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## HLA-B\*39:06 Efficiently Mediates Type 1 Diabetes in a Mouse Model Incorporating Reduced Thymic Insulin Expression<sup>1</sup>

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### Abstract

Type 1 diabetes (T1D) is characterized by T cell-mediated destruction of the insulin-producing  $\beta$  cells of the pancreatic islets. Among the loci associated with T1D risk, those most predisposing are found in the MHC region. HLA-B\*39:06 is the most predisposing class I MHC allele and is associated with an early age of onset. To establish an NOD mouse model for the study of HLA-B\*39:06, we expressed it in the absence of murine class I MHC. HLA-B\*39:06 was able to mediate the development of CD8 T cells, support lymphocytic infiltration of the islets, and confer T1D susceptibility. Because reduced thymic insulin expression is associated with impaired immunological tolerance to insulin and increased T1D risk in patients, we incorporated this in our model as well, finding that HLA-B\*39:06-transgenic NOD mice with reduced thymic insulin expression have an earlier age of disease onset and a higher overall prevalence as compared to littermates with typical thymic insulin expression. This was despite virtually indistinguishable blood insulin levels, T cell subset percentages, and TCR V $\beta$  family usage, confirming that reduced thymic insulin expression does not impact T cell development on a global scale. Rather, it will facilitate the thymic escape of insulin-reactive HLA-B\*39:06-restricted T cells which participate in  $\beta$  cell destruction. We also found that in mice expressing either HLA-B\*39:06 or HLA-A\*02:01 in the absence of murine class I MHC, HLA transgene identity alters TCR V $\beta$  usage by CD8 T cells, demonstrating that some TCR V $\beta$  families have a preference for particular class I MHC alleles.

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## INTRODUCTION

Type 1 diabetes (T1D)<sup>3</sup> is characterized by T cell-mediated destruction of insulin-producing  $\beta$  cells (1). Both CD4 and CD8 T cells are important for T1D pathogenesis, with CD8 T cells requiring the presentation of  $\beta$  cell epitopes by class I MHC molecules in order to interact with, and eliminate, the  $\beta$  cells (2, 3). It is thus unsurprising that while multiple genetic loci have been found to contribute to T1D development, those most predisposing to T1D can be found in the MHC region (4). Several class I MHC alleles have been found to be predisposing to T1D, including HLA-A\*02:01 and HLA-B\*39:06 (5–9). While the presentation of  $\beta$  cell epitopes by HLA-A\*02:01 has long been known and extensively studied (10), HLA-B\*39:06 has only more recently gained attention as a T1D-associated allele, and much remains to be understood about its ability to confer T1D risk.

While T1D associations have been observed at all HLA class I loci (9), HLA-B\*39:06 is the most predisposing HLA class I allele (7, 9) and, importantly, is associated with an early age of onset (11). Furthermore, HLA-B\*39:06 is most common among the Latin American population (12), where T1D incidence has been rising (13–15). Development of an HLA-B\*39:06-transgenic mouse model is thus of the utmost importance in order to understand the relationship between HLA-B\*39:06, genetic risk background, and T1D pathogenesis. A transgenic model is also essential for the preclinical testing of HLA-B\*39:06-targeted treatments.

Given the multiple risk factors associated with T1D predisposition, it is important to study HLA-B\*39:06 in a translationally relevant manner. The NOD mouse is considered by many to be a good model for human T1D (16, 17). For example, the NOD class II MHC H2-A<sup>g7</sup> shares striking similarities with several T1D-associated human class II MHC alleles such as HLA-DQ8 (18). Among other similarities, both NOD mice and human T1D patients display reduced regulatory T cell function and reduced IL-2 signaling (17, 19, 20). Most importantly, T cells from HLA-transgenic NOD mice may target similar or even identical  $\beta$  cell epitopes to those found in T1D patients (21–23). However, to most accurately model HLA-B\*39:06 in the context of human T1D, it is preferable to incorporate additional human non-MHC risk alleles. In humans, the non-MHC locus that confers the most susceptibility to T1D is the variable number of tandem repeats (VNTR) region of the insulin gene (24–26). Shorter VNTR sequences are known as class I, while longer VNTR sequences are known as class III. Class I VNTR sequences are associated with T1D risk and with a decrease in thymic insulin mRNA levels compared with the longer class III VNTR alleles, which are protective (24). The decrease in thymic insulin expression correlates with impaired negative selection of high-avidity insulin-specific T cells in humans (27).

The reduced thymic insulin expression associated with class I VNTR sequences in humans has been modeled in mice through introduction of two *Insulin 2* (*Ins2*) knockout (KO) alleles (28–32). Mice possess two insulin genes, *Insulin 1* (*Ins1*) and *Ins2*. Although expressed in the pancreas, little (28, 33) to no (34, 35) *Ins1* expression occurs in the thymus.

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<sup>3</sup>Abbreviations used in this paper  $\beta$ 2m,  $\beta$ 2-microglobulin; Het, heterozygous; HHD, human  $\beta$ 2m/HLA/H2-D<sup>b</sup>; Hom, homozygous; Ins2, insulin 2; KO, knockout; MFI, mean fluorescence intensity; T1D, type 1 diabetes; VNTR, variable number of tandem repeats; WT, wild-type.

In contrast, *Ins2* is expressed in both the thymus and the pancreas (28). Upon *Ins2* ablation, both NOD mice and mouse strains not prone to T1D exhibit diminished T cell tolerance to insulin, as evidenced by enhanced T cell reactivity to insulin and insulin-derived peptides (28–30, 32). We have shown that NOD mice even just heterozygous (Het) for the *Ins2*<sup>KO</sup> allele exhibit decreased thymic insulin expression as seen in human T1D patients (36). In the context of HLA-A\*02:01, we have previously found that NOD mice with reduced thymic insulin expression display increased T1D incidence, islet infiltration, and CD8 T cell responses to insulin-derived peptides (30, 36), speaking to the importance of examining multiple risk alleles simultaneously.

Here we have developed HLA-B\*39:06-transgenic NOD mouse models and have demonstrated that HLA-B\*39:06 is able to independently mediate the development of CD8 T cells required for T1D onset. We found that in the context of reduced immunological tolerance to insulin (*i.e.*, *Ins2* ablation), HLA-B\*39:06-transgenic NOD mice develop T1D at an accelerated rate compared to mice with wild-type (WT) thymic insulin expression. We excluded secondary causes for the enhanced disease by verifying that blood insulin levels were normal even though *Ins2* was ablated, as has been observed elsewhere (37), and that no gross alterations in lymphocyte composition or TCR V $\beta$  family usage were exhibited. Rather, with a decrease in thymic insulin expression, HLA-B\*39:06 will be less able to negatively select insulin-specific CD8 T cells. Thus, by generating HLA-B\*39:06-transgenic NOD mice in the presence of reduced thymic insulin expression, we show here the development of models that will provide excellent tools for the examination of HLA-B\*39:06's impact on T1D and for the preclinical testing of HLA-B\*39:06-targeted therapies.

## MATERIALS AND METHODS

### Mice

To develop HLA-B\*39:06-transgenic NOD mice, we prepared a monochain chimeric HLA-B\*39:06 construct, comprising the  $\alpha$ 1 and  $\alpha$ 2 peptide binding domains of HLA-B\*39:06 linked to the  $\alpha$ 3 CD8 binding and transmembrane domains of H2-D<sup>b</sup> with human  $\beta$ <sub>2</sub>-microglobulin ( $\beta$ 2m) linked covalently to the  $\alpha$ 1 domain. Chimeric constructs of this design are designated human  $\beta$ 2m/HLA/H2-D<sup>b</sup> (HHD) (38). This HLA-B\*39:06 HHD construct was injected into NOD zygotes, and founder mice were identified by PCR of tail-tip DNA using these HLA-B\*39:06 primers: 5'-CTTCATCTCAGTGGGCTAC-3' and 5'-CGGTCAGTCTGTGTGTTGG-3'. Positive progeny were further assessed for HLA-B\*39:06 expression on their peripheral blood leukocytes by flow cytometry using anti-HLA-A, B, C (W6/32; BioLegend). Founder 45, with the highest expression of HLA-B\*39:06, was selected for further investigation and was crossed with an NOD mouse. Progeny of this cross were assessed for the presence of the transgene by PCR of tail-tip DNA; mice hemizygous for the transgene were designated NOD.HLA-B\*39:06<sup>Hemi</sup>. To maintain this strain, NOD.HLA-B\*39:06<sup>Hemi</sup> mice were crossed with NOD littermates. NOD.HLA-B\*39:06<sup>Hemi</sup> females were also crossed with male mice from NOD. $\beta$ 2m<sup>KO</sup> (39) or NOD. $\beta$ 2m<sup>KO</sup>.*Ins2*<sup>KO</sup> strains (36). To fix the HLA-B\*39:06 transgene to homozygosity, the resulting progeny were interbred as appropriate to generate HLA-B\*39:06 homozygous

mice (HLA-B\*39:06<sup>Hom</sup>) with the following genotypes: NOD.HLA-B\*39:06<sup>Hom</sup>. $\beta$ 2m<sup>KO</sup>, NOD.HLA-B\*39:06<sup>Hom</sup>. $\beta$ 2m<sup>KO</sup>.Ins2<sup>Het</sup>, and NOD.HLA-B\*39:06<sup>Hom</sup>. $\beta$ 2m<sup>KO</sup>.Ins2<sup>KO</sup>. As we and others have found that female NOD. $\beta$ 2m<sup>KO</sup> mice breed poorly (40), we crossed male  $\beta$ 2m<sup>KO</sup> mice with female  $\beta$ 2m<sup>Het</sup> mice whenever possible. The WT and KO  $\beta$ 2m and WT and KO Ins2 alleles were identified by PCR using the following primer pairs:  $\beta$ 2m<sup>WT</sup>: 5'-GAAACCCCTCAAATTCAAGTATACTCA-3' and 5'-GACGGTCTTGGGCTCGGCCATACT-3';  $\beta$ 2m<sup>KO</sup>: 5'-GAAACCCCTCAAATTCAAGTATACTCA-3' and 5'-TCGAATTCGCCAATGACAAGACGCT-3'; Ins2<sup>WT</sup>: 5'-GGCAGAGAGGAGGTGCTTTG-3' and 5'-AGAAAACCACCAGGGTAGTTAGC-3'; Ins2<sup>KO</sup>: 5'-GGCAGAGAGGAGGTGCTTTG-3' and 5'-ATTGACCGTAATGGGATAGG-3'. NOD.HLA-A\*02:01 (HHD). $\beta$ 2m<sup>KO</sup> mice have been previously described (21).

