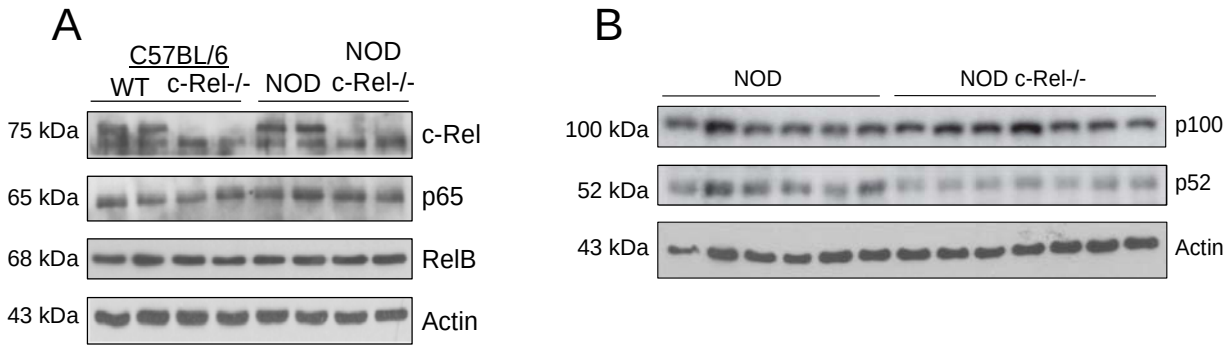
SUPPLEMENTARY DATA

Supplementary Figure 1. A. Expression of NF-κB family members in lungs of c-Rel deficient C57BL/6 and NOD mice. Total cell lysates were analyzed as in Fig 1. Two representative samples for each strain are shown from an N ≥ 4 mice for each strain. **B**. Total cell lysates of thymocytes from 6 NOD and 7 NOD c-Rel$^{-/-}$ mice were probed using anti-p52 antibody to examine the modest decrease in p52 levels in c-Rel$^{-/-}$ NOD mice.



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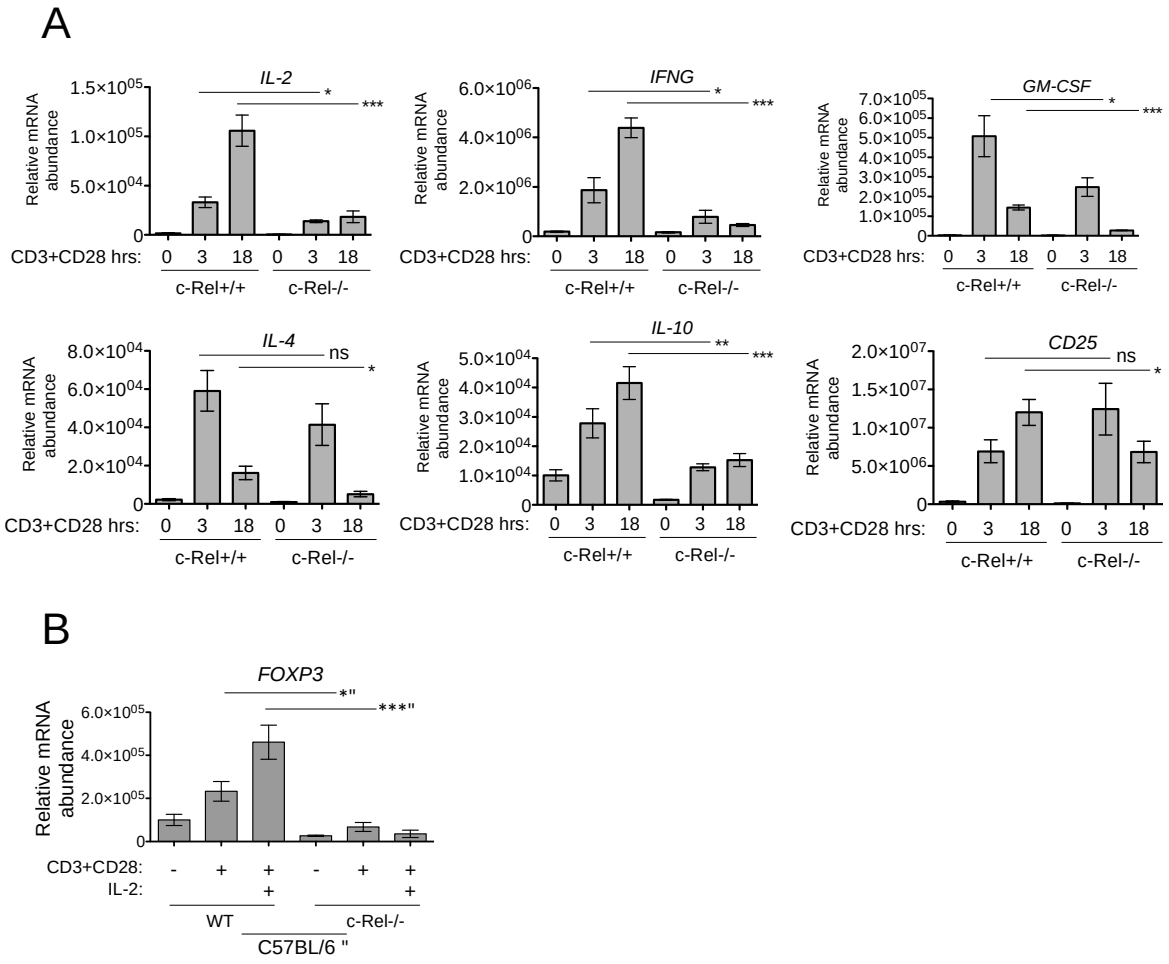
Supplementary Figure 2. **A**. Removal of T reg cells decreases the expression of selected cytokines in $CD4^+$ T-cell pool. $CD25^+$ depleted $CD4^+$ T-cells were purified from c-$Rel^{+/+}$ and c-$Rel^{-/-}$ mice by magnetic-activated cell sorting. Cells were stimulated and gene expression was analyzed by real-time PCR as in Fig 4**.** Data represents mean of duplicates from at least 4 mice from each group. **B**. Exogenous IL-2 does not rescue *FOXP3* expression in c-Rel deficient C57BL/6 mice. $CD4^+$ T-cells were treated as in Fig 4, and the effect of exogenous IL-2 (50 Units/ml) on *FOXP3* expression was analyzed by real-time PCR. Data represents mean of triplicates from three mice per genotype.



