Supplementary Table 1

Details of recall antigen clones used in suppression assays.

Clone	Specificity	HLA restriction element
RATT6	tetanus toxoid	HLA-DRB1*0407
RAHA5	haemagglutinin	HLA-DRB1*0101
TTTT6	tetanus toxoid	HLA-DRB1*0401

Supplementary Table 2
Details of external (ex) and internal (in) primer pairs used for single cell multiplex RT-PCR.

Template	ex/in	Sense primer	Anti-sense primer
CD3ε	ex	ATAGGCGGTGATGAGGATGA	TAGTCTGGGTTGGGAACAGG
CD3ε	in	ATGTCTGCTACCCCAGAGGA	TGGCCTCTCCTTGTTTTGTC
IL-10	ex	GAGAACAGCTGCACCCACTT	GGCCTTGCTCTTGTTTTCAC
IL-10	in	TGCCTTCAGCAGAGTGAAGA	GGGAAGAAATCGATGACAGC
granzyme A	ex	GAACAAAAGGTCCCAGGTCA	GCACGAGTCTCTTCCACCTC
granzyme A	in	TTGGGGCTCACTCAATAACC	GGCTTCCAGCACAAACCATA
granzyme B	ex	CCCTGGGAAAACATTCACAC	ATTACAGCGGGGGCTTAGTT
granzyme B	in	TGAAGATGACAGTGCAGGAAG	CGTTTCATGGTTTTCTTTATCCA
perforin	ex	CATGTAACCAGGGCCAAAGT	CCGAACAGCAGGTCGTTAAT
perforin	in	GCTGGACGTGACTCCTAAGC	AGGGCCCTCTTGAAGTCAG

Figure S1: Expression of activation and regulatory T-cell markers by islet specific Tregs. Resting Treg clones (21 days post peptide stimulation) were stained with anti-CD69, anti-HLA-DR, anti-CD25, anti-FoxP3, anti-CD39, anti-ICOS, anti-GITR, anti-CD62L or anti-CD127 antibodies (open histograms) or appropriate isotype control monoclonal antibodies (shaded histograms) as indicated.

