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Spontaneous Development of a Pancreatic Exocrine Disease in CD28-Deficient NOD Mice

Craig Meagher,* Qizhi Tang,† Brian T. Fife,* Helene Bour-Jordan,* Jenny Wu,* Cecile Pardoux,* Mingying Bi,* Kristin Melli,† and Jeffrey A. Bluestone2*

Autoimmune pancreatitis (AIP) is a heterogeneous autoimmune disease in humans characterized by a progressive lymphocytic and plasmacytic infiltrate in the exocrine pancreas. In this study, we report that regulatory T cell-deficient NOD.CD28KO mice spontaneously develop AIP that closely resembles the human disease. NOD mouse AIP was associated with severe periductal and parenchymal inflammation of the exocrine pancreas by CD4+ T cells, CD8+ T cells, and B cells. Spleen CD4+ T cells were found to be both necessary and sufficient for the development of AIP. Autoantibodies and autoreactive T cells from affected mice recognized a ~50-kDa protein identified as pancreatic amylase. Importantly, administration of tolerogenic amylase-coupled fixed spleen cells significantly ameliorated disease severity, suggesting that this protein functions as a key autoantigen. The establishment and characterization of this spontaneous pancreatic amylase-specific AIP in regulatory T cell-deficient NOD.CD28KO mice provides an excellent model for the study of disease pathogenesis and development of new therapies for human autoimmune pancreatitis. The Journal of Immunology, 2008, 180: 7793–7803.

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1 Abbreviations used in this paper: AIP, autoimmune pancreatitis; Treg, regulatory cell; T1D, type 1 diabetes; BGL, blood glucose level; PDC, primary pancreatic ductal cell; ZG, zymogen granule; SI, stimulation index; PVDF, polyvinylidene difluoride; ECDI, ethylenecarbodiimide; IB, immunoblot; SI, stimulation index; HEL, hen egg lysozyme; SHAM, BSA-coupled ECDI-fixed splenocytes.

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several mouse models of autoimmune diseases (29). Importantly, mutation of the Treg-lineage specific transcription factor Foxp3 leads to a Treg deficiency and autoimmunity similar to that observed by transferring Treg-depleted CD4+ T cells to lymphopenic hosts (30, 31). Thus, Tregs are essential in maintaining immune homeostasis. We have previously investigated the role of costimulatory molecules in the development of autoimmunity and described that the costimulatory receptor CD28 appears to be more important for tolerance induction than immunogenenicity even though CD28 deficiency inhibits T cell proliferation, Ig class switching, germinal center formation, and Th2 responses (32–34). This is due to the critical role of CD28 in thymic development and peripheral maintenance of Tregs (35). Consequently, NOD mice deficient in CD28 have limited numbers of Tregs, resulting in the exacerbation of autoimmune type 1 diabetes (T1D) and other NOD-prone autoimmune syndromes.

In this study, we establish that a reduction of Tregs in NOD.CD28KO mice leads to the development of spontaneous AIP. Development of AIP was associated with atrophied acinar cells and lymphocytic infiltration of parenchymal tissue. To further define the role of the immune system in this disease, we determined that the exocrine infiltrate was composed predominantly of CD4+ T cells and B cells, with CD4+ T cells being necessary and sufficient for development of AIP. Indirect immunofluorescence, immunoblotting, and cell-based studies using sera and spleen cells from affected animals led to the identification of a ~50-kDa autoantigen, pancreatic amylase, which localized to granular structures in the exocrine pancreas. Finally, we show that tolerance to amylase using an Ag-coupled fixed APC protocol is a promising approach for ameliorating the development of AIP.

Materials and Methods

Mice and insulin therapy

NOD, C57BL/6, and BALB/c mice were purchased from Taconic Farms. BDC2.5 TCR-transgenic, NOD.RAGKO, and NOD.CD28KO mice were bred and housed in a pathogen-free barrier facility at the University of California, San Francisco. All animal experiments were approved by the University of California, San Francisco, Animal Care and Use Committee. Diabetic NOD.CD28KO mice were implanted s.c. with a slow-release insulin pellet (LinBit for mice; LinShin Canada) at the time of diabetes onset as determined by a blood glucose level (BGL) of ≥250 mg/dl for two consecutive readouts. On average, mice were implanted every 10–14 days. Following implantation, BGLs were monitored daily and mice having a BGL >350 mg/dl were administered 1.5 U of human NPH insulin (Eli Lilly).

In vitro expansion of BDC2.5 Tregs

Isolation and expansion of BDC2.5 Tregs was performed using the same conditions as described previously by our laboratory (36).

Isolation of pancreatic zymogen granules (ZG) and culture of pancreatic duct cells (PDC)

ZG were isolated from the pancreas of NOD.RAGKO mice as described previously (37). PDC were prepared using the same conditions as described previously (38). Protein concentration was determined using the bicinchoninic acid protein assay kit (Pierce).

