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In Autoimmune Diabetes Unique Autoreactive T Cells Recognize Insulin Peptides Generated in the Islets of Langerhans

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Abstract

Besides the genetic framework, there are two critical requirements for the development of tissue-specific autoimmune diseases. First, autoreactive T cells need to escape thymic negative selection. Second, they need to find suitable conditions for autoantigen presentation and activation in the target tissue. We show here that these two conditions are fulfilled in diabetic NOD mice. A set of autoreactive CD4⁺ T cells specific for an insulin peptide, with the noteworthy feature of not recognizing the insulin protein when processed by the antigen presenting cells (APC) escape thymic control, participate in diabetes and can cause disease. We also find that APCs situated in close contact with the beta cells in the islets of Langerhans bear vesicles with the antigenic insulin peptides and activate the peptide-specific T cells. These findings may be relevant for other cases of endocrine autoimmunity.

Although autoimmune diabetic NOD mice exhibit a wide range of autoreactivities¹, the one directed against the insulin molecule is prominent². T cells reactive to insulin were identified in NOD mice and shown to transfer diabetes into non-diabetic mice^{3–7}. T cells from T cell receptor (TCR) transgenic mice specific for insulin were also diabetogenic⁸. Additional findings point to insulin as an important primary autoantigen for disease initiation. The amplitude of insulin expression in the thymus was linked to diabetes incidence^{9–12}, and high expression of insulin in APCs using a transgene ablated diabetes development^{13,14}. Moreover, mice expressing a mutant insulin gene product not recognized by T cells did not develop diabetes⁷. It is noteworthy that the T cell response to the insulin molecule is highly focused on a segment of the β -chain, encompassing residues 9-23 (B: 9-23)^{15–18}. This peptide binds poorly (μ M affinity and has a high dissociation rate) to the class II major histocompatibility complex (MHC) molecule I-A^{g7} (ref^{18,19}). How a small protein that yields a very weak binding peptide and circulates at η M concentrations can function as a significant autoantigen is surprising, and raises a number of important

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Author Contributions. J.F.M., M.G.L. and E.R.U. designed and evaluated the experimental work. J.F.M., M.G.L. and J.W.H. carried out most of the cellular experiments. Immunofluorescence and confocal microscopy was done by B.C. S.J.P. isolated insulin granules for mass spectrometry and quantified insulin content within the granules. J.F.M. and E.R.U. wrote the manuscript

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considerations regarding the molecular and cellular basis of autoreactivity. The current view is that weak binding MHC epitopes, such as those from the insulin B:9-23 peptide¹⁸ or the insulin C-peptide²⁰, may favor the development of autoreactivity because they may escape thymic selection^{21,22}.

Two sets of CD4⁺ T cells have been identified by studying CD4 T cell responses to hen-egg white lysozyme (HEL)^{23,24}. One set, the conventional T cells, termed 'type A', respond to the protein and to the peptide presented by APC. The second set, termed 'type B', have the unique features of responding only to peptides offered exogenously to the APC, but not to the identical peptide derived from the processing of the protein. The peptides were identical, but had different conformations when bound to the class II molecules. It was suggested that 'type B' T cells directed to autologous proteins participate in autoreactivity induction²³⁻²⁷. Here we show that 'type B' T cells specific for insulin appear spontaneously in the diabetic NOD mice, along with weakly reactive 'type A' T cells. The 'type B' T cells react with dendritic cells (DC) containing the B:9-23 peptides and induce diabetes.

