mt-Nd2^{a} Modifies Resistance Against Autoimmune Type 1 Diabetes in NOD Mice at the Level of the Pancreatic β-Cell

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OBJECTIVE—To investigate whether a single nucleotide polymorphism (SNP) in the mitochondrial gene for NADH dehydrogenase 2 (*mt-Nd2*) can modulate susceptibility to type 1 diabetes in NOD mice.

RESEARCH DESIGN AND METHODS—NOD/ShiLtJ mice conplastic for the alloxan resistant (ALR)/Lt-derived *mt-Nd2*^{a} allele (NOD.mt^{ALR}) were created and compared with standard NOD (carrying the *mt-Nd2*^{c} allele) for susceptibility to spontaneous autoimmune diabetes, or to diabetes elicited by reciprocal adoptive splenic leukocyte transfers, as well as by adoptive transfer of diabetogenic T-cell clones. β-Cell lines derived from either the NOD (NIT-1) or the NOD.mt^{ALR} (NIT-4) were also created to compare their susceptibility to cytolysis by diabetogenic CD8^{+} T-cells in vitro.

RESULTS—NOD mice differing at this single SNP developed spontaneous or adoptively transferred diabetes at comparable rates and percentages. However, conplastic mice with the *mt-Nd2*^{a} allele exhibited resistance to transfer of diabetes by the CD4^{+} T-cell clone BDC 2.5 as well as the CD8^{+} AI4 T-cell clones from T-cell receptor transgenic animals. NIT-4 cells with *mt-Nd2*^{a} were also more resistant to AI4-mediated destruction in vitro than NIT-1 cells.

CONCLUSIONS—Conplastic introduction into NOD mice of a variant *mt-Nd2* allele alone was not sufficient to prevent spontaneous autoimmune diabetes. Subtle nonhematopoietic type 1 diabetes resistance was observed during adoptive transfer experiments with T-cell clones. This study confirms that genetic polymorphisms in mitochondria can modulate β-cell sensitivity to autoimmune T-cell effectors. ***Diabetes*** **60:355–359, 2011**

Type 1 diabetes is a complex disease regulated by multiple genetic, metabolic, and environmental factors. In both human and animal models, genetic contributions to autoimmune diabetes have been linked to loci both in the nuclear as well as in mitochondrial genome (mtDNA) (1–5). While mtDNA polymorphisms can severely impair energy metabolism and lead to diabetes (6), only a single nucleotide polymorphism (SNP) in the mitochondrial gene for NADH dehydrogenase 2 (*mt-ND2*) has been associated with autoimmune diabetes in NOD mice and in humans (3–5).

Crossing the genetically related diabetes-prone NOD/ShiLt and diabetes-resistant alloxan resistant (ALR)/Lt mouse models (7,8) we previously mapped ALR-derived resistance against spontaneous diabetes to three nuclear loci and, by reciprocal outcrosses, to a mtDNA SNP in *mt-Nd2* (4). ALR mice were selected for resistance to alloxan, a free radical generator and selective β-cell toxin (7). This selection process resulted in mice with unusually elevated cellular defenses both systemically and at the islet level, providing resistance to both free radicals (9,10) and autoimmune effectors (5,11). In an additional genetic study to define loci or genes that provided diabetes resistance at the β-cell level, the ALR-derived *mt-Nd2*^{a} was significantly associated with resistance against alloxan-induced diabetes (9,12).

Sequence analysis of mtDNA revealed a SNP that distinguishes the ALR strain from NOD and all other strains whose mtDNA has been sequenced (5,13). ALR mice harbor a C to A nucleotide transversion and an amino acid substitution from leucine to methionine (5). In humans, there is a corresponding C to A SNP in *mt-ND2* resulting in an identical amino acid substitution. The human *mt-ND2*^{c} allele has also been reported to persist at a higher frequency in patients than control subjects (3). Allelism in *mt-ND2*^{a} was initially proposed to alter scavenging of reactive oxygen species (ROS) (3). However, our studies did not substantiate a role in ROS dissipation, but rather we have established that the resistance allele of this gene reduces basal mitochondrial ROS production by ~30% (14,15). Hence, it might be predicted that this change in mitochondrial ROS may significantly alter β-cell death. In the present study, we confirm that *mt-Nd2*^{a} protects against β-cell death mediated by single T-cell clones but not against the full array of autoimmune effector mechanisms.

