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Mapping I-A^{g7} restricted epitopes in murine G6PC2

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Abstract

G6PC2, also known as islet specific glucose 6-phosphatase catalytic subunit related protein (IGRP), is a major target of autoreactive CD8⁺ T cells in both diabetic human subjects and the non-obese diabetic (NOD) mouse. However, in contrast to the abundant literature regarding the CD8⁺ response to this antigen, much less is known about the potential involvement of IGRP-reactive CD4⁺ T cells in diabetogenesis. The single previous study that examined this question in NOD mice was based upon a candidate epitope approach and identified three I-A^{g7}-restricted epitopes that each elicited spontaneous responses in these animals. However, given the known inaccuracies of MHC class II epitope prediction algorithms, we hypothesized that additional specificities might also be targeted. To address this issue we immunized NOD mice with membranes from insect cells overexpressing full-length recombinant mouse IGRP, and measured recall responses of purified CD4⁺ T cells using a library of overlapping peptides encompassing the entire 355aa primary sequence. Nine peptides representing 8 epitopes gave recall responses, only 1 of which corresponded to any of the previously reported sequences. In each case proliferation was blocked by a monoclonal antibody to I-A^{g7}, but not the appropriate isotype control. Consistent with a role in diabetogenesis, proliferative responses to 4 of the 9 peptides (3 epitopes) were also detected in CD4⁺ T cells purified from the pancreatic draining lymph nodes of pre-diabetic female animals, but not from peripheral lymph nodes or spleens of the same animals. Intriguingly, one of the newly identified spontaneously reactive epitopes (P8 [IGRP_{55–72}]) is highly conserved between mice and man, suggesting that it might also be a target of HLA-DQ8-restricted T cells in diabetic human subjects, an hypothesis that we are currently testing.

Keywords

Autoimmune diabetes; NOD; IGRP; epitope

INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease characterized by selective destruction of the beta cells of the islets of Langerhans and consequent life-long absolute insulin insufficiency (1). It is one of the most common endocrinopathies of childhood in the US (2) and for at least the last 20 years has been increasing in incidence throughout the developed world, especially in younger children (3, 4). Although its etiology remains incompletely

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understood (5, 6), it is generally accepted that autoreactive T cells are critical participants in diabetogenesis (7). Accordingly, most therapies currently under investigation are directed towards modulating these subsets (8). Although some success has been obtained in phase I/II clinical trials with non-selective agents such as short-term treatment with antibodies to CD3 (9, 10), the “holy grail” of T1D research remains the development of therapies that selectively restore organ specific tolerance with minimal immunosuppression, which will likely require a significant antigen-specific element (11). Studies in the non-obese diabetic (NOD) mouse, the most widely used animal model of spontaneous T1D (12), suggest that islet autoimmunity is initially directed to a single epitope in insulin (13), but subsequently spreads to encompass a large number of additional epitopes in this and other β -cell proteins (14). Similarly, longitudinal studies of genetically at risk children indicate that the appearance of autoantibodies to multiple molecular targets is highly predictive of subsequent progression to clinical disease (1). Consequently, it is generally accepted that a successful therapy for active T1D will need to control autoreactive T cells specific to a variety of β -cell targets. Although the process of infectious tolerance might permit a successful therapy to be based on the therapeutic targeting of a single specificity (15), given the apparently heterogeneous etiology of the disease in humans, uncertainty as to whether or not insulin is always the initiating target (16), and potential involvement of pathogenic T cells restricted to multiple MHC class I and II molecules, it seems more reasonable to expect that multiple specificities from multiple targets will actually need to be targeted to provide a universally efficacious therapy. Thus a significant amount of current T1D research is still devoted to identifying immunodominant epitopes within islet autoantigens, with the expectation that such knowledge will promote the development of improved therapeutics.

G6PC2, also known as islet specific glucose 6-phosphatase catalytic subunit related protein (IGRP), is a 355aa integral membrane protein localized primarily to the endoplasmic reticulum of pancreatic β -cells (17). It is highly hydrophobic with 9 predicted transmembrane helices and approximately 70% of its amino acids embedded in the membrane (18). The mRNA encoding IGRP was originally identified in the β TC3 mouse insulinoma cell line (19) using subtractive hybridization against RNA from non-beta cell lines (20), and subsequent sequencing of a full-length clone (18). At the protein level IGRP shows extensive sequence homology to liver glucose 6-phosphatase (G6PC), and is catalytically active, albeit at a much reduced level than the liver enzyme (18). The precise mechanism of action of IGRP remains uncertain, but it is believed to function in regulating glucose stimulated insulin secretion (18, 21–25). Evidence for a role of IGRP in autoimmune diabetes was first provided by the discovery that it is the target of the diabetogenic NY8.3 NOD mouse CD8⁺ T cell clone (26–28). Indeed T_H cells recognizing the immunodominant epitope IGRP_{206–214} can account for almost 50% of the total CD8 insulinitic infiltrate in these animals (29), and subsequent studies indicated that numerous other epitopes in mouse IGRP are also targeted by CD8⁺ T cells in pre-diabetic and newly diabetic NOD mice (30). Despite this, autoreactivity to IGRP is not essential for diabetogenesis in NOD mice, with animals genetically ablated for this autoantigen developing disease with similar kinetics and incidence to wild-type littermates (31), suggesting it is likely a consequence of epitope spreading. Consistent with the hypothesis of conservation of organ-specific autoimmune targets between species, the human ortholog is also a significant target of CD8⁺ T cells in diabetic HLA-A2⁺ subjects (32–36).

