Online Supplementary data

InsB9-23 gene transfer to hepatocytes-based combined therapy abrogates recurrence of type 1 diabetes after islet transplantation.

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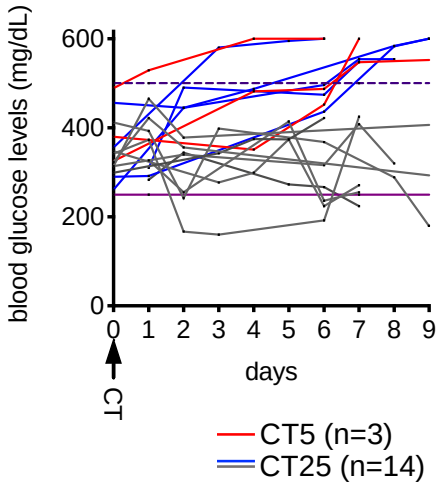
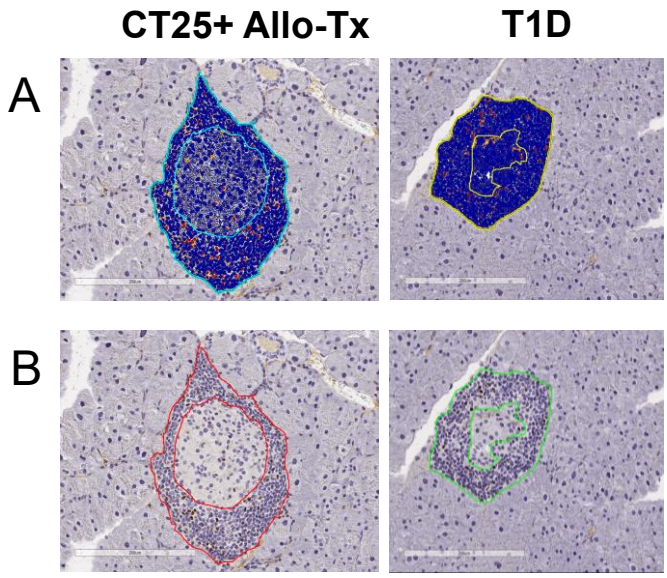
Supplementary data list:

