Supplemental data

to manuscript "**Impaired Oral Tolerance Induction in Diabetes Prone but not in Diabetes Resistant Mice Revealed by Cholera Toxin Subunit B-Peptide Fusion Proteins"**

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Supplemental Table S1, referring to Figure 2
B: Absolute number of A^{g7}/2.5mi tetramer$^+$ CD4$^+$ T cells of NOD mice immunized i.p. as indicated[a]

Treatment	n	2.5mi$^+$ Teff	2.5mi$^+$ Treg
CTB-2.5mi	6	10401 ± 1950 ***	498 ± 114**
s.p. 2.5mi	6	736 ± 154	21.9 ± 14
Naive	6	98.9 ± 23	7.6 ± 2

[a] Values indicate the mean of tetramer$^+$ cells ± SEM per 4x10^5 CD4$^+$ T cells. One way ANOVA and Bonferroni post-test: ***$p < 0.0001$, **$p < 0.001$ compared with s.p. 2.5mi. Cell numbers were normalized to 4x10^5 CD4$^+$ T cells, the average number collected per mouse.

Supplemental Table S2, referring to Figure 3 A: Absolute numbers of tetramer$^+$ CD4 T cells in NOD mice receiving the indicated type of treatment[a]

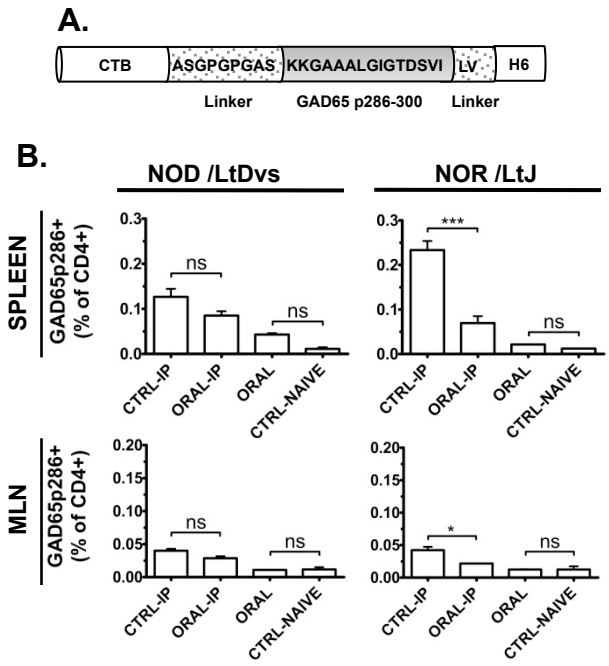
Treatment	n	$CD4^+$ $2.5mi^+$ T cells
CTRL-Naive	6	126 ± 19
ORAL	6	254 ± 33
CTRL-IP	6	3578 ± 397
ORAL-IP	6	2693 ± 292

[a] Values indicate the mean of tetramer$^+$ cells ± SEM per $4x10^5$ $CD4^+$ T cells. Cell numbers were normalized to $4x10^5$ $CD4^+$ T cells, the average number collected per mouse. CTRL-IP vs ORAL-IP were not statistically significant.

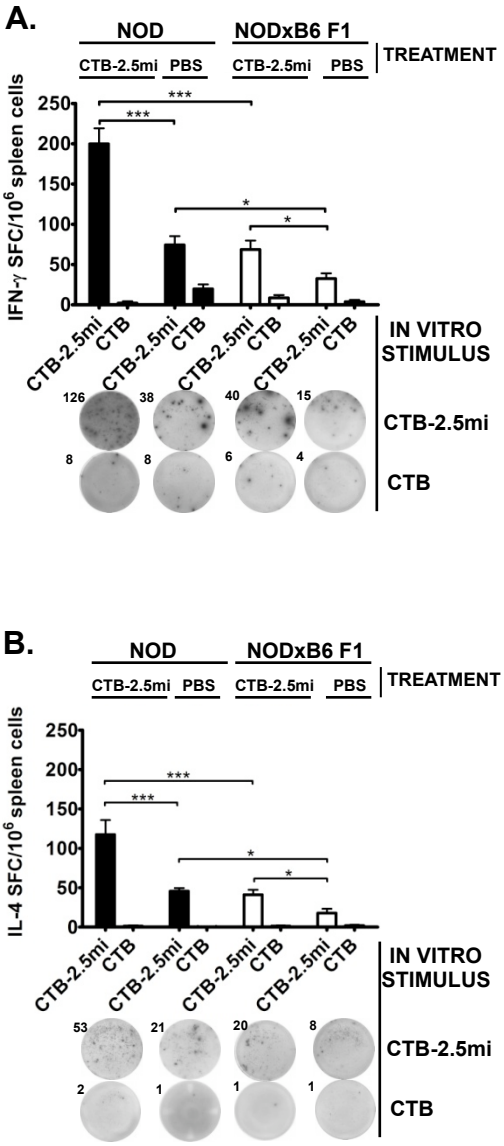
Supplemental Table S3, referring to Figure 7**:** Absolute numbers of tetramer^{+} CD4 T cells of the indicated subsets receiving the indicated type of treatment [a)]