Autoantibodies and immunoblotting

Pancreas, adipose, heart, salivary gland tissue, pancreatic ZG, and PDC were prepared from NOD.RAGKO mice and homogenized in ice-cold gland lysis buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 1% CHAPS, and complete protease inhibitors (Roche). Protein concentration was determined using the bicinchoninic acid protein assay kit (Pierce). Aliquots of pancreas lysate (100 µg of total protein) were added to SDS sample buffer, loaded onto a single-well 10% polyacrylamide minigel, electrophoresed, and transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore). Membranes were blocked in TBST (25 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 0.1% Tween 20) supplemented with 4.0% BSA (Sigma-Aldrich), then fitted into a MiniPROTEAN II Multiscreen apparatus (Bio-Rad). Ags were detected by incubating membrane with serum samples (1/600 dilution) or rabbit anti-human amylase (1:2,500; Sigma-Aldrich) followed by HRP-conjugated goat anti-mouse IgG (1:2,000; Jackson ImmunoResearch Laboratories) or donkey anti-rabbit IgG (1:2800; Amersham Biosciences), respectively. For comparing autoantibody reactivity between separate tissues, purified porcine pancreatic amylase (1 µg; USB Chemicals) and aliquots (20 µg) of each tissue extract were added to SDS–sample buffer and fractionated on a 4–20% gradient Criterion acrylamide gel (Bio-Rad), transferred to a PVDF membrane, blocked, and immunoblotted using the conditions described above. Loading of an equivalent amount of protein and adequate membrane transfer was confirmed by staining the PVDF membrane with PROACT Membrane stain (Amresco). In all experiments, signal was detected using the SuperSignal West Femto electrochemiluminescence reagent (Pierce) and autoradiography.

Indirect immunofluorescence

Autoantibodies reacting with pancreas were detected by indirect immunofluorescence as described previously (39). All slides were examined with an Axioskop microscope (Zeiss) using the ×40 and ×100 objectives and were imaged with a Hamamatsu ORCA 100 digital camera and Openlab software.

Immunostaining

Pancreata were embedded in OCT freezing medium and 8-µm sections were prepared. Immune cell infiltrates were visualized in the pancreas by immunohistochemistry using anti-CD4 (RM4-5), anti-CD8 (53-6.7), and anti-IgG (11-26c.2a) Abs from BD Biosciences and a 3,3′-diaminobenzidine staining kit (Vector Laboratories).

Adoptive transfer

Spleen cells depleted of CD4+ T cells were prepared using rabbit complement as described elsewhere (33). Purified spleen CD4+ T cells were isolated by staining splenocytes with anti-CD4-FITC (GK1.5) conjugated in our laboratory and sorting on a Mo-Flo Cytometer (DakoCytomation). For each cell population, 3 × 10^6 cells were transferred i.v. into each NOD.RAGKO recipient that was euthanized at 16 wk posttransfer for tissue histopathology. Beginning on days 0 and 4 posttransfer, thence weekly afterward, recipients of spleen cells were treated i.p. with 0.4 mg of rat IgG (Jackson ImmunoResearch Laboratories) and recipients of spleen CD4+ T cells and spleen cells depleted of CD4+ T cells were treated with 0.4 mg of anti-CD8 (YTS169.4) and anti-CD4 (GK1.5) to prevent expansion of residual CD8+ and CD4+ T cells, respectively.

Histopathology

Tissues were fixed in buffered 10% formalin, embedded in paraffin, and 8-µm sections were stained with H&E and scored for immune infiltrates. For autoimmune pancreatitis, 0–75% of pancreatic lobes were damaged and moderate acinar infiltrates were evident, no acinar cell destruction; 1 = periductal infiltrates and focal acinar infiltrates with limited tissue destruction; 2 = periductal infiltrates and moderate acinar infiltrates (<50% of acinar tissue destroyed in one or more pancreatic lobes), fat necrosis observed; and 4 = the majority of exocrine tissue destroyed in most pancreatic lobes, extensive fat necrosis. For autoimmune thyroiditis, 0 = no infiltration; 1 = <25% of thyroid lobe is infiltrated; 2 = 25–50% of thyroid lobe is infiltrated; 3 = 50–75% of thyroid lobe is infiltrated; and 4 >75% of thyroid lobe is infiltrated and little follicular architecture is observed.

Proliferation assays

Cells were cultured in 96-well microtiter plates (Corning) at 5 × 10^3 cells/well in complete HL-1 medium supplemented with Ag. Cells were pulsed with 1 µCi of [3H]TdR (MP Radiochemicals) during the last 18 h of a 96-h culture, and [3H]TdR uptake was detected using a Packard TopCount microplate scintillation counter (Packard Instrument). Anti-CD3 (145-2C11) Ab (0.5 µg/ml) was the positive control. Data are expressed either as mean proliferation for six wells ± SD or as a stimulation index (SI) defined as mean cpm (stimulated response)/mean cpm (unstimulated response).

