Igrp and insulin vaccination induce CD8$^+$ T cell mediated autoimmune diabetes in the RIP-CD80GP mouse

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Summary

Autoimmune diabetes is characterized by autoantigen-specific T cell-mediated destruction of pancreatic islet beta cells and $CD8^+$ T cells are key players during this process. We assessed whether the bitransgenic RIP-CD80 x RIP-LCMV-GP (RIP-CD80GP) mice may be a versatile antigen specific model of inducible $CD8^+$ T cell mediated autoimmune diabetes. Antigen-encoding DNA, peptide loaded dendritic cells, and antigen plus incomplete Freund's adjuvant were used for vaccination. Of 14 pancreatic proteins tested by DNA vaccination, murine pre-proinsulin 2 (100% of mice; median time after vaccination, 60 days), and Igrp (77%, 58 days) could induce diabetes. DNA vaccination with zinc transporter 8, Ia-2, Ia-2β, Gad67, Chromogranin A, IAPP, and Nkx2.2 induced diabetes development in 25-33% of mice, and with Gad65, Sgne1, Pdx1, Cel, glucagon, and control HBsAg in <20% of mice. Diabetes induction efficiency could be increased by DNA vaccination with a vector encoding a ubiquitin-antigen fusion construct. Diabetic mice had florid T cell islet infiltration. $CD8^+$ T cell targets of Igrp were identified with a peptide library based ELISpot assay, and diabetes could also be induced by vaccination with MHC class I restricted Igrp peptides loaded on mature dendritic cells. Vaccination with antigen plus incomplete Freund's adjuvant, which can prevent diabetes in other models, led to rapid diabetes development in the RIP-CD80GP mouse. We conclude that RIP-CD80GP mice are a versatile model of antigen specific autoimmune diabetes

and may complement existing mouse models of autoimmune diabetes for evaluating $CD8^+$ T cell-targeted prevention strategies.

Introduction

In type 1 diabetes (T1D), a self-directed immune response leads to the destruction of insulin producing beta cells in the pancreatic islets. There is good evidence from animal models of disease that autoreactive T cells are the critical effectors of beta cell destruction [1-4]. Moreover, studies with samples from cadaveric T1D donors show that pancreatic infiltrates contain and are sometimes dominated by $CD8^+$ T cells [5-10], and include beta cell antigen specific cells [11]. Furthermore, insulin directed $CD8^+$ T cells cloned from T1D patients can efficiently kill autoantigen expressing cells [12].

The relevance of $CD8^+$ T cells in human T1D is currently poorly represented in animal models. The RIP-CD80 mouse model, established almost two decades ago, transgenically expresses the CD80 costimulatory molecule on islet beta cells to facilitate a break in self tolerance when $CD8^+$ T cells directed against a beta cell specific self protein are present [13]. Sole vaccination with plasmid DNA encoding for murine or human insulin is sufficient to induce $CD8^+$ T cell mediated beta cell destruction and diabetes development [14]. Diabetes induction upon DNA vaccination with insulin is independent of $CD4^+$ T cells in this model but critically depends on IFNγ secreting diabetogenic $CD8^+$ T cells that specifically recognize the K^b restricted insulin A-chain epitope $A_{12\text{-}21}$ [15]. RIP-CD80 mice that are crossbred to RIP-LCMV-GP [16] transgenic mice (RIP-CD80GP) can additionally be rendered diabetic by vaccination with a plasmid encoding for the

neo-self antigen lymphocytic choriomeningitis virus glycoprotein (LCMV-GP) and also show a higher sensitivity towards insulin DNA vaccination [17].

Here, we examined the versatility of the RIP-CD80GP mouse as a model of antigen induced $CD8^+$ T cell-mediated autoimmune diabetes. We screened a set of additional islet proteins for their potential to induce diabetes upon DNA vaccination, multiple antigen vaccination strategies, and exemplarily tested targeting to the antigen processing machinery.

Material and Methods

Mice - The generation of RIP-murine CD80 (B7.1) transgenic mice backcrossed for >15 generations to the C57BL/6 (H-2^b) background as well as the generation of RIP-LCMV-GP on the C57BL/6 background has been previously described [13, 16]. For experimental studies, heterozygous F1 animals (RIP-CD80 x RIP-LCMV-GP), single transgenic RIP-CD80 or C57BL/6 were used. All animal studies were conducted with institutional board approval in accordance with the Federal German Animal Protection Law (55.2-1-54-2531-154-08; 24-9168.11-1/2010-39).

