

# 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

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**OBJECTIVE**—Islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP), now known as G6PC2, is a major target of autoreactive T cells implicated in the pathogenesis of type 1 diabetes in both mice and humans. This study aimed to determine whether suppression of *G6pc2* gene expression might therefore prevent or delay disease progression.

**RESEARCH DESIGN AND METHODS**—*G6pc2*<sup>-/-</sup> mice were generated on the NOD/ShiLtJ genetic background, and glycemia was monitored weekly up to 35 weeks of age to determine the onset and incidence of diabetes. The antigen specificity of CD8<sup>+</sup> T cells infiltrating islets from NOD/ShiLtJ *G6pc2*<sup>+/+</sup> and *G6pc2*<sup>-/-</sup> mice at 12 weeks was determined in parallel.

**RESULTS**—The absence of *G6pc2* did not affect the time of onset, incidence, or sex bias of type 1 diabetes in NOD/ShiLtJ mice. Insulinitis was prominent in both groups, but whereas NOD/ShiLtJ *G6pc2*<sup>+/+</sup> islets contained CD8<sup>+</sup> T cells reactive to the *G6pc2* NRP peptide, *G6pc2* NRP-reactive T cells were absent in NOD/ShiLtJ *G6pc2*<sup>-/-</sup> islets.

**CONCLUSIONS**—These results demonstrate that *G6pc2* is an important driver for the selection and expansion of islet-reactive CD8<sup>+</sup> T cells infiltrating NOD/ShiLtJ islets. However, autoreactivity to *G6pc2* is not essential for the emergence of autoimmune diabetes. The results remain consistent with previous studies indicating that insulin may be the primary autoimmune target, at least in NOD/ShiLtJ mice. *Diabetes* 60:2922–2927, 2011

The *G6PC* gene family is composed of three members, *G6PC*, *G6PC2*, and *G6PC3* (1), each encoding multispansing membrane proteins located in the endoplasmic reticulum that catalyze the hydrolysis of glucose-6-phosphate (G6P) to glucose and inorganic phosphate. *G6PC* (*G6Pase*) is predominantly expressed in liver and kidney, where it catalyzes the terminal step in the gluconeogenic and glycogenolytic pathways

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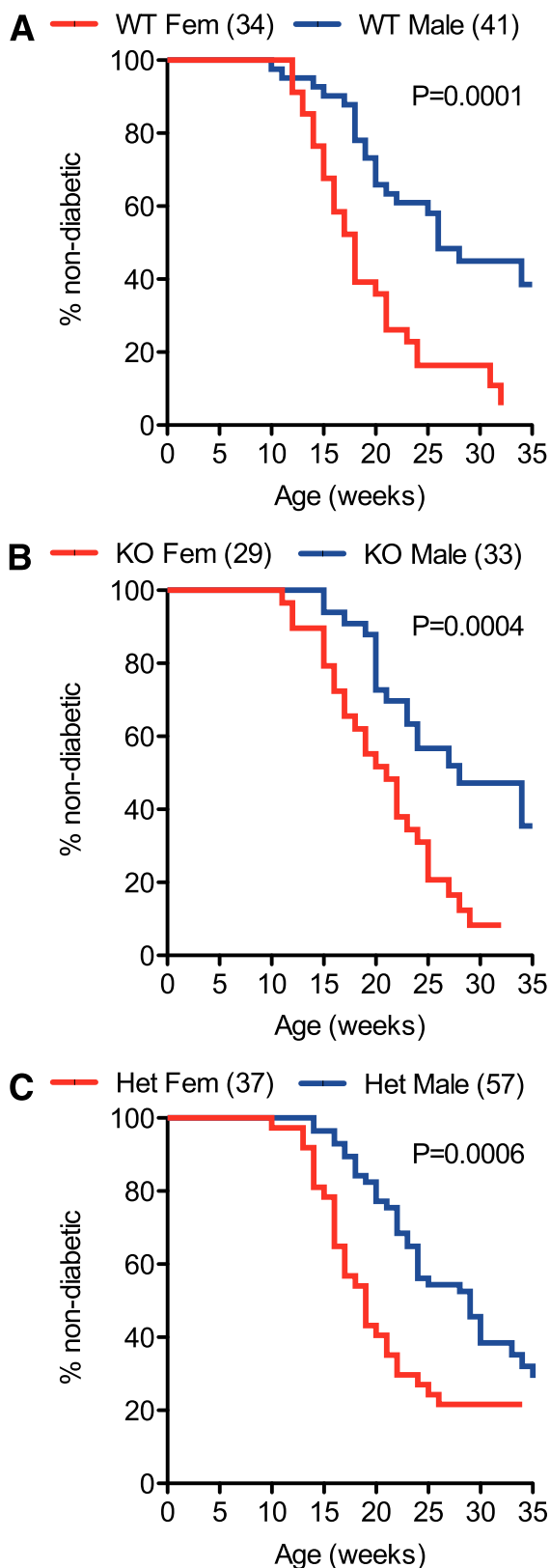
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(1). *G6PC3* (*UGRP*; *G6Pase-β*) is ubiquitously expressed, although its function in vivo is unclear (1). *G6PC2* (*IGRP*) is expressed specifically in pancreatic islet β-cells (1). *G6PC2* is proposed to act as a negative component of the β-cell glucose sensor, opposing the action of glucokinase and thereby modulating glucose-stimulated insulin secretion (2). Accordingly, *G6pc2*<sup>-/-</sup> mice exhibit a 15% reduction of fasting blood glucose but are otherwise metabolically and developmentally indistinguishable from wild-type littermates. Likewise, genome-wide association studies have linked polymorphic variants in *G6PC2* to variations in fasting blood glucose levels in humans (3,4).

