Pancreatic islets communicate with lymphoid tissues via exocytosis of insulin peptides

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Tissue-specific autoimmunity occurs when selected antigens presented by susceptible alleles of the major histocompatibility complex are recognized by T cells. However, the reason why certain specific self-antigens dominate the response and are indispensable for triggering autoactivity is unclear. Spontaneous presentation of insulin is essential for initiating autoimmune type 1 diabetes in non-obese diabetic mice1,2. A major set of pathogenic CD4 T cells specifically recognizes the 12–20 segment of the insulin B-chain (B:12–20), an epitope that is generated from direct presentation of insulin peptides by antigen-presenting cells3,4. These T cells do not respond to antigen-presenting cells that have taken up insulin that, after processing, leads to presentation of a different segment representing a one-residue shift, B:13–21. CD4 T cells that recognize B:12–20 escape negative selection in the thymus and cause diabetes, whereas those that recognize B:13–21 have only a minor role in autoimmunity3–5. Although presentation of B:12–20 is evident in the islets6,7, insulin-specific germline cells can be formed in various lymphoid tissues, suggesting that insulin presentation is widespread3,5. Here we use live imaging to document the distribution of insulin recognition by CD4 T cells throughout various lymph nodes. Furthermore, we identify catabolized insulin peptide fragments containing defined pathogenic epitopes in β-cell granules from mice and humans. Upon glucose challenge, these fragments are released into the circulation and are recognized by CD4 T cells, leading to an activation state that results in transcriptional reprogramming and enhanced diabetogenicity. Therefore, a tissue such as pancreatic islets, by releasing catabolized products, imposes a constant threat to self-tolerance. These findings reveal a self-recognition pathway underlying a primary autoantigen and provide a foundation for assessing antigenic targets that precipitate pathogenic outcomes by systemically sensitizing lymphoid tissues.

On the basis of previous studies demonstrating constrained T-cell migration during limited antigen recognition8–12, we investigated insulin presentation in peripheral lymph nodes by two-photon microscopy of lymph-node explants following transfer of insulin-specific T cells (Fig. 1a). These were transferred together with wild-type CD4 T cells as a control; each population of transferred cells was labelled with a different fluorescent probe (Fig. 1b). We monitored the individual trajectories of transplanted T cells within the same region of the lymph nodes and quantified their motility (Extended Data Fig. 1a). We first performed this assay with a control CD4 T cell (10E11), which recognizes hen egg lysozyme (HEL). These experiments confirm that limited antigen recognition that is insufficient to trigger cell division can be detected by a decrease in the mean velocity of T cells (Fig. 1c, Extended Data Fig. 1b, Supplementary Video 1).

Widespread presentation of insulin peptides was demonstrated by reduced motility of the B:12–20-specific 8F10 T cells in the pancreatic (pLN), inguinal (iLN), mesenteric (mLN) and axillary (aLN) lymph nodes of non-obese diabetic (NOD) mice, relative to wild-type CD4 T cells (Fig. 1d, Supplementary Video 2). Motility was reduced to a similar degree on day 1 or day 5 of imaging (Extended Data Fig. 1c), and was unaffected by switching the labelling of the fluorescent probes (Extended Data Fig. 1d). The diffuse, rather than clustered, pattern of motility arrest indicates that presentation of insulin peptides was limiting and was not restricted to selected antigen-presenting cells (APCs). Motility of 8F10 T cells was also reduced in μMT and Batf3−/− mice, which are deficient in B cells and XCR1+ dendritic cells, respectively (Extended Data Fig. 1e).

We performed three experiments to interrogate key parameters of antigen recognition by 8F10 T cells. First, we examined B16A mice, which are deficient in both Ins1 and Ins2 but express a proinsulin transgene with a Tyr16Ala substitution in the B chain7. This mutant insulin is bioactive but is not immunogenic to B:12–20- or B:13–21-specific T cells. There was no reduction in motility of 8F10 T cells in the B16A mouse recipients, demonstrating that the effects on T cell motility require specific epitope recognition by the 8F10 T cells (Fig. 1e, Supplementary Video 3). Second, we investigated whether prior recirculation through the pLN was required for insulin recognition in other sites. Surgical removal of pLNs did not influence the motility arrest of 8F10 T cells in the iLNs (Fig. 1f). Third, we detected motility arrest of 8F10 T cells in diabetes-resistant B6 mice harbouring the I-Aβ haplotype (B6g7) (Fig. 1g) but not in NOD mice with the H2b haplotype (Extended Data Fig. 1f). Therefore, peripheral insulin presentation to 8F10 T cells requires I-Aβ and is not restricted to the NOD strain. The motility of 4F7 T cells, which specifically recognize the B:13–21 epitope, was also markedly reduced in the pLNs and iLNs of NOD recipients (Extended Data Fig. 1g). By contrast, the 8.3 CD8 T cells, which recognize the islet-specific glucose-6-phosphatase-related protein (IGRP)13, a protein that is expressed in the endoplasmic reticulum of β-cells, exhibited reduced motility in the pLN but not in the iLN (Extended Data Fig. 1h). Therefore, epitopes of insulin, but not those from IGRP, a cell-associated antigen, are systemically available.