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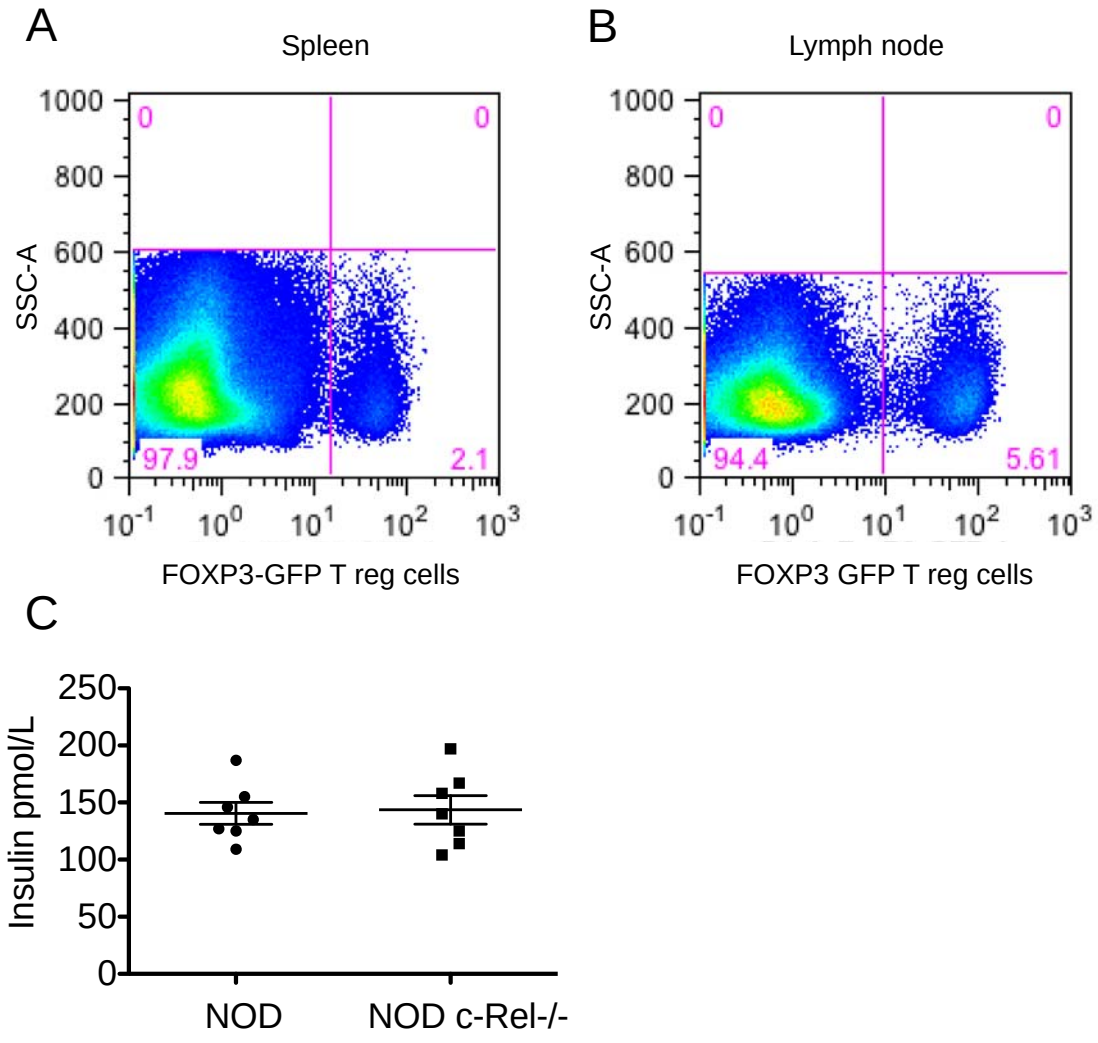
Supplementary Figure 3. **A**. Analysis of GFP$^+$ T reg cells in spleen (left) and lymph node (right) of *FOXP3-IRES-GFP* NOD mice by flow cytometry. FACS plot is representative of flow cytometry performed using total single cell isolates from the indicated organs from three mice each per genotype. **B**. c-Rel deficiency does not affect insulin production in NOD mice. Mice were fasted for 6 hours and blood was collected by tail vein bleeding, allowed to clot and the serum was analyzed for insulin levels by ELISA. Data represents the mean of duplicates from 7 animals per group.