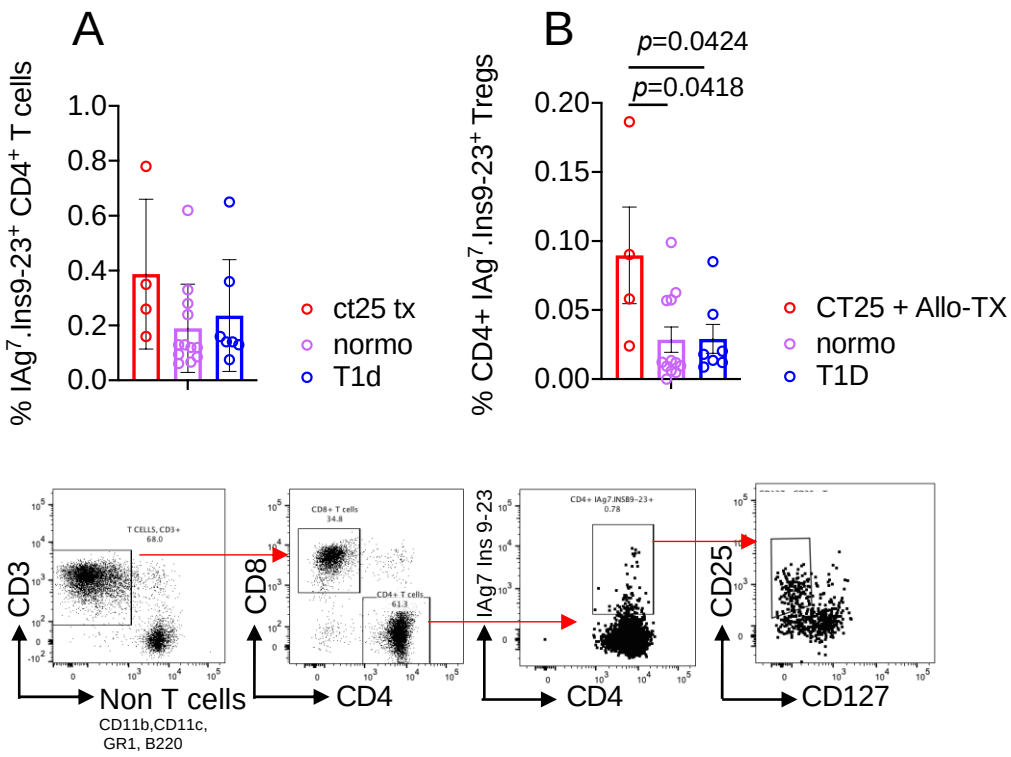
Supplementary figure 1



Supplementary figure 1. Optimized LV.InsB and Anti-CD3 mAb Combined therapy (CT25) administration modulates T1D progression.
To postpone the islet transplantation we compared the ability of CT5 (LV.InsB + 1X5µg anti-CD3 mAb) and CT25 (LV.InsB + 1X25µg anti-CD3 mAb) to prevent further endogenous β cells loss and the consequent increase of blood glucose levels. Diabetic NOD mice (n=17) were treated with CT5 (n=3, baseline bgl 398±48mg/dL) or CT25 (n=14, baseline bgl 331±13mg/dL) and glycemia was monitored to evaluate disease progression. Glycemia of each mouse is plotted in time in red and blue were highlighted mice that reached values above the upper bound of disease progression (bgl=500).

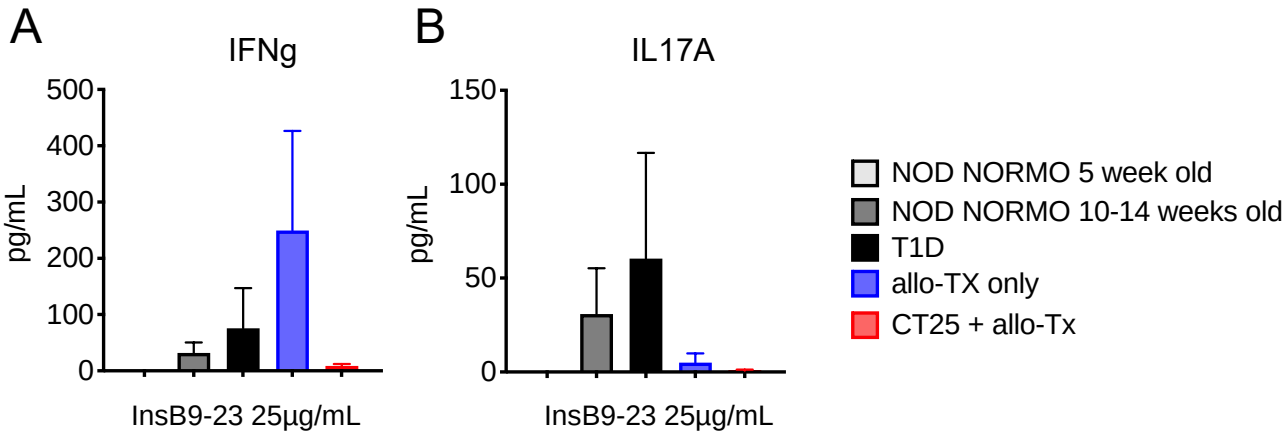


Supplementary figure 2. Unbiased histological analysis to determine FoxP3+ Tregs infiltration of pancreatic islets.
The presence of FoxP3 expressing cells within islets infiltrated areas has been quantified by Immuno-histochemistry staining of Foxp3. The algorithm for positive pixel counts by Aperio Scan Software System (Leica) was applied to determine the area covered by the FoxP3 staining signal and to measure total area of leucocytic infiltration of pancreatic islets (1 pixel= $2.53512X10^{-7}$ mm^2). The ratio [area Foxp3 : infiltration area] for each single islet has been used as quantification of Treg density in the islets. Of note, analogous unbiased measurement of leucocytic infiltration of pancreatic islets (Insulitis reported in figure 3) was used to determine the percentage of infiltration of each single islets identified by hematoxylin-eosins staining in the pancreas of experimental and control NOD mice.



