Supporting Information

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SI Materials and Methods

Mice. Our nonobese diabetic (NOD) mouse colony was originally derived from Jackson Laboratories. All NOD mice were bred and maintained in specific pathogen-free barrier facilities at Yale University. All animal experiments were approved by the institutional animal care and use committee of Yale University. Only female NOD mice were used for experiments. Female NOD mice develop diabetes at approximately 12 wk of age with an incidence of approximately 90% by 24 to 30 wk. BDC2.5 NOD mice were originally obtained from Diane Mathis (Harvard Medical School, Boston, MA). RIP-CCL2 transgenic (tg) mice on the C57BL/6 background were generated as previously described (1). RIP-CCL2 tg mice were backcrossed more than 10 times onto the NOD background. Experiments were mostly performed at backcross 17 and beyond. Genotyping primer sequences of WT and tg NOD mice are available upon request.

Immunohistochemistry and Assessment of Insulitis. Insulitis was assessed by histologic analysis. Pancreata were prepared frozen on dry ice in optimal cutting temperature compound (Miles Laboratories) or fixed in 10% formalin before embedding in paraffin as described previously (1). Paraffin-embedded pancreata were sectioned and stained with H&E to assess presence and severity of insulitis by light microscopy. Frozen sections were analyzed by immunohistochemistry by using biotinylated monoclonal antibodies against CD4 (Life Technologies), CD8 (Life Technologies), B220 (Caltag), F4/80 (Serotec), CD11b, CD11c, insulin (BioGenex), and a polyclonal anti-glucagon antibody (BioGenex). Microscopic images were recorded with an Axio Imager.A1 microscope (Carl Zeiss Microimaging), AxioCam MRc5 camera, and AxioVision 4.7.1 imaging software (Carl Zeiss Microimaging).

Diabetes Assessment. Diabetes was assessed by weekly measurements of urine glucose with Diastix (Ames). Onset of diabetes was confirmed by blood glucose measurements. Mice were considered diabetic if three consecutive measurements of blood glucose were greater than 250 g/dL. Mice without glucosuria beyond 32 wk of age were considered protected.

Cell Isolations and Flow Cytometric Analysis. Single cell suspensions of spleens and inguinal and pancreatic lymph nodes were prepared by glass-slide disruption. Cells were stimulated with phorbol 12-myristate 13-acetate (50 nM) and ionomycin (500 nM) for 4 h for intracellular cytokine analysis. Cytokines were detected following the manufacturer's protocol (BD Pharmingen). Pancreatic tissue was disrupted in Bruff medium containing 2 mM EDTA followed by removal of debris with a filter. Mononuclear cells were separated by Ficoll density gradient centrifugation. In separate experiments, islet cells were isolated freshly from pancreata by the Yale Diabetes Endocrinology Research Center Islet Isolation Sub-core following standard procedures including collagenase digestion of tissues and handpicking of pancreatic islets as previously described (2, 3). Cells isolated from the various tissues were counted after trypan blue staining. Single cell suspensions were analyzed by flow cytometry by using FACS SCAN, FACSCalibur, or LSRII and CELLQuest software, FACS Diva software (BD Biosciences), or FlowJo software (Tree Star), respectively. Cell sorting and counting of absolute numbers of defined subpopulations was performed by using a FACSAria cell sorter (BD Biosciences). The following monoclonal antibodies (all from BD Pharmingen except when noted otherwise) were used: CD3, CD4, CD8α, CD11b, CD19, CD25, Foxp3, CD44, CD62L, CD127 (eBioscience), B220, CD40, MHC II (IA^b/IA^d), CD80, CD86, Gr-1/Ly6C, and IL-2, -4, and -17, and IFN-γ. Cells stained with biotinylated monoclonal antibodies were incubated with fluorochrome-conjugated streptavidin-PE, streptavidin-PerCP, streptavidin-APC, and streptavidin-APC-Cyc7 (BD Pharmingen). All fluorescence intensity plots are shown in log scales. The purity of sorted cell populations used for in vivo transfer experiments was always at least 95%.

