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Supplemental Information

Efficient Presentation of Multiple Endogenous Epitopes to Both $CD4^+$ and $CD8^+$ Diabetogenic T Cells for Tolerance

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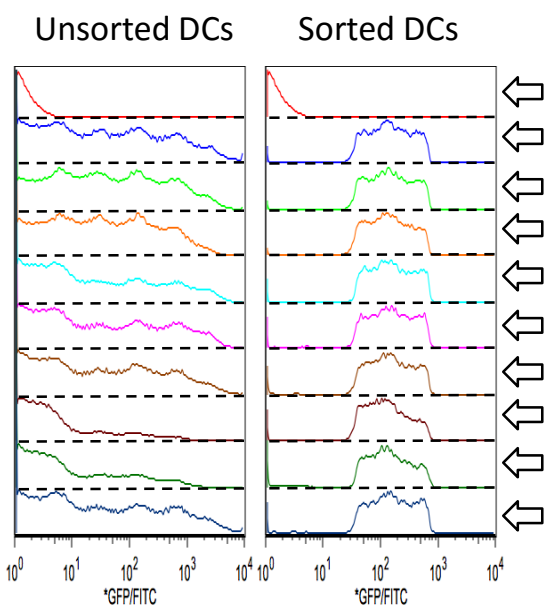
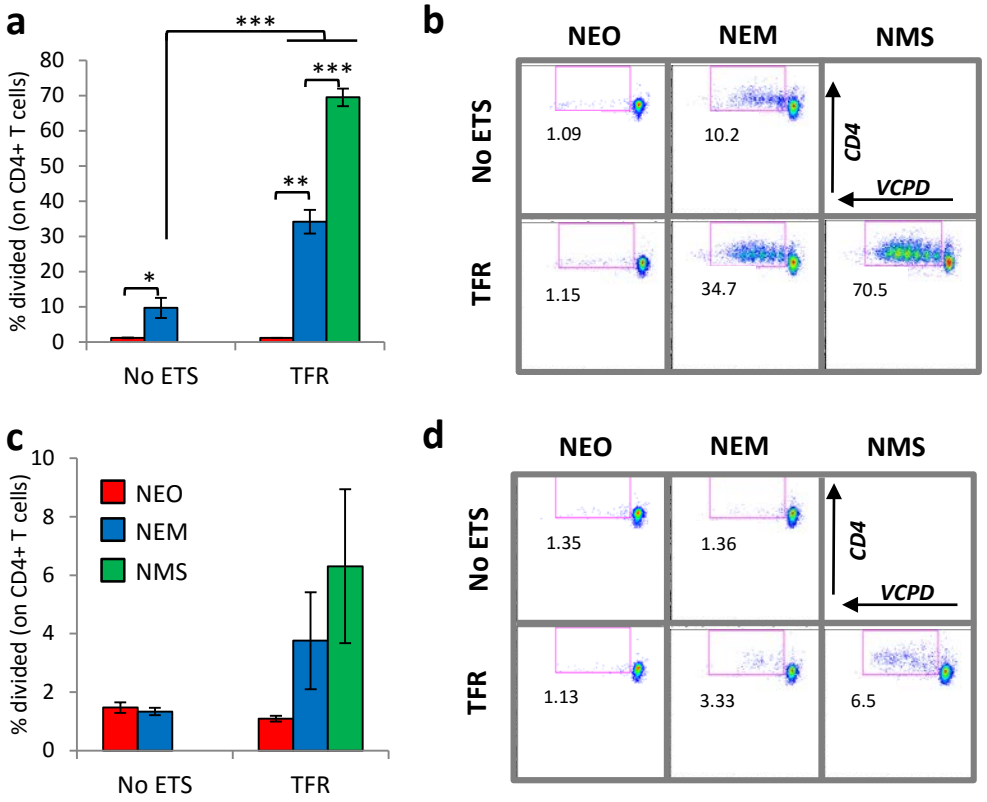
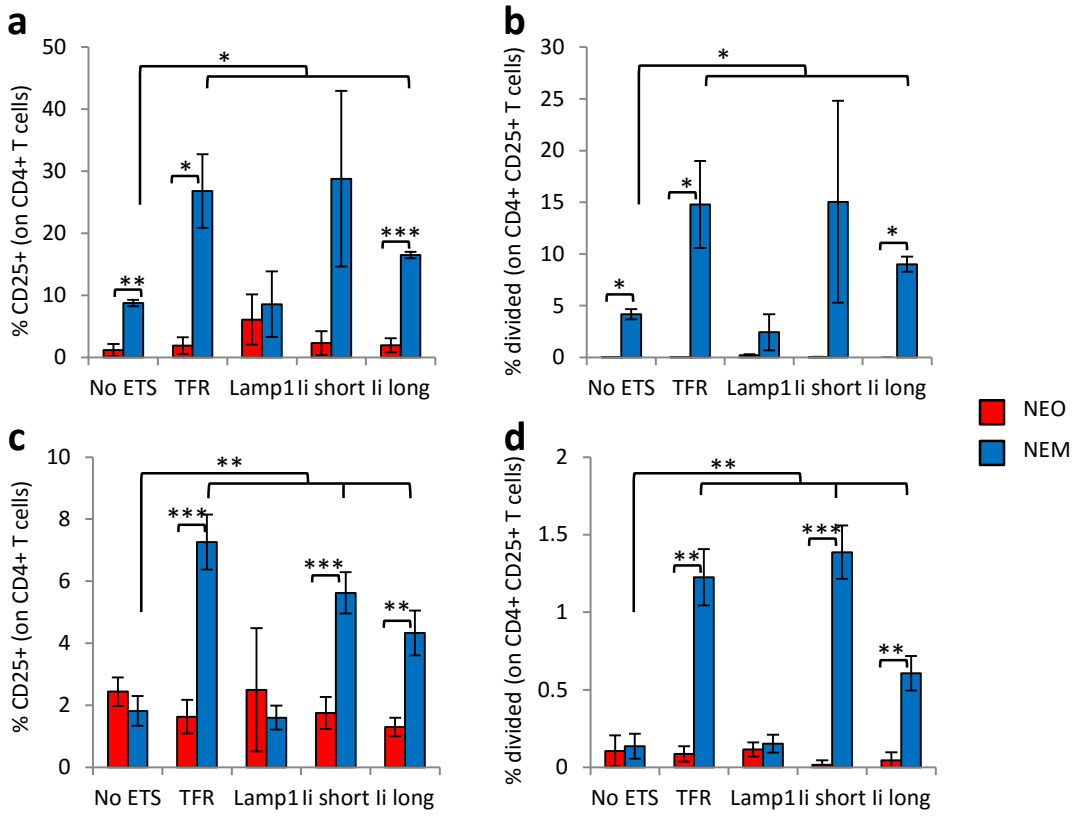


