Prevention of “Humanized” Diabetogenic CD8 T-Cell Responses in HLA-Transgenic NOD Mice by a Multipeptide Coupled-Cell Approach

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OBJECTIVE—Type 1 diabetes can be inhibited in standard NOD mice by autoantigen-specific immunotherapy targeting pathogenic CD8+ T-cells. NOD.β2mnull.HHD mice express human HLA-A2.1 but lacking murine major histocompatibility complex class I molecules develop diabetes characterized by CD8 T-cells recognizing certain autoantigenic peptides also targeted in human patients. These include peptides derived from the pancreatic β-cell proteins insulin (INS1/2 A210, and INS1 B3.14) and islet-specific glucose-6-phosphatase catalytic subunit–related protein (IGRP265–273) and IGRP228–236. Hence, NOD.β2mnull.HHD mice represent a model system for developing potentially clinically translatable interventions for suppressing diabetogenic HLA-A2.1–restricted T-cell responses.

RESEARCH DESIGN AND METHODS—Starting at 4–6 weeks of age, NOD.β2mnull.HHD female mice were injected intravenously with syngeneic splenocytes to which various admixtures of the four above-mentioned peptides were bound by the cross-linking agent ethylene carbodiimide (ECDI).

RESULTS—Treatment with such cells bearing the complete cocktail of INS and IGRP epitopes (designated INS/IGRP-SPs) significantly inhibited diabetes development in NOD.β2mnull.HHD recipients compared with controls receiving splenocytes coupled with an irrelevant HLA-A2.1–restricted Flu16 peptide. Subsequent analyses found syngeneic splenocytes bearing the combination of the two ECDI-coupled IGRPs but not INS peptides (IGRP-SPs or INS-SPs) effectively inhibited diabetes development in NOD.β2mnull.HHD mice. This result was supported by enzymelinked immunospot (ELISPOT) analyses indicating combined INS/IGRP-SPs diminished HLA-A2.1–restricted IGRP but not INS autoreactive CD8+ T-cell responses in NOD.β2mnull.HHD mice.

CONCLUSIONS—These data support the potential of a cell therapy approach targeting HLA-A2.1–restricted IGRP autoreactive CD8 T-cells as a diabetes intervention approach in appropriate human patients. Diabetes 60:1229–1236, 2011

It has been long recognized that in both humans and NOD mice the autoimmune destruction of insulin-producing pancreatic β-cells causing type I diabetes development requires pathogenic CD4 T-cell responses mediated by particular major histocompatibility complex (MHC) class II molecules (1–3). However, studies in NOD mice have led to a more recent appreciation that, when expressed in the proper genetic context, some quite common MHC class I variants can acquire an aberrant ability to mediate autoreactive CD8 T-cell responses also essential to diabetes development (4–9). Moreover, CD8 T-cells that recognize various pancreatic β-cell peptides in the context of some particular MHC class I variants can also be detected in the peripheral blood of human diabetic patients (10–15). One relatively common MHC class I variant that can contribute to diabetes susceptibility in humans is HLA-A2.1 (16). Importantly, NOD.β2mnull.HHD mice expressing human HLA-A2.1 but no murine MHC class I molecules generate diabetes-inducing autoreactive CD8 T-cell responses (17,18). NOD.β2mnull.HHD mice have been found to generate HLA-A2.1–restricted autoreactive CD8 T-cell responses against three peptides each derived from the pancreatic β-cell proteins islet-specific glucose-6-phosphatase catalytic subunit–related protein (IGRP228–236, IGRP265–273, and IGRP337–345) and insulin (INS1 L3–11, INS1 B5–14, and INS1/2 A2–10) (17,18). Significantly, the homologous human peptides for Ins1/2 A2–10, Ins1 B5–14, IGRP228–236, and IGRP265–273 are also recognized by NOD.β2mnull.HHD CD8 T-cells (17,18). At least, the IGRP228–236 and IGRP265–273 epitopes have also been found to be the targets of HLA-A2.1–restricted CD8 T-cells in human diabetic patients (10,19,20). For these reasons, NOD.β2mnull.HHD mice would appear to represent an ideal model for developing potentially clinically translatable interventions for suppressing diabetogenic HLA-A2.1–restricted T-cell responses.