Preparation of α-amylase-coupled splenocytes

Spleen cells were isolated from naive female NOD mice and coupled to porcine α-amylase (USB Chemicals) or BSA (Sigma-Aldrich; SHAM control) with the chemical cross-linker ethylene carbodiimide monobutyl ester (ECI) as previously described (40). Induction of peripheral tolerance was induced by i.v. injection of 5 × 10^7 coupled splenocytes into NOD.CD28KO mice ±1 wk of age.
Statistical analysis

All statistical analyses were performed using GraphPad Prism version 5.00. Statistical comparison of stimulation indexes and disease severity was performed using an unpaired two-tailed Mann-Whitney U test (p values ≤0.05 were considered significant).

Results

NOD.CD28KO mice treated with islet Ag-specific BDC2.5 Tregs or aged on insulin therapy develop AIP

NOD.CD28KO mice develop exacerbated diabetes due to a dramatic reduction in Tregs and compromised Th2 responses (33, 34). We have shown previously that ex vivo-expanded BDC2.5 Tregs transferred into NOD.CD28KO mice could prevent and even reverse diabetes (36). In Fig. 1A, when 4–5 × 10⁵ BDC2.5 Tregs were transferred into NOD.CD28KO mice before 7 wk of age, 100% of the recipients are protected from T1D. Histological analyses showed that insulitis was attenuated in BDC2.5 Treg-treated NOD.CD28KO mice (data not shown), leaving sufficient β cell mass to maintain normal glycemia. Strikingly, exocrine pancreatitis (AIP) was observed to develop in 100% of these islet-protected NOD.CD28KO mice (Fig. 1B). AIP was first observed in mice as young as 8 wk, progressing to 100% incidence in older animals, and was associated with atrophied acinar cells and focal or widespread parenchymal destruction with complete destruction of the exocrine pancreas in the most severe cases. At 8 wk of age, a mild infiltrate was observed in 50% of both male and female mice and was predominantly localized to periductal structures but not within acinar tissue. By 12 wk of age, mononuclear cell infiltrates generally surrounding perivascular ductal structures were observed in 100% of islet-protected NOD.CD28KO mice and disease severity had significantly increased (p = 0.03). The severity of tissue infiltration continued to increase significantly at 16 wk of age (p = 0.009) where the immune infiltrates were more diffuse with widespread destruction of the parenchyma, small regions of adipose tissue replacing degenerated regions of acinar tissue, and acinar cells appeared shrunken or atrophied (Fig. 1B, white arrow). Finally, by ≥24 wk of age, the majority of exocrine tissue was destroyed in the most severe cases. Fat necrosis/degredation (Fig. 1B, black arrow) and thickening of blood vessels were evident in most mice as was a limited amount of periductal fibrosis. Remarkably, despite the severe inflammatory immune response directed at the exocrine pancreas, islets directly adjacent to the mononuclear cell infiltrate remained sufficiently intact to maintain normal glycemia as a consequence of the immune regulation mediated by the adoptively transferred islet Ag-specific Tregs. To rule out the possibility that BDC2.5 Tregs mediate development of pancreatitis after transfer into NOD.CD28KO mice, diabetic NOD.CD28KO mice were treated with insulin until at least 20 wk of age to prevent mortality and were histologically evaluated for AIP. Similar to BDC2.5 Treg-treated NOD.CD28KO mice, 100% of NOD.CD28KO mice (male and female) maintained on insulin therapy spontaneously developed AIP (data not shown), thus indicating that BDC2.5 Tregs were not a prerequisite for development of AIP. Together, these results suggest that elimination of a majority of Tregs in the NOD background led to an additional autoimmune syndrome, AIP.

The exocrine pancreas of NOD.CD28KO mice is infiltrated with CD4⁺ lymphocytes that can transfer disease

Initial flow cytometric analyses showed that B and T cells are the predominant infiltrating cell populations into the exocrine pancreas in affected animals. In addition, a significant number of macrophages and myeloid dendritic cells were noted. There were minimal numbers of additional innate system cells including neutrophils and NK cells (data not shown). These observations were confirmed and cells localized using immunohistochemistry (Fig. 2). Significant numbers of CD4⁺ T cells (Fig. 2A), B cells
(Fig. 2B), and CD8<sup>+</sup> T cells (Fig. 2C) were observed infiltrating the exocrine pancreas and localized to interacinar spaces and around islets. A rat IgG control Ab was used to demonstrate staining specificity (Fig. 2D). These results are similar to those reported for human AIP where CD4<sup>+</sup> T cells are the major lymphocyte subset found in the pancreas (17). To determine which cellular subset was capable of transferring disease, individual spleen cell subsets were harvested from severely diseased 24-wk-old islet-protected NOD.CD28KO mice and transferred into NOD.RAGKO recipients. As shown in Fig. 3, 100% of NOD.RAGKO mice receiving either 3 × 10<sup>6</sup> spleen cells (group A; A) or sorted CD4<sup>+</sup> T cells (group B; B) developed a similar diffuse pancreatitis at 16 wk posttransfer with the majority of acinar cells being replaced by adipose tissue (black arrow). In contrast, spleen cells depleted of CD4<sup>+</sup> T cells (group C; C) did not transfer severe AIP in comparison to nondepleted spleen cells (p = 0.0043) and CD4<sup>+</sup> T cells (p = 0.0048). Importantly, to ensure removal of residual CD4<sup>+</sup> or CD8<sup>+</sup> T cells following transfer, recipient mice transferred with spleen cells depleted of CD4<sup>+</sup> T cells or enriched CD4<sup>+</sup> T cells were treated biweekly with anti-CD4 and anti-CD8 mAb, respectively. Rat IgG control Ab administered to recipients transferred with spleen cells did not alter disease course. Overall, CD4<sup>+</sup> T cells were necessary and sufficient for the transfer of AIP.