	MFI GFP⁺ unsorted	MFI GFP⁺ sorted
Untransduced DCs control		
GFP	159	150
NEO	134	148
NEM	108	141
NEO/TFR	153	160
NEM/TFR	167	157
NMS/TFR	155	153
NEO/Ii short	74	129
NEM/Ii short	90	146
NMS/Ii short	167	161

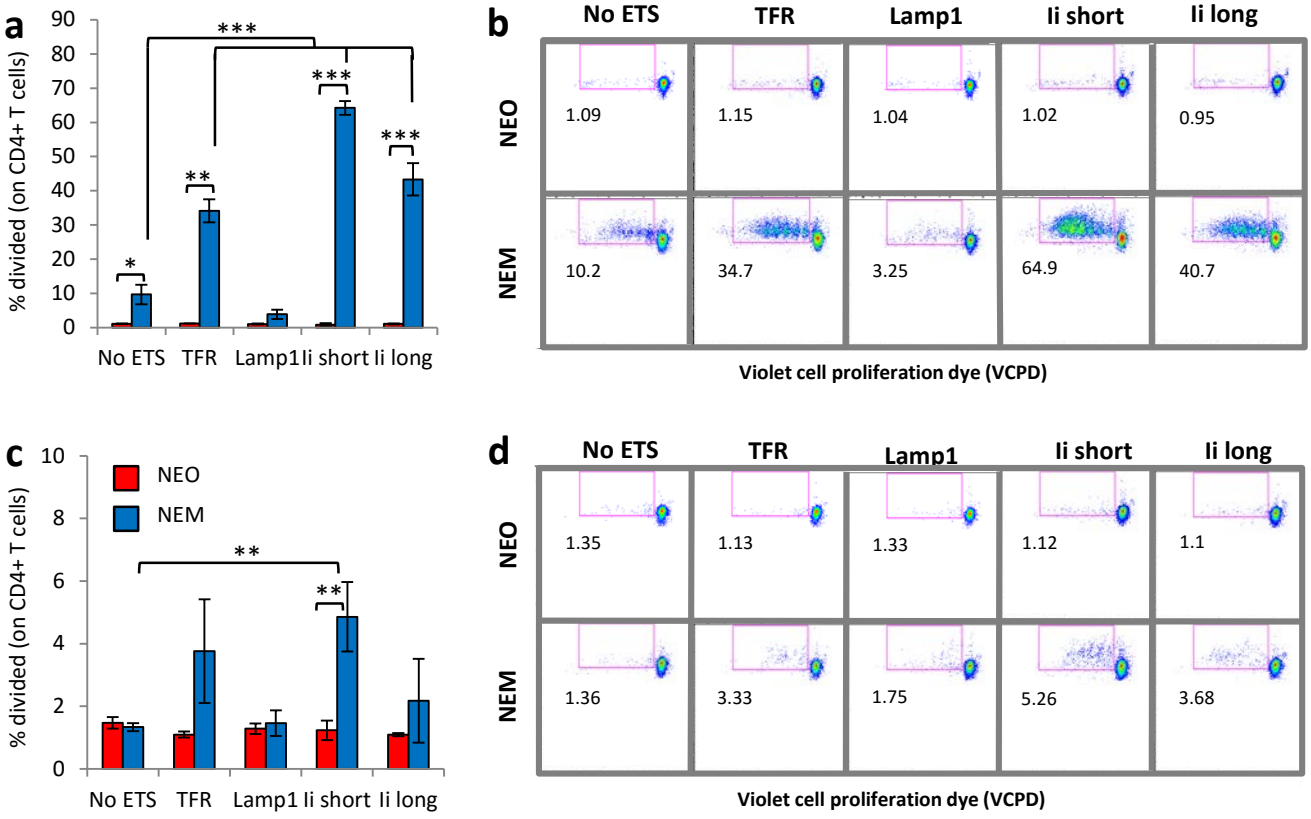
Supplementary Figure S1. Sorting of DCs at intermediate GFP levels. DCs were sorted 2 days after transduction such that all groups of DCs have a comparable level of transgene expression based on GFP MFI before co-culture with T cells. The data are representative of 4 experiments, wherein transduction efficiencies varied and was thereby corrected.



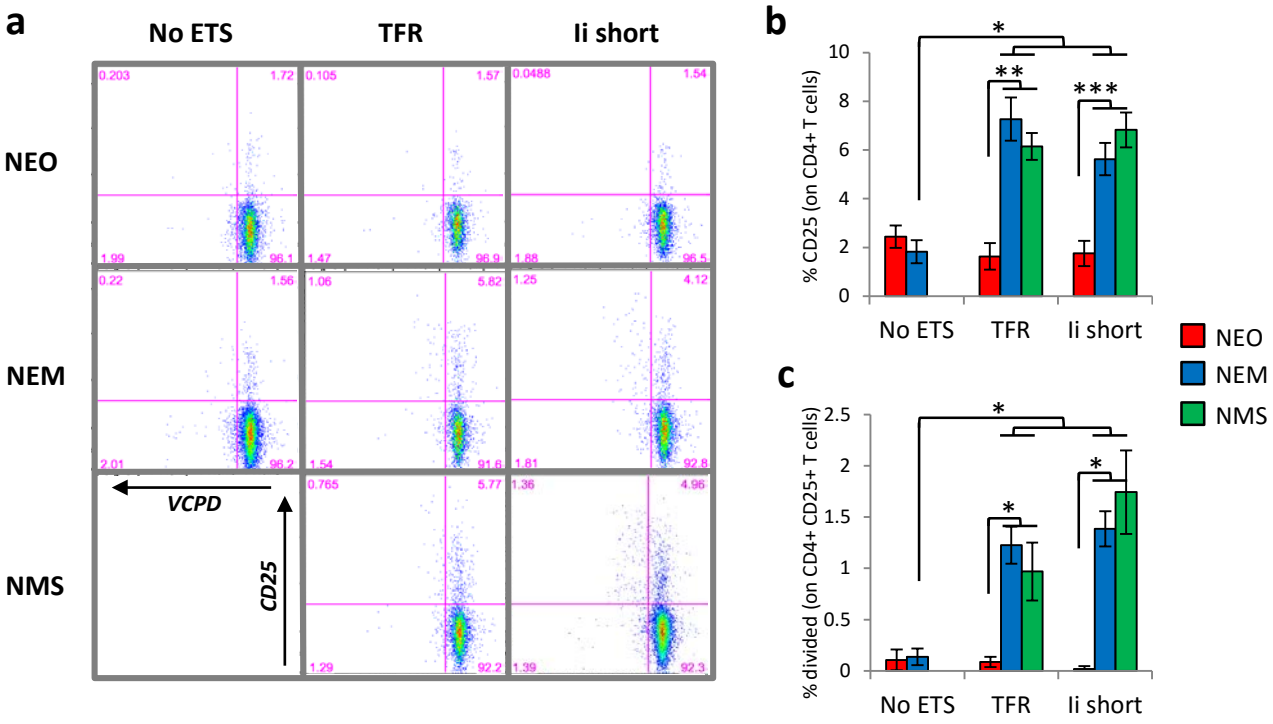
Supplementary Figure S2. Stimulation of CD4⁺ T cells from BDC2.5 mice (a,b) and BDC12-4.1 mice (c,d). DCs were lentivirally transduced to express constructs containing no ETS or $TFR_{1\text{-}118}$ ETS. Stimulation was measured by T cell division. (**a,c**) Mean ±SD from three technical replicates. T-test analysis: * $p<0.05$; ** $p<0.01$; *** $p<0.005$. (**b,d**) Representative FACS plots of Violet Cell Proliferation dye versus CD4.



