Transgenic rescue implicates β_2-microglobulin as a diabetes susceptibility gene in nonobese diabetic (NOD) mice

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Type 1 diabetes in both humans and nonobese diabetic (NOD) mice results from T-cell-mediated autoimmune destruction of insulin-producing pancreatic β cells. Linkage studies have shown that type 1 diabetes in NOD mice is a polygenic disease involving more than 15 chromosomal susceptibility regions. Despite extensive investigation, the identification of individual susceptibility genes either within or outside the major histocompatibility complex region has proven problematic because of the limitations of linkage analysis. In this paper, we provide evidence implicating a single diabetes susceptibility gene, which lies outside the major histocompatibility complex region. Using allelic reconstitution by transgenic rescue, we show that NOD mice expressing the β_2 microglobulin (β_2M)^a allele develop diabetes, whereas NOD mice expressing a murine β_2M^b or human allele are protected. The murine β_2M^a allele differs from the β_2M^b allele only at a single amino acid. Mechanistic studies indicate that the absence of the NOD β_2M^a isoform on nonhematopoietic cells inhibits the development or activation of diabetogenic T cells.

The protein β_2M is encoded on human chromosome 15 and mouse chromosome 2 (1, 2). It is a 12-kDa protein with an amino acid sequence homologous to a single Ig domain. Apart from its association with the products of class I major histocompatibility complex (MHC) responsible for presentation of peptides to the immune system, β_2M is associated with a number of homologues of MHC molecules that have diverse roles. These include presentation of lipid antigens (CD1), transport of immunoglobulins (neonatal F_c receptor), regulation of iron metabolism (HFE), and deception of the host immune system (by viral homologues) (3). β_2M is relatively nonpolymorphic. Although seven alleles have been identified in mice, they differ only in a limited number of residues (4, 5). Of note, the three isoforms that have been identified in inbred strains differ only at a single residue, 85 (4, 6). Lack of polymorphism in β_2M has led to the view that β_2M has purely structural roles in ensuring correct protein folding and transport to the cell surface (7), although there is some evidence that β_2M polymorphism may influence antigen presentation (8).

The development of type 1 diabetes (IDDM) in nonobese diabetic (NOD) mice results from the destruction of pancreatic β cells by autoreactive T-cell responses that are mediated by both the class I (K^d, D^b) and class II (A^{g7}) gene products of the $H2^{g7}$ MHC haplotype (9). In addition to the $H2^{g7}$ MHC haplotype, genes within a minimum of 14 other chromosomal regions also contribute to IDDM development in NOD mice (10, 11). *β_2M* maps within the 24-centimorgan segment on chromosome 2 originally defined as *Idd13* on the basis of recessive IDDM resistance in NOD mice congenic for this genomic interval derived from the related nonobese resistant (NOR) strain (12). More recent studies on NOD mice that carry NOR-derived subcongenic intervals of *Idd13* have shown that this region consists of at least two allelically variable genes that contribute to IDDM susceptibility or resistance, although the identity of these genes has remained unknown (13). One of the NOR-derived subcongenic intervals that is associated with a decreased incidence of IDDM in NOD mice contains the β_2M locus. On the basis of the role of β_2M in MHC class I and CD1 expression, we investigated *β_2M* as a candidate susceptibility gene. The ideal approach would be direct replacement of the NOD β_2M allele with another variant through an embryonic stem (ES) cell-based gene "knock-in" strategy. However, this was not possible, because no ES cell lines of NOD origin with efficient germ line transmission capacity are yet available. A β_2M knock in could be done by using ES cells of 129 strain origin, but in the course of transferring this allele to the NOD background, other linked genes of 129 origin would also be cotransferred, which could affect IDDM development. Hence, we chose the best currently available alternative approach of determining whether transgenic rescue of β_2M-deficient NOD mice with various β_2M isoforms engendered IDDM susceptibility or resistance. An advantage of this approach over the knock in strategy by using non-NOD ES cells is that the parental β_2M-deficient NOD genotype used for transgenic rescue is internally controlled i.e., the various β_2M transgenic NOD mice were generated in the same parental strain.

Methods

Mice. C3H.H-2^o(K^d, D^k) NOD/Lt, $\beta_2M^{-/-}$ NOD and β_2M transgenic NOD mice were maintained under specific pathogen-free conditions at the John Curtin School of Medical Research. NOD/Lt, $\beta_2M^{-/-}$ NOD, human β_2M (hβ_2M) transgenic NOD, and NOD-severe combined immunodeficient (SCID) mice were maintained under specific pathogen-free conditions at The Jackson Laboratories.

Sequencing. The β_2M exon and promoter regions were amplified from NOD/Lt mice by PCR by using Pwo polymerase. DNA from three independent PCR products was sequenced by using ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin–Elmer Applied Biosystems), according to the manufacturer's instructions.

Generation of β_2M Transgenic Mouse Lines. The previously described hβ_2M transgene (two to four copies) (14) was backcrossed for nine generations to the previously described N11 stock of NOD.$\beta_2M^{-/-}$ mice (15). Backcross segregants carrying the hβ_2M transgene were identified by flow cytometry for positive staining of peripheral blood leukocytes with the FITC-

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Abbreviations: β_2M, β_2-microglobulin; hβ_2M, human β_2M; MHC, major histocompatibility complex; IDDM, type 1 diabetes; SCID, severe combined immunodeficient.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AY048122).