By avoiding the potential complications of generalized immunosuppression, antigen-specific tolerance induction therapies may ultimately represent a desirable diabetes intervention approach in humans (21). Early support for such a possibility was provided in an article by Amrani et al. (22) that a free peptide injection approach, which deleted high-avidity IGRP-reactive CD8 T-cells, blocked progression to overt diabetes in standard NOD mice. Han et al. (23) subsequently found that diabetes development was more readily inhibited in NOD mice by a soluble IGRP analog peptide treatment protocol that only depleted CD8 T-cells with high T-cell receptor avidity for this antigen rather than one eliminating all such effectors. Even though these soluble peptide treatments are effective, they are particularly dependent on time of injection and antigenic doses. An alternative antigen-specific method to induce T-cell tolerance is intravenous treatment with syngeneic splenocytes bearing ethylene carbodiimide (ECDI) cross-linked peptides (peptide-SPs) or whole proteins. Treatment with peptide-SPs that induce antigen-specific tolerance has been reported to inhibit experimental autoimmune diabetes.

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encephalomyelitis in mice (24,25). Syngeneic spleen cells bearing whole insulin as an ECDI-coupled autoantigen have also been reported to exert a diabetes-protective effect in standard NOD mice (26). A peptide-SP approach has also been shown to attenuate the activity of diabeticogenic BDC2.5 clonotypic CD4 T-cells in NOD mice (26). However, there have been no evaluations of whether a peptide-SP approach could attenuate the activity of diabeticogenic CD8 T-cells, particularly those recognizing HLA-A2.1-restricted antigenic epitopes that may be of potential high clinical relevance in humans. We addressed this question in the current study by determining whether syngeneic splenocytes bearing any ECDI-coupled combination of the HLA-A2.1-restricted Ins1/2A2–10, Ins1B2–15, IGRP228–236, and IGRP265–273 epitopes could suppress diabetes development in NOD.β2mnull.HHD mice by modulating pathogenic CD8 T-cell responses.

**RESEARCH DESIGN AND METHODS**

Mice. Previously described NOD.β2mnull.HHD mice (17) are maintained by sibling matings at The Jackson Laboratory. MHC class I-deficient NOD.β2mnull mice have also been previously described (4). Some experiments used an N10 backcross generation NOD stock congenically expressing the CD45.2 rather than the CD45.1 variant leukocyte marker. NOD.β2mnull.HHD mice in which antigen-presenting cells (APCs) specifically express an MHC class II promoter (H2-Eb)–driven mouse pronasin 2 transgene (NOD.β2mnull.HHD.PP) were generated by crossing NOD.β2mnull.HHD with previously described NOD.PP mice (27). The pronasin transgene is maintained in a heterozygous state. The institutional Animal Care and Use Committee at The Jackson Laboratory approved all animal experiments.

**Pepitides and antibodies.** Synthetic peptides Ins1/2 A2–10 (VLFGLGFAI), Ins1B2–15, IGRP228–236, IGRP265–273 (VLFGLGFAAI), and Flu16 (Flu MP58.S1) were purchased from Mimotopes Pty, Melbourne, Australia. Monoclonal antibodies specific for CD11c (clone N14S15), CD8 (clone 53.6.7), and Flu16 (Flu MP58) were purchased from BD Biosciences, San Jose, CA and eBiosciences, San Diego, CA.

**ECDI peptide-coupled cell treatment.** ECDI peptide-coupled splenocyte (peptide-SPs) treatment was carried out as previously described (28). Briefly, spleens were removed from syngeneic female mice, collagenase D treated (Roche Diagnostics, Mannheim, Germany), and the erythrocytes lysed. The splenocytes were incubated with ECDI (150 mg/3.2 × 10^6 cells [Calbiochem, La Jolla, CA]), and as indicated, a mixture of INS and/or IGRP peptide(s) (1 mg/mL each) on ice for 1 h, hand-shaking every 10 min. The peptide-SPs were washed, centrifuged, filtered to remove cell clumps, and resuspended in PBS. NOD.β2mnull.HHD female mice (4-6 weeks old) received 50 × 10^6 peptide-loaded spleen cells by intravenous injection. Controls consisted of NOD.β2mnull.HHD female mice treated with the Flu16 peptide. Three injections at 2-week intervals were followed by treatments once every 3 weeks. The mice were monitored for type 1 diabetes development.