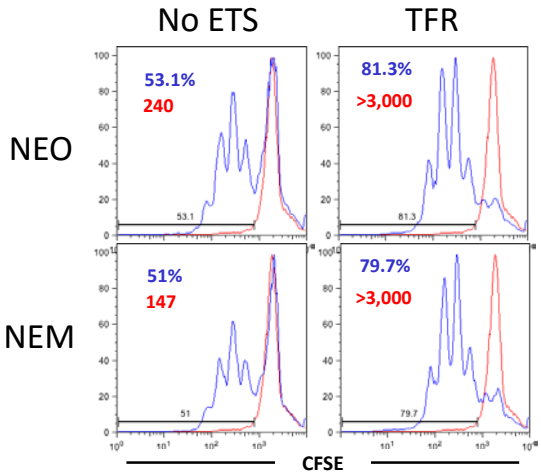
Supplementary Figure S3. Stimulation of CD4⁺ T cells from BDC2.5 mice (a,b) and BDC12-4.1 mice (c,d). DCs were lentivirally transduced to express constructs containing no ETS or one of four tested TS. Stimulation was measured by CD25 upregulation (**a,c**) and T cell division (**b,d**). Data show the mean ±SD from three technical replicates (**a,b**, representative of 3 out of 4 experiments) or five biological replicates (**c,d**, representative of four experiments). T-test analysis: * p<0.05; ** p<0.01; *** p<0.005.



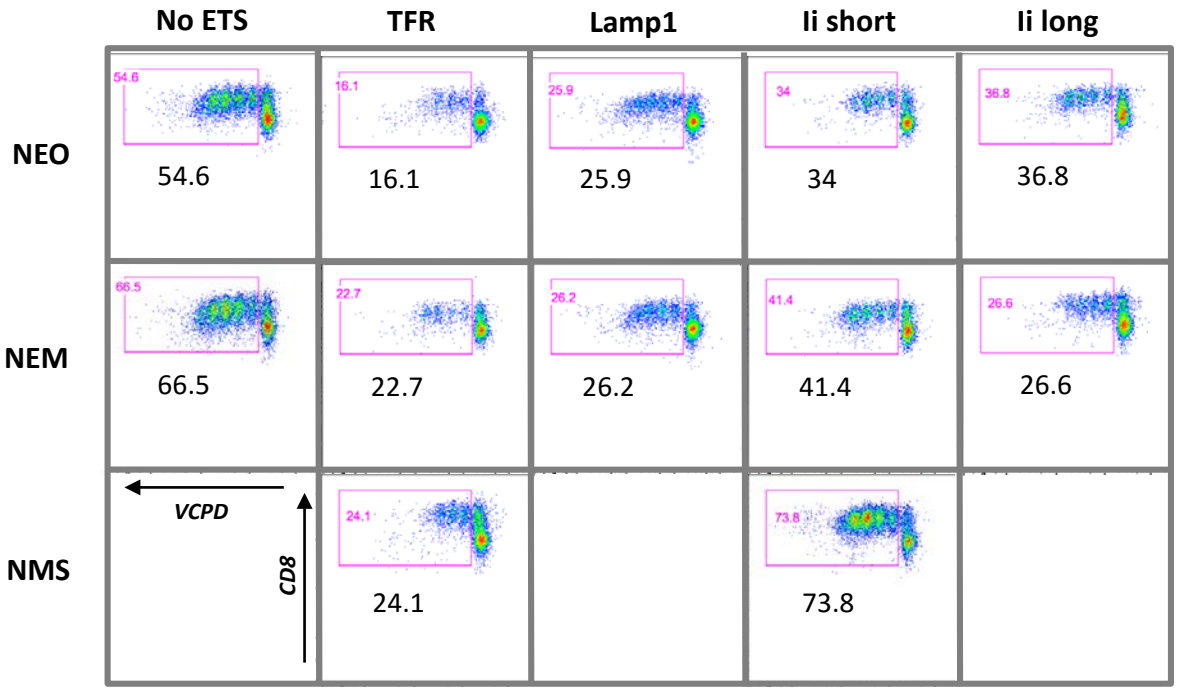
Supplementary Figure S4. Stimulation of CD4⁺ T cells from BDC2.5 mice (a,b) and BDC12-4.1 mice (c,d). DCs were lentivirally transduced to express constructs containing no ETS or one of four tested TS. Stimulation was measured by T cell division. Data show the mean ±SD from three technical replicates (**a,c**) and representative plots (**b,d**; CD4 on y axis). Data are representative of 3 out of 4 experiments (**a,b**) or 4 experiments (**c,d**). T-test analysis: * $p<0.05$; ** $p<0.01$; *** $p<0.005$.



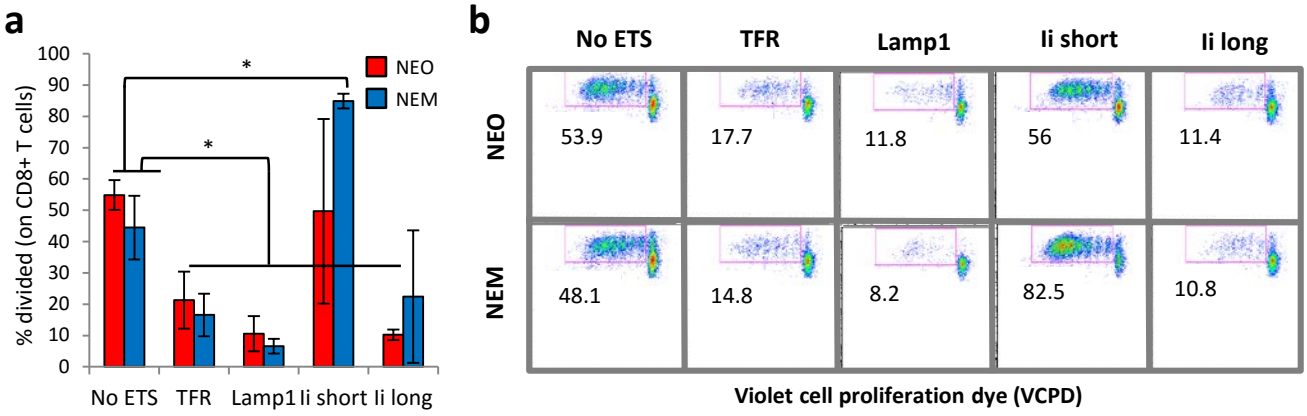
Supplementary Figure S5. Stimulation of CD4⁺ T cells from BDC12-4.1 mice. DCs were lentivirally transduced to express constructs containing no ETS, $TFR_{1\text{-}118}$ ETS or $Ii_{1\text{-}80}$ ETS. Stimulation was measured by CD25 upregulation and T cell division. (a) Representative dot plots of Violet Cell Proliferation Dye against CD25. (**b,c**) Mean ±SD (% CD25 on panel **b**; % divided on panel **c**) from at least three technical replicates (representative of four experiments, except NMS/Ii short, 2 experiments). T-test analysis: * p<0.05; ** p<0.01; *** p<0.005. Stimulation with latex beads coated with anti-CD3/CD28 gave >90% proliferation (not shown).