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conjugated mAb BM63 (Sigma). By using previously described methods (16), this NOD.$\beta_2M^{-/-}$.hβ_2M transgenic stock was shown to be homozygous for linkage markers delineating all known *Idd* loci of NOD origin.

The β_2M^b gene derived from the plasmid pKC7-β_2M^b (17) was converted to the β_2M^a form of the gene by excision of the *Eco*RI-*Bgl*I fragment of exon II, and replacement with a 53-bp oligonucleotide identical to the excised fragment except the codon GCC of β_2M^b was changed to the GAC of β_2M^a. The integrity of the insertion site was checked by sequencing, as described above. To distinguish the transgenic β_2M gene from the endogenous gene, 150-bp dormant *lox*P sites were inserted into the *Nde*I site in intron A and the *Bam*HI site in intron C. The converted β_2M^a gene and the unmodified β_2M^b gene were used to generate NOD transgenic mice according to standard methods (18). Transgenic founders were crossed to $\beta_2M^{-/-}$ NOD mice, which were at the 11th backcross, and then intercrossed for homozygosity. β_2M^a transgenic offspring (two to three copies) were genotyped by a PCR, which detected the 150-bp insertion into intron A by using the oligonucleotides TCAATTGTCATGGTCCTCACATCTC and GCAGGCGTATGTATCAGTCTCAGT. β_2M^b transgenics (one to two copies) were genotyped by *Bgl*I digestion of a PCR product amplified from exon 2 by using the oligonucleotides GAGAATGGGAAGCCGAACATACT and TTAAAGTCAAGAAACCTACCATCAT. The *Bgl*I site is unique to exon II of β_2M^b (4), and both the β_2M^a and β_2M^b PCRs differentiate the disrupted β_2M gene, which has a 1.1-kb insert in exon 2 (19).

Disease Assessment in β_2M Transgenic Mice. Female $\beta_2M^{+/-}$ NOD, β_2M^a transgenic $\beta_2M^{-/-}$NOD, β_2M^b transgenic $\beta_2M^{-/-}$NOD, and β_2M^b transgenic $\beta_2M^{+/-}$NOD, described in Fig. 3*a*, were regularly tested for glycosuria by using TesTape (Lilly Research Laboratories, Indianapolis) and were scored as diabetic after two positive readings 2 days apart. After 250–300 days or development of diabetes, mice were killed and pancreata fixed and stained with hematoxylin/eosin. Islets were scored as: 0, no infiltrate; 1, peri-insulitis; 2, circumferential insulitis; 3, intraislet infiltration; and 4, severe structural derangement. Between 10 and 100 islets from each mouse were examined. Alternatively pancreatic tissue was frozen in Tissue-Tek OCT compound (Bayer, Elkhart, IN), and acetone-fixed sections were stained with primary antibodies as indicated, followed by horseradish peroxidase-conjugated anti-rat Ig (Dako). Color was developed with diaminobenzidine tetrahydrochloride, and sections were counterstained with hematoxylin.

Female NOD and hβ_2M transgenic NOD mice, described in Fig. 3*b*, were maintained at The Jackson Laboratory and were monitored weekly for the development of glycosuria with Ames Diastix (kindly supplied by Miles Diagnostics, Elkhart, IN), and were scored as diabetic after two positive readings at least 2 days apart.

Flow-Cytometric Analysis. Islets were isolated as previously described (20), then cultured for 18 h in RPMI 1640 culture medium containing 100 units of γIFN, 10% FCS, penicillin, gentamicin, and streptomycin at 37°C with 10% CO_2. Single-cell islet suspensions were made by digestion for 10 min with trypsin, followed by passage through a 20-gauge needle five times. Islet, spleen, mesenteric lymph node, thymus, and peripheral blood lymphocyte cells were stained for H2-K^d with SF1–1.1 and for H2-D^b with 28–14-8 (PharMingen). Dead cells were excluded by propidium iodide staining, islet β cells were identified by sorting before fixation with acetone and immunohistochemical staining for insulin by using peroxidase-antiperoxidase methodology (Dako), and lymphocytes were identified by anti-CD3 staining.

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NOD          CTAAG GGTTGAGTTC TGCCAGTTAA TGCTCTTAAT TGTCCTGGCT
β2Ma         ----- ---------- ---------- ---------- ----------
                                                    ******
50     TTAGTTTTCA AGATTGCAAA CTTCAGGTCC TAAGTCCTTT TCTGAGTGGG
       ---------- ---------- ---------- ---------- ----------

       ********** ********** ********** ********** **********
100    ATATTGTCAG CAATTGAATA AATGAAGGCG GTCCCAGGCT GAACGACCAG
       ---------- ---------- -----/---- ---------- ----------

       ********** ********** ********** ********** **********
150    ATACACCAAA CTCAAGAGCA CACCCTAGAT AGTAGGGCAC CAAGGGTCCA
       ---------- ---------- ---------- ---------- ----------

