Online Methods

Mice

NOD, NOD.CB17-*Prkdc*^{scid}/J (NOD.SCID), NOD.129S7(B6)-*Rag*1^{tm1Mom}/J (NOD.*Rag1*$^{-/-}$), and NOD.Cg-Tg(Ins2*Y16A)1Ell *Ins1*^{tm1Jja} *Ins2*^{tm1Jja}/GseJ (B16:A-dKO mice) were purchased from the Jackson Laboratory and maintained under SPF conditions. All animal studies were approved by the Washington University animal study committee.

~~**Identify the instituitional committee that approved live animal studies**~~

Comment [eru1]: Added istuitional committee statement

Immunizations and T cell response

Human insulin (Sigma Aldrich) and B:9-23 peptide synthesized in the laboratory were used for immunizations. 10 ηmoles of antigen was emulsified in Complete Freund's Adjuvant (Difco) and injected subcutaneously into the hind footpads of mice. Specific T cell responses were determined by IL-2 ELISPOT assays (BD Biosciences) seven days later.

Generation of hybridomas

T cell hybridomas were generated from the draining lymph nodes of NOD mice immunized with B:9-23 peptide in CFA or from infiltrating T cells found in dispersed islets of pre- or early diabetic NOD mice (12-20 weeks old). Islets were isolated and dispersed as described[28]. After boosting *ex vivo* for 72 h with irradiated splenocytes plus B:9-23 and IL-2, cells were fused using standard protocols[26] .

T Cell lines and adoptive transfers

T cell lines were generated by B:9-23 peptide immunization of NOD mice. Draining lymph node cells were grown in 96 well plates with B:9-23 peptide. After 7 days, growth positive wells were expanded with B:9-23 peptide, irradiated splenocytes, and IL-2 (50 U/ml). Cytokine production of primary lines was measured 48 h after anti-CD3/anti-CD28 (BD Biosciences) stimulation of 1×10^5 cells using the Th1/Th2 Cytometric Bead Array kit (BD Biosciences). For adoptive transfer studies 5×10^6 activated T cells were injected i.v. into the lateral tail vein of 6 week old NOD.SCID mice. Blood glucose was monitored in recipients: two consecutive readings of $\geq$250 mg/dl was considered to be diabetic. Mice were followed for 30 weeks.

B:9-23 peptide specific antibody generation

NOD mice were immunized with 10 ηmoles B:9-23 in complete Freund's adjuvant subcutaneously and boosted 4x with 10 μg of B:9-23 in pyrogen free saline subcutaneously. Splenocytes were fused by standard procedures. Supernatants from growth positive hybridomas were screened by plate bound B:9-23 ELISA.

Antigen presentation assays

T cell assays were performed in 96-well tissue culture plates. T cell hybridomas (5×10^4 per well) were cultured with APC cells (5×10^4 or 1×10^5 per well) in the presence of indicated doses of antigen. Unless indicated, all assays were performed with the C3.G7 APC cell line which has been previously described[17]. For soluble I-A^{g7} presentation assays, I-A^{g7}-CLIP protein was expressed using a baculovirus system[23]. Briefly, I-A^{g7}-CLIP was treated with thrombin to remove CLIP from the protein and subsequently loaded with B:9-23 peptide overnight. Peptide-MHC complexes were coated onto 96 well plates and washed extensively to remove free peptide. 5×10^4 hybridoma T cells were added per well. In all assays, after incubation for 18 h the culture supernatant from each well were assayed for IL-2 production by using the IL-2 dependant cell line CTLL-2. Proliferation of CTLL-2 cells was measured by ^{3}H thymidine incorporation.

