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Supplementary Information for

Revealing the specificity of regulatory T cells in murine autoimmune diabetes

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Supplemental Experimental Procedures

Mice: Female NOD/ShiLtJ, (Jackson laboratories) mice were housed and bred under specific pathogen free conditions in accordance with the UCSF (San Francisco, CA) Animal Care and Use Committee guidelines. NOD.Foxp3[mRFP(tm1flv)] [1, 2], NOD.Foxp3[eGFP] [3], NOD.Rag2^{-/-} [4] and NOD.CD28^{-/-} [5] mice have been previously described. Nur77[GFP] mice [6] were received from Art Weiss and backcrossed at least 10 generations onto the NOD background.

***In vitro* primary T cell stimulation assays:** For quantification of Nur77[GFP] MFI, pooled LNs were isolated and stimulated with plate bound anti-CD3 (145-2C11) at the indicated concentrations for 16 hours. Cells were stained for CD4, CD8, fixable viability dye APC-eFluor780 (eBioscience) and analyzed on a Fortessa flow cytometer (BD Biosciences). For Nur77[GFP] decay experiments, CD4^{+}Foxp3^{+} Tregs or Foxp3^{-} Tconv cells were flow sorted and stimulated or not with plate-bound anti-CD3 (3.05μg/mL) clone 145-2C11 and anti-CD28 (1μg/mL) clone PV1 for 24 hours in complete RPMI with 2,000 U/mL human IL2 (Treg) or 200 U/mL human IL2 (Tconv). Cells were removed from CD3/28 stimulation and maintained in media and IL2 for an additional 6 days analyzed for Nur77[GFP] MFI every 24 hours.

Flow cytometry: Islet and lymph node single-cell suspensions were prepared as previously described [7]. The following antibodies were used to stain the cells: CD103-FITC or Pacific Blue (2E7), ICOS-APC (C398.4A), Ki67-PE-Cy7 (SolA15), TIGIT-PerCPeFluor710 (MBSA43), CD5-APC (53-7.3), Foxp3-eFluor450 (FJK-16s), (eBiosciences), anti-CD4-PE or APC (RM4-5) (eBioscience or Tonbo Biosciences). CD8-Pacific Orange (5H10) (Life Technologies), CTLA4-PE (UC10-4F10-11), Thy1.1-PerCP (OX-7) (BD Biosciences). For intracellular staining, cells were first fixed and permeabilized per manufacturer's instructions (eBioscience or Tonbo Biosciences). Analyses were performed on a LSRII or Fortessa flow cytometer (BD Biosciences) with FACSDiva (BD Biosciences) and Flowjo software.

Clonality calculation: Clonality was calculated using the inverse of normalized Shannon's diversity index:

$$Normalized\ Shannon's\ entropy = \frac{-\sum_{i=1}^{S}(p_i \ln p_i)}{\ln S}$$

where S = total number of unique species (TCR sequences) and p_i = proportion of S made up of the ith species.

Morisita-Horn diversity index: The Morisita-Horn diversity index was calculated using the following equation:

$$Morisita - Horn\ diveristy = \frac{2\sum_{i=1}^{S} x_i y_i}{(\sum_{i=1}^{S}\frac{x_i^2}{X^2} + \sum_{i=1}^{S}\frac{y_i^2}{Y^2})XY}$$

where S = total number of unique species (TCR sequences), x_i = the number of times species i is represented in the total X from one sample, and y_i = the number of times species i is represented in the total Y from one sample.

Single-cell TCRαβ primer design: All nested TCR primers were designed to amplify Vα, Vβ, Cα and Cβ, and base degeneracy was incorporated into the primers to account for TCR polymorphism and ensure amplification of all known functional regions identified in the IMGT database (http://www.imgt.org/). V-region primers were designed to target the leader sequence and near the 5' end of of each Vα, Vβ to include of the entire V(D)J region. All primers for the second PCR reaction contain the IlluminaTM common paired-end sequences, which enables further amplification with barcoding primers during the third reaction. Each primer for the third reaction consists of the appropriate Illumina adapter, an 8-nt plate ID sequence, and a 7-nt well ID sequence.