       ********** ********** ********** ********** **********
200    GCCCAGGCTG TTTGAAATAT CACGGGACTT TATAAGAACA TGAAACTGAA
       ---------- ---------- ---------- ---------- ----------

       ********** ********** ********** ********** **********
250    AATGGGAAAG TCCCTTTGTA ACCTAGTTCA GCATTAACAG CTAGGAGACT
       ---------- ---------- ---------- ---------- ----------

       ********** ********** *
300    GGTGACGACC TCCGGATCTG AGTCCGGATT GGCTGTGAGT TCAGGAACTA
       ---------- ---------- ---------- ---------- ----------

350    TATAAGAGCG CGCGCCCTGG CTGG.CTCTC ATTTCAGTAG GCTGCTACTC
       ---------- --------A- ----T----- --------/- ----------

400    GGCGCTTCAG TCGCGGTCGC TTCAGTCGTC AGCATG
       ---------- ---------- ---------- ------
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Fig. 1. Sequence of the NOD β_2M promoter. TATA box and ATG are underlined. The 226-bp fragment reported to have enhancer activity (30) is marked with stars. The previously published β_2M^a sequence is aligned beneath the NOD sequence (30).

^{51}Cr Release Cytotoxicity Assay. C3H.H-2^o, NOD, $\beta_2M^{+/-}$ NOD, β_2M^a transgenic $\beta_2M^{-/-}$ NOD, β_2M^b transgenic $\beta_2M^{-/-}$NOD, and $\beta_2M^{-/-}$ NOD male mice greater than 12 weeks of age were immunized for 7 days with 10^7 plaque-forming units of vaccinia virus or 10^4 haemagglutination units of influenza virus strain A/JAP. Splenocytes were then restimulated for 5 days with nuclear protein peptide (NPP) or vaccinia virus, as described (21), to generate secondary cytotoxic T lymphocytes. P815 ($H2^d$, β_2M^a), H2-K^d-transfected RMA-S (H-2^b-K^d, β_2M^b), and fibroblasts OH (K^d,D^k, β_2M^a) and HTG (K^dD^b) targets were labeled with ^{51}Cr and treated with synthetic NPP, A/JAP, vaccinia virus, or mock peptide, as described (21). ^{51}Cr release cytotoxicity assay was performed as described with an assay length of 18 h. Percentage specific lysis was calculated by the formula: percentage specific lysis = (experimental release—medium release)/(maximum release—medium release).

Generation of Bone Marrow Chimeras. Females from the indicated strains were lethally irradiated (1,200 R from a ^{137}Cs source) at 4 weeks of age, and then reconstituted, as previously described (13), with 5×10^6 bone marrow cells isolated from the indicated 8-week-old female donors. Bone marrow chimeras were then monitored through 21-week postreconstitution for the development of IDDM, as assessed by the appearance of glycosuria. In some experiments, splenic leukocytes were isolated from the indicated bone marrow chimeras at 6 weeks postreconstitution and injected i.v. into 4- to 6-week-old female NOD-SCID mice (10^7 cells/recipient). The NOD-SCID recipients were then monitored for IDDM development for up to 15 weeks. Recipients were assessed for CD4 and CD8 T cell repopulation by flow cytometric analyses of splenocytes at the onset of IDDM or at the end of the 15-week observation period.

Results and Discussion

The Promoter Region of the NOD β_2M^a Allele Does Not Contribute to IDDM. The NOD β_2M gene was sequenced to determine whether either the promoter or the coding region carried a rare mutation. Although we found the coding sequence to be identical to that of the common "a" isoform, there were four changes in the promoter region that differed from published sequences (Fig. 1).

