

Role of ITGAE in the development of autoimmune diabetes in non-obese diabetic mice

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Abstract

There is compelling evidence that autoreactive CD8⁺T cells play a central role in precipitating the development of autoimmune diabetes in non-obese diabetic (NOD) mice, but the underlying mechanisms remain unclear. Given that ITGAE (CD103) recognizes an islet-restricted ligand (E-cadherin), we postulated that its expression is required for initiation of disease. We herein use a mouse model of autoimmune diabetes (NOD/ShiLt mice) to test this hypothesis. We demonstrate that ITGAE is expressed by a discrete subset of CD8⁺T cells that infiltrate pancreatic islets before the development of diabetes. Moreover, we demonstrate that development of diabetes in *Itgae*-deficient NOD mice is significantly delayed at early but not late time points, indicating that ITGAE is preferentially involved in early diabetes development. To rule out a potential contribution by closely linked loci to this delay, we treated WT NOD mice beginning at 2 weeks of age through 5 weeks of age with a depleting anti-ITGAE mAb and found a decreased incidence of diabetes following anti-ITGAE mAb treatment compared with mice that received isotype control mAbs or non-depleting mAbs to ITGAE. Moreover, a histological examination of the pancreas of treated mice revealed that NOD mice treated with a depleting mAb were resistant to immune destruction. These results indicate that ITGAE⁺ cells play a key role in the development of autoimmune diabetes and are consistent with the hypothesis that ITGAE⁺ CD8⁺T effectors initiate the disease process.

Key Words

- ▶ diabetes
- ▶ autoimmune
- ▶ immune system
- ▶ mouse

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Introduction

Type 1 diabetes is a T cell-mediated autoimmune disease caused by the destruction of insulin-producing β cells of the islets of Langerhans in the pancreas, resulting in a hyperglycemic state. Non-obese diabetic (NOD) mice (NOD/ShiLt) represent an experimental model of type 1 diabetes (Delovitch & Singh 1997), which mimics key aspects of human disease. Studies of both NOD mice and humans indicate that autoreactive CD4 T cells play a key

role in the development of diabetes, in direct destruction of islets and/or in providing help for B and T cell responses to islet autoantigens (Shizuru *et al.* 1988, Haskins & Cooke 2011). However, there is compelling evidence that recognition of autoantigens by CD8 $\alpha\beta$ ⁺TCR $\alpha\beta$ ⁺ cells (CD8⁺T cells) plays a pivotal role in the initiation of diabetes. In both diabetic NOD mice and humans, there is evidence that MHC I alleles (the target of CD8 T cells)

contribute to development of diabetes (Katz *et al.* 1993, Serreze *et al.* 1994, Wicker *et al.* 1994, Serreze *et al.* 1997, Jarchum *et al.* 2007, Nejentsev *et al.* 2007, Tsai *et al.* 2008). Experiments in which spleen cells from prediabetic NOD mice are transferred into immunodeficient NOD-SCID recipients document an absolute requirement for CD8 cells in the initial phases of development of diabetes (DiLorenzo *et al.* 1998). This requirement for CD8 cells is a robust finding reproduced in a variety of experimental models including transfers into non-irradiated young mice (Bendelac *et al.* 1987), adult irradiated mice (Miller *et al.* 1988), athymic nude mice (Kano *et al.* 1992, Matsumoto *et al.* 1993), and NOD-SCID mice (Christianson *et al.* 1993, Rohane *et al.* 1995). Indeed, depletion of CD8 cells in young NOD mice prevents the development of diabetes (Wang *et al.* 1996). These data are consistent with a model in which autoreactive CD8⁺T cells initiate the disease process leading to diversification of the CD4 T cell response and eventual development of a pathogenic CD4 T cell response (Serreze *et al.* 1997).