### Assessment of HLA-B\*39:06 homozygosity by real-time PCR

Mouse tails were numbed with ethyl chloride (Gebauer) and the tail tips were removed. Tails were digested in 200  $\mu$ l proteinase K (Roche) solution overnight at 56°C. The reaction was stopped by placing tails at 95°C for 10 min. The resultant DNA (1  $\mu$ l) was mixed with PrimeTime Gene Expression Master Mix (IDT) and each of the following primers and TaqMan probes: HLA-B\*39:06 primers (5'-TTCATCTCAGTGGGCTACG-3' and 5'-TGTGTTCCGGTCCCAATATTC-3') and probe [5'-(6-FAM)-TCGCTGTGCAACCTCACGAACCTG-(Zen probe with Iowa Black)-3']; internal positive control primers (5'-CACGTGGGCTCCAGCATT-3' and 5'-TCACCAGTCATTTCTGCCTTTG-3') and probe [5'-(Cy5)-CCAATGGTCGGGCACTGCTCAA-(Black Hole Quencher 2)-3']. Real-time quantitative PCR was performed in triplicate using an iQ5 Real-time PCR Detection System (Bio-Rad). Amplification was carried out as follows: initial denaturing at 94°C for 2 min, followed by 38 cycles of 20 s at 94°C, 15 s at 60°C and 10 s at 72°C. Copy numbers were calculated using the 2<sup>-Ct</sup> method.

### Assessment of T1D

Mice were monitored weekly from 4–30 wks for glucosuria using Diastix reagent strips (Bayer). Mice were considered diabetic following two consecutive positive tests. The first positive test was recorded as the date of diabetes onset.

### Histology

Pancreata were fixed in Bouin's solution, sectioned at three non-overlapping levels, and stained with aldehyde fuchsin and hematoxylin and eosin. Islets were scored for insulinitis by a blinded observer as previously described (41): 0, no visible lesions; 1, peri-insular or non-invasive leukocytic aggregates; 2, <25% islet destruction; 3, 25–75% islet destruction; 4, >75% islet destruction. A mean insulinitis score was determined for each mouse by dividing the total score for each pancreas by the total number of islets examined. Diabetic mice were assigned a score of 4.

### Blood collection and staining of peripheral blood leukocytes

Blood (10  $\mu$ l) was collected from the mouse tail vein and added to 50  $\mu$ l PBS (pH 7.2, Gibco) with 1 mM EDTA (Sigma). Samples were mixed well and erythrocytes were lysed for 2–3 min with 200  $\mu$ l ACK lysis buffer (Lonza). Plates were centrifuged at 700xg for 3 min and ACK lysis was repeated. Following centrifugation, samples were washed twice with PBS containing 1% FBS (HyClone) and 0.1% (w/v) sodium azide. All subsequent washes and dilutions were performed using this buffer. Cells were stained with Fc Block (BD Biosciences), followed by anti-CD8 $\alpha$  (53–6.7; BD Biosciences) and anti-HLA-A, B, C (B9.12.1; Beckman Coulter) and incubated on ice for 15–20 min. Samples were washed twice, suspended in 1  $\mu$ g/ml DAPI and incubated on ice for 15–30 min. Samples were filtered through a 35- $\mu$ m cell strainer prior to data collection on a BD LSRII flow cytometer with five lasers (355 nm, 405 nm, 488 nm, 561 nm and 640 nm). Data were analyzed using FlowJo software (version 8.8.6).

### Serum collection and insulin ELISA

Blood (20–40  $\mu$ l) was collected from the mouse tail vein and allowed to clot at room temperature for 1 h. Samples were centrifuged for 15 min at 960xg at 4°C. Serum was stored in aliquots at –20°C. Blood insulin levels were measured using the Mouse Ultrasensitive Insulin ELISA (ALPCO). Absorbance of each well at 405 nm was detected using an Emax precision microplate reader (Molecular Devices) and the results were analyzed using GraphPad Prism 7 software.

### Preparation of splenocytes and pancreatic lymph node cells and flow cytometry

Mice were euthanized using CO<sub>2</sub> asphyxiation, followed by cervical dislocation. Spleens and, for some experiments, pancreatic lymph nodes were harvested and placed in ice-cold RPMI (Gibco) supplemented with 10% FBS, 1% sodium pyruvate (Gibco), 1% non-essential amino acids (Gibco), 50 U/ml penicillin and 50  $\mu$ g/ml streptomycin (Gibco). Organs were crushed, passed through a 40- $\mu$ m cell strainer and washed with RPMI. Samples were centrifuged at 486xg for 5 min. Erythrocytes were lysed in ACK lysis buffer (Lonza) for 4 min at room temperature and washed with RPMI. The resultant cells were centrifuged and washed twice with PBS. Prior to the final wash, samples were passed through a 40- $\mu$ m cell strainer. Cells were counted and suspended in PBS. Samples prepared in the above manner were added to a V-bottomed plate and centrifuged at 486xg for 5 min. Samples were washed once in PBS containing 2% FBS (HyClone). This buffer was used for all subsequent washing and dilution steps. Cells were stained with Fc Block (BD Biosciences) on ice for 10 min and washed once. For monitoring of class I MHC expression, cells were incubated on ice for 20 min with labeled anti-HLA-A, B, C (B9.12.1; Beckman Coulter), anti-pan murine class I MHC (M1/42; The Jackson Laboratory), or an appropriate isotype control antibody (mouse IgG2a for B9.12.1 and rat IgG2a/ $\kappa$  for M1/42). For analysis of splenic immune cell populations, cells were stained with labeled anti-CD19 (6D5; BioLegend), anti-TCR $\beta$  (H57–597; BD Biosciences), anti-CD8 $\alpha$  (53–6.7; BD Biosciences), anti-CD4 (GK1.5; BD Biosciences), and anti-CD25 (PC61.5; eBioscience). For study of TCR V $\beta$  usage, an anti-mouse TCR V $\beta$  screening panel was used (BD Biosciences) in conjunction with labeled anti-CD19 (6D5; BioLegend), anti-CD3e (145-2C11; BD Biosciences), anti-CD8 $\alpha$  (53–6.7;

BD Biosciences), anti-CD4 (GK1.5; BD Biosciences), and anti-CD25 (PC61.5; eBioscience). Samples were washed twice, incubated in 1  $\mu\text{g/ml}$  DAPI for 15 min on ice, and filtered through a 35- $\mu\text{m}$  cell strainer prior to data collection. Data were collected on a BD LSRII flow cytometer with five lasers (355 nm, 405 nm, 488 nm, 561 nm and 640 nm) and analyzed using FlowJo (version 8.8.6) and GraphPad Prism 7 software.

## RESULTS

### NOD mice transgenic for HLA-B\*39:06 are susceptible to T1D

To begin to study the association of HLA-B\*39:06 with T1D, we first developed NOD.HLA-B\*39:06 mice using a monochain HLA-B\*39:06 construct. We tracked these mice for susceptibility to disease to ensure that the integration of HLA-B\*39:06 did not interfere with T1D development. We found no decrease in disease susceptibility compared to non-transgenic littermates in either females (Fig. 1A) or males (Fig. 1B). The earliest age of onset among female mice was 14 wks, with 82% diabetic by 30 wks. In males, the earliest age of onset was at 13 wks, though as expected, incidence was reduced compared to females, with only 57% converting to disease by 30 wks of age. Because females were more susceptible to disease than males, we used female mice for our subsequent experiments.