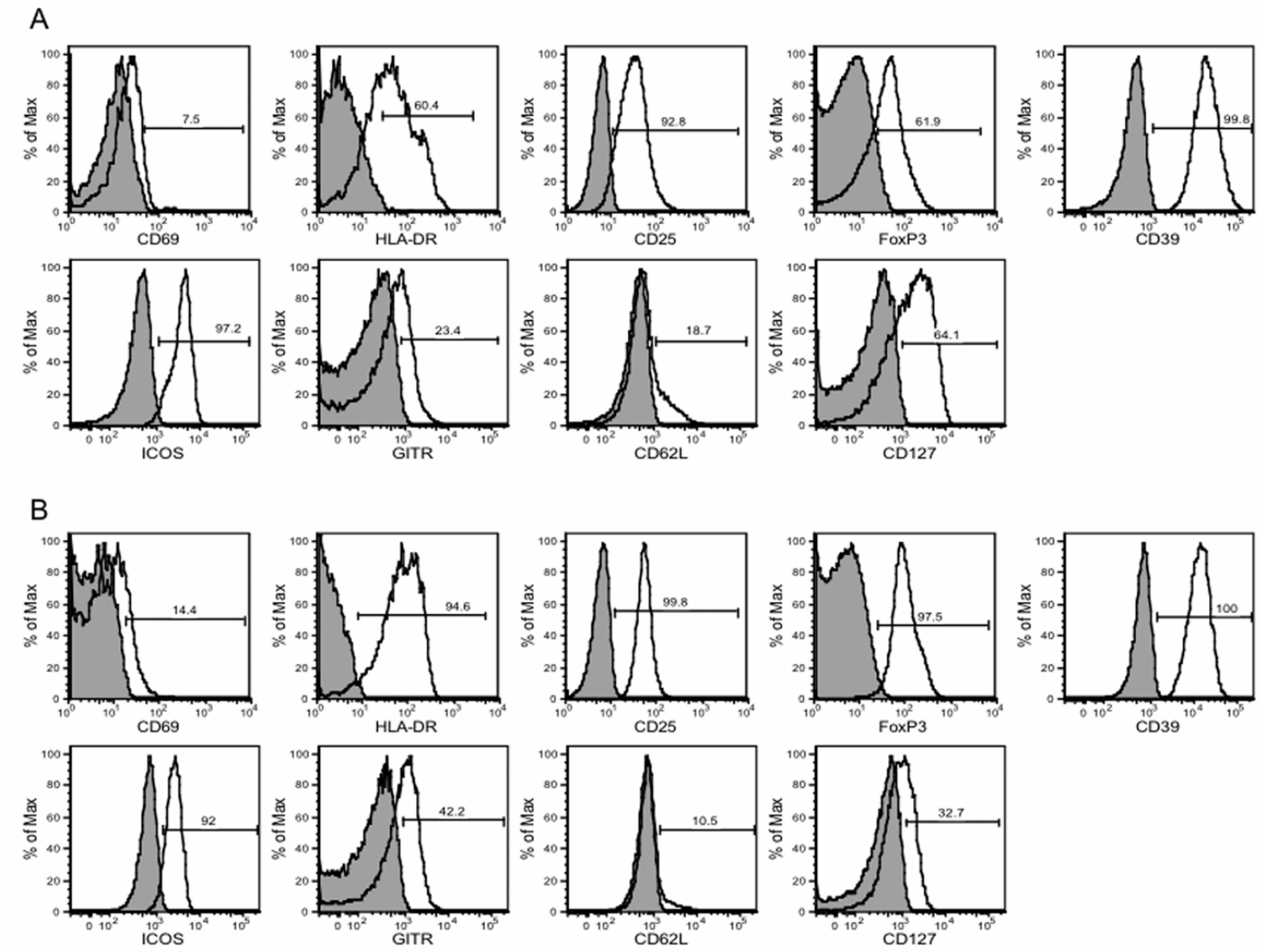


Figure S2: Islet specific Tregs but not recall antigen specific Th1 clones express the gut-specific homing receptor α4β7 integrin. Resting Treg clones MHB10.3 (**A**) and RAR5.3 (**B**) or Th1 clones TATT6 (**C**) and RAHA5 (**D**) were stained with anti-α4 integrin and anti-β7 integrin (contour plots) or appropriate isotype control monoclonal antibodies (dot plots).