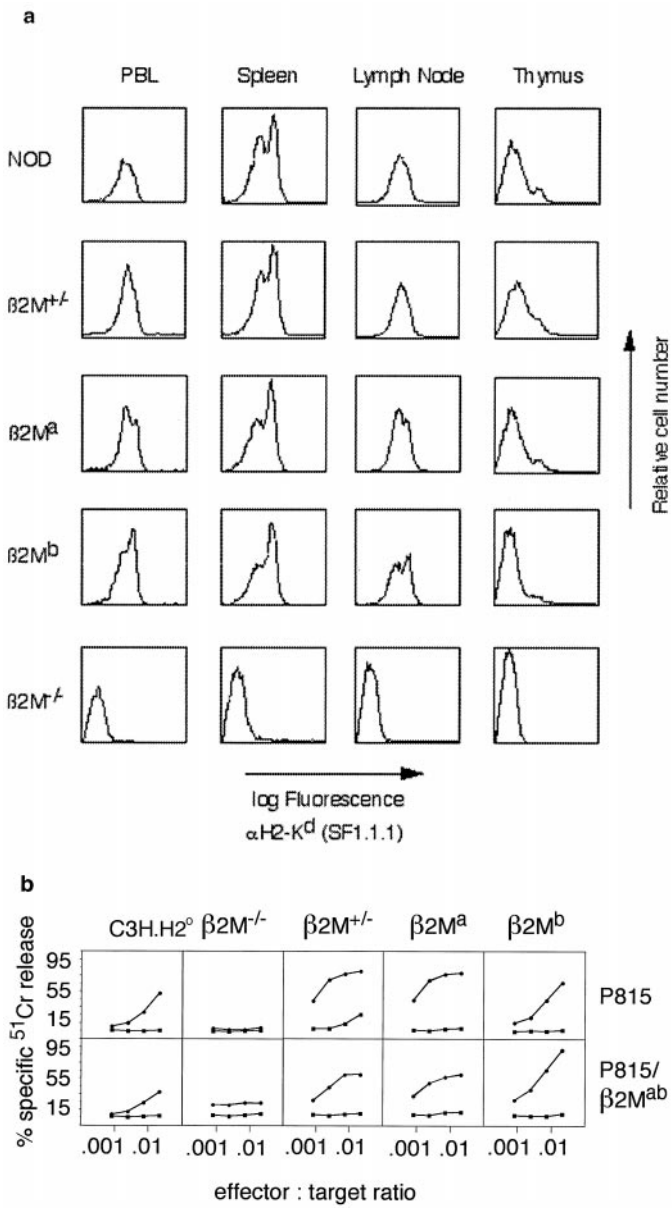

Fig. 2. (*a*) Expression of transgenic mouse β_2M is normal. Single-cell suspensions were made from peripheral blood, spleen, mesenteric lymph nodes, and thymus of NOD, $\beta_2M^{+/-}$, β_2M^a transgenic, β_2M^b transgenic, and $\beta_2M^{-/-}$ NOD female 10- to 17-week-old mice. Cells were stained for H2-K^d and analyzed by FACS. One of several experiments is shown. (*b*) Secondary cytotoxic T lymphocyte (CTL) responses to NPP. CTL activity of cells from primed C3H.H2°, $\beta_2M^{-/-}$ NOD, $\beta_2M^{+/-}$ NOD, β_2M^a transgenic, $\beta_2M^{-/-}$ NOD, and β_2M^b transgenic $\beta_2M^{-/-}$ NOD mice was assessed ($n = 2$ per group, one of two experiments). P815 target killing was reduced in β_2M^b transgenic derived cytotoxic cells, compared with β_2M^a derived cytotoxic cells. The lower cytotoxicity of β_2M^b transgenic derived cells was corrected by inclusion of β_2M^b on the target cells, showing a differential peptide binding between the two β_2M isoforms.

Three of these changes were found in a cluster between the TATA box and the start codon.

To test whether the changes found in the NOD β_2M promoter could confer susceptibility to IDDM, we replaced the β_2M^a gene in NOD mice with a β_2M^a gene derived from a nondiabetes prone strain. We made NOD mice transgenic for the nondiabetes prone β_2M^a construct and crossed this transgene into $\beta_2M^{-/-}$ NOD mice, which completely lack expression of β_2M. Resultant β_2M^a transgenic $\beta_2M^{-/-}$ NOD mice were shown to express MHC class I in a pattern, and at a level that is not significantly different from NOD mice (Fig. 2*a*). The β_2M^a molecule was shown to function normally in terms of its ability to pair with MHC class I and to select for CD8 T cells. β_2M^a transgenic $\beta_2M^{-/-}$ NOD mice were shown to have normal CD8 T-lymphocyte function by their ability to reject class I disparate skin grafts (data not shown) and their ability to clear viral infection (Fig. 2*b*).

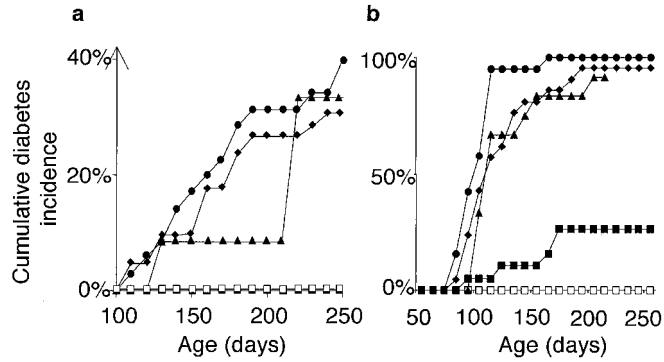

Fig. 3. (*a*) Incidence of diabetes in the John Curtin School of Medical Research colony of female β_2M^a and β_2M^b transgenic NOD mice. β_2M^a transgenic $\beta_2M^{-/-}$ NOD (diamonds, $n = 11$), β_2M^b transgenic $\beta_2M^{-/-}$ NOD (squares, $n = 27$), littermate control β_2M^b transgenic $\beta_2M^{+/-}$ NOD (circles, $n = 34$), $\beta_2M^{-/-}$ NOD (open squares, $n = 16$), and $\beta_2M^{+/-}$ NOD mice (triangles, $n = 12$) were followed for 250 days for development of diabetes. The incidence in β_2M^b transgenic $\beta_2M^{-/-}$ NOD mice was dramatically reduced ($P < 0.002$ vs. $\beta_2M^{+/-}$ NOD mice). (*b*) Incidence of diabetes in The Jackson Laboratory colony of female hβ_2M transgenic NOD mice. hβ_2M transgenic $\beta_2M^{-/-}$ NOD mice (squares, $n = 19$), $\beta_2M^{-/-}$ NOD mice (open squares, $n = 11$), hβ_2M transgenic NOD mice (circles, $n = 19$), $\beta_2M^{+/-}$ NOD (triangles, $n = 12$), and NOD mice (diamonds, $n = 21$) were followed for 250 days for development of diabetes. Diabetes incidence in hβ_2M transgenic NOD $\beta_2M^{-/-}$ mice was 25% that of NOD or hβ_2M transgenic NOD mice ($P < 0.0001$).