In contrast to the considerable amount of information that has been obtained regarding IGRP-specific CD8⁺ T cell responses, much less is known about potential CD4⁺ T cell autoreactivity to this antigen. Thus, to date only two studies have reported responses in mouse (37) and human (38) respectively. In each case a candidate epitope approach was followed, based on prediction of likely I-A^{g7}, HLA-DR3, and HLA-DR4 binding peptides. Despite significant recent improvements it is generally accepted that the performance of

even the best MHC class II prediction algorithms remains sub-optimal (39). We therefore hypothesized that additional disease relevant epitopes might also be present. To begin to test this hypothesis we conducted an unbiased analysis of I-A^{g7}-restricted responses to IGRP in NOD mice. We now report the identification of 7 additional epitopes, 1 of which is conserved between mouse and human and might potentially be clinically relevant.

METHODS AND MATERIALS

Mice

NOD/bdc mice were obtained from the University of Colorado Health Sciences Center Diabetes and Endocrinology Research Center bioresources core. All experimental procedures were conducted in accordance with protocols previously approved by the Institutional Animal Care and Use Committee of the University of Colorado Anschutz Medical Campus.

Peptides

A library of 45 mixed 16 and 18mer peptides overlapping by 8 or 10 residues spanning the entire 355 aa primary sequence of IGRP was obtained from Sigma Genosys, St. Louis, MO (supplementary table 1). Purified peptides (>95%) were obtained from GenScript, Piscataway, NJ. Peptides were dissolved in DMSO to a final concentration of 10 mg/ml and aliquots diluted to 200 µg/ml with PBS and sonicated immediately prior to use. Final concentrations in proliferation assays were 20 µg/ml (0.2% DMSO).

IGRP-expressing insect cells

A cDNA encoding full-length mouse IGRP (18) lacking the stop codon was cloned into pMT/V5-HisB (Invitrogen, Carlsbad, CA) in frame with the C-terminal V5-His epitope tag. The resulting plasmid was used to co-transfect *Drosophila* S2 cells (40) (Invitrogen) with pCoHygro (Invitrogen) at a 19:1 ratio according to the manufacturer's recommendations. Stably transfected cells were selected with hygromycin (300 µg/ml) and cloned by limiting dilution. Expression of IGRP in cloned cells was monitored 24h after induction with 0.5 mM CuSO₄ (41) by SDS-PAGE and western blotting. Transferred proteins were detected with Ponceau S (Sigma, St. Louis, MO) prior to immunostaining with HRP-conjugated mouse anti-V5 (Invitrogen) and detection by enhanced chemiluminescence (GE Healthcare Bio-Sciences, Piscataway, NJ). A representative clone (2a3) was used for all subsequent experiments. To prepare membrane fractions, parental and clone 2a3 cells (1.2×10^6 /ml) were incubated for 24h at 28°C in Schneider's S2 medium (Invitrogen) containing 2 mM L-glutamine, 10% heat inactivated FBS, and 0.5 mM CuSO₄. Cells were harvested by centrifugation, washed twice with PBS, then resuspended at 1×10^7 cells/ml in 50mM HEPES pH 7.4, containing 1mM EGTA and 1x protease inhibitor cocktail III (EMD Chemicals Inc, Gibbstown, NJ) and homogenized with 40 strokes of a Dual tissue grinder (Kontes, Vineland, NJ). The homogenates were centrifuged at 800g for 10 min at 4°C to remove nuclei and unbroken cells, and the resulting supernatants then re-centrifuged at 48,384g_{max} for 30 min at 4 °C (Beckman JA-25.50 rotor; 20,000rpm). The supernatants were discarded, and pellets resuspended in PBS and again centrifuged at 48,384g_{max} for 30 min at 4 °C. The final pellets were resuspended in PBS at a protein concentration of ~2 mg/ml, and aliquots stored at -80 °C until use.

Immunization of male NOD mice and proliferation assays

Groups of 3–5 male NOD mice (6–8 weeks old) were immunized with 50µg of membrane fractions, from either parental cells or induced clone 2a3 cells, emulsified 1:1 in complete Freund's adjuvant subcutaneously at the base of the tail. Ten to fourteen days later the