α_E (ITGAE)/ β_7 (herein referred to as ITGAE) is a T-cell integrin that confers specificity for the epithelial-cell-specific ligand, E-cadherin (Cepek *et al.* 1994, Karecla *et al.* 1995). Results from studies using transplant models indicate that ITGAE promotes destruction of epithelial compartments by alloreactive CD8⁺T cells (Feng *et al.* 2002, El-Asady *et al.* 2005, Yuan *et al.* 2005). These findings potentially provide insights into mechanisms of diabetogenesis because the ITGAE ligand, E-cadherin, is highly expressed by pancreatic β -cells (Cirulli *et al.* 1994, Meda 2013), the critical targets in diabetes. Several lines of evidence indicate that autoreactive ITGAE⁺CD8⁺ cells play a key role in initiation of diabetes. For instance, ITGAE is preferentially expressed by CD8⁺T cells (Hadley *et al.* 1997), a subset thought to play a central role in triggering the development of diabetes. Moreover, CD8⁺T cells unable to express ITGAE (i.e., from mice with targeted disruption of ITGAE) are deficient in the capacity to reject pancreatic islet allografts (Feng *et al.* 2002). Histological analyses have revealed that CD8⁺T effectors in *Itgae*-deficient hosts successfully traffic to the general graft site but completely fail to accumulate within the islet allograft itself (Feng *et al.* 2002). This histological picture is strikingly similar to the peri-insulinitis stage in the development of diabetes. Thus, these results raise the possibility that acquisition of expression of ITGAE by diabetogenic CD8 effector/memory cells may be a key checkpoint in progression from peri-insulinitis to frank diabetes. Therefore, we postulated that blockade of ITGAE

might have the capacity to slow the development of autoimmune diabetes. The goal of this study was to test this hypothesis.

Itgae deficient mice were originally developed in 1999 (Schon *et al.* 1999) on the Balb/c background. In these mice, exon 10 of the integrin alpha E (*Itgae*) locus was replaced with a neomycin resistance gene. The deficiency was detected via PCR, fluorescence-activated cell sorting (FACS) analysis, and immunoprecipitation as an insertion of approximately 200 bp. Results from previous studies have indicated that *Itgae* deficiency does not have a significant effect on host weight, reproduction, survival (Schon *et al.* 1999), or defense against pathogens (Fousteri *et al.* 2009). In the present studies, the *Itgae*^{-/-} genotype was backcrossed onto the NOD background. We herein describe the development of *Itgae*-deficient mice on the NOD background and demonstrate that such mice exhibit delayed development of diabetes. We further document that depleting monoclonal antibodies (mAbs) to ITGAE have a blocking effect in WT NOD mice indicating that the decrease in diabetes development is not due to genes closely linked to *Itgae*. The interpretation of these results and their therapeutic implications are discussed.

Materials and methods

PCR

DNA was isolated from a tail clip using the DNeasy Blood & Tissue Kit (Qiagen), as per the manufacturer's instructions. The concentration of DNA was measured using a Nanodrop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA). PCR was performed with HotStar Taq Polymerase (Qiagen) on a Veriti Thermocycler (Applied Biosystems). Primers for the detection of *Itgae* deficiency were as follows: F: GCAACAACGCATCGTTCATATGGA and R: GTGCTCTGTCTATTGTTCCCCTCCTT. The PCR product of the disrupted allele is expected to be 1100 bp, while the product for the WT allele is 900 bp (Schon *et al.* 1999). Primers for the detection of D11Mit320 were as follows: F: CCCATATAGTGAAGCAAGAAACG and R: TTATAGTGATGCATCCAGGTGTG. Amplification of DNA from NOD mice yields a 124 bp product, whereas mice on a Balb/c background will produce a 140 bp product. Primers for the detection of D11Mit325 were as follows: F: CCTCCACCTGAAAATTCATACT and R: GGTTAGAGGATGATGATGATGATG. Amplification of DNA from NOD mice yields a 110 bp product and a 124 bp product from Balb/c mice.

Mice

NOD/ShiLtJ and Balb/cJ mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). To develop *Itgae*-deficient mice on a diabetic background, we interbred WT NOD mice with *Itgae*^{-/-} mice on the Balb/c background (C.129S2-*Itgae*^{tm1Cmp/J}), and then backcrossed the resulting offspring to NOD mice for 12 generations. Diabetes in NOD/ShiLtJ mice is characterized by insulinitis, a leukocytic infiltration of the pancreatic islets with marked decreases in pancreatic insulin content occurring in females at approximately 12 weeks of age and several weeks later in males (Makino *et al.* 1980). Before development of diabetes, blood glucose (BG) levels were tested once per week using a Bayer Glucometer Elite. A mouse was deemed diabetic after two consecutive BG readings of >250 mg/dl. Mice were housed in accordance with the Ohio State University's requirements and regulations for animal care and use. Mice were maintained under pathogen-free housing conditions with free access to food and water.