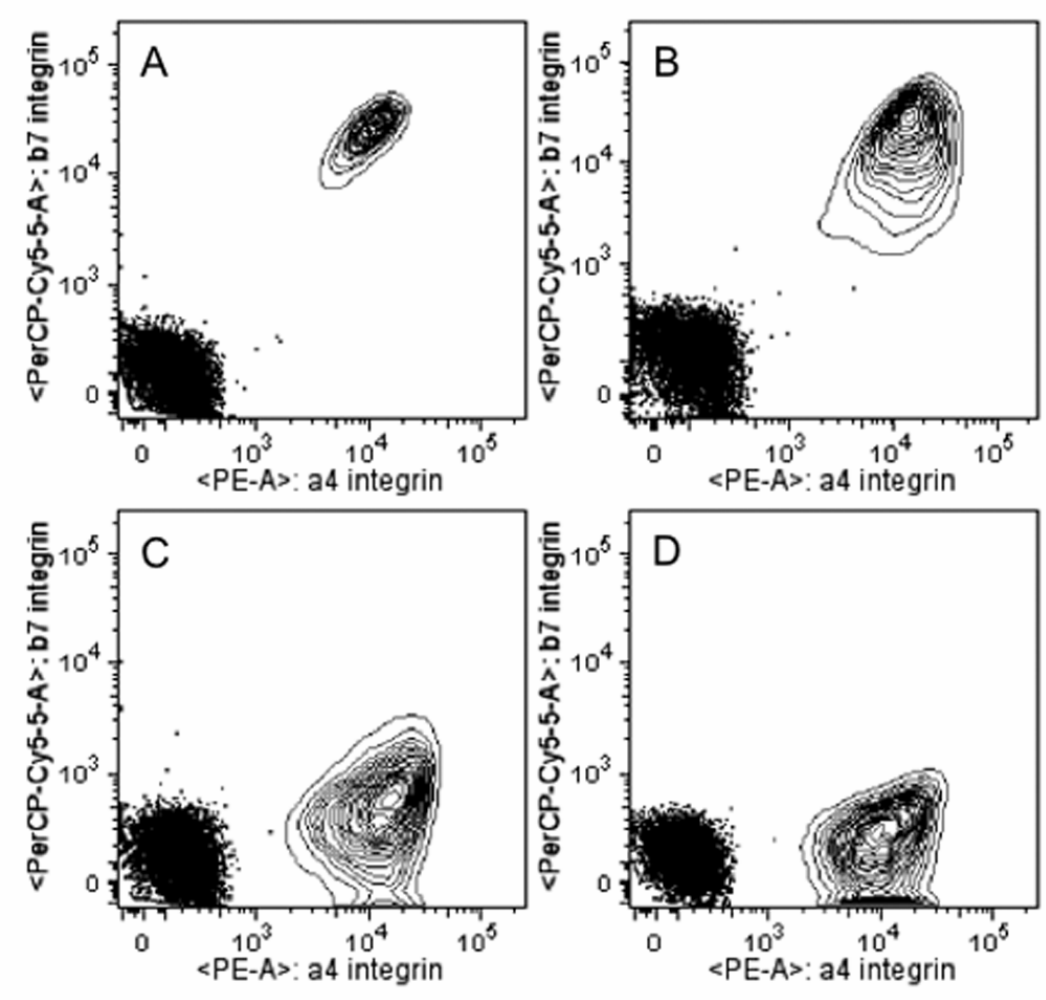


Figure S3: Regulation of responder T-cell responses by IL-10 secreting islet specific T cell clones is not dependant upon IL-10 or TGF-β under conditions suboptimal for regulatory activity. PBMCs from RA were labeled with CFSE and stimulated with recombinant influenza haemagglutinin (HA; 45ng/ml) in the presence or absence of optimal ($3x10^4$, indicated as hi) or suboptimal ($6x10^3$, indicated as lo) numbers of DDAO labeled RAR5.3 IL-10 secreting islet specific T cell clone; optimal (5µg/ml indicated as lo) or sub-optimal (0.5µg/ml, indicated as lo) concentrations of IA-2 709-736 as indicated in the presence of anti-IL-10R and anti-TGF-β antibody (black bars) or isotype control (white bars). Proliferation of CD4+ and CD8+ T-cells were assessed after 5 days by flow cytometry. Numbers represent the % cells divided, error bars represent the SEM of duplicate cultures. Data are representative of two independent experiments. Similar results were obtained with clone MHB10.3.

