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Enhanced Anti-Serpin Antibody Activity Inhibits Autoimmune Inflammation in Type 1 Diabetes

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Intracellular (clade B) OVA-serpin protease inhibitors play an important role in tissue homeostasis by protecting cells from death in response to hypo-osmotic stress, heat shock, and other stimuli. It is not known whether these serpins influence immunological tolerance and the risk for autoimmune diseases. We found that a fraction of young autoimmune diabetes-prone NOD mice had elevated levels of autoantibodies against a member of clade B family known as serpinB13. High levels of anti-serpinB13 Abs were accompanied by low levels of anti-insulin autoantibodies, reduced numbers of islet-associated T cells, and delayed onset of diabetes. Exposure to anti-serpinB13 mAb alone also decreased islet inflammation, and coadministration of this reagent and a suboptimal dose of anti-CD3 mAb accelerated recovery from diabetes. In a fashion similar to that discovered in the NOD model, a deficiency in humoral activity toward serpinB13 was associated with early onset of human type 1 diabetes. These findings suggest that, in addition to limiting exposure to proteases within the cell, clade B serpins help to maintain homeostasis by inducing protective humoral immunity. The Journal of Immunology, 2012, 188: 6319–6327.

Type 1 diabetes mellitus (T1D) is thought to be primarily a T cell-mediated disease that results from destruction of the insulin-producing β cells in the pancreatic islets (1–3). The incidence of this condition has increased significantly in developed countries over the last decade (4, 5) and hygiene has been added to the growing list of potential contributors to this worrying trend. One hypothesis for the role of hygiene in the risk for T1D is based on the assumption that adequate hygiene causes a change in exposure to certain pathogens and leads to reduced innate immunity and the output of regulatory T cells with anti-inflammatory properties (6–8). According to an alternative model, hygiene may contribute to exacerbation of destructive autoimmunity by decreasing the total amount of tissue damage and impeding the development of a protective autoimmune response.

We examined the role of protective autoimmunity in the risk for T1D by focusing on intracellular molecules of the clade B family, also known as OVA-serine proteinase inhibitors (serpins) (9, 10). We hypothesized that serpins can stimulate an immune response that could influence the severity of autoimmune inflammation. To investigate this possibility, we studied the immune response against clade B serpins during the immune-mediated destruction of pancreatic islets in NOD mice (1, 2). We chose this model because the cathepsin proteases have been implicated in the pathogenesis of autoimmune diabetes (11–15), and clade B serpins are potent inhibitors of these proteases (16, 17).

In this study, we focused on a novel autoantibody against serpinB13. We found that, in contrast to the autoantibodies that are associated with an elevated risk for T1D, anti-serpinB13 autoantibody supports protective outcomes, including a diminished inflammatory response in the pancreatic islets. The identification of this autoantibody provides new information regarding the etiology of T1D and contributes to our understanding of interrelationships between the immune system and other biological pathways.

Materials and Methods

Human subjects

Patients with recent-onset T1D (n = 55) and healthy controls (n = 53), aged 3–20 y, were recruited consecutively by the Belgian Diabetes Registry. After obtaining written informed consent from each subject or the subject’s parents, the investigators collected blood samples and stored them at −80°C until they could be analyzed for serpinB13 serum-binding activity. The study was approved by the Institutional Review Board at the Belgian Diabetes Registry and Yale University.

Mice

NOD, NOR, NOD SCID, BALB/c, and C57BL/6J mice were used as donors of T cells, serum, and pancreatic islets. NOD/Caj mice were kindly provided by Dr. L. Wen (Yale University). NOD/LtJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and used to study the effects of treatment with anti-serpinB13 mAb on blood glucose levels. Mice were considered diabetic after two consecutive blood or urine glucose levels >200 and 250 mg/dl, respectively. The University Animal Care and Use Committee at Yale and University Committee on Animal Resources at the University of Rochester approved all mouse experiments.