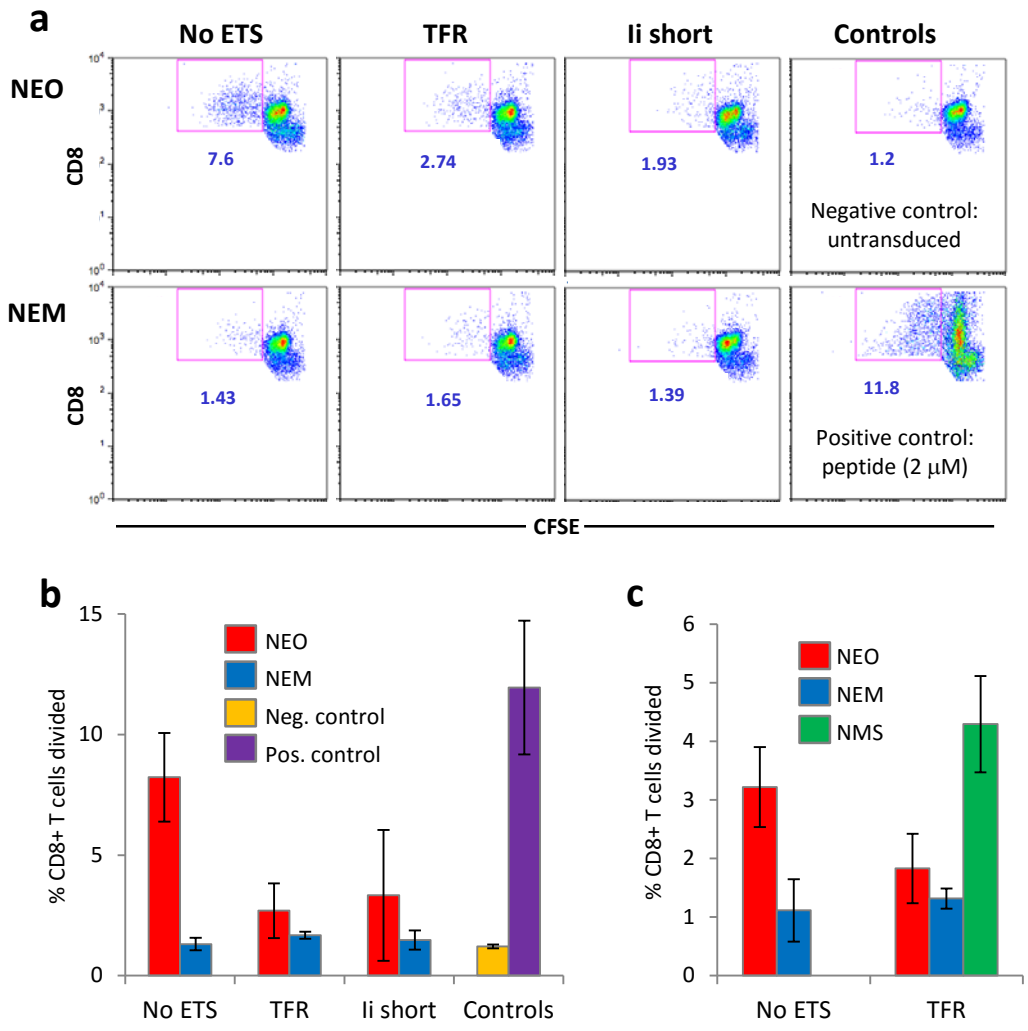
Supplementary Figure S6. Stimulation of GAD65$_{286-300}$-reactive CD4$^+$ T cells from G286 mice. T cells were cultured for 3 days with transduced DCs (blue histograms) or untransduced DCs (red histograms). The percentage of divided T cells is indicated in blue, along with the concentration of IL-2 measured (pg/ml) for each condition in red (the background IL-2 measured with untransduced DCs was 4 pg/ml). Stimulation of these T cells could only be performed once, as the only known colony for these mice, from which we obtained spleens, became lost before we could repeat the experiment.



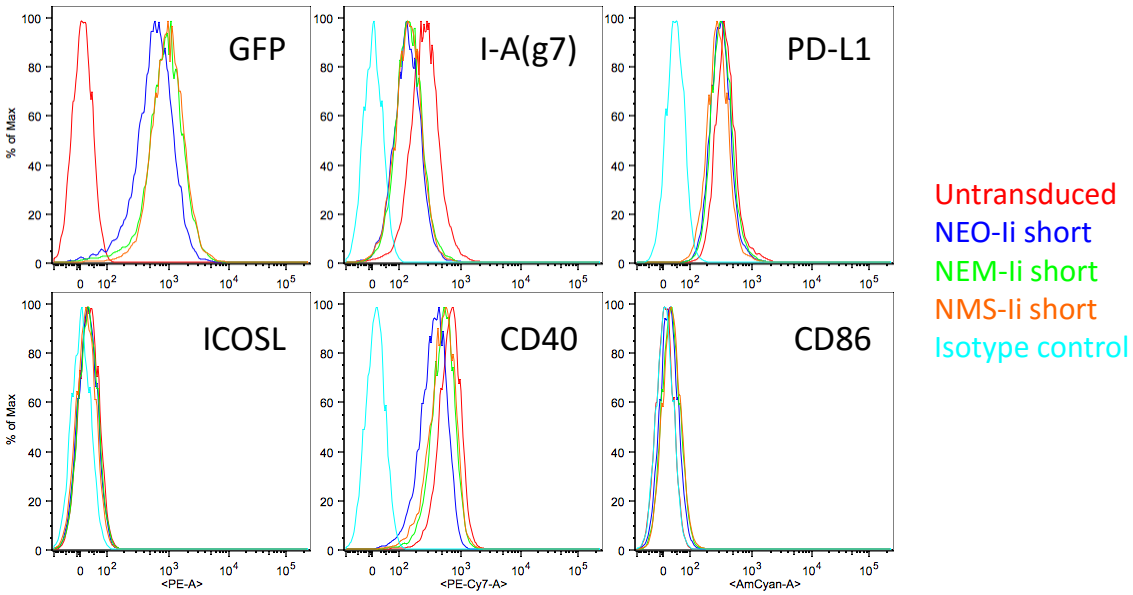
Supplementary Figure S7. Stimulation of CD8⁺ T cells from NY8.3 mice. DCs were lentivirally transduced to express constructs containing no ETS or one of four tested TS. Stimulation was measured by T cell division. Representative dot plots are for the graphs shown in **Fig. 3c,d**.