Flow cytometry

Fluorochrome-conjugated mAbs to mouse ITGAE (2E7 and M290), CD4 (GK1.5), CD8a (53-6.7), CD8b (H35-17.2), CD3e (145-2C11), and CD44 (IM7) and the respective species- and isotype-matched negative control mAbs were purchased from BD Biosciences Pharmingen (San Jose, CA, USA). Flow cytometry was performed using a FACSCalibur (BD Biosciences) and data were analyzed using the FlowJo Software (BD Bio Sciences Pharmingen). The percentage of positive cells for a given marker was based on cutoff points chosen to exclude >99% of the negative control population. Pancreas-infiltrating lymphocytes for flow cytometry experiments were obtained by mincing and then digesting mouse pancreata for 30 min in DMEM/F12 (50:50) media supplemented with 0.1% collagenase (type IV; Worthington, Freehold, NJ, USA), 0.1% soybean trypsin inhibitor (Sigma-Aldrich), and 0.01% DNase I (Boehringer Mannheim Corp., Indianapolis, IN, USA). Following vigorous agitation, the resulting cell suspension was centrifuged on Lympholyte-M (Accurate Chemical & Scientific Co., Westbury, NY, USA) to isolate lymphocytes and then stained immediately for analyses.

mAb development and treatment

The ITGAE mAbs used in these studies were engineered variants of the well-characterized rat anti-mouse ITGAE

mAb, M290 (Kilshaw & Baker 1988). To produce the mAbs, standard recombinant DNA technology was used to render M290 effector competent in mice by replacing the rat IgG1 Fc domain with a murine IgG2a domain (herein referred to as ChM290-IgG2a), which is anticipated to bind Fc receptors with a high affinity and be an effector competent in mice. To further promote its depleting capacity, ChM290-IgG2a was grown in CHO cells with targeted disruption of α 1,2 fucosyltransferase (Potelligent, Kyowa Hakko Kirin, Tokyo, Japan) that produces a low-fucose antibody, which has been shown to improve Fc receptor binding and enhance effector function (Shinkawa *et al.* 2003). For a blocking but non-depleting mAb, we generated a variant (ChM290-IgG1) that contains the same antigen-binding regions as ChM290-IgG2a but carries an IgG1 Fc domain. A specific point mutation (at position N297Q, Kabat numbering) was introduced within the murine IgG1 constant domain to eliminate the N-glycosylation site, rendering the mAb essentially unable to bind Fc receptors and recruit effector cells (Jefferis 2009). Starting at 2 weeks of age, we treated NOD pups with ChM290-IgG2a afucosyl ($n=13$) or ChM290-IgG1 agly ($n=16$) at 1 mg/kg i.p. once per week through 5 weeks of age. The dose of antibody was determined empirically by measuring the *in vivo* dose of M290 required to block adhesion of mouse primary lymphocytes to an E-cadherin Fc fusion protein. At a dose of 1 mg/kg, the predicted peak serum concentration was in the range of 10–100 μ g/ml, well above this value. As a negative control, a group of NOD pups ($n=17$) were treated in parallel with P1.17, a mouse Ig2a isotype control antibody of irrelevant specificity (Neuwelt *et al.* 1994, Remsen *et al.* 1996, Metzner *et al.* 2005). As determination of sex was not possible at the initial time of injection (2 weeks of age), all pups were treated with the respective reagent, and once sex was determined males were killed. BG levels were then measured through 30 weeks twice weekly (unless the mouse developed diabetes at an earlier time point) with diabetes defined as two consecutive readings >250 mg/dl BG. Serum and pancreas were collected following diabetes development.

Statistical analyses

A Kaplan–Meier analysis was performed using GraphPad Prism (La Jolla, CA, USA) and significance of pairwise comparisons calculated by the log rank (Mantel-Cox) test using the SPSS Software (IBM, Armonk, NY, USA). A value of $P<0.05$ was considered to be statistically significant.