draining lymph nodes (inguinal and periaortic) and spleens were harvested (42). In some experiments lymph node cells were used directly and in others CD4⁺ T cells were purified from lymph node cells by negative selection (Mouse CD4⁺ T Cell Isolation Kit, R&D Systems, Minneapolis, MN). To analyze spontaneous responses lymphocytes were isolated from the pancreatic draining lymph nodes, peripheral lymph nodes (axillary and cervical), and spleens of pooled groups of 4–12 female NOD mice aged 4–12 weeks, or spleens from individual newly diabetic animals (within 24h of diagnosis), and processed as described above. Aliquots of cells ($5\text{--}20 \times 10^4$) were co-cultured in duplicate with 1×10^6 irradiated (35Gy) splenocytes $\pm$ antigen in a final volume of 200 μ l of RPMI 1640 supplemented with 10% FBS, 50 μ M 2-mercaptoethanol, 10mM HEPES, 100U/ml penicillin and 100 μ g/ml streptomycin for 72h at 37°C. ³H-methyl-thymidine (0.5 μ Ci/well; Perkin Elmer, San Jose, CA) was added for the final 16h before harvest, and the incorporated radioactivity determined by liquid scintillation counting (Perkin Elmer Wallac). A stimulation index (SI; mean of cpm of Ag/mean of cpm of vehicle alone) of > 3 was considered positive. Representative experiments are shown. In some experiments purified IgG from monoclonal clone 10-3.6.2 (anti-I-A^k but cross-reactive with I-A^{g7}) (43) or MOPC-173 (IgG2a isotype control; BD Biosciences, Franklin Lakes, NJ) was added to a final concentration of 50 μ g/ml. In others porcine insulin (Sigma) was added to a final concentration of 100 μ g/ml.

RESULTS

I-A^{g7} restricted responses in IGRP-immunized NOD mice

In order to try to conduct as comprehensive and unbiased an analysis of potentially diabetes-relevant I-A^{g7} restricted epitopes in IGRP as possible we elected to follow a two stage strategy comprising an initial determination of the recall responses in immunized animals followed by a more focused study of spontaneous responses to the immunodominant epitopes consequently identified. A key question thus became the format of the initial immunogen. As previously discussed, IGRP is a glycoprotein integral to the endoplasmic reticulum of pancreatic β -cells (18), with 9 predicted transmembrane helices (Fig 1A). Given that the presence of the lipid bi-layer and single N-linked oligosaccharide chain might each influence processing of the antigen we therefore chose to use a membrane fraction prepared from S2 insect cells induced to over-express the protein (Fig 1B, C). Critical to this process was the utilization of a tightly controlled inducible system (41), since even prolonged low level constitutive over-expression of IGRP led to apoptosis of the insect cells, likely due to ER stress (44). In contrast, tightly controlled stable clones continued to grow for at least 24h post-induction, and could be maintained in continuous culture for more than 3 months.

Following immunization with membrane fractions prepared either from parental or induced cells a library of overlapping peptides encompassing the entire 355aa primary sequence of mouse IGRP (supplementary table 1 and inset to Fig 2) was used to investigate antigen-specific recall responses in purified CD4⁺ T cells. As shown in Fig 2 (open bars) although robust responses to both immunogens was observed, no recall response to the peptide library was detectable in animals immunized with the parental membranes. In contrast, animals immunized with the IGRP-containing membranes consistently responded both to the 2 immunogens, and to 9 of the 45 peptides (Fig 2, filled bars). Only 2 of the peptides that elicited recall responses overlapped (P40 and P41) suggesting the existence of at least 8 I-A^{g7} restricted epitopes in murine IGRP. An essentially identical result was obtained when recall responses were measured in animals immunized with the entire peptide library, rather than IGRP-containing membrane fractions (data not shown).

Surprisingly, only the epitope contained in P17 (IGRP_{125–142}) corresponded to one of the 3 previously reported epitopes (IGRP_{123–145}; (37)), whereas no significant response was

observed to either P1 (IGRP₁₋₁₈) or P2 (IGRP₉₋₂₆) that should contain the IGRP₄₋₂₂ epitope, nor to P26 (IGRP₁₉₅₋₂₁₀) or P27 (IGRP₂₀₁₋₂₁₈) that overlap with IGRP₁₉₅₋₂₁₄. It should be noted that P27 (IGRP₂₀₁₋₂₁₈) also contains the immunodominant H2-K^d restricted epitope IGRP₂₀₆₋₂₁₄ (26–28). Thus our failure to detect any response to this peptide strongly suggests that the small population of CD8⁺ cells contaminating our responder populations (<5%; data not shown) did not contribute to the specific responses we observed. However, to confirm that this was indeed the case we conducted blocking experiments with monoclonal 10-3.6.2 (43) (anti-I-A^k but cross-reactive with I-A^{g7} (45, 46)). As expected, no blockade of the response to the polyclonal activator concanavalin A was observed (Fig 3). In contrast, inclusion of 10-3.6.2 (striped bars) but not the isotype control MOPC-173 (solid bars), significantly inhibited proliferation in response to each of the antigenic peptides tested. Essentially equivalent results were obtained using unfractionated lymph node cells (data not shown).

Spontaneous I-A^{g7} restricted responses to IGRP in pre-diabetic and newly diabetic NOD mice