Construction of DNA Expression Vectors - Antigens were selected on the basis of known targets of autoantibodies in patients with type 1 diabetes and their pancreatic islet-specific expression and abundance [18]. cDNA of pancreatic islet antigens was generated by RT-PCR from C57BL/6 murine islet cell RNA. Subsequently for each antigen encoding cDNA PCR primers containing appropriate restriction sites as well as a uniform Kozak consensus GTAGGCATG were used to allow ligation of the PCR product into the multiple cloning site of pcDNA3.1V5HisB (Invitrogen). NotI/XbaI restriction sites were used for cloning of Iapp (GENE ID: 15874 Iapp; 93 amino acids), ChgA (GENE ID: 12652 Chga; 463aa), Igrp (GENE ID: 14378 G6pc2; 355aa), Nkx2.2 (GENE ID: 18088 Nkx2-2; 273aa), Gad67 (GENE ID: 14415 Gad1; 593aa), Cel (GENE ID: 12613 Cel; 599aa), Pdx-1 (GENE ID: 18609 Pdx1; 284aa), ppIns2 (GENE ID: 16334 Ins2; 110aa), Gad65 (GENE ID: 14417 Gad2; 585aa), Ia-2 i.c. (GENE ID: 19275 Ptprn;

381), Ia-2β i.c. (GENE ID: 19276 Ptprn2; 379). NotI/BstBI restriction sites were used for cloning of HBsAg (HBsAg AS1-AS226 EF103285), ZnT8 (GENE ID: 239436 Slc30a8; 367aa), Sgne 1 (GENE ID: 20394 Scg5; 212aa), LCMV-GP (LCMV-GP AS1-AS498, NM _AF186080), and Glucagon (GENE ID: 14526 Gcg; 180aa). All antigen encoding vectors used in this study are based on the pcDNA3.1 V5HisB backbone except for pCI/ppinsN110A which was kindly provided by Reinhold Schirmbeck, Ulm, Germany, and uses the pCI backbone. The construction of pCI/ppinsN110A has been previously described [19]. For cloning of Ia-2 and Ia-2β intracelluar region (i.c.) constructs, start codons were introduced into the sense primers accordingly. The Ia-2 i.c. construct encodes for Ia-2 amino acids 599-979, the Ia-2β i.c. construct encodes for amino acids 623-1001. For construction of Ubiquitin-ZnT8 (Ubi-ZnT8) or IgG-signal peptide (IgG-SP) fusion containing plasmids, ubiquitin (GENE ID: 22190 Ubc; 76aa) or IgG-SP (GENE ID: 692179 aa1-21) encoding DNA was PCR amplified from murine islet cDNA with primers that allow homologous recombination of ubiquitin or IgG-SP with the above mentioned ZnT8 construct upstream of ZnT8. For the Ubi-ZnT8 encoding construct the antisense primer was designed to remove the start codon of ZnT8 and to introduce a G76V mutation in ubiquitin resulting in stable ubiquitin fusion to ZnT8 after translation. Subsequently, Red/ET recombination technology [20] was carried out using recombineering proficient competent *E.coli* GB2005 harboring the pSC101-BAD-gbaA plasmid, as previously described [21]. Primer details are listed in the supplementary material. Construct driven expression of

protein was tested by transfection in HEK293FT cells and western blot (Supplementary data).

Vaccination and follow-up – For DNA vaccination, 50 µg of plasmid DNA (dissolved in 50 µl saline) was administered intramuscularly into each tibialis anterior muscle of 10 to 12 week-old male or female mice. No pretreatment or adjuvants were used. For peptide vaccination, bone marrow cells were isolated from B6.129S7-Rag^{tm1Mom}/J mice, cultivated in medium containing IL-4 and GM-CSF and stimulated with LPS from day 5. On day 6, fractions of cells were incubated for 1 hr in medium containing 10 µM of the respective peptides (0.1 µM in case of LCMV-GP$_{33-41}$). Cells were washed in PBS and intraperitoneally injected at 200,000 cells/per mouse. LCMV-GP$_{33-41}$ (KAVYNFATM), LCMV-GP$_{276-286}$ (SGVENPGGYCL), Igrp$_{225-233}$ (LRLFGIDLL), Igrp$_{241-249}$ (KWCANPDWI) and LCMV-NP$_{396-404}$ (FQPQNGQFI) were synthesized by Bio-Synthesis (Lewisville, TX, USA). For immunization with antigen and incomplete Freunds adjuvant (IFA), mice (n=24) were injected subcutaneously with 100 µg insulin (human, recombinant, Sigma-Aldrich, 91077C) dissolved in water (1 mg/ml) and emulsified 1:1 in IFA (Sigma-Aldrich, F5506) or with IFA alone at 4, 5, 6, and 7 weeks of age. At age 11 weeks, the mice received 100 µg ppIns2 DNA as described or were left untreated. In all mice, urine glucose levels were measured twice weekly (Diastix, Bayer HealthCare LLC). Diabetes was defined as two consecutive urine glucose values >5.5 mmol/l and blood glucose levels >13.9

mmol/l (Glucometer Elite, Bayer Diagnostics). If not stated differently, mice were followed for 140 days post final vaccination.