We hypothesized that presentation of the low concentrations of circulating insulin (about 40 pM) might require insulin receptor-mediated uptake by APCs. To test this, we examined the effects of S961, an insulin receptor antagonist14. In assays on cultured cells, S961 impaired the ability of concanavalin A (ConA)-activated macrophages to present insulin (Extended Data Fig. 1i). In vivo blockade of insulin receptor by infusion of mice with S961 via osmotic pump caused a sustained increase in blood glucose levels (Extended Data Fig. 1j), permitting two-photon microscopy analysis (Extended Data Fig. 1k). A significant reduction in mean velocity of transferred 4F7 T cells was observed in lymph nodes of control mice infused with phosphate-buffered saline (PBS) (Fig. 1h, Supplementary Video 4). Although the motility of 4F7 T cells was also arrested after S961 infusion, the magnitude of the reduction was significantly smaller than with PBS (Fig. 1h, Supplementary Video 4). Therefore, blockade of insulin receptor-mediated uptake of insulin partially abrogated recognition by the 4F7 T cells, suggesting that free insulin peptides are an additional source of the B:13–21...
epitope. By contrast, the motility arrest of 8F10 T cells remained at a comparable level in recipients infused with PBS or S961 (Fig. 1i, Supplementary Video 5), indicating that the presence of B:12–20 is independent of insulin receptor-mediated uptake of insulin. This epitope must therefore derive from insulin peptides that reach the peripheral lymphoid organs. Of note, APCs expressing autoimmune

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**Fig. 1 | Peripheral insulin presentation is systemic, epitope-specific, and occurs physiologically.** a. Specificities of the antigen-specific T cells. b. The scheme of the transplant and two-photon imaging experiments using CFSE and CMTMR labels for transgenic and wild-type (WT) T cells, respectively. LN, lymph node. c–i. Mean track velocities of 10E11 and wild-type (WT) CD4 T cells in mice given PBS or the indicated amounts of HEL (c); 8F10 and wild-type CD4 T cells in NOD (d) or B16A (e) mice; 8F10 and wild-type CD4 T cells in NOD mice after surgical removal of the pancreatic lymph nodes (pLNrem) or sham surgery (f); 8F10 and wild-type CD4 T cells in B6g7 mice 24 h after transfer (g); 4F7 and wild-type (h) or 8F10 and wild-type (i) CD4 T cells in NOD mice infused with S961 or PBS. pLN, pancreatic lymph node; iLN, inguinal lymph node; mLN, mesenteric lymph node; aLN, axillary lymph node. Data are pooled results from at least three independent experiments. Each dot represents the velocity of one T cell, and the bar denotes the mean of all T cells in the group. **P < 0.001; ****P < 0.0001; one-way ANOVA with Sidak's multiple comparisons test. NS, not significant.

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**Fig. 2 | Generation of insulin peptides in β-cell granules.** a, b. Immunofluorescence of isolated islets stained for B:9–23 (a) or B:1–30 (b), CD11c and insulin. Data are representative of 50 islets per group in three independent experiments. c. Immunogold electron microscopy showing antibodies against B:1–30 (large gold) and insulin (small gold) in a representative β-cell. d, e. A representative granule that contains both B:1–30 and insulin (d) or insulin only (e) is shown. The arrowhead in d indicates the B:1–30 peptide. Data are representative of 317 granules analysed in three independent experiments. f. Competitive ELISA showing quantification of insulin, B:1–30 and B:9–23 in granules isolated by centrifugation of islets from B6g7 mice at 5,000g (5k) or 25,000g (25k). Each line represents one paired experiment using 4–8 mice. *P < 0.05; **P < 0.01; two-tailed paired Student's t-test. g. Peptide coverage of insulin B chain by sequences identified in 25k (red) and 5k (blue) β-cell granules using nLC–MS/MS analysis. Each line represents the alignment of individual peptides with insulin-2 B:1–30. Data are from four independent analyses using islets from 8–10 mice per strain. h. Box plot of log_{2}(mass spectrometry peak area), showing the abundance of individual insulin B-chain peptides (purple) in the 25k and 5k granules relative to all insulin peptides, including the C-peptides (box). Boxes with dashed outlines denote B:1–30 with a high abundance. Box plots show the median, box edges represent the first and third quartiles, and the whiskers extend to 1.5 × interquartile range.
Immunofluorescence with AIP showed a punctate pattern of B:9–23 staining in β-cells from NOD.Rag1−/− or B6g7.Rag1−/− mice (Fig. 2a). By contrast, B:1–30 staining using 6F3.B8 was more diffuse in nearly all the β-cells and co-stained with insulin (Fig. 2b). Using double immunogold-labelling antibodies, we detected B:1–30 in granules containing insulin (Fig. 2c). Many granules (106 out of 317, 33%) contained both B:1–30 and insulin (Fig. 2d), and the rest contained only insulin (Fig. 2e). AIP did not stain islets satisfactorily after labelling with immunogold.

Regular secretory granules obtained by centrifugation at 25,000g (25k) contained significantly higher amounts of insulin than the fraction obtained at 5,000g (5k), which includes the crinophagic bodies (Fig. 2f). B:1–30 was primarily found in the 25k fraction and not in the 5k fraction, but in concentrations about one tenth that of insulin (Fig. 2f). By contrast, B:9–23 was significantly more abundant in the granules in the 5k fraction (Fig. 2f).

We analysed the peptidomes of granules prepared from B6g7 mice, B6 mice or 3-week old female NOD mice, by nanoFlow liquid chromatography–tandem mass spectrometry (nLC–MS/MS). In all three strains, granules from the 25k fraction mostly contained sequences from the insulin C-peptide, the intact B:1–30, and a few small peptides from the B chain (Fig. 2g, h, Supplementary Table 1). By contrast, the granules from the 5k fraction, contained more diverse short sequences from throughout the B chain (Fig. 2g, h, Supplementary Table 1). Peptides derived from the 9–23 region, such as B:9–23 and B:11–23 (Extended Data Fig. 3b), were identified exclusively in the 5k granules of all three mouse strains. Manual interrogation of unassigned spectra only identified two putative hybrid peptides in the 5k granules (Extended Data Fig. 3c), a C-peptide–islet amyloid polypeptide (IAPP) fusion, and a fusion of the N terminus of the C-peptide of insulin-2 and the C terminus of the C-peptide of insulin-1. Peptides from other proteins were present at much lower levels in comparison to those from insulin.

Examination of β-cell granules from human islets revealed a striking similarity in the segregation of peptides between 5k and 25k fractions to that from the mouse islets (Extended Data Fig. 4a, Supplementary Table 1). The human 25k granules contained the intact B chain and a limited number of short sequences. The 5k fraction contained many short peptides, including a sequence representing B:11–30 (Extended Data Fig. 4b), containing the HLA(DQ8)-binding B:11–23 determinant, which is recognized by peripheral T cells in patients with type 1 diabetes.