In Vivo Transfer Experiments. Pancreatic LNs were isolated from 10 pooled 3-wk-old RIP-CCL2/BDC2.5 NOD mice and their BDC2.5/NOD littermates. $CD11c^+$ $CD11b^+$ DCs, $CD11b^+$ $CD11c^-$ macrophages/monocytes, $CD4^+$ T cells, and $CD4^+$ $CD25^+$ T cells were sorted by FACS. One million cells of each sorted subpopulation were injected i.v. into female NOD mice at 3 wk of age (n = 9–10 per each group). Disease incidence was monitored as described in *Diabetes Assessment* until 12 and 18 wk, respectively (Fig. S6 illustrates the setup of the transfer experiment).

Cell Culture and in Vitro Proliferation Experiments. In vitro cocultures of BDC2.5 TCR tg NOD $CD4^+$ T cells with WT or RIP-CCL2 tg NOD $CD11c^+$ $CD11b^+$ dendritic cells (DCs) were performed in Bruff medium supplemented with 10% FCS, 2 mM L-glutamine, 1% penicillin/streptomycin, and 1 mM nonessential amino acids. $CD4^+$ T cells (1×10^5) from BDC2.5/NOD mice were sorted. A total of 10^3, 10^4, and 10^5 sorted DCs obtained from pancreatic LN of 12 wk old RIP-CCL2 tg NOD mice or control littermates were cocultured in vitro with BDC2.5 peptide (termed 1040–55; 30 μg/mL) and TCR tg T cells. Positive control DCs and $CD4^+$ T cells from C57BL/6 mice were stimulated with αCD3 (5 μg/mL) and soluble αCD28 (2 μg/mL) for 24 to 48 h. Cell proliferation was measured by [^{3}H]thymidine incorporation during the last 6 to 8 h of coculture. Proliferation was expressed as cpm.

In Vitro Stimulation of DCs and Detection of Cytokine Secretion. $CD11c^+$ $CD11b^+$ DCs were isolated from spleen and pancreatic LN as described earlier. DCs were stimulated ex vivo with LPS (100 ng/mL) for 36 h to induce cytokine secretion. Supernatants were collected at the end of the culture period, and IL-10, IL-6, and TNF-α were measured by standard ELISA (ELISA kit; BD Biosciences).

RNA Extraction and RT-PCR. RNA isolation and reverse transcription was performed to test if *Grail* mRNA is detectable in pancreatic LN $CD4^+$ T cells. In brief, total RNA was isolated from pancreatic LN $CD4^+$ T cells by using an RNA Mini Prep kit (Stratagene) followed by cDNA synthesis with oligo dT primer and SuperScript II RT (Invitrogen). The PCR was performed with *Hprt* and *Grail* primers, which are available upon request. The band area size was quantified using the open source software ImageJ (http://rsbweb.nih.gov/ij/).

Statistical Analysis. Data are presented as mean ± SEM. Statistical significance was assessed by using the two-sided Student t test and log-rank test for cumulative diabetes incidence curves, respectively. Statistically significant P values were those lower than 0.05, as marked with a asterisk in the figure legends or stated specifically.

1. Grewal IS, et al. (1997) Transgenic monocyte chemoattractant protein-1 (MCP-1) in pancreatic islets produces monocyte-rich insulitis without diabetes: Abrogation by a second transgene expressing systemic MCP-1. *J Immunol* 159:401–408.
2. Faveeuw C, Gagnerault MC, Lepault F (1995) Isolation of leukocytes infiltrating the islets of Langerhans of diabetes-prone mice for flow cytometric analysis. *J Immunol Methods* 187:163–169.
3. Green EA, Eynon EE, Flavell RA (1998) Local expression of TNFalpha in neonatal NOD mice promotes diabetes by enhancing presentation of islet antigens. *Immunity* 9: 733–743.

