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The type 1 diabetes candidate gene *Dexi* does not affect disease risk in the nonobese diabetic mouse model

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Abstract

Genome wide association studies have implicated more than 50 genomic regions in type 1 diabetes (T1D). A T1D region at chromosome 16p13.13 includes the candidate genes *CLEC16A* and *DEXI*. Conclusive evidence as to which gene is causal for the disease-association of this region is missing. We previously reported that *Clec16a* deficiency modified immune reactivity and protected against autoimmunity in the nonobese diabetic (NOD) mouse model for T1D. However, the diabetes-associated SNPs at 16p13.13 were described to also impact on *DEXI* expression and others have argued that *DEXI* is the causal gene in this disease locus. To help resolve whether *DEXI* affects disease, we generated *Dexi* knockout (KO) NOD mice. We found that *Dexi* deficiency had no effect on the frequency of diabetes. To test for possible interactions between *Dexi* and *Clec16a*, we intercrossed *Dexi* KO and *Clec16a* knockdown (KD) NOD mice. *Dexi* KO did not modify the disease protection afforded by *Clec16a* KD. We conclude that *Dexi* plays no role in autoimmune diabetes in the NOD model. Our data provide strongly suggestive evidence that *CLEC16A*, not *DEXI*, is causal for the T1D association of variants in the 16p13.13 region.

INTRODUCTION

The risk of type 1 diabetes (T1D) is modulated by more than 50 genomic regions[1]. Most of these regions include several genes, and exactly how disease-associated genetic variants affect islet autoimmunity is largely unresolved. The region at chromosome 16p13.13 contains many T1D-associated SNPs, the most significant of which are located in introns 8, 10, and 19-22 of *CLEC16A*[2]. Owing to the location of these SNPs, *CLEC16A* had initially been suggested as the causal gene for the disease association of 16p13.13[3,4]. Gene expression analyses subsequently provided evidence that disease SNPs affect *CLEC16A*

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AUTHOR CONTRIBUTIONS

J.M.N.B. performed experiments, analyzed data and wrote the manuscript. B. K. performed experiments. C.S. helped with experimental design and data interpretation. S.K. supervised the study, analyzed data and wrote the manuscript. S.K. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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expression[5,6]. Notably, a significant effect was attributed to rs12708716 that is associated with both T1D and multiple sclerosis, and this SNP was described to modify *CLEC16A* expression in human thymus[5]. We previously reported that *Clec16a* deficiency in thymic epithelial cells modified T cell selection, impacted immune function and was protective against autoimmune diabetes[7]. Despite functional data that support *CLEC16A* as the causal gene for the association of the 16p13.13 locus, it was argued that *DEXI* is instead a more likely candidate because disease-associated SNPs also modify *DEXI* expression[2,8,9]. A recent publication suggested that *DEXI* participates in the type I interferon pathway and modulates beta cell inflammation[10]. However, whether this gene has any role in autoimmunity remains unresolved. In our earlier report, we described that *Clec16a* knockdown (KD) was strongly protective against diabetes in the nonobese diabetic (NOD) mouse model for type 1 diabetes[7]. In the present study, we tested if *Dexi* deficiency alone or in combination with *Clec16a* KD would modify disease risk in NOD mice. To this end, we generated *Dexi* knockout (KO) NOD mice by CRISPR-Cas9 genome editing. We found that *Dexi* KO had no effect on the frequency of diabetes in this model, and that it also did not affect the strong protective effect of *Clec16a* KD. Our data provides strongly suggestive functional evidence that *CLEC16A* and not *DEXI* is causal for the association of the 16p13.13 region.

MATERIALS AND METHODS

Mice

NOD *Dexi* KO mice were generated by CRISPR-Cas9 genome editing in nonobese diabetic (NOD/ShiLtJ) mice (Jackson Laboratory). PCR genotyping was performed using two distinct primer pairs to distinguish homozygote and heterozygote mice, with primers A1 amplifying a large region that spans the *Dexi* coding region, and primers A2 that amplify smaller region near the start the coding region (Table 1). Mice were cared for and maintained as approved by the Joslin IACUC (Protocol #2014-01).

Genome editing

Two guide RNAs (gRNAs, Table 1) were selected to flank exon 1 *Dexi*, using a published algorithm (http://crispr.dfci.harvard.edu/SSC/)[11], and synthesized as described in ref. 12 using the pX330 vector (Addgene). gRNAs were generated with the Megashortscript T7 kit (Life Technologies) and purified using the Megaclear clean-up kit (Life Technologies) prior to microinjection into the pronucleus of NOD zygotes together with Cas9 mRNA (Trilink Technologies).