The results described in Figs 2 and 3 clearly demonstrate that NOD mice can mount MHC class II restricted responses to at least 8 naturally processed epitopes from IGRP (responses to P40 and P41 likely reflect a single epitope). However they do not demonstrate which, if any, are likely to have significance in diabetogenesis. To address this key issue we isolated secondary lymphoid tissues from groups of “pre-diabetic,” or individual newly diabetic, female mice from the NOD/bdc colony. In our colony insulinitis is typically evident from 3–5 weeks of age, with animals progressing asynchronously to persistent hyperglycemia beginning at 12–14 weeks such that ~80% of females have become diabetic before they are 40 weeks of age (data not shown). Accordingly, to investigate responses in “pre-diabetic” animals, we pooled tissues from groups of mice aged 4–12 weeks, and isolated CD4⁺ T cells by negative selection. Pooling was necessary to obtain sufficient numbers of cells. As shown in Fig 4 (open bars) proliferative responses to 4 of the 9 tested peptides (P8 [IGRP₅₅₋₇₂], P36 [IGRP₂₇₁₋₂₈₈], P40 [IGRP₃₀₁₋₃₁₈] and P41 [IGRP₃₀₉₋₃₂₆]) could be detected in cells from the pancreatic draining lymph nodes of these mice, but not from cells from peripheral lymph nodes (horizontally striped bars) or spleen (solid bars) of the same animals. Increasing numbers of autoreactive T cells home to the bone marrow and spleen of NOD mice during diabetogenesis (47–49), and consistent with these observations proliferative responses to the same 4 peptides could also be detected in the spleens of newly diabetic animals shortly after onset of persistent hyperglycemia (Fig 4 diagonally striped bars). In each case positive responses were selectively inhibited by inclusion of the blocking monoclonal antibody (data not shown). Similar results were obtained with the positive control antigen insulin (INS).

DISCUSSION

The results described above clearly indicate that IGRP is a target for both induced and spontaneous CD4⁺ T cell responses in NOD mice. As such they are in broad agreement with the prior study by Mukherjee and colleagues (37). However, there is considerable discrepancy between the epitopes identified in the current study and those previously reported, with only 1 being common to both investigations. At present the reasons for the observed inconsistencies are not entirely certain, but likely reflect variations in the methodology used. Thus, in contrast to the comprehensive nature of the current study, Mukherjee *et al* adopted a candidate epitope approach, focusing solely on 7 peptides encompassing 6 epitopes that they identified using bioinformatics (37). However it seems probable that the algorithm that they used to identify likely I-A^{g7} peptide-binding motifs in murine IGRP was inadequate. This is evidenced both by its failure to predict the 7 novel

epitopes uncovered in the current study, which were consequently not previously tested, and by the fact that half of the peptides that the algorithm predicted to contain I-A^{g7} epitopes failed to elicit a recall response in immunized animals (37).

Given the apparent promiscuity of peptide binding to I-A^{g7} (50), and known difficulties in deriving algorithms capable of accurately predicting all MHC class II epitopes within an antigen (51), in the current study we instead strove to take an unbiased approach. To achieve this we initially used membrane fractions containing full-length recombinant IGRP in order to mimic as closely as possible the natural processing of the antigen, and screened the resulting induced responses with a systematic library of overlapping peptides. The library was originally designed to have sequential peptides overlapping by 10 residues in order to include all possible combinations of core nonamers. However due to the highly hydrophobic nature of the antigen it was modified slightly based upon the recommendations of the manufacturer and so contained 5 pairs of peptides that overlap by only 8 residues and 1 pair by 9 (supplementary table 1). While these gaps in coverage cannot directly account for our inability to detect the previously reported IGRP₄₋₂₂ and IGRP₁₉₅₋₂₁₄ epitopes, it is known that residues flanking the core sequences can also significantly influence peptide binding to MHC class II molecules (52, 53). This raises the possibility that our library design did not achieve the comprehensive coverage that we envisaged, and that a greater degree of overlap between sequential peptides, was in fact required. Thus our inability to detect the previously reported epitopes could be explained if it is assumed that more than one or two N or C terminal flanking residues are required to provide sufficient binding affinity to activate autoreactive T cells specific to these sequences under the conditions we used. The requirement for additional flanking residues beyond the central core may be particularly relevant in the context of I-A^{g7}, which is generally regarded as a poor peptide binder (54), and thus the choice of overlapping 16–18mers is a potential limitation of the approach we used. Similarly, our ability to detect responses to low affinity peptides may have been compromised by the decision to use a relatively modest concentration of peptide (20 μg/ml), which was selected in view of the relative insolubility of many of the peptides within the library, and the necessity of limiting the concentration of DMSO added. Nonetheless, our overall experimental design was generally successful, identifying 7 previously unreported epitopes in IGRP, at least 3 of which may be relevant to disease. However, it is also apparent that the approaches used in the both the previous and the current studies (*in silico* prediction and library screening respectively) each have distinct limitations, and consequently we believe that the results obtained should be regarded as essentially complementary, rather than contradictory, in nature.

At first glance, perhaps more surprising than the aforementioned apparent unresponsiveness of either P1, P2, P26, or P27 in our recall experiments was the fact that, although we were able to replicate the previous observation that the epitope contained within IGRP₁₂₃₋₁₄₅ induces responses in immunized animals, in contrast to the previous report (37), we did not conclude that there were spontaneous responses to this peptide in either pre-diabetic or newly diabetic mice. However, a more detailed analysis reveals that this apparent discrepancy is merely a consequence of differences in the cut-off for positivity chosen between the two studies. For the current study we selected a stimulation index of 3 as a cut-off value, and hence regarded the SIs of 2.0–2.5 that we obtained for P17 in our analysis of spontaneous proliferation as negative. However, Mukherjee and colleagues only observed levels of proliferation to the equivalent peptides in un-immunized animals that exceeded our cut-off at the highest peptide concentrations they tested (50–100 μg/ml), and obtained similar values to our own at equivalent doses (37). Thus our results are actually in agreement. Given that peptides that elicit the greatest spontaneous responses at the lowest doses likely contain the most immunodominant epitopes, we would conclude that the newly described P8, P36,

and P40/41 epitopes should therefore be considered dominant, whilst P17 be classified as sub-dominant.