Peptides

Two peptide libraries were purchased from ProlImmune, each consisting of 38 overlapping peptides representing the first 200 aa of OVA (peptides 1–19), moth cytochrome c (peptides 20–38), and the entire sequence of...
serpinB13. The overlap between peptides was 10 aa in length. The pMOG sequence (MEVGWYRSPFSRVHLYRNGK) was synthesized in the Keck Facility at Yale University.

**Serpins**

Purified mouse serpinB13 and serpinB8 expressed in baculovirus were obtained from GeneScript.

**Abs**

The 2C11 anti-CD3 mAb was used to stimulate CD4 T cells isolated from various mouse strains and to treat diabetic mice. An anti-His6 epitope tag (Rockland) and anti-α-tubulin Abs (Cell Signaling, Millipore, Billerica, Mass) were used to coat the Luminex beads and stain the Western blots. PE- and allophycocyanin-conjugated Abs were used against CD4 (RM4-5), CD11b (M1/70), CD11c (HL3), CD45 (30-F11), B220 (RA3-6B2), and TCR (H57-597) (BD Biosciences, Billerica, Mass).

**Production of anti-serpinB13 mAb**

A single dose of 20 µg purified mouse serpinB13 resuspended in 200 µl CFA was injected s.c. into a 6-wk-old female NOD mouse. Two weeks later, the same animal was injected with the same amount of Ag resuspended in IFA. The spleen was harvested 3 d after the second injection and was used to generate hybridoma fusions. After 2 wk of selection with hypoxanthine-aminopterin-thymidine media supplement (Sigma-Aldrich, St. Louis, MO), colonies were visible in 30 wells. These wells were screened for evidence of anti-serpinB13 Ab secretion using a Luminex assay. The supernatant obtained from the culture in one well demonstrated strong anti-serpinB13-binding activity. Hybridoma cells derived from this well were subcloned by serial dilution and retested for their ability to produce the Ab and its IgG isotype. In addition to the anti-serpinB13, we produced an IgG mAb with unknown specificity. This Ab was generated in a NOD mouse and used as an IgG control in studies described in Fig. 8.

**Luminex assays**

Luminex-based technology was used to measure the serum-binding activity of serpins, GFP, and secretagogin. Beads labeled with distinct dyes and blocked with a 1% BSA solution were precoated with anti-His6 rabbit polyclonal Ab overnight at 4°C using a Bio-Rad (Hercules, CA) labeling kit. The beads were then washed twice and incubated for 24–48 h at 4°C with lysates of 293T cells overexpressing individual His6-tagged proteins. The beads then underwent extensive washing and were pooled, incubated for 2 h at room temperature with mouse serum samples (final dilution: 1:12), and then stained with biotinylated goat anti-mouse IgG polyclonal Ab (dilution: 1:100; Invitrogen/Molecular Probes, Grand Island, NY). To determine the IgG subclass of anti-serpinB13 autoantibodies, the beads were precoated with mouse serum samples and stained separately with biotinylated anti-mouse IgG1 (clone A85-1), IgG2a (clone R19-15), IgG2b (clone R12-3), or IgG3 (clone R40-82). These mAbs were purchased from BD Pharmingen (San Diego, CA) and used at a dilution of 1:500. The secondary reagents were preabsorbed with rabbit IgG (dilution: 1:100; Invitrogen/Molecular Probes) in a 1% BSA solution before use. The final step was to incubate the beads with streptavidin (dilution: 1:200; Catlog catalog # SA1004-4; Invitrogen) for 10 min at room temperature. Fluorescence intensity (FI) was measured using Luminex 100 and BioRad-Bioplex software. Specific binding to serpinB13 was calculated as FI units after subtracting FI due to serum-binding activity in the presence of beads precoated with a control lysate (293T cells transfected with GFP [mouse] or GFP + secretagogin [human]). To demonstrate the specificity of Ab binding to serpinB13, representative mouse serum samples producing high (n = 12) or low (n = 13) FI were subjected to competitive inhibition with soluble serpinB13 (1 µg/ml) in a fashion similar to that described for human serum samples (in the following paragraph).

