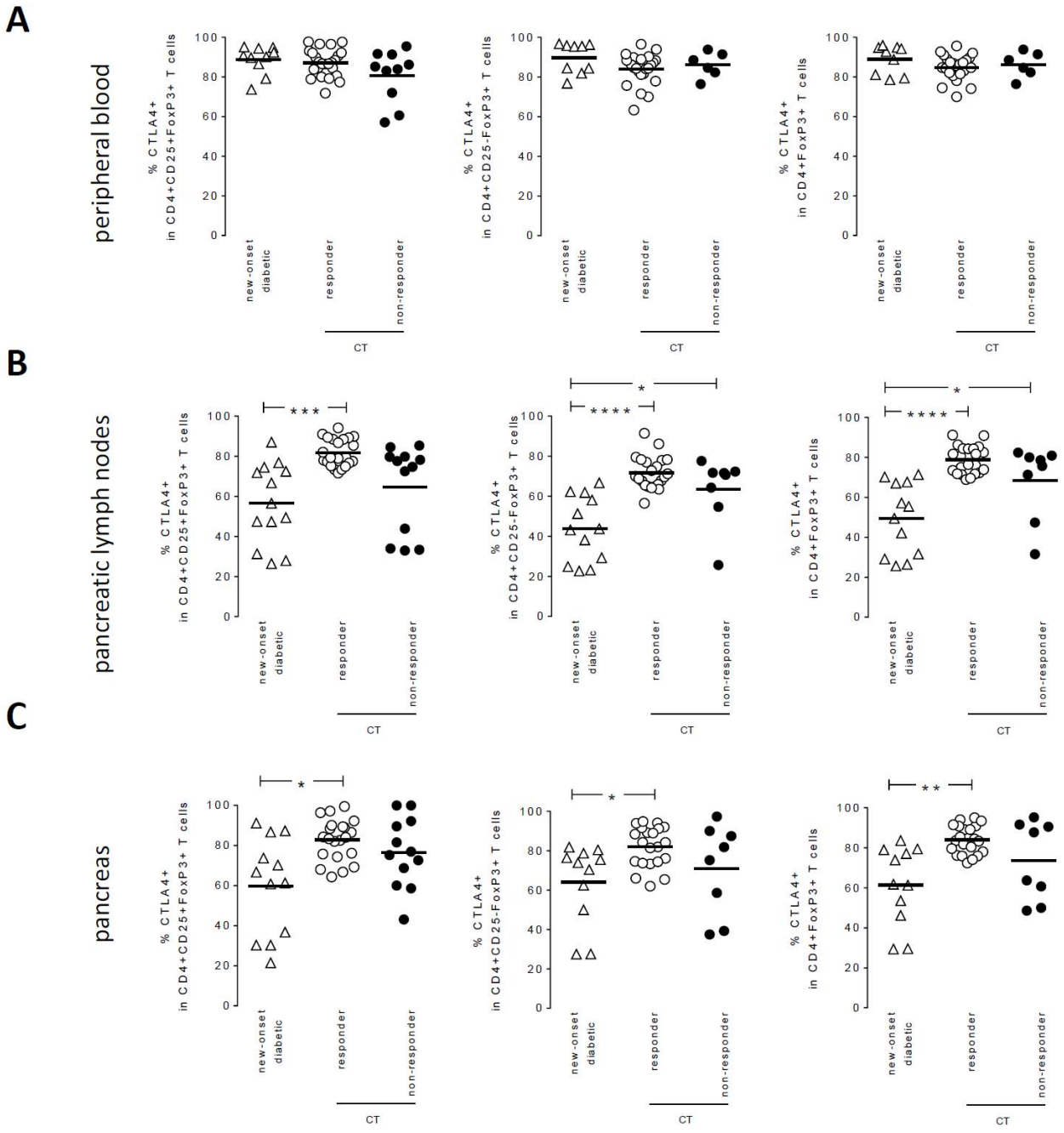
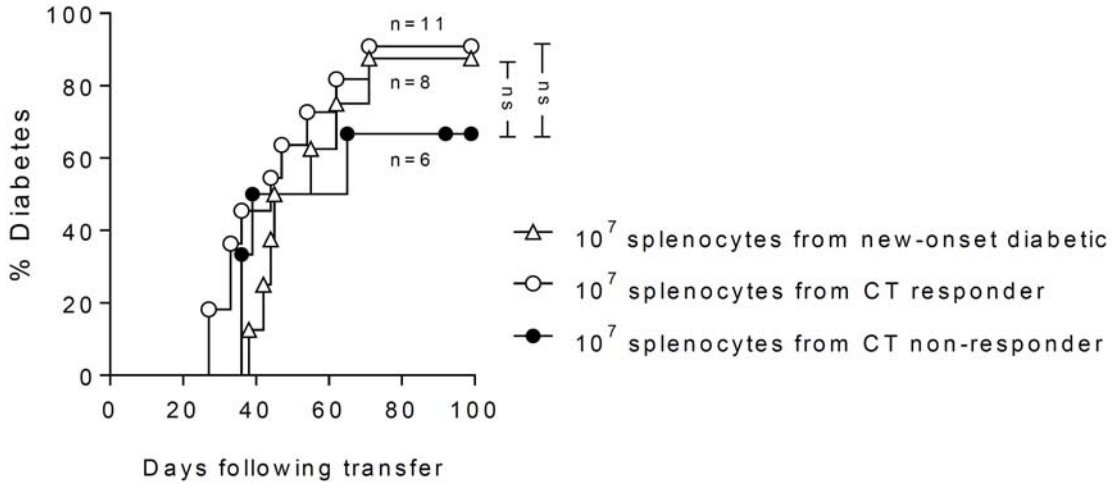
SUPPLEMENTARY DATA