In the current study none of the tested peptides elicited spontaneous proliferative responses significantly above background in CD4⁺ T cells isolated from the spleens of 4–12 week old “pre-diabetic” female NOD mice, despite several giving positive signals in the PLNs of the same animals. In contrast, 4/9 peptides induced SI values >3 for CD4⁺ T cells isolated from the spleens of newly diabetic animals. One possible explanation for this dichotomy is that it merely reflects increasing accumulation of IGRP-reactive T cells in the spleen during diabetogenesis (47, 48). However an alternative, non-mutually exclusive, explanation is that it reflects a change in the quality of the autoreponse. For human CD4⁺ T cells reactive with an HLA-DR4 restricted epitope in GAD65 a process of avidity maturation has been observed (55), and similarly for spontaneous CD8⁺ T cell responses to IGRP in NOD mice (56), suggesting the possibility that a similar process also occurs for CD4⁺ responses to the same antigen in these animals. More detailed analysis of the frequency of individual T cell receptors specific to these epitopes will be required to resolve this issue.

A key question that remains unanswered by the current study is the role that CD4⁺ T cells specific to the newly identified IGRP epitopes play in diabetogenesis in NOD mice. As stated above, the fact that NOD mice genetically ablated for IGRP expression develop overt disease with kinetics and incidence equivalent to wild type animals (31), indicate that neither IGRP-specific CD8⁺ nor CD4⁺ T cells are essential to diabetogenesis. However this does not preclude them from playing a significant, albeit redundant, role in progression, as opposed to initiation, of the “natural” disease. Indeed T cells specific to other redundant islet autoantigens such as IAPP can cause accelerated disease when adoptively transferred into young NOD recipients (57), and we anticipate that this will also prove to be the case for T cells specific to the spontaneously reactive epitopes now identified. Such investigations were beyond the scope of the current study, but our preliminary data that indicate that IFN- γ secreting cells specific for P8 can be detected within islet infiltrates of prediabetic mice (not shown) lend credence to this hypothesis. Similarly, the ability of other IGRP derived peptides that bind I-A^{g7} to prevent spontaneous T1D in NOD mice (37) also suggests that IGRP-specific CD4⁺ T cells might play a significant role. In contrast to the other epitopes, the likely core nonamer of P8 [IGRP_{57–65}] (58) is completely conserved between human and mouse, raising the possibility that it might also be targeted by human T cells restricted to the susceptibility molecule HLA-DQ8, which has a similar peptide specificity (59). Experiments to address this possibility are currently underway, and if successful, could provide an additional therapeutic target in man, which is the ultimate goal of this study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Bluestone JA, Herold K, Eisenbarth G. Genetics, pathogenesis and clinical interventions in type 1 diabetes. *Nature*. 2010; 464:1293–300. [PubMed: 20432533]