Single-cell TCRαβ sequencing: Cells were sorted directly into 11µl of sort buffer (8.5µl of water and 2.2µl of 5x One-step RT PCR buffer (Qiagen) and 0.3µl of Ribolock RNAse inhibitor (ThermoFisher). For the first RT-PCR reaction, reverse transcription and preamplification were performed with multiple Vα and Vβ region primers (final concentration 0.12µM each) and Cα and Cβ region primers (final concentration 0.6µM each) in a 15µl reaction. A 25 cycle RT-PCR reaction was performed according to the manufacturer's instructions. For PCR2 and PCR3 reactions, amplification was done using 2x HotStarTaq Master Mix Kit (Qiagen). In the second PCR reaction, 1µl of the RT-PCR product was added as a template to a 15µl PCR reaction with the same final primer concentrations and the following cycling conditions: 95°C 15 min; 94°C 30 s, 68°C (TCRα) or 63°C (TCRβ) 1 min, 72°C 1 min, x 30 cycles; 72°C 5 min; 4°C. For the third PCR reaction, 1µl of the second PCR product was used as a template for a 20µl PCR reaction, which incorporates the barcode primers and enables sequencing on the IlluminaTM MiSeq platform. In the third PCR reaction, amplification was done for 36 cycles using unique plate ID barcoding primers (0.2µM), well ID barcoding primers (0.2µM), and Illumina adapter primers (1µM). The cycling conditions are as follows: 95°C 15 min; 94°C 30 s, 66°C 1 min 72°C 1 min x 36 cycles; 72°C 5 min; 4°C. The final PCR products were pooled at equal proportion by volume, followed by Ampure XP bead wash (Beckman Coulter), run on a 1% agarose gel, and a band around 450 to 500 bp was excised and gel purified using a Qiaquick gel extraction kit (Qiagen). DNA QC was determined by the Agilent 2100 Bioanalyzer and the final library was sequenced. Raw sequencing data were processed and demultiplexed according to the unique plate and well ID combination. To assign V(D)J families, data was sent to the IMGT HighV-QUEST (www.imgt.org/HighV-QUEST) database to predict germline allele usage, germline sequence recombination, and mutations relative to the germline sequence.

Hybridoma generation and stimulation: TCRs of interest were cloned into the pMSCV-IRES-mCherry retroviral vector (Addgene). The hen egg lysozyme (HEL)-specific TCR, PA21.14H4 TCR, was obtained from Dr. Dario Vignali [8]. TCR expressing virus was generated using Phoenix-ECO packaging cells and used to transduce the TCR-deficient 58α-β- hybridoma cell line, which was modified to express GFP downstream of an NFAT promoter [9, 10]. Three days after transduction

TCRβ⁺mCherry⁺ cells were sorted. For cultures with splenic DCs, either commercially synthesized peptide (GenScript) at 10 µM [11-19], islet lysate (1.87µg), or metabolically inactive human insulin, B25D-insulin (Novo Nordisk, 20µg) was added to hybridoma cultures. In some experiments, hybridomas were stimulated with 2.5 µg/mL plate bound tetramer of I-Ag⁷ bound to HLVERLYLCGEEG (Insulin peptide 8E) [11]. As a positive control, hybridomas were stimulated in wells coated with 3 □g/ml anti-CD3 antibody (clone 17A2, Tonbo, or 145-2C11). Cells were stimulated overnight in complete RPMI at 37°C and analyzed the following day for NFAT^GFP expression.

Islet lysate: NOD.Rag2⁻/⁻ [4] islets were resuspended in PBS and frozen in dry ice and thawed at 37°C twice. Then, mixture was homogenized in a 2mL dounce homogenizer followed by several passages through a 25-gauge needle. Protein quantification was performed with the Pierce BCA Protein Assay Kit (Thermo fisher).

Diabetes monitoring: Diabetes incidence was monitored by weekly blood glucose measurements. Mice were considered diabetic after 2 sequential blood glucose readings exceed 300 mg/dL.