To detect anti-serpinB13 Abs in human serum, these samples were divided into two halves and then incubated for 12 h at 4°C with either soluble serpinB13 or serpinB8 as specific and nonspecific competitor, respectively. After incubation, the beads were precoated with individual Ags, added to the samples, which were incubated for 2 h at room temperature on a shaker (final serum dilution of serum: 1:10), and stained using a mixture of biotinylated mouse anti-human κ-chain and λ-chain mAbs (BD Biosciences; dilution: 1:300). The final steps of the assay were performed exactly as for the detection of mouse Abs. The samples were evaluated based on the degree of inhibition of binding to serpinB13-coated beads by soluble serpinB13 compared with soluble serpinB8. Serum samples, for which the degree of inhibition was 25–49% or 50–100%, were considered positive (+) and strongly positive (++), respectively.

**Insulin autoantibody (IAA) levels were measured using color-coated Luminex beads covalently conjugated with human insulin (Roche Molecular Biochemicals, Mannheim, Germany). The coupling of insulin to these beads was carried out according to a protocol provided by the vendor (BioPlex Amine Coupling Kit 171-406001; Bio-Rad). To demonstrate Ag specificity, we divided each serum sample into two smaller aliquots that were preincubated with either free insulin (final concentration 30 µg/ml), to serve as a binding competitor, or without free insulin. The rest of the assay was similar to that described above.**

**Western blots**

To verify the expression of His-tagged proteins, including clade B serpins, 293T cell transfectants were lysed in a buffer containing 1% Nonidet P-40, 150 mM NaCl, 50 mM Tris (pH 7.4), 1 mM Na3VO4, 1 mM PMSF, and a commercial protease inhibitor (Roche Diagnostics, Indianapolis, IN). Protein samples from precleared cell lysates were fractionated under reducing conditions on a 9% SDS-polyacrylamide gel. After electrophoresis, the proteins were electroblotted onto nitrocellulose membranes (Bio-Rad), blocked with 5% nonfat dry milk, and probed with anti-His6 rabbit Ab (1 µg/ml) or anti-serpinB13 mAb (1 µg/ml), followed by HRP-linked protein A (1:2,000; Sigma) and goat anti-mouse secondary Ab (1:10,000; GE Healthcare, Uppsala, Sweden), respectively. The immunoblots were developed using an ECL detection system (GE Healthcare-Amersham Biosciences).

**Molecular constructs**

cDNA samples encoding mouse plasminogen activator inhibitor 2 (also known as serpinB2), serpinB3a, serpinB3b, serpinB13 (both mouse and human), cathepsin L, secretagogin, and GFP were obtained from Open Biosystems (Thermoscientific, Lafayette, CO) as IMAGE clones and subcloned into a pcDNA3.1 Directional V5-His-TOPO vector. The sequence of every construct was verified at the Keck Facility at Yale University.

**Transfections**

To obtain 293T cells expressing serpins, secretagogin, or GFP, we transfected individual cDNAs using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions. The cells were harvested 48 h after transfection and were used to make the lysates.

**Preparation of CD4 T cells and APCs**

CD4+ T cells were isolated by negative selection using mAb to CD8 (TIB-210), MHC class II (10.2.16), FcR (2.4.G2), NK cells (HB1.19), and B cells (TIB164) and then incubated with anti-mouse and rat Ig-coated magnetic beads (QIAGEN, Valencia, CA). The purity of the recovered CD4+ T cells was 85–90%, as determined by staining with anti-CD4 mAb (PK1.5). T cell-depleted APCs were prepared by Ab-mediated complement lysis of NOD splenocytes. Briefly, spleen cells were depleted of erythrocytes by centrifugation on lymphocyte-separation medium (MP Biomedicals, Solon, OH) and then incubated, first with a mixture of anti-Thy 1 (Y-19), anti-CD3 (TIB-105), and anti-CD4 (PK1.5) mAbs and then with low-toxicity rabbit complement and 50 µg/ml mitomycin C (Sigma-Aldrich). Purity of the APCs was 90–95%, as determined by staining with anti-MHC class II mAb.