**Statistical analyses.** Data were evaluated using Prism 5 software (GraphPad Software). The log-rank test was used to compare diabetes incidence curves and the nonparametric unpaired test to compare antigen-reactive CD8 T-cell numbers between different treatment groups.

**RESULTS**

Splenocytes bearing ECDI-coupled peptides ablate IGRP-specific CD8 T-cell responses in vitro. CD8 T-cells were enriched from pooled spleens of 9- to 16-week-old NOD.β2mnull.HHD mice and cocultured in vitro with syngeneic splenocytes bearing an ECDI-coupled cocktail of the HLA-A2.1-restricted Ins1/2A2–10, Ins1B2–15, IGRP228–236, and IGRP265–273 β-cell autoantigenic peptides (INS/IGRP-SPs) and assessed the next day for responsiveness to each individual epitope by IFN-γ ELISPOT analyses. Compared with those exposed to the control Flu16 peptide (Flu-SPs), CD8 T-cells from NOD.β2mnull.HHD mice cocultured with INS/IGRP-SPs displayed significantly decreased responses to restimulation by IGRP epitopes (Fig. 1A). Levels of reactivity to the HLA-A2.1-restricted INS peptides was low among control CD8 T-cells exposed to Flu16 and not further influenced by previous coculture with INS/IGRP-SPs (Fig. 1A). Coincubation with syngeneic splenocytes bearing an ECDI cross-linked mixture of the two INS peptides (INS-SPs) did not diminish CD8 T-cell responsiveness to INS or IGRP epitopes (Fig. 1B). However, preincubation with IGRP-SPs did significantly diminish the ability of CD8 T-cells from NOD.β2mnull.HHD mice to respond to restimulation by IGRP but not INS epitopes (Fig. 1B). IL-10 ELISPOT analyses also indicated that regardless of preincubation conditions, no CD8 T-cells from NOD.β2mnull.HHD mice responding to INS or IGRP stimulation produced this immunosuppressive cytokine. On the basis of the design of these collective data, we subsequently assessed syngeneic INS/IGRP-SPs as a possible diabetes intervention approach in NOD.β2mnull.HHD mice.

**Syngeneic splenocytes bearing ECDI-coupled autoantigenic IGRP, but not INS peptides, inhibit diabetes development in NOD.β2mnull.HHD mice.** Initial analyses found that repeated injections of a soluble mixture of the four INS and IGRP peptides (25 μg each) did not inhibit diabetes development in NOD.β2mnull.HHD mice (data not shown). Thus, given the in vitro results shown in Fig. 1, we assessed whether a single intravenous injection of INS/IGRP-SPs given at 4-6 weeks of age could protect NOD.β2mnull.HHD female mice from diabetes development. Diabetes development was inhibited in mice treated with the INS/IGRP-SPs, compared with controls
receiving Flu-SPs (Fig. 2). We next determined which peptide(s) were responsible for the diabetes protective effects. NOD.β2mnull.HHD mice were injected with syngeneic Flu-SPs or INS/IGRP-SPs (bearing the complete mixture of IGRP265-273, IGRP228-236, INS1/2 A2-10, and INS1 B5-14 peptides). The next day, recovered CD8 T-cells were cocultured for 48 h with 1 μmol/L of each of the individual INS or IGRP peptides, and antigen reactivity was assessed by ELISPOT analyses of IFN-γ production. Controls were injected with syngeneic Flu-SPs, IGRP-SPs (mixture of IGRP265-273 and IGRP228-236), or INS-SPs (mixture of INS1/2 A2-10 and INS1 B5-14). T-cell reactivity to individual IGRP or INS peptides was then determined as described above. All samples were evaluated in triplicate. Bars represent mean numbers of IFN-γ spots ± SEM. P values are based on comparison with Flu-SPs. *P < 0.01, **P < 0.05; P values INS-SPs compared with IGRP-SPs, ***P < 0.01, ##P < 0.001.