**FIGURE 2.** Invasion of the exocrine pancreas by lymphocytes in NOD.CD28KO mice. Immunohistochemistry of frozen pancreas serial sections using anti-CD4 (A), anti-IgD (B), anti-CD8 (C), or IgG control (D) identifies CD4<sup>+</sup> T cells and IgD<sup>+</sup> B cells as the predominant mononuclear cells invading the exocrine pancreas. Significant numbers of CD8<sup>+</sup> T cells are also observed.

**FIGURE 3.** CD4<sup>+</sup> T cells are necessary and sufficient for development of autoimmune pancreatitis in NOD.CD28KO mice. Autoimmune pancreatitis can be transferred with spleen cells (group A, ■) and spleen CD4<sup>+</sup> cells (group B, ▲), but not effectively with spleen cells depleted of CD4<sup>+</sup> cells (group C, ●) into NOD.RAGKO recipient mice. Cell populations were isolated from pooled islet-protected NOD.CD28KO mice at 24 wk of age and 3 × 10<sup>6</sup> cells were transferred i.v. into each recipient mouse. All donor mice were protected against T1D, pancreata were harvested 16 wk posttransfer, and results from one of two independent experiments are presented. Representative histological pancreas sections for immunodeficient recipient mice transferred with spleen cells (A), spleen CD4<sup>+</sup> T cells (B), and spleen cells devoid of CD4<sup>+</sup> cells (C) are presented. Statistical comparison of disease severity was performed using the Mann-Whitney U test (*, p = 0.0043; **, p = 0.0048).
in Fig. 4, A and B, a limited number of antigenic specificities were detected using serum isolated from mice at 16 and 20 wk of age, respectively. The majority of serum samples detected an Ag migrating at ~50 kDa. To determine whether the ~50-kDa Ag was specifically targeted during the autoimmune response against the exocrine pancreas, control sera from C57BL/6 mice, which were disease...
free, were used in immunoblotting and failed to react with pancreatic lysate. As an additional negative control, serum from NOD.RAGKO in combination with secondary Ab (denoted RAGKO) did not detect any proteins in the pancreas. These results indicated that autoimmune responses against the exocrine pancreas in NOD.CD28KO mice primarily target an autoantigen of \(~50\) kDa.

**Antigenic targets in the pancreas predominantly localize to granular structures in the exocrine pancreas**

Indirect immunofluorescence was used to localize Ags recognized by autoantibodies within the exocrine pancreas. Initially, a panel of serum samples collected from islet-protected NOD.CD28KO mice at ages \(\geq 24\) wk of age and with a disease score of \(>2\) were tested. Most sera showed a punctate staining pattern in the exocrine pancreas with only one exception (mouse 1953) displaying reactivity to ductal-like structures (Fig. 5A). A combination of high and low magnifications is shown to appreciate both the granular and ductal patterns as well as the widespread nature of the staining pattern in specific Ab but not control Ab (BALB/c)-stained tissues. Importantly, further analysis of sera specificity showed a correlation between the staining pattern and the immunoblot analysis (Fig. 5, A and B). Those sera showing a punctate pattern of immunofluorescence (e.g., m0436) recognized an \(~50\)-kDa protein by IB, whereas a minority of sera (m1953) that lacked this staining pattern, or demonstrated a ductal staining pattern, did not. Subsequent analysis of a larger panel of serum samples from NOD.CD28KO mice \(\geq 24\) wk of age showed that a majority (69.2%; \(n = 9\) of 13) of serum samples demonstrated a punctate staining pattern, whereas a small percentage of sera (15.3%; \(n = 2\) of 13) reacted against ductal-like structures (Fig. 5C). Some serum samples (15.3%; \(n = 2\) of 13) failed to detect a pancreatic Ag by indirect immunofluorescence similar to BALB/c control sera (\(n = 5\) of 5).