*G6pc2* was implicated as a major autoantigen in the NOD mouse model of type 1 diabetes, following its identification as the molecular target of a CD8<sup>+</sup> T-cell clone (NY8.3) isolated from NOD/ShiLtJ mice (5,6). This clone, cognate mimotope peptides, and 8.3 T cell receptor (TCR) transgenic mice have provided insight into the role of CD8<sup>+</sup> T cells in type 1 diabetes and the importance of phenomena such as TCR avidity and maturation as determinants of autoimmunity and escape from immune tolerance (6). In NOD/ShiLtJ mice, up to 40% of the CD8<sup>+</sup> cells infiltrating the islet were found to recognize the cognate peptide of the 8.3 TCR (*G6pc2* amino acids 206–214) (5). Subsequent reports have shown that *G6pc2* also is recognized by CD4<sup>+</sup> T cells in NOD/ShiLtJ mice (7). Most importantly, T-cell responses to *G6PC2* peptides have been reported in human type 1 diabetes (8,9) and in humanized NOD/ShiLtJ mice (10). The observation that the administration of *G6pc2*-derived peptides abrogate or delay the disease process in NOD/ShiLtJ mice (7,11) offers the prospect that the protein or its encoding DNA, or the suppression of *G6PC2* gene expression, might be used as tolerogenic therapeutic strategies to delay or prevent the onset of type 1 diabetes in humans. A key question in this context is where *G6PC2* stands in the sequence of immunological events that characterize the initiation of autoimmunity, epitope spreading, and skewing of effector and regulatory T-cell subsets that characterize the progression of the disease from benign peri-insulinitis to infiltrating β-cell destruction. We address this issue here by investigating the incidence and progression of type 1 diabetes in NOD/ShiLtJ mice lacking *G6pc2*.

## RESEARCH DESIGN AND METHODS

**Animal care.** The animal housing and surgical facilities used for the mice in these studies meet the Association for Assessment and Accreditation of Laboratory Animal Care International standards. All animal protocols were approved by the Vanderbilt University Medical Center Animal Care and Use Committee. **Generation and genotyping of NOD/ShiLtJ *G6pc2*<sup>-/-</sup> mice.** The generation of *G6pc2* knockout mice on a mixed 129/SvEv<sup>Brd</sup> × C57BL/6 background has



**FIG. 1.** Comparison of the incidence and time of onset of type 1 diabetes in wild-type NOD/ShiLtJ and NOD/ShiLtJ *G6pc2*<sup>-/-</sup> mice: the effect of sex. The incidence and time of onset of type 1 diabetes in male and female wild-type NOD/ShiLtJ, NOD/ShiLtJ *G6pc2*<sup>-/+</sup>, and NOD/ShiLtJ *G6pc2*<sup>-/-</sup> mice was compared starting at the age of 10 weeks. Clear differences were observed between male and female wild-type NOD/ShiLtJ mice (A), between male and female NOD/ShiLtJ *G6pc2*<sup>-/+</sup> mice (B), and between male and female NOD/ShiLtJ *G6pc2*<sup>-/-</sup> mice (C). The

been previously described (2). The NOD/ShiLtJ *G6pc2*<sup>-/-</sup> congenic strain was developed using a speed congenic breeding strategy (12) combined with the analysis of single nucleotide polymorphisms (SNPs) that distinguished between the NOD/ShiLtJ, 129/SvEv, and C57BL/6 genomes. Using this approach, backcrossing was complete after eight generations, although diabetes appeared after four generations and backcrossing continued for nine generations. Mice were genotyped as previously described (2). Wild-type animals for these studies were generated by interbreeding NOD/ShiLtJ *G6pc2*<sup>+/+</sup> mice.