**T cell culture and activation**

All CD4+ T cell cultures were maintained in Click’s medium containing 2-ME, supplemented with 10% FCS, 10 mM HEPES, and antibiotics. Purified CD4+ T cells were incubated with APCs and stimulated with either peptide pools (final concentration 10 µg/ml each), single peptides, or 2C11 mAb (1 µg/ml). In some experiments (Fig. 1), T cells were subjected to several rounds of stimulation at 2-wk intervals.

**Proliferation assay**

To measure proliferative activity, T cells were stimulated with APCs and the peptide in round 96-well plates for 56 h, pulsed for the next 16 h with [3H]thymidine, and then harvested to count cells that had incorporated the isotope.

**Isolation of islets**

Pancreatic islets were isolated using the collagenase/DNase I digestion method and handpicked under a stereomicroscope. Islet cell suspensions were obtained by treating the islets with Cellstripper buffer (cat. # 25-056-C1; Invitrogen) for 5 min at 37°C. We used 100 µl tissue digest devoid of the islets to analyze protease activity in the exocrine pancreas.
Immunological response against clade B serpins is induced in NOD mice at a young age

In the first attempt to determine whether NOD mice develop immunity to clade B serpins, we studied their immunological response to chicken OVA, a founding member of this protein family (18). Following in vitro stimulation, the proliferation of CD4+ T cells isolated from 2-mo-old NOD mice was observed in all of the analyzed animals in response to pool 16 (p16) OVA peptides (aa 121–200). This response was not observed with CD4+ T cells isolated from NOR, BALB/c, or B6 mouse strains, including B6 mice immunized with myelin oligodendrocyte glycoprotein peptide (pMOG) 36–50 (Fig. 1A). These results suggest that NOD mice have a relatively high frequency of OVA-specific CD4+ T cells and that stimulation of the immune system with a random Ag (e.g., pMOG), without coexisting pathologic changes in the peripheral tissues, is not likely to induce anti-clade B serpin immunity.

To identify the specific OVA peptide responsible for the CD4+ T cell response in NOD mice, we stimulated NOD T cells with individual peptides from p16 (p16.1–p16.8). We found that the amino acid sequence QARELINSWVESQTNGIIRN (p16.3) was the best candidate (Fig. 1B). This sequence shares strong similarity with other members of the clade B serpin family, including serpinB3a, serpinB3b, plasminogen-activated inhibitor (PAI)-2, and anti-serpinB13 (Fig. 1C) (19). Thus, the CD4+ T cell response to p16.3 may represent the ability of NOD T cells to recognize several distinct clade B serpins.

To determine whether NOD mice develop a humoral response against overserpins, we used Luminex-based technology to assay the serum-binding activity of PAI-2, serpinB3b, and serpinB13 simultaneously in serum samples taken from 5-wk-old NOD mice. The specificity of this assay was verified by competitive inhibition with soluble serpinB13 (Fig. 2). The binding activity of serum samples (which were considered positive for anti-serpinB13 auto-antibodies) to the beads precoated with serpinB13 was effectively blocked by preincubation of these samples with soluble serpinB13 (Fig. 2C, p = 0.0103). By contrast, the binding activity in sera that were considered weak-positive was less affected by preincubation with soluble serpinB13 (Fig. 2B, p = 0.0724) and completely unaffected by this treatment in negative-serum samples (Fig. 2A, p = 0.5007).

We found that the binding activity was significantly higher for serpinB13, which is expressed in the pancreas (Supplemental Fig. 1), compared with PAI-2, serpinB3b (Fig. 3A), or cathepsin L, which is inhibited by serpinB13 (16, 17) (Fig. 3B). Importantly, the Ab response to serpinB13 in NOD mice was associated with pancreatic tissue damaged by diabetogenic splenocytes (Fig. 3C), rather than passive acquisition from NOD mothers during the feeding period (Fig. 3D). These findings demonstrate that both T and B cell-mediated immunity against clade B serpins is active during the prediabetes stage in NOD mice and that immune-mediated injury of pancreatic islets plays a role in promoting this immunological response.