APC isolation and cell lines

Thymic epithelial cells were isolated as previously described[35]. Briefly, serial collagenase digestions were performed to dissociate thymi. A single cell suspension was obtained and $CD45^+$ cells were removed using anti-CD45 magnetic beads (Miltenyi Biotec). Peritoneal exudate cells were isolated from mice 3 days post Con A (Sigma Aldrich) i.p. injection (100 μg). $CD11c^+$ cells were increased in NOD mice treated with Flt-3L (10 μg) for 3 days. 10 days after the first injection splenocytes were isolated and $CD11c^+$ cells were enriched using anti-$CD11c^+$ magnetic beads (Miltenyi Biotec). Intra-islet $CD11c^+$ cells were isolated from dispersed islets with anti-$CD11c^+$ magnetic beads (Miltenyi Biotec). M12.C3.G7β9-23 cells were a generous gift from Norio Abiru and George Eisenbarth[15]**.** Nit-1 cells were a generous gift from Ed Leiter**.**

ELISPOT Assays

All ELISPOT assays were performed according to manfucturer's protocol (BD Biosciences). Briefly, anti-IL-2 capture antibody was coated onto a 96 well Elispot plate overnight. Subsequently, draining lymph node cells (1 $x10^6$ per well) were plated and incubated in the presence of exogenous antigen overnight. The following day plates were washed, stained with detection antibody and analyzed with CTL Immunospot Analyzer plate reader and software (Cellular Technology Ltd.). In $I\text{-}A^{g7}$ blocking assays, 2 μg/well of antibody was added to lymph node cells 1 h prior to the addition of exogenous antigen.

Islet isolation and staining

Islets were isolated from pancreata perfused with a collagenase solution as previously described[28]. Islets were washed and subsequently handpicked or separated by ficoll gradient. Islets were initially stained with anti-CD11c Alexa Fluor® 647 (eBioscience), then fixed and permeabilized with Cytofix/Cytoperm reagents (BD Biosciences). Islets were stained intracellularly with anti-B:9-23 (20 µg/ml) directly conjugated to Alexa Fluor® 488 (Invitrogen). Insulin staining was performed using a rabbit monoclonal antibody (Cell Signaling, Technology, Inc.) following the manufacturer's protocol and Alexa Fluor® 555 donkey anti-rabbit antibody (Invitrogen). LAMP-1 Alexa Fluor® 647 antibody was purchased from eBioscience. The I-A^{g7} was generated in the laboratory and has been previously described. Competitive staining assays were performed with the addition of exogenous B:9-23 peptide, proinsulin (R&D Systems) or insulin (Sigma Aldrich) at a concentration of 0.2mg/ml during intracellular staining.

Insulin granule isolation and mass spectrometry analysis

Insulin granules were isolated from Nit-1 cells and primary beta cells as previously described[36] with modifications. Briefly, Nit-1 cells were homogenized by passing through a 21 and 25 gauge needles. Primary beta cells were homogenized with a dounce homogenizer followed by repeated passing through a 30 gauge needle. Unlysed cells, cell debris and nuclei were removed by low speed centrifugation (1000 x g for 10 min). High-speed centrifugation (25,000 x

g for 20 min) was used to isolate organelles from the cytosolic fraction. The organelle fraction was snap-frozen and thawed 5x to burst open the granules and centrifuged again (25,000 x g for 20 min) to separate granule contents from the membrane material. Insulin content was determined by radioimmunoassay using the kit from Linco Research Co. Content of the B:9-23 peptide was determined using the monoclonal antibody described above. For mass spectrometry experiments, peptides were separated from proteins using a Centricon YM-3 filter device (Millipore) (molecular weight cut off of 3 kDa). Peptide mixtures were examined in the Mass Spectrometry Resource Laboratory at Washington University. The material was loaded directly onto a 75 um ID Picofrit column (New Objective) packed with Magic C18 media (5 um particles with 200 Å pores, Michrom BioResources), eluted and directed into the LTQ-FT mass spectrometer (ThermoFisher). Data was collected using Xcalibur 2.0 software. Spectra were searched against the NCBInr database (version 20090105) using Mascot (Matrix Science) and X!Tandem with Scaffold (Proteome Software) using a parent ion mass tolerance of 10 ppm and a fragment ion mass tolerance of 0.70 Da. Relative abundances are normalized to the most abundant identified insulin B chain peptide.