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 single-nucleotide polymorphisms (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* 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 β-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 T1D [7]. In the present study, we tested whether *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 NOD (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 Institutional Animal Care and Use Committee (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 were 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 reverse-transcription 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.

Protein isolation and 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 1 mM phenylmethylsulfonyl fluoride (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 h.

Western blotting

Samples mixed with 4× Laemmli buffer (Bio-Rad) supplemented with 2-Mercaptoethanol (Sigma-Aldrich) at a 3:1 (sample:buffer) ratio were incubated at 65°C for 5 min 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 horseradish peroxidase-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 (2 g/kg body weight).

Insulitis

Pancreata were fixed in 10% paraformaldehyde in phosphate-buffered saline (PBS) overnight at 4°C, processed, sectioned, mounted, and stained with hematoxylin and eosin. Insulitis was scored blindly as having no, moderate, or severe infiltration as shown in representative images.

Differentiation of bone marrow-derived macrophages

Bone marrow from the femur and tibia was differentiated in high glucose (4 g/L) Dulbecco’s modified Eagle medium (DMEM) containing sodium pyruvate and L-glutamine, supplemented with 10% fetal bovine serum, 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 collected using cold 2 mM EDTA-containing PBS and resuspended 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 0.5 μg. Bone marrow-derived macrophages (BM-DMs; 2.5 × 10^5/well) 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 >250 mg/dL. Mice were checked weekly and thrice weekly for spontaneous and cyclophosphamide (CY)-accelerated (250 mg/kg at day 0 and 21, Sigma-Aldrich) diabetes, respectively.

Statistics

Data were analyzed using the Prism software (GraphPad). Quantitative PCR (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 without 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 encircles 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 ~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

Table 1 gRNA, PCR, and qPCR primer sequences

| Name                     | Sequence                                     |
|--------------------------|----------------------------------------------|
| mDexiKO-g1-Forward       | 5′-CACCGATGGGCAGTGACCTGCGG-3′                |
| mDexiKO-g1-Reverse       | 5′-AAACCCCGAGGCTACGTCCGACATCCATC-3′         |
| mDexiKO-g2-Forward       | 5′-CACCGGGATGGCGACCCAGGAAG-3′               |
| mDexiKO-g2-Reverse       | 5′-AAACCTTCCTGGGCTCCATCCATC-3′              |
| T7_mDexiKO-g1-Forward    | 5′-TTAATACGCTACATATAGGGATGGCGAGTGGCCTGCAGG-3′ |
| T7_mDexiKO-g2-Forward    | 5′-TTAATACGCTACATATAGGGGATGGCGAGTGGCCTGCAGG-3′ |
| T7_mDexiKO-Reverse       | 5′-AAAAGCAGCCGACCTGCAGGAC-3′                |
| mDexiKO_gen01-Forward    | 5′-ACAAAGGTGTCTGTAACCCG-3′                  |
| mDexiKO_gen01-Reverse    | 5′-TGGCAATGTGCAATCCAGG-3′                   |
| mDexiKO_gen02-Forward    | 5′-CTTTTCACCAGCAGCATATT-3′                  |
| mDexiKO_gen02-Reverse    | 5′-TTGACACCCCGAGATGCT-3                     |
| mActb-Forward            | 5′-GGCTGTATTCCCCTCCTCGG-3′                  |
| mActb-Reverse            | 5′-CCAGTTGTTAACATCGCATTG-3′                 |
| mDexi-Forward            | 5′-CTGCTGTCCCTATATGTTACGC-3′                |
| mDexi-Reverse            | 5′-GCCAAGGTCTGAAAGTACGC-3′                  |
| mClec16a-Forward         | 5′-CCTGATTGGGCGCCAAATCAAA-3′                |
| mClec16a-Reverse         | 5′-CATACAGGCTGATATGCTGCGG-3′                |
| mSOCS1-Forward           | 5′-CTGCCGCTCCTATGGGGGCAC-3′                 |
| mSOCS1-Reverse           | 5′-AAAAGCAGTCGAAGTCTCGG-3′                  |
| mCIITA-Forward           | 5′-TGCGTGTGATGGGTAATGACG-3′                 |
| mCIITA-Reverse           | 5′-CCAAAGGGATGGTGCTCAGG-3′                  |
**Fig. 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 8 bp of exon 1 remain, followed by a 3 bp insertion and a 544 bp 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** Quantification 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 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** Quantification of *Clec16a (e)*, *Socs1* (f), and *Ciita* (g) mRNA by quantitative PCR in the 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-β 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.
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. As 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 (Fig. 1e–g). 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 frequency 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 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 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 prediabetic 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 et al. [10] may stem from our use of a different cell type (macrophages vs. β-cells) or species (mouse vs. rat and human) in these experiments, even though the interferon response is known to be conserved [13].

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, similar to that of Clec16a [7, 14], 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 et al. [15] suggest that Dexi mutation increased disease in female NOD mice, although 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 the 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 expression quantitative trait loci (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 T1D.

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Author contributions JMNB performed experiments, analyzed data, and wrote the manuscript. BK performed experiments. CS helped with experimental design and data interpretation. SK supervised the study, analyzed data, and wrote the manuscript. SK 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.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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