Additional characterization of anti-serpin humoral responses revealed that the serum-binding activity to serpinB13 usually occurred at a titer <1:100 (Supplemental Fig. 2A), was predominantly IgG2b (Supplemental Fig. 2B), and was at a lower level in NOR mice compared with NOD mice, although the difference between these animals was not statistically significant (Fig. 4). In contrast, the autoantibody response to serpinB13 was significantly lower in B6 mice compared with NOD mice (p = 0.0354) but not with NOR mice. Although these observations suggest that an inflammatory lesion in pancreatic tissue helps to induce the anti-serpin response, male NOD mice, which usually develop diabetes at a much lower rate, had similar levels of anti-serpinB13 autoantibodies compared with age-matched female NOD mice (Supplemental Fig. 2C).
Secretion of anti-clade B serpin autoantibodies is associated with protection from early-onset T1D

To determine whether anti-clade B serpin immunity plays a role in the pathogenesis of autoimmune diabetes, we compared the expression of anti-serpinB13 autoantibodies with that of anti-IAAs, a known marker of an elevated risk for autoimmune diabetes (20, 21). We found that a strong IAA response was associated with a relatively weak anti-clade B serpin autoantibody response, whereas a strong anti-clade B serpin autoantibody response was associated with a relatively weak expression of IAAs (Fig. 5A, left panel). This inverse relationship could be explained by different kinetics for anti-insulin versus anti-serpinB13 autoantibody responses. We found that the secretion of anti-serpinB13 autoantibodies was strongest early in life (i.e., at age 4 wk) and declined over time, whereas IAAs levels peaked at age 12 wk (Fig. 5A, right panels). We also found that a small subset of mice (~16%; Supplemental Table I) generated high levels of anti-serpinB13 Abs, and that these mice showed a tendency toward a later onset of disease. Specifically, almost half of the animals with late-onset diabetes (age > 24 wk) had a strong anti-serpinB13 autoantibody response when they were 4 wk old. In contrast, none of the NOD mice that developed early-onset diabetes (age < 16 wk) produced high levels of the anti-serpinB13 autoantibody at a young age (<16 wk versus >24 wk; p = 0.0249) (Fig. 5B, Supple-mental Table I). This observation suggests the existence of a protective mechanism mediated through anti-clade B serpin immunity. We found additional support for this role when we compared T cell responses in NOD mice with different levels of anti-serpinB13 autoantibodies. T cells isolated from pancreatic lymph nodes in NOD mice with high levels of anti-serpin autoantibodies proliferated defectively in response to stimulation with diabetogenic Ags, including insulin (22) (Fig. 6A) and BDC2.5 mimotope (data not shown) (23). The number of islet-associated CD4 T cells was also reduced in these animals (Fig. 6B).

Abs against serpinB13 demonstrate anti-inflammatory and therapeutic properties

To determine whether Ab contributes directly to the protective process in autoimmune diabetes, we injected anti-serpinB13 mAb that was produced in our laboratory (Fig. 7) into NOD mice that secreted relatively low levels of autoantibodies against this molecule. The response was similar to that observed in mice with high levels of endogenous anti-serpinB13 autoantibodies (i.e., the expression of anti-serpinB13 autoantibodies with that of anti-IAAs, a known marker of an elevated risk for autoimmune diabetes (20, 21). We found that a strong IAA response was associated with a relatively weak anti-clade B serpin autoantibody response, whereas a strong anti-clade B serpin autoantibody response was associated with a relatively weak expression of IAAs (Fig. 5A, left panel). This inverse relationship could be explained by different kinetics for anti-insulin versus anti-serpinB13 autoantibody responses. We found that the secretion of anti-serpinB13 autoantibodies was strongest early in life (i.e., at age 4 wk) and declined over time, whereas IAAs levels peaked at age 12 wk (Fig. 5A, right panels). We also found that a small subset of mice (~16%; Supplemental Table I) generated high levels of anti-serpinB13 Abs, and that these mice showed a tendency toward a later onset of disease. Specifically, almost half of the animals with late-onset diabetes (age > 24 wk) had a strong anti-serpinB13 autoantibody response when they were 4 wk old. In contrast, none of the NOD mice that developed early-onset diabetes (age < 16 wk) produced high levels of the anti-serpinB13 autoantibody at a young age (<16 wk versus >24 wk; p = 0.0249) (Fig. 5B, Supplemental Table I). This observation suggests the existence of a protective mechanism mediated through anti-clade B serpin immunity. We found additional support for this role when we compared T cell responses in NOD mice with different levels of anti-serpinB13 autoantibodies. T cells isolated from pancreatic lymph nodes in NOD mice with high levels of anti-serpin autoantibodies proliferated defectively in response to stimulation with diabetogenic Ags, including insulin (22) (Fig. 6A) and BDC2.5 mimotope (data not shown) (23). The number of islet-associated CD4 T cells was also reduced in these animals (Fig. 6B).