A colony of these mice was established and assessed for disease in terms of mononuclear cell infiltrate into the islet tissue (insulitis) and in terms of hyperglycemia. We found that the β_2M^a transgene was able to restore both insulitis and diabetes to the normally resistant $\beta_2M^{-/-}$ NOD mice. There was no difference in the extent of insulitis or the incidence of hyperglycemia (Fig. 3*a*) between $\beta_2M^{-/-}$ NOD mice carrying the β_2M^a transgene and the $\beta_2M^{+/-}$ littermates, which carried a copy of the endogenous NOD β_2M^a gene. We therefore conclude that, although the promoter for the β_2M gene in NOD mice is different from the published sequence, it does not contribute to IDDM susceptibility.

The NOD β_2M^a Structural Variant Confers Dominant Susceptibility to IDDM. Although some of the susceptibility loci that contribute to diabetes may represent rare mutations that confer aberrant gene products, it is becoming increasingly clear that many of the susceptibility genes contributing to this polygenic disorder may represent common allelic variants that manifest a diabetes phenotype only in a combinatorial way (22). Having established that the coding region of the β_2M gene in NOD mice is identical to the published sequence for the common "a" isoform of β_2M, we next assessed the possibility that this particular isoform was associated with diabetes susceptibility. We made NOD mice transgenic for the "b" isoform of the β_2M gene by using the same nondiabetes-prone promoter that was used for the β_2M^a transgenic NOD mice. The "b" isoform characterizing the IDDM-resistant NOR strain differs from the "a" isoform only at amino acid 85, in that it has an alanine instead of an aspartate. Similarly, NOD mice were made transgenic for the hβ_2M, which is ≈70% homologous with murine β_2M^a. Both the β_2M^b and hβ_2M

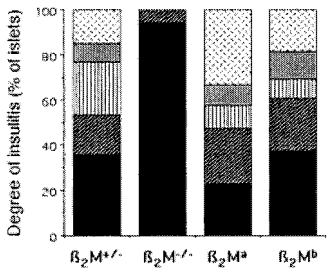

Fig. 4. Degree of insulitis is not reduced in infiltrated β_2M^a or β_2M^b transgenic mice. The mean scores of female $\beta_2M^{+/-}$ ($n = 8$), $\beta_2M^{-/-}$ ($n = 7$), β_2M^a $\beta_2M^{-/-}$ ($n = 6$), and β_2M^b $\beta_2M^{-/-}$ ($n = 9$, mice with infiltrate only) NOD mice are shown. Black bars, 0; diagonal stripes, 1; vertical stripes, 2; gray, 3; and hatched, 4. The proportion of islets exhibiting each level of insulitis severity was not significantly different between β_2M^a, β_2M^b, and $\beta_2M^{+/-}$ mice.

transgenes were then crossed into $\beta_2M^{-/-}$ NOD mice, as was done for the β_2M^a transgene. Both β_2M^b (Fig. 2*a*) and hβ_2M transgenic $\beta_2M^{-/-}$ NOD mice expressed functional β_2M, as evidenced by normal β_2M-dependent MHC class I expression. Staining for MHC class I expression in NOD and hβ_2M transgenic $\beta_2M^{-/-}$ NOD mice, respectively, showed a mean fluorescence intensity of 191 ± 7 and 235 ± 17 (K^d) and 121 ± 7 and 160 ± 10 (D^b). Expression of MHC class I was significantly higher in hβ_2M transgenic $\beta_2M^{-/-}$ NOD than NOD mice ($P < 0.01$). Both hβ_2M and β_2M^b transgenic $\beta_2M^{-/-}$ NOD mice were also shown to have normal CD8 T-lymphocyte numbers and function. Splenocytes from hβ_2M transgenic $\beta_2M^{-/-}$ NOD responded normally in an MHC class I-specific mixed lymphocyte reaction (data not shown). β_2M^b transgenic $\beta_2M^{-/-}$ NOD mice rejected class I disparate skin grafts (data not shown) and were able to mount a normal cytotoxic response to both vaccinia virus and influenza virus (Fig. 2*b*).