2. Libman I, Songer T, LaPorte R. How many people in the U.S have IDDM? *Diabetes Care*. 1993; 16:841–2. [PubMed: 8495628]
3. Patterson CC, Dahlquist GG, Gyurus E, Green A, Soltesz G. Incidence trends for childhood type 1 diabetes in Europe during 1989–2003 and predicted new cases 2005–20: a multicentre prospective registration study. *Lancet*. 2009; 373:2027–33. [PubMed: 19481249]
4. Soltesz G, Patterson CC, Dahlquist G. Worldwide childhood type 1 diabetes incidence--what can we learn from epidemiology? *Pediatr Diabetes*. 2007; 8 (Suppl 6):6–14. [PubMed: 17727380]
5. Todd JA. Etiology of type 1 diabetes. *Immunity*. 2010; 32:457–67. [PubMed: 20412756]
6. Atkinson MA, Bluestone JA, Eisenbarth GS, Hebrok M, Herold KC, Accili D, et al. How does type 1 diabetes develop?: the notion of homicide or beta-cell suicide revisited. *Diabetes*. 2011; 60:1370–9. [PubMed: 21525508]
7. Roep BO. The role of T-cells in the pathogenesis of Type 1 diabetes: from cause to cure. *Diabetologia*. 2003; 46:305–21. [PubMed: 12687328]
8. van Belle TL, Coppieters KT, von Herrath MG. Type 1 diabetes: etiology, immunology, and therapeutic strategies. *Physiol Rev*. 2011; 91:79–118. [PubMed: 21248163]
9. Kaufman A, Herold KC. Anti-CD3 mAbs for treatment of type 1 diabetes. *Diabetes Metab Res Rev*. 2009; 25:302–6. [PubMed: 19319985]
10. Chatenoud L. Immune therapy for type 1 diabetes mellitus-what is unique about anti-CD3 antibodies? *Nat Rev Endocrinol*. 2010; 6:149–57. [PubMed: 20173776]
11. Peakman M, von Herrath M. Antigen-specific immunotherapy for type 1 diabetes: maximizing the potential. *Diabetes*. 2010; 59:2087–93. [PubMed: 20805382]
12. Shoda LK, Young DL, Ramanujan S, Whiting CC, Atkinson MA, Bluestone JA, et al. A comprehensive review of interventions in the NOD mouse and implications for translation. *Immunity*. 2005; 23:115–26. [PubMed: 16111631]
13. Nakayama M, Abiru N, Moriyama H, Babaya N, Liu E, Miao D, et al. Prime role for an insulin epitope in the development of type 1 diabetes in NOD mice. *Nature*. 2005; 435:220–3. [PubMed: 15889095]
14. von Herrath M, Sanda S, Herold K. Type 1 diabetes as a relapsing-remitting disease? *Nat Rev Immunol*. 2007; 7:988–94. [PubMed: 17982429]
15. Cobbold SP, Adams E, Nolan KF, Regateiro FS, Waldmann H. Connecting the mechanisms of T-cell regulation: dendritic cells as the missing link. *Immunol Rev*. 2010; 236:203–18. [PubMed: 20636819]
16. DiLorenzo TP. Multiple antigens versus single major antigen in type 1 diabetes: arguing for multiple antigens. *Diabetes Metab Res Rev*. 2011; 27:778–83. [PubMed: 22069259]
17. Shieh JJ, Pan CJ, Mansfield BC, Chou JY. The islet-specific glucose-6-phosphatase-related protein, implicated in diabetes, is a glycoprotein embedded in the endoplasmic reticulum membrane. *FEBS Lett*. 2004; 562:160–4. [PubMed: 15044018]
18. Arden SD, Zahn T, Steegers S, Webb S, Bergman B, O'Brien RM, et al. Molecular cloning of a pancreatic islet-specific glucose-6-phosphatase catalytic subunit-related protein. *Diabetes*. 1999; 48:531–42. [PubMed: 10078553]
19. Efrat S, Linde S, Kofod H, Spector D, Delannoy M, Grant S, et al. Beta-cell lines derived from transgenic mice expressing a hybrid insulin gene-oncogene. *Proc Natl Acad Sci U S A*. 1988; 85:9037–41. [PubMed: 2848253]
20. Neophytou PI, Muir EM, Hutton JC. A subtractive cloning approach to the identification of mRNAs specifically expressed in pancreatic beta-cells. *Diabetes*. 1996; 45:127–33. [PubMed: 8549854]
21. Bouatia-Naji N, Rocheleau G, Van Lommel L, Lemaire K, Schuit F, Cavalcanti-Proenca C, et al. A polymorphism within the G6PC2 gene is associated with fasting plasma glucose levels. *Science*. 2008; 320:1085–8. [PubMed: 18451265]
22. Dos Santos C, Bougneres P, Fradin D. A single-nucleotide polymorphism in a methylatable Foxa2 binding site of the G6PC2 promoter is associated with insulin secretion in vivo and increased promoter activity in vitro. *Diabetes*. 2009; 58:489–92. [PubMed: 18984742]