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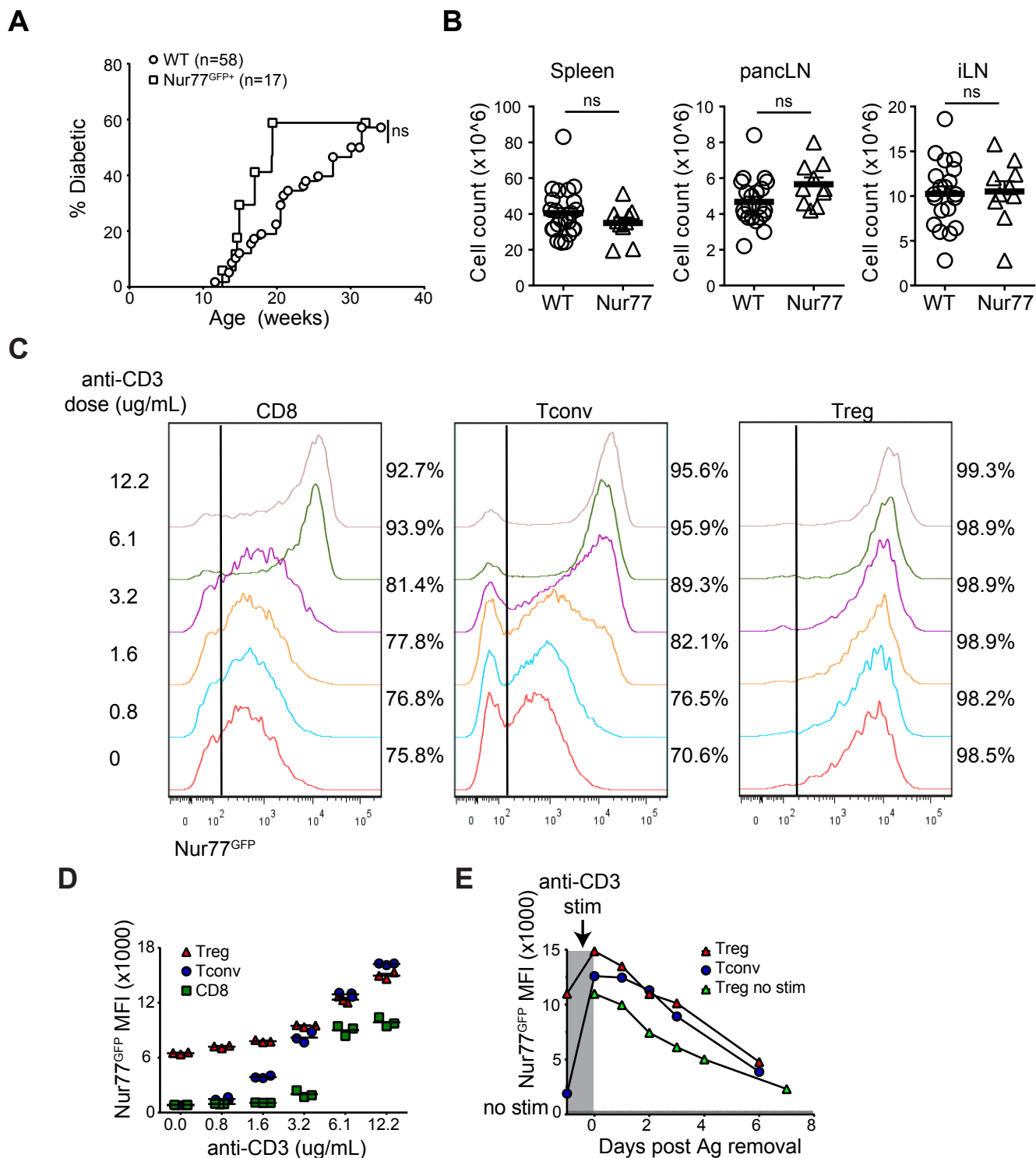
Figure S1. Characterization of NOD.Nur77GFP reporter mouse. **(A)** Wild type (WT) NOD and Nur77GFP mice at backcross 13 were followed for >30 weeks for incidence of diabetes. Statistical significance was calculated using either the Log-rank (Mantel-Cox) test or the Gehan-Breslow-Wilcoxon test, ns, not significant. **(B)** Cellularity of spleen, pancLN, and iLN of pre-diabetic WT NOD and Nur77GFP mice. Each circle represents a single mouse and the mean and SD of the group are shown. Statistical significance was determined using a student's t test, ns, not significant. **(C)** Pooled