### HLA-B\*39:06 allows for the selection of CD8 T cells in NOD mice

To examine the influence of HLA-B\*39:06 on T1D without the complicating factor of the concomitant expression of murine class I MHC molecules, we developed a model in which the transgenic HLA-B\*39:06 was expressed in the absence of murine  $\beta 2\text{m}$  by breeding with the NOD. $\beta 2\text{m}^{\text{KO}}$  strain (39). Because the transgenic HLA-B\*39:06 HHD molecules contain covalently bound human  $\beta 2\text{m}$ , HLA-B\*39:06 can fold without reliance on murine  $\beta 2\text{m}$ , whereas the endogenous H2-K<sup>d</sup> and H2-D<sup>b</sup> cannot. To maximize the expression of HLA-B\*39:06 and the thymic selection of CD8 T cells, we sought to fix the HLA-B\*39:06 transgene to homozygosity (HLA-B\*39:06<sup>Hom</sup>). To do so, we first examined the level of human class I MHC on peripheral blood leukocytes from female NOD.HLA-B\*39:06. $\beta 2\text{m}^{\text{KO}}$  mice (Fig. 2A). While all mice tested expressed human class I MHC, there appeared to be two groups of mice, one with high levels of class I MHC, with an average geometric mean fluorescence intensity (MFI) of 1534, and one with low class I MHC levels, with an average MFI of 607, suggesting that the mice with increased human class I MHC levels were HLA-B\*39:06<sup>Hom</sup>. We used real-time PCR for the HLA-B\*39:06 transgene to ensure that the high expressers were indeed homozygous for HLA-B\*39:06 (Fig. 2B). We found that the average copy number of the low expressers was 1.6. This value was consistent with previous experiments with NOD.HLA-B\*39:06. $\beta 2\text{m}^{\text{KO}}$  mice that were known to be hemizygous (data not shown), confirming that the human class I MHC-low mice were HLA-B\*39:06<sup>Hemi</sup>. Human class I MHC-high mice had a copy number of 3.1, nearly double what was seen in the HLA-B\*39:06<sup>Hemi</sup> mice and indicating that these mice were, in fact, homozygous for HLA-B\*39:06. We hypothesized that HLA-B\*39:06<sup>Hom</sup> mice would be capable of developing increased amounts of CD8 T cells relative to HLA-B\*39:06<sup>Hemi</sup> mice. We therefore examined the percent of blood CD8 T cells in female NOD.HLA-B\*39:06. $\beta 2\text{m}^{\text{KO}}$  mice (Fig. 2C). HLA-B\*39:06<sup>Hemi</sup> mice had 1.5% CD8 T cells among their peripheral blood leukocytes, while HLA-B\*39:06<sup>Hom</sup> mice had nearly double that

amount with 2.5% CD8 T cells, indicating that increased HLA-B\*39:06 expression can mediate the development of a higher percentage of CD8 T cells. Mice homozygous for HLA-B\*39:06 were used for all subsequent experiments.

Having observed CD8 T cells in the peripheral blood of NOD.HLA-B\*39:06. $\beta$ 2m<sup>KO</sup> mice, we next sought to confirm the lack of cell-surface expression of murine class I MHC on splenocytes using the pan murine class I MHC antibody M1/42. Splens from NOD and NOD. $\beta$ 2m<sup>KO</sup> mice, and the previously characterized NOD.HLA-A\*02:01. $\beta$ 2m<sup>KO</sup> strain (21), were also examined. As expected, only NOD splenocytes showed expression of murine class I MHC (Fig. 3A). The absence of murine class I MHC in NOD. $\beta$ 2m<sup>KO</sup> mice results in a lack of CD8 T cells (Fig. 3B, 3C), as reported previously (39). However, we observed a partial restoration of CD8 T cell development in the NOD.HLA-B\*39:06. $\beta$ 2m<sup>KO</sup> strain (Fig. 3B, 3C), demonstrating that HLA-B\*39:06 is indeed able to mediate CD8 T cell development.

### HLA-B\*39:06 mediates T1D in NOD mice lacking murine $\beta$ 2m

We next examined the ability of HLA-B\*39:06 to mediate the development of T1D. NOD. $\beta$ 2m<sup>KO</sup> mice are protected from T1D because they lack CD8 T cells (39, 42–44). However, homozygous expression of HLA-B\*39:06 in NOD. $\beta$ 2m<sup>KO</sup> mice partially restored a disease phenotype (Fig. 4A), with the earliest age of onset at 20 wks and with 17% of NOD.HLA-B\*39:06. $\beta$ 2m<sup>KO</sup> mice diabetic at 40 wks. We therefore show here for the first time that HLA-B\*39:06 is able to independently lead to the development of T1D in mice. As previously reported (21), homozygous expression of HLA-A\*02:01 (HHD) also allowed for partial restoration of disease susceptibility (Fig. 4A). The incidence curves for the two strains were statistically indistinguishable ( $p = 0.17$ ) (Fig. 4A), and examination of insulinitis in non-diabetic mice of each strain revealed similar amounts of islet infiltration ( $p = 0.15$ ) (Fig. 4B). Representative islets from HLA-A\*02:01 and HLA-B\*39:06 mice are shown in Fig. 4C and 4D, respectively. Consistent with previous results (36), the majority of NOD.HLA-A\*02:01. $\beta$ 2m<sup>KO</sup> mice displayed insulinitis (Fig. 4B). Similarly, histological examination of islets from 40-wk-old NOD.HLA-B\*39:06. $\beta$ 2m<sup>KO</sup> mice revealed that, despite not all progressing to overt T1D, all mice displayed some degree of insulinitis, with 81% of mice fully infiltrated (Fig. 4B).

### Decreased thymic insulin expression results in earlier T1D onset in HLA-B\*39:06-transgenic mice

NOD.*Ins2*<sup>KO</sup> mice are characterized by greatly diminished thymic insulin expression (32). Compared to WT littermates, they display accelerated T1D onset, increased insulinitis, and impaired immunological tolerance to insulin manifested as increased T cell reactivity to insulin and insulin-derived peptides (30–32). The impact of *Ins2* deficiency on disease is dependent on the genetic context, as NOD.HLA-A\*02:01. $\beta$ 2m<sup>KO</sup>.*Ins2*<sup>KO</sup> mice have a faster disease onset than NOD.*Ins2*<sup>KO</sup> mice (45), indicating that the effects of multiple risk alleles can combine to increase risk. We find that, in conjunction with HLA-B\*39:06, *Ins2* deficiency leads to a rapid onset of disease (Fig. 5A, 5B), demonstrating the importance of examining T1D in the context of multiple risk factors. Female NOD.HLA-B\*39:06. $\beta$ 2m<sup>KO</sup> mice had an earliest age of onset of 25 wks, with 58% diabetic at 30 wks. In contrast,

NOD.HLA-B\*39:06.β2m<sup>KO</sup>.Ins2<sup>KO</sup> mice had an earliest age of onset of 12 wks, with 100% of this strain being diabetic by 16 wks. We have previously noted that Ins2<sup>Het</sup> NOD mice exhibit a modest decrease in thymic insulin expression compared to WT NOD mice (36). Comparison of incidence curves (Fig. 5A) and age at onset (Fig. 5B) showed a trend for NOD.HLA-B\*39:06.β2m<sup>KO</sup>.Ins2<sup>Het</sup> mice to exhibit a disease phenotype intermediate between that of their KO and WT counterparts, though the differences between the Het and WT mice did not reach statistical significance with the sample sizes available.

### Differing amounts of thymic insulin expression do not grossly alter lymphocyte populations

It was likely that the increased disease susceptibility seen in the NOD.HLA-B\*39:06.β2m<sup>KO</sup>.Ins2<sup>KO</sup> mice was due solely to the impaired T cell tolerance to insulin that is well known to be associated with *Ins2* ablation (28–30, 32). However, to exclude a possible secondary cause which had not been explored in prior work, *i.e.*, gross changes in lymphocyte populations, we examined the impact of differing amounts of thymic insulin expression on splenic B cell, CD8 T cell, and CD4 T cell populations (Fig. 6A). As previously reported (21), NOD.HLA-A\*02:01.β2m<sup>KO</sup> mice have a reduced percentage of splenic CD8 T cells and an increased percentage of B cells and CD4 T cells relative to NOD mice, and this was also observed in the NOD.HLA-B\*39:06.β2m<sup>KO</sup> strain (Fig. 6B). However, when HLA-B\*39:06 mice with differing *Ins2* genotypes were compared, no significant changes in splenocyte subset percentages were observed (Fig. 6B). Furthermore, the percentage of CD4<sup>+</sup>CD25<sup>+</sup> T cells was consistent across all groups, suggesting that the change seen in disease susceptibility was not due to a differing proportion of largely regulatory T cells. Together, these data suggest that the increase in disease incidence seen in the NOD.HLA-B\*39:06.β2m<sup>KO</sup>.Ins2<sup>KO</sup> mice, compared to their Ins2<sup>Het</sup> and Ins2<sup>WT</sup> counterparts, was not due to gross changes in lymphocyte composition.

### Thymic insulin expression does not alter TCR Vβ usage, but HLA transgene identity does

We next wished to exclude the unlikely possibility that changes in TCR Vβ usage accompanied the enhanced disease observed in the NOD.HLA-B\*39:06.β2m<sup>KO</sup>.Ins2<sup>KO</sup> mice. For this purpose, splenocytes from these mice and their Ins2<sup>WT</sup> counterparts were stained with a panel of anti-mouse TCR Vβ antibodies. Separate examination of CD8 and CD4 T cells revealed no significant differences in TCR Vβ usage between these two strains of mice (Fig. 7A, 7B). When the CD4<sup>+</sup>CD25<sup>+</sup> (largely regulatory) T cell population was examined individually, the Ins2<sup>KO</sup> mice showed a small but significant increase in the use of TCR Vβ8.1/2 when compared to Ins2<sup>WT</sup> mice, but no other changes were noted (Fig. 7C).

The availability of both NOD.HLA-B\*39:06.β2m<sup>KO</sup> and NOD.HLA-A\*02:01.β2m<sup>KO</sup> mice presented a unique opportunity to examine the influence of HLA transgene identity on TCR Vβ usage. Examination of CD8 T cells revealed significant differences in usage of Vβ2 and Vβ11 (higher in the presence of HLA-B\*39:06) and Vβ6 and Vβ8.1/2 (higher in the presence of HLA-A\*02:01) (Fig. 7A). In contrast, there were no differences in the usage of these TCR Vβ families when CD4 T cell populations were studied (Fig. 7B, 7C). These findings demonstrate that some TCR Vβ families have a preference for particular class I MHC alleles.