RESULTS

Insulin-reactive CD4⁺ 'Type B' T cells

CD4⁺ T cells reactive against insulin were identified in islets and the peri-pancreatic lymph nodes of pre-diabetic/early diabetic mice and T cell hybridomas were generated. Forty-two T cell hybridomas (7% of total) from 6 different fusions, were reactive to insulin and/or to the B:9-23 peptide (617 hybridomas were screened of which 32 were further characterized). A number of T cell hybridomas, the conventional 'type A', responded to insulin as well as to the B:9-23 peptide. Several T cells hybridomas, the 'typeB', only recognized the peptide B:9-23 but not the insulin molecule (Fig. 1a). The distribution of 'type A' and 'type B' T cells was equal: among the 32 insulin-reactive CD4⁺ T cells which were characterized, 16 responded to peptide but not to insulin ('type B'), and an equal number, 16, responded to both ('type A'). The number is too small to derive definitive conclusions on their relative incidence. However, most of 'type A' T cells reacted weakly (in the low μ M range) with either insulin or the peptide. About half of the 'type A' T cells had a 10–100-fold lower affinity to insulin than to the B:9-23 peptide, while the remaining reacted equally to both (Fig. 1b,c). Both 'type A' and 'type B' T cell subsets were found in the peri-pancreatic node as well as within the infiltrated islets. These results imply that 'type B' T cells were specifically recruited to the islets together with weakly reactive 'type A' T cells, suggesting the involvement of both 'type B' and low affinity 'type A' T cells in the early disease process.

Mice respond to immunization with B:9-23 peptide

To examine in detail the immune response to insulin or to the B:9-23 peptide, mice were immunized with one or the other and the incidence of 'type A' and 'type B' insulin reactive CD4⁺ T cells were examined. When NOD mice were immunized with insulin, few, if any, insulin-specific T cells were detected in the draining lymph nodes (Fig. 1d). Previous studies detected a weak insulin T cell proliferative response¹⁴. In contrast, a prominent CD4⁺ T cell response was found upon immunization with the B:9-23 peptide. The response

was entirely blocked by an anti-I-A^{g7} antibody (Fig. 1e,f). These T cells were peptide specific, because they responded only to B:9-23 peptide, but not to the insulin protein. Of 351 T cell hybridomas generated from the draining lymph nodes of the immunized mice, the majority (74%) were peptide specific, while only a small number (1.7%) recognized both peptide and the insulin molecule (Fig. 1a–c and Supplementary Table 1). The remaining hybridomas were either non-responsive (17.7%) or autoreactive (6.6%). The ‘type B’ T cells also recognized denatured insulin, indicating that they were not recognizing an artificial determinant arising from the synthetic peptide (Supplementary Fig. 1a).

We next compared the insulin-reactive T cells from NOD wild-type and NOD mice with genetic deletions of both insulin genes but expressing a transgene encoding a mutant proinsulin⁷. The mutant transgene encodes an insulin molecule containing a single tyrosine to alanine amino acid substitution at residue 16 of the insulin beta-chain, referred to as B16:A-dKO. In the absence of native insulin genes, the transgenic mutant insulin restored biological activity, however, the substitution abrogated recognition by insulin-specific T cells⁷. Unlike NOD mice, B16:A-dKO mice immunized with insulin generated a T cell response specific to insulin protein (Fig. 1g and d). Immunization of B16:A-dKO mice with the B:9-23 peptide generated a peptide response and a weak response to insulin (Fig. 1h). In addition, ‘type A’ hybridomas generated from B16:A-dKO mice immunized with insulin displayed a much greater sensitivity for insulin compared to hybridomas derived from NOD mice (Fig. 1i). This indicates that in the NOD mouse the high affinity T cells to insulin are normally deleted. T cells found in peripheral tissue are less reactive, reflecting that they escaped negative selection and bear the stamp of tolerance.

Differential recognition of covalent Peptide-MHC complexes

The explanation for the different recognition of the B:9-23 peptide-I-A^{g7} complex between the two sets of T cells is presently unclear. Initial studies indicate that in contrast to the HEL system, H2-DM did not affect the development of ‘type A’ or ‘type B’ peptide-MHC (pMHC) complexes (data not shown). Moreover, H2-DM favored the assembly of B:9-23 to I-A^{g7}. We have searched extensively for changes in the peptide or for post-translational modifications that may account for recognition by one or the other set of T cells and have failed to identify any (data not shown). Both ‘type A’ and ‘type B’ CD4⁺ T cells reacted to purified I-A^{g7} molecules incubated with the identical B:9-23 peptide (Supplementary Fig. 1b). The differential recognition of the B:9-23 peptide by the two T cells types most likely reflects different conformational states of the pMHC complex. In support of this interpretation, ‘type A’ and ‘type B’ T cells recognized differently a covalently-linked pMHC complex. M12.C3 cells engineered to express I-A^{g7} with the B:9-23 peptide covalently tethered¹⁵ activated the ‘type A’ T cells, including those that reacted weakly with insulin, but not the ‘type B’ T cells (Fig. 1j and Supplementary Table 1). Thus, the covalently-linked complex was presented in a state or a conformation that allowed the TCR of ‘type A’ T cells to recognize it, but not the TCR of ‘type B’ T cells. It is probable that the intracellular pathway by which insulin reaches the class II molecule and the site within the APC are the two critical factors in generating a unique complex that T cells recognize.