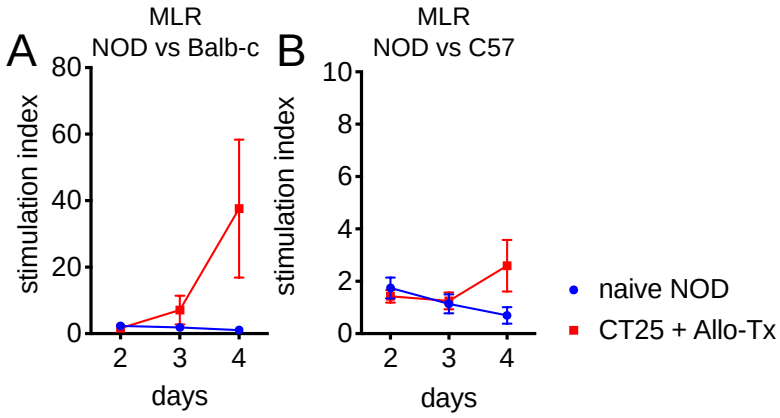
Supplementary figure 3. Ins9-23 specific Tregs are expanded in PLN of normo-CT25+Allo-Tx. The frequency of Ins9-23-specific CD4+ T cells was determined by FACS analysis of the PLN cell isolated from normo-CT25+Allo-Tx (n=4) and normoglycemic (n=12) and diabetic (n=7) untreated controls. IAg7.InsB9-23 class II tetramer have been used to label Ins B9-23 specific CD4 T cells (**A**) and accordingly to positivity for CD25 and low/neg expression of CD127 determine the frequency of CD4+ IAg7.InsB9-23+ Tregs (**B**). Single measurements and mean% ±SE from each group are reported, Mann-Whitney test. A representative analysis is reported, including the gating strategy.