To determine whether certain V $\beta$  families were enriched among beta cell-specific CD8 T cells in our HLA-transgenic models, we attempted to perform TCR V $\beta$  analysis on islet infiltrates directly *ex vivo*. However, these experiments proved unfeasible due to limited cell numbers. As an alternate approach, we investigated whether certain V $\beta$  families were enriched among CD8 T cells in the pancreatic lymph nodes of the mice, where beta cell-specific CD8 T cells can be primed and induced to expand (46). For the NOD.HLA-B\*39:06. $\beta$ 2m<sup>KO</sup> strain, Ins2<sup>Het</sup> mice were used, as they trend toward an intermediate disease phenotype compared to Ins2<sup>KO</sup> and Ins2<sup>WT</sup> mice (Fig. 5A). CD8 T cells in the pancreatic lymph nodes utilized a broad repertoire of TCR V $\beta$  families, as observed for splenic CD8 T cells, and no V $\beta$  families were enriched in the pancreatic lymph nodes relative to the spleens of the same animals (Fig. 8A). Similarly, the TCR V $\beta$  family usage among CD8 T cells from the pancreatic lymph nodes of NOD.HLA-A\*02:01. $\beta$ 2m<sup>KO</sup> mice was indistinguishable from that in the spleens (Fig. 8B). These results suggest that beta cell-specific CD8 T cells in both HLA-transgenic models may utilize a broad TCR repertoire.

### Despite altered thymic insulin expression, HLA-B\*39:06-transgenic NOD mice retain typical blood insulin levels

Because of the compensatory changes observed in pancreatic *Ins* gene expression when the total number of *Ins* genes is reduced from four to two (28), we considered it unlikely that insufficient pancreatic insulin expression contributed to the earlier age of disease onset seen in the NOD.HLA-B\*39:06<sup>Hom</sup>. $\beta$ 2m<sup>KO</sup>.Ins2<sup>KO</sup> mice. To confirm this, we measured blood insulin levels in young mice (5–6.5 wks old), well prior to disease onset (Fig. 9). We found that the level of insulin expression was statistically indistinguishable between NOD.HLA-B\*39:06<sup>Hom</sup>. $\beta$ 2m<sup>KO</sup> mice and their Ins2<sup>Het</sup> and Ins2<sup>KO</sup> counterparts, with an average concentration of 0.8 ng/ml, consistent with previous reports for other mouse strains (37, 47). This supports the notion that the changes in disease onset are solely due to diminished T cell tolerance to insulin associated with *Ins2* ablation (28–30, 32), and not also influenced by an inherently decreased ability to produce insulin.

## DISCUSSION

Multiple loci are associated with T1D risk, including a number of class I and class II MHC alleles (4). Among these, HLA-B\*39:06 is not only the most predisposing class I HLA allele in T1D patients (7, 9), but also leads to an earlier age of disease onset (11). However, due to the rarity of this allele among the populations studied thus far, investigation of the direct impact of HLA-B\*39:06 on T1D pathogenesis has not been possible (6, 7). It is important to note that HLA-B\*39:06 is more common among Latin American populations, with allele frequencies of 0.03 among Mexican Americans, 0.02–0.06 among Hispanic Americans, and 0.01–0.09 among Mexican individuals (12). The Venezuela Perja Mountain Bari population has an allele frequency of 0.24. While T1D is relatively rare within Latin American countries, incidence is rising worldwide and new patients from these populations can be expected (14, 15). Similarly, patients carrying this genetic variant can increasingly be found in countries where T1D incidence is highest (48). While genetic background is important, environment is as well; when individuals from areas with low T1D incidence move to areas with high incidence, they assume some of the risk of their new environment (49, 50).

Therefore inclusion of HLA-B\*39:06-positive patients in treatment studies is essential. As such, the development of a mouse model for the study of HLA-B\*39:06 is important as this resource can provide a useful preclinical tool for the testing of HLA-B\*39:06-directed treatments in the absence of sufficiently powered patient studies.

We have previously used an NOD. $\beta 2m^{KO}$ -based model to study the contribution of HLA-A\*02:01 to T1D development (21). In the current study, we found that NOD.HLA-B\*39:06<sup>Hom</sup>. $\beta 2m^{KO}$  mice develop similar amounts of CD8 T cells as their HLA-A\*02:01-transgenic counterparts (Fig. 3B, 3C), suggesting that HLA-B\*39:06 is as efficient at leading to CD8 T cell development as the more well-studied HLA-A\*02:01. Indeed, we show here for the first time that HLA-B\*39:06 can directly mediate T1D in an NOD. $\beta 2m^{KO}$  model (Fig. 4A, 5A). However, unlike HLA-A\*02:01 (21, 51), when expressed in the presence of the NOD class I MHC alleles H2-D<sup>b</sup> and H2-K<sup>d</sup>, HLA-B\*39:06 did not accelerate disease (Fig. 1). While this may be due to strain-specific differences (*e.g.*, transgene integration site), it also may speak to the importance of other aspects of the genetic environment which are known to be important for HLA-B\*39:06-related susceptibility in humans. HLA-B\*39:06 has been found to exert its effect on T1D risk in patients with specific class II MHC haplotypes, namely HLA-DR8/DQ4 (6, 52). Depending on the population studied, these class II MHCs may be independently predisposing, in which case HLA-B\*39:06 accelerates disease progression, or may have a neutral impact on T1D, in which case HLA-B\*39:06 lends risk to such patients. It has been well established that H2-A<sup>g7</sup>, the NOD class II MHC, bears great similarity to the human T1D-associated HLA-DQ8 (18). HLA-DR8, part of a haplotype associated with HLA-B\*39:06, has similar peptide binding characteristics to both H2-A<sup>g7</sup> and HLA-DQ8 (53). While HLA-DR8 has been hypothesized to be the T1D-causative allele in the HLA-DR8/DQ4 haplotype, other evidence suggests that HLA-DQ4 is associated with risk of greater disease progression (54–56). Given that genetic context is important for the association between HLA-B\*39:06 and T1D risk, the lack of a class II MHC molecule similar to HLA-DQ4 in the NOD mouse model may explain why similar diabetes incidence curves were observed for NOD and NOD.HLA-B\*39:06 mice (Fig. 1). However, despite potentially not having an ideal genetic environment, HLA-B\*39:06 is still able to mediate disease and islet infiltration, as confirmed by our findings in the NOD.HLA-B\*39:06. $\beta 2m^{KO}$  strain (Fig. 4A, 4B, 4D, 5A). It is important to appreciate that while HLA-B\*39:06 allows the development of a diabetogenic CD8 T cell repertoire when expressed in NOD mice (Fig. 1A, 1B, 5A), not all HLA class I molecules can do so. For example, when expressed along with H2-D<sup>b</sup> and H2-K<sup>d</sup> in NOD mice, HLA-B\*27 inhibits the development of T1D (51).

To more accurately model the genetic background of patients with T1D, we incorporated reduced thymic insulin expression into the NOD.HLA-B\*39:06. $\beta 2m^{KO}$  model. We found that NOD.HLA-B\*39:06. $\beta 2m^{KO}$ .*Ins2*<sup>KO</sup> mice are susceptible to disease at a younger age compared to their *Ins2*<sup>WT</sup> counterparts (Fig. 5). Based on our findings that the gross lymphocyte populations (Fig. 6B), TCR V $\beta$  usage (Fig. 7), and blood insulin levels (Fig. 9) do not differ dramatically between these strains, the earlier age of onset in the context of reduced insulin expression is likely to be solely explained by the decrease in insulin tolerance that is associated with *Ins2* ablation (28–30, 32). CD8 T cells are necessary for the development of T1D (2, 39, 42–44). As the expression of HLA-B\*39:06 restores T1D

susceptibility to NOD. $\beta 2m^{KO}$  mice (Fig. 4A, 5A) and is enhanced in the Ins2 $^{KO}$  mice (Fig. 5A), it is likely that reduced thymic insulin expression results in an increase in CD8 T cell reactivity towards insulin, as it has been shown to do in NOD.Ins2 $^{KO}$  and NOD.HLA-A\*02:01. $\beta 2m^{KO}$ .Ins2 $^{KO}$  mice. However, increased CD4 T cell reactivity to insulin could also be a contributing factor. We propose that an increased HLA-B\*39:06-restricted reactivity to insulin may also contribute to the earlier age of onset seen in HLA-B\*39:06-positive patients. These points will be clarified by future investigations.

The NOD.HLA-B\*39:06. $\beta 2m^{KO}$  model can be used in a variety of ways to probe the influence of HLA-B\*39:06 on T1D susceptibility. As we have successfully done for HLA-A\*02:01 (21, 22, 30), the model will allow us to identify the beta cell peptides recognized by HLA-B\*39:06-restricted T cells without the potentially confounding presence of murine class I MHC molecules. That the peptide-binding motif for HLA-B\*39:06 has recently been identified may simplify the identification of HLA-B\*39:06-restricted epitopes (57, 58). We have previously identified HLA-A\*02:01-restricted epitopes in an NOD. $\beta 2m^{KO}$ -based model (21, 22, 30); these epitopes were the same or similar to those found in human T1D patients expressing this class I variant (21, 23). Thus the use of the NOD.HLA-B\*39:06. $\beta 2m^{KO}$  models could provide a direct translational impact. Identification of HLA-B\*39:06-restricted epitopes can enable their use in epitope-directed therapies; these are an attractive option as they can allow for treatments targeted at specific epitopes without the risk of off-site effects. Furthermore, knowledge of targeted epitopes permits the tracking of response to therapy, *e.g.*, through the use of peptide-MHC tetramers. Such therapies require preclinical testing, representing another future use of the NOD.HLA-B\*39:06. $\beta 2m^{KO}$  models.

