Curative and β cell regenerative effects of α1-antitrypsin treatment in autoimmune diabetic NOD mice

Maria Koulmanda*†, Manoj Bhasin*‡, Lauren Hoffman*§, Zhigang Fan*†, Andi Qipo*†, Hang Shi*§, Susan Bonner-Weir*¶, Prabhakar Putheti*†, Nicolas Degauque*†, Towia A. Libermann*‡, Hugh Auchincloss Jr.*, Jeffrey S. Flier*§, and Terry B. Strom*†**

*Department of ●●●, Harvard Medical School, Boston, MA 02115; ‡Genomics Center, †Transplant Research Center, and §Division of Endocrinology, Beth Israel Deaconess Medical Center, Boston, MA 02115; and ¶Joslin Diabetes Center, Boston, MA 02115

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Invasive insulitis is a destructive T cell-dependent autoimmune process directed against insulin-producing β cells that is central to the pathogenesis of type 1 diabetes mellitus (T1DM) in humans and the clinically relevant nonobese diabetic (NOD) mouse model. Few therapies have succeeded in restoring long-term, drug-free euglycemia and immune tolerance to β cells in overtly diabetic NOD mice, and none have demonstrably enabled enlargement of the functional β cell mass. Recent studies have emphasized the impact of inflammatory cytokines on the commitment of antigen-activated T cells to various effector or regulatory T cell phenotypes. Inflammation has also been linked to insulin resistance and defective insulin signaling in the NOD diabetes model. Hence, we tested the hypothesis that inflammatory mechanisms trigger insulitis, insulin resistance, faulty insulin signaling, and the loss of immune tolerance to islets. We demonstrate that treatment with α1-antitrypsin (AAT), an agent that dampens inflammation, does not directly inhibit T cell activation, ablates invasive insulitis, and restores euglycemia, immune tolerance to β cells, normal insulin signaling, and insulin responsiveness in NOD mice with recent-onset T1DM through favorable changes in the inflammation milieu. Indeed, the functional mass of β cells expands in AAT-treated diabetic NOD mice.

autoimmunity | type 1 diabetes

A destructive T cell-dependent autoimmune process directed against insulin-producing β cells causes type 1 diabetes mellitus (T1DM) in humans and the nonobese diabetic (NOD) mouse model (1, 2). Although many therapeutic interventions, including viral-mediated gene transfer of human α1-antitrypsin (AAT) (3), can prevent T1DM or resolve the T cell-rich, β cell-invasive insulitis lesion in prediabetic hosts, surprisingly few therapies have succeeded in restoring long-term drug-free euglycemia and immune tolerance to β cells in overtly diabetic NOD mice (4–8). Although each of these successful therapies directly targets T cells, each bears an element that may dampen proinflammatory responses or their consequences upon target tissues.

Inflammatory cytokines direct the commitment of antigen-activated $CD4^+$ T cells to specific effector or Foxp3+ regulatory phenotypes (9–11). In addition, islets are sensitive to proinflammatory cytokines (12–15). Adverse inflammation in muscle and fat causes faulty insulin signaling and insulin resistance (16) in type 2 diabetes mellitus (13) and, as recently shown, T1DM (7, 17). Hence, we have tested the hypothesis that treatment with AAT, an acute-phase reactant with known antiinflammatory and antiapoptotic effects (18–21) including effects on islets (21, 22), is effective in NOD mice with overt new-onset T1DM. In short, we are probing the hypothesis that inflammatory mechanisms trigger T1DM.

Results

AAT Does Not Inhibit T Cell Activation. Purified carboxyfluorescein diacetate succinmidyl ester (CFSE)-labeled C57BL/6 mouse T cells were stimulated with plate-bound anti-CD3 plus soluble anti-CD28 mAbs. AAT did not impair T cell proliferation or acquisition of an activated phenotype ($CD25^{high}$ $CD44^{high}$ $CD62L^{low}$) [supporting information (SI) Fig. S1] in accord with the reported failure of AAT to bind to T cells (23) or inhibit T cell proliferation (24, 25).

Short-Term AAT Treatment Restores an Enduring Euglycemic State in New-Onset Diabetic (DIA) NOD Mice. A short (2 mg i.p. every 3 days $\times$ 5) course of human AAT was given to new-onset (>10 days) T1DM NOD mice whose three times repeated blood glucose levels ranged from 300 to 450 mg/dl, creating a postinfusion 2-fold rise in total AAT (mouse plus human) levels with levels falling to baseline before each dose (21). All untreated diabetic NOD mice remained hyperglycemic (Fig. 1), and most died within 7 weeks despite insulin treatment. In contrast, euglycemia, maintained indefinitely (>270 days), was achieved in 21 of 24 AAT-treated diabetic NOD mice despite cessation of insulin and AAT therapy (Fig. 1).