LNs were stimulated for 16 hours with plate-bound anti-CD3 at concentrations listed. The percentage of Nur77GFP+ cells was measured for each population. Vertical black line denotes GFP+ staining based on trangene negative control. **(D)** Quantification of Nur77GFP MFI of the cells stimulated in C. Results represent at least 2 independent experiments. **(E)** Pooled LNs were sorted for CD4+Foxp3+ (Tregs) or CD4+Foxp3- (Tconv) and then stimulated or not with plate-bound anti-CD3 and anti-CD28 for 24 hours. Cells were removed from stimulation and Nur77GFP MFI was measured for 6 days. Results represent at least 2 independent experiments.
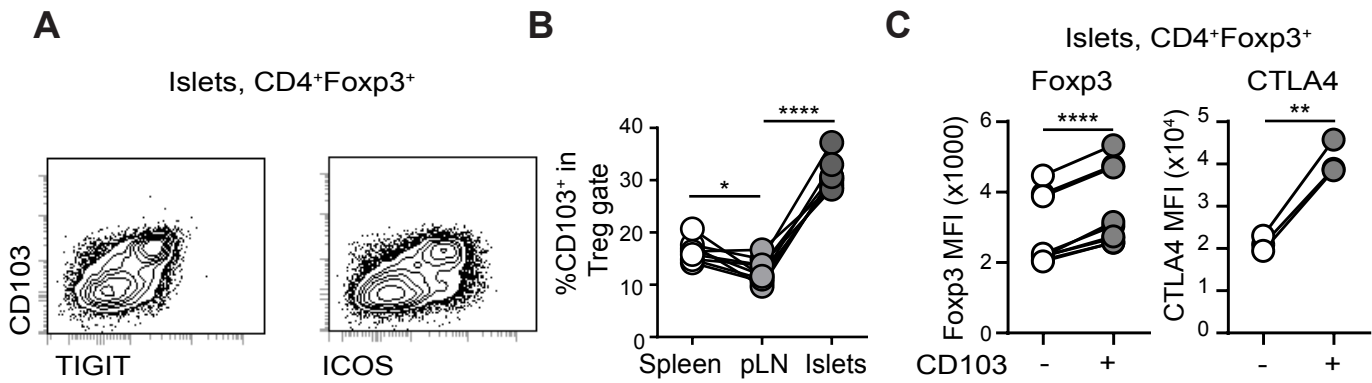
A