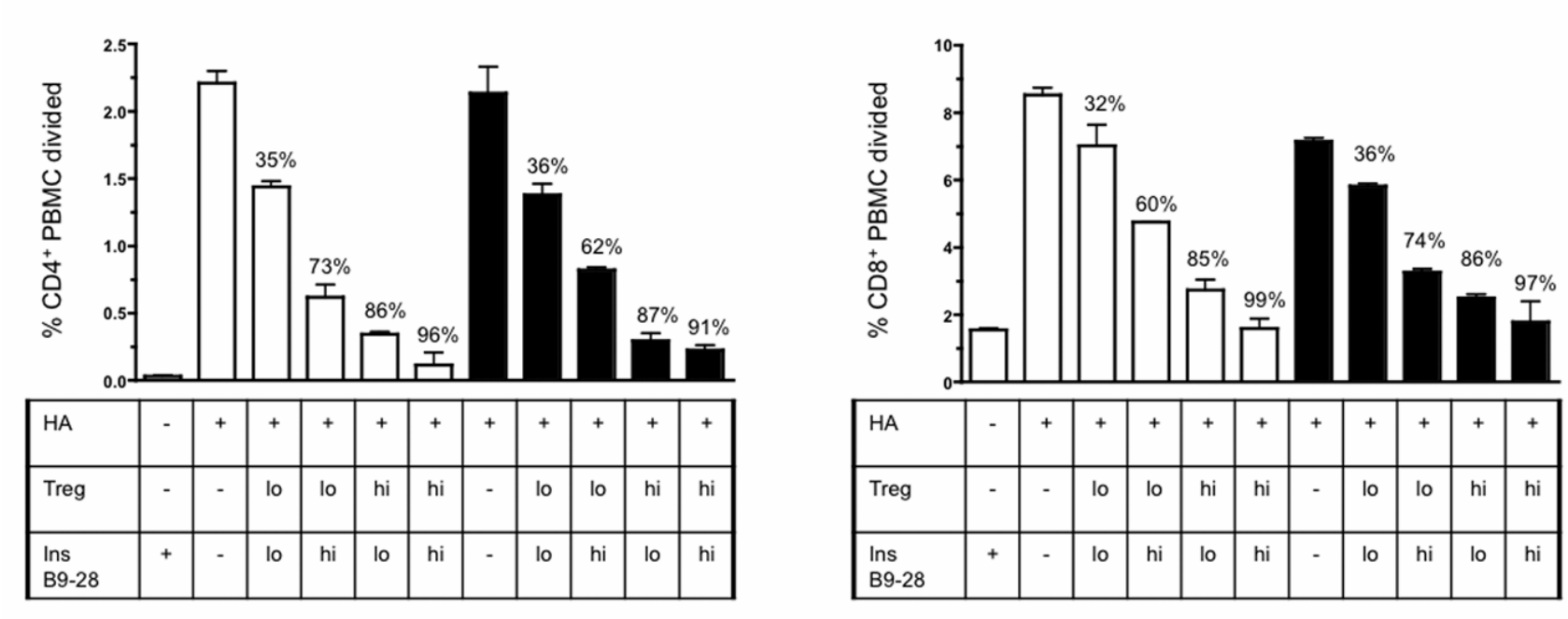
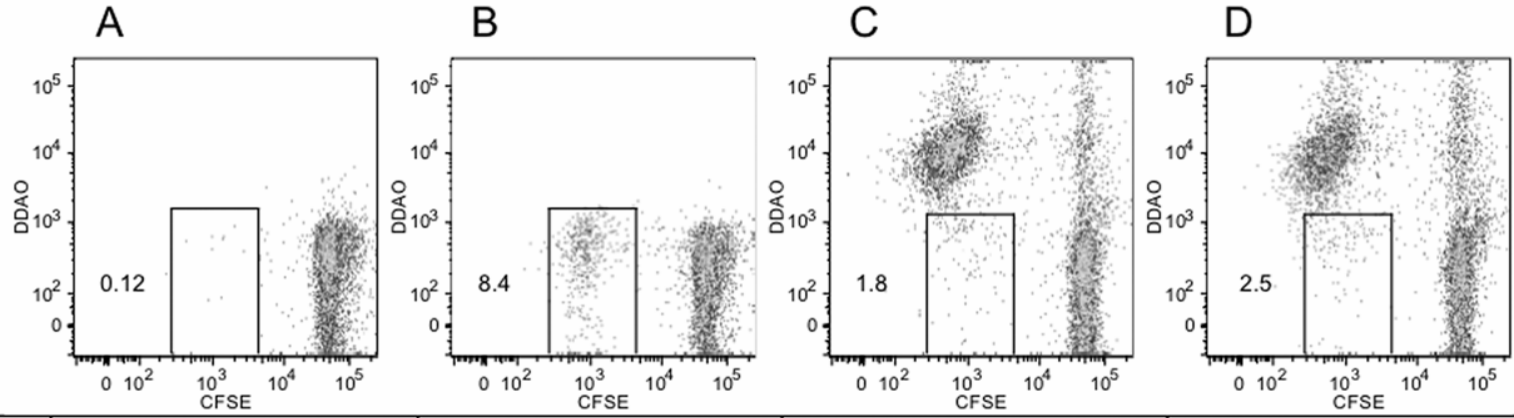