**Determination of disease status.** Intercrossed NOD/ShiLtJ *G6pc2*<sup>-/+</sup> mice were monitored for the development of diabetes from 10 weeks of age by weekly testing for morning glycosuria (Chemstrip 2 GP urinalysis strips; Roche). Mice with urine glucose concentrations >250 mg/dL, subsequently confirmed 24 h later, were designated as having diabetes, at which time the mice were killed.

**Insulinitis scoring.** Serial paraffin sections from wild-type and NOD/ShiLtJ *G6pc2*<sup>-/+</sup> mice were stained using immunoperoxidase methods for the presence of insulin and glucagon before counterstaining with methyl green. Slides were then scanned in Aperio Scanscope (Aperio Technologies, Vista, CA), and varying degrees of insulinitis were visualized and scored on ImageScope software using an arbitrary scale (0 = no insulinitis, 1 = peri-islet insulinitis, 2 = intermediate insulinitis, 3 = intraislet insulinitis, and 4 = atrophic islets [residual glucagon cells only]) in a double-blind study.

**Isolation of pancreatic islets and analysis of antigen-specific CD8<sup>+</sup> T cells using major histocompatibility complex–class I peptide tetramer staining.** Islet isolation following collagenase perfusion of the common bile duct was carried out by the Islet Procurement and Analysis Core of the Vanderbilt Diabetes Research and Training Center (<http://www.mc.vanderbilt.edu/diabetes/drtc>). Isolated islets were washed and then resuspended in Hanks' buffered salt solution containing DNase I (Worthington Biochemical, Lakewood, NJ) and handpicked using a silinized micropipet. Isolated islets were resuspended in RPMI-1640 medium supplemented with 10% FBS (HyClone, Logan, UT) and 50 units/mL human recombinant interleukin-2 (BD Pharmingen, San Diego, CA) and cultured intact in 24-well tissue-culture plates (~50 islets per well) for 9 days.

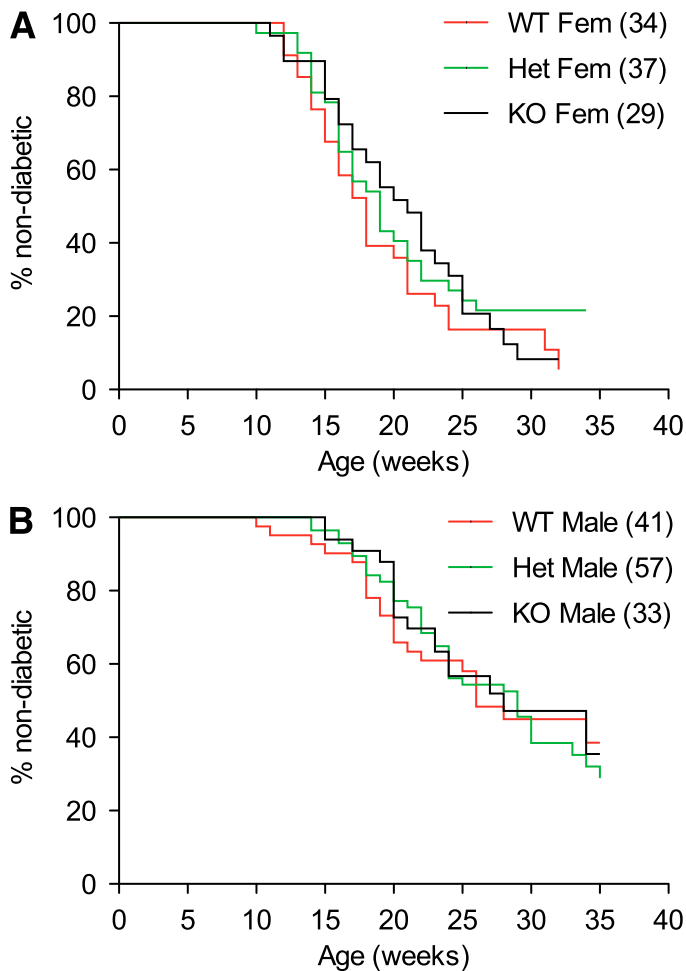
The following phycoerythrin (PE)-conjugated, peptide-loaded major histocompatibility complex (MHC)-class I tetramers were obtained from the National Institutes of Health Tetramer Core Facility (Emory University, Atlanta, GA): MimA2, a mimotope peptide of dystrophin myotonic kinase that is recognized by AI4-like T cells (13,14) (YAIENYLEL)/H-2D<sup>b</sup>; NRP-V7, a peptide mimetic of *G6pc2*<sub>206–214</sub> (5) (KYNKANVFL)/H-2K<sup>d</sup>; INS-L9, the G9 L variant of murine insulin B<sub>15–23</sub> (LYLVCGERL)/H-2K<sup>d</sup>; Flu-NP<sub>366–374</sub> (ASNNEMTM)/H-2D<sup>b</sup>; and Flu-NP<sub>147–155</sub> (TYQRTRALV)/H-2K<sup>d</sup>. Fluorescein isothiocyanate (FITC)-conjugated anti-CD8 $\alpha$  was purchased from BD Pharmingen. Cells were stained with tetramer and anti-CD8 $\alpha$  antibody in a fluorescence-activated cell sorter (FACS) buffer (PBS containing 3% FBS, 1 mmol/L EDTA, and 10 mmol/L HEPES) in a 96-well U-bottom plate at 37°C for 15 min and an additional 15 min at room temperature. Flow cytometry was performed using a FACS Calibur instrument (BD Pharmingen), and the acquired data were analyzed using FlowJo software (Tree Star, Ashland, OR).