**FIGURE 6.** Identification of pancreatic amylase as an autoantigen recognized by NOD.CD28KO mouse serum. A, Using serum from individual NOD.CD28KO mice (left panel), the \(~50\)-kDa autoantigen is only detected by immunoblotting lysates prepared from tissues or tissue compartments that contain amylase (pancreas, purified ZG, salivary gland (SG)) and not in amylase-deficient lysates (heart, adipose, primary PDC). Purified porcine pancreatic amylase was used as a positive control. Results are representative of four independent experiments. B, The same blot was stripped and probed with an anti-human/mouse pancreatic amylase Ab (right panel). C, Autoantibodies from individual NOD.CD28KO mice recognize pancreatic amylase. Serum samples were preincubated with increasing concentrations of porcine pancreatic amylase or lactoferrin before use as primary Abs in a multiscreen immunoblot against pancreas extract. Lactoferrin, a potential autoantigen in the pancreas of patients afflicted with pancreatitis, was included as a control. Results are representative of three independent experiments.

**FIGURE 7.** Spleen cells from NOD.CD28KO mice spontaneously proliferate in response to purified porcine pancreatic amylase. A, Spleen cells from NOD.CD28KO of at least 16 wk of age and C57BL/6 mice (control strain) were tested for their ability to respond to porcine pancreatic amylase or HEL control protein. A representative proliferative response as assessed by \([3H]\)thymidine incorporation is presented. Results are expressed as the mean cpm from six wells \(\pm\) SD. B, Summary of spleen cell proliferative responses in the presence of 200 \(\mu\)g/ml Ag. Results are expressed as the calculated SI for each mouse analyzed. The following range of background proliferation for medium controls for mice tested in each group were: 492–3330 cpm for NOD.CD28KO, 677–2862 cpm for C57BL/6 mice, and 2047–4331 cpm for heterozygous NOD.CD28\(^{+/−}\) mice. A SI \(\geq 3\) was considered the threshold of significance (black line). Significant differences were determined in the proliferative response to porcine pancreatic amylase and HEL for NOD.CD28KO mice (*, \(p < 0.0001\)) and between NOD.CD28KO and C57BL/6 (**, \(p = 0.010\)) and NOD.CD28\(^{+/−}\) (***, \(p = 0.008\)) spleen cells in response to porcine pancreatic amylase.
Of note, sera from 80% of age-matched female NOD mice produced a punctate staining pattern and reacted against the ~50-kDa Ag by IB (Fig. 5), but in comparison to NOD.CD28KO mice, sera reactivity was weaker. Additionally, 40% of NOD sera produced a nuclear staining pattern, whereas sera from NOD.CD28KO mice did not, suggesting that CD28 may be necessary for the generation of antinuclear Ab. Overall, our results suggest that NOD mice recognize autoantigens in the exocrine tissue but have self-limited disease possibly until long after the development of diabetes.

Direct demonstration that amylase is a specific autoantigen involved in exocrine pancreatitis

Potential autoantigens reported by serological analyses to be linked to the development of AIP include carbonic anhydrase II, lactoferrin, pancreatic secretory trypsin inhibitor, pancreas-specific protein disulﬁde isomerase, antinuclear Abs, and rheumatoid factor (15, 18–20). In addition, another study showed that amylase—but not carbonic anhydrase II—nor lactoferrin-sensitized T cell lines were sufﬁcient to transfer AIP into immunocompetent rat strains (41). Together with our immunofluorescent and IB results (Figs. 4 and 5), amylase was likely to be an autoantigen in the NOD model and, therefore, we next tested reactivity to amylase.

Protein lysates from whole tissues or tissue compartments of NOD.RAGKO mice that express amylase, including the pancreas, salivary gland, and purified pancreatic ZG were prepared. Lysates were also prepared from primary PDC in addition to adipose and heart tissue that do not express amylase as a negative control (www.ncbi.nlm.nih.gov/UniGene/ESTProfileViewer.cgi?uglist=Mm.439729). As shown in Fig. 6A, sera from islet-protected NOD.CD28KO mice reacted with an ~50-kDa Ag only within tissue lysate containing amylase (pancreas, salivary gland, and ZG), as well as purified porcine amylase used as a positive control. The same protein band was detected when the blots were stripped and reprobed with a rabbit anti-human pancreas amylase Ab (Fig. 6B). Of note, since the isoform of amylase expressed in the salivary gland is structurally distinct and less immunogenic than pancreatic amylase (42), it was not surprising that sera reactivity to amylase in the salivary gland was generally weak and only detected following extended exposure (data not shown). Secondary anti-mouse and anti-rabbit Ab alone did not detect any pancreatic protein (data not shown).