Islets stimulated with 25 mM glucose secreted insulin (Fig. 3a) along with lower concentrations of peptides that were recognized by 6F3.B8 (Fig. 3b) or AIP (Fig. 3c). Secretion of insulin or insulin peptides was not affected when glucose challenge was carried out in the presence of protease inhibitors (Extended Data Fig. 5a), indicating that the peptides were not generated extracellularly.

We characterized insulin peptides secreted by β-cells using nLC–MS/MS. Most of these peptides were derived from the C-peptide, along with B-chain-derived sequences related to the 9–23 region and spanning the B–C–peptide (B–C) junction (Fig. 3d, Supplementary Table 2). Many of the peptides were identical to or contained pathogenic epitopes that were identified using diabetogenic T cells as probes (Fig. 3e, Extended Data Fig. 5b–e). The intact B chain contained identical sequences to peptides identified in the 25k granules, whereas B:9–23 and B:11–23 were identical to peptides in the 5k granules (Fig. 3e, Supplementary Table 2). Synthetic versions of peptides associated with B:9–23 activated T cells specific for B:12–20 as well as those specific for B:13–21 (Extended Data Fig. 6). In general, these potentially immunogenic peptides were present at low relative abundance (Fig. 3f, Supplementary Table 2).

We identified a form of B:9–23 containing cysteine oxidized to cysteic acid in mouse urine using antibody capture (Fig. 3g); this is a modification that can occur during sample preparation (Extended Data Fig. 7a). This finding indicates that B:9–23 is present in the circulation. Indeed, fluorochrome-labelled B:9–23 was rapidly displayed in the circulation.
by I-Aαβ-expressing APCs in spleen but not in thymus following intravenous injection (Extended Data Fig. 7b, c).

The widespread presentation of insulin peptides in lymphoid tissues influences the biology of T cells. We generated a bone marrow chimera model in which we transferred a small number of bone marrow stem cells from CD45.2 8F10 mice deficient in T-cell receptor alpha chain (8F10α/β-/-) into non-lethally irradiated NOD or B16A hosts (CD45.1) (Fig. 4a). This resulted in the development of a small number of 8F10 T cells (0.5–2%) among the endogenous CD4 T-cell repertoire (Extended Data Fig. 8a). We performed RNA sequencing (RNA-seq) on isolated 8F10 T cells from iLNs of both hosts (Fig. 4a).

Hierarchical clustering using Pearson’s correlation revealed differences between the transcriptomes of 8F10 T cells sourced from NOD (8F10-NOD) and B16A (8F10-B16A) hosts (Fig. 4b). Gene set enrichment analysis (GSEA) showed significant correlations between transcripts that were upregulated in the 8F10-NOD samples with three immunological signature datasets depicting T cell activation and effector function. 

In summary, peptide exocytosis is a normal response of β-cells that represents a mechanism of communication with the lymphoid tissues. Similar mechanisms may apply to other endocrine organs that also contain crinophagic granules. Examining the released peptides may enable better-targeted identification of T cell responses; a set of responses that could be extensive, given the diversity of exocytosed moieties. Previous studies have shown that ablation of all lymph nodes eradicates the pathogenic T cell repertoire and abolishes diabetes30 emphasizing the importance of the entire lymphatic system in interactions with T cells. Finally, the biological outcomes described here for 8F10 T cells may vary for other insulin-reactive T cells with divergent TCR affinities. Comprehensive understanding of these outcomes will require analysis of the entire insulin-reactive T cell pool at different stages of the disease.

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Author contributions X.W. and E.R.U. conceived the project, designed the experiments and analysed the results. B.H.Z. and X.W. performed two-photon microscopy and analysed the data. A.N.V. and X.W. prepared samples for mass spectrometry. C.F.L. performed the nLC–MS/MS studies and analysed the data. A.N.V. performed cell biology experiments. P.N.Z. and X.W. analysed the RNA-seq data. M.S.A., R.T. and L.S. provided key reagents and interpreted the results. B.H.Z. and X.W. performed two-photon microscopy and analysed the data. C.F.L. performed the nLC–MS/MS studies and analysed the data. A.N.V. performed cell biology experiments. P.N.Z. and X.W. analysed the RNA-seq data. M.S.A., R.T. and L.S. provided key reagents and interpreted the results. X.W., C.F.L. and E.R.U. wrote the manuscript.

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Methods

Mice. NOD/ShiLtJ (NOD), NOD.129S7-B6-Rag1tm1GtmJ/J (NOD.Rag1−/−), NOD.129S7-Br1-Ins2+/+ Tg(Ins2×Y16A)1Eilln J (both from B6.A), NOD.Cg-Tg(Tfrc1bcr8.3)Pesa/Dvl (83), NOD.129S2-B6-Il10−/−/−Oa/Dv (μMT), NOD.CrCtcrb(Nprc1-D1Mit262)Wehell (NOD.CD45.2, NOD.B10sn-H2b/J (NOD.JH2b) and B6-NOD(17Mit21-17Mit10)Lt/j (B6g7) mice were originally obtained from the Jackson Laboratory. NOD.10E11 TCR transgenic mice (TCRα: TRAV5-4/TRA1-43, TCRβ: TRBV3-5/31 TRBDR2/1-9) were generated using a previous protocol. NOD.F47 TCR transgenic mice, NOD.Aire−/−; and NOD.TCRα−/− mice were generated by M.S.A. The 8F1 or 10E11 mice expressing the CD45.2 allele were generated by intercrossing the original TCR transgenic line (CD45.1) with the NOD. CD45.2 mice, and the CD45.2.NOD.8F10 mice were further crossed with the NOD. TCRα−/− mice to generate the CD45.2.8F10(α/−)−/−. B6. Rag1−/− mice were used to intercross with B6g7 mice to generate B6g7.Rag1−/− mice. All mice were housed and maintained under specific pathogen-free conditions in our animal facility. All experiments were approved by the Division of Comparative Medicine of Washington University School of Medicine in St. Louis (Accreditation number A3811-01).