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numbers of islet CD45<sup>+</sup> cells decreased in the islets considerably when this mAb was administered soon after weaning) (Fig. 8A).

In addition, and in support of another report that the decrease in β cell replication is reduced during diabetes remission (24), we found that the mAb-mediated reduction in lymphocyte levels in the peri-islet region was associated with a reduction in the proliferation of insulin-producing cells (data not shown). In light of these results, we decided to examine the possibility that enhanced anti-serpinB13 humoral immunity slows down the progression of autoimmune diabetes. NOD mice that received serum containing high levels of anti-serpinB13 autoantibodies exhibited a delayed onset of diabetes compared with animals that received serum containing low levels of these autoantibodies (Fig. 8B). Moreover, although the anti-serpinB13 mAb injection did not appear to influence the rate of diabetes reversal in animals that developed the disease at an early age, up to 60% of mice with later-onset diabetes (age > 12 wk) responded to this autoantibody with a transient or sustained reduction in blood glucose levels (p = 0.0108) (Fig. 8C, middle and lower panels combined). Finally, to determine whether normoglycemia could be achieved sooner through the coadministration of anti-serpinB13 mAb and another agent with a proven antidiabetic effect, we added an anti-CD3 mAb (25–27) to the treatment regimen in these animals. We found that coadministration of anti-serpinB13 mAb and a suboptimal dose of anti-CD3 mAb (28) in NOD mice with severe diabetes at onset (blood glucose > 300 mg/dl) was followed by the accelerated restoration of a normoglycemic state (Fig. 8D). Together, these data show that an enhancement of anti-serpinB13 humoral immunity can be therapeutically beneficial.

Expression of anti-serpinB13 Abs in humans with T1D

To determine whether an immunological response to serpinB13 is associated with the pathogenesis of autoimmune diabetes in humans, we measured anti-serpin autoantibody levels in patients with recent-onset T1D (Table I, Supplemental Table II). We found that the anti-serpin activity increased significantly with age in these patients (p = 0.02) but decreased with age in healthy controls, although not to a statistically significant degree (p = 0.07). The test of age-by-disease status interaction in the logistic model was highly significant (p = 0.003), suggesting that the relationship between age and anti-serpin humoral activity differs between patients with T1D and healthy controls. These data, together with

![FIGURE 4.](image1.png)

**FIGURE 4.** Immune response to serpinB13 in various mouse strains. Serum binding activity of serpinB13 in NOD (n = 20), NOR (n = 9), and B6 (n = 8) mice. The assay was performed exactly as described in Fig. 3. GEEs were used to model the relationship of Ab secretion among the three groups indicated. The overall χ² test was significant (p = 0.0354) as was the post hoc comparison of NOD versus B6 (p = 0.0001).