Figure S4. Regulation of autologous T-cell responses by RAR5.3 expanded in the presence or absence of anti-IL10R antibodies. **A-D** PBMCs from RA were labeled with CFSE and stimulated with of recombinant influenza haemagglutinin (HA; 45ng/ml) and IA-2 709-736 (5µg/ml) in the presence or absence of DDAO labeled RAR5.3 which had previously been expanded in the presence of anti-IL-10R antibody (10µg/ml clone 37607.11, R&D Systems, Abingdon, UK) or isotype control clone (10µg/ml MOPC31C; Sigma Chemical Corporation, Poole, UK). or isotype control as indicated. Proliferation of CD3+CD4+ T-cells was assessed after 7 days by flow cytometry. Numbers represent the % responder cells divided as a percentage of CD3+CD4+ DDAO-ve cells as indicated by the gated area. Data are representative of two independent experiments.



PBMC (CFSE)	+	+	+	+
RAR5.3 (DDAO)	-	-	+ (isotype control)	+ (anti-IL-10R)
Stimulus	-	HA + IA-2 709-736	HA + IA-2 709-736	HA + IA-2 709-736

Figure S5. IL-10 secreted by islet specific Tregs upregulates B7-H4 expression on CD14+ monocytes. Untouched monocytes were isolated from peripherial blood using the Miltenyi monocyte isolation kit II according to the manufactures protocol and incubated with tissue culture supernatants from peptide stimulated or unstimulated MHB10.3 cells in the presence (black bars) or absence (white bars) of anti-IL-10R antibodies as indicated. After 48hr monocytes were harvested, stained with anti-CD14 and anti B7-H4 antibodies (or appropriate isotype control) and analysed by flow cytometry.

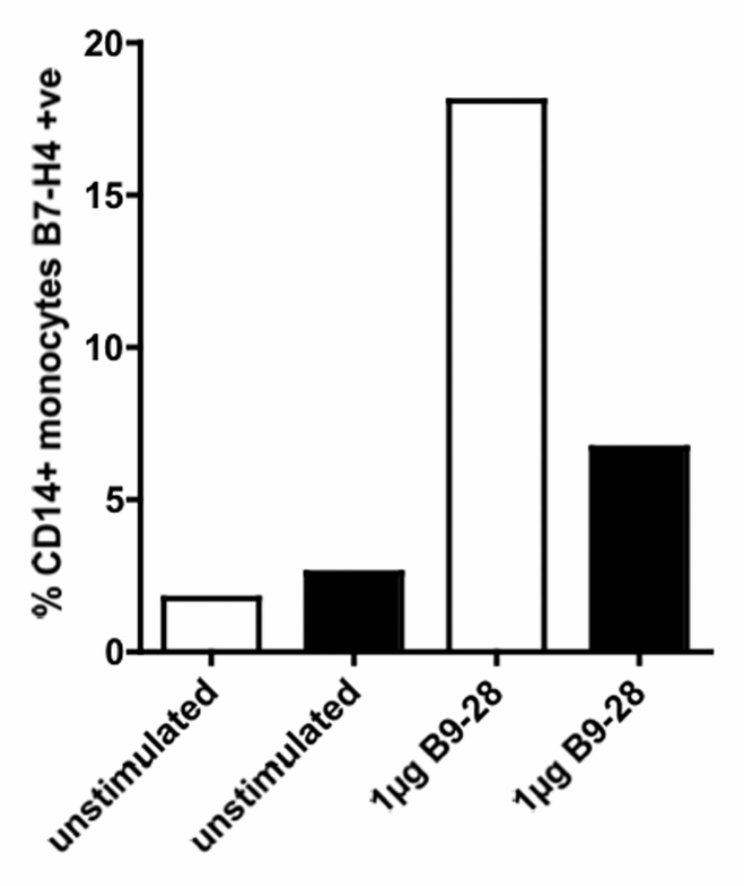
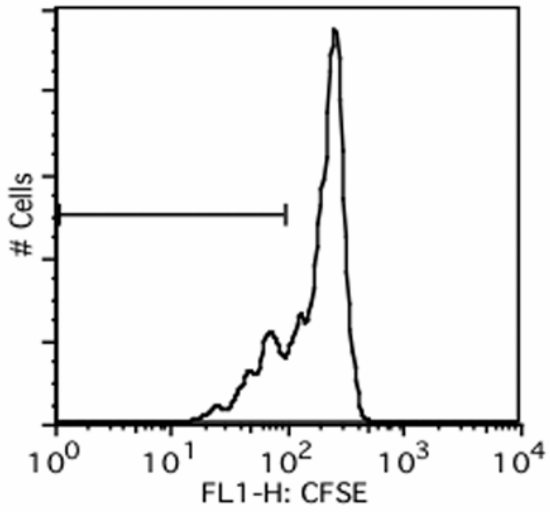
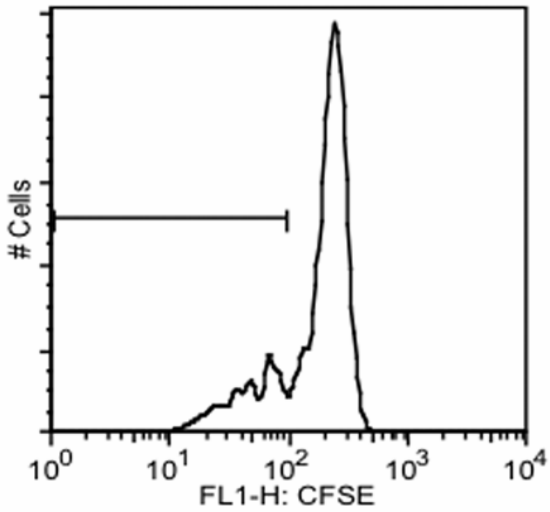