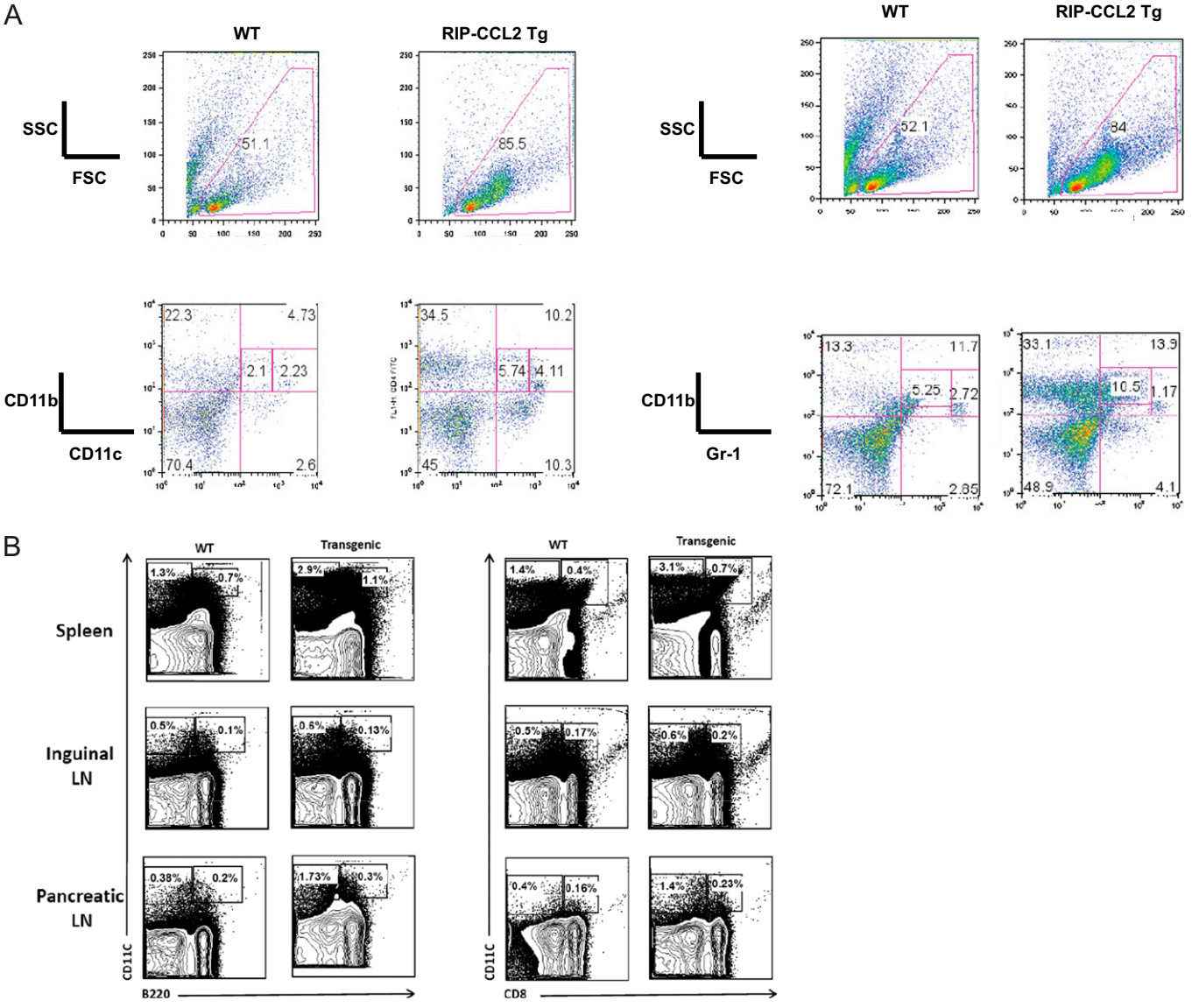

Fig. S1. Relative cell numbers of DC subpopulations in pancreas, pancreatic LNs, peripheral LNs, and spleens of RIP-CCL2 tg and WT NOD mice. (*A* and *B*) Cells from pancreas, spleen, peripheral LN, and pancreatic LN were isolated, processed and stained for flow cytometry as described in *SI Materials and Methods*. (*A*) Relative numbers of $CD11c^+/CD11b^{+/-}$ DCs (*Left*) as well as $CD11b^+$ and $CD11b^+/Gr\text{-}1^+$ macrophages (*Right*) within pancreatic mononuclear cells were analyzed by FACS. *Upper*, Forward/sideward scatter (FSC/SSC) gating on live mononuclear cells after pancreatic tissue isolation; *Lower*, Scatter plots of CD11c/CD11b and CD11b/Gr-1 double staining of mononuclear cells. Percentages of DC subpopulations and macrophages are shown within the plots. Results are representative of eight pooled WT and seven pooled RIP-CCL2 tg NOD mice, respectively. (*B*) $CD11c^+$ $B220^+$ and $CD11c^+$ $CD8\alpha$+ DCs within each tissue were analyzed by FACS. Contour plots from one representative among three independent experiments are depicted. Percentages of $B220^+$ and $B220^-$ as well as $CD8\alpha^+$ and $CD8\alpha^-$ subpopulations within $CD11c^+$ cells are shown within the plots.