We did not find differences in TCR V $\beta$  gene usage among splenic CD8 T cells when comparing NOD.HLA-B\*39:06. $\beta 2m^{KO}$  mice that were KO, het, or WT for Ins2 (Fig. 7A). However, when we compared the TCR V $\beta$  usage among splenic CD8 T cells in NOD.HLA-B\*39:06. $\beta 2m^{KO}$  and NOD.HLA-A\*02:01. $\beta 2m^{KO}$  mice, we found that four TCR V $\beta$  families were differentially expressed (Fig. 7A). Specifically, usage of V $\beta 2$  and V $\beta 11$  was increased in the presence of HLA-B\*39:06, while usage of V $\beta 6$  and V $\beta 8.1/2$  was decreased (Fig. 7A). This was initially a surprising finding, as until recently it was not generally thought that a given TCR V $\beta$  family had any preference for a particular MHC allele (59). Recently, however, usage of TCR V $\beta$  (and V $\alpha$ ) genes has been found to be associated with MHC genotype in humans, leading to the proposal that different TCR V gene products may indeed have a bias toward particular MHC alleles (60). Our results using the NOD.HLA-B\*39:06. $\beta 2m^{KO}$  and NOD.HLA-A\*02:01. $\beta 2m^{KO}$  strains (Fig. 7A) support this view and represent a valuable system to explore this phenomenon further. As it would be most relevant to examine this question in the context of human TCRs, previously described mice expressing a polyclonal human TCR repertoire (61) would enhance such studies. In the case of HLA-A\*02:01-transgenic mice, human TCRs have been shown to better select CD8 T cells than murine TCRs (61), and the same could hold true for HLA-B\*39:06 mice as well.

In sum, we have established that HLA-B\*39:06 can directly mediate T1D in the NOD mouse model, confirming the results seen in multiple genome-wide association studies (5, 7). We have furthermore developed models for HLA-B\*39:06 in a genetic context more

relevant to human disease by incorporating reduced thymic insulin expression. These models will allow a detailed investigation of the influence of HLA-B\*39:06 on T1D development.

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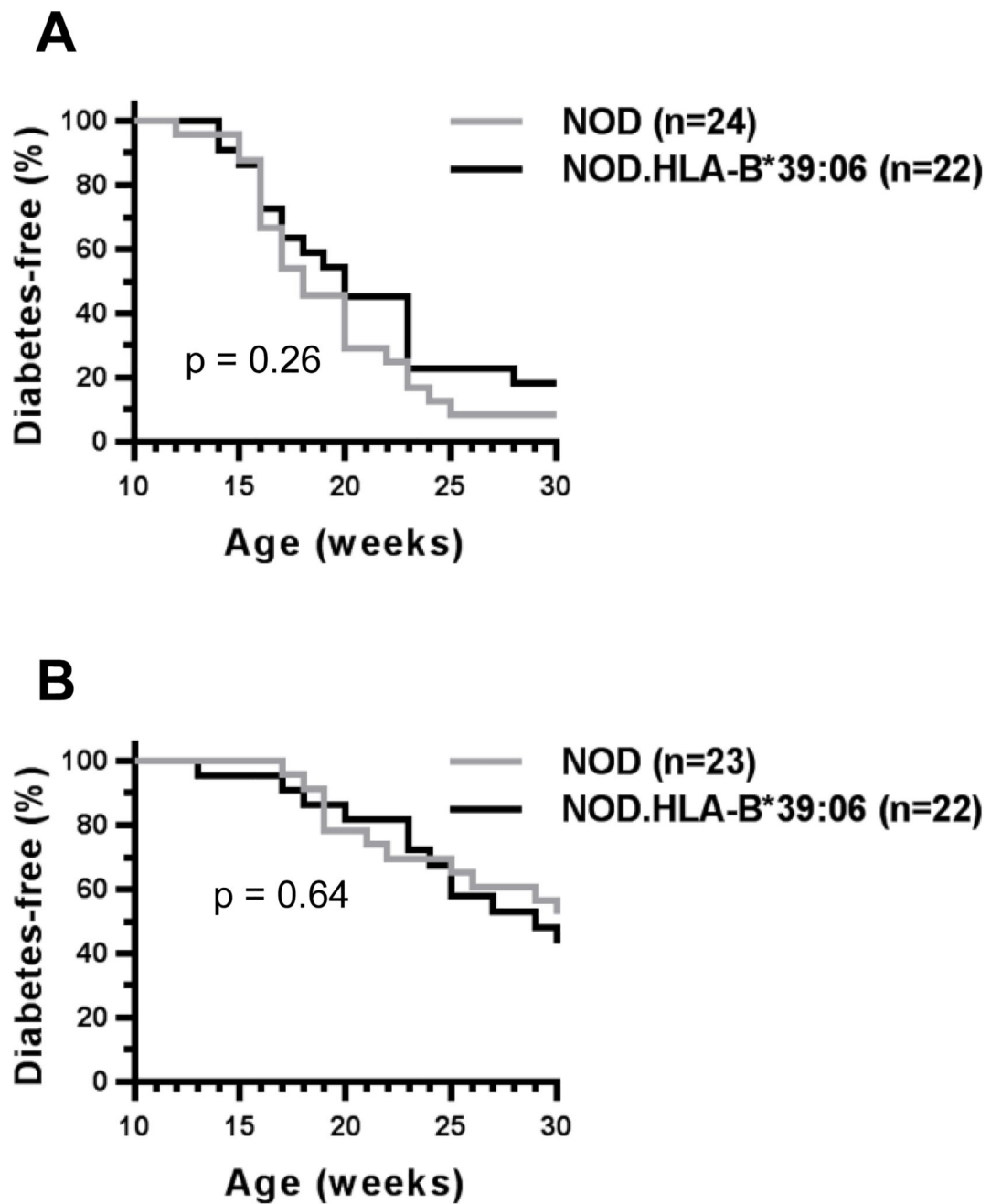
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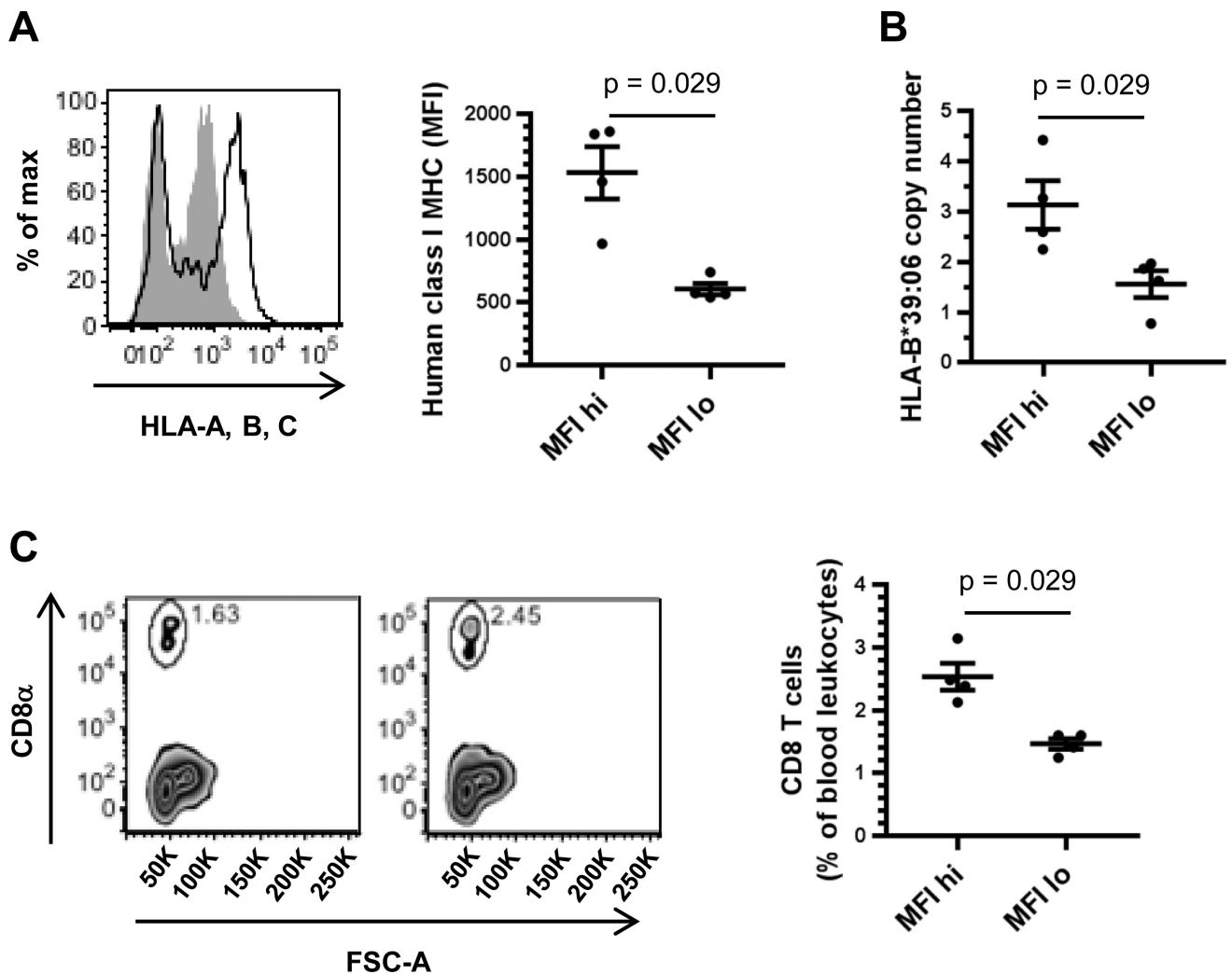
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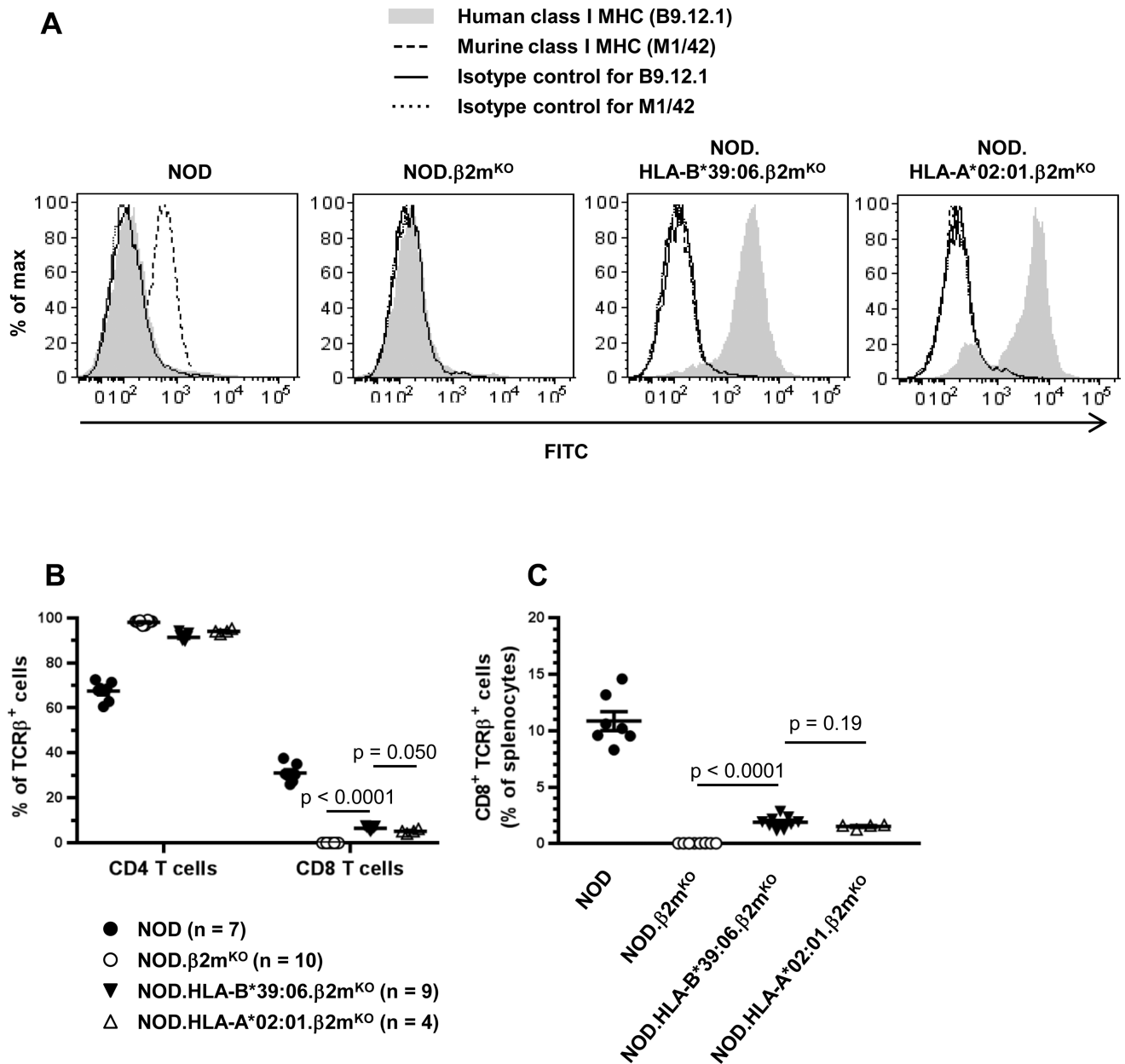
**Figure 1. HLA-B\*39:06-transgenic NOD mice are susceptible to T1D**  
Diabetes incidence curves for female (A) and male (B) NOD.HLA-B\*39:06 mice and non-transgenic NOD littermates are shown. (A)  $p = 0.26$ , Mantel-Cox; (B)  $p = 0.64$ , Mantel-Cox.