RESEARCH DESIGN AND METHODS

NOD/ShiLtJ (NOD), ALR/LtJ (ALR), NOD.mt^{ALR} (15), and NOD.129S7(B6)-*Rag1*^{tm1Mom}/J (NOD-*Rag1*) were bred in our mouse facility. Immunodeficient conplastic mice, NOD.mt^{ALR}-*Rag1*, were created by mating NOD.mt^{ALR} females to male NOD-*Rag1*, followed by backcrossing to NOD-*Rag1* males and selecting mice homozygous for the disrupted allele of *Rag1*. To ensure the comparability of recipients for diabetogenic T-cells, we created an immunodeficient ALR/LtJ [ALR-*Rag1*] by outcrossing to NOD-*Rag1* and then backcrossing for 10 generations to ALR. The congenic interval is from *D2Mit15* to *D2mit190*. Genotyping for *Rag*1$^{-/-}$ was performed as described (www.jax.org). NOD.Cg-Tg(Ins2-TAg)1Lt *Prkdc*^{scid}/DvsJ [NOD.RIP-Tag], NOD.Cg-*Rag1*^{tm1Mom}Tg(*TcraAI4*)1Dvs/DvsJ [NOD-AI4a], and NOD.Cg-*Rag1*^{tm1Mom}Tg(*TcrbAI4*)1Dvs/DvsJ [NOD-AI4b] were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred in our mouse facility. F1 hybrid progeny from matings of NOD-AI4a with NOD-AI4b [NOD-AI4a/b] develop diabetes at 3–5 weeks of age. All mice were housed in specific

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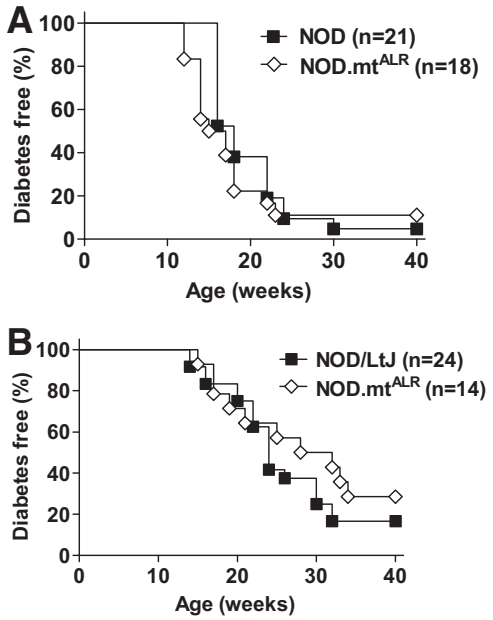

FIG. 1. There is no significant difference in spontaneous type 1 diabetes incidence in either female (*A*) or male (*B*) NOD mice (*mt-Nd2*^{c} allele) compared with NOD.mt^{ALR} conplastic mice with the ALR-derived *mt-Nd2*^{a} allele. Incidence studies were performed independently at The Jackson Laboratory and The Children's Hospital of Pittsburgh using both female and male NOD and NOD.mt^{ALR} conplastic mice. These two studies obtained equivalent incidence results, and the combined analysis is presented.

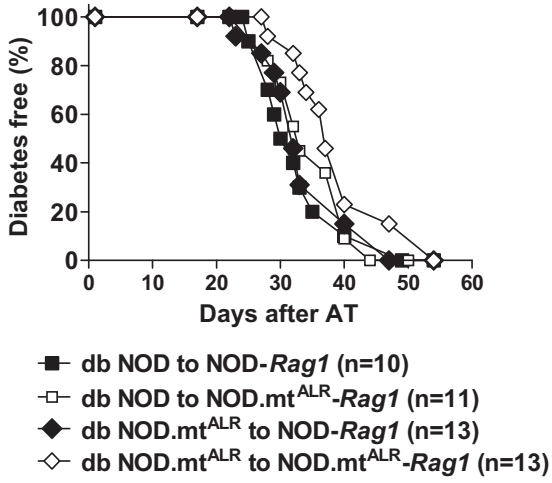

FIG. 2. Reciprocal adoptive transfers (ATs) were performed into immunodeficient NOD-*Rag1* females expressing either *mt-Nd2* allele as recipients and splenic leukocytes from diabetic NOD or diabetic conplastic donors (NOD.mt^{ALR}). Splenocytes from these donor mice were collected and erythrocytes removed using hypotonic solution treatment. Cells were injected intravenously (tail vein) at 2×10^{7} cells/mouse into age-matched female recipients. The source of the mitochondrial population in either diabetic donor splenocytes or in *Rag1* recipients did not significantly affect rate of diabetes development in NOD recipients.

pathogen-free facilities and approved by the relevant institution's Animal Care and Use Committee.