Supplementary Figure S1 – *L. lactis*-based combination therapy induces higher percentage of CTLA4$^+$ Tregs in the pancreas of responder mice. The percentages of CTLA4$^+$ cells within the CD4$^+$CD25$^+$Foxp3$^+$ (left), CD4$^+$CD25$^-$Foxp3$^+$ (middle), and CD4$^+$Foxp3$^+$ (right) T cell population in peripheral blood (**A**), pancreatic draining lymph nodes (**B**) and pancreas (**C**) of new-onset diabetic and *L. lactis*-based combination therapy (CT)-treated mice (both responders and non-responders). Each symbol represents one mouse, and horizontal bars indicate the median value. Statistical significance was calculated using Mann-Whitney t-test; * P < 0.05, ** P < 0.01, ***, P < 0.001; ****, P < 0.0001.



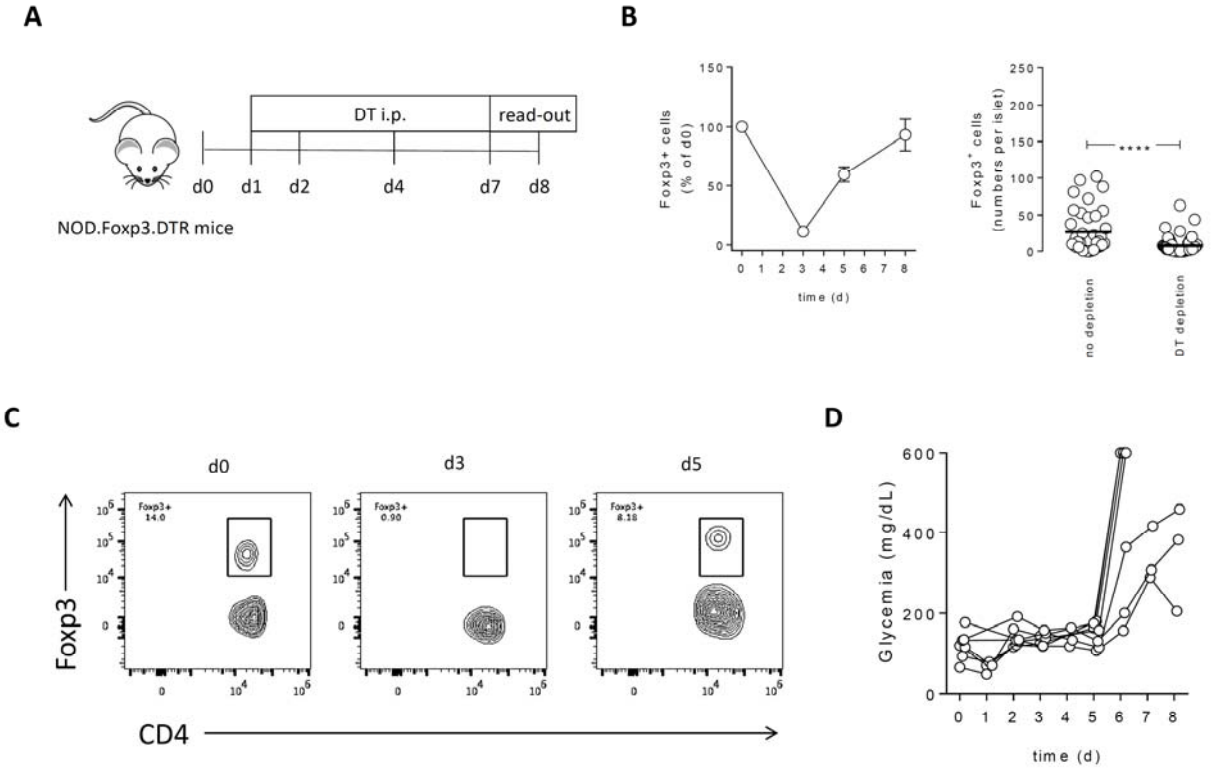
SUPPLEMENTARY DATA

Supplementary Figure S2 – *L. lactis*-based combination therapy-tolerized mice are not depleted in pathogenic T effector cells. Adoptive transfer of total splenocytes (1×10^7) isolated from overtly diabetic (white diamonds), combination therapy (CT) responders (white circles) or non-responders (crossed circles). Statistical calculation was done using Mantel-Cox log-rank test, ns: not significant.



SUPPLEMENTARY DATA

Supplementary Figure S3. Depletion of Foxp3$^+$ cells with DT in unmanipulated NOD.Foxp3.DTR mice. After four consecutive i.p. DT injections (on day (d) 1, 2, 5 and 7)(40 μg/kg body weight/d) as indicated in the scheme (**A**), mice (n=6) were killed on day 8, and peripheral blood and pancreas were removed. (**B**) Flow cytometric analysis of peripheral blood demonstrated efficient depletion of Foxp3$^+$ cells in DT-treated NOD.Foxp3.DTR mice. Foxp3 staining of pancreas sections showed effective depletion of islet-resident Foxp3$^+$ cells in DT-treated NOD.Foxp3.DTR mice. (**C**) Representative flow cytometric profiles showing the percentage of CD4$^+$ T cells positive for Foxp3 and the DTR-GFP fusion protein before (d0) and after two consecutive DT injections (d3 and 5) of NOD.Foxp3.DTR mice (left panel). (**D**) Rapid diabetes onset upon acute Foxp3$^+$ Treg depletion in NOD.Foxp3.DTR mice.