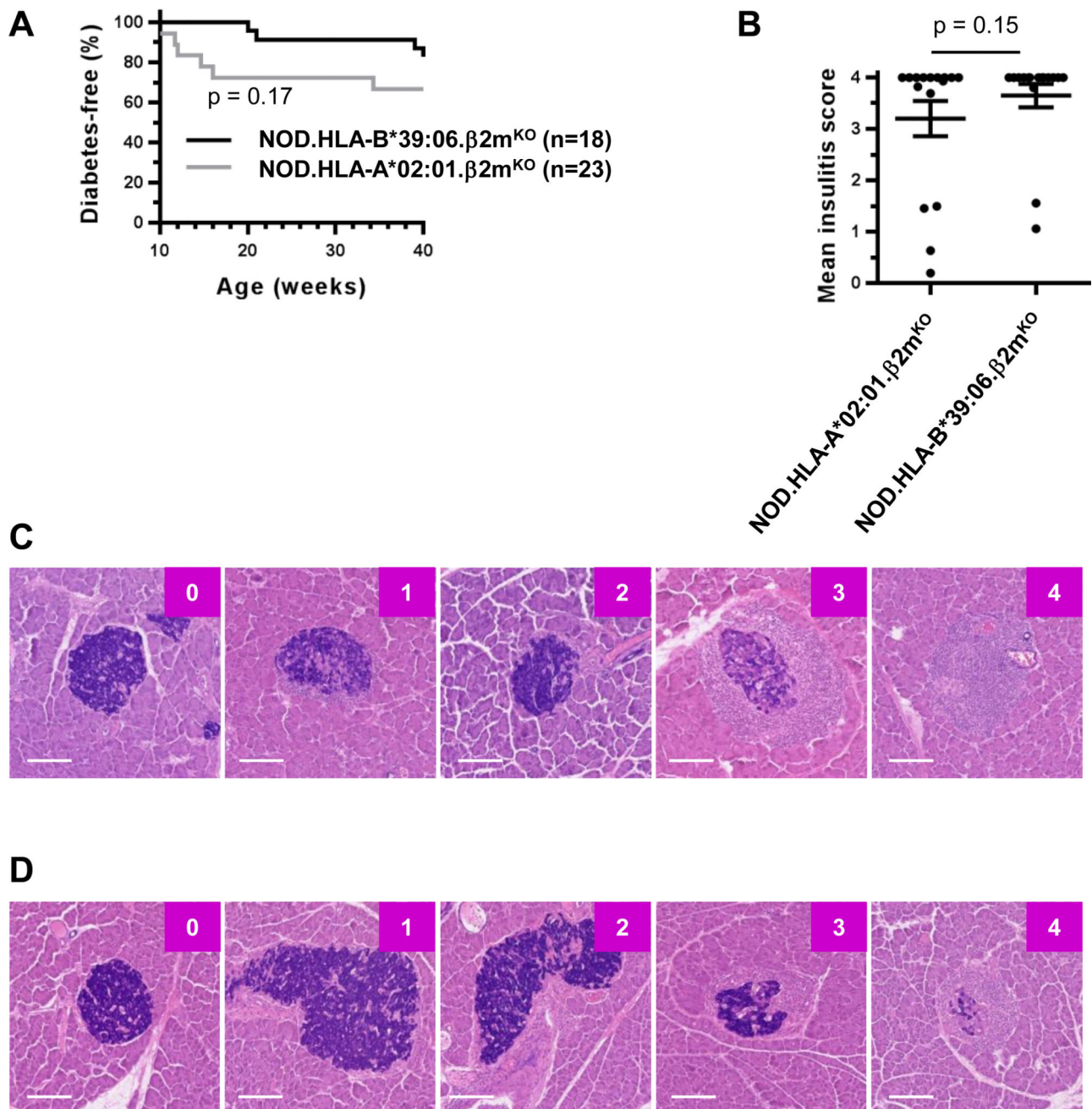


**Figure 2. HLA-B\*39:06 expression and CD8 T cell development in NOD.HLA-B\*39:06. $\beta$ 2m<sup>KO</sup> hemizygous and homozygous mice**

(A) Peripheral blood leukocytes from eight NOD.HLA-B\*39:06. $\beta$ 2m<sup>KO</sup> mice were analyzed by flow cytometry for expression of human class I MHC. Left panel: representative histograms for an MFI-high (black line) and an MFI-low mouse (filled gray) are shown. Right panel: geometric MFI of the positive population for each mouse is displayed. Each circle represents an individual mouse. Lines denote mean  $\pm$  SEM ( $p = 0.029$ , Mann-Whitney). (B) DNA from four mice per group was assessed for HLA-B\*39:06 copy number by quantitative PCR. Each circle represents an individual mouse. Lines denote mean  $\pm$  SEM ( $p = 0.029$ , Mann-Whitney). (C) The percent of CD8 T cells among peripheral blood leukocytes was assessed for four mice per group by flow cytometry. Left panels: representative plots for an MFI-low (left) and an MFI-high mouse (right) are shown. Right panel: The percent of CD8 T cells among blood leukocytes is shown. Each circle represents an individual mouse. Lines denote mean  $\pm$  SEM ( $p = 0.029$ , Mann-Whitney).