**Analysis of interferon- $\gamma$  secretion by islet-resident CD8<sup>+</sup> T cells.** Islets were cultured in complete medium as described above, and the culture supernatant was collected at day 9 for measurement of interferon (IFN)- $\gamma$  by ELISA (BD Biosciences). Alternatively, for intracellular detection of IFN- $\gamma$ , cells were harvested and stimulated with ionomycin (1  $\mu$ mol/L) and phorbol myristate acetate (100 ng/mL) for 5 h in the presence of Golgi Plug (BD Biosciences). The cells were then surface stained with anti-CD8-FITC, followed by intracellular staining with anti-IFN $\gamma$ -PE, according to the manufacturer's protocol (BD Biosciences).

**Peptide immunization of mice.** Wild-type or *G6pc2*<sup>-/-</sup> mice were immunized with NRP-V7 peptide (100  $\mu$ g per mouse) emulsified in complete Freund's adjuvant. Two weeks later, splenic and draining lymph-node cells were prepared and stained with anti-CD8 $\alpha$ -FITC together with PE-labeled NRP-V7 or control Flu-NP tetramer, as described above.

**Statistical analyses.** Progression to diabetes and diabetes incidence were calculated using the product-limit method (Kaplan Meier analysis). Statistical significance of the difference between pairs of curves was determined by the log-rank test (Mantel-Cox test using Prism 5 software; GraphPad). *P* values  $\leq 0.05$  were considered statistically significant. Data derived from T-cell studies were analyzed with a two-tailed Student *t* test. *P* values  $\leq 0.05$  were considered statistically significant.

number of animals studied is indicated in parentheses. Statistical significance between curves was determined by the log-rank (Mantel-Cox) test. Het, heterozygous; KO, knockout; WT, wild-type.