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Reagents and Antibodies

Antibodies against mouse CD3ε (145-2C11) and CD28 (37.51) used for cell stimulations were from Biolegend (San Diego, CA). Antibodies against c-Rel, p65, RelB, p105/p50 and Actin were from Santa Cruz Biotechnology (Dallas, TX). Anti-p100/p52 antibody was from Cell Signaling Technology (Danvers, MA). Fluorescence conjugated antibodies against CD3, CD4, CD8, TCRβ, TCRγδ, FOXP3, CD11b, CD11c, CD19, F4/80 and NK1.1 for flow cytometric analysis were purchased from eBiosciences or Biolegend (San Diego, CA). Anti-Ki67 antibody was from BD Biosciences (San Jose, CA).

Animals

c-Rel knockout mice were kindly provided by Dr. Hsiou-Chi Liou, Weill Medical College of Cornell University, New York, USA. C57BL/6 mice were from a colony maintained in-house. NOD/ShiLtJ mice (#0001976) were purchased from Jackson Laboratories. Breeding pairs for *FOXP3-IRES-GFP* NOD mice expressing GFP in their T reg cells were obtained from Diane Mathis and Christophe Benoist laboratory, Harvard Medical School. All mice used in experiments were 6-10 weeks old except those used to study diabetogenesis, which were housed up to 35 weeks of age. Mice were housed in the animal facility and handled in accordance with National Institute of Health guidelines under Institutional Animal Care and Use Committee approved protocols.

Generation of c-Rel deficient NOD mice

c-Rel knockout mice in C57BL/6 background were mated with NOD mice. The heterozygous offsprings were backcrossed successively with NOD mice following the breeding strategy as described (Fig 1A). The initial breeding was set up using C57BL/6 c-Rel knockout mice and NOD mice. The heterozygous c-Rel$^{+/-}$ NOD offspring were mated with NOD mice to obtain N2 generation and breeding with NOD mice was continued for 6 more generations. We utilized the single nucleotide polymorphism (SNP) based genome scanning service at Jackson laboratories to identify the incorporation of NOD genome in the c-Rel deficient congenic strain. Tail snips of up to fifteen pups born in each generation was genotyped at Jackson laboratory to identify animals with maximum enrichment of the NOD genome (Fig 1B). We reached 99.99% of NOD genome in a c-Rel deficient background by the N5 generation. In order to fix the NOD Y chromosome in the congenic strain, we used NOD males as breeding partners in the N2 and N6 generations. The heterozygous c-Rel$^{+/-}$ male and female mice born in the N6 generation were mated to obtain c-Rel$^{-/-}$ NOD mice.

Magnetic- and Fluorescence-activated cell sorting

Magnetic sorting kits for the isolation of mouse CD4$^+$ T-cells (L3T4), CD8$^+$ T-cells (CD8a$^+$) and T reg cells were from (Miltenyi Biotec, Bergisch Gladbach, Germany) and cells were isolated following the manufacturer's instructions. Total CD4$^+$ T-cells were isolated by positive selection magnetic sorting. T reg depleted CD25$^-$ CD4$^+$ T-cells were isolated by negative selection or positive selection after depletion of CD25$^+$ cells. CD8$^+$ cells were selected by positive selection magnetic sorting.