Histology - Pancreatic cryosections (5 µm) were fixed in 4% formalin and stained for c-peptide or glucagon using polyclonal rabbit anti-c-peptide (Cell Signaling) or polyclonal rabbit anti-glucagon (Dako) followed by Alexa Fluor 488 labeled polyclonal goat anti-rabbit IgG (Invitrogen) antibodies. Subsequently detection of CD4 or CD8 was carried out using rat anti-CD4 (clone RM4-5) or rat anti-CD8 antibodies (clone 53-6.7; both BD Pharmingen) followed by staining with Alexa Fluor 568 labeled polyclonal goat anti-rat IgG secondary antibodies (Invitrogen). DAPI staining of DNA was used to visualize cell nuclei. All images were acquired with a Leica SP5 upright Laser Scanning confocal microscope.

ELISpot assays – IFNγ ELISpot assays were carried out with purified $CD8^+$ T cells from vaccinated mice and T cell depleted splenocytes as antigen presenting cells according to the previously described protocol [22]. In short, $CD8^+$ T cells were pre-enriched from cells of spleen, popliteal and pancreatic lymph nodes using MACS technology and $CD8^+$ T cells were sorted to high purity on a BD Aria II Sorp. For antigen presentation, splenocytes from healthy RIP-CD80 mice were freshly isolated and depleted of T cells using biotinylated anti-CD3 (clone 145-2C11), anti-CD4 (clone GK1.5) and anti-CD8 antibodies (clone 53-6.7, all eBioscience) followed by streptavidin coupled microbeads and MACS LD separation columns (Milteny Biotech). Per well, 1.8×10^5 antigen presenting cells

were used together with 3 x 10^4 purified viable $CD8^+$ T cells. Igrp 15mer library peptides were synthesized in screening grade by Mimotopes (Clayton Victoria, Australia), peptides were dissolved with DMSO and used at a concentration of 10 µg/ml. GP_{33-41} (KAVYNFATM) and $ppIns_{101-110}$ (A_{12-21}) (SLYQLENYCN) peptide were purchased from PANATecs (Tübingen, Germany) at a purity >98% and used in the assay at a final concentration of 10 µg/ml. Stimulation with anti-CD3/anti-CD28/anti-CD137 (Invitrogen) coupled beads was used as positive control. Incubation was for 24 hours in 96-well ELISpot MultiScreen Filter plates (Merck Millipore) and IFNγ specific antibody pairs from U-Cytech (Utrecht, The Netherlands) were used for spot detection. Plates were processed according to manufacturer's instructions and as previously described [22].

Statistical analysis – Prism 5.03 GraphPad Software was used for statistical analyses. Time to event analysis (Kaplan-Meier) was used for estimating diabetes-free survival in mice. Comparisons of survival curves between groups were made using the log rank test. For all analyses, a two-tailed p value <0.05 was considered significant. P values were not corrected for multiple comparisons.

Results

DNA vaccination identifies Igrp as diabetogenic antigen in RIP-CD80GP mice

As previously reported, vaccination with a construct encoding for mutated murine pre-proinsulin 2 (mut ppIns, n=12 mice) or the model autoantigen LCMV-GP (n=17 mice) led to diabetes in all vaccinated RIP-CD80GP mice after a median of 18 days (Fig.1A). In contrast, only one of 16 mice developed diabetes upon vaccination with a vector encoding for the non-endogenously expressed Hepatitis B surface antigen (HBsAg).

A further 14 antigens found in pancreatic islets were tested. Seven of these were secretory granule proteins (Fig.1B). DNA vaccination with vectors encoding for wild type pre-proinsulin 2 induced diabetes in all mice (ppIns; n=10; $p<0.0001$ as compared to HBsAg control) at a median of 60 days post vaccination. Of note, vectors encoding for wild type and mutated pre-proinsulin 2 also induced diabetes in single transgenic RIP-CD80 mice, although with delayed disease onset and at a lower frequency (40% and 50%, respectively, data not shown). In RIPCD80GP, cases of diabetes were also observed in mice vaccinated with constructs encoding for zinc transporter 8 (ZnT8; 3 of 12 mice), the intracellular domain of the receptor tyrosine phosphatase family members Ia-2 or Ia-2β (Ia-2 i.c.; Ia-2β i.c.; each 4 of 16 mice), Chromogranin A (Chga; 3 of 11 mice), or the α-cell peptide hormone glucagon (2 of 12 mice). Frequencies of diabetes were not, however, different to that of the HBsAg control group. Vaccination with Sgne1,

which is highly and specifically expressed in beta cells resulted in no cases of diabetes.