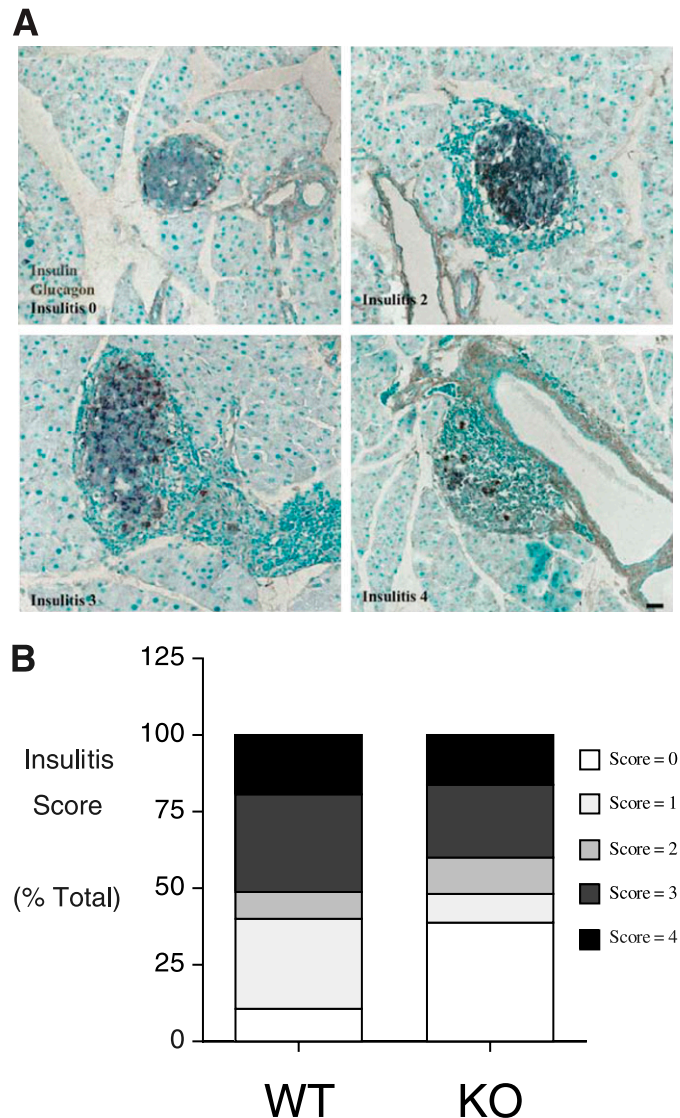


**FIG. 2.** Comparison of the incidence and time of onset of type 1 diabetes in wild-type NOD/ShiLtJ and NOD/ShiLtJ *G6pc2*<sup>-/-</sup> mice: the effect of *G6pc2* gene deletion. The cumulative incidence of type 1 diabetes in male and female wild-type NOD/ShiLtJ, NOD/ShiLtJ *G6pc2*<sup>+/-</sup>, and NOD/ShiLtJ *G6pc2*<sup>-/-</sup> mice was compared starting at the age of 10 weeks. No differences were observed between female wild-type, NOD/ShiLtJ *G6pc2*<sup>+/-</sup>, and NOD/ShiLtJ *G6pc2*<sup>-/-</sup> mice (A) or between male wild-type, NOD/ShiLtJ *G6pc2*<sup>+/-</sup>, and NOD/ShiLtJ *G6pc2*<sup>-/-</sup> mice (B). The number of animals studied is indicated in parentheses. Statistical significance between curves was determined by the log-rank (Mantel-Cox) test. Het, heterozygous; KO, knockout; WT, wild-type.

## RESULTS

**Generation and analysis of diabetes in congenic NOD/ShiLtJ *G6pc2*<sup>-/-</sup> mice.** A previously described modified *G6pc2* allele, generated by replacement of exons 1–3 plus 10 bp of the third intron by a LacZ/Neo cassette (2), was backcrossed onto the NOD/ShiLtJ genetic background using a speed congenic strategy followed by the analysis of specific SNPs surrounding the *G6pc2* locus. Intercrosses of heterozygous animals generated 259 pups (137 male) with an approximating Mendelian genotype distribution at 3 weeks (78 *G6pc2*<sup>+/+</sup>, 116 *G6pc2*<sup>+/-</sup>, and 65 *G6pc2*<sup>-/-</sup>).