Results

Expression of ITGAE by CD8⁺T cells in the NOD pancreas during development of diabetes

To determine the extent to which ITGAE is expressed by CD8 cells during the course of diabetes, we conducted FACS analyses of lymphocytes isolated from the pancreata, lymph nodes (LNs), and spleens of female NOD mice at different points in disease progression. In these experiments, we monitored ITGAE expression by CD8⁺T cells of effector/memory phenotype (gated CD8b⁺CD44^{hi} cells) beginning at 7 weeks through development of hyperglycemia, which occurred at 17 weeks of age (on average) in our colony. ITGAE expression is constant in spleen and LNs over time. There is a drop in ITGAE expression in pancreas-infiltrating lymphocytes at week 10.5, with a return to previous levels by the final time point. Small numbers of CD8b⁺CD44^{hi}T cells (0.2×10^6 cells/pancreas) were present in the pancreas of female NOD mice as early as 7 weeks of age and the total number increased progressively with age, reaching maximal numbers at the time of overt diabetes (BG >250 mg/dl) (0.5×10^6 cells/pancreas). As shown in Fig. 1, ITGAE was expressed by a subset of CD8 T effectors (CD8⁺CD44^{hi} cells) at all time points examined. These data are consistent with a role for the ITGAE⁺CD8⁺ effector/memory subset in transition from benign peri-insulinitis to invasive insulinitis. Note that while the proportion of CD8⁺CD44^{hi} cells expressing ITGAE is relatively small, this percentage is similar to that of CD8⁺T cells isolated from rejected pancreatic islet allografts, a scenario where

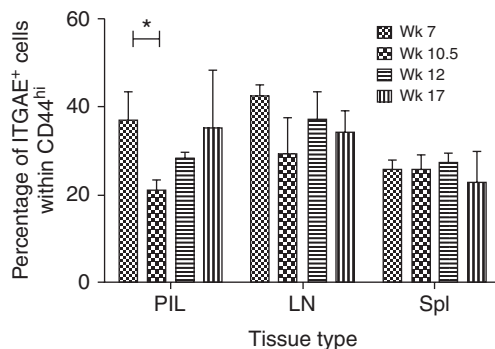


Figure 1
ITGAE expression by gated CD8b⁺ CD44^{hi} lymphocytes isolated from pancreata (PIL), lymph nodes (LN), and spleens (Spl) of female NOD mice of the indicated ages and stages of diabetes development ($n=3$). Cells are stained with mAbs to CD8b (H35-17.2) and CD44 in combination with mAbs to ITGAE (M290). All mice at week 17 are hyperglycemic.

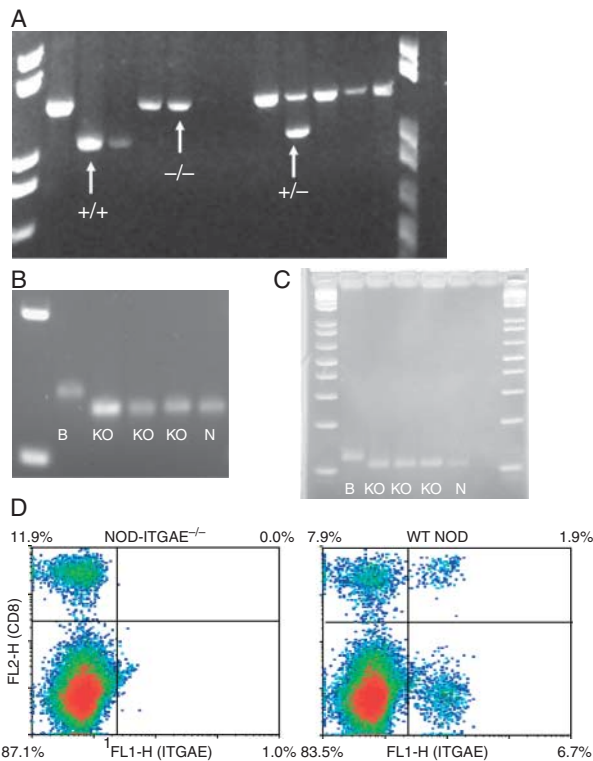
ITGAE⁺CD8⁺T cells play a critical role in β -cell destruction (Feng *et al.* 2002).

Itgae deficiency delays the development of type 1 diabetes

To test the hypothesis that autoreactive ITGAE⁺CD8⁺ effector/memory cells play a pivotal role in triggering diabetes development, we compared the incidence of diabetes in NOD mice homozygous for the *Itgae* knockout allele with that of WT NOD littermates. For this, backcross offspring were monitored for the presence of the *Itgae* knockout allele, which as shown in Fig. 2A, is readily distinguished from the WT allele by standard PCR methodology. Only mice with two disrupted copies of *Itgae* were included in this study. Moreover, only females were used in these experiments because of the earlier development and higher incidence of diabetes when compared with male mice. To confirm the absence of the ITGAE protein, lymphocytes infiltrating the host pancreas were stained for ITGAE expression (Fig. 2D).