Quantitative PCR analyses

RNA was isolated using the NucleoSpin® RNA Plus Kit (Macherey-Nagel). cDNA was synthesized using the SuperScript™ III First-Strand Synthesis System Kit (Invitrogen) or the AzuraQuant™ cDNA Synthesis Kit (Azura Genomics). Quantitative RT-PCRs were performed using the *Power* SYBR™ Green PCR Master Mix (Applied Biosystems) or the AzuraQuant™ Green Fast qPCR Mix HiRox (Azura Genomics). Primers used are described in Table 1.

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Protein Isolation & Immunoprecipitation

Organs were prepared using TissueLyserII (Qiagen) in 1X Cell Lysis Buffer (Cell Signaling Technology) supplemented with protease inhibitors (cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail, Roche) and 1mM PMSF (Cell Signaling Technology). Protein content was quantified using a Pierce™ BCA Protein Assay Kit (Thermo Scientific). Protein lysates were incubated with DEXI Antibody (NOVUS) overnight then with Protein A Agarose Beads (Cell Signaling Technology) for 3-4 hours.

Western Blotting

Samples mixed with 4X Laemmli buffer (Bio-Rad) supplemented with 2-Mercaptoethanol (Sigma-Aldrich) at a 3:1 (sample:buffer) ratio were incubated at 65°C for 5 minutes before loading onto a 15% SDS-PAGE gel, followed by a transfer onto a nitrocellulose membrane (Bio-Rad). Protein were detected using Rabbit DEXI (NOVUS) and rabbit β-Actin (Cell Signaling Technology) antibodies followed by HRP-conjugated anti-rabbit antibody (Cell Signaling Technology).

Glucose Tolerance Test

Blood glucose concentration of mice fasted overnight was determined using a Contour blood glucose monitor (Bayer) before and after intraperitoneal injection of glucose (2g/kg body weight).

Insulitis

Pancreata were fixed in 10% paraformaldehyde in PBS overnight at 4°C, processed, sectioned, mounted and stained with Hematoxylin & Eosin. Insulitis was scored blindly as having no, moderate or severe infiltration as shown in representative images.

Differentiation of Bone Marrow Derived Macrophages (BM-DM)

Bone marrow from femur and tibia was differentiated in high glucose (4g/L) DMEM containing sodium pyruvate and L-glutamine, supplemented with 10% FBS, 1% Penicillin and Streptomycin, 1% L-glutamine and 1% Sodium Pyruvate with 30% L-929 M-CSF conditioned media (kind gift from Charles Evavold, Harvard Medical School) for 7 days then harvested using cold 2mM EDTA-containing PBS and re-suspended in DMEM supplemented with 5% L-929 M-CSF conditioned media.

PolyI:C Treatment

Polyinosinic–polycytidylic acid sodium salt (PolyI:C) (Millipore Sigma) was resuspended in ultrapure non-pyrogen containing water and used for treatment at a final concentration of 0.5μg. 2.5×10^6 / well BM-DMs were transfected with PolyI:C using FuGENE® 6 Transfection Reagent (Promega). Gene expression was measure after 24 h.

Diabetes measurements

Glycosuria was measured using Diastix (Bayer). Mice were considered diabetic with two consecutive readings of >250mg/dL. Mice were checked weekly and thrice weekly for

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spontaneous and cyclophosphamide-accelerated (250mg/kg at day 0 and 21, Sigma-Aldrich) diabetes, respectively.

Statistics

Data were analyzed using the Prism software (GraphPad). qPCR data were compared using a two-sided unpaired t-test. Insulitis was compared using Fisher's exact test. Diabetes frequencies were compared by Mantel-Cox Log-rank test. Age of onset was compared by Mann Whitney test. All data were obtained from age- and sex-matched contemporary mice. $P < 0.05$ was considered statistically significant. Sufficient sample size was estimated withouth the use of a power calculation. No samples were excluded from the analysis. No randomization was used for animal experiments. Data analysis was not blinded, except for histological scoring of insulitis.