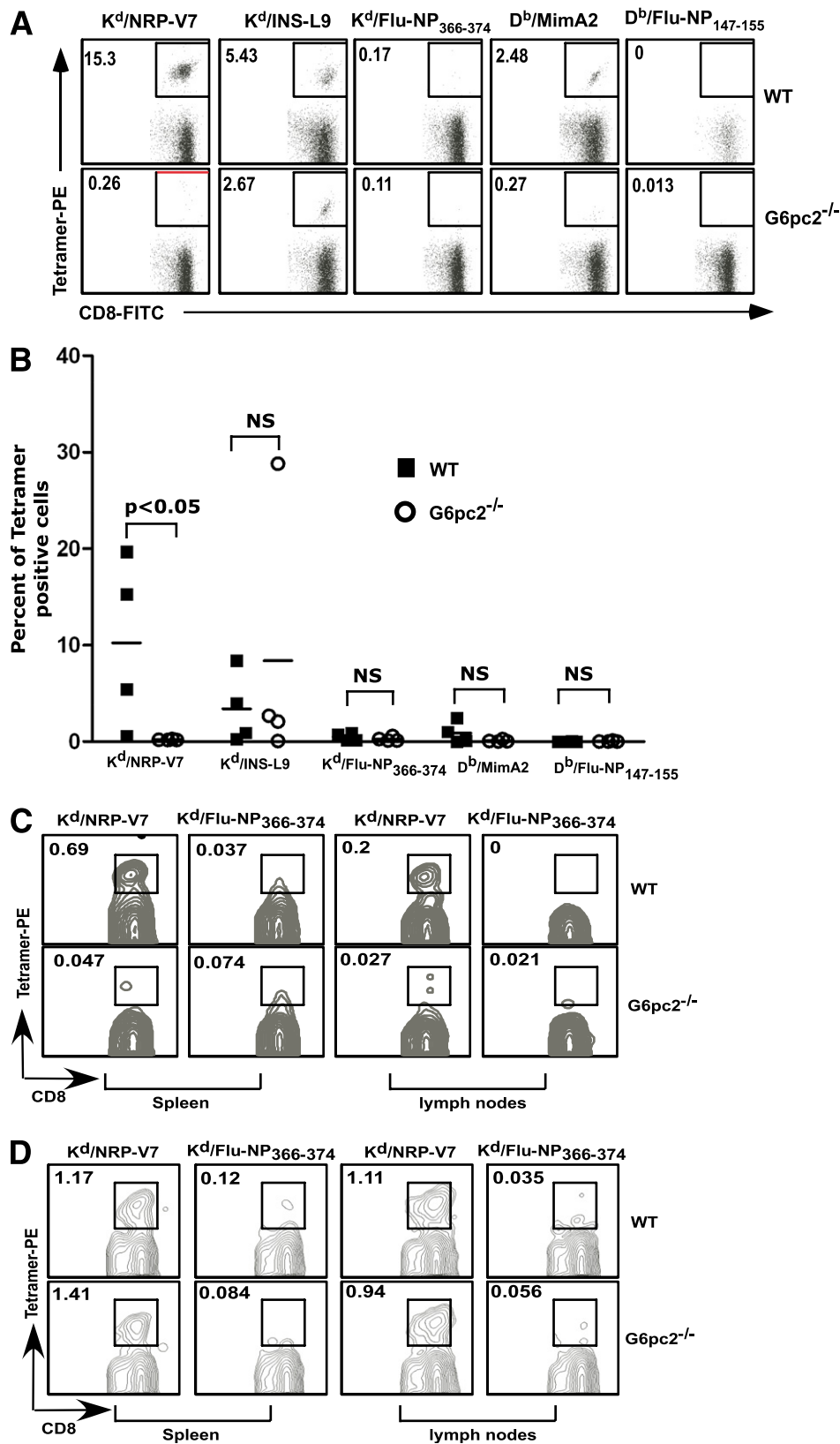
Monitoring for diabetes in this mouse colony began at 10 weeks of age. Figures 1 and 2 show survival analysis data. A clear sex difference was evident between female and male mice in the median onset of disease in *G6pc2*<sup>+/+</sup> (18 vs. 26 weeks,  $P = 0.0002$ ), *G6pc2*<sup>+/-</sup> (19 vs. 29 weeks,  $P < 0.0001$ ), and *G6pc2*<sup>-/-</sup> (21 vs. 28 weeks,  $P = 0.0021$ ) animals (Fig. 1), which was in accord with reported studies on wild-type NOD/ShiLtJ mice (15). However, the key observation was



**FIG. 3.** Insulinitis in normoglycemic wild-type and *G6pc2*-deficient NOD/ShiLtJ mice. Paraffin sections of pancreata removed from 25-week-old normoglycemic male animals were stained by immunoperoxidase methods for the presence of insulin (gray) and glucagon (brown). Varying degrees of peri-islet mononuclear cell infiltration were evident, as visualized by the methyl green counter stain. Representative images (scale bar = 50  $\mu$ m) are shown in A, whereas insulinitis scores from five wild-type and five NOD/ShiLtJ *G6pc2*<sup>-/-</sup> mice are shown in B, based on the examination of a total of 127 wild-type and 151 *G6pc2*<sup>-/-</sup> islets. KO, knockout; WT, wild-type. (A high-quality digital representation of this figure is available in the online issue.)

that neither the absence of *G6pc2* nor *G6pc2* gene dosage affected the median onset of diabetes or final incidence at 35 weeks. Post hoc analysis of data up to 24 weeks indicated a weak trend ( $P = 0.0924$ ) toward retarded onset of diabetes in male *G6pc2*<sup>-/-</sup> animals versus wild-type animals; however, this was not apparent in females ( $P = 0.6470$ ).