The *Itgae* locus is located at 45.22 cM on mouse chromosome 11 (MGI website). To confirm that the mice were on the NOD background, we genotyped two marker sites that differed between NOD and Balb/c, flanking *Itgae*. Figure 2B shows that the NOD-*Itgae*^{-/-} mice expressed the NOD allele at the D11Mit320 locus (43.21 cM) and Fig. 2C demonstrates that the knockout mice expressed the NOD allele at the D11Mit325 locus (51.88 cM). The presence of the NOD allele at D11Mit320 and D11Mit325 provided compelling evidence that the resulting strain was predominantly of NOD origin. As shown in Fig. 2D, NOD-*Itgae*^{-/-} mice completely failed to express ITGAE, whereas samples from WT NOD mice contained readily detectable populations of ITGAE-expressing CD8⁺ and CD8⁻T cells.

As shown in Fig. 3, *Itgae*^{-/-} mice on the NOD background (open circles, $n=60$) were delayed by 5–6 weeks in the development of diabetes when compared with WT NOD littermates (filled circles, $n=42$) (3% incidence for knockout when compared with 43% for WT mice at 20 weeks). While the greatest effects of *Itgae* deficiency on development of diabetes were observed at early time points (weeks 17–21), the difference persisted through to the end of the observation period. There was a strong significant difference between the *Itgae*^{-/-} and WT groups ($P=8 \times 10^{-9}$). These results indicated that the absence of ITGAE delays development of diabetes in NOD mice but does not prevent development of diabetes,

**Figure 2**

Development and characterization of *Itgae*-deficient NOD mice. (A) Expression of ITGAE by backcross offspring. Genomic DNA from backcross offspring was amplified with primers to *Itgae*. Data presented are obtained from a typical screening assay to detect backcross offspring carrying the *Itgae* KO allele. Lanes 1 and 12 are DNA ladders. Lane 2: Balb/c-*Itgae*^{-/-}; Lane 3: WT Balb/c; Lane 4: WT NOD; Lanes 5–11: 12th generation backcross offspring. The three possible genotypes are indicated by arrows. Lane 3 shows two copies of the WT allele (+/+); lane 6 shows a full knockout with two disrupted copies of *Itgae* (-/-); lane 10 shows a mouse carrying one copy of the WT allele and one disrupted allele (+/-). The PCR product for the disrupted allele is expected to be 1100 bp, while the product for the WT allele is 900 bp. (B) Genomic DNA amplified with Mit320 primers. Lane 1: DNA ladder; lane 2: Balb/c (B); lanes 3–5: 12th generation NOD-*Itgae*^{-/-} (KO); lane 6: WT NOD (N). In this assay, a mouse on a Balb/c background shows a band at 140 bp, while the other on a NOD background shows a band at 124 bp. (C) Genomic DNA amplified with Mit325 primers. Lanes 1 and 8 contain DNA ladder; Lane 2: WT Balb/c (B); Lanes 3–5: 12th generation NOD-*Itgae*^{-/-} (KO); Lane 6: WT NOD (N); Lane 7: no-template control. Samples on a NOD background amplify a 110 bp product, while those on a Balb/c background amplify a product of 124 bp. (D) 2D FACS density plot of CD8 versus ITGAE expression of gated CD3⁺ pancreas-infiltrating lymphocytes from a representative NOD-*Itgae* knockout mouse (left) when compared with a WT NOD mouse (right).

indicating that expression of ITGAE is not absolutely required for development of diabetes in the NOD mouse.

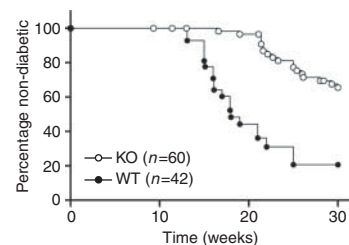
Treatment of WT NOD pups with depleting mAbs to ITGAE decreases the incidence of diabetes