Colonies of β_2M^b transgenic and hβ_2M transgenic $\beta_2M^{-/-}$ NOD mice and appropriate control mice were independently established at the John Curtin School of Medical Research and The Jackson Laboratories and were assessed for disease in terms of insulitis and hyperglycemia. Although control $\beta_2M^{+/-}$ NOD mice and β_2M^a transgenic $\beta_2M^{-/-}$ NOD mice, both of which expressed the "a" isoform of β_2M, developed diabetes, the β_2M^b transgenic and hβ_2M transgenic $\beta_2M^{-/-}$ NOD mice, which lacked the β_2M^a isoform, showed markedly reduced susceptibility to diabetes (Fig. 3). That diabetes susceptibility is markedly reduced in both non-β_2M^a lines of NOD mice analyzed indicates such protection is not because of a nonspecific position effect of transgene insertion. Furthermore, the finding that diabetes protection was not observed when the β_2M^b or hβ_2M transgenes were expressed on a background in which the endogenous NOD β_2M^a allele was coexpressed (Fig. 4) indicates the former alleles do not provide dominant resistance, which also supports the fact they are unlikely to confer protection through a nonspecific position effect. Thus, β_2M^a is implicated as a dominant diabetes susceptibility gene in NOD mice.

Replacing β_2M^a with Other Variants Does Not Impair MHC Class I Expression, but Does Limit Insulitis Development in NOD Mice. In an attempt to understand the role of β_2M in the disease process, the severity of the insulitis lesion was compared histologically between β_2M^a and β_2M^b transgenic $\beta_2M^{-/-}$ NOD mice. Although 100% of β_2M^a transgenic $\beta_2M^{-/-}$ NOD mice and $\beta_2M^{+/-}$ NOD mice develop insulitis, only 38% of β_2M^b transgenic $\beta_2M^{-/-}$ NOD mice showed signs of infiltration; the remainder were completely free of insulitis. Of those mice that did develop insulitis, it was no less severe than that seen in β_2M^a transgenic $\beta_2M^{-/-}$ NOD mice (Fig. 4). Further, no differences were found

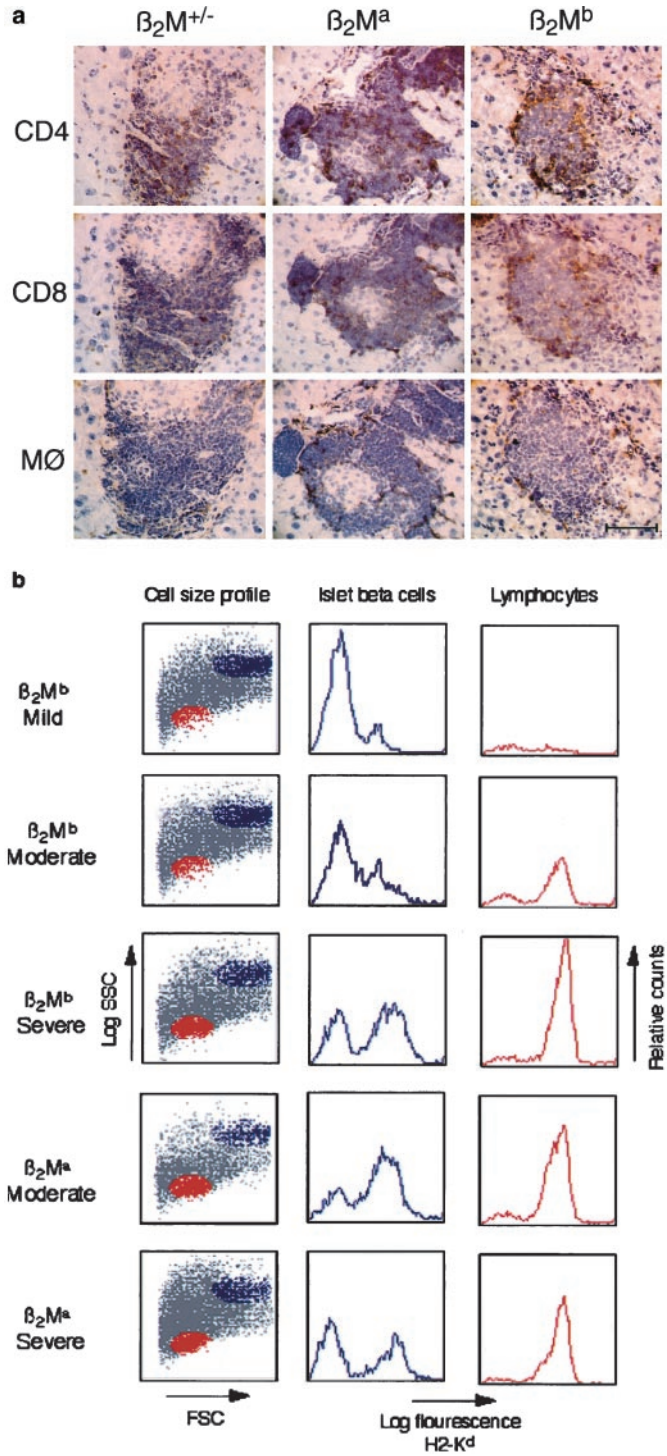

Fig. 5. (*a*) Content of lymphocytic infiltrate in β_2M transgenic mice. Consecutive frozen sections taken from 250- to 300-day-old female mice were stained with the antibodies 53.6 (CD8 specific), GK1.5 (CD4 specific), and F4/80 (macrophage specific). Sections from $\beta_2M^{+/-}$ NOD, β_2M^a transgenic $\beta_2M^{-/-}$ NOD, and β_2M^b transgenic $\beta_2M^{-/-}$ NOD mice are shown. (Bar = 10 mm.) (*b*) Expression of transgenic β_2M on islet β cells is normal and correlates with level of lymphocytic infiltration. Islet cells from female, 13- to 17-week-old β_2M^b transgenic $\beta_2M^{-/-}$ NOD mice, and age-matched control β_2M^a transgenic $\beta_2M^{-/-}$ NOD mice were analyzed for H2-K^d expression. The β-cell population was identified by sorting, followed by immunohistochemical insulin staining, and the lymphocyte population was identified by double staining for CD3. Several experiments were performed, and a typical result is shown.