Islets, CD4⁺Foxp3⁺

CD103 / TIGIT

CD103 / ICOS

B

%CD103⁺ in Treg gate

Spleen — pLN — Islets

* (Spleen vs pLN)

**** (pLN vs Islets)

C

Islets, CD4⁺Foxp3⁺

Foxp3

Foxp3 MFI (x1000)

CD103 − +

****

CTLA4

CTLA4 MFI (x10⁴)

CD103 − +

**

Figure S2. Activation phenotype and localization of islet Tregs expressing CD103, ICOS, and TIGIT. (**A**) Representative flow plot showing staining of islet Tregs for CD103, TIGIT, and ICOS from at least 10 independent experiments. (**B**) Percentage of Tregs expressing CD103 in different organs of pre-diabetic NOD mice (ages 15-18wks.) Results shown are a summary of 3 independent experiments. Statistical significance was determined using a repeated measures one-way ANOVA followed by Tukey's multiple comparison test, *p<0.05, ****p<0.0001. (**C**) Foxp3 and CTLA4 MFI in islet Treg subsetse from pre-diabetic NOD mice. Foxp3 (n=6) results shown are a summary of 3 independent experiments, CTLA4 (n=3) results are representative of at least 2 independent experiments. Statistical significance was determined using a paired student's t-test, **p<0.01, ****p<0.0001.
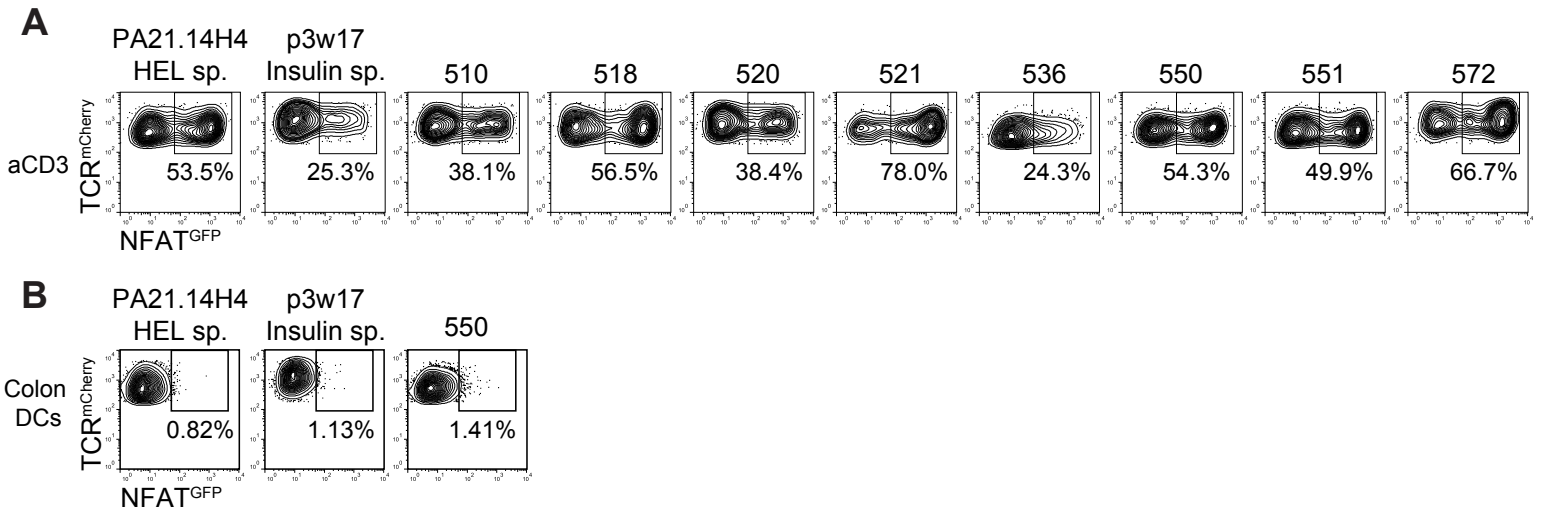
A

B

Figure S3. Related to Figure 4. TCR hybridoma response to colon DCs. (**A**) Hybridomas were stimulated with plate bound anti-CD3 and reactivity was assessed as described in Figure 4. (**B**) Hybridomas were stimulated with colon DCs and reactivity was assessed as described in Figure 4.

Figure S4. Insulin-specific Tregs in NOD mice. Quantification of HEL and Insulin tetramer staining in pancLN and Islet Tconv (left) and Tregs (right). Each circle represents one mouse and columns represent the mean and SD of the group. Results shown are a summary of 6 independent experiments. Each circle represents one group of 2 mice pooled and divided evenly between HEL and Insulin tetramer stain, pancLN n=8 and Islet n=24. Statistical significance was determined using a paired student's t test, *p<0.05, ****p<0.0001, ns, not significant.

Table S1. Summary of TCRβ sequencing results.