23. Wang Y, Martin CC, Oeser JK, Sarkar S, McGuinness OP, Hutton JC, et al. Deletion of the gene encoding the islet-specific glucose-6-phosphatase catalytic subunit-related protein autoantigen results in a mild metabolic phenotype. *Diabetologia*. 2007; 50:774–8. [PubMed: 17265032]
24. Heni M, Ketterer C, Hart LM, Ranta F, van Haeften TW, Eekhoff EM, et al. The impact of genetic variation in the G6PC2 gene on insulin secretion depends on glycemia. *J Clin Endocrinol Metab*. 2010; 95:E479–84. [PubMed: 20826583]
25. Hu C, Zhang R, Wang C, Yu W, Lu J, Ma X, et al. Effects of GCK, GCKR, G6PC2 and MTNR1B variants on glucose metabolism and insulin secretion. *PLoS One*. 2010; 5:e11761. [PubMed: 20668700]
26. Nagata M, Santamaria P, Kawamura T, Utsugi T, Yoon JW. Evidence for the role of CD8+ cytotoxic T cells in the destruction of pancreatic beta-cells in nonobese diabetic mice. *J Immunol*. 1994; 152:2042–50. [PubMed: 7907110]
27. Santamaria P, Utsugi T, Park BJ, Averill N, Kawazu S, Yoon JW. Beta-cell-cytotoxic CD8+ T cells from nonobese diabetic mice use highly homologous T cell receptor alpha-chain CDR3 sequences. *J Immunol*. 1995; 154:2494–503. [PubMed: 7868915]
28. Lieberman SM, Evans AM, Han B, Takaki T, Vinnitskaya Y, Caldwell JA, et al. Identification of the beta cell antigen targeted by a prevalent population of pathogenic CD8+ T cells in autoimmune diabetes. *Proc Natl Acad Sci U S A*. 2003; 100:8384–8. [PubMed: 12815107]
29. Anderson B, Park BJ, Verdager J, Amrani A, Santamaria P. Prevalent CD8(+) T cell response against one peptide/MHC complex in autoimmune diabetes. *Proc Natl Acad Sci U S A*. 1999; 96:9311–6. [PubMed: 10430939]
30. Han B, Serra P, Amrani A, Yamanouchi J, Maree AF, Edelstein-Keshet L, et al. Prevention of diabetes by manipulation of anti-IGRP autoimmunity: high efficiency of a low-affinity peptide. *Nat Med*. 2005; 11:645–52. [PubMed: 15908957]
31. Oeser JK, Parekh VV, Wang Y, Jegadeesh NK, Sarkar SA, Wong R, et al. Deletion of the G6pc2 gene encoding the islet-specific glucose-6-phosphatase catalytic subunit-related protein does not affect the progression or incidence of type 1 diabetes in NOD/ShiLtJ mice. *Diabetes*. 2011; 60:2922–7. [PubMed: 21896930]
32. Ouyang Q, Standifer NE, Qin H, Gottlieb P, Verchere CB, Nepom GT, et al. Recognition of HLA class I-restricted beta-cell epitopes in type 1 diabetes. *Diabetes*. 2006; 55:3068–74. [PubMed: 17065344]
33. Standifer NE, Ouyang Q, Panagiotopoulos C, Verchere CB, Tan R, Greenbaum CJ, et al. Identification of Novel HLA-A*0201-restricted epitopes in recent-onset type 1 diabetic subjects and antibody-positive relatives. *Diabetes*. 2006; 55:3061–7. [PubMed: 17065343]
34. Mallone R, Martinuzzi E, Blancou P, Novelli G, Afonso G, Dolz M, et al. CD8+ T-cell responses identify beta-cell autoimmunity in human type 1 diabetes. *Diabetes*. 2007; 56:613–21. [PubMed: 17327428]
35. Jarchum I, Nichol L, Trucco M, Santamaria P, DiLorenzo TP. Identification of novel IGRP epitopes targeted in type 1 diabetes patients. *Clin Immunol*. 2008; 127:359–65. [PubMed: 18358785]
36. Martinuzzi E, Novelli G, Scotto M, Blancou P, Bach JM, Chaillous L, et al. The frequency and immunodominance of islet-specific CD8+ T-cell responses change after type 1 diabetes diagnosis and treatment. *Diabetes*. 2008; 57:1312–20. [PubMed: 18305140]
37. Mukherjee R, Wagar D, Stephens TA, Lee-Chan E, Singh B. Identification of CD4+ T cell-specific epitopes of islet-specific glucose-6-phosphatase catalytic subunit-related protein: a novel beta cell autoantigen in type 1 diabetes. *J Immunol*. 2005; 174:5306–15. [PubMed: 15843527]
38. Yang J, Danke NA, Berger D, Reichstetter S, Reijonen H, Greenbaum C, et al. Islet-specific glucose-6-phosphatase catalytic subunit-related protein-reactive CD4+ T cells in human subjects. *J Immunol*. 2006; 176:2781–9. [PubMed: 16493034]
39. Nielsen M, Lund O, Buus S, Lundegaard C. MHC class II epitope predictive algorithms. *Immunology*. 2010; 130:319–28. [PubMed: 20408898]
40. Schneider I. Cell lines derived from late embryonic stages of *Drosophila melanogaster*. *J Embryol Exp Morphol*. 1972; 27:353–65. [PubMed: 4625067]