Monitoring for spontaneous autoimmune diabetes onset and histological analysis. Female and male mice were monitored for diabetes onset as previously described (4). For histological studies, pancreata were removed from female mice and fixed, embedded, and scored for insulitis as previously described (4,11).

Adoptive transfers. Splenocytes from donor mice (diabetic female NOD, diabetic female NOD.mt^{ALR}, or young [3–5 weeks old] NOD-AI4a/b F1 hybrids) were collected, had the erythrocytes removed using hypotonic solution treatment, and were injected intravenously (tail vein) at 2×10^{7} cells/mouse into age-matched female recipients (NOD-*Rag1*, ALR-*Rag1* or NOD.mt^{ALR}-*Rag1*). For BDC2.5 T-cell clone transfers (kindly provided by Dr. Katie Haskins, University of Colorado, Denver), cells were prepared and injected as previously described into NOD, NOD.mt^{ALR}, or ALR/LtJ mice (16). Mice were followed for diabetes development after injection as described previously (11).

Generation of β-cell lines and cell-mediated lysis. A novel β-cell line NIT-4 was derived in a similar fashion to NIT-1 (17) by mating NOD.mt^{ALR} females to NOD-RIP-Tag males. Islets were isolated from an 8-week-old female F1 offspring, dissociated, and cultured in Dulbecco's modified Eagle's medium modified for NIT-1 culture (17). Colonies were picked using cell cloning rings. NIT-1 and NIT-4 cells were genotyped for *mt-Nd2* by pyrosequencing as described previously (5) (supplementary Fig. 1 in the online appendix available at http://diabetes.diabetesjournals.org/cgi/content/full/db10-1241/DC1). For the included experiments, NIT-1 and NIT-4 cells were used at passage numbers P11-P20 and cultured as described (17). To test the sensitivity of β-cell lines to killing by AI4 T-cells in vitro, cell-mediated lysis (CML) assays were performed and calculated as described previously (18).

Statistical analysis. Survival analysis and Student *t* test were performed using Prizm-v5a (GraphPad Software, LaJolla, CA).

RESULTS

The *mt-Nd2*^{a} allele alone does not affect the overall incidence of spontaneous diabetes in NOD mice. Comparable rates of type 1 diabetes incidence were recorded for NOD and NOD.mt^{ALR} (Fig. 1). Although there was a trend toward slower incidence in male NOD.mt^{ALR}, the difference was not significant. A study of insulitis development in female NOD and NOD.mt^{ALR} mice from 4 to 16 weeks of age was also performed. Consistent with diabetes incidence, there were no differences in the histological scores comparing age-matched NOD with NOD.mt^{ALR} mice (data not shown).

Adoptive transfer of splenic leukocytes. To identify effects of *mt-Nd2*^{a} on immune functions versus an effect on pancreatic β-cells, reciprocal adoptive transfers were performed. Donors were either diabetic NOD females or diabetic NOD.mt^{ALR} females. All recipients were age-matched immunodeficient *Rag1*$^{-/-}$ females separately expressing each *mt-Nd2* allotype. Neither allotype expressed by mitochondria in splenocytes from diabetic donors significantly affected adoptive transfer (Fig. 2). Similarly, the *mt-Nd2* allotype expressed by the recipients had no significant effect on the adoptive transfer kinetics regardless of the allotype expressed by the transferred leukocytes. Thus, in the absence of other ALR-protective nuclear genes, the ALR-derived *mt-Nd2*^{a} allele cannot deviate the attack mediated by the plethora of autoreactive T-cells present in spleens of diabetic donors.