RESULTS AND DISCUSSION

To investigate the role of *Dexi* in autoimmune diabetes, we deleted this gene in the NOD mouse model for T1D using CRISPR-Cas9 genome editing. We microinjected gRNAs and Cas9 mRNA into NOD zygotes to generate double-stranded DNA breaks at either end of exon 1 that encompasses the entire protein coding sequence (Fig. 1A). Among the seven pups born following microinjection, we identified one mutant mouse. Unexpectedly, this founder carried two separate mutant alleles in addition to the wild-type (WT) sequence at the targeted region of *Dexi*. Upon breeding, the mutant alleles each segregated into approximately 25% of the progeny, with the remaining pups carrying only WT alleles (Fig. 1B). These data indicate that the original founder was chimeric, with the gene editing event occurring at the two-cell stage, giving rise to two mutant alleles that we termed Allele #1 and #2. DNA sequencing established that Allele #1 comprised a near complete deletion of the exon 1 (Fig. 1A). In contrast, the deletion in Allele #2 was very short and preceded the start codon (not shown), likely resulting from a single double-stranded DNA break caused by the gRNA 5' of the coding region. We proceeded to verify that Allele #1 caused the loss of *Dexi* expression. After intercrossing Allele #1 mutant mice, we measured *Dexi* levels in homozygous mutants by qPCR (Fig. 1C) and western blotting (Fig. 1D). The results of these analyses confirmed that *Dexi* mRNA and protein were absent in *Dexi* KO mice.

DEXI is a candidate gene for a region that includes three additional candidates, *CIITA*, *CLEC16A* and *SOCS1*[2]. Because this chromosomal region is conserved between mouse and human, all three genes are also in close proximity to *Dexi* in the mouse genome. We established that *Dexi* deletion had no effect on *Ciita*, *Clec16a* or *Socs1* expression. (1E, 1F and 1G). Of interest, it was reported that *Dexi* modulates type I interferon expression in response to poly I:C, a synthetic viral double-stranded RNA[10]. Unexpectedly, *Dexi* deletion had no effect on this pathway in our model. We found that *Dexi* KO and WT cells had comparably robust interferon responses to poly I:C stimulation (Fig. 1H).

Having established that *Dexi* KO mice had the expected loss of *Dexi* expression without affecting the expression of nearby genes, we tested the frequeny of autoimmune diabetes in both male and female mice. We reported previously that *Clec16a* KD was protective in the NOD model[7]. In addition to exploring a role for *Dexi* in diabetes susceptibility, we tested for

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a possible interaction between *Clec16a* and *Dexi* by intercrossing *Dexi* KO mice with *Clec16a* KD animals to generate a cohort of double-deficient NOD mice. The *Clec16a* KD is mediated by a lentiviral transgene that is not located within proximity of the *Dexi/Clec16a* region and can be combined with the *Dexi* mutant allele by breeding.

We first tested the diabetes susceptibility of male cohorts using the cyclophosphamide (CY)-accelerated model. As reported earlier, *Clec16a* KD protected NOD mice against CY-induced diabetes (Fig. 2A). In contrast, *Dexi* KO did not affect the frequency of diabetes on its own and also had no independent effect when combined with *Clec16a* KD. *Dexi* KO also did not change the day of disease onset (median: day 29 for both WT and *Dexi* KO groups, P=0.42, Mann Whitney test)

We proceeded to measure the frequency of spontaneous diabetes in female cohorts. Again, *Dexi* KO neither increased nor decreased disease risk either alone or in combination with *Clec16a* KD (Fig. 2B). Again, *Dexi* KO had no significant effect on the age at diabetes onset (WT vs. *Dexi* KO: P=0.4, Mann Whitney test). Of note, *Dexi* deficiency also had no effect on glucose tolerance in pre-diabetic mice (Fig. 2C) and did not affect the severity of islet infiltration that precedes disease onset (Fig. 2D). Collectively, our data indicate that *Dexi* plays no significant role in autoimmune diabetes in the NOD model.

The ongoing debate over which gene is causal for the T1D association of the 16p13.13 region stems from the ambiguous effect of disease-associated SNPs on gene expression[2,5,6,8,9] and limited functional data for *DEXI*[10]. Of note, our experiments with *Dexi* KO cells did not replicate the previously reported effects of *Dexi* inhibition on the type I interferon signaling pathway[10]. The difference between our results and those of Dos Santos and colleagues may stem from our use of a different cell type (macrophages vs. beta cells) or species (mouse vs. rat and human) in these experiments, even though the interferon response is known to be conserved[14].