Islet Histology, β Cell Mass (BCM), and Circulating Insulin Levels. Histologic analysis of islets at the onset of overt hyperglycemia reveal that (*i*) most islets are atrophic with few β cells remaining, (*ii*) a minority of islets retain many β cells, (*iii*) ●●● normal numbers of α cells (Fig. 2 *A* and *B*), (*iv*) leukocytes invade the islets (Fig. 2 *A* and *B*), and (*v*) β cells are partially degranulated (Fig. 2*A*). In contrast, islet histology of diabetic NOD mice rendered euglycemic by human AAT treatment at least 35 days after cessation of AAT treatment (Fig. 2 *C* and *D*) show (*i*) prominent β cell re-granulation, (*ii*) larger islands of β cells than at T1DM onset, and (*iii*) β cell-rich islets are surrounded, but not invaded, by lymphocytes (Fig. 2 *C* and *D*).

As compared with the situation at onset of overt T1DM, BCM of AAT-treated new-onset T1DM NOD mice increased (two-tailed unpaired Mann–Whitney *t* test; $P < 0.01$). For comparison, nondiabetes-prone NOD.SCID mice at 13 and 18 weeks of age had a BCM of 1.36 ± 0.12 mg ($n = 26$) (26) (Table 1). The BCM of recent onset diabetic NOD mice was ≈10% of adult NOD.SCID mice, whereas the BCM in normoglycemic AAT-treated mice quickly rose to 45% of the normal BCM for NOD.SCID mice (Fig. 2). Using a dual staining technique for insulin and Ki67, a marker for cell proliferation, there was no evidence of β cell proliferation in the pancreases of AAT-treated

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**To whom correspondence should be addressed. E-mail: strom@bidmc.harvard.edu.

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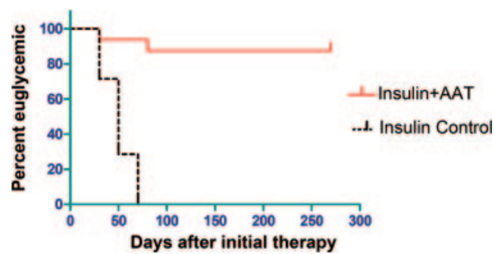

Fig. 1. Short-term AAT treatment of DIA NOD mice restores euglycemia. Spontaneous DIA NOD mice (NOD-sp) were treated with insulin alone or treated with a short term of AAT plus insulin therapy. In the insulin control group all animals stayed diabetic (150/150). In contrast 14 of 16 mice treated with AAT and insulin became and remained normoglycemic. AAT-treated animals were compared with insulin control mice by using Wilcoxon signed ranked test ($P < 0.0001$).

hosts examined at 50 days after initiation of treatment (data not shown). The mass of glucagon-positive α cells was stable after AAT treatment. Moreover, circulating fasting insulin levels rose in euglycemic AAT-treated NOD mice as compared with newly diagnosed diabetic NOD mice (Table 2; $P = 0.0312$; Wilcoxon signed-rank test).

AAT Treatment Aborts Diabetogenic Autoimmunity and Induces Specific Immune Tolerance to β-Cells in NOD Mice with New-Onset T1DM. Despite the lack of a direct effect on T cells (refs. 23–25 and Fig. S1) in an accessory-free system, AAT treatment tilted the overall balance of antiislet immunity toward tolerance as affirmed through experiments in which syngeneic islets were placed into DIA hosts that had been successfully treated with AAT and thereby rendered euglycemic. Control untreated new-onset T1DM NOD recipients destroyed syngeneic islet grafts 4–21 days posttransplantation (Fig. 3). To determine whether euglycemic AAT-treated NOD mice were rendered tolerant to their islets, we chemically destroyed their remnant β cells through administration of streptozotocin (STZ)

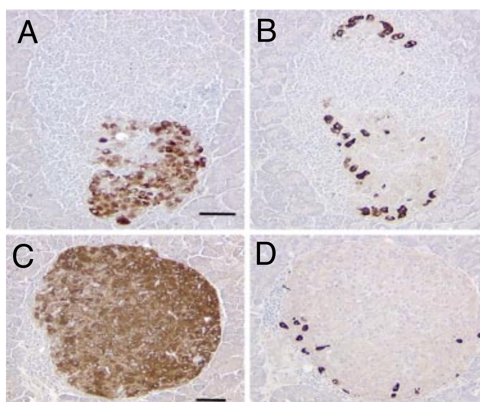

Fig. 2. Islet histology of spontaneous diabetic NOD mice at recent onset of disease and treatment with AAT (100 days after onset). NOD pancreases are analyzed at onset of diabetes (*A* and *B*) and after treatment with AAT (*C* and *D*). *A* and *C* show pancreases that are immunostained for insulin, and *B* and *D* show pancreases that are immunostained for glucagon. (*A*) At the onset of overt hyperglycemia, most islets are atrophic with few β cells remaining (unstained central cells); even so some islets remain that have substantial number of beta cells and a massive lymphocyte infiltrate. The remaining beta cells are partially degranulated. (*B*) The same islet stained for glucagon is seen. (*C* and *D*) After treatment of diabetic NOD mice as determined by three consecutive pretreatment blood glucose levels of 300–350 mg/dl, islets have similar atrophic appearance with occasional large, β cell-rich islets that are less degranulated and have greater proportion of β to α cells than at onset, and are surrounded, not invaded, by lymphocytes. (Scale bars = 50 μm.)