Type B T cells cause diabetes

To determine if 'type B' insulin T cells are diabetogenic, eleven primary T cell lines were generated from the draining lymph nodes of B:9-23 immunized NOD mice, expanded and adoptively transferred into NOD.SCID recipients. All T cell lines were 'type B', meaning they recognized the B:9-23 peptide, but not the insulin molecule (Supplementary Fig. 2). None of them recognized the covalently linked pMHC complex on M12.C3.G7 β 9-23 cells (data not shown). All T cell lines induced extensive insulinitis, with 7 of 11 lines also inducing diabetes (Fig. 2a,c,d and Supplementary Fig. 3a). A number of these lines also accelerated diabetes in young NOD mice (Supplementary Fig. 3a-d). The extent of disease transfer varied among the clones and was irrespective of their cytokine profiles (Fig. 2b and Supplementary Fig. 3a). Thus, the peptide reactive, 'type B' T cells could damage the islets and induce the same histopathology as other diabetogenic T cells, suggesting that they participate in the autoimmune process.

Presentation of B:9-23 by islet DC

In order to address where insulin peptide presentation leading to 'type B' T cells activation occurs, antigen presenting cells (APC) from various tissues were examined. We could not detect antigen presentation to T cells by APCs from spleen, inguinal or peri-pancreatic lymph nodes from pre-diabetic mice, suggesting that circulating insulin was not the source of peptide. In addition, neither dendritic cells (DC) nor epithelial cells isolated from thymi activated 'type A' hybridomas, indicating that presentation in the thymus is quite low. Of all tissues examined the only APCs that presented insulin peptides to either 'type A' or 'type B' T cells were the intra-islet DC (Fig. 3a). In all mouse strains, the islets of Langerhans harbor an average of about 10 DC per islet, most of which are constitutively expressing pMHC complexes derived from beta cell proteins, irrespective of the diabetic status, islet inflammation or beta cell death²⁸. Whole islets cell fractions containing DC, or purified intra-islet DC from NOD.*Rag1*^{-/-} mice constitutively presented antigen to both 'type A' and 'type B' T cells in the absence of inflammation or overt cellular death (Fig. 3b,c). Furthermore, when Nit-1 insulinoma cells, which do not express MHC class II molecules, were incubated with purified splenic DC, uptake of insulin granules by DC was detected by electron microscopy (Fig. 3d). These DC were capable of activating both the 'type A' and 'type B' insulin-reactive T cells (Fig. 3e,f), showing that both peptide-MHC conformers are presented by APC within close contact to beta cells.

The secretory granules of beta cells can constitute a source of the B:9-23 peptide, due to the fact each granule is comprised of about 10⁶ insulin molecules, as well as catabolic products from its processing²⁹. Purified secretory granules from primary beta cells incubated with splenic DC led to robust activation of both 'type A' and 'type B' hybridomas (Fig. 3g). To assess whether these granules contain insulin peptides, we generated a monoclonal antibody that specifically binds to the B:9-23 peptide but not to insulin or proinsulin molecules (Supplementary Fig. 4). Specific granule staining was observed in whole islets or purified beta cells from NOD or NOD.*Rag1*^{-/-} mice (Fig. 4a,d). The reaction was blocked by free peptide (Fig. 4b,e), but not by an excess of insulin or proinsulin (Supplementary Fig. 4). Cells containing B:9-23 positive granules represented a subset of total beta cells. The majority of granules inside beta cells were positive for insulin but negative for B:9-23 and

did not contain LAMP-1 (Fig. 4c and Supplementary Fig. 5). In contrast, all vesicles containing the B:9-23 peptide in beta cells were positive for LAMP-1, and only very few of these vesicles (less than 10%), were also positive for insulin (Supplementary Fig. 5).