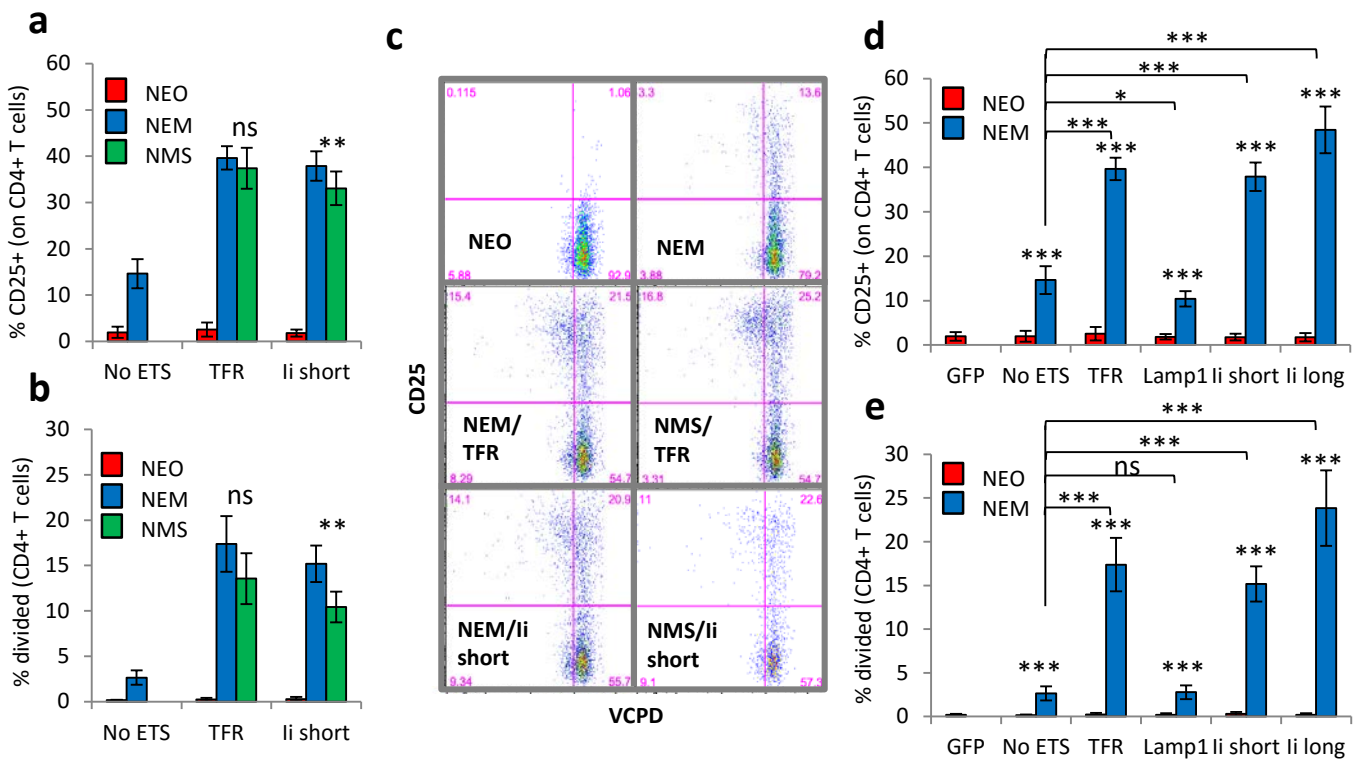
Supplementary Figure S8. Stimulation of CD8⁺ T cells from NY8.3 mice. DCs were lentivirally transduced to express constructs containing no ETS or one of four tested TS. Stimulation was measured by T cell division. (**a**) Mean ±SD from three technical replicates (representative of 3 out of 5 experiments). T-test analysis: * $p<0.05$. (**b**) Representative plots (CD8 on y axis).



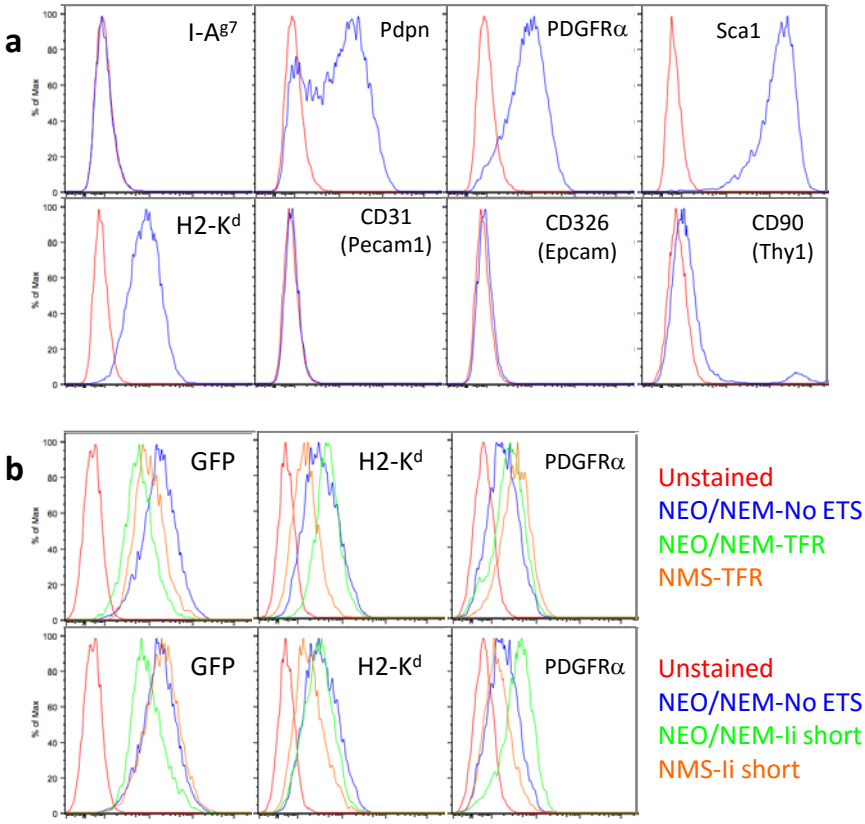
Supplementary Figure S9. Stimulation of CD8⁺ T cells from G9C8 mice. DCs were lentivirally transduced with tandem epitope constructs containing no ETS, $TFR_{1\text{-}118}$ ETS or $Ii_{1\text{-}80}$ ETS. (**a**) Percentage of divided CD8⁺ T cells gated on live CD8⁺ cells (representative plots for data depicted on panel **b**), using T cells isolated from a fresh G9C8 spleen shipped to us. Soluble peptide at 2 μM was used as positive control. (**b**) Mean ±SD from triplicate; T-test NEO vs NEM: $p<0.05$, NEO vs NEO/TFR: $p<0.05$, NEO vs NEO/Ii short: $p=0.069$. (**c**) Data obtained using T cells isolated from frozen splenocytes; mean ±SD from triplicate; T-test NEO vs NEM: $p<0.05$, NEO vs NEO/TFR: $p=0.097$, NEO vs NMS: $p=0.23$