Supplementary figure 4



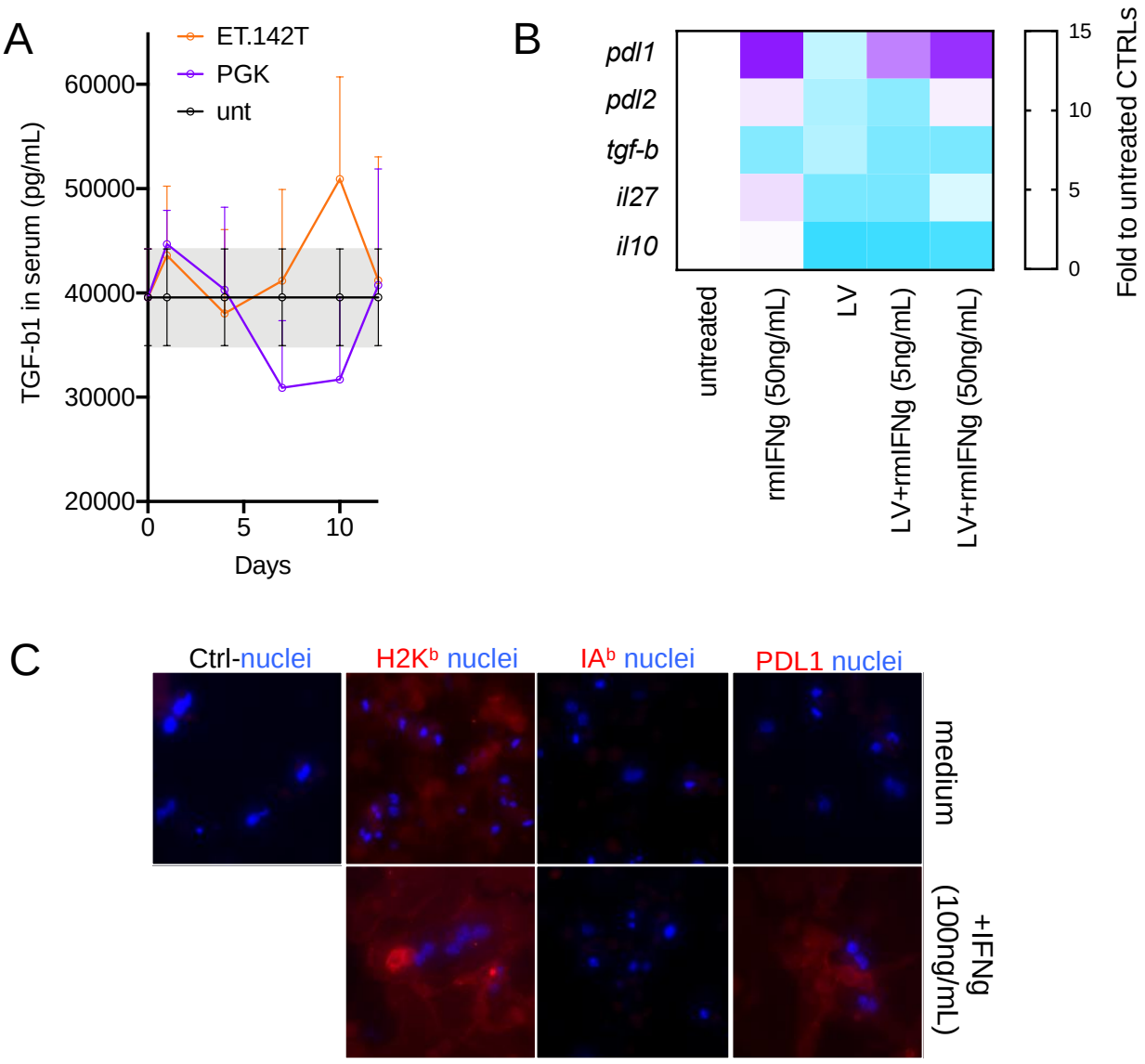
Supplementary figure 4. InsB9-23-driven IFNg and IL17A release by T cells is severely impaired in NOD mice cured by CT25 plus allogeneic Islets.
Splenocytes isolated from CT25-treated and transplanted after at least 100 days of normoglycemia (n=5), transplanted (n=3), untreated diabetic (n=9), normoglycemic 10-14 week-old (n=13), and normoglycemic 5 week-old (n=4) NOD mice were stimulated *in vitro* for 72-96hrs with 25µg/mL of InsB9-23 peptide. Culture supernatants were collected to determine the concentration of IFNg (**A**) and IL17A(**B**), mean of concentration in pg/mL ±SE is reported for each group.