Table 1. Replacement of the murine β_2M^a isoform with a human variant inhibits diabetes development in NOD mice at the level of nonhematopoietically derived cells

Marrow donor	Recipient (1,200 R) treated	% IDDM 21-wk postreconstitution
NOD	NOD	91.6% (11/12)
NOD	hβ_2M transgenic $\beta_2M^{-/-}$ NOD	30/0% (3/10)*
hβ_2M transgenic $\beta_2M^{-/-}$ NOD	NOD	81.3% (13/16)

Female recipients were lethally irradiated (1,200 R) at 4 weeks of age and reconstituted with 5×10^6 T-cell-depleted bone marrow cells from the indicated donors. The chimeras were then monitored for IDDM development through 25 weeks of age.

*Significantly less ($P < 0.005$, χ^2 analysis) than in NOD recipients of syngeneic marrow.

in the relative proportions of CD8 T lymphocytes, CD4 T lymphocytes, or macrophages in the insulitis lesion (Fig. 5*a*). The absence of insulitis in the majority of β_2M^b mice suggests that β_2M^a confers susceptibility at a very early stage of autoimmunity.

The level of MHC class I expression on islet β cells isolated from β_2M^a and β_2M^b transgenic mice was compared. Islet cells and the associated insulitis lesions were isolated and cultured *in vitro* with γIFN before analyzing their relative levels of MHC class I by FACS. Consistent with our earlier analysis of the lymphoid population, we found that the γIFN-induced level of class I expression on lymphoid cells from the insulitis lesion was no different between β_2M^a and β_2M^b transgenic NOD mice. This was also true for the γIFN-induced level of class I expression on islet β cells from the two groups. However, we did find that β_2M^b transgenic NOD mice generally had fewer islet β cells with up-regulated class I than β_2M^a transgenic NOD mice, and this was directly correlated with the severity of insulitis (Fig. 5*b*). It has previously been shown that MHC class I expression on β cells increases with severity of insulitis (23). Hence, the quantitative reduction in infiltrating leukocytes most likely accounts for the overall lower levels of MHC class I expression on β cells from β_2M^b transgenic NOD mice. However, it is important to note that MHC class I expression was equivalent on β cells from β_2M^a and β_2M^b transgenic NOD mice with matched levels of insulitis.

Replacement of β_2M^a with Another Isoform Inhibits the Development of Diabetogenic T-Cell Responses in NOD Mice. Previous studies indicated IDDM resistance in a stock of NOD mice that express β_2M^b rather than β_2M^a, because of a homozygous NOR-derived chromosome 2 congenic interval, results from a reduced ability of nonhematopoietically derived cell types to support the development of diabetogenic T cells and/or target them to pancreatic β cells (13). However, it was possible that this decrease in the development or targeting of diabetogenic T cells resulted not from the loss of β_2M^a but rather through the effects of some other NOR-derived gene(s) in the chromosome 2 congenic interval. Thus, we tested whether the IDDM resistance induced in NOD mice by transgenic replacement of β_2M^a by another isoform also results from a reduced ability of nonhematopoietically derived cell types to support development of diabetogenic T cells or target them to pancreatic β cells. This was initially done by comparing the rate of IDDM development in reciprocal bone marrow chimeras between female standard NOD mice and the hβ_2M transgenic $\beta_2M^{-/-}$ NOD stock. Controls consisted of female NOD mice reconstituted with syngeneic bone marrow. As expected, IDDM developed in 91.6% (11/12) of NOD control females over a 21-week period after reconstitution with syngeneic marrow (Table 1). In contrast, reconstitution of hβ_2M transgenic $\beta_2M^{-/-}$ NOD recipients with NOD marrow elicited a significantly lower incidence of IDDM (30.0%, 3/10). Conversely, reconstitution of standard NOD recipients with hβ_2M transgenic $\beta_2M^{-/-}$ NOD bone marrow elicited a very high rate of IDDM (81.3%, 13/16). Thus, the IDDM resistance that results from replacing the NOD β_2M^a variant with another isoform can be mechanistically explained by alternations in nonhematopoietically derived cell types that inhibit the development and/or targeting of β-cell autoreactive T cells.