SUPPLEMENTARY DATA

Supplementary Research Design and Methods

Construction of clinical-grade self-containing L. lactis secreting human PINS and IL10.

sAGX0407, engineered to secrete human PINS along with human IL10 (secreting 1.75 ng/ml PINS; 17.5 ng/ml IL10) was generated by replacement of the chromosomally-located thymidylate synthase (thyA) gene in an MG1363 parental strain by an expression cassette for human PINS and IL10 as described (1; 2). In brief, the method to introduce changes in the *L. lactis* chromosome makes use of double homologous recombination. A conditionally replicative carrier plasmid derived from pORI19 and containing an erythromycin selection marker, was constructed in the repA+ *L. lactis* strain LL108. Carrier plasmids are designed in such way that the cargo of interest is cloned in between up to 1 kb cross over (XO) areas, identical to the ones flanking the wild type sequence on the bacterial chromosome. This plasmid is introduced in MG1363 or any of its derivatives (repA-), resistant colonies are selected on agar plates containing erythromycin and a first homologous recombination either at the 5' or 3' target sites is verified by PCR screening. Release of erythromycin selection will enable the excision of the carrier plasmid from the bacterial chromosome by a second homologous recombination, at either the 5' or 3' target site. The final genetic structure of the clinical-grade strain is extensively documented by both Sanger and Illumina full genome sequencing. There are no plasmids or residual erythromycin resistance in the final clinical strain.