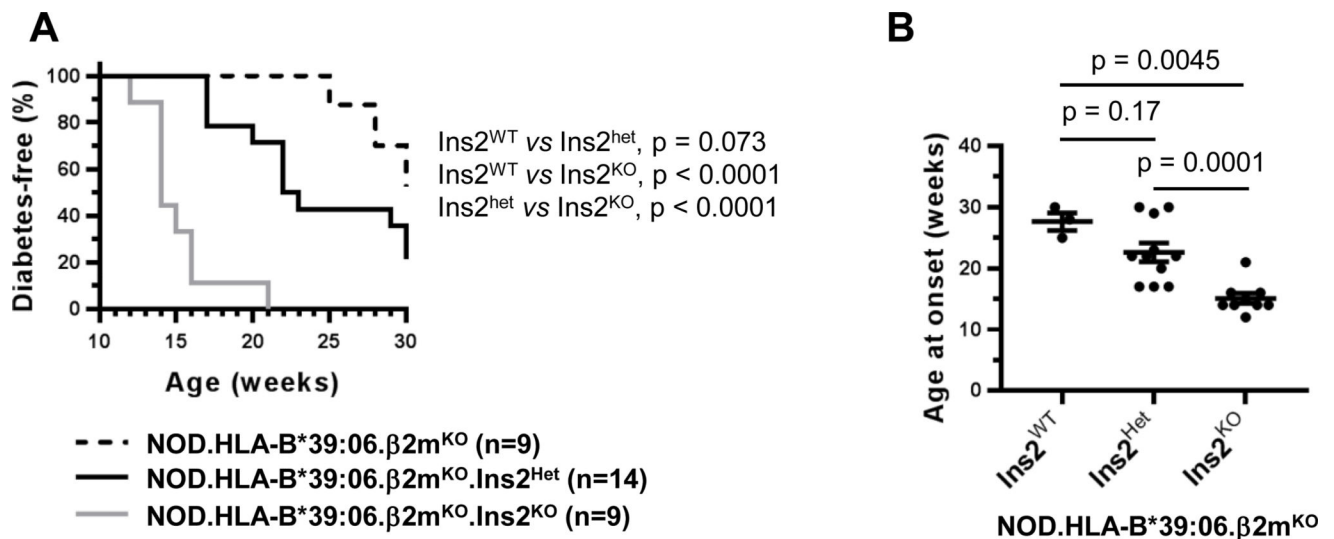


**Figure 3. HLA-B\*39:06 allows for the development of CD8 T cells in NOD mice**  
 Splenocytes from the indicated mouse strains were analyzed by flow cytometry for human and murine class I MHC expression (A) and percentages of the indicated T cell subsets among TCRβ<sup>+</sup> cells (B) or splenocytes (C). (B–C) Lines denote mean ± SEM; p values are indicated (Mann-Whitney).



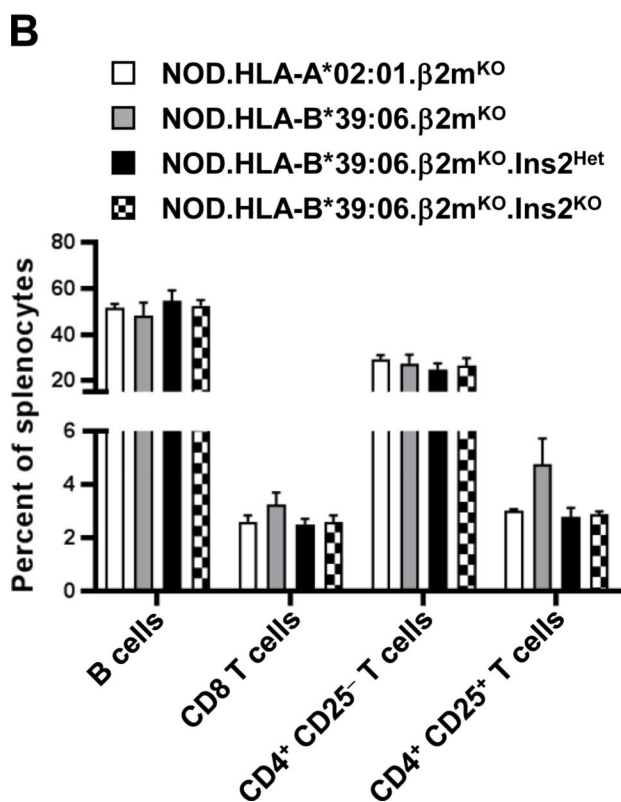
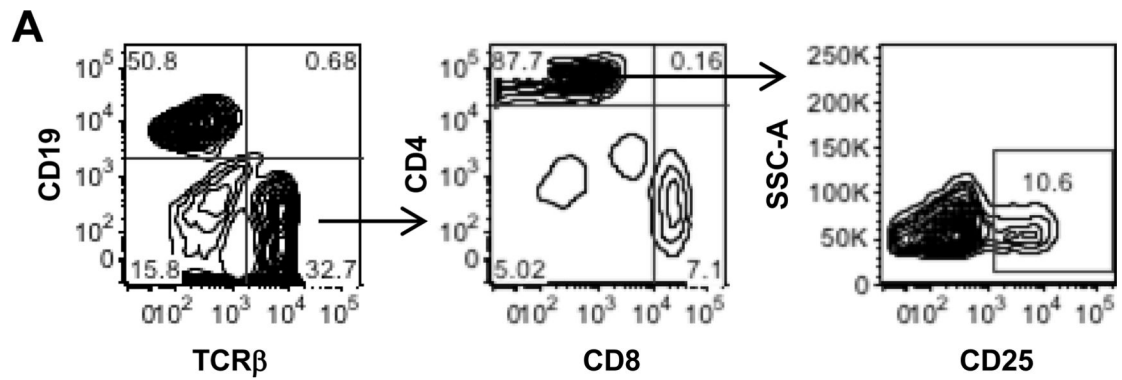
**Figure 4. NOD.HLA-B\*39:06<sup>Hom</sup>.β2m<sup>KO</sup> mice are susceptible to T1D**

(A) The results of diabetes incidence studies performed at The Jackson Laboratory using female NOD.HLA-B\*39:06<sup>Hom</sup>.β2m<sup>KO</sup> and NOD.HLA-A\*02:01.β2m<sup>KO</sup> mice are shown ( $p = 0.17$ , Mantel-Cox). (B) Female non-diabetic NOD.HLA-A\*02:01.β2m<sup>KO</sup> ( $n = 16$ ) and NOD.HLA-B\*39:06<sup>Hom</sup>.β2m<sup>KO</sup> mice ( $n = 16$ ) were euthanized at 40 wks and mean insulinitis scores determined as described in *Materials and Methods*. Each circle represents an individual mouse. Lines represent mean  $\pm$  SEM ( $p = 0.15$ , Mann-Whitney). Representative islets with their assigned scores for the female non-diabetic NOD.HLA-A\*02:01.β2m<sup>KO</sup> (C) and NOD.HLA-B\*39:06<sup>Hom</sup>.β2m<sup>KO</sup> mice (D) are shown. Scale bar represents 100  $\mu$ m.



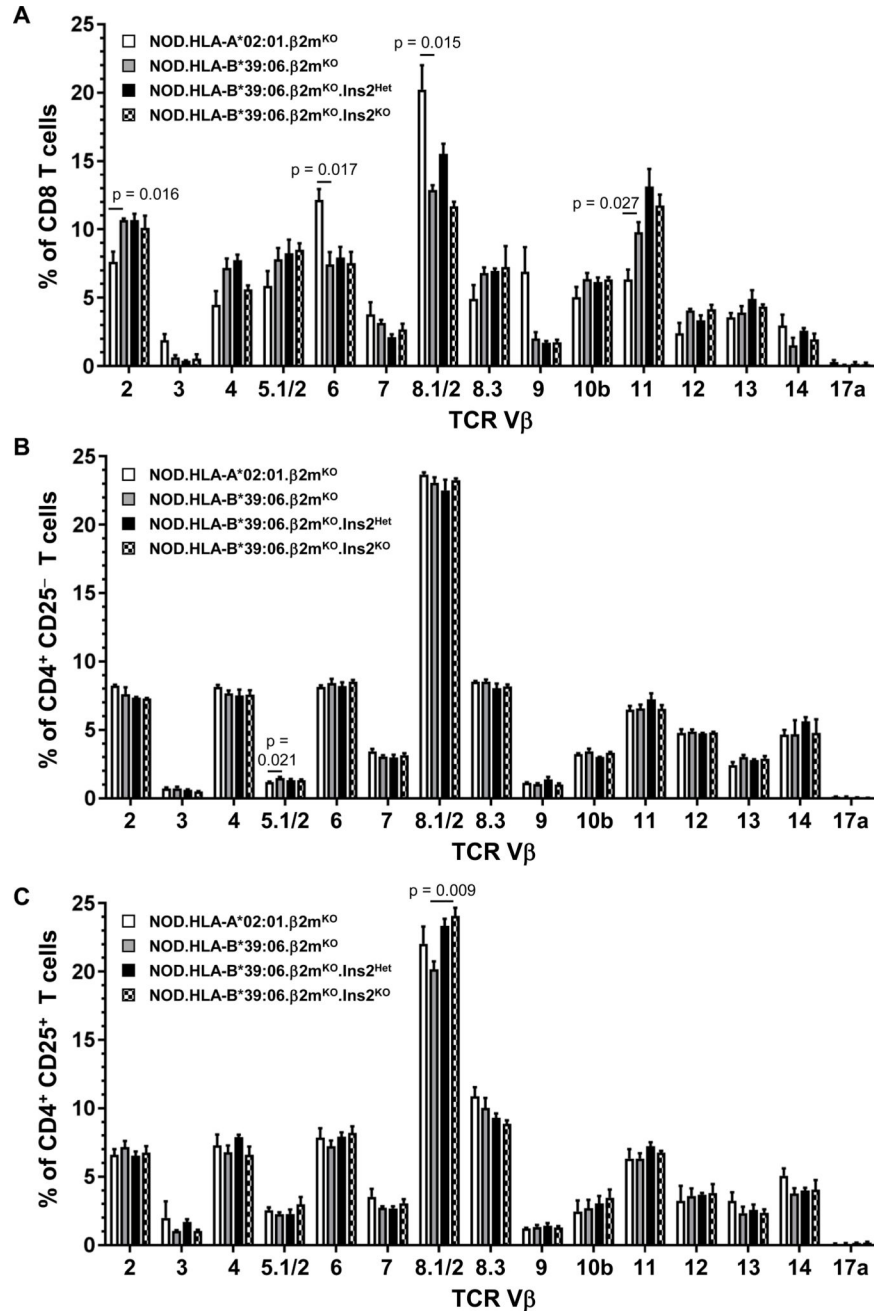
**Figure 5. HLA-B\*39:06<sup>Hom</sup> mice display increased diabetes development in the context of reduced thymic insulin expression**