Mouse #	Organ	CD4+ T cell Population	Cell #	Unique TCRβ sequences	TCRβ Read #	Reads per unique TCRβ
1	Spleen	Treg CD103+	40,000	8,724	736,716	84.4
2	Spleen	Treg CD103+	29,000	7,235	803,340	111.0
3	Spleen	Treg CD103+	110,000	10,639	693,392	65.1
4	Spleen	Treg CD103+	89,000	15,157	744,578	49.1
1	Spleen	Treg CD103-	250,000	43,968	660,839	15.0
2	Spleen	Treg CD103-	235,000	52,167	463,872	8.8
3	Spleen	Treg CD103-	604,000	62,607	435,820	6.9
4	Spleen	Treg CD103-	436,000	54,959	514,760	9.3
2	pancLN	Treg CD103+	15,000	3,131	475,729	151.9
3	pancLN	Treg CD103+	22,000	5,969	565,116	94.6
4	pancLN	Treg CD103+	36,000	9,804	594,185	60.6
1	pancLN	Treg CD103-	47,000	13,097	663,220	50.6
2	pancLN	Treg CD103-	54,000	13,722	598,528	43.6
3	pancLN	Treg CD103-	90,000	9,485	297,741	31.3
4	pancLN	Treg CD103-	107,000	35,612	777,471	21.8
1	iLN	Treg CD103+	60,000	11,264	470,873	41.8
2	iLN	Treg CD103+	56,000	11,195	461,328	41.2
3	iLN	Treg CD103+	55,000	12,603	417,697	33.1
1	iLN	Treg CD103-	335,000	59,124	429,293	7.2
2	iLN	Treg CD103-	373,000	57,429	536,558	9.3
3	iLN	Treg CD103-	262,000	57,629	577,195	10.0
4	iLN	Treg CD103-	245,000	45,376	572,640	12.6
1	Islet	Treg CD103+Nur77^{GFP-hi}	3,800	1,419	575,346	405.4
2	Islet	Treg CD103+Nur77^{GFP-hi}	8,600	1,577	696,886	441.9
3	Islet	Treg CD103+Nur77^{GFP-hi}	6,400	1,638	732,321	447.0
4	Islet	Treg CD103+Nur77^{GFP-hi}	3,500	1,317	681,828	517.7
1	Islet	Treg CD103-Nur77^{GFP-hi}	1,600	1,144	627,703	548.6
2	Islet	Treg CD103-Nur77^{GFP-hi}	3,600	1,261	451,152	357.7
1	Islet	Treg CD103-Nur77^{GFP-lo}	5,000	1,626	578,388	355.7
2	Islet	Treg CD103-Nur77^{GFP-lo}	12,000	5,225	658,562	126.0
3	Islet	Treg CD103-Nur77^{GFP-lo}	8,000	2,863	739,148	258.1
4	Islet	Treg CD103-Nur77^{GFP-lo}	5,900	1,601	682,404	426.2
1	Islet	Tconv Nur77^{GFP-hi}	4,100	1,372	613,096	469.6
2	Islet	Tconv Nur77^{GFP-hi}	11,000	3,077	505,504	170.7
3	Islet	Tconv Nur77^{GFP-hi}	18,000	2,103	301,760	155.4
4	Islet	Tconv Nur77^{GFP-hi}	8,900	1,441	360,779	262.3

Treg populations were sorted as described in Fig 2A. Cells from various organs of up to 4 individual mice were isolated using FACS. Numbers of cells recovered from FACS are listed to provide reference to the abundance of each cell subset in a mouse. RNA extracted from these cells were used for TCR sequencing analyses after quality assurance and archiving, thus the actual number of cells that were sequenced were lower than listed. **pancLN:** pancreatic LN; **iLN:** inguinal LN; **Tconv:** Conventional T cells (CD4+Foxp3-).