41. Bunch TA, Grinblat Y, Goldstein LS. Characterization and use of the *Drosophila* metallothionein promoter in cultured *Drosophila melanogaster* cells. *Nucleic Acids Res.* 1988; 16:1043–61. [PubMed: 3125519]
42. Kelemen K, Gottlieb PA, Putnam AL, Davidson HW, Wegmann DR, Hutton JC. HLA-DQ8-associated T cell responses to the diabetes autoantigen phogrin (IA-2 beta) in human prediabetes. *J Immunol.* 2004; 172:3955–62. [PubMed: 15004204]
43. Oi VT, Jones PP, Goding JW, Herzenberg LA. Properties of monoclonal antibodies to mouse Ig allotypes, H-2, and Ia antigens. *Curr Top Microbiol Immunol.* 1978; 81:115–20. [PubMed: 567555]
44. Shamel A, Yamanouchi J, Thiessen S, Santamaria P. Endoplasmic reticulum stress caused by overexpression of islet-specific glucose-6-phosphatase catalytic subunit-related protein in pancreatic Beta-cells. *Rev Diabet Stud.* 2007; 4:25–32. [PubMed: 17565413]
45. Boitard C, Bendelac A, Richard MF, Carnaud C, Bach JF. Prevention of diabetes in nonobese diabetic mice by anti-I-A monoclonal antibodies: transfer of protection by splenic T cells. *Proc Natl Acad Sci U S A.* 1988; 85:9719–23. [PubMed: 3264405]
46. Burtles SS, Trembleau S, Drexler K, Hurtenbach U. Absence of T cell tolerance to pancreatic islet cells. *J Immunol.* 1992; 149:2185–93. [PubMed: 1355504]
47. Bendelac A, Carnaud C, Boitard C, Bach JF. Syngeneic transfer of autoimmune diabetes from diabetic NOD mice to healthy neonates. Requirement for both L3T4+ and Lyt-2+ T cells. *J Exp Med.* 1987; 166:823–32. [PubMed: 3309126]
48. Wicker LS, Miller BJ, Mullen Y. Transfer of autoimmune diabetes mellitus with splenocytes from nonobese diabetic (NOD) mice. *Diabetes.* 1986; 35:855–60. [PubMed: 3525284]
49. Li R, Perez N, Karumuthil-Meilethil S, Vasu C. Bone marrow is a preferential homing site for autoreactive T-cells in type 1 diabetes. *Diabetes.* 2007; 56:2251–9. [PubMed: 17596402]
50. Stratmann T, Apostolopoulos V, Mallet-Designé V, Corper AL, Scott CA, Wilson IA, et al. The I-Ag7 MHC class II molecule linked to murine diabetes is a promiscuous peptide binder. *J Immunol.* 2000; 165:3214–25. [PubMed: 10975837]
51. Gowthaman U, Agrewala JN. In silico tools for predicting peptides binding to HLA-class II molecules: more confusion than conclusion. *J Proteome Res.* 2008; 7:154–63. [PubMed: 18034454]
52. Moudgil KD, Sercarz EE, Grewal IS. Modulation of the immunogenicity of antigenic determinants by their flanking residues. *Immunol Today.* 1998; 19:217–20. [PubMed: 9613039]
53. O'Brien C, Flower DR, Feighery C. Peptide length significantly influences in vitro affinity for MHC class II molecules. *Immunome Res.* 2008; 4:6. [PubMed: 19036163]
54. Carrasco-Marin E, Shimizu J, Kanagawa O, Unanue ER. The class II MHC I-Ag7 molecules from non-obese diabetic mice are poor peptide binders. *J Immunol.* 1996; 156:450–8. [PubMed: 8543793]
55. Standifer NE, Burwell EA, Gersuk VH, Greenbaum CJ, Nepom GT. Changes in autoreactive T cell avidity during type 1 diabetes development. *Clin Immunol.* 2009; 132:312–20. [PubMed: 19482555]
56. Amrani A, Verdager J, Serra P, Tafuro S, Tan R, Santamaria P. Progression of autoimmune diabetes driven by avidity maturation of a T-cell population. *Nature.* 2000; 406:739–42. [PubMed: 10963600]
57. Delong T, Baker RL, Reisdorph N, Reisdorph R, Powell RL, Armstrong M, et al. Islet amyloid polypeptide is a target antigen for diabetogenic CD4+ T cells. *Diabetes.* 2011; 60:2325–30. [PubMed: 21734016]
58. Chang KY, Suri A, Unanue ER. Predicting peptides bound to I-Ag7 class II histocompatibility molecules using a novel expectation-maximization alignment algorithm. *Proteomics.* 2007; 7:367–77. [PubMed: 17211830]
59. Suri A, Walters JJ, Gross ML, Unanue ER. Natural peptides selected by diabetogenic DQ8 and murine I-A(g7) molecules show common sequence specificity. *J Clin Invest.* 2005; 115:2268–76. [PubMed: 16075062]

Figure 1. Expression of epitope-tagged IGRP in stably transfected insect cells

Parental and IGRP transfected S2 insect cells were induced with CuSO_4 and fractionated as described in methods. Aliquots of whole cells (WC) and the respective membrane fractions (MF) were analyzed by SDS-PAGE and western blotting. **A.** Schematic representation of IGRP showing the predicted 9 transmembrane helices and single N-linked oligosaccharide (18). The C-terminal V5-His epitope tag is indicated by the white rectangle. **B.** Transferred proteins were detected with Ponceau S. **C.** Epitope tagged IGRP was detected using HRP-conjugated anti-V5 and enhanced chemiluminescence.

Figure 2. Recall responses to immunization with membrane fractions from IGRP-expressing S2 cells

Male NOD mice were immunized subcutaneously with membrane fractions from parental (open bars) or IGRP-expressing (closed bars) S2 cells. Ten days later the draining lymph nodes were harvested and lymphocytes cultured for 3 d with the primary immunogens or a library of overlapping peptides spanning the entire 355 aa sequence of IGRP. ^3H -thymidine was added for the final 16h, and incorporated radiolabel determined by liquid scintillation counting. A representative experiment is shown, with results expressed as stimulation indices (SI) relative to the DMSO (vehicle) control. An SI of > 3.0 (dotted lines) was considered positive. The inset illustrates the distribution of the peptides relative to the predicted transmembrane regions (shaded bars) of IGRP.

Figure 3. Blockade of recall responses by treatment by with anti-I-A^{g7}

Lymphocytes from the draining nodes of immunized NOD mice were cultured for 3 d in the presence of selected peptides, vehicle, or concanavalin-A (Con A) either in media alone (open bars), media + anti-I-A^{g7} (hatched bars), or media + isotype control (filled bars) as described in methods. ³H-thymidine was added for the final 16h, and incorporated radiolabel determined by liquid scintillation counting.

Figure 4. Spontaneous responses to IGRP peptides in pre-diabetic and newly diabetic NOD mice
Lymphocytes were harvested from the pancreatic draining lymph nodes (open bars), peripheral lymph nodes (horizontally striped bars), or spleen (filled bars) of 12–14 week old female NOD mice, or spleen (diagonally striped bars) of a newly diabetic mouse. Cells were cultured with IGRP peptides or human insulin as described in methods, with ^3H -thymidine added for the final 16h. A representative experiment is shown, with results expressed as stimulation indices (SI) relative to the DMSO (vehicle) control. An SI of > 3.0 (dotted line) was considered positive.