Confocal microscopy showed that the majority of DC in the examined islets had taken up B:9-23 positive and/or insulin granules (Fig. 4f). Screening of purified islet DC showed that about 80% of them contained insulin positive granules with an average of 7 granules per DC. Many of these DC stained positive for both insulin containing vesicles and B:9-23 containing vesicles. Granules within islet DC co-localized with LAMP-1 and I-A^{g7} (Fig. 4g), consistent with trafficking to cellular compartments involved in antigen processing and presentation. These observations suggest that the B:9-23 peptides presented by I-A^{g7} could be derived from beta cell vesicles containing peptides of the insulin β -chain and/or from the processing of insulin granules within DC. Evidence for granules within intra-islet DC under physiological conditions was presented previously by electron microscopy²⁶. In addition, granules isolated from Nit-1 cells contained 8.8 η moles of anti-insulin-reactive material and 12 ρ moles of anti-9-23 reactive material. Initial mass spectrometry studies on purified secretory granules showed they contained C-peptide (data not shown), but also peptides from the B:9-23 segment, which included peptides centered on the 12-21 residues (VEALYLVGGE) of the insulin β -chain (Supplementary Fig. 6). These observations suggest that peptides immunogenic for 'type B' T cells are produced during the physiological processing of mature insulin molecules, and are not dependent on any prior inflammation.

DISCUSSION

Three sets of insulin-reactive T cells were distinguished in this study. One set had the unique feature of being specific to exogenous insulin peptide, but unreactive to insulin. The peptide that activated these 'type B' T cells was normally derived from the insulin secretory granule and was loaded on MHC class II in the intra-islet DC. These T cells were capable of transferring diabetes and were found spontaneously in infiltrated islets, indicating their participation in the autoimmune process. These T cells are compatible with the 'type B' T cells described before²³⁻²⁶.

The two other sets of T cells were insulin and peptide-reactive T cells, but differed in their sensitivity to insulin. We refer to them as 'type A' T cells, identified in the NOD or in the B16:A-dKO mice, respectively. In the B16:A-dKO mice 'type A' T cells were readily found after insulin immunization and showed a higher response to the insulin molecule or the B:9-23 peptide compared to 'type A' T cells from the NOD mouse. The strong insulin reactivity found in B16:A-dKO mice supports the conclusion reached by others⁹⁻¹² that native insulin expression in the thymus influences the selection of insulin-reactive T cells, and that partial tolerance or unresponsiveness to the insulin molecule exists in NOD mice. Thus, the few insulin reactive T cells found in NOD mice had an imprint of tolerance, with weak reactivity to insulin, which might explain why they escaped negative selection.

The frequency of 'type B' T cells is markedly greater than 'type A' T cells in the precursor population of NOD mice. However, because both 'type A' and 'type B' T cells were isolated from the islets, we cannot conclude which set is the major participant, or if they act

synergistically in disease development. More than one factor can influence the diabetogenicity of transferred T cell cells, such as time in culture and expression of adhesion molecules³⁰. Due to this variability, strict comparisons between the diabetogenicity of 'type A' and 'type B' T cells were not done systematically. Our results and previous reports^{5, 8} indicate that both 'type A' and 'type B' insulin-specific T cells are diabetogenic.

The B:9-23 peptide could be generated following DC handling of insulin upon capture of the insulin granules or from uptake of the granules that already contained the peptide. The finding that islet DC contain insulin granules and B:9-23 peptide, and the observation that feeding DC with purified secretory granules results in strong presentation explains another conundrum concerning insulin presentation. There is a relatively poor yield of peptide selected by MHC molecules after protein processing³¹. Furthermore, due to the low binding affinity of the B:9-23 peptide for I-A^{g7} and the rapid dissociation of the peptide from I-A^{g7} (ref 18), a high concentration of insulin within the APC is required for effective presentation to T cells. The concentration of insulin in blood and tissue is in the low nM level. In antigen presentation assays, the concentration of insulin or B:9-23 peptide required for presentation is in the low μM level which is unphysiological in the whole mouse. If all these aspects are taken into consideration, it is most likely that the islet DC have two essential roles: to migrate into the pancreatic draining lymph node to initiate the sensitization process³²; and to present antigen to sensitized T cells in the effector stage of diabetogenesis.

