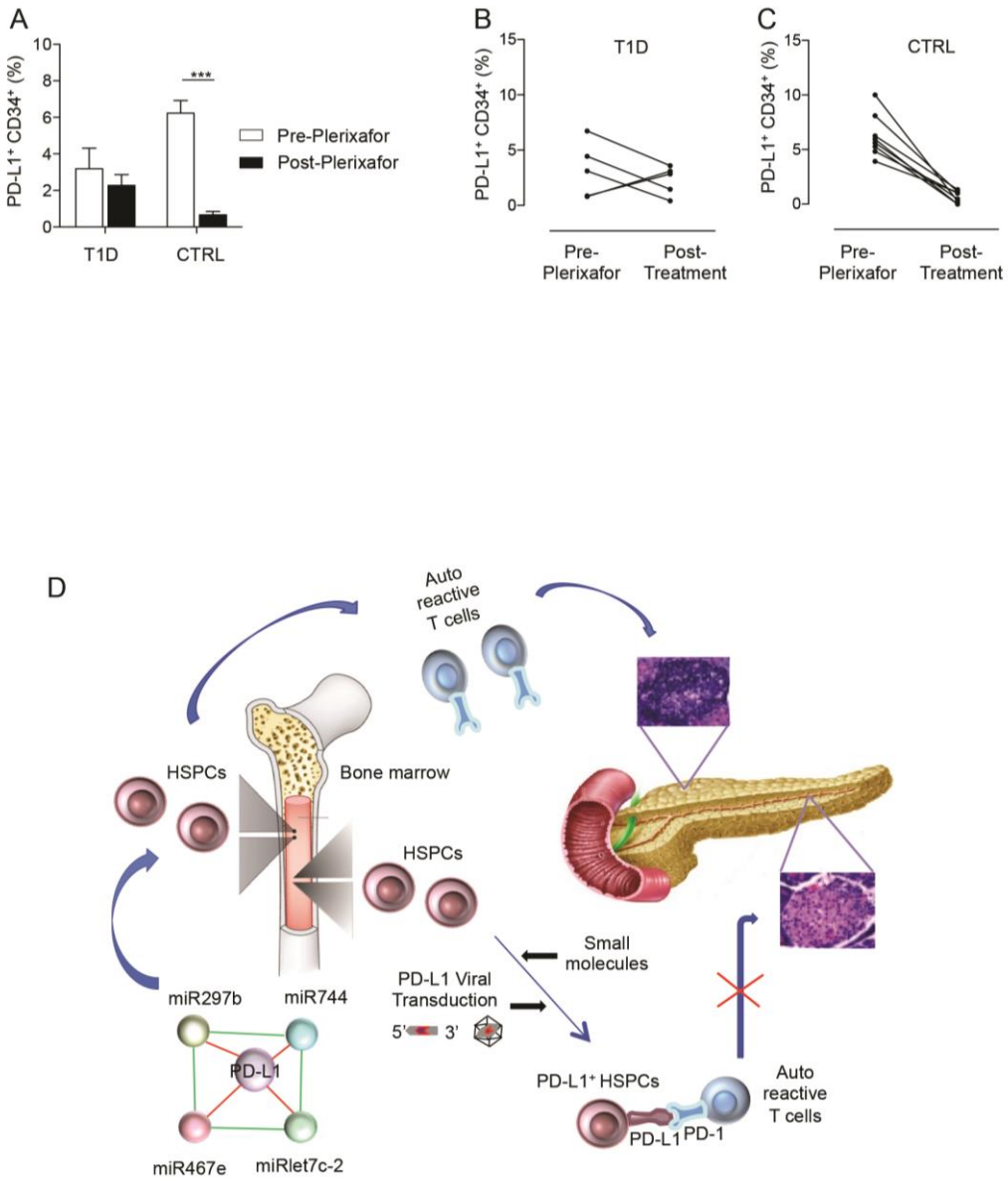


Fig. S8. CD34$^+$ cell mobilization with plerixafor and working hypothesis. (**A-C**) The impact of CD34$^+$ cell mobilization with Plerixafor on the peripheral PD-L1$^+$CD34$^+$ cells frequencies in T1D patients (n=5 samples) and in healthy controls (n=8 samples) was evaluated. (**D**) Schematic representing the defect in PD-L1 in HSPCs in T1D, as well as the effect of PD-L1 genetic/pharmacological restoration. Data are expressed as mean±standard error of the mean

(SEM) and statistical analysis was performed using two-tailed unpaired t-test with Welch's Correction. *P<0.05; **P<0.01; ***P<0.0001.