Amongst the 7 non-secretory granule antigens, vaccination with plasmid DNA encoding for the islet specific glucose-6-phosphatase Igrp induced diabetes from as early as 20 days post vaccination; 17 (77%) of 22 Igrp vaccinated animals developed diabetes within the observation period with a diabetes onset comparable to ppIns (median 58 days; $p<0.0001$ as compared to HBsAg control; Fig.1C). Additionally, diabetes development was observed in 25-33% of mice vaccinated with constructs encoding for Gad67 (4 of 12 mice), Iapp (3 of 12 mice), Nkx 2.2 (3 of 12 mice), Gad65 (2 of 12 mice), Cel (2 of 12 mice), and Pdx-1 (1 of 12 mice). Again, frequencies of diabetes were not different to that of the HBsAg control group.

For both ppIns and Igrp-vaccinated mice, florid lymphocyte infiltrates were observed in pancreatic islets along with reduced beta cells within islets at diabetes onset (Fig.1D). Infiltrates contained both $CD8^+$ and $CD4^+$ T cells. Islets were unaffected and remained free of lymphocytes in non-diabetic mice immunized with a low diabetes inducing DNA and sacrificed 140 days after vaccination.

In order to increase diabetogenicity of the low diabetes inducing antigens, we targeted antigen delivery to the antigen processing compartments. To this end we used homologous recombination to either insert a noncleavable ubiquitin or IgG signal peptide upstream of ZnT8 in the ZnT8 encoding DNA vaccination

vector. Five of 12 mice developed diabetes using ER-targeting (p=0.03 vs. HBsAg control) and 7 of 12 mice (p=0.003 vs. HBsAg control) upon DNA vaccination with the vector encoding for the ubiquitin fusion construct (Fig. 1E). We did not test HBsAg with ubiquitin or IgG signal peptide and cannot exclude that the approach may increase diabetes induction by irrelevant antigen.

Identification of Igrp CD8$^+$ T cell targets in DNA vaccinated mice

$CD8^+$ T cells from spleens and lymph nodes of Igrp DNA vaccinated diabetic mice were tested against an Igrp 15mer peptide library to identify peptides targeted by $CD8^+$ T cells upon DNA vaccination with Igrp encoding vector. Strong responses could be detected towards the adjacent 15mer peptides $Igrp_{237-251}$, $Igrp_{241-255}$ that contain the H-$2D^b$ restricted 9mer epitope $Igrp_{241-249}$ (Fig. 2A). Additionally, lower responses were detected against the peptide $Igrp_{245-259}$ that partially overlaps the same epitope and against $Igrp_{273-287}$, which does not contain any previously described epitopes of Igrp. $CD8^+$ T cell responses could also be detected towards the insulin epitope $ppIns_{101-110}$ (A_{12-21}) as well as against the dominant LCMV-GP epitope GP_{33-41} suggesting that antigen-spreading had occurred within these mice. Separately, non-transgenic C57BL/6 mice were immunized with Igrp encoding plasmid and splenocytes tested for Igrp reactivity 7 weeks post vaccination. These mice showed low responses (<10 spots per 100,000 $CD8^+$ T cells) to only few Igrp peptides suggesting that the strong responses observed in Igrp vaccinated, diabetic RIP-CD80GP mice had been amplified in the islet pathology region.

Summarizing, these data suggest that in RIP-CD80GP mice, $Igrp_{241-249}$ is targeted by $CD8^+$ T cells upon vaccination with Igrp encoding DNA and we identify $Igrp_{273-287}$ as new target region of Igrp directed $CD8^+$ T cells.

Diabetes can be induced with Igrp-peptide loaded BMDCs

$Igrp_{225-233}$ and $Igrp_{241-249}$ have been described as H-$2D^b$ restricted epitopes of Igrp [23] and may represent targets of $CD8^+$ T cells in RIP-CD80GP mice. We applied a bone marrow-derived dendritic cell (BMDC) vaccination approach to examine whether diabetes can be initiated by BMDCs pulsed with either of the two epitopes. As reported previously [24], all mice (n=10) vaccinated with BMDCs loaded with immunodominant peptide GP_{33-41} of the model autoantigen LCMV-GP developed diabetes after a median of 8 days (Fig. 2B). Mice vaccinated with BMDC/$GP_{276-286}$ (n=11) developed diabetes after median 53 days. Vaccination with BMDC/$Igrp_{225-233}$ induced diabetes in 8 of 12 mice (p=0.025 as compared to BMDC/$NP_{396-404}$ vaccinated control mice) demonstrating that vaccination with a MHC class I restricted Igrp epitope can suffice to induce diabetes development in RIP-CD80GP mice. Vaccination with BMDC/$Igrp_{241-249}$ led to diabetes development in two mice.