Collectively, these results suggest that self-reactive 'type B' T cells and low affinity 'type A' T cells escape negative selection and give rise to pathogenic T cells in autoimmune diabetes. A situation akin to insulin autoimmunity may also be found in other endocrine autoimmune diseases such as in autoimmune thyroiditis in which DC are found surrounding the follicles, some of which have class II-positive epithelial cells^{33, 34}.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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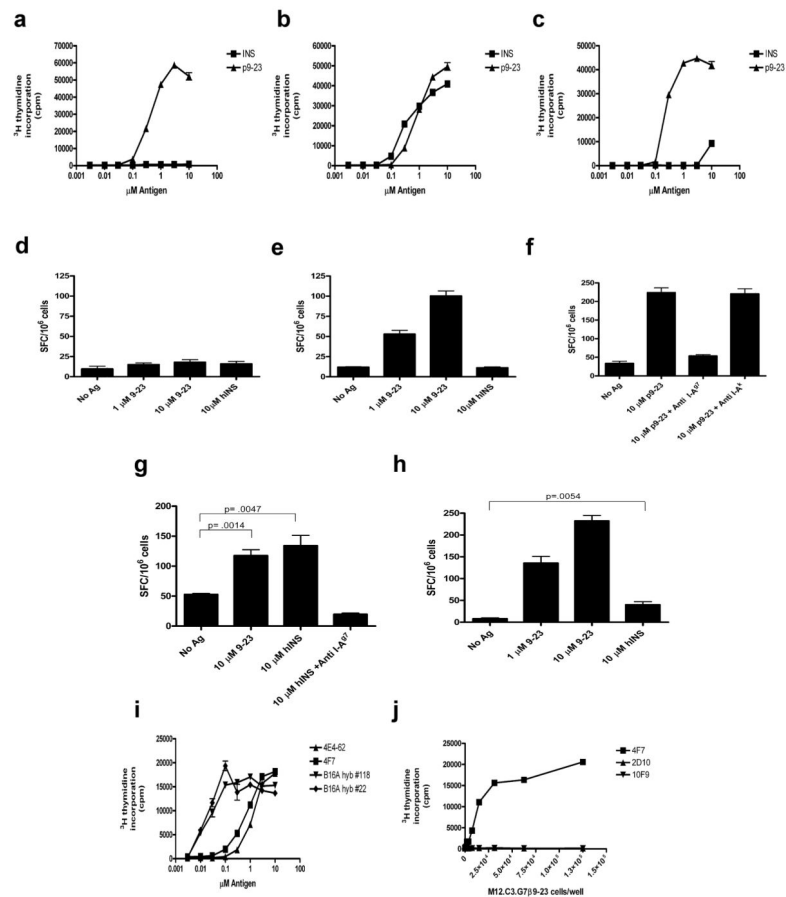


Figure 1.