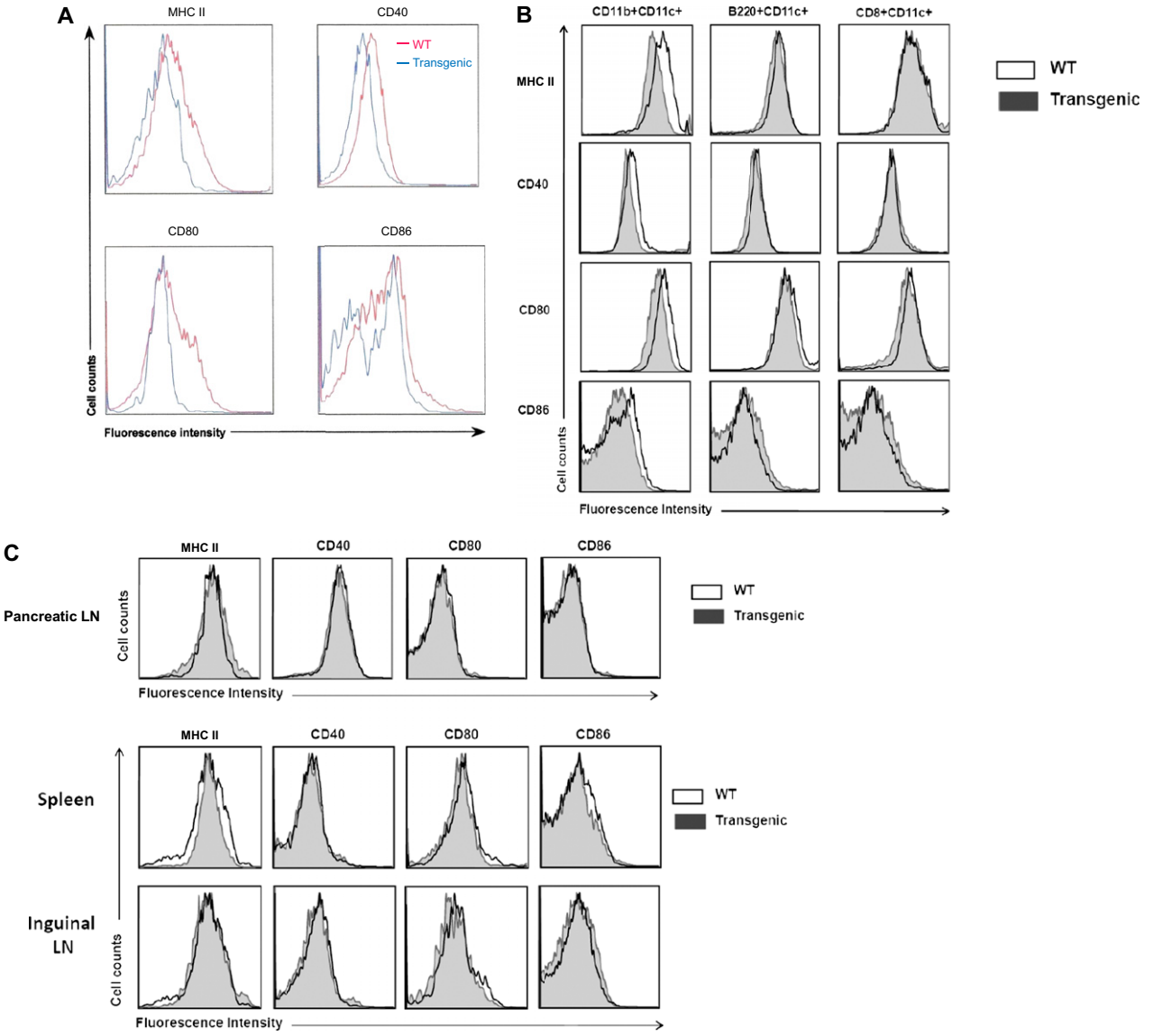

Fig. S2. Activation and costimulatory markers of DCs and macrophages from WT and RIP-CCL2 tg NOD mice. (*A*) Activation and costimulatory markers expressed on $CD11c^+$ $CD11b^+$ DCs from WT and RIP-CCL2 tg NOD mice. $CD11c^+$ $CD11b^+$ DCs from PLNs of 12-wk-old WT and tg NOD mice were stained ex vivo for MHC class II, CD40, CD80, and CD86. All markers are down-regulated in RIP-CCL2 tg NOD mice (blue lines) compared with WT (red lines). Shown are FACS histograms from one representative experiment among three independent experiments. Average geometric mean fluorescence intensities from all three experiments are shown in Fig. 3. (*B*) Surface expression of activation markers and costimulatory molecules from three DC subsets (as indicated) from pancreatic LNs of 12-wk-old WT NOD mice (white histograms) and RIP-CCL2 tg NOD mice (gray histograms). Mean fluorescence intensities did not change significantly in $CD11c^+$ $B220^+$ and $CD11c^+$ $CD8\alpha^+$ DCs. Shown are FACS histograms from one of three independent experiments. (*C*) Surface expression of activation markers and costimulatory molecules on $CD11b^+$ $CD11c^-$ macrophages from pancreatic LN, spleen, and inguinal LN of 12-wk-old WT NOD mice (white histograms) and RIP-CCL2 tg NOD mice (gray histograms). Expression and mean fluorescence intensities of any of the surface markers tested were unchanged in WT vs. tg macrophages from pancreatic LN, spleen, or inguinal LN. Shown are FACS histograms from one of three independent experiments.