Supplementary figure 5

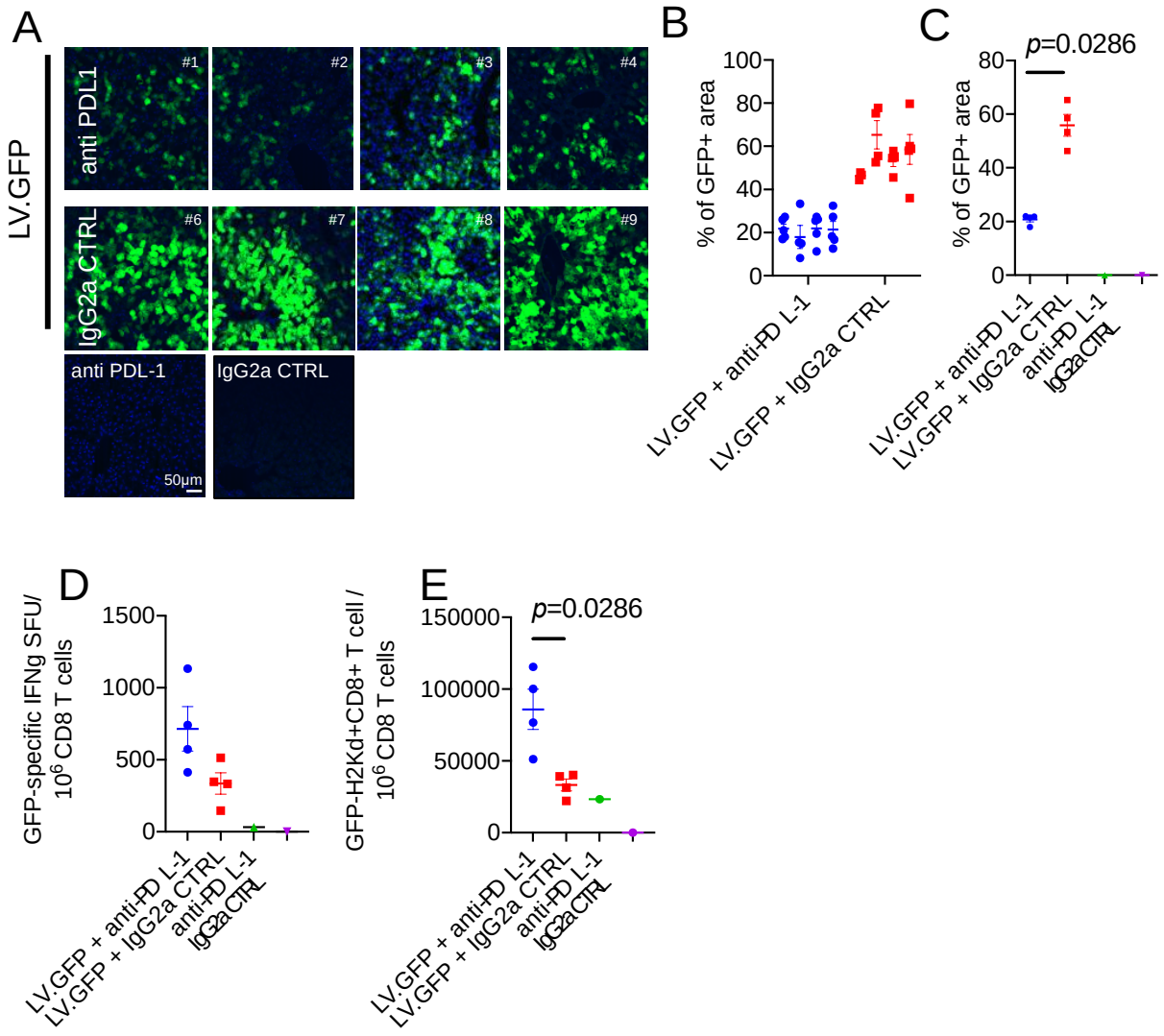


Supplementary figure 5. Priming to Balb-c allo-Ags occurred *in vivo* in transplanted and CT25-treated NOD mice.