![FIGURE 5.](image2.png)

**FIGURE 5.** Immunological response to serpinB13 is associated with prevention of early-onset autoimmune diabetes. (A) Left panel, Hierarchical clustering of anti-IAA (upper five rows) and anti-serpinB13 autoantibody (SBA; lower five rows) in a cohort of NOD mice (n = 96) from whom blood samples were taken at age 4 wk and then every 4 wk thereafter until the onset of diabetes. Both Abs were measured using Luminex technology. Right panels, The kinetics of anti-IAA and SBA responses based on the data displayed in the left panel. GEEs were used to model the response variable in association with the relationship of Ab type and mouse age, using the robust sandwich estimator to accommodate correlations introduced by the longitudinal data. Pair-wise comparisons of interest were carried out as indicated. (B) Distribution of anti-serpinB13 serum-binding activity in 4-wk-old NOD mice (n = 96) by age of disease onset. GEEs were used to model the relationship of Ab response and age at diabetes onset.
our observations in the NOD mouse, demonstrate that early-onset autoimmune diabetes is associated with a relatively low level of anti-serpin immunity.

Discussion

T1D is thought to be primarily a T cell-mediated disease (1–3); however, B cells (29–32) and Abs (33–35) have also been implicated in its pathogenesis. Remarkably, certain autoantibodies were found to be associated with protection from T1D (36–38). For example, vertical autoantibody transmission from mothers to their progeny significantly lowers the risk for the subsequent development of multiple islet autoantibodies and diabetes (38, 39). In this study, we demonstrated the existence of a novel autoantibody that recognizes serpinB13 and is associated with the protection against T1D. Experiments with a mAb against serpinB13, which was produced in our laboratory, suggest an active defense mechanism that slows down progression to clinical diabetes. However, the exact molecular events following enhancement of anti-serpin immunity remain to be determined. One possibility is that binding serpinB13 to its Ab impairs its inhibitory effect and consequently allows the activity of its protease targets to increase. In turn, upregulated proteases may cause damage to inflammatory cells that accumulate in the pancreatic islets; alternatively they may increase the rate of conversion of molecules with a potential to regulate cell survival and islet regeneration. These hypothetical scenarios are currently being tested in our laboratory.

It is interesting to know whether anti-serpin B cell immunity occurs in disease models other than NOD mice. Based on the preliminary work, we found that B6 mice produce anti-serpin autoantibodies following s.c. injection of pMOG autoantigen coupled with i.v. injection of pertussis toxin (this treatment leads to experimental autoimmune encephalomyelitis [EAE]). In contrast, exposure to pMOG alone does not result in EAE and fails to induce the secretion of these autoantibodies. These observations, together with other data described in this article, suggest a two-step model. Step 1 consists of the formation of an inflammatory lesion, which helps to induce the anti-serpin response; step 2, which follows the generation of these Abs, helps to confer protection against further progression of inflammation. Of note, our observations regarding the protective role of anti-serpin humoral activity primarily relate to autoimmune diabetes. We do not know whether anti-serpin Abs secreted during the weeks preceding the onset of EAE are protective or harmful.

In addition to their induction following an inflammatory tissue injury, anti-serpin autoantibodies may be derived from B1 B cells. These cells are responsible for the very early Ab response and are known to be active in NOD mice (40). In our study of CDS5+ B1 B cells isolated from the peritoneal cavity and spleen in NOD mice, we found no detectable levels of anti-serpinB13 Igs in the culture supernatants after stimulating these cells with LPS (data not shown). Thus, it is not very likely that B1 B cells are the main source of anti-serpin activity. Consistent with this conclusion is a recent report that B1 B cells promote, rather than inhibit, the inflammatory response in the pancreas (41).