Table 1. ●●●

Mouse group	*N*	BCM, mg	α Cell mass, mg
DIA	6	0.17 ± 0.05	0.31 ± 0.04
AAT-treated	4	0.61 ± 0.09	0.52 ± 0.12
NOD.SCID	26	1.36 ± 0.12	ND

ND, not determined.

after (200–300 days) cessation of AAT (Fig. 3). Subsequently, syngeneic islet grafts were transplanted into successfully AAT-treated NOD mice whose diabetic state was rekindled with STZ administration (Fig. 3). Without reinstitution of immunosuppressive therapy in hosts previously treated with AAT, all STZ-treated recipients of syngeneic islets became normoglycemic permanently (Fig. 3). In contrast, allogeneic islets were quickly rejected (Fig. 3). AAT treatment creates specific, drug-free tolerance to syngeneic insulin-producing β cells.

AAT Treatment Alters the Balance of Immunity and Inflammation in the Pancreatic Lymph Node (PLN). Using targeted real-time PCR (RT-PCR) analysis, we compared transcriptional profiles of PLN samples obtained from mice rendered euglycemic by AAT treatment with samples from insulin-treated DIA NOD mice (chronic diabetic group), but not AAT, for 3–5 weeks. In PLN from AAT-treated NOD mice, dampened expression of the guanylate nucleotide binding protein-1 ($P = 0.05$) and C-reactive protein (CRP) ($P = 0.08$) acute-phase reactant genes was evident (Fig. 4*A*). As amplified expression of genes encoding acute-phase reactants arises within inflamed tissues, reduced expression of these genes may signify dampened inflammation. In these samples, reduced expression of the IFN-γ ($P = 0.03$), IL-6 ($P = 0.01$), and IL-1β ($P = 0.08$; data not shown) proinflammatory cytokine genes was detected (Fig. 4*B*). Although AAT-induced change in CRP and IL-1 gene expression are statistically marginal, the overall trend toward diminished expression of proinflammatory cytokines in AAT-treated T1DM NOD mice is clear. Expression of T helper 1 (Th1)-specific T-bet and Th17-specific retinoic acid-related orphan receptor γt (RORγt) transcription factors were markedly decreased in AAT-treated new-onset T1DM NOD mice as compared with new-onset T1DM NOD mice. As compared with chronic diabetic new-onset NOD mice, Foxp3 expression was markedly increased in AAT-treated mice, but not increased in comparison with newly diagnosed T1DM NOD mice (Fig. 4*C*). In short, AAT initially tilted the balance of expression of proinflammatory to antiinflammatory cytokines and the balance T cell Th1/ Th17 effector (dramatically down-regulated) to regulatory T cell genes sharply toward predominance of antiinflammatory and regulatory T cell gene expression. AAT did not alter expression of the suppressor of cytokine signaling1 (SOCS) 1, SOCS2, TNF-α, and TGF-β genes within the PLN. No additional unreported gene expression events were analyzed by RT-PCR.

Table 2. Circulating fasting insulin levels in successfully treated new-onset T1DM NOD mice

Animal no.	Day*	Insulin, ng/ml	Day†	Insulin, ng/ml
1	0	1.051	6	1.606
2	0	0.549	6	0.835
3	0	1.18	7	1.654
1	0	1.508	15	2.128
2	0	0.835	15	1.606

The insulin levels of AAT-treated animals were compared before and after treatment by using Wilcoxon signed ranked test ($P = 0.0312$).
*Day at which time AAT treatment commenced in new-onset T1DM NOD mice.
†Day after initiation of therapy at which time restoration of normoglycemia was evident.

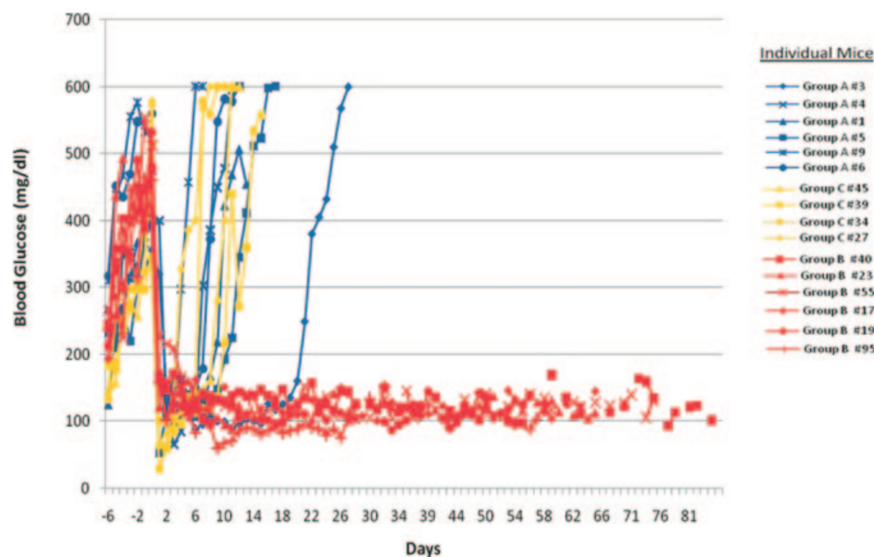

Fig. 3. Short-term treatment of T1DM NOD mice with AAT specifically restores immune tolerance to α cells. Group A, NOD.SCID (donor), NOD-sp (spontaneous DIA NOD mice; recipient). Group B, NOD.SCID (donor), NOD-sp/stz (a STZ-induced diabetic state was induced in NOD recipients; recipient). Group C, C57BL/6 (donor), NOD-sp/stz (recipient). Groups B and C have prior treatment with AAT. Spontaneously diabetic NOD mice were previously restored to a euglycemic after onset of diabetes by AAT therapy. These mice remained (groups B and C) euglycemic 200–300 days after the cessation of treatment. Syngeneic (groups A and B) NOD.SCID islet or allogeneic C57BL/6 (group C) islet grafts were transplanted into NOD recipients.