It remained possible that the delayed development of diabetes in *Cd103*-deficient hosts reflected the action of

non ITGAE encoded loci mapping close to *Itgae*. We have previously reported that the existing mAbs to murine ITGAE are non-depleting and that our anti-ITGAE immunotoxin is extremely hepatotoxic (Zhang *et al.* 2009). In the present studies, we developed a ITGAE-depleting mAb that lacks the extreme hepatotoxicity of our anti-ITGAE immunotoxin. As shown in Fig. 4, ChM290-IgG2a afucosyl was highly effective at eliminating ITGAE⁺CD8⁺T cells from the small intestinal epithelia and spleens of 8-week-old NOD mice given a single injection at a dose of 1 mg/kg. In contrast, ChM290-IgG1 agly was completely ineffectual in this regard with levels of ITGAE⁺CD8⁺T cells in the spleens and intraepithelial lymphocyte compartments similar to those of NOD mice treated with the isotype control mAb, P1.17. Notably, a second anti-mouse ITGAE mAb, 2E7 – which is directed to an independent ITGAE epitope (Lefrancois *et al.* 1994) – was used for detection of ITGAE in these experiments, hence the absence of ITGAE-expressing cells was not due to masking of the determinant by residual mAbs. Based on these results, we conclude that ChM290-IgG2a afucosyl depletes ITGAE-expressing cells in NOD mice.

We then used this antibody to deplete ITGAE-expressing cells in female WT NOD mice and monitored the development of diabetes through 30 weeks of age. Treatment began at 2 weeks of age, with a weekly injection through to week 5 (four injections in total). The three groups included were as follows: ChM290-IgG2a afucosyl (depleting, *n* = 13), ChM290-IgG1 aglycosylated (agly) (non-depleting, *n* = 16), and isotype control (P1.17, IgG2a, *n* = 17).

Results presented in Figure 5 indicate that treatment with ChM290-IgG2a decreased the incidence of diabetes compared with ChM290-IgG1 or the isotype-matched negative control mAb (P1.17, IgG2a). Although the pairwise comparison between development of diabetes in the WT and IgG2a treatment groups did not display a

**Figure 3**

Incidence of diabetes in NOD-*Itgae*^{-/-} mice (open circles, *n* = 60), vs WT NOD mice (filled circles, *n* = 42). Blood glucose levels were measured once per week via tail vein laceration. Both the 12th generation backcross NOD-*Itgae*^{-/-} offspring and WT NOD mice were bred in-house.

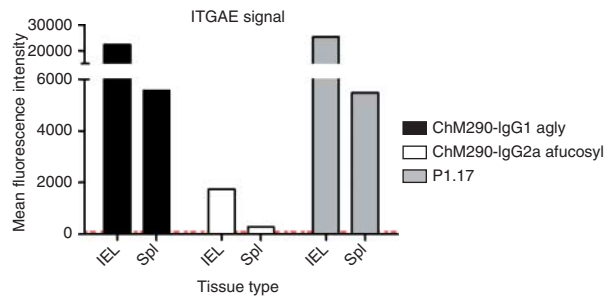


Figure 4

FACS analysis of depletion of ITGAE⁺ (gated on CD3⁺, CD8⁺ cells) intraepithelial lymphocytes (IELs) and splenic lymphocytes (Spl) in 8-week-old NOD mice following a single i.p. injection of the following at 1 mg/kg: aglycosylated ChM90-IgG1, afucosylated ChM90-IgG2a, or an isotype-matched negative control mAb (P1.17, mIgG2a). After 4 days, lymphocytes were harvested and stained as described previously (Zhang *et al.* 2009).

statistically significant difference, there was a trend toward significance ($P=0.07$), reflecting a decreased incidence of diabetes in the IgG2a-treated group compared with untreated mice.

While we have demonstrated an effect of treatment at weeks 2 and 8, later-stage treatment was ineffective. We treated mice ($n=3$) aged 16 weeks with injections of either ChM290-IgG2a afucosyl or P1.17. By week 20, two mice in the IgG2a group and one in the P1.17 group had developed diabetes.

A histological examination of the insulin-producing cells in the various groups revealed that mice treated with depleting mAbs to ITGAE (ChM290-IgG2a afucosyl) were resistant to destruction of cells by islet-infiltrating leukocytes as evidenced by a lack of injury to the insulin-producing cells (Fig. 6A and B). This histology was highly similar to that of mice treated with the isotype control mAb (P1.17) that failed to develop autoimmune diabetes (Fig. 6C and D). Notably, the pancreata of both mice exhibited the hallmark characteristics of autoimmune diabetes with massive infiltration of residual insulin-producing cells of the pancreas (β cells) with leukocytes. In diabetic mice treated with ChM290-IgG1 agly or the isotype control mAb, the insulin-producing cells were completely destroyed and thus they did not display residual cells (results not shown). These data provide direct evidence that depletion of ITGAE-expressing cells can moderate the course of autoimmune diabetes.