The reasons why treatment with anti-serpinB13 mAb inhibited late-onset diabetes and endogenous anti-serpinB13 autoantibodies were associated with protection from the early-onset diabetes in our study remain unclear. The existence of distinct mechanisms of protection that are regulated differently by anti-serpin Abs in young...
versus old mice may be responsible for this observation. For example, one may argue that anti-serpin Abs are more effective in mice with late-onset disease because islet inflammation progresses less aggressively in these animals compared with mice that develop early-onset T1D. In contrast, during the first weeks of life, anti-serpin Abs may, in addition to dampening the inflammatory response in islets, enhance the regeneration of insulin-producing cells. Consistent with this model, and with the notion that islet regeneration is more active early in life, the presence of anti-serpin activity during that early period would mainly benefit animals that are destined to develop early-onset (rather than late-onset) T1D. Our observations in the NOD mouse model are relevant to human disease, because we have found that children with T1D onset during the first decade of life are deficient in anti-clade B serpin Abs compared with age-matched healthy counterparts. Interestingly, the opposite is true for individuals who developed T1D during the second decade of life. Specifically, anti-serpin activity is seen more frequently in healthy young children (0–10 y; 46%) compared with young children with recent-onset diabetes (21%) or healthy adolescents (11–20 y; 26%). The reason for this finding is not clear. Perhaps the secretion of anti-serpin Abs is a normal response to tissue damage caused by environmental factors. Indeed, certain members of the OVA-serpin family are upregulated by Th2-type cytokines in bronchial epithelial cells, and their expression is augmented in the bronchial lesions of patients with asthma.

Table I. Analysis of anti-serpinB13 Abs in patients with recent-onset T1D

<table>
<thead>
<tr>
<th>Age at Onset (y)</th>
<th>Controls</th>
<th>Diabetic Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>SBA Positive (%)</td>
</tr>
<tr>
<td>0–5</td>
<td>7</td>
<td>71</td>
</tr>
<tr>
<td>6–10</td>
<td>19</td>
<td>37</td>
</tr>
<tr>
<td>11–15</td>
<td>9</td>
<td>33</td>
</tr>
<tr>
<td>16–20</td>
<td>18</td>
<td>22</td>
</tr>
<tr>
<td>Trend p*</td>
<td></td>
<td>0.07</td>
</tr>
</tbody>
</table>

Data are expressed as the percentages of subjects in each age group that were positive for anti-serpin Abs (SBA).

*Computed using the logistic-regression model with age as a continuous covariate; age-by-disease status interaction, p = 0.003.

FIGURE 8. Protective properties of the anti-serpinB13 mAb. (A) FACS analysis of CD45+ cells (stained with 30-F11 mAb) in islets from mice treated with anti-serpinB13 mAb. Four-week-old female NOD mice received four injections of mAb (n = 4) or the IgG mAb control (n = 4) (100 μg/injection) over 10 d. The animals were sacrificed at 5–6 wk of age. Right panel, The average of three independent experiments is shown. (B) Diabetes-free survival in NOD mice treated with serum containing anti-serpinB13 autoantibodies. The recipient mice were prescreened for low anti-serpinB13 autoantibody levels and were injected every 3 d with a 100-μL sample of serum containing either a high (n = 7) or low (n = 7) level of anti-serpin autoantibodies. A total of 10 injections was made, the first at 3.5 wk of age. After the last injection, the animals were followed weekly for evidence of glycosuria. (C) Blood glucose levels in NOD mice following treatment with anti-serpinB13 mAb. Animals were injected four times with anti-serpinB13 mAb (right panels) or IgG mAb control (left panels) (100 μg/injection), starting on the day of diagnosis (open circles), and their glucose levels were measured every 5 d thereafter. Data are displayed according to age at onset of diabetes: early onset (<12 wk, upper panels), intermediate onset (12–16 wk, middle panels), and late onset (>16 wk, lower panels). The trajectory of each mouse toward the development of disease is shown in a different color. In groups treated with anti-serpinB13 mAb, responders are depicted in green, orange, and brown, and nonresponders are shown in black and purple. To determine the difference between control and treatment groups that develop diabetes after 12 wk, animals from intermediate- and late-onset groups were combined. The Fisher exact test was used for the statistical analysis. (D) Timing of recovery from diabetes. Animals were treated with 2C11 mAb (10 μg/injection) and IgG mAb control (n = 10) or 2C11 and anti-serpinB13 mAb (n = 14). This injection regimen was similar to that described in (C). The data are from one experiment and are representative of two to three independent experiments.
References