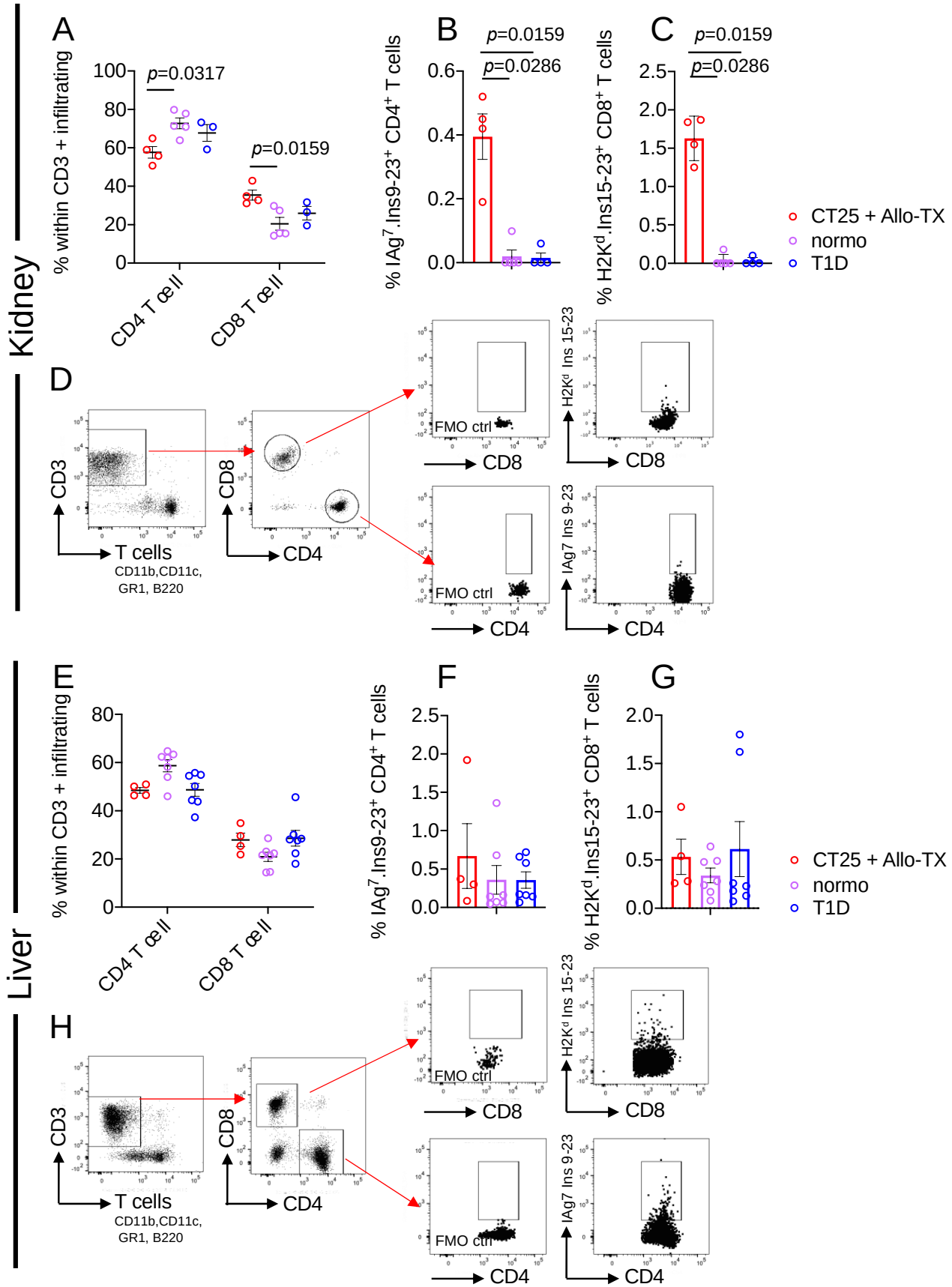
At 100 days of normoglycemia after transplantation and CT25 treatment NOD mice were euthanized and splenocytes were tested in a conventional mixed leucocytes reaction (MLR). Naïve Balb-c- or C57Bl6-derived splenocytes were irradiated (30 Gy) and used as stimulator of splenocytes from naïve NOD (n=6) or transplanted and CT25-treated NOD mice (n=4). Proliferative responses to Balb-c or C57Bl6 cells were measured at different timepoints by ^{3}H-thymidine incorporation and plotted as stimulation index (cpm stimulated / cpm unstimulated ctrl) (**AB**). Mean of stimulation index ±SE from each group is reported at the indicated time points.