Human pancreatic islets. Deidentified human primary islets isolated from deceased donors were obtained from ProBio Laboratories. Experiments were judged to be not human subject research by Washington University Human Research Protection Office (IRB ID # 201810113; Federalwide Assurance #FWA000022844). In total, islets from three donors were used: donor 1 (female, 57 years, BMI 21.35), donor 2 (female, 49 years, BMI 33), donor 3 (male, 28 years, BMI 34.7). Purity of the islets was between 85 and 98%. Islets were cultured in CMRL medium supplemented with 10% FBS and 10% L-cell conditioned medium for recovery. The growth medium was replaced every 1500 islets after 1–3 days of culture.

Antibodies. The following frequently conjugated antibodies were purchased from BioLegend: anti-B220 (RA3-682), anti-CD11c (N418), anti-CD4 (RM4-5), anti-CD45 (30-F11), anti-CD45.1 (A20), anti-CD45.2 (104A), anti-CD8a (53-6.7), anti-F4/80 (BM8), anti-V-18.1/2 (KJ16-133.18), anti-CD4 (IM7), anti-CD62L (MEL-14), anti-CD25 (GC5.6.1) and anti-TNFα (MP6-XT22). Unconjugated or Alexa Fluor 647-labelled Rabbit anti-insulin monoclonal antibody (C2729) was purchased from Cell Signaling Technology. Unconjugated mouse anti-insulin monoclonal antibody (EI1D7) was purchased from Millipore. Alexa Fluor 594 F(ab)2 donkey anti-mouse IgG and HRP-conjugated goat anti-mouse IgG (Fc-specific) were purchased from Jackson Immunoresearch.

Flow cytometry and cell sorting. Flow cytometry analysis was done as previously described. The samples were examined using a FACSCanto II (BD Biosciences) and the data were analysed using FlowJo software (Tree Star Software). CD4+ T cells from iLNs were enriched using the CD4+ T cell isolation kit (Miltenyi Biotech), the 8F10 T cells were sorted as CD45.2+CD45.1−CD4+CD8−B220−CD11c− using FACSaria II (BD Biosciences).

CFSE and CMTMR labelling. For two-photon imaging, naive CD4 T cells were pulsed with CFSE (5 μM) by incubating the cells for 15 min at 37 °C. Un conjugated cells or CMTMR labelled cells were stained with 1 μM of the dye (both from ThermoFisher Scientific), respectively. In brief, T cells (10 6/ml in PBS) were incubated with 10 μM CFSE or 8 μM CMTMR for 25 min at 37 °C with a gentle shake after 10 min. Under these conditions, T cells were labelled with satisfactory intensities without significant cell death. Ice-cold PBS was then added to quench the labelling.

Adoptive transfer. CFSE- or CMTMR-labelled T cells were mixed 1:1 and were injected intravenously. For all the two-photon experiments, 2 × 10 6 T cells with either label were transferred. Varying T cell numbers in preliminary experiments determined that this amount resulted in a stable 0.5–0.8% reconstitution of the transferred T cells in the endogenous CD4 T cell pool, which was sufficient for two-photon imaging without causing obvious intracranial competition. All the recipient mice were 3–4-week-old female mice unless otherwise mentioned. For experiments in 4. FACs-sorted 8F10 T cells from pooled iLNs of 8–10 NOD or B16A mice were adoptively transferred intravenously into 4–6-week old NOD. Rag1−/− mice. CD4+ T cells. The data were then stained with Alexa Fluor 647 anti-mouse IgG (30 μg/ml), Alexa Fluor 647 Rabbit anti-insulin (20 μg/ml), and Alexa Fluor 488 anti-mouse CD11c (40 μg/ml) for 45 min on ice and mounted using the Prolong Diamond mountant (ThermoFisher). The samples were viewed using the Eclipse E800 microscope (Nikon) equipped with the EXI Blue fluorescence camera (Qimaging).

Electron microscopy with Immunogold. Mouse islets were isolated as previously described. The islets were blocked with normal goat serum, fixed with 4% methanol-free formaldehyde, permeabilized with 0.2% saponin (Sigma), and stained with AIP (63F.88 (50 μg/ml)) for 45 min on ice. The samples were then stained with Alexa Fluor 594 F(ab)2 donkey anti-mouse IgG (30 μg/ml), Alexa Fluor 647 Rabbit anti-insulin (20 μg/ml), and Alexa Fluor 488 anti-mouse CD11c (40 μg/ml) for 45 min on ice and mounted using the Prolong Diamond mountant (ThermoFisher). The samples were viewed using the Eclipse E800 microscope (Nikon) equipped with the EXI Blue fluorescence camera (Qimaging).

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BSA with 2.5 mM or 25 mM glucose. After 1 h incubation, the culture supernatants were collected for the competitive ELISA assay or mass spectrometry analysis.

β-cell granule isolation. Mouse and human islets were dispersed using non-enzymatic dispersion solution (Sigma). Cells were resuspended in PBS and lysed by passing them through a cell homogenizer (Isobiotec). The lysate was centrifuged twice for 10 min at 500g, 4 °C to pellet cell debris. The supernatant was centrifuged for 10 min at 5,000g, 4 °C. The 5,000g spin was repeated on the supernatant and the two pellets were combined. This fraction was highly enriched in peptide-containing vesicles compatible with the crinophagic bodies, and as such have been labelled. This fraction may also contain organelles other than the insulin-containing ones. The supernatant after the 5,000g spin was centrifuged for 30 min at 25,000g, 4 °C to pellet secretory granules. This supernatant was discarded, and the 25000g pellet was suspended in 100 µl PBS. The microcentrifuge used for granule isolation was an Eppendorf 5417R (Eppendorf) with a FA45-24-11 fixed angle rotor. Fractions were frozen at −80 °C and thawed at 37 °C for five cycles to release the contents of granules. After freeze-thaw, complete protease inhibitor cocktail was added to the sample which was then concentrated by SpeedVac to <100 µl. The sample was passed through C18 ZipTips (Pierce) and peptides then were eluted in 0.1% formic acid/95% acetonitrile and then dried with a SpeedVac.