Table S2: TCR pairs selected from single-cell TCR sequencing of islet Tregs

A. Treg single-cell sorted TCRs

Seq ID	Features of interest	Vα	CDR3α	Vβ	CDR3β
510	Present in multiple mice, Va similar to insulin-specific T cells	5D-4	CAASVSGGSNYKLTF	5	CASSQGTGGTEVFF
518	Found twice (2.82%) in one mouse	16	CAMRGVNSGGSNYKLTF	13-3	CASSDLGAYEQYF
520	Present in multiple mice, Va similar to insulin-specific T cells	5D-4	CAASATGNTRKLIF	19	CASTGVQNTLYF
521	Va similar to insulin-specific T cells; Found twice (2.82%) in one mouse	5D-4	CAASATGSGGKLTF	13-1	CASSGDSGNTYLF
536	Found twice (2.82%) in one mouse	7N-4	CAVRNSGGSNAKLTF	3	CASSLNRDEQYF
550	Found 4 times (5.63%) in one mouse	10	CAASRTGNYKYVF	2	CASSQLGGLEQYF
551	Found twice (2.82%) in one mouse	16	CAMRQGTGSKLSF	15	CASSLDRAGNTLYF
572	Found twice (2.82%) in one mouse	9-1	CAVSYNNRIFF	1	CTCSAIGGAHEQYF

B. Known specificity TCR controls

Name	Specificity	Vα	CDR3α	Vβ	CDR3β
p3w17	Insulin	5D-4	CAASKGGSALGRLH	1	CTCSADGGGAQEQYF
PA21. 14H4	HEL	5D-4	CAASEQGTGSKLSF	19	CASSIGGTGGYEQYF

A. CD4^{+}Foxp3^{+}ICOS^{+}TIGIT^{+} Tregs from islet infiltrates were single-cells sorted and TCRα and β chains were sequenced. Clones of interest were selected based on either present in multiple times in one mouse or in more than one mouse.

B. Information on the control TCRs with known specificities is listed.

Table S3. Islet specific peptides used for stimulating hybridomas.

Peptide name	Sequence	Responding TCRs	Ref
Ins B:9-23	PHLVEALYLVCGERG	p3w17	
Ins B:9-23 Register 1	HLREALYLVAEE	N/A	
Ins B:9-23 Register 2	LYRALYLVAGER	521	(1-3)
Ins B:9-23 Register 3A	VERLYLVAGEEG	p3w17	
Ins B:9-23 Register 3B	VERLYLVAGGEG	p3w17	
Ins B: 1-15	FVKQHLCGSHLVEAL	N/A	(4)
Ins B: 24-36	FFYTPMSRREVED	518	(5)
Ins A: 1-15	GIVDQCCTSICSLY	N/A	(4)
Ins A: 7-21	CTSICSLYQLENYCN	N/A	(6)
2.5HIP	EVEDPQVAQLELGGGPGAGDLQT LALWSRMDQLAKELTAE	N/A	
6.9HIP	EVEDPQVAQLELGGGPGAGDLQT LALNAARDPNRESLDFLLV	N/A	(7)
BDC mimotope	YVRPLWVRME	N/A	(8)
BDC pS3 mimotope	SRLGLWVRME	N/A	(2)
ZnT8 212-225	SVRAAFVHALGDVF	N/A	
ZnT8 313-336	ILSVHVATAASQDS	N/A	
ZnT8 330-344	RTGIAQALSSFDLHS	N/A	(9)
ZnT8 345-359	LTIQIESAADQDPSC	N/A	
GAD 206-220	TYEIAPVFVLLEYVT	N/A	
GAD 471-490	VDKCLELAEYLYNIIKNREG	N/A	(10)
IGRP P1	TAALSYTISRMEESSVTL	N/A	
IGRP P2	LHRSGVLIIHHLQEDYRTY	N/A	
IGRP P3	WYVMVTAALSYTISRMEESSVTL	N/A	(11)
IGRP P7	HTPGVHMASLSVYLKTNVFL	N/A	
IAPP K20	KCNTATCATQRLANFLVRSS	N/A	(12)
IA-2b Phogrin 2	KLSGLGADPSADATEAYQEL	N/A	
IA-2b Phogrin 7	QREENAPKNRSLAVLTYDHASRI	N/A	(13)

Peptides were added to splenic DCs at 10µM to examine hybridoma reactivity as described in Figure 3 and Figure S3.

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