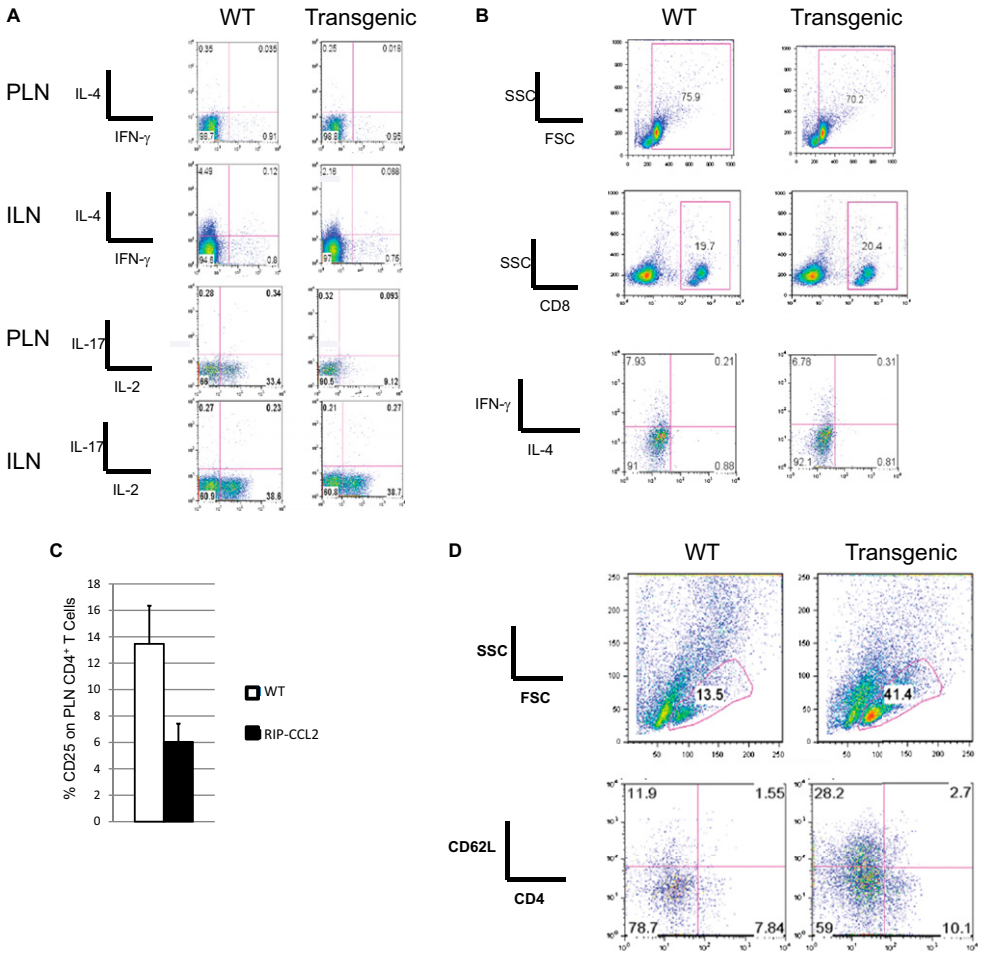

Fig. S3. T helper cytokine secretions by pancreatic LN CD4$^+$ and CD8$^+$ T cells, CD25 expression on pancreatic LN CD4$^+$ T cells, and CD62L expression on islet CD4$^+$ T cells. (*A*) Pancreatic LN (PLN) and inguinal LN (ILN) cells from 7- to 8-wk-old RIP-CCL2 tg NOD mice and littermate controls were isolated as described in *SI Materials and Methods*. LN CD4$^+$ T cells were activated with PMA/ionomycin and stained intracellularly for IFN-γ, IL-4, IL-17, and IL-2 as described in *SI Materials and Methods*. FACS scatterplots are shown for each staining. Percentages of relative numbers are depicted within each quadrant, which represent the average of five or six pooled mice for each group, respectively. (*B*) Pancreatic LN CD8$^+$ T cells were isolated and stained as described in *A* and analyzed for IFN-γ and IL-4 secretion. Percentages of relative numbers are depicted within each quadrant, which represent the average of three to five pooled mice for each group. (*C*) Pancreatic LN (PLN) cells were isolated from WT and RIP-CCL2 tg NOD mice as described in *A* and analyzed for CD25 surface expression on CD4$^+$ T cells by FACS. Bar graphs are shown for an average of CD25 expression from three independent experiments. Error bars represent SEM. (*D*) Islet cells were isolated from pancreata of 7- to 8-wk-old RIP-CCL2 tg NOD mice and littermate controls as described in *SI Materials and Methods*. *Upper*: FACS plots for FSC and SSC of total cells. Percentages of live mononuclear cells (gated based on FSC/SSC of lymph node mononuclear cells) are shown after a collection of a total of 20,000 events in both WT and Tg samples. *Lower*: FACS scatterplots of CD62L and CD4 costained mononuclear cells. Percentages of relative numbers are depicted within each quadrant, which represent the average of three to six pooled mice for each group, respectively.