The AAT Treatment Ablates Insulin Resistance in New-Onset T1DM NOD Mice. As an insulin-resistant state in NOD mice exists in new-onset T1DM (7, 17), we analyzed the effect of AAT treatment on the sensitivity of NOD mice to insulin-driven disposal of blood glucose. After an i.p. injection of insulin, blood glucose levels in 10-week-old DIA mice remained stable for 15 min and slowly decreased over a 1-h period (37% decrease at 30 min). In contrast, blood glucose disposal returned to normal in the AAT-treated group (Fig. 5). Human albumin control injections failed to alter insulin resistance (data not shown). Thus, AAT treatment ablates insulin resistance, thereby normalizing the response of host tissues to insulin.

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AAT Treatment Restores *in Vivo* Insulin Signaling in Diabetic NOD Mice. As insulin resistance in DIA NOD mice is accompanied by defective *in vivo* insulin signaling in fat and muscle (7), we examined the effects of AAT on insulin signaling in skeletal muscle of DIA NOD mice *in vivo*. Insulin-stimulated tyrosyl phosphorylation of the insulin receptor (IR) and the IR substrate-1 (IRS-1) were markedly diminished in new-onset T1DM NOD mice (Fig. 6). The impact of short-term AAT therapy on tyrosine phosphorylation patterns was compared with those obtained in mice rendered euglycemic from the time of diagnosis of T1DM with intense insulin therapy delivered with osmotic pumps. AAT therapy, unlike osmotic insulin pump therapy, does not immediately render the treated mice euglycemic. As AAT-treated mice remain hyperglycemic for up to 3–5 weeks, we temporarily used nonintensive, conventional insulin therapy delivered i.p. in AAT-treated hosts to prevent extreme hyperglycemia until the advent of euglycemia (at which time insulin therapy is discontinued). Unlike AAT, intense osmotic pump delivered insulin or conventional insulin (chronic diabetic group) treatment did not fully restore tyrosine phosphorylation of IR and IRS-1 in new-onset T1DM NOD mice.

AAT Treatment Exerts an Antiinflammatory Effect on Critical Insulin-Sensitive Tissues. Using RT-PCR methodology, a hypothesis-driven targeted transcriptional profile for select inflammation-associated gene expression events known to influence insulin sensitivity within fat, a key tissue for insulin-driven disposal of blood glucose, was compiled in NOD mice (Fig. 7). As AAT-treated mice remain hyperglycemic for 3 weeks, we temporarily used nonintensive, conventional (i.p.) insulin therapy in AAT-treated hosts to prevent extreme hyperglycemia until the advent of euglycemia (at which time insulin therapy was discontinued). We analyzed insulin-sensitive tissues by RT-PCR in new-onset T1DM mice treated by conventional insulin treatment for 3 weeks (chronic diabetic group). Hyperexpression of SOCS1 and/or SOCS2 and TNF-α by insulin-sensitive tissues creates insensitivity to insulin-driven disposal of blood glucose (13, 16, 27). Hence, we analyzed the expression of TNF-α and SOCS genes in the fat of AAT-treated and control T1DM NOD mice. As compared with control chronic diabetic NOD mice, expression of TNF-α, SOCS1, and SOCS2 genes was reduced in AAT-treated diabetic mice (Fig. 7).

Microarray and Network-Based Analysis. To identify the overall transcriptional changes induced by AAT treatment, we performed genomewide transcriptional analysis on fat obtained from normal, diabetic, and AAT-treated animals. A total of 649 transcripts were significantly differentially expressed [lower confidence bound (LCB) > 2] in the fat of diabetic mice as compared with control normal mice (Table S1). A hierarchical cluster of differentially expressed transcripts is shown in Fig. S2*A*. K-means clustering of differentially expressed transcripts to 20 bins identified 348 of 649 transcripts (differentially expressed diabetic vs. normal) that were counterregulated by AAT treatment. Fig. S2*B* reveals K-means clustering patterns that depict the different degree of counter regulation induced by AAT on differentially expressed transcripts. We performed system biology analysis on these 348 transcripts to identify biological networks resulting from the AAT-induced reversal pattern, i.e., genes whose expression in fat resembles those of normal mice after, but not before, AAT treatment. Thirteen interactive gene networks were identified that achieved a score $> = 15$ (Table S2). To understand the underlying biological mechanism specifically related to immune response and metabolism, we merged three networks with functions in immune response, inflammatory disease, and metabolism. A merged network resulting from these three networks along with annotation of dysregulated functional processes is shown in Fig. S2*C*. The network-based analysis identified inflammation-related genes (TNF-α, IL-4, NFκB) forming the regulatory or highly connected nodes, which serve as focus hubs in the networks. The focus hub-forming genes are considered better targets as they are critical for overall function of the network. The reversal effect induced by AAT on genes of merged network is shown as a cologram in Fig. S2*D*. For example, CCR2 and INSIG1 are up-regulated in diabetic vs. normal mice (Fig. S2*C*) and are counter-regulated or down-regulated by AAT (Fig. S2*D*). This