Figure S6. IL-10 secreting islet specific Tregs suppress Th1 clone proliferation. CFSE labelled RATT6 Th1 clone cells were stimulated with tetanus toxoid (25 ng/ml) and insulin B 11-30 (10µg/ml) in the presence or absence of DDAO labeled MHB10.3 as indicated. DDAO labeled irradiated PBMCs were used as a source of antigen presenting cells as indicated. Proliferation of RATT6 (DDAO-ve cells) was assessed after 4 days by flow cytometry





Tresp	+	+
Treg	-	+
APC	HLA-DRB1*0301-0101 HLA-DRB1*00407-0101	HLA-DRB1*0301-0101 HLA-DRB1*00407-0101

Figure S7. Expression of cytotoxic molecules by IL-10 secreting islet-specific Tregs. Resting (dashed lines) or peptide stimulated (solid lines) IA-2-specific RAR5.3 T-cells were stained with (**A**) anti-granzyme A, (**B**) anti-granzyme B and (**C**) anti-perforin monoclonal antibodies. Staining of cells with isotype control monoclonal antibodies are indicated by filled histograms. Similar results were obtained with the MHB10.3 clone. Percentage of activated cells positive for each molecule are indicated.

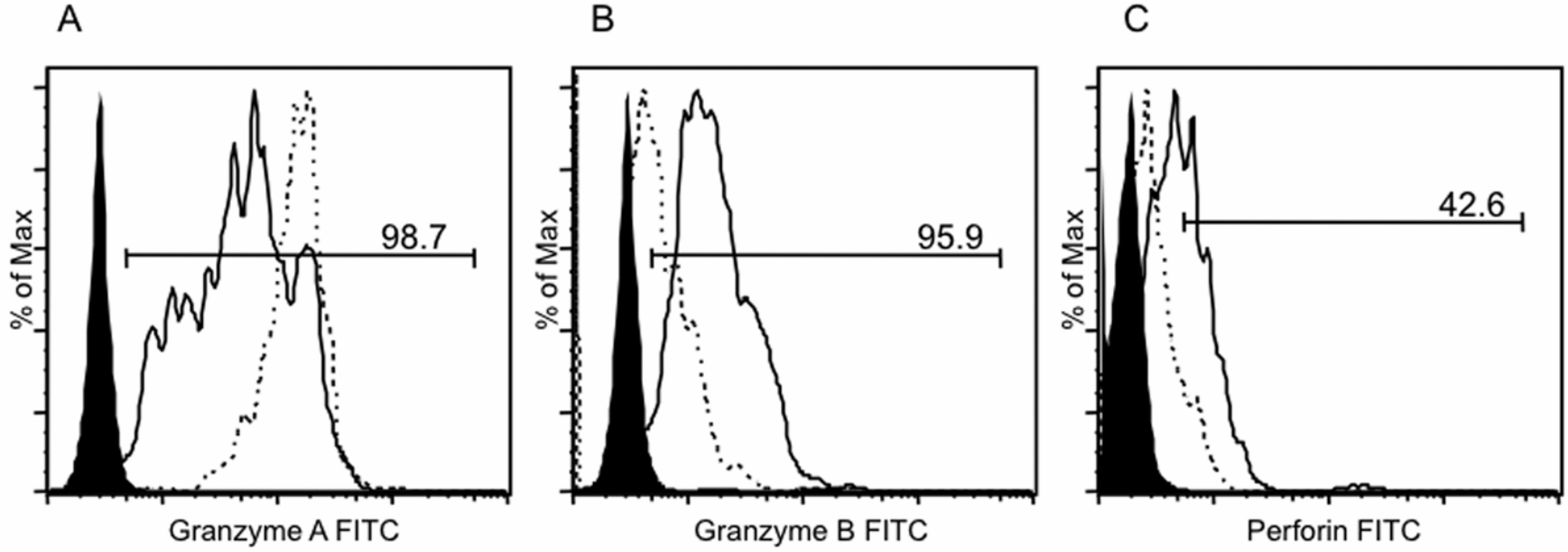


Figure S8. Viability of responder T cell clones upon suppression. The haemagglutinin specific Th1 clone RAHA5 was labeled with CFSE and stimulated with HLA-matched DDAO labeled irradiated PBMCs pulsed with combinations of recombinant haemagglutinin (45ng/ml) and insulin B9-28 (1µg/ml) in the presence or absence of DDAO labeled MHB10.3 as indicated. After 3 days of culture, the viability of non-dividing Th1 clones (DDAO-ve $CFSE^{hi}$ cells) was assessed by staining with 7-AAD and analysis by flow cytometry. Pulsing of antigen presenting cells with peptide and staining with 7-AAD was performed as described for cytotoxitiy assays.