We next determined whether T cells originally generated in an environment where β_2M^a vs. hβ_2M was expressed on nonhematopoietically derived cell types differed in ability to subsequently transfer IDDM to lymphocyte-deficient NOD-SCID mice in which the MHC class I molecules expressed on β cells would all be associated with β_2M^a. At 6 weeks postreconstitution, splenic leukocytes from reciprocal chimeras between standard NOD mice and the hβ_2M transgenic $\beta_2M^{-/-}$ NOD stock were transferred into NOD-SCID recipients. As expected, splenocytes from NOD mice that had been reconstituted with marrow from the hβ_2M transgenic stock efficiently transferred IDDM to 7/10 NOD-SCID recipients (Table 2). In contrast, splenocytes from hβ_2M transgenic $\beta_2M^{-/-}$ NOD mice that had been reconstituted with standard NOD marrow failed to transfer IDDM to any NOD-SCID recipients (0/9). Equivalent levels of CD4 and CD8 T-cell repopulation were observed in NOD-SCID recipients of splenocytes from each type of reciprocal chimera. Collectively, these results demonstrate that replacing the NOD β_2M^a variant

Table 2. Replacement on nonhematopoietic cells of the murine β_2M^a isoform with a human variant blocks the selection, rather than the targeting, of autoreactive diabetogenic T cells

Chimeric splenocyte donor*	% IDDM in NOD-SCID recipients by 15-wk postsplenocyte repopulation	Splenic CD4 T levels in NOD-SCID recipients (%±sem)†	Splenic CD8 T levels in NOD-SCID recipients (%±sem)†
NOD BM→hβ_2M Tg $\beta_2M^{-/-}$ NOD	0% (0/9)	33.2 ± 1.4 (n = 9)	5.8 ± 0.4 (n = 9)
hβ_2M Tg $\beta_2M^{-/-}$ NOD BM→NOD	70.0% (7/10)	28.5 ± 1.2 (n = 10)	7.4 ± 0.5 (n = 10)

Splenocytes were pooled from the indicated reciprocal bone marrow chimeras (five each) and injected i.v. into 4 to 6-week-old female NOD-SCID mice (10^7 cells/recipient) that were then monitored for diabetes development over a 15-week followup period.

*Donor splenocytes from the NOD BM→hβ_2M Tg $\beta_2M^{-/-}$ NOD chimeras contained 31.1% CD4 and 9.0% CD8 T cells. Donor splenocytes from the hβ_2M Tg $\beta_2M^{-/-}$ NOD BM→NOD chimeras contained 20.4% CD4 and 5.2% CD8 T cells.

†Levels of splenic CD4 and CD8 T-cell repopulation in the secondary NOD-SCID recipients were assessed at the onset of diabetes or at the end of the 15-week observation period.

with another isoform induces alterations in nonhematopoietically derived cell types that inhibit the development or activation, rather than the targeting, of autoreactive diabetogenic T cells.

Conclusion

Using the technique of allelic reconstitution by transgenic rescue, we have implicated β_2M^a as one of the diabetes susceptibility genes within the NOD *Idd13* region on chromosome 2. Because β_2M pairs with a number of functional molecules, there are many possible explanations for its role in susceptibility to IDDM. It is possible that β_2M confers susceptibility through its interaction with viral products or neonatal Fc receptor or, perhaps less likely, through its interaction with HFE. More likely is its role in pairing with either CD1 or the heavy chain of classical MHC class I molecules, both of which have an established role in IDDM via interaction with NKT cells and CD8 T lymphocytes, respectively (24, 25). However, we found no differences between β_2M^a and β_2M^b transgenic NOD mice in thymic NKT cell numbers. Furthermore, because IDDM resistance elicited by transgenic β_2M^b or hβ_2M isoforms is not dominant in mice that also express β_2M^a and is not mediated by hematopoietically derived antigen-presenting cells, protection is unlikely to result from the clonal deletion (26) or anergy (27) of autoreactive T lymphocytes. Instead, our results indicate that replacing the NOD β_2M^a variant with another isoform elicits its effect through nonhematopoietically derived cell types that are unable to support the development of β-cell autoreactive T-cell responses. This might be explained by the previously published finding that, when dimerized with different isoforms of β_2M, MHC class I molecules could present an altered peptide repertoire to CD8 T lymphocytes (8). Such alterations in antigen presentation may be because of alterations in the structural conformation of NOD MHC class I molecules upon dimerization with hβ_2M or β_2M^b rather than the β_2M^a isoform. Regardless of the mechanism, we have implicated β_2M as a susceptibility gene in NOD mice.

It is not yet possible to determine whether subtle variations in β_2M may also contribute to autoimmune diabetes in humans, because the extent of polymorphism within this gene has yet to be extensively investigated. However, that the β_2M^a allele that we have implicated as a dominant diabetes susceptibility gene in NOD mice is not a biologically aberrant variant has a potentially important implication for the pathogenic basis of this disease in humans. This implication is that our current findings strongly support the hypothesis that many diabetes susceptibility genes may actually represent common physiologically normal alleles, which exert pathogenic functions only in certain combinatorial contexts (22). Although not yet definitively proven, further support for this concept is that strong linkage disequilibrium implicates a number of other physiologically normal cytokine variants as candidate susceptibility genes (28, 29). Our current findings implicating β_2M as a susceptibility gene in NOD mice, in combination with these strong candidates, suggests that the search for type 1 diabetes susceptibility genes in humans should not be restricted solely to functionally defective variants.

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