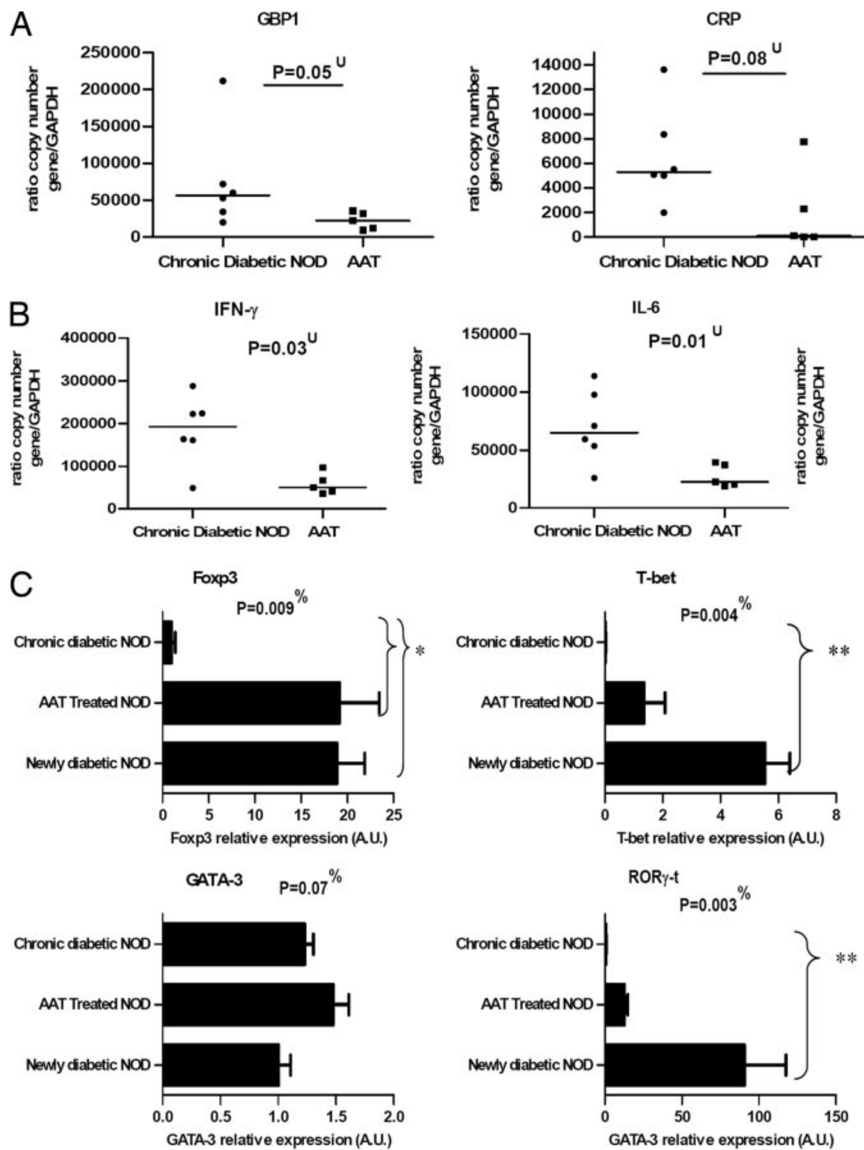

Fig. 4. A RT- PCR based analysis of AAT effects ongene expression with PLNs comparing AAT-treated NOD mice with chronic diabetic NOD mice. Analysis of gene transcription was performed according to the absolute quantification method as described by the manufacturer (Applied Biosystems). GAPDH was used as endogenous control to normalize for mRNA levels. Results are expressed as intrasample target: GAPDH mRNA copy number ratio (*A* and *B*) or as target gene relative expression (*C*). A.U., arbitrary unit. Data represent mean of five independent experiments, and error bars represent SEM. U denotes that analysis has been performed by using two-tailed Mann—Whitney test. % denotes *P* value using Kruskal-Wallis test. *, $P < 0.05$; **, $P < 0.01$ (Dunn's Post Hoc test after Kruskal-Wallis test)

finding provides an insight about the role of AAT in reversal of gene expression events that play significant roles in inflammation, immune response, and lipid/nucleic acid metabolism.