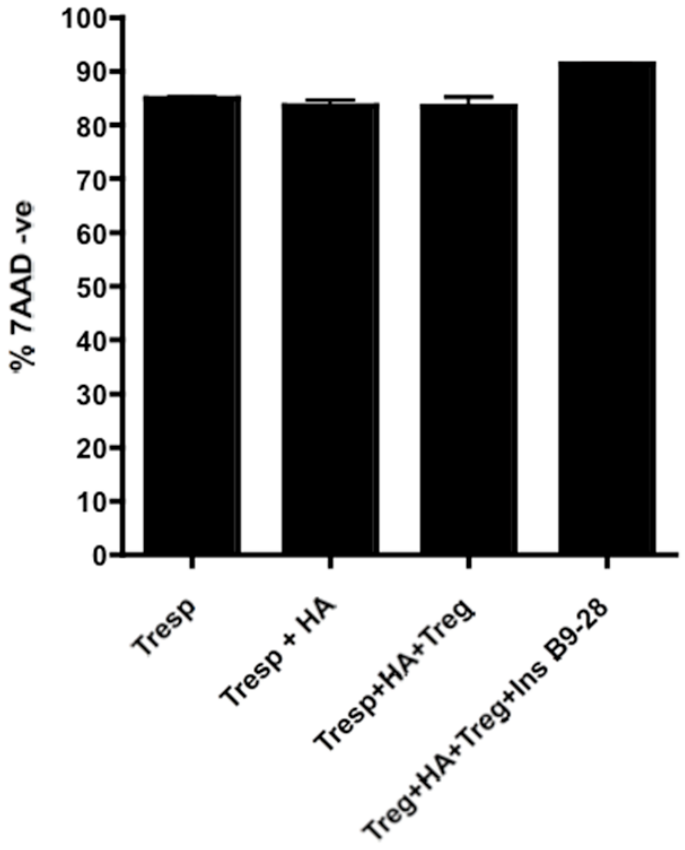
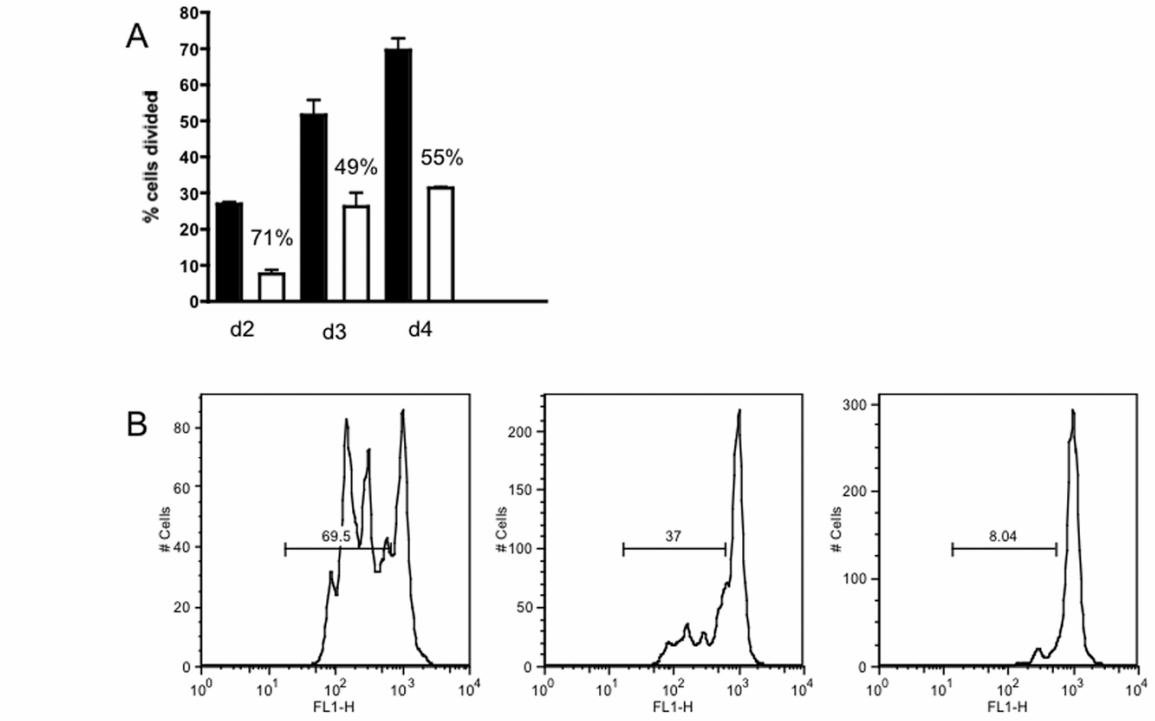


Figure S9. Kinetics of suppression by IL-10 secreting islet specific Tregs. **A.** The Th1 clone RAHA5 was labeled with CFSE and stimulated with haemagglutinin (45ng/ml) in the presence (open bars) or absence (black bars) of DDAO-labeled RAR5.3 Treg clone and IA-2 709-736 (5µg/ml) as indicated. HLA matched DDAO labeled irradiated PBMCs were used as a source of antigen presenting cells. Proliferation of the Th1 responder clone (DDAO-ve cells) was assessed after 2-4 days by flow cytometry. Numbers indicate % suppression and error bars indicate standard error from the mean for triplicate cultures. **B-D** The tetanus toxoid specific Th1 clone RATT6 was labeled with CFSE and stimulated with tetanus toxoid (100ng/ml) and insulin B 11-30 (10µg/ml) in the presence or absence of DDAO labeled MHB10.3 as indicated. DDAO matched PBMC as a source of antigen presenting cells (APC) were present in all cultures. In panel C responder T cells (RATT6), Treg (MHB10.3) and APC were added to the cultures simultaneously; in panel D Treg and APC were incubated for 24hr prior to the addition of responder T cells. Proliferation of responder T cells (DDAO-ve cells) was measured after they had been in culture for 3 days numbers indicate the percentage of responder T cells divided.



Cell type	Time of addition to culture		
APC	0hr	0hr	0hr
Tresp	0hr	0hr	24hr
Treg	-	0hr	0hr