Insulin-IFA immunization does not prevent but induces diabetes in RIP-CD80GP mice

A model in which disease can be induced antigen specifically offers the opportunity to study antigen-specific disease pre- and intervention. Insulin plus

IFA injection can prevent autoimmune diabetes in NOD mice if given at age 4-10 weeks [25, 26]. Young mice received either four vehicle/IFA or insulin/IFA immunizations before standard DNA vaccination with ppIns encoding vector. Six of 7 mice that received vehicle/IFA immunization and all 5 mice that received insulin/IFA immunization developed diabetes after a median of 62 days and 63 days after ppIns DNA vaccination indicating no protection by insulin/IFA immunization (Fig. 3). Control mice that received either vehicle/IFA or insulin/IFA treatment without subsequent ppIns DNA vaccination confirmed that insulin/IFA immunization could induce diabetes in RIP-CD80GP mice; none of 7 vehicle/IFA immunized mice and 4 of 5 insulin/IFA immunized mice developed diabetes ($p=0.0036$).

Discussion

Experimental models of antigen-induced autoimmune diabetes are few, and most involve adoptive transfer of transgenic T cells. Here, we expand the versatility of the RIP-CD80GP mouse as an experimental antigen-induced autoimmune diabetes model. We show that in addition to insulin and the transgenically expressed LCMV-GP protein, the beta cell antigen Igrp strongly induces diabetes in this model, in a manner that is consistent with $CD8^+$ T cell-mediated disease. Moreover, we show that antigen vaccination strategies can be tuned so that other proteins including antigens relevant to human T1D can also induce diabetes in this model. A variety of immunization strategies were effective in inducing

diabetes, demonstrating robustness of disease induction, and similar to spontaneous models of autoimmune diabetes and human T1D, immunization with a single antigen led to spreading of T cell responses to other beta cell antigens.

Similar to the NOD mouse, both insulin and Igrp appear to be strong antigen targets in this experimental model suggesting that they can induce diabetes on diverse MHC backgrounds. Studies have shown that the insulin antigen is likely to be an essential target and Igrp a non-essential target for diabetes in the NOD mouse [27-29]. Of note we saw that insulin- and LCMV-GP-specific $CD8^+$ T cell responses were also generated by vaccination with Igrp. Thus it is possible that targeting insulin and/or the LCMV-GP may also be required for diabetes development in the RIP-CD80GP model. We did not test vaccination with multiple antigens, nor did we examine T cell responses in mice developing diabetes after vaccination with minor antigens, both of which may have been helpful to understand whether insulin is a required target in the RIP-CD80GP model or whether the differences in diabetes observed between antigens reflect variation in antigenicity or ability of effector T cells to destroy beta cells.

A range of vaccination strategies was able to induce diabetes in this model. DNA vaccination with model LCMV-GP antigen and insulin had previously been demonstrated to induce diabetes, as had immunization with the LCMV glycoprotein peptide-loaded dendritic cells [17, 24]. We extended the peptide loaded BMDC to include also Igrp peptides as diabetes inducers. For the peptide-loaded DC vaccination, the peptides with greatest diabetes induction potential did not correlate with the major $CD8^+$ T cell peptide

responses observed after DNA vaccination. It is possible, therefore that the vaccination strategies tested result in diverse immunodominant epitopes. We also found that vaccination with insulin plus IFA, a treatment that protects against diabetes development in the NOD mouse, induced diabetes in this model. It is unclear why insulin plus IFA induced disease in this model and protects against disease in the NOD mouse. Insulin plus IFA is known to induce a strong antibody response [25] and likely a transient inflammatory response. It is therefore possible that the transgenic RIP-CD80GP islets are sensitive to humoral immune factors that promote beta cell death and activation of $CD8^+$ T cells at the pancreas. Additionally, it has been described that peptides emulsified in IFA can trigger effector cell immunity and especially vaccination with longer peptides or short proteins (as is the case for insulin) can promote cytotoxic T cell responses [30]. Thus the RIP-CD80GP mouse could represent a stringent model with respect to identifying strategies that interfere with diabetes induction. Identification of antigen delivery modes that could prevent the development of diabetes by antigen DNA vaccination in this model are expected to have powerful tolerance-inducing mechanisms.