(A) The results of diabetes incidence studies performed at Albert Einstein College of Medicine using female NOD.HLA-B\*39:06<sup>Hom</sup>.β2m<sup>KO</sup> mice (n = 9) and their Ins2<sup>Het</sup> (n = 14) and Ins2<sup>KO</sup> (n = 9) counterparts are shown; p values are indicated (Mantel-Cox). (B) The ages at onset for all mice in (A) that became diabetic during the incidence study are plotted. Each circle represents an individual mouse. Lines denote mean ± SEM; p values are indicated (Mann-Whitney). Ins2<sup>WT</sup>, n = 3; Ins2<sup>Het</sup>, n = 11; Ins2<sup>KO</sup>, n = 9.

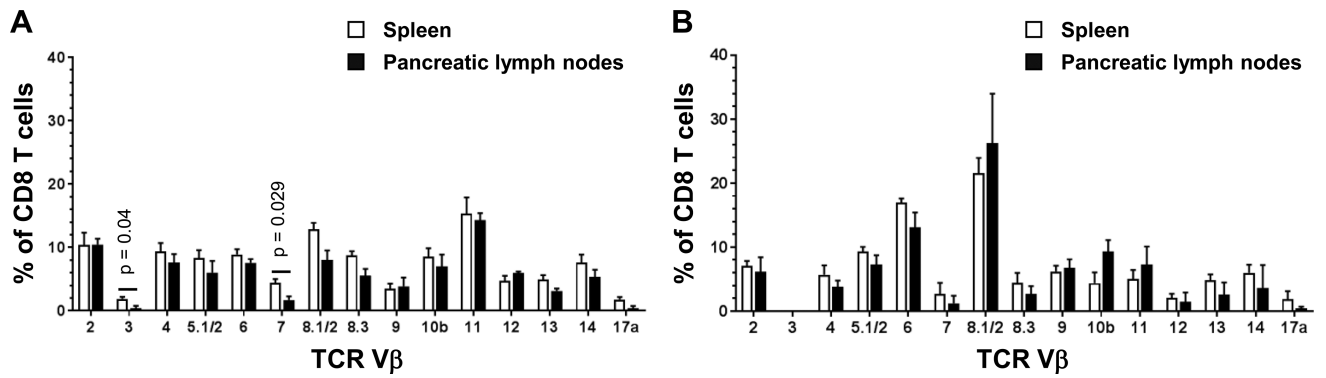


**Figure 6. Splenocyte composition does not differ among NOD.HLA-B\*39:06<sup>Hom</sup>. $\beta$ 2m<sup>KO</sup> mice regardless of Ins2 genotype**

Splenocytes from three female mice per group (16–25 wks of age) were analyzed by flow cytometry for percentages of lymphocyte populations. (A) Gating strategy. (B) Percentages of the indicated cell populations are shown. Graph depicts mean + SEM.

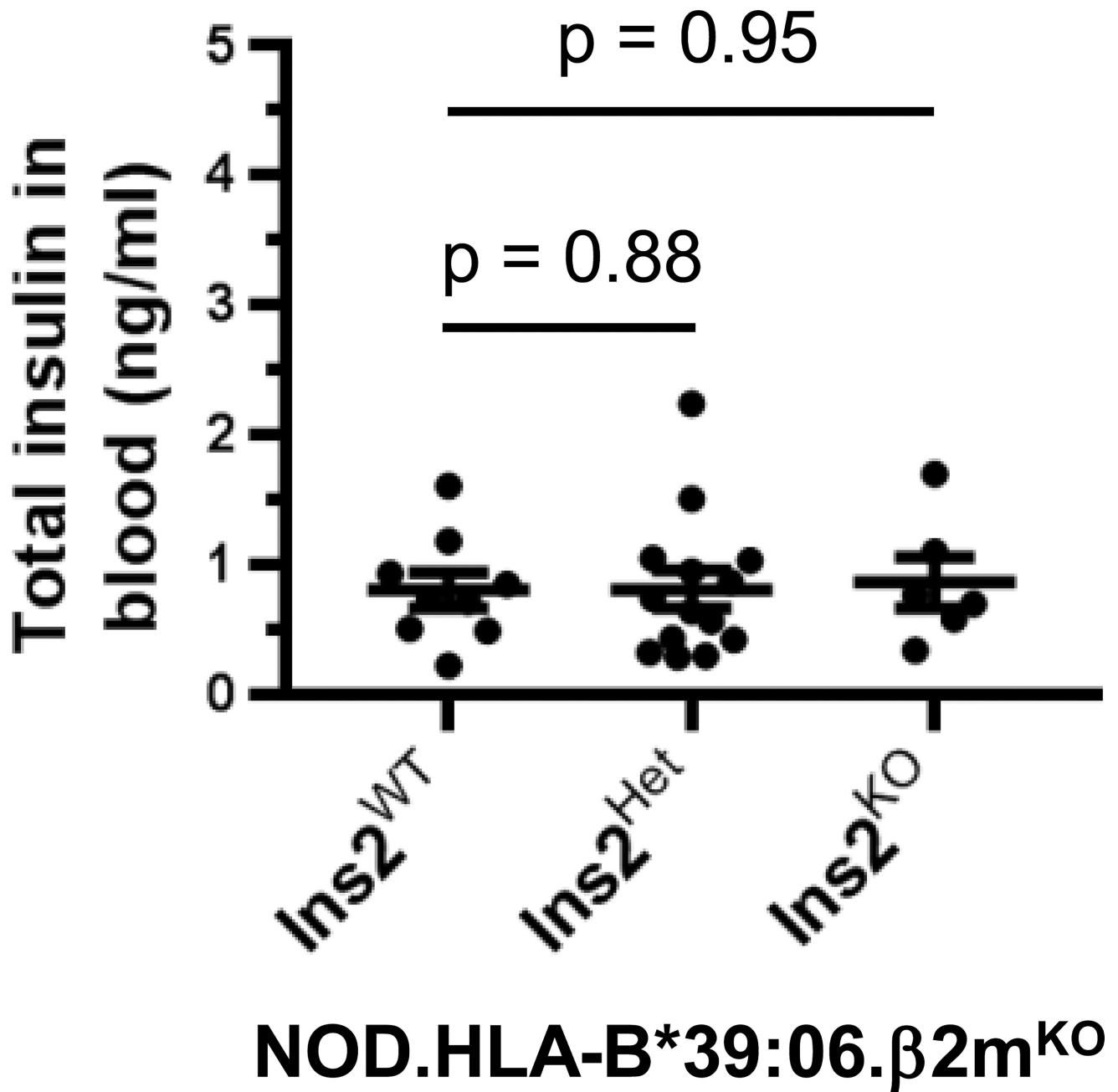


**Figure 7. Class I HLA transgene identity dramatically alters TCR Vβ usage among CD8 T cells, but thymic insulin expression does not**  
 Splenocytes from three female mice per group were analyzed by flow cytometry for TCR Vβ usage. (A) CD8 T cells, (B) CD4 T cells, and (C) CD4<sup>+</sup>CD25<sup>+</sup> T cells were considered separately. Graphs depict mean + SEM; p values are indicated (t test). Significant differences between NOD.HLA-B\*39:06.β2m<sup>KO</sup> and NOD.HLA-A\*02:01.β2m<sup>KO</sup> mice are indicated with p values, as are those between NOD.HLA-B\*39:06.β2m<sup>KO</sup> and NOD.HLA-B\*39:06.β2m<sup>KO</sup>.Ins2<sup>KO</sup> mice.



**Figure 8. TCR Vβ usage among CD8 T cells from pancreatic lymph nodes does not show enrichment of any Vβ families compared to spleen**

CD8<sup>+</sup> splenocytes and pancreatic lymph node cells from (A) three female NOD.HLA-B\*39:06.β2m<sup>KO</sup>.Ins2<sup>Het</sup> mice or (B) three to four NOD.HLA-A\*02:01.β2m<sup>KO</sup> mice were analyzed by flow cytometry for TCR Vβ usage. Graphs depict mean + SEM. Significant differences between CD8<sup>+</sup> splenocytes and pancreatic lymph node cells within a strain are indicated with p values (t test). In (B), the bars for Vβ3 are not visible.



**Figure 9. Blood insulin levels in young NOD.HLA-B\*39:06.β2m<sup>KO</sup> mice do not vary regardless of Ins2 genotype**

Serum was collected from NOD.HLA-B\*39:06<sup>Hom</sup>.β2m<sup>KO</sup> mice (5–6.5 wks old) having the indicated Ins2 genotypes (Ins2<sup>WT</sup>, n = 9; Ins2<sup>Het</sup>, n = 14; Ins2<sup>KO</sup>, n = 6) in the morning, and insulin levels were measured by ELISA. Each circle represents an individual mouse. Lines denote mean ± SEM; p values are indicated (Mann-Whitney).