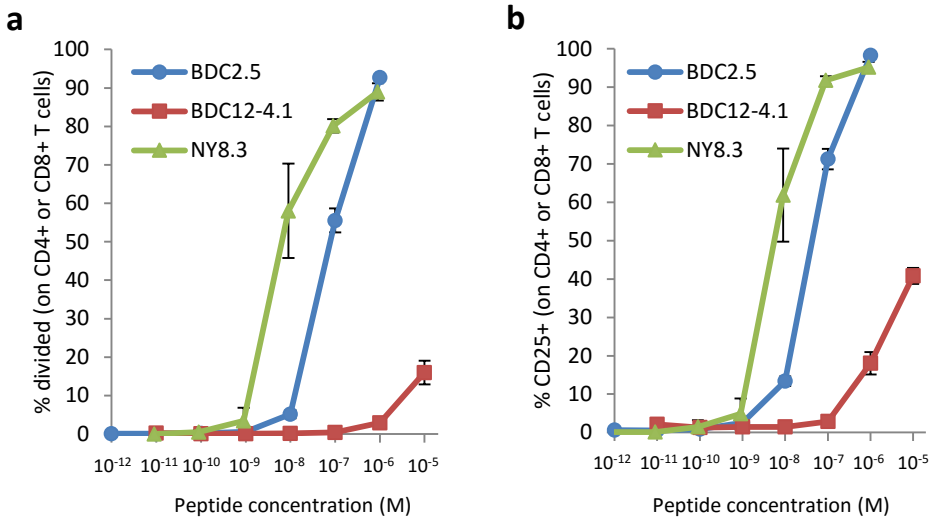
Supplementary Figure S10. Phenotype of DAPg7 cells. Expression of GFP and cell surface markers were compared between the original cell line (untransduced) and the modified, antigen-expressing lines (lines with the different constructs containing "Ii short" as ETS are shown as representative examples). All antibodies used were from Biolegend.



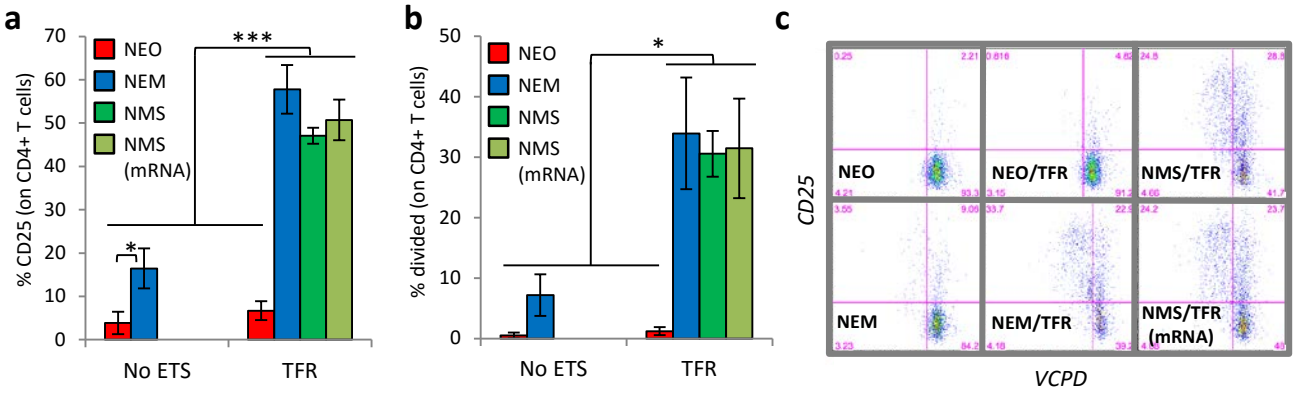
Supplementary Figure S11. Stimulation of BDC12-4.1 (TCRαKO) CD4⁺ T cells by transduced DAPg7 cells. Comparison of constructs with mixed epitopes (NEO, NEM) and segregated epitopes (NMS) for CD25 upregulation (**a**) and T cell division (**b**), with representative dot plots (**c**), gated on live CD4⁺ singlets. Comparison of mixed epitope constructs (NEO and NEM) without or with four types of TS for CD25 upregulation (**d**) and T cell division (**e**). Data show the mean ±SD from five biological replicates (five donor transgenic mice) and from one of three similar experiments. Paired T-test analysis: *** p<0.005.



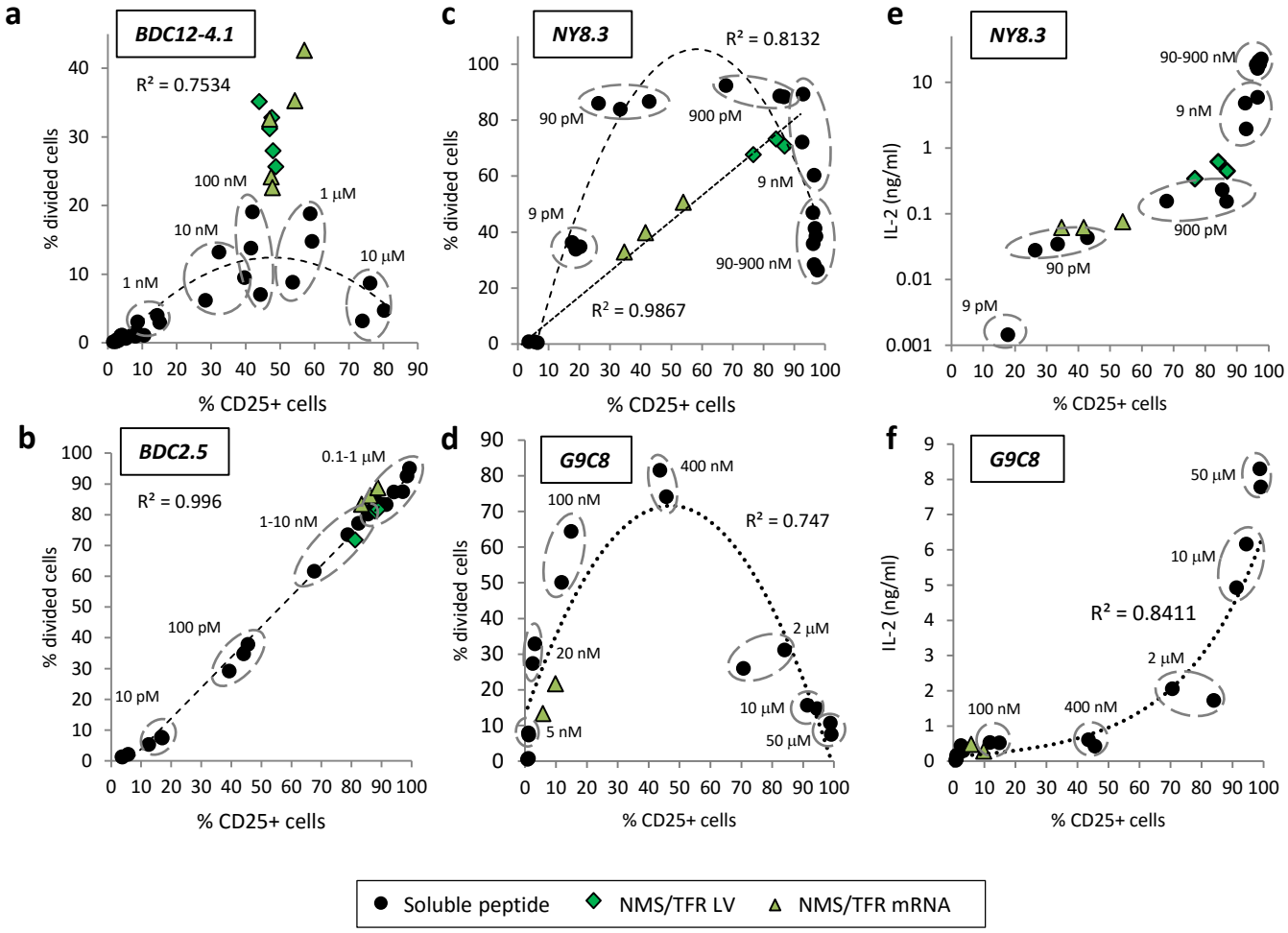
Supplementary Figure S12. Phenotype of PCRC-5 cells. (**a**) Expression of cell surface markers on PCRC-5 cells (red histograms: isotype control; blue histograms: specific marker staining). (**b**) Expression of GFP, K^d and PDGFRα on transduced (antigen-expressing) PCRC-5 lines (selected lines shown as example). All antibodies used were from Biolegend.