Sample preparation for mass spectrometry analysis. Biological samples were treated with 2.5% trifluoroacetic acid (TFA) to a final concentration of 0.36% (v/v), and the peptides were purified using the C18 ZipTips, eluted with 0.1% formic acid in 95% acetonitrile, and lyophilized. For peptide capture, TFA-affected mouse urine (12 ml) was cleaned up using C18 Sep Pak cartridges (Waters). The analytes retained by the cartridge sorbent were eluted with methanol, lyophilized, and reconstituted with 2 ml sterile PBS. The material was then incubated with a 1:1 mixture of sepharose pre-conjugated with AIP or 6F3.88 monoclonal antibodies (1 ml slurry total) for 72 h at 4 °C with gentle rotation. The urine–sepharose mixture was poured into a Bio-Rad Econo column, and after extensive washing, the antibody-bound material was eluted with 10% acetic acid and lyophilized.

Mass spectrometry. A Dionex UltiMate 1000 system (Thermo Scientific) was coupled to an Orbitrap Fusion Lumos (Thermo Scientific) through an EASY-Spray ion source (Thermo Scientific). Peptide samples were loaded (30 µl/min, 1 min) onto a trap column (100 µm × 2 cm, 5 µm Acclaim PepMap 100 C18, 50 °C), eluted (300 µl/min) onto an EASY-Spray PepMap RSLC C18 column (2 µm, 25 cm × 75 µm ID, 50 °C, Thermo Scientific) and separated with the following gradient, all % Buffer B (0.1% formic acid in ACN): 0–40 min, 2–22%; 40–50 min, 22–35%; 50–60 min, 35–95%; 60–70 min, 95–2%; 70–71 min, 95–2%, 71–85 min, isotropic at 2%. Spray voltage was 1900 V, ion transfer tube temperature was 275 °C, and RF lens was 30 ms. Mass spectrometry scans were acquired in profile mode and MS/MS scans in centroid mode, for ions with charge states 2–7, with a cycle time of 3.3 s. For HCD, mass spectra were recorded from 375–1500 Da at 120K resolution (at m/z = 200), and MS/MS was triggered above a threshold of 2.5 × 10^5, with quadrupole isolation (1.6 Da) at 30K resolution, and collision energy of 30%. Dynamic exclusion was used (35 s). For high SA EthCD mass spectra were acquired from 350–1500 Da at 60K resolution, and MS/MS spectra were triggered for ions above a threshold of 5 × 10^4 with quadrupole isolation (0.7 Da) at 15K resolution. Fragmentation employed calibrated charge-dependent ETD, with SA (40%) applied in the HCD cell. Dynamic exclusion was used (60 s). For low SA EthCD, mass spectra were recorded from 375–1500 Da at 120K resolution (at m/z = 200), and MS/MS spectra were acquired for ions above a minimum intensity threshold of 2.5 × 10^4 at 15K resolution. ETD reaction time was fixed at 100 ms, with SA (15%) applied in the HCD collision cell.

Mass spectrometry data analysis. Data files were uploaded to PEAKS 8.0 (Bioinformatics Solutions) for processing, de novo sequencing and database searching. Resulting sequences were searched against the UniProt Mouse Proteome database (downloaded 8 June 2017; 25,144 entries) with mass error tolerances of 20 ppm and 0.02 Da for parent and fragment, respectively, no enzyme specificity and no fixed or variable modifications. The Common Repository for Adventitious Proteins database (www.thegpm.org/crap/) was used to identify contaminant proteins. FDR estimation was enabled. Peptides were filtered for −10log P ≥ 20, and proteins were filtered for −10log P ≥ 30 and one unique peptide. For all experiments, this gave an FDR of <1% at the peptide-spectrum match level. Peptides matching to insulin-1 and insulin-2 were manually verified by visual inspection. For relative quantification, peak areas for all manually verified peptides were exported from PEAKS, normalized to the total ion current, and log, transformed.

T cell stimulation and antigen presentation assay. In Extended Data Fig. 1i, ConA-stimulated peritoneal macrophages were treated with 0.2 or 1 µl S961 for 1 h at 37 °C and were then cultured with the IIT-3 T cell hybridoma that recognizes the 13–21 peptide, in the presence of serially diluted insulin (I9278; Sigma). In Extended Data Fig. 2e, C3g7/7 cells were treated with chloroquine for 2 h at 37 °C, washed, pulsed with the antigens, and cultured with T cell hybridomas. After incubation for 18 h, the culture supernatants were assayed for IL-2 production.

Bone marrow chimera. The female donor 8F10.TCRα–/− (CD45.2) mice were injected intraperitoneally with fluorouracil (200 mg/kg), and bone marrow cells were isolated from the femur and tibia on day 5. The cells were adoptively transferred into sublethally irradiated (600 rads) 3-week-old female NOD or B16A hosts (10^4/mouse).

RNA-seq analysis. Total RNA was isolated using the Ambion RNAqueous-Micro kit (Thermo Fisher Scientific). RNA-seq library preparation and sequencing was performed as previously described20. The differential expression analysis was done with the DESeq2 package (version 1.18.1). Multifactor analysis was used to account for donor effect. Specifically, paired 8F10-NOD and 8F10-B16A samples from one isolation (four pairs in total) were treated as one donor group. Gene set enrichment pathways analysis was done using the Broad Institute's GSEA software and MSigDB Hallmark or C7 immunological signatures databases. The latter included datasets: GSE2872624, GSE1000001_1577_200_UP25, GSE965026 and GSE3202528. All heat maps are in log2 scale. The gene expression matrix counts were adjusted for donor effect with Combat (sva package) only for heat maps and clustering.

Statistics. Mice were age and gender matched. Among the mice with matched ages and genders, they were randomized and distributed equally into experimental groups. Power analysis was used to estimate the sample size in some experiments, as described in the Reporting Summary. The investigators were not blinded to allocation during experiments and outcome assessment. One-way ANOVA with Sidak's multiple comparisons test was used to determine significant differences among multiple groups with unpaired biological replicates. The two-tailed unpaired Student’s t-test was used to determine significant differences between two groups with unpaired biological replicates. The two-tailed paired Student’s t-test was used to calculate P values of each pair of independent experiments. The log-rank test was used to determine the significant difference of diabetes incidence.