Insulin reactive T cells in NOD mice. (a–c) IL-2 production in response to insulin or B:9-23 peptide stimulation by three representative CD4⁺ T cell hybridomas using a standard CTLL proliferation bioassay. (a) ‘type B’ T cell (2D10), no response to insulin. (b) ‘type A’ T cell (4F7), equal response to insulin and peptide. (c) ‘type A’ T cell (IIT-9) very poor response to insulin. (d) ELISPOT of IL-2 production in NOD mice immunized with insulin and re-stimulated with insulin or the B:9-23 peptide. Results represent number of IL-2 secreting cells per 10⁶ draining lymph node cells. SFC, spot forming cells. (e) ELISPOT of IL-2 secretion in NOD mice immunized with B:9-23 peptide and restimulated with peptide or insulin. (f) ELISPOT of IL-2 secretion in NOD mice immunized with B:9-23 peptide and restimulated with peptide in the presence of specific (anti-I-A^{g7}) blocking antibody. (g) ELISPOT of IL-2 secretion in B16:A-dKO mice immunized with insulin and re-stimulated as indicated. (h) ELISPOT of IL-2 secretion in B16:A-dKO mice immunized with B:9-23 peptide and re-stimulated as indicated. (i) IL-2 production in two T cell hybridomas derived from the B16:A-dKO mice (#118 & #22) and two hybridomas derived from NOD mice (4F7 & 4E4-62) in response to insulin presented by the C3.G7 cell line. (j) IL-2 production in ‘type A’ (4F7) and ‘type B’ (2D10 & 10F9) hybridomas incubated with the C3.G7 cell line bearing a B:9-23 peptide-I-A^{g7} covalent complex. P values in experiments were determined using one-tailed unpaired Student’s *t* test. Error bars, s.e.m.

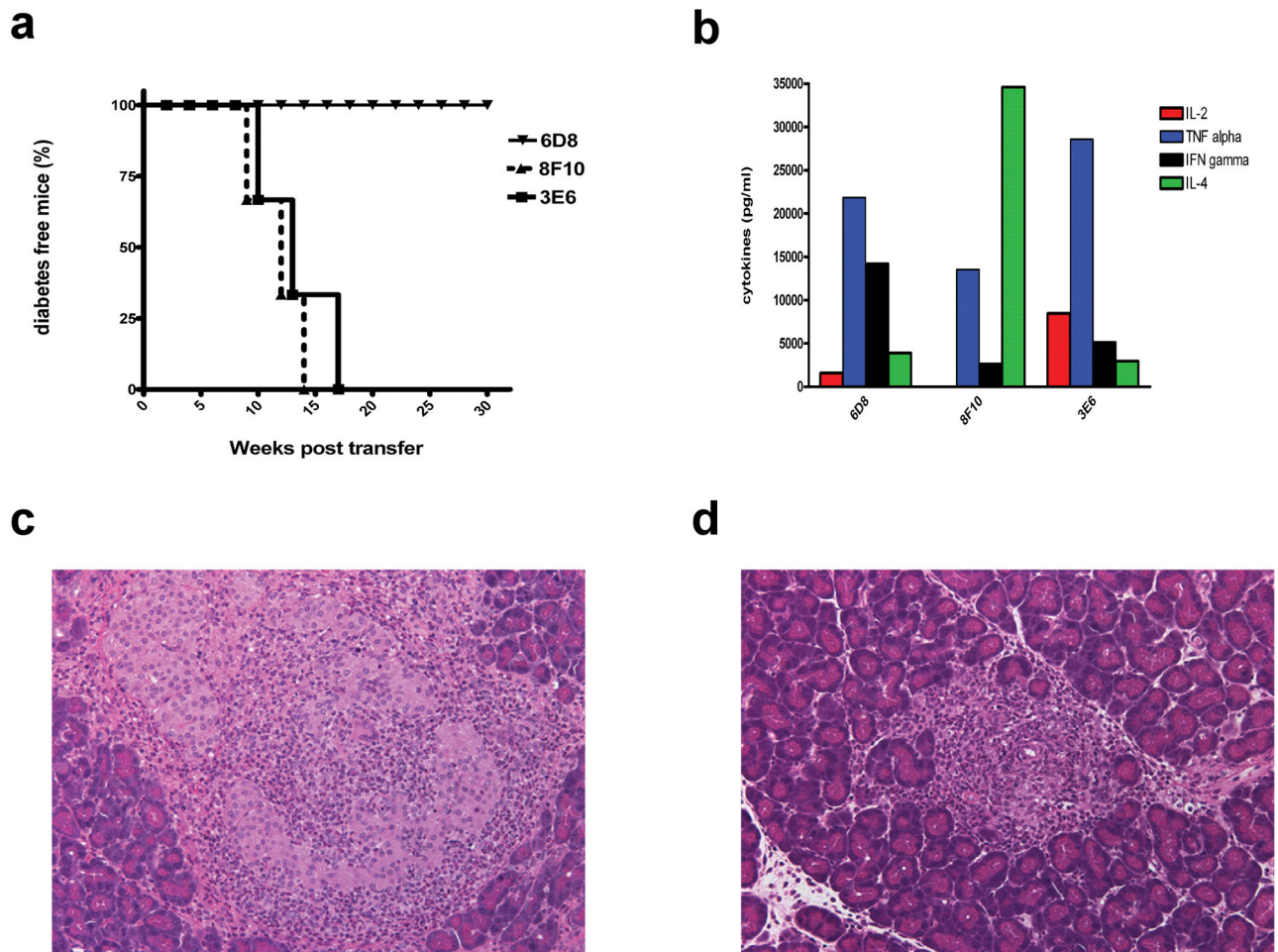
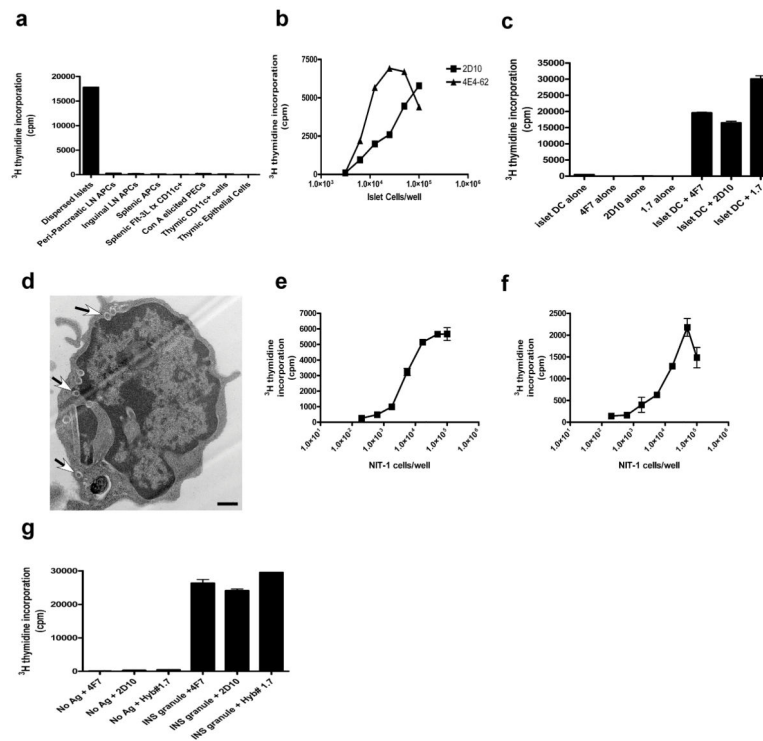