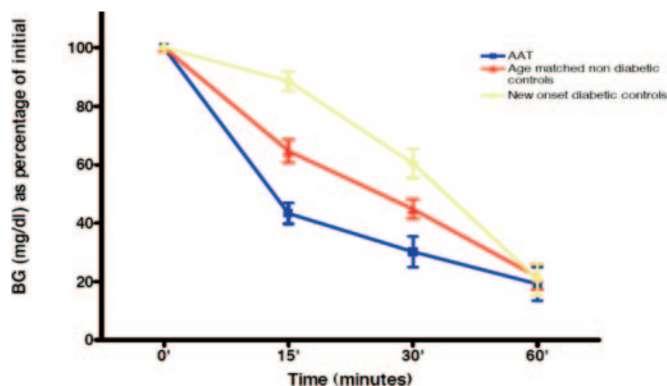

Fig. 5. The AAT treatment ablates insulin resistance in diabetic NOD mice. ITT was performed in age-matched spontaneous DIA NOD mice (NOD-sp, n = 10); AAT-treated spontaneous new-onset NOD mice (AAT, n = 8); and nondiabetic NOD mice (n = 10).

Discussion

In the clinically relevant NOD model (1, 2), the loss of immune tolerance to β cells leads to autoimmune-mediated destruction of insulin-producing β cells. Few T cell-directed therapies have succeeded in restoring euglycemia and self-tolerance to islets in overtly diabetic NOD mice (4–8). We suspect that the inability of many of these failed T cell-directed treatments owes to their inability to quench non-T cell-mediated proinflammatory responses. Proinflammatory responses can indirectly, but powerfully, influence the commitment of Ag-activated T cells to various protective (Treg) or cytopathic phenotypes (Th1, Th2, Th17) and proinflammatory cytokines directly create β cell damage and insulin resistance. To directly test this hypothesis, we treated new-onset overtly diabetic mice with a short course of human AAT, an acute-phase reactant with serine proteinase inhibitor (28, 29) and antiinflammatory and antiapoptotic effects (18–22, 29). Because serum levels of AAT rise sharply in response to inflammation (30), one may speculate that the function of AAT is to limit the duration and magnitude of inflammation.

Despite the absence of direct action on accessory cell-independent T cell activation (Fig. S1) AAT therapy induces

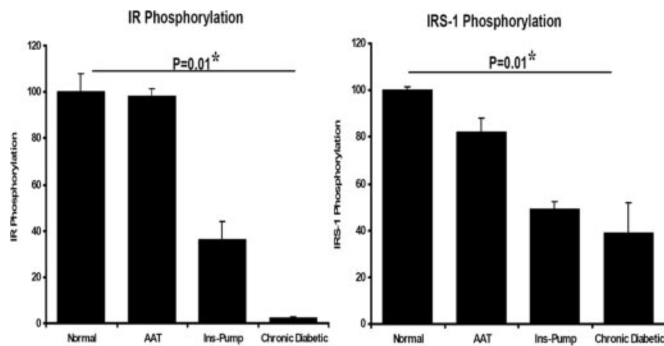

Fig. 6. AAT treatment aborts insulin resistance in DIA NOD mice. Mice were fasted overnight and injected with human insulin (20 units/kg body weight i.p.) to acutely stimulate insulin signaling. Mice were killed 10 min later. Skeletal muscle (gastrocnemius) obtained (50 days posttreatment) was dissected and frozen in liquid nitrogen for immunoblotting analysis of insulin signaling proteins. Group 1, control nondiabetic NOD mice. Group 2, AAT-treated NOD mice at 50 days. Group 3, acute diabetic NOD mice rendered euglycemic by delivery of insulin via a osmotic pump for 10 days. Group 4, chronic diabetic NOD mice treated with conventional insulin therapy. *, Kruskal-Wallis test.

tolerance to allogeneic islet transplants (24). AAT also confers antiapoptotic effects on islets (22). Although human AAT is immunogenic in mice (21), 14 days of AAT monotherapy halts invasive and cytodestructive insulitis type autoimmunity in the NOD model. Both euglycemia and immune tolerance to β cells are restored. The ability of AAT therapy to modify the molecular context in which autoantigen is recognized by T cells may play an important role in quenching destructive autoimmunity. The cytokine and inflammatory texture of the environment in which naïve CD4$^+$ T cells recognize antigen dictates the initial commitment of these cells to various effector (Th1, Th2, Th17) or Foxp3+ regulatory phenotypes (9–11). After AAT therapy an islet-invasive form of insulitis was supplanted by circumferential insulitis that is often associated with tolerance to islets (1, 2). Indeed, AAT-treated NOD mice are rendered tolerant to syngeneic islets. The rapid ablation of invasive insulitis and the marked decrease in proinflammatory-type, lineage-specific T-bet and RORγt, but not Foxp3, transcripts within the PLN suggest that AAT-triggered alterations in inflammation serve to rapidly alter the fundamental nature of T cell-dependent autoimmunity in the NOD model. The cytopathic Th17 phenotype can be destabilized with consequent acquisition of antiinflammatory properties by the changes in the inflammatory milieu (31), and AAT treatment results in a marked favorable change in the balance of FOXP3 to RORγT and T-bet gene expression. Hence, a marked decrease in expression of proinflammatory, but not antiinflammatory, cytokines is associated with and probably causal for restoration of immune tolerance to islets.