Along with the versatility of the model demonstrated in the study, the RIP-CD80GP model has limitations. Diabetes induction is strongly facilitated by the transgenic expression of the co-stimulatory molecules CD80 specifically on beta cells. This renders the model susceptible to spontaneous diabetes development in 5-16% of older mice ([17] and own observations), and low frequencies of diabetes can be induced by vaccination with irrelevant antigen, possibly as a result of generalized inflammation. Nevertheless, experimental antigen-specific

models have distinct benefits. The inducing antigen is known, thereby providing the ability to intervene against the primary disease-inducing antigen and monitor responses to this antigen. Such models have provided preclinical evaluation of therapies in immune-mediated diseases such as multiple sclerosis and allergy [31, 32]. The RIP-CD80GP model also has the advantage that peptides targeted by $CD8^+$ T cells can be used to induce and potentially drive disease and therefore strategies to interfere with $CD8^+$ T cell-mediated disease can be tested. Finally, in contrast to the NOD mouse and more similar to man, diabetes could be induced equally in both males and females. We therefore propose the experimental autoimmune diabetes RIP-CD80GP mouse to be considered in the repertoire of autoimmune disease models that should be used for preclinical evaluation of immune tolerance-inducing therapies.

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Conflict of Interest

The authors declare no conflict of interest.

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Figure Legends

Figure 1: Diabetes induction by vaccination in RIP-CD80GP mice. Ten to 12 week old mice were vaccinated with DNA vectors encoding for established (mutated ppIns, LCMV-GP) and control (HBsAg) antigens in this model (**A**), secretory granule associated islet proteins (**B**) or other islet proteins (**C**) and followed for diabetes development shown by life table analysis. Numbers of mice vaccinated per group are indicated. Immunohistological images (**D**) of islets from vaccinated non-diabetic mice (Nkx2.2 vaccinated, 140 days post-vaccination), and diabetic mice at diabetes onset (ppIns vaccinated – 61 days after vacciantion, Igrp vaccinated – 45 days after vaccination). Islets from diabetic mice had few c-peptide positive cells and were infiltrated with $CD4^+$ and $CD8^+$ T lymphocytes. Scale bars=50 µm. Vaccination was also performed with antigen targeted to the antigen processing machinery (**E**). Ten to 12 week old RIP-CD80GP mice were vaccinated with standard ZnT8 encoding DNA vector (blue line, n=12), vectors encoding for ubiquitin-ZnT8 (red line, n=12) or IgG-Signal peptide-ZnT8 (green line, n=12) fusion constructs, or vector encoding control HBsAg (black line, n=16). Mice were followed for diabetes development shown as a life table analysis.

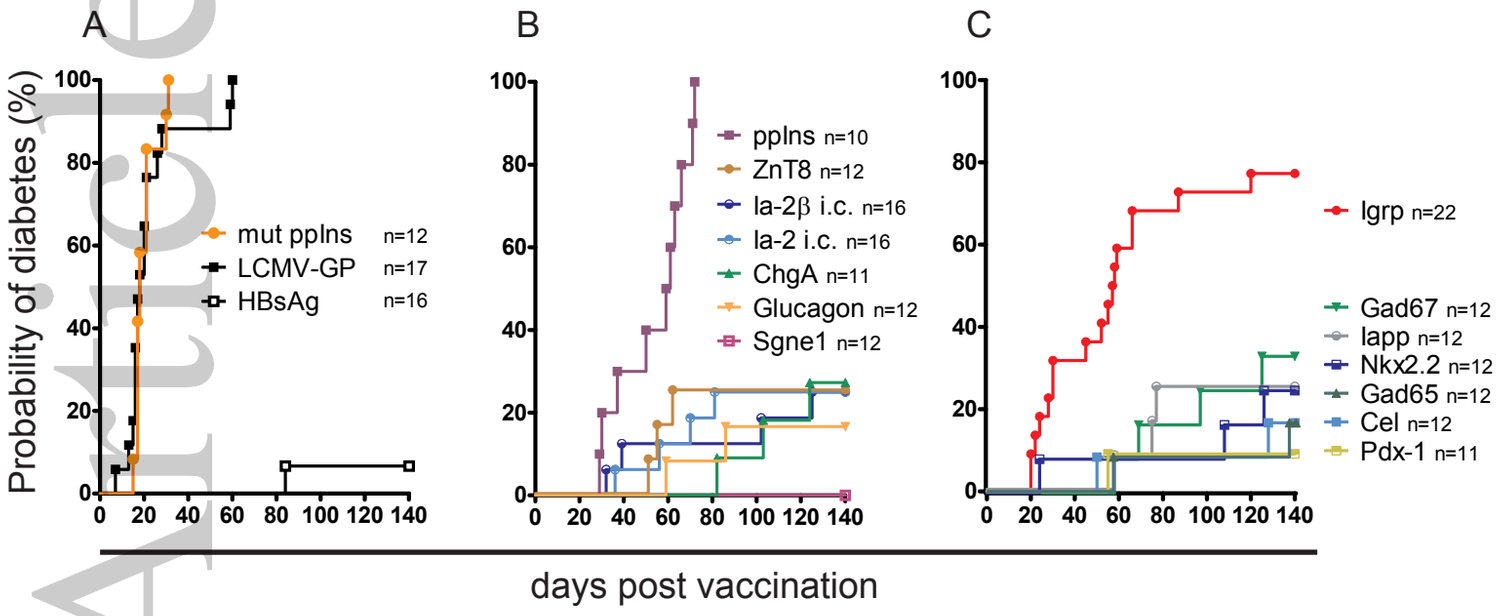
Figure 2: $CD8^+$ T cell targets in RIP-CD80GP mice. (**A**) Identification of Igrp peptide regions targeted by $CD8^+$ T cells in Igrp vector vaccinated, diabetic mice. Representative data of an IFNγ ELISpot assay performed with sorted $CD8^+$ T