Adoptive transfer study using diabetogenic CD8^{+} T-cells. All NOD-*Rag1* recipients developed diabetes after transfer of activated AI4a/b CD8^{+} T-cells (Fig. 3*A*). Not surprisingly, ALR-*Rag1* mice were resistant to disease transfer by AI4 T-cells (Fig. 3*A*), consistent with published data showing that ALR/LtJ islets resist lysis by AI4 cells (11). NOD.mt^{ALR}-*Rag1* mice, although clearly susceptible to AI4-mediated diabetes transfer, nevertheless exhibited a statistically significant retardation in disease development compared with NOD-*Rag1* controls ($P = 0.0036$). Thus, when the T-cell attack is limited to recognition of a single β-cell autoantigen (AI4 recognizes an epitope of dystonia myotonica kinase), the ALR-derived mitochondrial genome confers some resistance, albeit incomplete in the absence of additional protective ALR-derived nuclear genes.

CML. The only known genetic difference between NIT-1 and NIT-4 cell lines is the derivation of the mitochondrial population from ALR/Lt in the latter line. SNP typing has

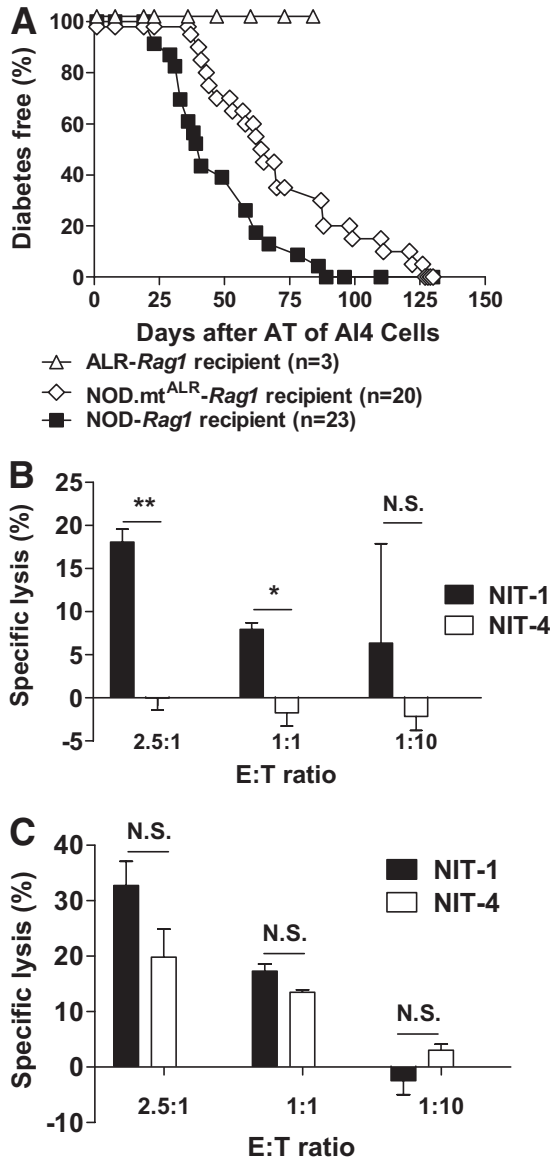

FIG. 3. The *mt-Nd2*^{a} allele confers resistance to killing by diabetogenic CD8$^+$ AI4 T-cells in vivo and in vitro. *A*: Type 1 diabetes onset is significantly retarded following A14 transfer into Rag1 recipients conplastic for the *mt-ND2*^{a} allele (NOD.mt^{ALR}-Rag). Splenocytes from young (3–5 weeks old) NOD-AI4a/b F1 hybrids were collected and erythrocytes removed using hypotonic solution treatment. Cells were injected intravenously (tail vein) at 2 × 10^7 cells/mouse into age-matched female recipients. Onset of diabetes was monitored using Diastix, with a diagnosis of type 1 diabetes called after positive tests on 2 sequential days. *B* and *C*: AI4-induced CML is significantly reduced in NIT-4 cells compared with NIT-1 cells in vitro. Effector cells, splenocytes from NOD-AI4a/b F1 mice were isolated, erythrocytes removed, and the T-cells activated for 3 days with 0.1 μmol/l AI4 mimotope (amino acid sequence YFIENYLEL) and 25 units/ml interleukin-2 in RPMI1640 supplemented with 10% FBS, 2 mmol/l L-glutamine, 1.5g/l sodium bicarbonate, 10 mmol/l HEPES, and 1.0 mmol/l sodium pyruvate. Target NIT-1 and NIT-4 cells were seeded at 1 × 105 cells/well in 96-well culture plates, labeled with 51Cr (Perkin Elmer) for 3 h, and washed; then activated AI4-effector cells were added at increasing effector-to-target (E:T) ratios, in triplicate. *B*: Cr-51–labeled NIT-1 and NIT-4 cells were cultured with preactivated AI4 cells at different E:T ratio for 16 h. Specific lysis of NIT-4 cells at the highest E:T ratio is only 40% that of NIT-1. *C*: NIT-1 and NIT-4 cells were primed with 1,000 U/ml IFN-γ for 24 h before adding T-cells. Both cell types were more sensitive to AI4 T-cell killing compared with unprimed. At the highest E:T ratio, NIT-4–specific lysis reached 61% that of NIT-1. ***P* = 0.0009; **P* = 0.0048. AT, adoptive transfer; NS, not statistically significant.