Reporting summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this paper.

Data availability. The RNA-seq data have been deposited in the Gene Expression Omnibus under accession number GSE114824. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD009919.

31. Aly, H. et al A novel strategy to increase the proliferative potential of adult human β-cells while maintaining their differentiated phenotype. PLoS One 8, e66131 (2013).
32. Ferris, S. T. et al. The islet-resident macrophage is in an inflammatory state and senses microbial products in blood. J. Exp. Med. 214, 2369–2385 (2017).
Extended Data Fig. 1 | Probing peripheral antigen presentation by two-photon imaging: the motility assay. a, Representative 3D reconstructions of two-photon z-stacks visualizing CFSE-labelled anti-HEL 10E11 TCR transgenic and CMTMR-labelled wild-type CD4 T cells in an iLN explant on day 3 post transfer. Individual T cells were tracked in the area bound by the dashed line. Right, magnified views of this region, showing movement of T cells over a 7.5-min time interval. Quantification was performed over a 5-min interval. Cyan and purple tracks represent 10E11 and wild-type T cells, respectively. Mice were injected with $10^{-8}$ µg HEL. b, NOD mice (CD45.1) were injected intraperitoneally with indicated amounts of HEL. Six hours after injection, naive CFSE-labelled 10E11 (CD45.2) T cells were transferred. On day 3, CFSE dilution of the transferred T cells (CD45.2+CD45.1-CD4+V$\beta$8.1/8.2+) in the iLNs was measured by flow cytometry. Data are representative of two independent experiments. c, Mean track velocities of 8F10 and wild-type CD4 T cells in iLNs from NOD recipients on day 1 or day 5 post transfer. d, CFSE(8F10) plus CMTMR(WT) or CMTMR(8F10) plus CFSE(WT) T cells were separately transferred into two cohorts of NOD recipients, and their mean track velocities in iLNs on day 3 were compared by paired two-photon imaging analysis. e, Mean track velocities of 8F10 and wild-type CD4 T cells in NOD$\mu$MT or NOD.Baf5-/- recipients on day 3 post transfer. f, Mean track velocities of 8F10 and 10E11 T cells in NOD.H2b recipients 24 h post transfer. g, h, Mean track velocities of 4F7 and wild-type CD4 (g) or 8.3 and wild-type CD8 (h) T cells in NOD recipients on day 3 post transfer. i, Response (mean ± s.e.m.) of the B:13–21-specific IIT-3 T cells to ConA-activated peritoneal macrophages treated with or without S961 before insulin pulse. j, Blood glucose levels (mean ± s.e.m.) of 3-week old NOD mice infected with S961 or PBS via osmotic pumps. k, The scheme of the experiments in Fig. 1h, i, l. Mean track velocities of 8F10 and wild-type CD4 T cells in iLNs of Aire–/– recipients. Data summarize two (c, d, f, l) or three (e, g, h) independent experiments. Each dot represents individual T cell tracks, and the bar denotes the mean. ns, not significant; ****$P < 0.0001$; one-way ANOVA with Sidak’s multiple comparisons test (c, d, g, h) or two-tailed unpaired Student’s t-test (e, f, l).
Extended Data Fig. 2 | Analysis of insulin peptide-specific monoclonal antibodies and presentation of the intact B-chain. a–c, Competitive ELISA responses showing the binding of: anti-insulin monoclonal antibody (E11D7) to plate-bound insulin (a) anti-B:9–23 monoclonal antibody (AIP) to plate-bound B:9–23 (b), and anti-B:1–30 monoclonal antibody (6F3.B8) to plate-bound B:1–30 (c) in the presence of serial dilutions of the indicated soluble antigens as a competitive inhibitor. Inhibition by a specific soluble antigen indicates the specificity of the monoclonal antibody to this antigen. d, Competitive ELISA responses showing the binding of 6F3.B8 to plate-bound B:1–30 in the presence of soluble unmodified B:1–30 or B:1–30 in which the two cysteines were changed to serines (B:1–30 C to S). The results indicate the intrachain link formed by the cysteines does not influence the specificity of the 6F3.B8 monoclonal antibody. Data are means representing two independent experiments. e, Responses of the B:13–21-specific IIT-3 (left) or the B:12–20-specific 9B9 (right) T cell hybridoma to C3g7 APCs treated with or without 100 µM chloroquine for 2 h and pulsed with indicated antigens after extensive washes. C3g7 cells are a B cell lymphoma line expressing I-A^K, and are used as APCs. The results of the effects of chloroquine indicate that reactivity to insulin, but not to B:9–23 or B:1–30 require internal processing. Data are mean ± s.e.m., representative of two independent experiments.
Extended Data Fig. 3 | nLC–MS/MS analysis of mouse β-cell granules.

**a**, Mass spectra of mouse insulin-1 B:1–30 with intramolecular disulphide bonds (left) and mouse insulin-2 B:1–30 with oxidized methionine in position 29 (right).

**b**, Mass spectra of mouse insulin B:9–23 (left) and B:11–23 (right), which were exclusively identified in the 5k granules of B6g7, B6 and NOD mice.