Discussion

We herein provide evidence that ITGAE⁺CD8⁺T effectors initiate the development of autoimmune diabetes in

NOD mice. We initially determined that ITGAE is expressed by CD8⁺T effectors before the onset of disease in these mice (Fig. 1). Our results further indicate that NOD-*Itgae* knockout mice exhibit a significant delay in development of autoimmune diabetes (Fig. 3). We use a ITGAE-depleting mAb to exclude the possibility that this delay represents an effect of one or more of the large number of loci known to confer protection from diabetes (Fig. 4), some of which map to sites in close proximity to *Itgae*. Indeed, *Itgae* maps within the Idd4.2 locus (Ivakin *et al.* 2006), hence it was important to perform studies with a ITGAE-depleting mAb to exclude the possibility that the delayed development of diabetes was due to carryover of closely linked genes during backcrossing. We observed the strongest effect in the *Itgae* knockout mice, but a depleting anti-ITGAE mAb (ChM90-IgG2a afucosyl) also decreased the development of diabetes in WT NOD mice and protected insulin-producing cells from immune destruction, strongly indicating that the effects are due to *Itgae* itself.

It is not clear why targeted disruption of ITGAE on the NOD background was more effective than depletion of ITGAE-expressing cells from WT hosts at preventing the onset of diabetes. There are many potential technical explanations for this including the difference between the constitutive absence of ITGAE from birth in NOD-*Itgae*^{-/-} mice versus elimination of pre-existing ITGAE⁺ cells from WT NOD mice and an immune response to the antibodies, thereby neutralizing their effectiveness. Although the constant regions of ChM20-IgG2a are of mouse origin and are therefore probably

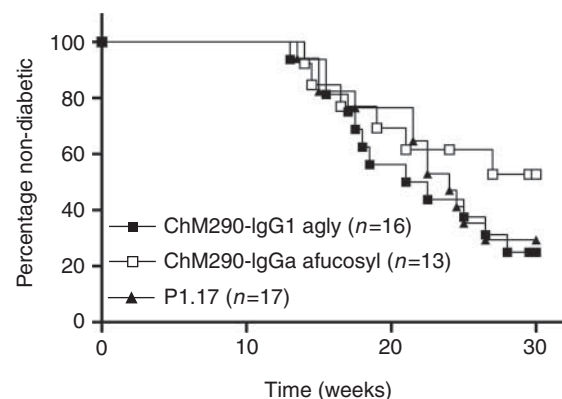


Figure 5

Incidence of diabetes in WT NOD mice treated with murinized anti-ITGAE mAbs engineered to express either an afucosylated IgG2a (open squares, $n=13$, ChM290-IgG2a) or aglycosylated IgG1 (filled squares, $n=16$, ChM290-IgG1 agly) Fc domain. A control group was treated with an isotype-matched negative control mAb (filled triangles, $n=17$, P1.17, IgG2a). Mice received injections from 2 through 5 weeks of age of an mAb at a dose of 1 mg/kg.