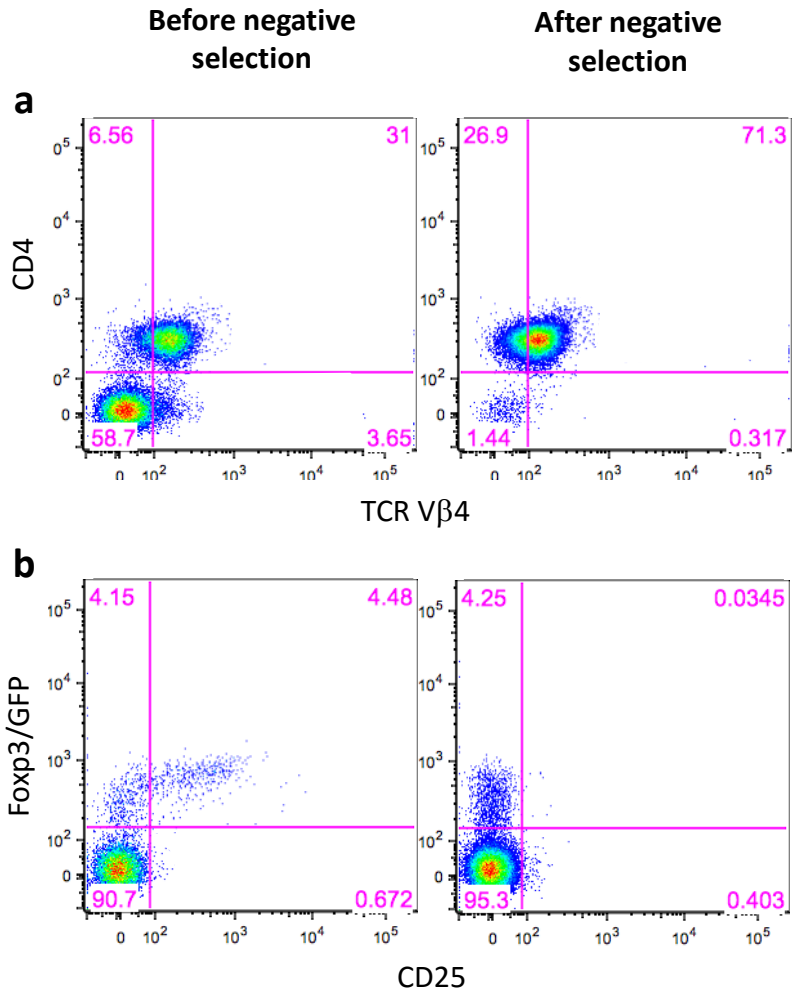
Supplementary Figure S13. T cell responses to exogenous peptide titration on stromal cells. T cell responses were measured as % divided cells (**a**) and % $CD25^+$ (**b**) using 1040-79 mimotope peptide (blue lines), $InsB_{9-23}$ R22E mimotope peptide (red lines) or $IGRP_{206-214}$ peptide (green lines) pulsed onto DAPg7 cells (blue and red lines) or PCRC-5 cells (green lines). Data show the mean ±SD from 3-5 biological replicates.