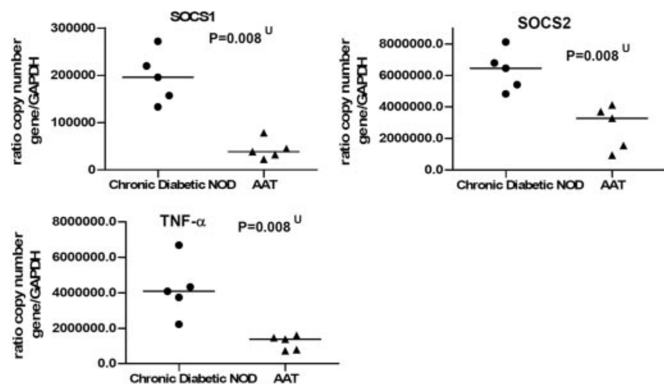

Fig. 7. RT- PCR results from fat comparing AAT-treated NOD mice with chronic diabetic NOD mice Analysis of gene transcription was performed according to the absolute quantification method as described by the manufacturer (Applied Biosystems). GAPDH was used as an endogenous control to normalize for mRNA levels. The results were expressed as intrasample target/GAPDH mRNA copy number ratio. U denotes that a two-tailed nonparametric Mann–Whitney test was used for data analysis.

The advent of overt diabetes occurs before the complete loss of β cells. Euglycemia is rapidly obtained in AAT-treated diabetic NOD mice. The rapid restoration of euglycemia, normal insulin sensitivity, and *in vivo* insulin signaling by AAT treatment is linked to reduced expression of proinflammatory molecules previously known to impair insulin responsiveness in tissues. Note that the functional, insulin-positive BCM expanded markedly and rapidly in AAT-treated hosts. These findings further authenticate the effective cytoprotective effects of AAT on islets (21, 22). Indeed, we demonstrate that the treatment of DIA mice leads to expansion of the autologous BCM. Using the Ki67 marker as a guide, there was no evidence of β cell proliferation in the pancreases of AAT-treated hosts at 50 days after initiation of AAT treatment. This study suggests that the expansion of BCM is caused by repair of damaged islets, although an early burst of β cell proliferation cannot be excluded.

The interactive network-based functional analysis of DNA microarray data of fat tissue obtained from normal, diabetic, and AAT-treated mice provides insight into the association between the effects of AAT treatment and the impact of treatment on biological processes related to nucleic acid/lipid metabolism, immune response, and inflammatory disease. The three networks (networks 1, 4, and 7) identified by the interactive network analysis predicted an effect of AAT treatment on metabolism, inflammation, and immune response. The merging of these significant networks reveals that three inflammation-related molecules (TNF-α, IL-4, and NFκB) form central regulatory nodes whose expression in the fat of diabetic mice was dampened and restored toward normal, as a consequence of AAT treatment. These data strongly suggest that AAT treatment restores insulin sensitivity and signaling as a consequence of treatment-induced effects on inflammation. Nevertheless, none of these genes (TNF-α, IL-4, and NFκB) were identified as differentially expressed on Affymetrix arrays. RT-PCR, a more sensitive technique, did detect a suppressive effect of AAT therapy on intrafat TNF-α, but not IL-4, gene expression. As the regulation of NFκB activation is posttranscriptional, we did not assess the effect of AAT treatment on gene expression.

In short, AAT, an acute-phase reactant, is a member of the serine protease inhibitor (serpin) family of proteins, a family that serves to maintain homeostatic balance between proteases and antiproteases (28, 29). In accord with data presented herein, AAT has been shown by Lewis *et al.* (21, 24) and others (reviewed in ref. 29) to exert potent antiinflammatory effects, including regulated expression of antiinflammatory cytokines. As we note, AAT treatment served to dampen expression of proinflammatory, but not antiinflammatory, cytokines. In parallel, enhanced expression of Foxp3 relative to T-bet and RORγt was noted. Hence, we hypothesize that the change in balance of proinflammatory to antiinflammatory cytokines that occurs in AAT-treated autoimmune NOD mice acts to restore immune tolerance to islets despite the absence of direct AAT effects on CD4$^+$ T cells. How can this possibly occur? The commitment of CD4$^+$ T cells to the Foxp3+ cytoprotective phenotype is favored within a milieu dominated by TGF-β, an antiinflammatory cytokine, whereas commitment of T cells to Th1, Th2, and Th17 cytodestructive phenotypes is driven in a microenvironment dominated by proinflammatory cytokines even in the presence of TGF-β (9–11). AAT ablates inflammation-driven insulin resistance. It seems likely that the 2-fold tolerance promoting and antiinflammatory effects of AAT treatment provide a

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microenvironment conducive to repair of damaged islets, thereby facilitating expansion of the functioning BCM.

Successful application of therapies that restore euglycemia in overtly diabetic NOD mice has predictive value for human T1DM (2). The excellent results achieved with anti-CD3 treatment in diabetic NOD mice have served as the basis for initiating successful clinical trials in which anti-CD3 mAb treatment slowed the progression to permanent diabetes in humans with new-onset T1DM (32, 33). Consequently, AAT may warrant attention as an agent worthy of clinical testing for individuals with new-onset T1DM and residual β cell function. We submit that adverse inflammation, potentially sensitive to AAT treatment, may play an important role in T1DM disease expression.