Here, we provide data implicating *Clec16a* but not *Dexi* in autoimmune diabetes. Both genes are conserved between species, and it is reasonable to assume that the function of *Dexi*, like that of *Clec16a*[7,13], is similar in mouse and human. Therefore, the finding that *Dexi* KO had no effect on the risk of diabetes in NOD mice is strongly suggestive that this gene plays no role in human T1D. Of note, unpublished data by Davison and colleagues[15] suggest that *Dexi* mutation increased disease in female NOD mice, though surprisingly not in males. However, the mutant strains used in this study carry incompletely characterized mutations that were not conclusively shown to eliminate *Dexi* expression[15], unlike our model in which the coding sequence for *Dexi* is completely deleted, leading to absence of both *Dexi* mRNA and protein. Even though disease-associated SNPs may well modify the expression of *Dexi* in some tissues[8], this does not imply that *Dexi* function impacts autoimmunity. Genetic association data, even when combined with eQTL analyses are insufficient to establish causality. Instead, functional studies are needed to provide convincing support for a gene's effect on disease. Data from our experimental model that combines both *Clec16a* and *Dexi* deficiency strongly suggest that *CLEC16A,* not *DEXI,* is causal for the effects of 16p13.13 in type 1 diabetes.

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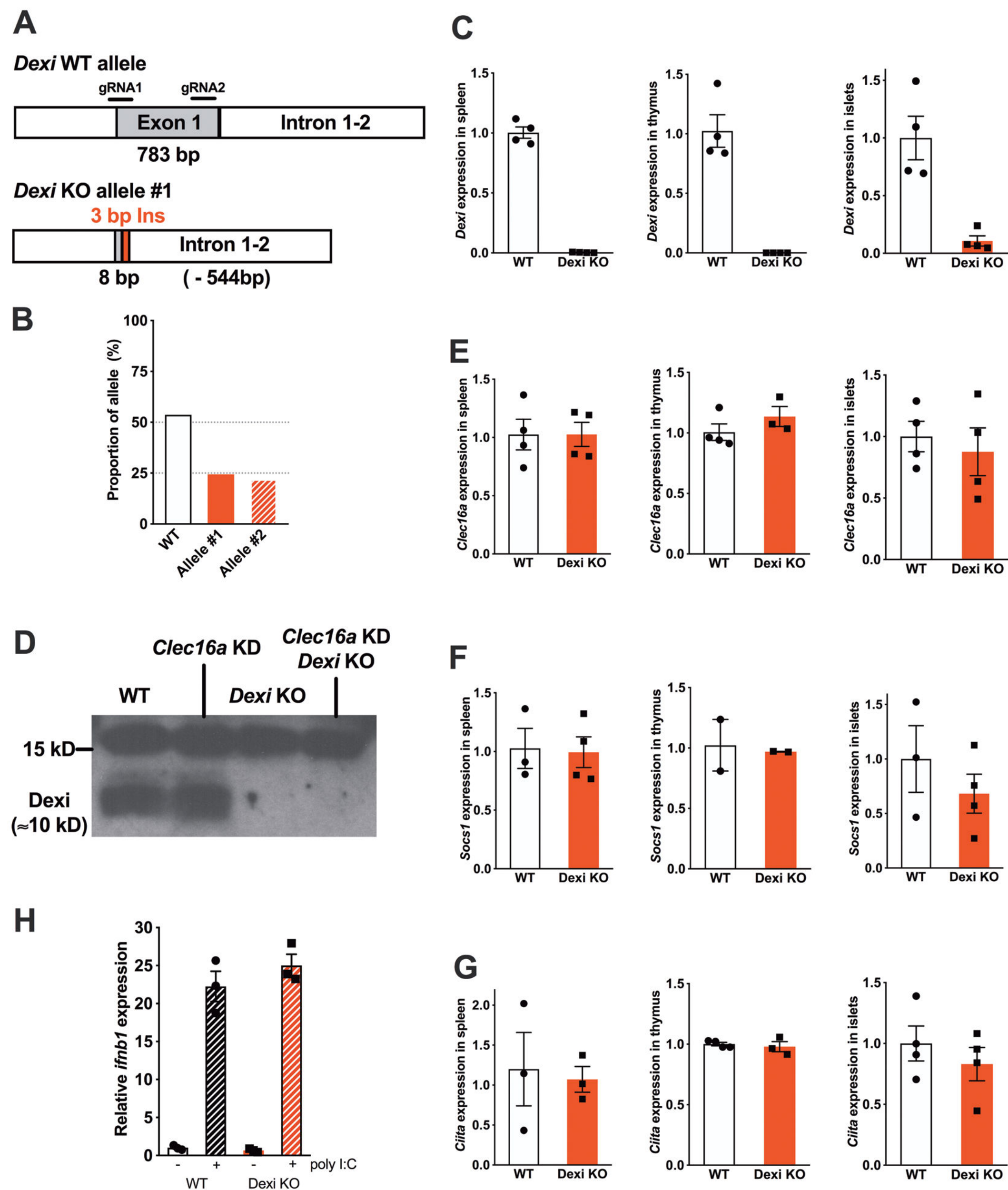

FIGURE 1.