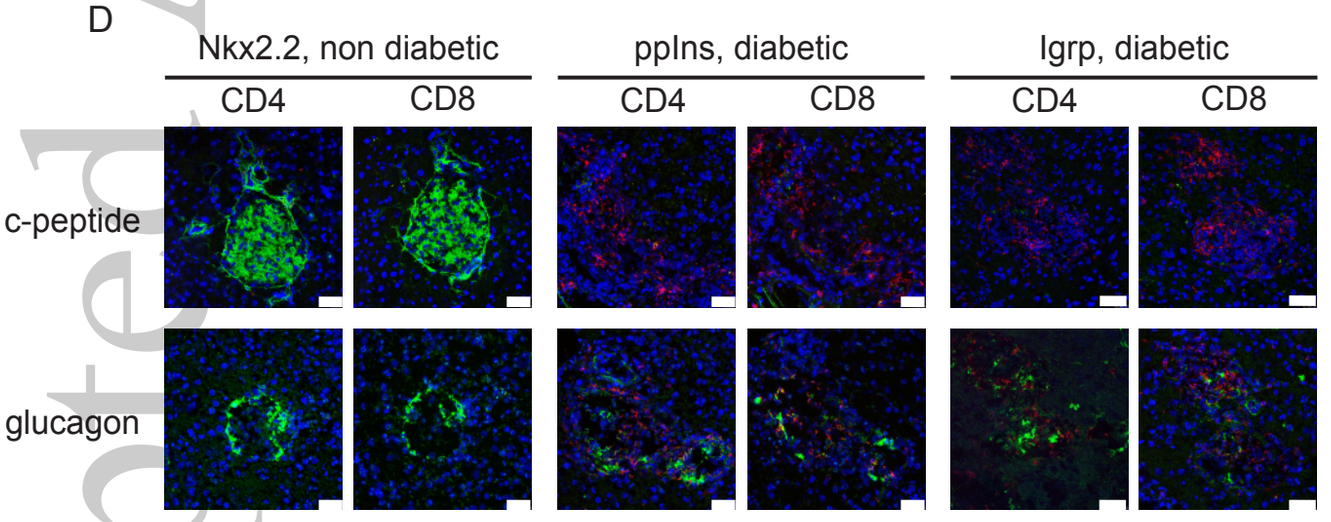
cells isolated from pooled splenocytes, popliteal and pancreatic lymph node cells of two mice is shown. T cell depleted splenocytes from non-vaccinated RIP-CD80 mice loaded with peptides of an overlapping Igrp 15mer peptide library were used for antigen presentation. As controls, cells were incubated with peptide solvent (DMSO), polyclonal T cell stimulatory anti-CD3/anti-CD28/anti-CD137 coated beads, and splenocytes loaded with immunodominant epitopes of the insulin A chain (A_{12-21}) and LCMV-GP (GP_{33-41}). Number of spots per 100,000 $CD8^+$ T cell (filled bars) and in the absence of T cells (open bars) is shown, as are the numbers of spots observed 7 weeks post Igrp vector vaccination in non-transgenic C57BL/6 mice (gray bars). (**B**) Diabetes induction upon vaccination with bone marrow-derived dendritic cells loaded with peptide. Ten to 12 week old mice were intraperitoneally injected with 200,000 BMDC pulsed with MHC class I restricted peptides of LCMV-GP (GP_{33-41}, black line, n=10; $GP_{276-286}$, blue line, n=11), LCMV-NP ($NP_{396-404}$, grey line, n=12) or Igrp ($Igrp_{225-233}$, red line, n=12; $Igrp_{241-249}$, green line, n=11) and followed for diabetes development shown as a life table analysis.

Figure 3: Insulin/IFA immunization in RIP-CD80GP mice. Mice received four weekly Insulin/IFA (solid lines, n=10) or vehicle/IFA (dashed lines, n=14) immunizations at 4-7 weeks of age. Half of the mice from each group were vaccinated at 10 weeks of age (21 days post final IFA vaccination) with pre-proinsulin (red lines) encoding DNA vector, the other half remained untreated

(black lines). All animals were followed for diabetes development shown as a life table analysis.