Flow cytometric analyses were performed using MACSQuant (Miltenyi Biotec) or accuri (BD Biosciences, San Jose, CA) flow cytometers and data were analyzed using MACSQuant (Miltenyi) and FlowJo software (Treestar, Ashland, OR). Pancreata were isolated, weighed and 125 mg of tissue from each mouse was minced and incubated in PBS containing collagenase P (2 mg/ml) at 37°C. Single cell suspension from the whole pancreata and pancreatic lymph nodes were prepared by teasing the tissue apart by pressing with the rubber capped plunger of a 3 ml syringe though a 40 μM cell strainer. Cells were washed and collected in PBS followed by red blood cell lysis in RBC lysis buffer (Biolegend), and total cells isolated from the pancreas and pancreatic lymph nodes were stained with the respective antibodies.

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For intracellular staining, thymocytes or total cells isolated from pancreas were first stained with fluorochrome-conjugated antibody against surface markers CD4 or CD8. Cells were then fixed and permeabilized using Cytofix/Cytoperm Kit (BD Biosciences) and stained with fluorochrome-conjugated anti-FOXP3 or anti-Ki67 antibodies. Respective fluorochrome-conjugated isotype control antibodies were used as controls in all the stainings.

T-cell proliferation assay

$CD4^+$ T-cells were purified from the spleens of NOD and c-$Rel^{-/-}$ NOD mice by magnetic sorting. Cells were stained with Cell Tracer CFSE (Life Technologies, Carlsbad, CA) and 150,000 cells/well were plated in a 96 well plate and incubated at 37^{o}C in 5% CO_2. Cells were stimulated with 2 μg/mL plate bound anti-CD3 and 1 μg/mL soluble anti-CD28 (eBioscience) antibodies in triplicates. Cell proliferation was studied after 48 hours by detecting CSFE dye dilution using an Accuri C6 flow cytometer.

T reg suppression assay

$CD4^+$ $CD25^+$ T reg cells were isolated from NOD mice and NOD c-$Rel^{-/-}$ mice and $CD4^+$ $CD25^-$ T-cells were isolated from NOD mice by magnetic sorting using the T reg cell isolation kit (Miltenyi Biotec). $CD25^+$ cells were positively selected and the $CD25^+$ depleted $CD4^+$ fraction was negatively selected from the total $CD4^+$ pool of cells. $CD4^+$ T-cells isolated from NOD mice were resuspended at 2 million cells/ml in PBS and labeled with 5μM CFSE. Labeling was done by mixing 1 ml of cells with 1 ml of PBS containing 10 μM CFSE slowly by vortexing to obtain a final concentration of 5 μM CFSE. Cells were incubated at 37°C for 10 minutes and washed with 5 volumes of ice-cold PBS and then with RPMI medium. Cells were resuspended in RPMI medium and 1 x 10^5 CFSE labeled $CD4^+$ cells were mixed at 2:1 ratio with 0.5 x 10^5 NOD or NOD c-$Rel^{-/-}$ T reg cells or unlabeled $CD4^+$ cells in the control sample with out T reg cells and plated in 96 well plate coated with anti-CD3 + anti-CD28 antibodies (2 μg/ml each). The cultures were incubated for 72 hours and cell proliferation was assessed by flow cytometry.

Histological examination of pancreas for insulitis

Pancreata were isolated and fixed in neutral buffered 10% formalin solution. Formalin fixed samples were paraffin embedded, sectioned and stained with hematoxylin and eosin by Pacific Pathology, Inc (San Diego, CA). The stained slides were blinded and two investigators scored pancreatic islet infiltrates independently. Representative islets were photographed using a Nikon Diaphot or Leica DM IL LED microscope.

ELISA

$CD4^+$ T-cells (3 million cells in 1.5 ml) were treated with plate bound anti-CD3 and anti-CD28 for 18 hrs. Culture supernatants were analyzed for IL-2 and IFNγ proteins by ELISA (R&D systems, Minneapolis, MN). Levels of insulin in sera from 6-7 week old NOD and NOD c-$Rel^{-/-}$ mice were determined by ELISA following the manufacturer's instructions (Thermo Scientific).

Examination of overt diabetes

Mice were restrained and blood was collected from tail vein by venipuncture. Blood sugar levels were examined weekly using ACCU-CHEK Advantage glucose meter. Three consecutive sugar readings of 250 mg/dl were considered as diabetic and the mouse was culled to minimize suffering.