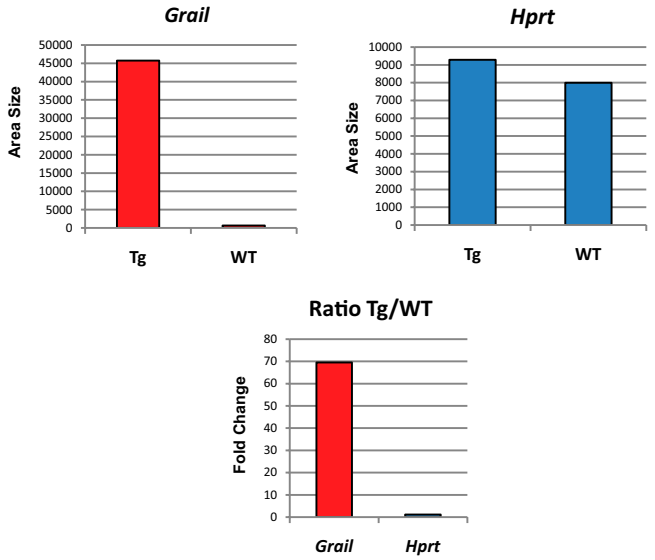

Fig. S4. Densitometric analysis of *Grail* and *Hprt* expression in PLN CD4$^+$ T cells. The density of bands shown in Fig. 5 was quantified by using ImageJ software as described in *SI Materials and Methods*. The area size for *Grail* and *Hprt* expressed in pancreatic LN CD4$^+$ cells from 10 RIP-CCL2 tg NOD mice (Tg) and 10 littermate controls (WT) is shown in bar graphs (*Upper*). *Lower*: Ratio of Tg to WT expression of *Grail* and *Hprt*, respectively. *Grail* is 69.53-fold higher in Tg compared with WT whereas *Hprt* is only 1.16-fold higher.

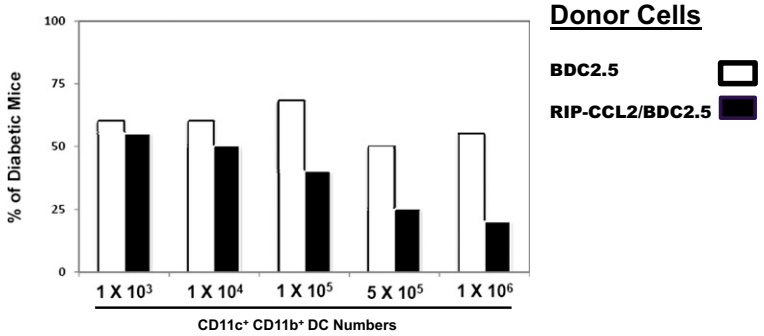

Fig. S5. Dose-dependent inhibition of diabetes by CD11c$^+$ CD11b$^+$ DCs from RIP-CCL2/BDC2.5 tg NOD mice upon transfer into female NOD mice. Pancreatic LN and spleen cells from 3-wk-old BDC2.5 (white columns) and RIP-CCL2/BDC2.5 double-tg (black columns) NOD mice were isolated as described in *SI Materials and Methods*. Increasing numbers of purified CD11c$^+$ CD11b$^+$ DCs were injected i.v. into 3-wk-old NOD mice as detailed in *SI Materials and Methods*, starting at 10^3 in 10-fold increments, up to 10^6 cells. Diabetes incidence was assessed at 12 wk. Average results are shown for a total of 10 pooled mice in each group.

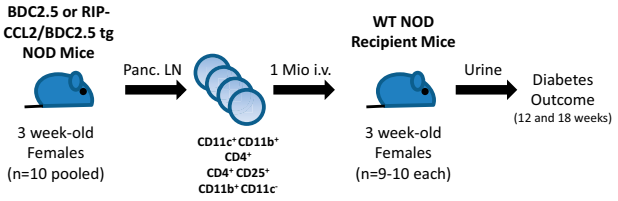

Fig. S6. Schematic overview of the in vivo transfer experiment using cell populations from RIP-CCL2/BDC2.5 tg NOD mice. Pancreatic LN cells from 10 pooled 3-wk-old BDC2.5 and RIP-CCL2/BDC2.5 double-tg NOD mice were isolated as described in *SI Materials and Methods*. Highly FACS-purified subpopulations as listed in the cartoon were injected i.v. into 3-wk-old WT NOD mice (n = 9–10 each) and assessed for diabetes development as described in *SI Materials and Methods*.

Table S1. Absolute cell numbers of pancreatic CD11c$^+$, CD11b$^+$, and CD11b/Gr-1 (Ly-6C)$^+$ cells of 7- to 8-wk-old RIP-CCL2 tg NOD mice and littermate controls*

Cell	WT	Tg	Fold change
CD11c	4,000	46,000	11.5
CD11b	41,000	205,000	5.0
CD11b/Gr-1	9,000	46,000	5.1

*Data are representative of an average of 10 pooled RIP-CCL2 tg NOD mice and littermate controls (WT), respectively. Fold change represents relative increase of absolute cell numbers in tg vs. WT.