Figure 1





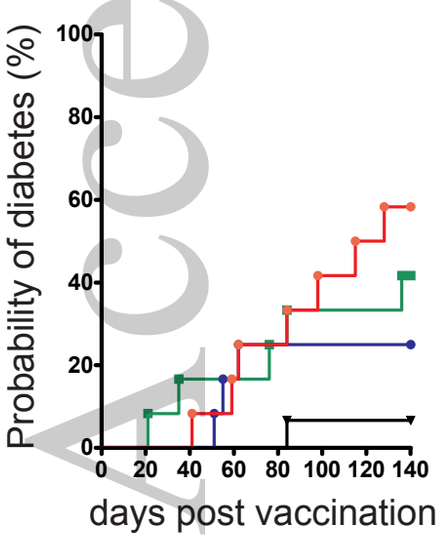
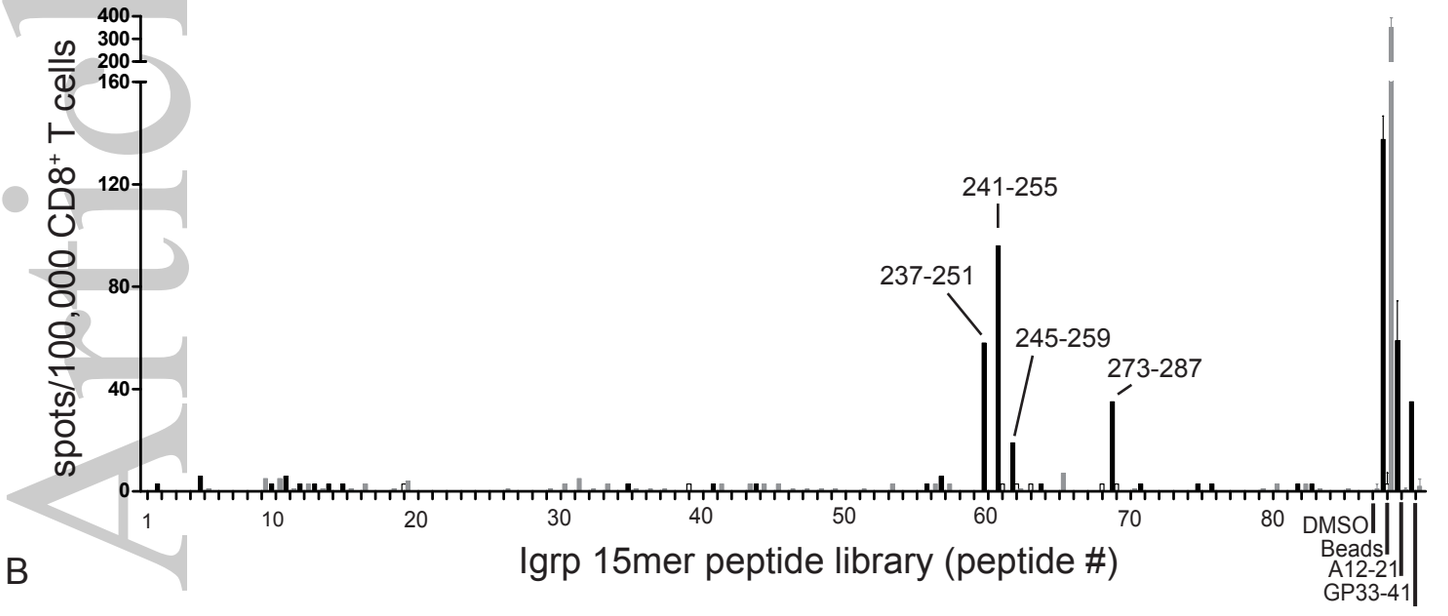


Figure 2

A



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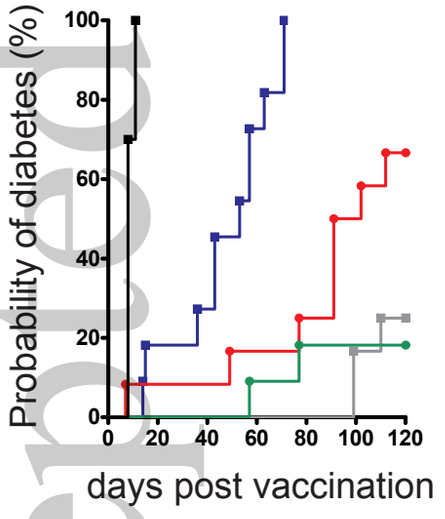

Figure 3