Histological analyses performed on a series of male *G6pc2*<sup>+/+</sup> and *G6pc2*<sup>-/-</sup> mice at 25 weeks of age that exhibited no glycosuria revealed insulinitis in both groups, which ranged from apparent normal islets, islets with pronounced peri-insulinitis yet strong insulin immunoreactivity, and islets with reduced insulin immunoreactivity and invasive mononuclear cell infiltrates (Fig. 3A and B). Such histopathological lesions typify the presence of



**FIG. 4.** Islet autoantigen-specific CD8<sup>+</sup> T-cell responses in wild-type and G6pc2-deficient NOD/ShiLtJ mice. **A** and **B**: Islets were isolated from individual female wild-type or G6pc2<sup>-/-</sup> NOD/ShiLtJ mice at 12 weeks of age and cultured in the presence of interleukin-2 for 9 days. Cells were then stained with FITC-labeled anti-CD8 $\alpha$  and PE-labeled MHC-class I tetramers loaded with the indicated peptides and analyzed by flow cytometry. Tetramers loaded with Flu-NP<sub>366-374</sub> or Flu-NP<sub>147-155</sub> were used as a negative control for tetramer staining. Data were electronically gated for CD8 $\alpha$ -expressing cells. **A**: Representative flow cytometry data for one wild-type mouse and one G6pc2-deficient mouse. Numbers indicate the percentage of cells within the gated area. **B**: Summary of the flow cytometry data for four wild-type and four G6pc2-deficient mice from two experiments. **C**: Splenic and lymph-node cells were prepared from female wild-type or G6pc2<sup>-/-</sup> NOD/ShiLtJ mice at 12 weeks of age, and the frequency of NRP-V7-specific CD8<sup>+</sup> T cells was determined by tetramer staining. Data were electronically gated for CD8 $\alpha$ -expressing cells.

autoimmune diabetes in NOD/ShiLtJ animals from as early as 8 weeks of age (16,17). No major differences were observed between *G6pc2*<sup>+/+</sup> and *G6pc2*<sup>-/-</sup> mice (Fig. 3B).

**Analysis of antigen-specific T-cell responses in congenic NOD/ShiLtJ *G6pc2*<sup>-/-</sup> mice.** Diabetes in the NOD/ShiLtJ mouse results from the specific destruction of pancreatic  $\beta$ -cells by a cell-mediated process involving an autoreactive antigen-specific CD4<sup>+</sup> and CD8<sup>+</sup> T-cell attack in an environment where regulatory T-cell responses are perturbed (6). Molecularly defined targets of pathogenic CD8<sup>+</sup> T cells have been well established (6) and include an H-2K<sup>d</sup>-restricted peptide of *G6pc2*<sub>206-214</sub> and its mimotope NRP-V7 (6) and a H-2K<sup>d</sup>-restricted peptide of the insulin B chain (insulin B<sub>15-23</sub> and its variant INS-L9), both of which have been implicated in disease pathogenesis and both of which are recognized by an appreciable proportion of CD8<sup>+</sup> T cells residing in islet infiltrates in NOD/ShiLtJ mice (6). We compared the prevalence of islet autoantigen-specific CD8<sup>+</sup> T cells in islets isolated from 12-week-old wild-type and NOD/ShiLtJ *G6pc2*<sup>-/-</sup> mice. Islets were cultured for 9 days ex vivo in the presence of interleukin-2 to expand CD8<sup>+</sup> T cells residing in them. Cells were then stained with a panel of peptide-loaded MHC-class I tetramers bearing the abovementioned *G6pc2* and insulin peptides, or influenza peptides as negative controls, and analyzed by flow cytometry. As previously reported, CD8<sup>+</sup> T cells reactive to NRP-V7 were the dominant population of CD8<sup>+</sup> T cells in wild-type mouse islets, accompanied by a variable, lesser percentage of insulin B<sub>15-23</sub>- and MimA2-specific T cells (Fig. 4A and B). In contrast, *G6pc2*<sup>-/-</sup> mouse islets completely lacked CD8<sup>+</sup> T cells reactive to NRP-V7 (Fig. 4A and B) but retained a high proportion of CD8<sup>+</sup> T cells specific for insulin B (Fig. 4A and B). The CD8<sup>+</sup> T cells in the islets of *G6pc2*<sup>-/-</sup> mice were functionally competent, as demonstrated by their capacity to secrete IFN- $\gamma$  in the culture supernatant (Supplementary Fig. 1A) and by their intracellular expression of IFN- $\gamma$  (Supplementary Fig. 1B).

Similar to what we observed in the islets, *G6pc2*<sup>-/-</sup> mice also lacked NRP-V7-reactive CD8<sup>+</sup> T cells in the spleen and lymph nodes (Fig. 4C). Nevertheless, these animals were capable of generating NRP-V7-specific CD8<sup>+</sup> T cells following immunization with NRP-V7 peptide emulsified in complete Freund's adjuvant (Fig. 4D).