To confirm the identity of the ~50-kDa autoantigen as amylase, we performed a competition immunoblot assay (Fig. 6C). Preincubation of serum from NOD.CD28KO mice with purified porcine pancreatic

FIGURE 8. Induction of tolerance to porcine pancreatic amylase ameliorates the development of autoimmune pancreatitis in NOD.CD28KO mice. A, NOD.CD28KO mice were administered 50 × 10⁶ porcine pancreatic amylase (●, n = 13) or SHAM control (○, n = 11)-coupled splenocytes i.v. before 11 wk of age. All recipient mice were diabetic at the time of treatment or developed diabetes following treatment and were maintained on insulin therapy until 26 wk of age when mice were euthanized. Pancreata of amylase-tolerized mice displayed significantly less infiltration and damage to the exocrine pancreas in comparison to SHAM-tolerized control mice (⁎, p = 0.038). All tissues were scored in a blinded fashion by three independent observers. B, No difference in the development of autoimmune thyroiditis (⁎, p = 0.16) was observed between treatment groups, demonstrating that the protective effect of inducing tolerance to pancreatic amylase is specific for the pancreas. C, Representative histological images of pancreas and thyroid from porcine pancreatic amylase and SHAM-tolerized NOD.CD28KO mice. Original magnification was ×200 except where indicated.
Amylase resulted in a dose-dependent reduction in the intensity of the ~50-kDa band as compared with serum preincubated with nothing or purified lactoferrin (another potential pancreas autoantigen in patients having AIP) (43). These results strongly indicate that amylase is the ~50-kDa autoantigen recognized in the pancreas by autoantibodies from NOD.CD28KO mice.

Spleen cells from NOD.CD28KO mice proliferate in response to pancreatic amylase

We performed in vitro T cell proliferation assays to analyze spleen cell responses to pancreatic amylase vs hen egg lysozyme (HEL) as a control Ag. The assays were performed in the presence of exogenous rIL-2 (20 IU/ml) to compensate for the CD28 deficiency. As shown in Fig. 7 spleen cells from NOD.CD28KO mice suffering from AIP proliferated specifically to amylase in a concentration-dependent manner with maximal amylase-specific proliferation observed at an Ag concentration of 200 μg/ml (Fig. 7B; *, p < 0.0001). In comparison, spleen cells from C57BL/6 mice (n = 5) did not respond to either amylase (Fig. 7B; **, p = 0.01) or HEL (Fig. 7B, n = 17). Additionally, heterozygous littermate NOD.CD28−/− mice, which exhibit equivalent proliferative responses and develop TID with similar kinetics to wild-type NOD mice (34), also failed to respond to amylase (n = 3) in comparison to NOD.CD28KO mice (***, p = 0.008). The lack of a proliferative response to amylase by NOD.CD28−/− mice is consistent with our serological results showing that although a majority of NOD mice exhibit reactivity to the 50-kDa Ag (Figs. 4 and 5), reactivity is weaker than in NOD.CD28KO mice. These results indicate that pancreatic amylase is an ~50-kDa autoantigen recognized by T cells in NOD.CD28KO mice.

Induction of amylase-specific tolerance prevents development of AIP

Given that amylase appeared to be a predominant Ag recognized by T cells from NOD.CD28KO with AIP, we next examined whether tolerizing mice to this autoantigen might ameliorate disease. We, and others, have previously shown that the i.v. administration of Ag-coupled ECDI-fixed spleen cells could be used to prevent and reverse autoimmune animal models of TID, experimental autoimmune encephalomyelitis, myocarditis, neuritis, uveitis, and thyroiditis (40, 44–48). In the present study, 5 × 10⁷ α amylase-coupled vs BSA-coupled (SHAM control) ECDI-fixed NOD splenocytes were administered to NOD.CD28KO recipient mice before (<11 wk of age) the development of extensive AIP. All recipient mice developed autoimmune TID with normal kinetics and were maintained on exogenous insulin until 26 wk, an age when AIP develops in all NOD.CD28KO mice, for histological examination of the pancreas. Importantly, the amylase-coupled fixed splenocyte-treated mice had reduced disease severity compared with SHAM-treated mice (p = 0.038; Fig. 8, A and C). In addition, the exocrine pancreas from the majority of amylase-tolerized mice (8 of 13) displayed limited mononuclear cell infiltration (scores ≤1), whereas SHAM-treated mice (8 of 11) exhibited more extensive mononuclear cell infiltration (scores ≥1). This protective effect in response to amylase tolerization was exocrine pancreas specific since there was no statistical difference in the severity of autoimmune thyroiditis (p = 0.16) when comparing treatment groups (Fig. 8, B and C). Importantly, the protective effect of amylase-coupled cell therapy was long-lasting as reduced disease severity was observed several months posttherapy. It should be pointed out that protection was not complete, most likely reflecting the difficulty in reversing disease once the pathology has begun.

Discussion

Over the past decade, we have used the NOD mouse strain as a mouse model of multiorgan autoimmunity. Strikingly, we have observed that multiple autoimmune manifestations are most prevalent when Tregs are eliminated and costimulatory pathways altered (32, 33, 49). In this report, we observed that Treg-deficient NOD.CD28KO mice develop spontaneous AIP. The disease occurs as the endocrine tissue is protected from destruction by islet Ag-specific Tregs and suggests a “compartmentalization” of autoimmune syndromes even in adjacent pancreatic tissue. Importantly, the data suggest that AIP is an independent disease and not a consequence of preexisting β cell autoimmunity. The disease has many similarities to human AIP and is similar to the pancreatitis observed in conjunction with “fulminant type 1 diabetes” described in some Japanese and Australian patients (50–52).