Abbreviations: CTRL, healthy controls; T1D, type 1 diabetes.

Supplemental Tables

Table S1. Transcriptomic profiling of murine KLS cells (provided as an Excel file). List of differentially expressed pro-/anti-inflammatory genes identified by transcriptomic profiling in Sca-1^{+}Lineage^{-}c-kit^{+} (KLS) cells from NOD as compared to those obtained from C57BL/6 mice. Related to Figures 1A and 1B.

Abbreviations: KLS; Sca-1^{+}lineage^{-}c-kit^{+} (provided as an Excel file).

Table S2. Genome-wide expression analysis of murine KLS cells (provided as an Excel file). List of differentially expressed genes identified by genome-wide expression analysis (GWAS) performed on Sca-1^{+}Lineage^{-}c-kit^{+} cells (KLS) from NOD as compared to those obtained from C57BL/6 mice (p<0.05). Related to Figures 2D-2G.

Abbreviations: KLS; Sca-1^{+}lineage^{-}c-kit^{+} (provided as an Excel file).

Table S3. Genome-wide expression analysis of Tg KL cells. List of differentially expressed genes identified by genome-wide expression analysis (GWAS) performed on Tg.KL cells as compared to Mock.KL cells isolated from NOD mice ($p<0.05$). Related to Figures 3F-3G.

Transcript Cluster ID	Tg	Mock	Fold Change	Gene Symbol	Description
TC1900000441.MM.1	14.12	5.77	327.59	Cd274 (PD-L1)	CD274 antigen
TC0600000577.MM.1	13.41	5.09	320.41	Gpnmb	glycoprotein (transmembrane) nmb
TC1200002584.MM.1	14.28	6.83	174.57	LOC544905	Ig heavy chain V region; immunoglobin heavy variable V9-3
TC0900003098.MM.1	15.11	8.16	123.97	Camp	cathelicidin antimicrobial peptide
TC0300000811.MM.1	17.5	11	90.29	S100a8	S100 calcium binding protein A8 (calgranulin A)
TC120000647.MM.1	12.62	6.29	80.58	Ighv8-12	Ig heavy chain V region; immunoglobin heavy variable V8-12
TC0700003570.MM.1	10.4	4.54	57.92	Anpep	alanyl (membrane) aminopeptidase
TC0900001461.MM.1	15.04	9.26	54.98	Ngp	neutrophilic granule protein
TC1500001728.MM.1	13.74	7.97	54.58	Ly6a	lymphocyte antigen 6 complex, locus A (lys6a), mRNA.
TC1000003214.MM.1	16.38	10.61	54.48	Lilrb4	leukocyte immunoglobulin-like receptor, subfamily B, member 4
TC0400001645.MM.1	5.82	11.6	-54.6	Rhd	Rh blood group, D antigen
TSU nmapped00000051.mm.1	11.85	17.78	-61.14	Ahsp	alpha hemoglobin stabilizing protein
TC0600000664.mm.1	7.43	13.67	-75.45	Aqp1	aquaporin 1
TC0400003418.mm.1	6.47	13.34	-117.38	Ermap	erythroblast membrane-associated protein
TC0500003382.mm.1	6.2	13.48	-155.47	Cldn13	claudin 13
TC0300001667.mm.1	11	18.4	-169.05	Car1	carbonic anhydrase 1
TC1700000817.mm.1	6.47	14.3	-228.24	Rhag	Rhesus blood group-associated A glycoprotein
TC0300000094.MM1	9.67	17.85	-290.12	Gm5843	predicted gene 5843; carbonic anhydrase 1 (car1) pseudogene

Abbreviations: KL, lineage$^-$c-kit$^+$; Tg, transgenic.

Table S4. Chemokine receptors expression in different groups of KL cells. List of differentially expressed chemokine receptors in PD-L1.Tg KL cells, pharmacologically-modulated KL cells (pKL) and unmodulated-KL cells (KL-Veh) isolated from bone marrow of normoglycemic NOD mice. Data are expressed as mean ± standard error (SEM). *mean statistically significant vs. others.