FIG. 2. INS/IGRP-SPs inhibit diabetes development in NOD.β2mnull.HHD mice. NOD.β2mnull.HHD female mice were injected intravenously at 4–6 weeks of age with INS/IGRP-SPs (bearing the ECDI-linked mixture of IGRP265-273, IGRP228-236, INS1/2 A2-10, and INS1 B5-14 peptides). Controls were injected with syngeneic Flu-SPs. Mice were monitored for diabetes development.
showed significantly decreased CD8 T-cell responses to both the IGRP228-236 and IGRP265-273 peptides (P = 0.05 and 0.002) (Fig. 4). These results further indicate that in the context of the human HLA-A2.1 class I variant, autoreactive CD8 T-cells recognizing the IGRP228-236 and/or IGRP265-273 peptides are of significant pathogenic importance during diabetes development in NOD β2mnull.HHD mice.

**Proinsulin is a pathogenic autoantigen in NOD β2mnull.HHD mice.** Previous studies have indicated that (pro)insulin is a key autoantigen for diabetes development in standard NOD mice (27,30–33). This was partly demonstrated by studies showing that MHC class II promoter-driven transgenic expression of proinsulin-2 in APCs of NOD mice inhibits insulitis and diabetes development (27,30). Hence, we used such a transgenic approach as an alternative means to test whether (pro)insulin may also be an autoantigen of pathogenic importance in NOD β2mnull.HHD mice. NOD β2mnull.HHD mice expressing the previously described (27) proinsulin-2 transgene in APCs (designated NOD β2mnull.HHD-PI) were generated and assessed for diabetes development. As shown in Fig. 5A, compared with nontransgenic controls, the rate of type 1 diabetes development was somewhat retarded in NOD β2mnull.HHD-PI female mice but did not quite achieve statistical significance (P = 0.08) by Kaplan-Meier analyses. However, as assessed by χ² analyses, the cumulative frequency of diabetes development by 35 weeks of age was significantly lower (P = 0.005) in NOD β2mnull.HHD-PI mice than in nontransgenic controls. Insulitis levels were also significantly lower in NOD β2mnull.HHD-PI mice than in nontransgenic controls (Fig. 5B). Baseline and primed levels of CD8 T-cell responses to the HLA-A2.1-restricted Ins2A2-10 or Ins1B6-14 epitopes were found not to differ in NOD β2mnull.HHD-PI mice and nontransgenic controls (data not shown). These findings do not
eliminate the possibility that like those targeting IGRP epitopes, HLA-A2.1–restricted insulin autoreactive CD8 T-cells are also important pathogenic contributors to diabetes development in NOD. **b2mnull.HHD** mice. However, these APC transgenic expression studies indicate that even if they do not represent autoantigens recognized by pathogenic HLA-A2.1–restricted CD8 T-cells, (pro)insulin derived epitopes are important targets of at least diabetogenic CD4 T-cells in NOD. **b2mnull.HHD** mice.

INS/IGRP-SPs do not have to share host MHC class I identity to tolerate IGRP-specific CD8 T-cells and to attenuate diabetes development in NOD. **b2mnull.HHD** mice. It has been previously reported that splenocytes bearing ECDI-coupled proteins or peptides do not directly induce tolerogenic responses by CD4 T-cells in an efficient manner, but rather do so indirectly following their uptake and processing by host-type APC (21). If this is also the case for inducing CD8 T-cell tolerance, we reasoned it should remain possible to inhibit diabetes development in the HLA-A2.1–expressing NOD. **b2mnull.HHD** stock by treatments with splenocytes from totally MHC class I–deficient NOD. **b2mnull** mice bearing the complete cocktail of ECDI cross-linked INS and IGRP epitopes (designated INS/IGRP-**b2mnull** SPs). Unlike those receiving Flu-**b2mnull** SPs, INS/IGRP-**b2mnull** SPs do not have to share host-type MHC class I identity to attenuate diabetes development in NOD. **b2mnull.HHD** mice. Beginning at 4–6 weeks of age NOD. **b2mnull.HHD** mice were injected intravenously at 5-week intervals with INS/IGRP-**b2mnull** SPs or Flu-**b2mnull** SPs. A: Mice were monitored for diabetes development. B: INS/IGRP-**b2mnull** SPs inhibit in vivo responses of HLA-A2.1–restricted IGRP but not INS autoreactive CD8 T-cells in NOD. **b2mnull.HHD** mice. Two days after the third treatment, mice were footpad primed with a mixture of the four INS/IGRP peptides. Ten days after priming, CD8 T-cells within the draining popliteal lymph nodes of nondiabetic mice were cocultured for 48 h with 1 μmol/L of each of the individual INS or IGRP peptides, and antigen reactivity was assessed by ELISPOT analyses of IFN-γ production.