Although we observed an extensive multilocular infiltrate in affected animals, the CD4⁺ T cell subset was the essential pathogenic population based on adoptive transfer experiments. This was supported by in vitro proliferation assays against the target autoantigen amylase. Even though CD4⁺ T cells are sufficient to transfer disease, the autoantibodies may also be pathogenic and synergize with T cells, especially early in the disease process. In our study, autoantibodies in the sera of affected animals provided a useful tool to identify target Ags. Distinctly, the diversity of proteins recognized by the antisera, based on staining patterns and immunoblot analyses, was restricted. In fact, the majority of sera recognized pancreatic amylase, an ~50-kDa protein produced by acinar cells in both the pancreas and salivary glands. These results led to the finding that this protein was indeed an important T cell autoantigen in the disease setting, a link previously observed for other autoantigens such as insulin in diabetes, acetylcholine receptor in myasthenia gravis and thyroglobulin in Graves’ disease, to name a few. Finally, we demonstrate that administration of amylase-coupled fixed splenocytes, an Ag-specific tolerogenic therapy in multiple animal models of autoimmunity, significantly inhibits the development of AIP in NOD.CD28KO mice. Together, these observations suggest that a relative Treg deficiency combined with genetic susceptibility leads to the manifestation of autoimmune diseases. These results have important implications in considering immunotherapies in general and specifically for the treatment of AIP in humans.

It is now clear that defects in critical pathways of peripheral or central processes of immune regulation lead to autoimmunity. Interestingly, the AIP observed in our studies is not unlike that observed in NOD.AIREKO mice (25). In the AIRE-deficient NOD mouse, the NOD.H-2^k haplotype is necessary but not sufficient for AIP to occur, synergy between the Idd3 and Idd5 diabetes susceptibility loci influences disease onset, and the NOD genetic background enhances disease penetrance (25). However, based upon published data (20, 25) and our unpublished data, the autoantibody repertoire specific to the pancreas is different among NOD.CD28KO and NOD.AIREKO mice and likely reflects different target Ags. In contrast to NOD.CD28KO mice that exhibit aberrant peripheral tolerance (33, 35), NOD.AIREKO mice exhibit defects in thymic negative selection (39, 53). Thus, it is intriguing to speculate that the differences in antigenic specificities reflect distinct T cell repertoires in the two settings. We hypothesize that AIRE-deficient mice develop a high-affinity autoreactive repertoire that seeds the periphery and, under the right conditions, promotes autoimmune pancreatitis that cannot be controlled by the resident Tregs. In contrast, the T cells that mediate disease in the NOD.CD28KO mice may be lower affinity and present normally in the NOD background, but escape regulation in the Treg-deficient

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setting. Although NOD mice do not spontaneously develop AIP, our results show that immune responses directed against the exocrine pancreas exist and are aggravatred by further skewing peripheral tolerance defects by removing CD28 and Tregs. However, this hypothesis does not fully explain why NOD.AIREKO mice develop AIP and not insulinis (20), while NOD.CD28KO mice develop both pathologies. Discovering how and why these divergent repertoires develop will be critical for unraveling the mechanisms shaping the autoimmune repertoire. Notably, our results highlight the extraordinary tissue specificity of Ag-specific Tregs, capable of discriminating neighboring tissue compartments within a single organ, and suggest that proposed bystander activity of Tregs must either be limited to individual tissues themselves or that lymph nodes responsible for initiating AIP are distinct from those involved in anti-islet cell autoimmunity.

Antigenic specificity of B and T cells against tissue Ags is often conserved during autoimmune responses (19, 39, 54). As such, we used autoantibodies from diseased mice to identify novel Ags within the exocrine pancreas with the goal of developing Ag-specific therapies aimed at preventing AIP. Strikingly, autoantibodies from a panel of diseased NOD.CD28KO mice exhibited oligoclonal reactivity against pancreatic extracts and a small number of specificities were comparable between different animals. A limited scope of tissue Ags is not unusual for organ-specific autoimmune diseases in NOD mice (25). The oligoclonal reactivity may be due to limitations in biochemical technology because certain Ags may not be recognized following denaturation during SDS-PAGE. Alternatively, mice deficient in CD28 produce significantly reduced autoantibody levels in comparison to wild-type mice which could limit the sensitivity of our approach to detect Ags (55, 56). That said, roughly 70% of the sera from affected mice that were tested produced a granular staining pattern by indirect immunofluorescence and a larger proportion of the sera reacted with an ~50-kDa protein, amylase, by immunoblotting. These results, combined with the T cell proliferation studies, strongly suggest that pancreatic amylase is a major autoantigen in this disease.