Bacteria and media.
The *L. lactis*-pT1NX is an MG1363 strain containing the empty vector pT1NX, and served as control. The plasmid-driven *L. lactis* strain (sAGX0328 secreting plasmid-encoded human PINS (4.25 ng/ml) and chromosomally-integrated IL10 (32.5 ng/ml)) was cultured as described (3). As growth and survival of thyA-deficient *L. lactis* strains depends on the presence of thymidine in the growth medium, the clinical-grade *L. lactis* (secreting chromosomally-integrated human PINS and IL10) was cultured in GM17T, i.e. M17 broth (BD, Franklin Lakes, NJ), supplemented with 0.5% glucose (Merck KGaA, Darmstadt, Germany) and 200 μM thymidine (Sigma, St. Louis, MO). For intragastric inoculations, stock suspensions were diluted 1000-fold in growth media and incubated for 16 hours at 30°C, reaching a saturation density of 2×10^9 cfu/ml. Bacteria were harvested by centrifugation and concentrated 10-fold in BM9 medium. Treatment doses consisted of 100 μl of this bacterial suspension.

Flow cytometry.
Peripheral blood and specified organs were harvested 6 weeks after treatment initiation, processed and incubated with fluorochrome-labeled antibodies or matching isotype controls for flow cytometric analysis. Tregs were stained with anti-mouse CD3 (145-2C11), CD4 (GK1.5), CD8a (53-6.7), CD25 (PC61.5 or 7D4 (BD, Erembodegem, Belgium)), and FR4 (eBio12A5) (all from eBioscience, San Diego, CA, unless specified) for 20 min on ice. Intracellular staining antibodies against Foxp3 (FJK-16s) and CTLA4 (UC10-4B9) were from eBioscience and used according to the manufacturer's instructions. Naïve, effector memory and central memory T cells were determined by staining with anti-mouse CD3 (145-2C11), CD4 (GK1.5), CD8a (53-6.7), CD44 (IM7), CD62L (MEL-14), CD69 (H1.2F3), and CCR7 (4B12). Cells were analyzed in a Gallios™ flow cytometer with Kaluza (Beckman Coulter, Suarlée, Belgium) or FlowJo software (Treestar, Ashland, OR).

In vitro suppressor assays.
Pathogenic $CD4^+CD25^-$ Teff cells were isolated from spleen cells of 10-week old NOD mice by negative selection using antibodies to CD25, CD8α, B220, CD11c, CD11b, MHC class II, and sheep anti-rat IgG Dynabeads (Invitrogen, Merelbeke, Belgium). $CD4^+CD25^+Foxp3^+$ and $CD4^+CD25^-Foxp3^+$ Tregs were isolated from pooled lymph node and spleen cells of NOD.Foxp3.hCD2 mice (harboring a human CD2-CD52 fusion protein, along with an intra-ribosomal entry site, into the 3' untranslated region of the endogenous foxp3 locus)(4). Briefly, cell

samples were passed through a 70-µm cell strainer and suspended in RPMI1640 medium, centrifuged for 5 min at 1,500 rpm and suspended in RPMI1640 medium. The resultant cell suspension was counted, washed and first depleted of $CD8\alpha^+$, $B220^+$, $CD11c^+$, $CD11b^+$ and MHC class$^+$, and adherent cells by panning and stained with biotin-conjugated anti-CD4 and anti-hCD2 antibodies. Cells were then incubated with anti-biotin microbeads ((Miltenyi Biotec B.V., Leiden, The Netherlands) and separated on LS or MS columns (Miltenyi Biotech). The resulting $hCD2^+$ or $hCD2^-$ cells were further purified with anti-CD25 antibodies. *In vitro* polyclonal suppressor assays were conducted as described (3). Cytokines (IFN-γ, IL10 and TGF-β) were measured in cell-free supernatants by multiplex immunoassay (Mesoscale Discovery, Rockville, MA) or flow cytometric bead array (Bender MedSystems FlowCytomix™, eBioscience) as described (3).

In additional set of experiments, blocking antibodies against CTLA4 (UC10-4F10; kind gift of Dr. Boon, Bioceros BV, Utrecht, The Netherlands), IL10 (clone JES5-2A5, BioXCell), and TGF-β (clone 1D11.16.8, which neutralizes all three mammalian TGF-β isoforms (β1, β2, and β3), BioXCell) were added to cultures at concentration of 10 µg/ml.

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