![Graph A](image1.png)

**FIG. 5.** Insulitis and diabetes development in NOD. **b2mnull.HHD-PI** mice. A: Incidence curve of diabetes development in NOD. **b2mnull.HHD-PI** female mice and nontransgenic littermates. The rate of diabetes development did not quite achieve a statistical difference (P = 0.08) by Kaplan-Meier analyses. The cumulative frequency of diabetes development by 35 weeks of age was significantly different (P < 0.005) as assessed by χ² analyses. B: Histological grading of insulitis in pancreas sections of 35-week-old female mice (NOD. **b2mnull.HHD-PI** mice, n = 14; NOD. **b2mnull.HHD** mice, n = 16).

![Graph B](image2.png)

**FIG. 6.** INS/IGRP-SPs do not have to share host-type MHC class I identity to attenuate diabetes development in NOD. **b2mnull.HHD** mice. Beginning at 4–6 weeks of age NOD. **b2mnull.HHD** mice were injected intravenously at 5-week intervals with INS/IGRP-**b2mnull** SPs or Flu-**b2mnull** SPs. A: Mice were monitored for diabetes development. B: INS/IGRP-**b2mnull** SPs inhibit in vivo responses of HLA-A2.1–restricted IGRP but not INS autoreactive CD8 T-cells in NOD. **b2mnull.HHD** mice.
SPs, diabetes development was completely abrogated in INS/IGRP-β2mnull SPs-treated NOD,β2mnull,HHD mice (Fig. 6A).

Two days after a third INS/IGRP-β2mnull SPs or Flu-β2mnull SPs treatment given at 5-week intervals, NOD,β2mnull,HHD mice were primed in the footpad with a cocktail of the four INS and IGRP peptides. Ten days postpriming, CD8 T-cells within the draining popliteal lymph nodes were assessed for reactivity to each individual peptide by IFN-γ ELISPOT analyses. CD8 T-cell responses to both the HLA-A2.1–restricted IGRP228–236 and IGRP265–273 peptides but again not the INS epitopes were significantly decreased in INS/IGRP-β2mnull SPs-treated NOD,β2mnull,HHD mice (Fig. 6B). These collective results indicate that in order to induce CD8 T-cell tolerance and to elicit diabetes protective effects in NOD,β2mnull,HHD recipients, donor cells bearing ECDI cross-linked HLA-A2.1 restricted IGRP autoantigenic peptides do not also have to express the relevant host-type MHC class I variant.

The results described above also indicated that rather than directing inducing tolerogenic responses inhibiting diabetogenic CD8 cell activity in NOD,β2mnull,HHD mice, INS/IGRP-SPs or IGRP-SPs instead do so in an indirect manner dependent on their uptake by host-type APC. To directly test this possibility, we compared the ability of ECDI-treated or untreated donor cells that were also

FIG. 7. More efficient uptake by host-type splenic CD8+ DCs of ECDI-treated than untreated donor cells. Splenocytes from NOD,CD45.2 congenic mice were labeled with the CFSE tracker dye and then subsequently treated or not with ECDI before being infused intravenously into standard NOD CD45.1-expressing recipients. The following day, viable host-type splenic DCs were assessed by flow cytometry for comparative uptake of CFSE-labeled ECDI-treated or untreated donor cells. A: Depiction of gating strategy to assess uptake by viable (propidium iodide negative) host-type CD45.1-expressing splenic DC subsets of CFSE-labeled donor-type leukocytes that had or had not been treated with ECDI. B: Mean proportions ± SEM of host-type splenic CD8+ DCs that had taken up CFSE-labeled ECDI-treated or untreated donor-type leukocytes (n = 3/group). Significantly greater uptake of ECDI-treated than untreated donor cells.
labeled with the carboxyfluorescein diacetate succinimidyl ester (CFSE) tracker dye to be taken up by host-type antigen-presenting dendritic cells (DCs). Donor- and recipient-type cells were also distinguished by respective expression of the CD45.2 versus CD45.1 pan-leukocyte marker variant. There was a significantly greater uptake by host-type splenic CD8+ DCs of ECDI-treated than untreated donor cells that had been intravenously infused 1 day earlier (Fig. 7A and B). ECDI fixation induces apoptotic death of treated cells (21). This likely explains why fewer ECDI-treated than untreated donor cells could be detected in the recipients, but with the apoptotic status of the former also allowing them to be more efficiently engulfed by host-type DCs. These results indicate the ability of donor cells bearing ECDI-coupled IGRP peptides to elicit diabetes protective effects likely entails their uptake by host-type DCs that then display the autoantigenic epitopes to CD8 T-cells in a tolerance-inducing manner.