Amylase is constitutively expressed by acinar cells, stored within ZG, and then released into pancreatic ducts in response to starch intake. Within the pancreas, immunohistochemical studies have localized amylase expression to various ducts, periductal cells, and acinar cells (57, 58). Additionally, in NOD mice, both periductal and acinar cells have been demonstrated to express each MHC in response to inflammation (24), indicating that both cell types may represent a location where lymphocytes encounter amylase. Interestingly, in our model, mononuclear infiltrates are localized to these regions of amylase and MHC expression in the pancreas. Inflammation appears to originate near ducts and/or venules in the pancreas, then disseminating out into the parenchyma leading to localized and, in a few cases, widespread destruction of acinar tissues across several lobes. The course of AIP progression is consistent with the notion of amylase as a key autoantigen for disease initiation and dissemination. These results are consistent with those describing amylase as a potential autoantigen for AIP in rats (41). However, in our model, amylase is not the only autoantigen involved in the development of AIP since there are clearly other specificities observed both by immunofluorescence and IB analyses, consistent with AIP being a chronic disease characterized by changes in the autoimmune repertoire over time (59). Finally, with additional autoantigens linked to development of AIP (15, 18, 19, 20), it is possible that human AIP encompasses several syndromes and NOD.CD28KO mice represent an amylase-specific example of disease.

In fact, characteristic pathology of human AIP, the predominance of T and B cells infiltrating the ductal and acinar parenchyma, acinar regression as well as a variable degree of fat necrosis, and oligoclonal lymphocytic responses suggest our model and human AIP are comparable (5, 60, 61). However, a few dissimilarities exist. First, although an intense inflammatory cell infiltrate is primarily found in the main and larger interlobular ducts in human AIP (1, 4, 62), an unbiased infiltrate targeting ductal structures is found in our model. Second, granulocytic infiltrates at ductal structures, consisting of neutrophils and eosinophils, are common in patients suffering from AIP (10, 61). Although some polymorphonuclear cells are observed surrounding ducts in the pancreas of our model, granulocytic epithelial lesions as pronounced as those associated with AIP in humans are not observed. Some of these incongruities may in part result from immune infiltrates being “patchy” throughout the pancreas of patients (61) and, consequently, results prepared from a large number of pancreactectomy specimens would provide for a more detailed comparison. Overall, CD28-deficient NOD mice provide a unique model to study how peripheral tolerance defects mediate the development of AIP.

The CD28/B7 costimulatory pathway plays an essential role in T cell activation and the development of autoimmune diseases (63). However, unlike initial expectations, the CD28 and CD80/CD86 knockout mice develop more aggressive disease due to CD28 playing a dominant role in thymic development and peripheral survival of Tregs (63). Thus, interrupting the CD28 pathway altered the balance between effector T cells and Tregs, resulting in accelerated autoimmunity (28, 33, 35). Unlike acute immune responses to nominal Ags which are exquisitely CD28/B7 dependent, the spontaneous autoimmune strain NOD was able to mount pathogenic chronic autoimmunity in this CD28-deficient setting. The reason for this is likely the ongoing exposure of T cell precursors to autoantigens because it has been shown that the continued presence of signal 1 alone, either through prolonged viral replication or repeated injection of peptide, prevents the induction of anergy and generates a functional T cell response in vivo (64). However, note that not all of the Tregs are gone and there are additional defects in both the CD4 and CD8 T cell compartments in NOD.CD28KO mice, which explains why these mice do not develop lethal lymphoproliferative disease like the Foxp3-deficient Scurfy mice (31). Thus, an altered balance of pathogenic and Tregs in NOD.CD28KO mice allows the long-term study of AIP.

Finally, the ultimate goal of using animal models to study autoimmunity is to characterize disease pathology allowing for the development of immunotherapies that can alter the course of the disease. In this study, we targeted amylase in an immunotherapy protocol designed to ameliorate disease and re-establish autoantigen-specific peripheral tolerance. For this study, our approach was modeled after the insulin-coupled spleen cell tolerance method used to prevent TID (40). This therapy has direct clinical applications for the treatment of AIP since coupled-cell tolerance has already been used successfully to tolerate Ag-specific human T cell responses (65). The tolerization protocol was successful in limiting the degree of mononuclear cell infiltration into the exocrine pancreas in comparison to SHAM-treated NOD.CD28KO recipient mice. We have recently shown that tolerance in this protocol is Treg independent and a result of programmed death 1, programmed death ligand 1, and CTLA-4-dependent inactivation of effector T cells (40), supporting the direct role of amylase as one major autoantigen in this disease. However, because some NOD.CD28KO mice treated with amylase-coupled spleen cells did develop significant immune infiltrates, probably reflecting the timing of treatment as some mice likely had already developed a pancreatic infiltrate when treated; additional autoactive T cell specificities must be involved in disease progression. Thus, although...
amylose-coupled splen cells represents a promising approach to hinder development of AIP, it is likely that a combination of these T effector inactivating therapies combined with more generalized immunoregulatory drugs will be needed to treat patients with ongoing disease.

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Disclosures
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