Figure 2.

'Type B' T cells are diabetogenic. (a) Diabetes incidence of three representative 'type B' T cell lines, when transferred into NOD.SCID recipients ($n=3$ for each); mice were considered diabetic after two consecutive blood glucose readings of ≥ 250 mg/dl. (b) Cytokine profile of representative $CD4^+$ T cell lines 48 h post stimulation with plate bound anti-CD3/CD28 antibodies. Haematoxylin and eosin stained pancreatic section of a NOD.SCID recipient that received 6D8 T cells and developed insulinitis but not diabetes (c) and a NOD.SCID recipient that received 3E6 T cells and developed overt diabetes (d).

**Figure 3.**

Intra-islet DC pulsed with secretory granules, present insulin peptides. **(a)** B:9-23 peptide presentation by a panel of cells from various tissues to a ‘type B’ hybridoma (Hyb #1.7). **(b)** IL-2 production from ‘type A’ (4E4-62) and ‘type B’ (2D10) hybridomas stimulated with dispersed islets from NOD.*Rag1*^{-/-} mice. **(c)** IL-2 secretion from ‘type A’ (4E4-62) and ‘type B’ (2D10) hybridomas stimulated with CD11c⁺ cells isolated from the islets of 8 week old NOD males. **(d)** Electron microscopy analysis of Flt-3L CD11c⁺ cell coincubated with NIT-1 insulinoma cells for 24 h (arrows highlight granules in DC). Scale bar 500 nm. **(e,f)** IL-2 production from ‘type B’ (2D10) **(e)** and ‘type A’ (4F7) **(f)** hybridomas stimulated with Nit-1 cells co-incubated with purified splenic CD11c⁺ DCs. **(g)** IL-2 production in ‘type A’ (4F7) and ‘type B’ (2D10 and Hyb #1.7) hybridomas stimulated with splenic CD11c⁺ cells co-incubated with purified insulin granules from primary dispersed islet cells. Error bars, s.e.m.