**c**, Mass spectra of two hybrid peptides identified in the 5k granules. The sequence (EVEDTPVRSGNFPQM, left) represents a C-peptide (underlined)–islet amyloid polypeptide (IAPP) fusion, and the sequence (EVEDPQVAEVARQ, right) represents a fusion of the N terminus of insulin-2 C-peptide (underlined) with the C terminus of insulin-1 C-peptide.
Extended Data Fig. 4 | nLC–MS/MS analysis of human β-cell granules. a, Peptide coverage of insulin B chain identified in human 25k (red) and 5k (blue) β-cell granules using nLC–MS/MS analysis. Shown is the alignment of individual peptides (each line) with the human insulin B:1–30 segment. Data summarizes results from four independent runs using human islets from three individual donors. b, A mass spectrum showing a sequence representing human insulin B:11–30 that was identified in the 5k granules. The cysteinylation in position 19 is indicated.
Extended Data Fig. 5 | Analysis of insulin peptides secreted from islets upon glucose challenge. a, Insulin secretion assay was performed as described in Fig. 3a–c, except that protease inhibitors were added during the 25-mM glucose challenge. The supernatants were then collected for the competitive ELISA assay. Data are mean ± s.e.m. from two independent experiments. b, Mass spectra of four secreted peptides that contain the B:12–20 and/or B:13–21 epitopes as listed in Fig. 3e. Secreted B:1–30 sequences are identical to those in Extended Data Fig. 3a, and B:9–23 and B:11–23 share identical sequences with those in Extended Data Fig. 3b. c, A mass spectrum of the secreted insulin B:15–23 MHC-I (K^d)-binding peptide. d, A mass spectrum of the secreted insulin A:14–20 MHC-I (D^d)-binding peptide. e, A mass spectrum showing a representative B–C-spanning peptide (B25–C23).
Extended Data Fig. 6 | T cell responses to B:9–23-associated peptides. Responses of three insulin-reactive T cell hybridomas to insulin peptides associated with the 9–23 region of the B chain as identified in Fig. 3e. The C3g7 cells were used as APCs. Data are mean ± s.e.m.
Extended Data Fig. 7 | Characterization of circulating B:9–23 and its localization into lymphoid organs. a, Unmodified synthetic B:9–23 (3 pmol) was spiked into 1 ml PBS, purified using C18 tips, lyophilized, and analysed by nLC–MS/MS. The data show the appearance of unmodified B:9–23 (left) together with oxidation of Cys19 to cysteic acid (right). b, c, Alexa Fluor 488-conjugated B:9–23 peptide (100 µg) was injected intravenously into 4-week old B6, B6g7 and NOD mice. An hour later, spleens and thymi were harvested, digested with liberase and DNase, and binding to splenic and thymic APCs was measured by flow cytometry. b, Representative FACS plots showing the binding of B:9–23 to splenic XCR1* and Sirpa* dendritic cell (DC) subsets and B cells (top). The bar graph summarizes cumulative results from individual mice (each point), pooled from three independent experiments. ns, not significant; **P < 0.05; ***P < 0.01; ****P < 0.005, two-tailed unpaired Student’s t-test. c, Representative FACS plots showing the binding of B:9–23 to thymic XCR1* and Sirpa* DC subsets and to CD45– cells expressing MHCII. Data are mean ± S.D from five individual mice per strain from two independent experiments.
Extended Data Fig. 8 | RNA-seq analysis of 8F10 T cells developed in NOD or B16A hosts. a, Representative FACS plots (top) showing the sorting strategy and recovery of 8F10 T cells from iLNs of NOD or B16A-recipient mice six weeks after adoptive transfer of bone marrow. The scatter plot (bottom) shows the percentage of recovered 8F10 T cells among total CD4 T cells from four independent experiments. ns, not significant; two-tailed paired Student’s t-test. b, Biological pathways that are significantly enriched in the 8F10-NOD versus 8F10-B16A samples using GSEA and Hallmark database. c, Heat maps of all enriched genes in individual metabolic pathways depicted in Fig. 4c.
Extended Data Fig. 9 | 8F10 T cells exhibit an effector phenotype, but no anergy or exhaustion phenotype, at the transcription level during peripheral antigen recognition. a, Heat maps showing all the enriched genes of the three immunological pathways illustrated in Fig. 4d. b, GSEA enrichment plots performed on differentially expressed genes in 8F10 T cells from the NOD-iLN versus B16A-iLN condition using datasets characterizing CD4 T cell anergy and CD8 T cell tolerance.
Extended Data Fig. 10 | Functional analysis of 8F10 T cells developed in NOD or B16A hosts. a–f, The bone marrow chimera was constructed as in Fig. 4a, and T cells were examined after 6 (a–c) or 9 (d–f) weeks. a, b, d, e, Bulk CD4+ T cells were purified from iLN of individual NOD or B16A mice (three per group) by two rounds of MACS negative selection. To examine cytokine repertoire (a, d), half of the individual T cell samples were combined. The remainder were kept as individual samples, labelled with CFSE (1.5 µM), and used to measure cell proliferation (b, e). In either case, T cells were mixed with NOD.Rag1−/− splenocytes (1:2 ratio) and stimulated with B:9–23 for 16 (a, d) or 72 (b, e) hours. a, Representative FACS plots showing intracellular cytokine staining of the 8F10 T cells from NOD-iLN or B16A-iLN, after stimulation with B:9–23 for 16 h (brefeldin A was added for the last 4 h). Production of IL-4, IL-17A, IL-5 and IL-10 was not detected. Data are representative of two independent experiments with 3 mice combined per experiment. b, Representative FACS plots (top) showing CFSE dilution of the 8F10 T cells stimulated by B:9–23 or the control HEL11–25 peptide for 72 h. The results of 6 individual mice from two independent experiments are summarized in the box plots (bottom). Box plots show the median, box edges represent the first and third quartiles, and the whiskers extend from the minimum to the maximum. **P < 0.01, two-tailed unpaired Student’s t-test. c, Representative FACS plots showing ex vivo surface staining of FR4 and CD73 as well as CD39 and TIGIT on endogenous CD4+ or 8F10 T cells in the iLN of NOD or B16A mice. Data are representative of three mice analysed in two independent experiments. d–f, Experiments were performed in week 9 following the procedures described in a–c. The data in d–f are from a single experiment.
Reporting Summary

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Statistical parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

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- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
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- Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
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- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
  Give P values as exact values whenever suitable.
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- Estimates of effect sizes (e.g. Cohen's d, Pearson's r), indicating how they were calculated
- Clearly defined error bars
  State explicitly what error bars represent (e.g. SD, SE, CI)

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection

- FACSDiva 8.0.1 (BD). XCalibur 4.0.27.42 (Thermo Fisher Scientific). Microplate Manager 6.2 (Bio-Rad). Wallac 1450 MicroBeta Workstation (Perkin Elmer)

Data analysis

- Graphpad Prism 6.0. PC. GSEA using Broad Institute software. Imaris software (Bitplane). PEAKS 8.0 (Bioinformatics Solutions). DESeq2 R package. Flowjo 7.5 and Flowjo 10.0.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.
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All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The RNAseq data have been deposited in the Gene Expression Omnibus under accession number GSE114824. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD009919 and 10.6019/PXD009919.