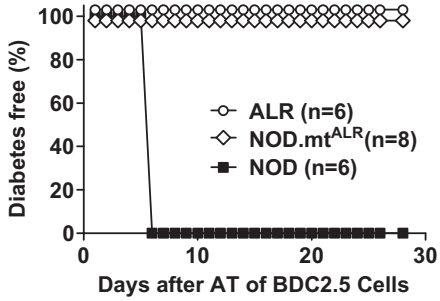

FIG. 4. NOD-mt^{ALR} mice are completely resistant to type 1 diabetes mediated by transfer of diabetogenic CD4$^+$ T-cell clone BDC2.5. BDC2.5 T-cell clones were injected into NOD, NOD.mt^{ALR}, or ALR/LtJ mice. Development of diabetes after injection was monitored using Diastix, with a diagnosis of type 1 diabetes called after positive tests on 2 sequential days. AT, adoptive transfer.

confirmed that the latter has the *mt-Nd2*^{a} allele and NIT-1 has the *mt-Nd2*^{c} allele (supplementary Fig. 1). NIT-1 cells were killed by activated AI4a/b CD8$^+$ T-cells in a dose-dependent fashion whereas NIT-4 cells were resistant (Fig. 3*B*). Live cell confocal microscopy time series confirmed the susceptibility of NIT-1 cells to AI4-mediated killing as well as the resistance of NIT-4 cells to AI4-mediated lysis (supplementary Videos 2–5). However, priming NIT-4 cells with γ-interferon (IFN-γ) for 24 h before adding AI4-effector cells resulted in heightened sensitivity. We observed no statistical difference comparing the CML results of IFN-γ–treated NIT-1 and NIT-4 cells (Fig. 3*C*).

Adoptive transfer of cloned diabetogenic BDC2.5 CD4$^+$ T-cell clones. The diabetogenic CD4$^+$ T-cell clone BDC2.5 transferred diabetes to all NOD mice within 5 days (Fig. 4). In marked contrast to NOD mice, all ALR and NOD.mt^{ALR} mice remained diabetes free after transfer of BDC2.5 cells for the 28-day follow-up period. This confirms that when the T-effector population is limited to a single β-cell autoantigen a significant contribution to resistance can be demonstrated.

DISCUSSION

Autoimmune diabetes is a polygenic disease. Major histocompatibility complex (MHC) and numerous non-MHC *IDD/type 1 diabetes/Idd* loci have been mapped in humans as well as rat and mouse models (rev. in 2). However, although multiple type 1 diabetes susceptibility and resistance loci have been identified, only a few of the responsible genes have been identified, and for the genes identified as responsible, the mechanism of action has yet to be elucidated. Using backcross and F2 hybrid breeding strategies in mouse models, we mapped the A allele of a SNP in the mitochondrially encoded *mt-Nd2* gene as protective against both autoimmune and alloxan-induced free radical–mediated diabetes. These data suggested that the *mt-Nd2*^{a} allotype provided protection at the β-cell level with ROS resistance as a potential mechanism (14). The current finding that substitution of the NOD allotype with its protective ALR/Lt counterpart failed to prevent spontaneous diabetes in NOD.mt^{ALR} mice (Fig. 1) is consistent with our previous observation with the alloxan model (9). This is not surprising given the complexity of autoimmune diabetes. In both the autoimmune (4) and alloxan-induced diabetes models (9), we identified multiple resistance loci, including *mt-Nd2*^{a}. The specific interaction of *mt-Nd2*^{a} with nuclear loci would provide a

model to study genetic interactions between the two cellular genomes—nuclear and mitochondrial.