## DISCUSSION

The NOD/ShiLtJ mouse has proven to be a robust and valuable experimental model for the investigation of the pathogenesis of type 1 diabetes and exhibits many features of the human clinical disease, including overlapping autoantigen specificity at the humoral and cellular level and shared genetic susceptibility, most notably the MHC-class II loci (16,17). In the current study, we used gene ablation to examine the role of *G6pc2* in the development of type 1 diabetes in NOD/ShiLtJ mice.

CD8<sup>+</sup> T cells infiltrating the islets of NOD/ShiLtJ *G6pc2*<sup>-/-</sup> mice failed to recognize the mimotope NRP-V7 peptide, indicating that the cognate peptide encoded by exon 5 is not expressed (Fig. 4). This also indicates that CD8<sup>+</sup> T-cell reactivity to NRP-V7 is not the result of reactivity or molecular

mimicry afforded by another gene family member or unrelated protein. However, the diabetes that emerged in *G6pc2*<sup>-/-</sup> and *G6pc2*<sup>-/+</sup> offspring on the NOD/ShiLtJ background was indistinguishable from that in *G6pc2*<sup>+/+</sup> animals in terms of its acute clinical onset (Fig. 2). Histo-pathological analysis showing monocytic infiltrates and attrition of  $\beta$ -cell numbers and insulin immunoreactivity were indicative of type 1 autoimmune disease (Fig. 3). It is thus apparent that *G6pc2*<sup>-/-</sup> animals progressed to invasive insulinitis. It is concluded that although *G6pc2*-reactive CD8<sup>+</sup> T cells are a prominent feature in wild-type NOD/ShiLtJ mice and the dominant population of CD8<sup>+</sup> T cells that invade islets (Fig. 4) (6), they are not necessary for the emergence of disease in the absence of autoreactivity to the protein. A similar conclusion was reached by Wang et al. (18), who examined the incidence and kinetics of diabetes in NOD/ShiLtJ mice expressing a nonimmunoreactive form of *G6pc2*.

Similar conclusions also have been reached from gene-ablation studies in NOD/ShiLtJ mice with GAD65, IA2, IA2 $\beta$  (phogrin), and IA2/IA2 $\beta$  double-knockout animals (16,17). In contrast, NOD/ShiLtJ mice with deletion of both the endogenous insulin I and insulin II alleles carrying an insulin transgene with an inactivated CD4<sup>+</sup> T-cell epitope do not develop diabetes (19). In addition, transgenic NOD/ShiLtJ mice tolerized to proinsulin do not develop proinsulin or *G6pc2* autoreactive T cells or diabetes; whereas although transgenic NOD/ShiLtJ mice tolerant to *G6pc2* do not develop *G6pc2* autoreactive T cells, they still develop proinsulin autoreactive T cells and diabetes (20). Even in transgenic mice with excess *G6pc2* autoreactive T cells, diabetes development is markedly reduced in the absence of proinsulin autoreactive T cells (21). These observations are consistent with the concept that proinsulin is the primary autoantigen in type 1 diabetes in the NOD/ShiLtJ mouse (15). Whether this also is the case in humans is another issue given the complexity of the MHC association with the disease and differences in the pathophysiology of type 1 diabetes in mice and humans (22).

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No potential conflicts of interest relevant to this article were reported.

J.K.O. performed genotyping and diabetes incidence analyses. V.V.P. performed isolated islet T-cell analyses and wrote parts of the manuscript. Y.W. performed genotyping

Representative flow cytometry data are shown. D: Wild-type or *G6pc2*<sup>-/-</sup> NOD/ShiLtJ female mice were immunized with NRP-V7 peptide (100  $\mu$ g per mouse) emulsified in complete Freund's adjuvant. Two weeks later, splenic and draining lymph-node cells were prepared and stained with anti-CD8 $\alpha$ -FITC and NRP-V7 or control Flu-NP tetramer and analyzed by flow cytometry. Data were electronically gated for CD8 $\alpha$ -expressing cells. Representative flow cytometry data are shown. NS, not significant; WT, wild-type. (A high-quality color representation of this figure is available in the online issue.)

and diabetes incidence analyses. N.K.J. performed diabetes incidence analyses. S.A.S. performed immunohistochemical analyses and wrote parts of the manuscript. R.W. performed speed congenic analyses. C.E.L. performed immunohistochemical analyses. L.D.P. performed diabetes incidence analyses. J.C.H. was the principal investigator for the immunohistochemical analyses and wrote parts of the manuscript. L.V.K. was the principal investigator for the isolated islet T-cell analyses and wrote parts of the manuscript. R.M.O. was the principal investigator for the diabetes incidence analyses and wrote parts of the manuscript.

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