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Life sciences

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size

For two-photon analysis, previous studies have shown that the mean track velocity ranges between 7 and 12 micrometers/min with a standard deviation of approximately 4. To have a power of 0.95 and an alpha 0.05, we require a minimal of ~50-60 samples. All of our calculations were derived from a minimal of 60 counted tracks. For competitive ELISA analysis, we have determined that the optical density measurement ranged from a min of 0.3 to 0.6 with a standard deviation of 0.05. To have a power of 0.95 and an alpha of 0.05, we require a minimal of 3 biological replicates, and in all the experiments we included 6 samples. For RNAseq, based on our previous studies we expected an average gene expression level standard deviation of 1-1.5. To have a power of 0.95 and an alpha of 0.05, we required 4 biological replicates per group. For diabetes incidence, with an equal number of animals monitored per group and a relative hazard ratio of 0.5, a minimal of 4 mice per group were required. We included 6 mice per group.

Data exclusions

We did not exclude any data from our studies.

Replication

For two-photon analysis, we analyzed 60-200 individual T cells from 2-4 mice per experiment, and the statistical analysis was based on 2-4 independent experiments. For ELISA, the statistical analysis was based on 6 independent experiments containing 4-8 mice per experiment. For mass spectrometry, we analyzed 16-24 mice per experiment. For RNAseq, we included 4 biological sample pairs and each pair was from a total of 8-10 mice. For diabetes incidence, we monitored 6 mice per group from 3 independent experiments.

Randomization

Mice were age and gender matched. Among the mice with matched ages and genders, they were randomized and distributed equally into experimental groups.

Blinding

All the measurements were performed by instruments or computing devices and therefore blinding is not necessary.

Reporting for specific materials, systems and methods

Materials & experimental systems

Methods

| Involved in the study | Involved in the study |
|-----------------------|-----------------------|
| n/a                   | n/a                   |
| ☒ Unique biological materials | ☒ ChiP-seq |
| ☒ Antibodies          | ☒ Flow cytometry      |
| ☒ Eukaryotic cell lines | ☒ MRI-based neuroimaging |
| ☒ Palaeontology       |                        |
| ☒ Animals and other organisms |                  |
| ☒ Human research participants |                |

Antibodies

The following fluorescently conjugated antibodies were purchased from BioLegend: anti-B220 (RA3-6B2), anti-CD11c (N418), anti-CD4 (RM4-5), anti-CD45 (30-F11), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD8a (53-6.7), anti-F4/80 (BM8), anti-Vβ8.1/8.2
(K16-133.18), anti-CD44 (IM7), anti-CD62L (MEL-14), anti-CD25 (PC61.5) and anti-TNFα (MP6-XT22). Unconjugated or Alexa Fluor 647-labelled Rabbit anti-insulin MoAb (C27C9) was purchased from Cell Signaling Technology. Unconjugated mouse anti-insulin MoAb (E11D7) was purchased from Millipore. Alexa Fluor 594 F(ab)2 donkey anti-mouse IgG and HRP-conjugated goat anti-mouse IgG (Fcy portion specific) were purchased from Jackson ImmunoResearch. All the commercial antibodies were validated by the vendor. The 6F3.B8 antibody recognizing mouse insulin B-chain was generated in the laboratory. The reactivity was confirmed using ELISA.

**Eukaryotic cell lines**

Policy information about cell lines

| Cell line source(s) | The T cell hybridomas used in this study were generated in the laboratory. |
|---------------------|------------------------------------------------------------------------|
| Authentication      | The reactivity of the T cell hybridomas to their specific antigens was tested using by their ability to produce interleukin-2 when stimulated by corresponding antigens presented by antigen presentation cells. |
| Mycoplasma contamination | All the cell lines were confirmed mycoplasma free. |
| Commonly misidentified lines | Not applicable. |

**Animals and other organisms**

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

| Laboratory animals | NOD/ShiLtJ (NOD), NOD.129S7(B6)-Rag1tm1Mom/J (NOD.Rag1-/-), NOD.Cg-Tg[Ins2*Y16A11EllIns1tm1JjaIns2tm1Jja/GseJ (NOD.B16A), NOD.Cg-Tg[TcraTcrbNY8.3]1Pesa/Dsvl (8.3), NOD.129S2(B6)-Ighm1c1gn/Doil (μMT), NOD.C-(Ptprc-01Mit262)/WehiJ (NOD.CD45.2), NOD.B10Sn-H2b/J (NOD.H2b) and B6.NOD-(D17Mit21-D17Mit10)/LtJ (B6g7) mice were originally obtained from the Jackson Laboratory. For most of the studies, 4-8 week old female or male mice were used. |
| Wild animals        | The study did not involve wild animals |
| Field-collected samples | The study did not involve field-collected samples |

**Flow Cytometry**

**Plots**

 confirms that:

- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

**Methodology**

| Sample preparation | Single cell suspensions were prepared and incubated with FcR blocking media containing 2.4G2 antibody for 30 min on ice. The cells were then stained for surface markers with corresponding fluorescent antibodies on ice for 25 min. |
| Instrument | The flow cytometry samples were collected using a FACSCanto II (BD Biosciences). T cell sorting was performed using FACSaria II (BD Biosciences). |
| Software | Data were collected using FACS DIVA (BD Biosciences) and were analyzed using Flowjo software (Tree Star Software). |
| Cell population abundance | The post sort was performed to confirm a purity above 99%. |
| Gating strategy | The starting cell population is gated on appropriate FSC/SSC with the default setting of the FACSDiva software. The singlet gate, based on FSH/SSW is applied in all the analyses to exclude doublets. The positive and negative boundaries were determined using isotype control staining or biological control samples. An example of gating strategy is shown in Extended data Figure 8a. |

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.