Supplementary figure 6. LV mediated gene transfer to hepatocytes directly and indirectly induces upregulation of tolerogenic factors. Serum level of TGF-b1 was determined at the indicated time points post LV (n=18, ET.142T hepato-specific), non-targeted gene transfer (n=21, PGK promoter ubiquitously active) and untreated controls (n=37, normal range defined by gray area) (**A**). The kinetic revealed a systemic upregulation of TGFb1 in immunocompetent mice starting from day 7 post LV.ET.142T injection. Although LV.ET.142T gene transfer induces active tolerance to the transgene expressing hepatocytes, *in vivo* LV administration results in the recruitment of IFNg releasing T cells (Annoni et al. Blood 2009). Therefore the expression of genes encoding for immunoregulatory molecules was studied to define the tolerogenic mechanism. Primary hepatocytes were cultured *in vitro* and RNA was isolated to determine induction of *pdl1, pdl2, il10, il27, tgf-b1* genes after exposure to LV and/or IFNg. Data showed that primary hepatocytes sense IFNg upregulating *pdl1* transcription and that LV transduction does not impact on *pdl1* expression (**B**). IFNg-dependent upregulation of PDL1 at protein level was confirmed by immunofluorescence on live cells in culture (**C**).



Supplementary figure 7. PD1-PDL1 co-stimulatory pathway blockade result in loss of tolerance induction triggered by hepatocytes directed gene transfer.
To assess the role of PD1-PDL1 co-stimulatory pathway in tolerance induction to the LV-encoded Ag immunocompetent mice were injected with LV.ET.GFP.142T and treated with anti PDL1 mAb or IgG2a isotype control (250µg every other day for 2 weeks). GFP expression in the liver was evaluated at confocal microscopy, and area covered by GFP+ hepatocytes was quantified (**A-C**). GFP-specific T cells were enumerated by IFNg elispot (**D**) and by H2-K^{d}GFP$_{200\text{-}208}$ pentamer staining (**E**). (n=4/group, mean ± SEM, Mann-Whitney test). These data demonstrate that PD1-PDL1 co-stimulatory pathway plays a crucial role in the maintenance of Ag-specific tolerance to transgene.


Kidney
A
% within CD3 + infiltrating
p=0.0317
p=0.0159
CD4 T cell
CD8 T cell
B
p=0.0159
p=0.0286
% IAg7.Ins9-23+ CD4+ T cells
C
p=0.0159
p=0.0286
% H2Kd.Ins15-23+ CD8+ T cells
CT25 + Allo-TX
normo
T1D
D
CD3
T cells
CD11b,CD11c,
GR1, B220
CD8
CD4
FMO ctrl
H2Kd Ins 15-23
IAg7 Ins 9-23
Liver
E
% within CD3 + infiltrating
CD4 T cell
CD8 T cell
F
% IAg7.Ins9-23+ CD4+ T cells
G
% H2Kd.Ins15-23+ CD8+ T cells
CT25 + Allo-TX
normo
T1D
H
CD3
T cells
CD11b,CD11c,
GR1, B220
CD8
CD4
FMO ctrl
H2Kd Ins 15-23
IAg7 Ins 9-23

Supplementary figure 8. Insulin-specific T cells are diluted at sites alternative to pancreas by the presence of the cognate Ag imposed by CT25+Allo-TX therapy.
Mononuclear cells infiltrating the kidney (site of islet transplantation) and the liver (site of InsB9-23 transgene expression) were isolated from normo-CT25+Allo-Tx (n=4) and normoglycemic (n=7) and diabetic (n=7) untreated controls.
The distribution of CD4 and CD8 within CD3+ T cell was determined (**A, E**). The frequencies Ins9-23-specific CD4+ T and Ins15-23-specific CD8+ T were quantified by using IAg7.InsB9-23 class II tetramer (**B, F**) and $H2K^d$.InsB9-23 class I tetramer (**C, G**), respectively. Single measurements together with mean% ±SE from each group are reported, Mann-Whitney test. A representative analysis is reported, including the gating strategy for both tissues.

Table 1. Percentages of tot T cell not Tregs, Tregs generating ratio reported in 4DE.