Materials and Methods

T Cell Activation Study. Highly purified T cells from spleen and lymph nodes were prepared as described in *SI Text* and labeled with the vital dye CFSE (Molecular Probes–Invitrogen) (34). T cells were cultured as described (9) in the presence or in the absence of AAT (0.5 μg/ml). CFSE profile was used to assess T cell proliferation as described in *SI Text*.

Mice. Female NOD (NOD/LtJx) mice and NOD.SCID (NOD.CB17-Prkdcscid/J) mice were purchased from Jackson Laboratories at 4 weeks of age and maintained under pathogen-free conditions at the Massachusetts General Hospital (Boston). All animal studies were approved by our institutional review board.

Blood Glucose Levels. NOD mice were monitored twice weekly with the Accu-Check blood glucose monitor system (Roche). When nonfasting blood glucose levels were in excess of 300 mg/dl on three consecutive measurements, a diagnosis of diabetes was made. For syngeneic islet transplant recipients, blood glucose levels were checked at the time of transplantation, then daily for 2 weeks, and then two to three times per week afterward.

Induction and Management of Diabetes. Successfully AAT-treated euglycemic NOD mice were rendered hyperglycemic with STZ (275 mg/kg i.p.) treatment 200–300 days after the restoration of euglycemia in treated and formerly spontaneously diabetic NOD. With the reemergence of hyperglycemia after STZ administration, the diabetic NOD mice were used as syngeneic or allogeneic islets graft recipients. Graft failure was declared on the first day of 3 consecutive days of blood glucose levels $>$250 mg/dl.

Islet Transplantation. NOD.SCID mice and C57BL/6 mice (10–12 weeks old) were used as donors for islet transplants. Islets were isolated by using a modification of the method of Gotoh *et al.* (35) as described in *SI Text*.

AAT Treatment Protocol. Aralast (human α1-proteinase inhibitor) is a serum serine-protease inhibitor that inhibits the enzymatic activity of neutrophil elastase, cathespin G, proteinase 3, thrombin, trypsin, and chymotrypsin. Aralast was purchased from Baxter and was given at a dose of 2 mg i.p. every 3 days for a total of five injections.

Insulin Tolerance Test (ITT). ITTs (36) were performed in age-matched NOD mice: spontaneous new-onset diabetic NOD mice (NOD-sp), AAT-treated spontaneous new-onset NOD mice (NOD-sp/AAT), and nondiabetic NOD mice as described in *SI Text*.

Morphometric Analysis of BCM. Immunostaining of islet sections (5 μm) for glucagon and insulin and measuring of BCM was performed as described in *SI Text* (37).

Quantitative RT-PCR Methods. mRNA was extracted, and reverse transcription was carried out with 1 μg of RNA (38) as described in *SI Text*. Two strategies for RT-PCR were used as described in *SI Text* (39).

Microarray Analysis of Gene Expression. The transcriptional profile of normal, DIA, and AAT-treated diabetic mice was characterized by oligonucleotide microarray analysis using the Mouse 430 2.0 Affymetrix GeneChip, according to previously described protocols for total RNA extraction and purification, cDNA synthesis, *in vitro* transcription reaction for production of biotin-labeled cRNA, hybridization of cRNA with Mouse 430 2.0 Affymetrix gene chips, and scanning of image output files (40). All of the experiments were performed in duplicate. After arrays quality analysis, all high-quality arrays were analyzed by using the Probe Logarithmic Intensity Error (PLIER) algorithm. Before the comparison, the data were preprocessed to reduce the false positive results (*SI Text*). When comparing normal vs. diabetic mice, we used the LCB of the fold change to identify differently expressed genes (41). To identify the transcripts that were counter-regulated out of differentially expressed transcripts we performed the K-means clustering using the Pearson correlation coefficient-based distance metric across normal, diabetic, and AAT-treated mice. Counter-regulation means that AAT treatment down-regulates the genes that are up-regulated in diabetic vs. normal mice and vice versa. Furthermore, to understand the biological mechanisms affected by the transcripts that are counter-regulated by drug treatment, we performed interactive networks, pathways, and functions analysis with the commercial System Biology oriented package Ingenuity Pathways Analysis (IPA 4.0) (www.ingenuity.com). The MIAME Compliant microarray data are available in the Gene Expression Omnibus repository at the National Center for Biotechnology Information (ID code GSE10478).

***In Vivo* Insulin Signaling Studies.** After a 16-h fast, mice were injected i.p. with 20 units/kg of human insulin (Eli Lilly) or saline and killed 10 min later. Skeletal muscle (gastrocnemius) were dissected and frozen in liquid nitrogen for immunoblotting analysis of insulin signaling proteins.

Immunoblotting. Whole-cell lysates from fat and skeletal muscle (gastrocnemius) from the *in vivo* insulin signaling studies were separated by SDS/PAGE as described in *SI Text*. Proteins were transferred to Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech), and Western blot analysis with rabbit polyclonal anti-IR (pY1162/1163) and anti-IRS-1 (pY612) antibodies were performed as described in *SI Text*.

Statistical Analyses. Statistical significance was calculated by using Prism software (GraphPad), with two-tailed Mann–Whitney tests when two groups were compared and Kruskal-Wallis tests when more than two groups were compared. The Wilcoxon signed-rank test was used to compare the insulin levels of AAT-treated animals before and after treatment.

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