Generation of *Dexi* KO NOD mice. (*A*) Schematic representation of the region targeted by CRISPR-Cas9 genome editing in the *Dexi* genomic (top) region and of the mutant allele #1 (bottom). Only the first 8bp of exon 1 remain, followed by a 3bp insertion and a 544bp deletion at the start of intron 1-2. (*B*) Inheritance pattern of the two mutant *Dexi* alleles (#1 and #2) present in the founder male NOD mouse. The proportion of wild-type and mutant alleles inherited from the founder in the F1 progeny (total 41 mice, of which 10 carried allele #1 and 9 carried allele #2) is shown. (*C*) Quantitation of *Dexi* mRNA in the spleen, thymus and pancreatic islets of WT and *Dexi* KO mice by quantitative PCR. n=4 mice per group, data show individual values and mean +/– SEM and are representative of at least three similar experiments. *** $P < 0.001$ (two-tailed t-test). (*D*) Detection of Dexi protein by

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western blotting following immune-precipitation with anti-Dexi antibody. Data are shown for WT, Clec16a KD, Dexi KO and Clec16a KD/Dexi KO mice and are representative for two similar experiments. (*E-G*) Quantitation of *Clec16a* (*E*), *Socs1* (*F*) and *Ciita* (*G*) mRNA by quantitative PCR in spleen, thymus and pancreatic islets. n=2-4 mice per group. Data show individual values, mean +/− SEM and are representative of at least two similar experiments. (*H*) Interferon beta expression in bone marrow-derived macrophages from WT and *Dexi* KO mice stimulated with poly I:C. Data show individual values, mean +/− SEM and are representative of two similar experiments.

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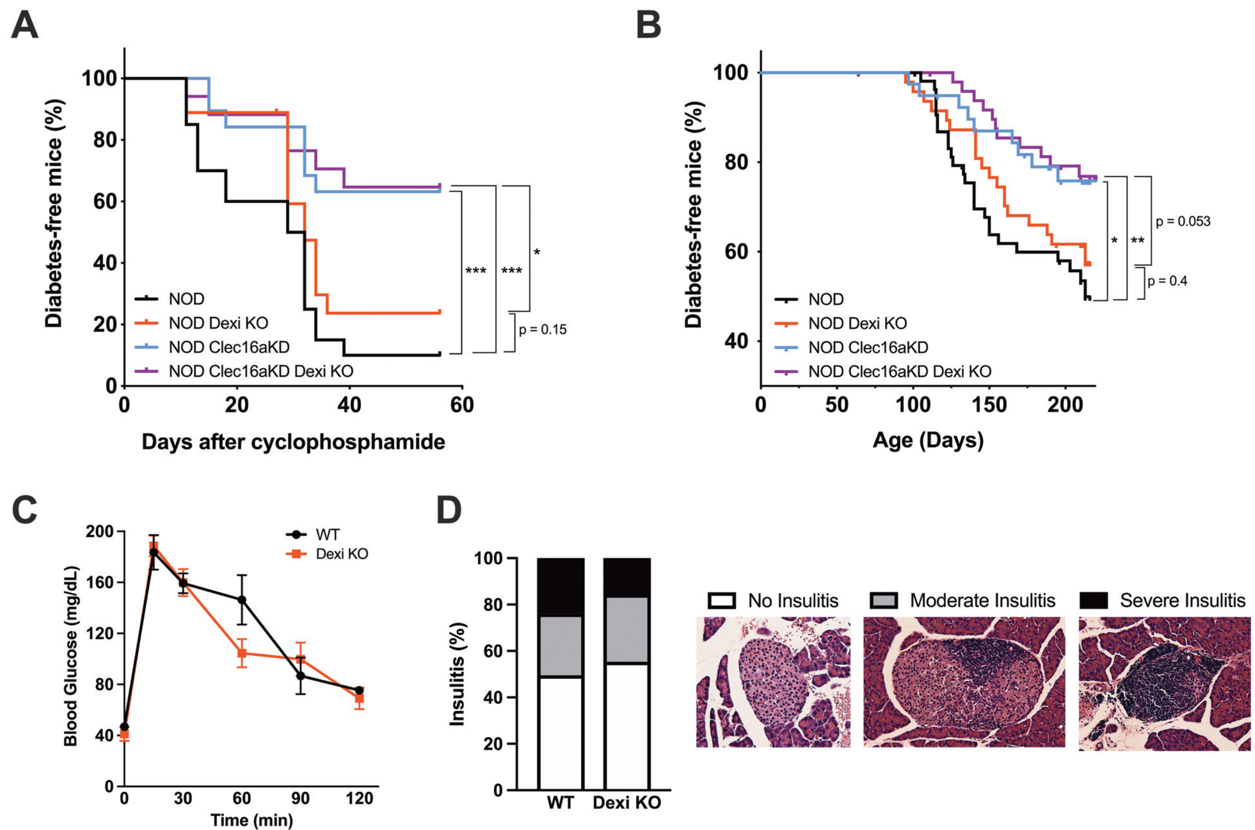