**DISCUSSION**

Our results indicate that treatment with INS/IGRP-SPs (bearing an ECDI cross-linked mixture of the Ins1/2 A2–10, Ins1 B5–15, IGRP228–236 and/or IGRP265–273 peptides) inhibits diabetes development in “humanized” NOD,β2mnull.HHD mice. We found IGRP-SPs to be more effective than INS-SPs in inhibiting diabetes development in NOD,β2mnull. HHD mice. Furthermore, the inhibition of diabetes development in NOD,β2mnull.HHD mice by INS/IGRP-SPs treatment was associated with an attenuation of IGRP but not INS-specific autoreactive CD8 T-cell responses. Treatment with IGRP228–236 or IGRP265–273 single peptide-SPs did not significantly inhibit diabetes development in NOD,β2mnull.HHD mice. This indicated that CD8 T-cell tolerance must be established to both the HLA-A2.1–restricted IGRP228–236 and IGRP265–273 epitopes in order to elicit diabetes-protective effects in NOD,β2mnull.HHD mice.

In standard NOD mice, insulin appears to be an earlier target than IGRP of CD4 and CD8 T-cells initiating diabetes development (31–33). It is unclear why INS/IGRP-SPs or INS-SPs treatment did not alter levels of HLA-A2.1–restricted CD8 responses against the Ins1/2 A2–10 or Ins1 B5–15 epitopes in NOD,β2mnull.HHD mice. However, NOD,β2mnull.HHD mice transgenically expressing pro-insulin-2 in APCs were largely protected from insulitis and diabetes development. These APC transgenic studies also indicated that even if diabetes development in NOD,β2mnull.HHD mice does not require HLA-A2.1–restricted CD8 responses targeting (pro)insulin epitopes, an important component of their disease susceptibility still entails CD8 T-cell responses against this pancreatic β-cell antigen.

It has been previously reported that although peptide-SPs can directly induce CD4 T-cell tolerance induction processes in an inefficient manner, they do so more efficiently through an indirect mechanism involving their uptake and processing by host-type APCs (25). Because of these alternative mechanisms, MHC compatibility between splenotype donor and host is not required in order to induce CD4 T-cell tolerance to ECDI-coupled antigens, although syngeneic donor cells are more efficient at doing so. We found INS/IGRP peptides ECDI coupled to completely MHC class I-deficient donor or splenocytes strongly inhibited IGRP-specific CD8 T-cell responses and diabetes development in NOD,β2mnull.HHD mice. Hence, donor-host MHC class I compatibility is not required to efficiently induce CD8 T-cell tolerance to ECDI-coupled self-antigenic peptides. These findings indicate that IGRP-SPs inhibit diabetes development in NOD,β2mnull.HHD mice by inducing CD8 T-cell tolerance through an indirect host-type APC-dependent pathway. Indeed, other data indicate that host-type DCs more efficiently take up donor ECDI-treated than untreated leukocytes.

A review by Luo et al. (21) discusses efforts by the Immune Tolerance Network to develop a clinical trial using ECDI insulin-coupled peripheral blood lymphocytes as a possible diabetes intervention in humans. Our current results indicate that the use of “humanized” NOD,β2mnull.HHD mice and other related strains may facilitate the development of clinically translatable peptide-based therapies for diabetic patients. In particular, currently available “humanized” mouse resources make it possible to determine which autoantigenic peptides when ECDI cross-linked to autologous leukocytes are most likely to attenuate HLA-A2.1–restricted CD8 T-cell responses that recent evidence (10–16) indicates may be important for diabetes development in many human patients.

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M.N. researched data and wrote the manuscript. A.E.G. researched data. M.M. created the NOD,β2mnull.HHD mice. T.W.H.K. created NOD.PI mice and contributed to discussion. D.L.G. contributed to discussion and reviewed and edited the manuscript. D.V.S. directed research and wrote the manuscript.

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