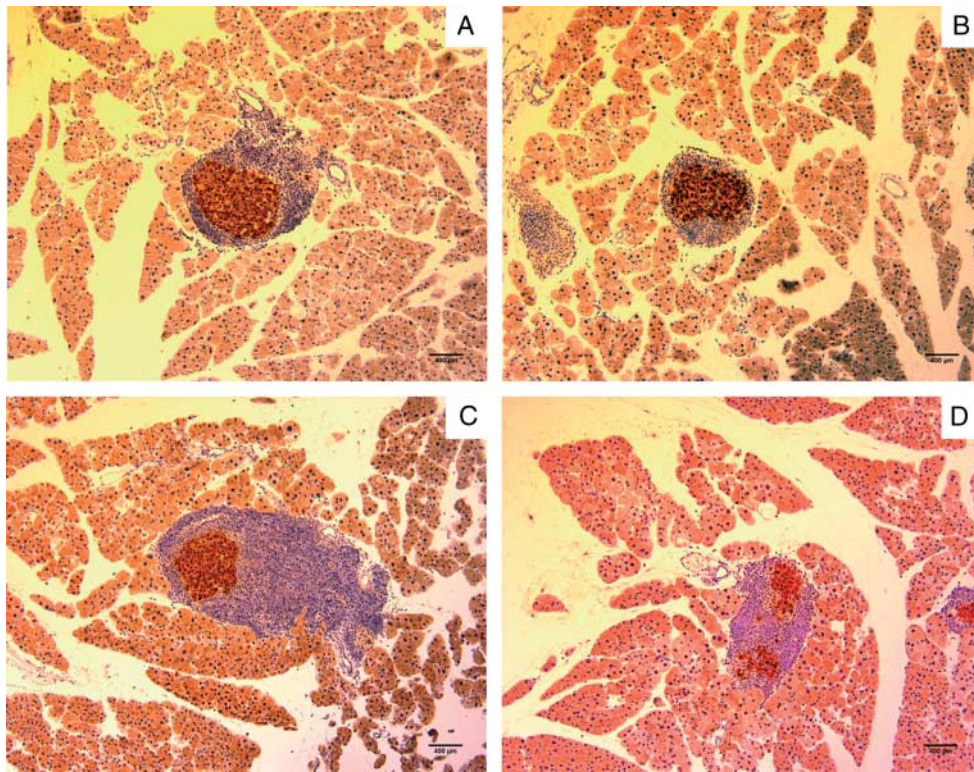


Figure 6

Immunohistochemistry of a pancreas from a diabetic mouse treated with ChM290-IgG2a afucosyl (A and B). As a control, similar sections from a non-diabetic NOD mouse treated with an isotype control mAb, P1.17, are shown (C and D). Insulin-producing cells are stained dark brown with

an anti-insulin Ab followed by diaminobenzidine (DAB); sections are counterstained (blue) with hematoxylin to highlight infiltrating leukocytes. Scale bars 400 μm .

non-immunogenic, the variable regions are still of rat origin and are thus potentially immunogenic.

In addition to CD8 T cells, ITGAE is potentially expressed by diverse leukocyte populations with important effector and regulatory functions, including dendritic cells (Fujimoto *et al.* 2011) and regulatory T cells (T regs) (Lehmann *et al.* 2002). Therefore, our results do not exclude the possibility that the latter cell populations contribute to the observed effects. ITGAE defines a subset of CD4⁺CD25⁺ T regs in the gut that exhibit potent immunosuppressive activity *in vitro* (Lehmann *et al.* 2002, Chen *et al.* 2008). Moreover, there is compelling evidence that the initiation of diabetes is controlled by T regs (You *et al.* 2008). However, our results indicating that neither elimination of ITGAE expression by gene knockout (Fig. 3) nor depletion of ITGAE-expressing cells (Fig. 4) increases the incidence of diabetes in NOD mice indicate that ITGAE⁺T regs do not contribute to this process. While there is evidence of a role for ITGAE-expressing dendritic cells in controlling the development of ITGAE-expressing T regs (Annacker *et al.* 2005), our results indicate that this

pathway is not operative during development of autoimmune diabetes in NOD mice. Rather, we proposed that the control of autoimmune diabetes is mediated by the ITGAE⁻ subset of T regs. To explain the transient delay in autoimmune diabetes observed in NOD-*Itgae*^{-/-} mice, we postulate that ITGAE expressed by CD8 T cells is required for initiation of diabetes, whereas at later time points the ITGAE-negative subset of T regs suppress this effect; leading to a biphasic requirement for expression of ITGAE during development of diabetes. However, as noted earlier in this study, our data do not exclude the possibility of a contribution by ITGAE-expressing dendritic cells to the development of autoimmune diabetes.

In summary, the expression of ITGAE by CD8⁺T cells that infiltrate the pancreatic islets before the development of diabetes (Fig. 1) indicated that blockade of ITGAE might be used to delay the onset of autoimmune diabetes, and our findings are consistent with this possibility (Figs 3 and 5). Our results indicating that *Itgae*-deficient hosts ultimately develop diabetes indicate that depleting antibodies to ITGAE may be ineffective in preventing

disease *per se*, but may be useful in inhibiting insulinitis and thereby slowing the progression of the disease.

Declaration of interest

P H W is an employee and shareholder of Biogen Idec, Inc.; none of the other authors have a conflict of interest.

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Author contribution statement

E S B wrote the manuscript and researched data. M L researched data. P H W researched data and contributed to the discussion. J B researched data. A R contributed to the discussion. C A contributed to the discussion. Q M researched data and contributed to the discussion. G A H wrote the manuscript and has access to all presented data and will take responsibility for the integrity of all data; he therefore is the guarantor of the study.

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