FIGURE 2.

Dexi KO does not modify the frequency of diabetes in NOD mice. (*A*) Cyclophosphamide-accelerated diabetes was measured in groups of WT (n=20), *Dexi* KO (n=18), *Clec16a* KD (n=19) and *Clec16a* KD/*Dexi* KO (n=17) male NOD mice injected with cyclophosphamide at age 9-10 weeks. (*B*) Spontaneous diabetes was measured in groups of WT (n=54), *Dexi* KO (n=47), *Clec16a* KD (n=39) and *Clec16a* KD/*Dexi* KO (n=50) female NOD mice. Differences between groups were measured using the Log-rank test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (*C*) WT and Dexi KO mice (9 weeks old) were injected with glucose intraperitoneally to test their glucose tolerance. Data show mean +/– SEM values from 6 mice per group. (*D*) Histological analysis was performed on 10 week-old WT and *Dexi* KO mice to quantify the degree of insulitis. Data show the proportion of WT islets (n=402) and *Dexi* KO islets (n=354) with no infiltration, moderate or severe insulitis from 3 mice per group. Fisher's exact test P=0.1 comparing the proportion of infiltrated islets in WT and *Dexi* KO mice.

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TABLE 1

gRNA, PCR and qPCR primer sequences

Name	Sequence
mDexiKO-g1-Forward	5’-CACCGATGGGCAGTGAGCCTGCGG-3’
mDexiKO-g1-Reverse	5’-AAACCCGCAGGCTCACTGCCCATC-3’
mDexiKO-g2-Forward	5’-CACCGGGATGGGACCCCAGGAAG-3’
mDexiKO-g2-Reverse	5’-AAACCTTCCTGGGGTCCCATCCC-3’
T7_mDexiKO_g1-Forward *(In Vitro Transcription)*	5’-TTAATACGACTCACTATAGGATGGGCAGTGAGCCTGCGG-3’
T7_mDexiKO_g2-Forward *(In Vitro Transcription)*	5’-TTAATACGACTCACTATAGGGGGATGGGACCCCAGGAAG-3’
T7_mDexiKO-Reverse *(In Vitro Transcription)*	5’-AAAAGCACCGACTCGGTGCC-3’
mDexiKO_genoA1-Forward	5’-ACAAAGGTGGTCTGTAAACCG-3’
mDexiKO_genoA1-Reverse	5’-TGGCAATGTTGGCAATCAGG-3’
mDexiKO_genoA2-Forward	5’-CTTTTCCACCCGGCATCATT-3’
mDexiKO_genoA2-Reverse	5’-TTGACACCCCGAGATGCT-3
mActb-Forward	5’-GGCTGTATTCCCCTCCATCG-3’
mActb-Reverse	5’-CCAGTTGGTAACAATGCCATGT-3’
mDexi-Forward	5’-CTGCTGCCCTCTATGTTCTACG-3’
mDexi-Reverse	5’-GCCAGGGTCTGAAAGTACGC-3’
mClec16a-Forward	5’-CCTGATTTGGGGCGATCAAAA-3’
mClec16a-Reverse	5’-CATAACGGCCTGATTTCTGCC-3’
mSOCS1-Forward	5’-CTGCGGCTTCTATTGGGGAC-3’
mSOCS1-Reverse	5’-AAAAGGCAGTCGAAGGTCTCG-3’
mCIITA-Forward	5’-TGCGTGTGATGGATGTCCAG-3’
mCIITA-Reverse	5’-CCAAAGGGGATAGTGGGTGTC-3