Because protection against diabetes was not observed when the *mt-Nd2* SNP was substituted with its protective counterpart, we assessed subphenotypes in which this SNP could influence disease. Immune cell profiles were strikingly similar when comparing the two strains (supplementary Fig. 2), suggesting that this SNP does not affect immune cell development. To isolate any effect of this SNP within immune cells, we used reciprocal adoptive transfers. These transfer experiments demonstrated no statistically significant differences in diabetes onset (Fig. 2), suggesting that this SNP does not affect immune cell function. However, when we challenged mice with single diabetogenic T-cell clones (either the $CD8^+$ AI4 or the $CD4^+$ BDC2.5), mice with the *mt-Nd2*^{a} allele responded differently than those with the *mt-Nd2*^{c} allele. NOD.mt^{ALR}-*Rag1* mice were more resistant to disease onset after the transfer of either diabetogenic T-cell clonotype (Figs. 3 and 4), suggesting this SNP modifies type 1 diabetes through nonhematopoietic factors.

$CD8^+$ cytotoxic T-lymphocytes destroy pancreatic β-cells directly by recognition of autoantigens in the context of class I MHC and initiation of cytotoxicity by fatty acid synthase (FAS)-Ligand/FAS pathways and release of granzyme and perforin (19). Subtle changes in mitochondrial pathways downstream of these death pathways may explain the partial resistance to transfer of diabetes by AI4 T-cells to *mt-Nd2*^{a} encoding NOD-mt^{ALR}-*Rag1* mice. Likewise, when preactivated diabetogenic $CD8^+$ AI4 T-cells were combined with β-cells in vitro the NOD-derived β-cell line NIT-1 was killed (Fig. 3*B* and supplementary Video 2), yet NIT-4 with the *mt-Nd2*^{a} allele were resistant (Fig. 3*B* and supplementary Video 4). Resistance was eliminated when target β-cell lines were primed with IFN-γ before adding AI4 cells (Fig. 3*C*). IFN-γ priming induces MHC class I and FAS on the β-cell surface (20). The change that *mt-Nd2*^{a} exerts on mitochondrial function through ROS modulation may alter ROS-dependent signaling of death receptor pathways as manifested as resistance of NIT-4 to autoreactive $CD8^+$ AI4 cells in vitro and retarded disease transfer by AI4 in vivo. However, the resistance conferred by this SNP at the β-cell level is overridden by IFN-γ priming.

Unlike $CD8^+$ T-cells, BDC2.5 $CD4^+$ T-cells do not directly kill β-cells, such that killing is cytokine-dependent (21). Upon transfer into recipient mice, BDC2.5 T-cells require activation to initiate pancreatic β-cell destruction. β-Cell expression of tumor necrosis factor (TNF) receptor 1(TNFR1)-P50 is critical for activation of BDC2.5 cells (22). When these diabetogenic $CD4^+$ T-cells were transferred to recipient mice, endogenous macrophages were recruited to the pancreas and activated to secrete cytokines (21). These cytokines include TNF-α. TNF-α induces both apoptosis and necrosis of cells through the TNFR1 (rev. in 23). During TNFR-induced cell death, mitochondria participate in a series of events including generating truncated BID locally, releasing cytochrome C, activating downstream caspases, and producing ROS (23). ROS production by mitochondria is an early event in TNFR-induced cell death (23). Therefore, mitochondrial participation through ROS production is likely indispensable in BDC2.5-mediated β-cell killing. The fact that a ROS scavenger inhibits the transfer of diabetes by BDC 2.5 clones (16) implicates ROS as mediators of β-cell death induced by BDC2.5.

In the current study, a substitution of a SNP in mitochondrially encoded *mt-Nd2* cannot alter the overall spontaneous diabetes in NOD mice. However, subtle changes caused by this SNP (15) account for the greater resistance of the NOD.mt^{ALR} mice to BDC2.5 and AI4-mediated diabetes. In conclusion, this study confirms that genetic polymorphisms in mitochondria can modulate β-cell sensitivity to autoimmune T-cell effectors and raises the possibility that the *mt-ND2*^{a} allotype in humans may provide similar protection.

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

J.C. and C.E.M. designed the research plan, performed experiments, analyzed data, and wrote the manuscript. A.M.G. performed experiments and revised the manuscript. J.P. performed the BDC2.5 adoptive transfers. E.H.L. participated in the experimental design and edited the manuscript.

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