PLN cells	% of CD45+												
normo 5 wks of age	tot T non Tregs	63,362	73,678	68,290	74,698								
	CD4+, FOXP3+ Tregs	4,438	4,522	4,610	4,802								
	[tregs/ tot T]ratio	***0,070***	***0,061***	***0,068***	***0,064***								
normo 10-14 wks of age	tot T non Tregs	34,718	72,521	65,550	72,255	63,683	59,965	74,056	64,600	71,873	73,637	74,707	67,450
	CD4+, FOXP3+ Tregs	3,352	6,679	6,550	5,545	5,417	3,135	4,944	4,900	6,927	6,563	6,093	5,750
	[tregs/ tot T]ratio	***0,097***	***0,092***	***0,100***	***0,077***	***0,085***	***0,052***	***0,067***	***0,076***	***0,096***	***0,089***	***0,082***	***0,085***
T1D	tot T non Tregs	64,370	62,407	65,067	47,971	38,240	54,217	48,012					
	CD4+, FOXP3+ Tregs	6,930	5,093	7,733	6,929	4,480	6,283	6,288					
	[tregs/ tot T]ratio	***0,108***	***0,082***	***0,119***	***0,144***	***0,117***	***0,116***	***0,131***					
CT25	tot T non Tregs	23,430	52,391	59,795									
	CD4+, FOXP3+ Tregs	2,711	4,809	3,605									
	[tregs/ tot T]ratio	***0,116***	***0,092***	***0,060***									
Allo-TX	tot T non Tregs	32,294	61,658	63,526									
	CD4+, FOXP3+ Tregs	1,406	2,742	3,474									
	[tregs/ tot T]ratio	***0,044***	***0,044***	***0,055***									
CT25 + Allo-TX	tot T non Tregs	4,116	53,945	64,477	3,930	29,706							
	CD4+, FOXP3+ Tregs	0,584	3,755	5,323	0,980	6,194							
	[tregs/ tot T]ratio	***0,142***	***0,070***	***0,083***	***0,249***	***0,208***							

Spleen cells	% of CD45+											
normo 5 wks of age	tot T non Tregs	37,551	38,679	42,460	37,301							
	CD4+, FOXP3+ Tregs	4,112	3,918	4,189	3,815							
	[tregs/ tot T]ratio	***0,109***	***0,101***	***0,099***	***0,102***							
normo 10-14 wks of age	tot T non Tregs	34,090	32,463	27,484	42,582	39,586	41,434	28,180	24,120	33,594	31,700	38,214
	CD4+, FOXP3+ Tregs	3,570	3,338	2,668	4,679	3,828	3,957	2,926	2,552	3,313	3,222	3,635
	[tregs/ tot T]ratio	***0,105***	***0,103***	***0,097***	***0,110***	***0,097***	***0,096***	***0,104***	***0,106***	***0,099***	***0,102***	***0,095***
T1D	tot T non Tregs	29,665	45,150	29,190	32,137	19,525	29,057	23,648	21,161			
	CD4+, FOXP3+ Tregs	2,913	4,537	3,545	3,106	2,269	3,403	2,707	2,526			
	[tregs/ tot T]ratio	***0,098***	***0,100***	***0,121***	***0,097***	***0,116***	***0,117***	***0,114***	***0,119***			
CT25	tot T non Tregs	31,308	32,867	31,743								
	CD4+, FOXP3+ Tregs	2,707	3,081	3,016								
	[tregs/ tot T]ratio	***0,086***	***0,094***	***0,095***								
Allo-TX	tot T non Tregs	32,491	25,091	30,758								
	CD4+, FOXP3+ Tregs	3,145	2,526	3,003								
	[tregs/ tot T]ratio	***0,097***	***0,101***	***0,098***								
CT25 + Allo-TX	tot T non Tregs	34,957	50,172	46,370	24,384	24,384						
	CD4+, FOXP3+ Tregs	3,429	5,027	4,859	2,617	2,346						
	[tregs/ tot T]ratio	***0,098***	***0,100***	***0,105***	***0,107***	***0,096***						