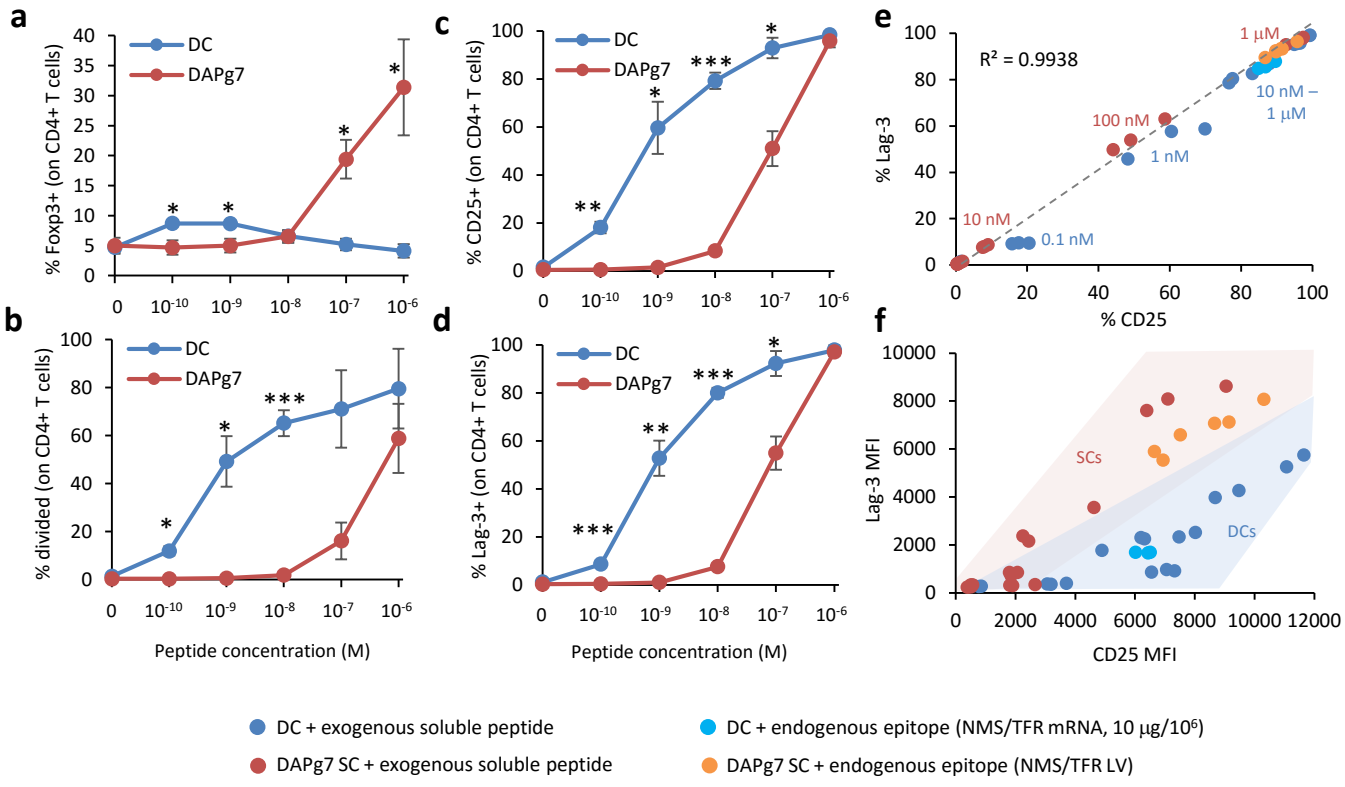
Supplementary Figure S14. Comparison between transduction and mRNA electroporation for the stimulation of diabetogenic T cells by DCs *in vitro*. The percentages of $CD25^+$ T cells (**a**) and of divided T cells (**b**) was measured 3 days after co-culture of $CD4^+$ T cells from BDC12-4.1.TCRαKO mice with transduced or transfected DCs (mean ±SD from five biological replicates; T-test analysis: * $p<0.05$; *** $p<0.005$). Representative dot plots are shown in panel **c**. DCs were either transduced with NMS/TFR LV were sorted based on intermediate GFP levels as before or electroporated with 1 μg NMS/TFR mRNA / 10^6 cells.



Supplementary Figure S15. Comparison between endogenously expressed epitopes and exogenous peptides for the stimulation of diabetogenic T cells by DCs *in vitro*. DCs were either transduced with NMS/TFR LV and sorted based on intermediate GFP levels as before, or electroporated with 1 µg NMS/TFR mRNA / 10^6 cells. These DCs were used to stimulate T cells from BDC12-4.1.TCRαKO mice (**a**), BDC2.5 mice (**b**) NY8.3 mice (**c,e**) and G9C8 mice (**d,f**), in parallel with control DCs pulsed with serial dilutions of exogenous peptide. Responses are plotted as % $CD25^+$ cells against % divided cells (gated on $CD4^+$ or $CD8^+$ T cells, panels **a-b**) or $CD25^+$ cells against IL-2 secretion (panels **e,f**). Each dot represents a biological replicate. Linear or polynomial trend lines with coefficient of correlation (R^2) on panels **a-d,f** for the soluble peptide titration and on panel **c** for endogenous epitopes are indicated.



Supplementary Figure S16. Isolation of BDC2.5.Foxp3/GFP CD4$^+$ T cells. Our negative selection protocol can enrich CD4$^+$ T cells to more than 98% (**a**) with depletion of CD25$^+$ cells to less than 1% (**b**); however CD25$^-$ Foxp3^{low} cells subsist.



Supplementary Figure S17. Stimulation of BDC2.5.Foxp3/GFP CD4⁺ T cells with DCs and DAPg7 cells. The T cell response to peptide-pulsed DCs and DAPg7 cells was measured in terms of % Foxp3/GFP⁺ (**a**), % cell divided (**b**), % CD25⁺ (**c**) and % Lag-3⁺ (**d**), 3 days after co-culture (mean ±SD from three biological replicates, from one experiment representative of two); Paired T-test analysis: * p<0.05; ** p<0.01; *** p<0.005). The % CD25⁺ and % Lag-3⁺ were plotted against each other in order to also display the response to endogenous epitopes (**e**), and showed a high correlation. In contrast, when CD25 MFI and Lag-3 MFI were plotted against each other, the responses separated based on APC type (higher Lag-3 expression with DAPg7 SCs), regardless of antigen source (exogenous or endogenous) (**f**).

SUPPLEMENTARY TABLE

Peptide	Antigen	Sequence	MHC	T cell	TCR	Mouse	Refs
B:9-23	Ins2	SHLVEALYLVCGERG	I-A^{g7}	CD4	Vβ2	BDC12-4.1	[53]
B:15-23	Ins2	LYLVCGERG	K^d	CD8	Vβ6 Vα18	G9C8	[48]
B:9-23 (R22E)	Mimotope	SHLVEALYLVCGEEG	I-A^{g7}	CD4	Vβ2	BDC12-4.1	[25,26]
358-371	ChgA	**WSRM**DQLAKELTAE	I-A^{g7}	CD4	Vβ4 Vα1	BDC2.5	[28]
1040-79	Mimotope	AVPPL**WVRM**E	I-A^{g7}	CD4	Vβ4 Vα1	BDC2.5	[8,9]
206-214	IGRP	VYLKTNVFL	K^d	CD8	Vβ8.1 Vα1	NY8.3	[54]
286-300	GAD65	KKGAAALGIGTDSVI	I-A^{g7}	CD4	Vβ1 Vα4.5	G286	[49]

Supplementary Table 1. Epitopes expressed, their source β cell antigen (or mimotope), their sequence, and their MHC restriction. The mimotope for $InsB_{9-23}$ differ by a single amino acid that favors anchoring of the peptide in a specific configuration.[25,26] The mimotope for ChgA was identified by peptide library screen[8] and the important conserved amino acids are highlighted in bold. The T cell clones specific for each epitope are then indicated, whether they are $CD4^+$ or $CD8^+$, their TCR usage and the TCR-transgenic mouse from which they are isolated.

References

53. Jasinski, J.M., Yu, L., Nakayama, M., Li, M.M., Lipes, M.A., Eisenbarth, G.S., and Liu, E. (2006). Transgenic insulin (B:9-23) T-cell receptor mice develop autoimmune diabetes dependent upon RAG genotype, H-2g7 homozygosity, and insulin 2 gene knockout. Diabetes 55, 1978–1984.

54. Verdaguer, J., Schmidt, D., Amrani, A., Anderson, B., Averill, N., and Santamaria, P. (1997). Spontaneous autoimmune diabetes in monoclonal T cell nonobese diabetic mice. J. Exp. Med. 186, 1663–1676.