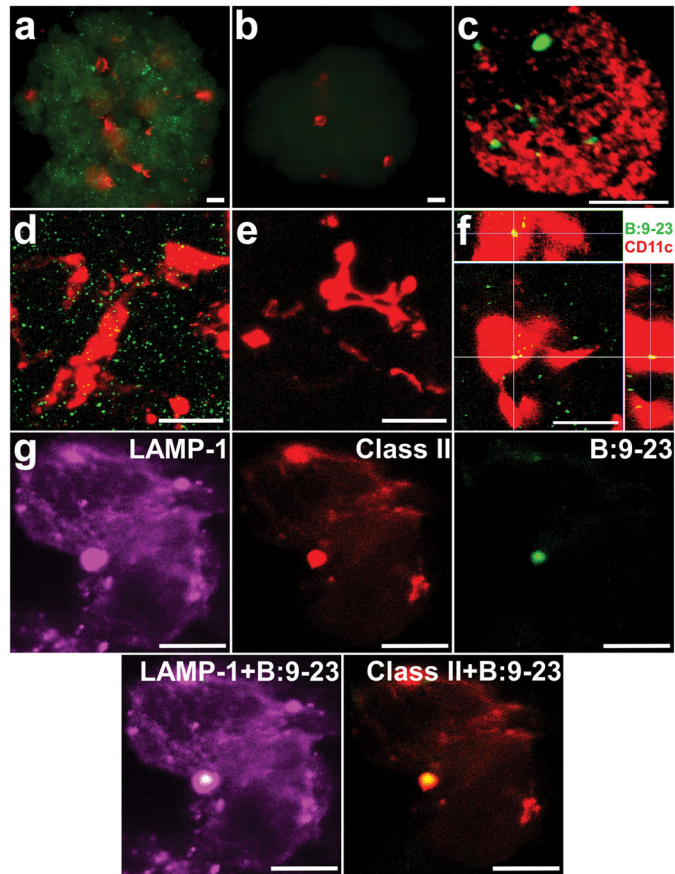


Figure 4. Secretory granules contain proteolytic fragments of the insulin β -chain in NOD.*Rag1*^{-/-} islets. (a) Immunofluorescence analysis at low magnification (20X) of an islet stained for CD11c⁺ (red) and intracellular B:9-23 (green). (b) Immunofluorescence analysis of an islet stained for CD11c⁺ (red) and B:9-23 (green) in the presence of competing exogenous B:9-23 peptide. Scale bars, 20 μ m. (c) Confocal microscopy of an isolated beta cell, stained for B:9-23 (green) and insulin (red). The reconstruction is from a single stack (1.5 μ m thickness). Scale bar, 15 μ m. (d, e) Reconstruction from a stack of 60 compiled optical sections acquired in 0.5- μ m increments stained for CD11c⁺ (red) and intracellular B:9-23 (green) in the absence (d) or presence of competing B:9-23 free peptide (e). Scale bars, 20 μ m. (f) Three-dimensional reconstruction of an islet by confocal microscopy stained for CD11c⁺ (red) and intracellular B:9-23 (green). The bottom left image is a projection along the z-axis (top view) from a stack of 30 optical sections acquired in 0.5 μ m increments. The top and right-sided images are zx- and zy-reconstructions (side view) of the same image stack (indicated as white lines). B:9-23 staining in the islet DC (yellow merge). Scale bar, 20 μ m. (g) Reconstruction of an isolated islet DC co-stained for B:9-23, class II MHC and for LAMP-1. Images represent a single stack with thickness of 1.5 μ m. Scale bars, 10 μ m.