Strain	Treatment	n	Naive Teff 2.5mi^{+}	CM Teff 2.5mi^{+}	EM Teff 2.5mi^{+}
NOD.*Foxp3^{EGFP}*	CTRL-PBS	6	48 ± 4	3 ± 1	4 ± 1
	ORAL-CTB-2.5mi	6	21 ± 3 (ns)	19 ± 9 (ns)	193 ± 81 **
NODxB6.*Foxp3^{EGFP}* F1	CTRL-PBS	6	49 ± 18	15 ± 8	21 ± 3
	ORAL-CTB-2.5mi	6	68 ± 14 (ns)	13 ± 6 (ns)	36 ± 18 (ns)

[a)] Three mice per treatment group were orally immunized with 200 µg of CTB-2.5mi or 200 µL of PBS, every 3-4 days with a total of 5 doses. Four days after the last oral dose Teff 2.5mi^{+} cells were analyzed for CD44 and CD62L expression. Cell numbers were normalized to 4x10^{5} CD4^{+} T cells, the average number collected per mouse. Values are shown as mean ± SEM of 2 independent experiments. Teff 2.5mi^{+}: CD4^{+}Foxp3-EGFP^{neg} 2.5mi^{+}. Naive: CD62L^{hi} CD44^{lo}, CM (Central Memory): CD62L^{hi} CD44^{hi}, EM (Effector Memory): CD62L^{lo} CD44^{hi}. Two way ANOVA and Bonferroni post-test: ORAL-CTB-2.5mi vs CTRL-PBS: ** $p < 0.01$, (ns) $p > 0.05$.

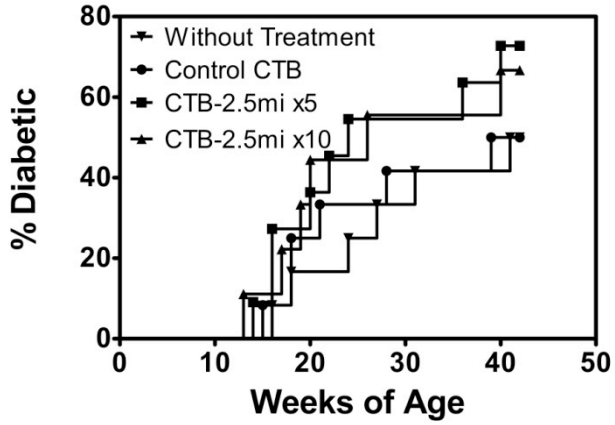


Supplemental Figure S1 NOR but not NOD mice mount oral tolarance against $GAD_{286-300}$.
(A) Schematic representation of the CTB-$GAD_{286-300}$ construct.
(B) Five-to-6-week-old NOD or NOR females were treated with 5 doses of 200 µg CTB-$GAD_{286-300}$ 3 to 4 days i.g. (ORAL) and analyzed 4 days after the last dose for $GAD_{286-300}$ specific T cell expansion in the spleen (A) and in mesenteric lymph nodes (B; MLN) as explained in the legend for Fig. 1 by tetramer staining and FACS analysis. Unmanipulated littermates served as baseline control (CTRL-Naive). Alternatively, 4 days after the last oral dose, mice received a boost immunization i.p. with 50 µg of CTB-$GAD_{286-300}$, were rested for an additional 4 days, and next analyzed as above (ORAL-IP). As controls, littermates were immunized i.p. without receiving the protein i.g. (CTRL-IP). Mean values ± SEM of 3 animals per group and treatment are shown. Values represent percentages of $GAD_{286-300}$ tetramer positive $CD4^+$ T cells within total $CD4^+$ T cells, gated on $B220^-$, $CD8^-$, PI^- and $CD4^+$ cells.



Supplemental Figure S2 Representative examples of the ELISPOTS of Fig. 6 B and C are shown (Th1 and Th2 cytokine analysis in NOD.Foxp3^{EGFP} and NODxB6.Foxp3^{EGFP} F1 mice.). Five to 6 week old NOD.Foxp3^{EGFP} and NODxB6.Foxp3^{EGFP} F1 females were treated with 5 doses of 200 µg CTB-2.5mi or PBS every 3 to 4 days i.g. Four days after the last dose, T cells were analyzed for cytokine production.

Total splenocytes from treated mice were incubated with 100 ng/ml of CTB-2.5mi or CTB and cytokine secretion quantified 2 days later (A, IFN-γ; B, IL-4). Values indicate spot forming cells (SFC) per 10^6 splenocytes; mean values ± SEM are indicated after subtraction of SFC in unstimulated wells. n=6 and 12, resulting from 2 and 4 independent experiments for NOD.Foxp3^{EGFP} and NODxB6.Foxp3^{EGFP} F1 mice, respectively (*p<0.05, **p<0.01, ***p<0.0001).



Supplemental Figure S3 Oral treatment with CTB-2.5mi does not prevent T1D development in NOD mice.
Five-week-old NOD females (n=10/treatment group) were treated with 5 or 10 doses of 200 µg each with either CTB (5 doses only), CTB-2.5mi every 3 to 4 days i.g. or left untreated and diabetes was onset monitored by weekly blood glucose measurements. Animals exceeding 200 mg/dl of blood glucose were considered diabetic. Curve differences are not statistically significant, Log-rank (Mantel-Cox) Test p = 0.5410.