Chemokine receptors	Expression on KL-Veh (%)	Expression on pKL (%)	Expression on PD-L1 Tg.KL (%)	P value
CCR2	6.1±0.1	5.2±0.0	2.9±0.1*	0.02
CCR4	1.4±0.1	3.1±0.2	11.6±1.1*	0.02
CCR5	0.7±0.0	0.0±0.0	0.0±0.0	ns
CCR6	2.1±0.3	8.6±0.7*	5.3±0.6	0.02
CCR7	2.7±0.3	0.2±0.0	0.7±0.3	ns
CCR8	2.0±0.4	9.8±0.4	2.0±0.1	ns
CXCR3	0.2±0.1	0.0±0.0	1.7±0.8	ns
CXCR4	41.7±0.3	37.0±2.5	61.6±1.9*	0.004
S1PR1	65.4±0.7	66.3±0.8	64.6±0.4	ns

Abbreviations: KL, lineage$^-$c-kit$^+$; Tg, transgenic; ns, not significant.

Table S5. Genome-wide expression analysis of pKL cells: up-regulated genes (provided as an Excel file). List of upregulated genes identified by genome wide expression analysis (GWAS) performed on pharmacologically-modulated KL cells (pKL) as compared to vehicle-treated KL cells isolated from normoglycemic NOD mice ($p<0.05$). Related to Figures 5K-5L.

Abbreviations: KL; lineage$^-$c-kit$^+$ (provided as an Excel file).

Table S6. Genome-wide expression analysis of pKL cells: down-regulated genes (provided as an Excel file). List of downregulated genes identified by genome wide expression analysis (GWAS) performed on pharmacologically-modulated KL cells (pKL) as compared to Vehicle treated-KL cells isolated from bone marrow of normoglycemic NOD mice ($p<0.05$). Related to Figures 5K-5L.

Abbreviations: KL; Lineage$^-$c-kit$^+$; pKL, pharmacologically-modulated KL cells (provided as an Excel file).

Table S7. Characteristics of patients enrolled in the study. Baseline demographic characteristics of patients enrolled in the study. Data are expressed as mean ± standard error (SEM).

	CTRL (n=12)	**T1D (n=12)**	**New-onset T1D (n=12)**
Sex (M/F)	4/8	6/6	4/8
Age (years)	34.8±2.3	39.6 ± 3.1	10.9± 1.1
Years of T1D	N/A	22.3±3.8	N/A
HbA1c % (mmol/mol)	5.0±0.05 (31±0.5)	9.2±0.4 (78±4.0)	12.3± 0.4 (111±4.9)
EIR (UI)	N/A	42.0±4.1	N/A
Concomitant Treatments	N/A	Levothyroxine (n=3) Statin (n=2)	N/A

Abbreviations. CTRL, healthy volunteers; T1D, type 1 diabetic patients,; HbA1c %, glycated hemoglobin A1c %; EIR, exogenous insulin requirement; N/A, not applicable.

Table S8. Characteristics of patients enrolled in the plerixafor mobilization study. Baseline demographic characteristics of patients enrolled in the plerixafor mobilization study. Data are expressed as mean ± standard error (SEM).

	CTRL (n=8)	**T1D (n=5)**	**P value**
Age (years)	44.1±5.1	39.0±3.9	0.494
Male/Female		4/1	Ns
Weight (kg)	76.7±6.7	76.2±6.1	0.961
Height (cm)	174.8±1.84	174.4±4.6	0.913
BMI (kg/mq)	25.0±2.1	25.0±1.5	0.988
HbA1c (%)	5.7±0.2	7.9±0.3	0.026
Duration (Years)	0.0	20.8±4.9	Ns
Hypertension (%)	37.5	0	0.139
Retinopathy (%)	0.0	20.0	0.742
Microalbuminuria (%)	0.0	0.0	Ns
Neuropathy (%)	0.0	0.0	Ns
Atherosclerosis (%)	0.0	20.0	Ns
White blood cells (10^9 per liter)	7.1±0.9	6.1±0.6	Ns

Abbreviations. CTRL, healthy controls; T1D, type 1 diabetes; HbA1c, glycated hemoglobin A1c.

Table S9. Transcriptome of pCD34$^+$ cells (provided as an Excel file). List of upregulated and downregulated inflammatory and costimulation-related genes identified by transcriptome profiling in pharmacologically-modulated CD34$^+$ cells as compared to unmodulated-CD34$^+$ cells obtained from T1D patients. Genes with statistically significant differences ($p<0.05$) are in italics. Related to Figures S6A and S6B.

Abbreviations: pCD34$^+$, human pharmacologically-modulated CD34$^+$cells; T1D, type 1 diabetes (provided as an Excel file).

Table S10. Primary data (provided as an Excel file).