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Increased Neutrophil Elastase and Proteinase 3 and Augmented NETosis Are Closely Associated with β-cell Autoimmunity in Patients with Type 1 Diabetes

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ABSTRACT

Type 1 diabetes (T1D) is an autoimmune disease resulting from self-destruction of insulin-producing β cells. Reduced neutrophil counts have been observed in patients with T1D. However, the pathological roles of neutrophils in the development of T1D remain unknown. Here we show that circulating protein levels and enzymatic activities of neutrophil elastase (NE) and proteinase 3 (PR3), both of which are neutrophil serine proteases (NSPs) stored in neutrophil primary granules, were markedly elevated in patients with T1D, especially those with disease duration of less than one year. Furthermore, circulating NE and PR3 levels increased progressively with the increase of the positive numbers and titers of the autoantibodies against β-cell antigens. An obvious elevation of NE and PR3 was detected even in those autoantibody-negative patients. Increased NE and PR3 in T1D patients are closely associated with elevated formation of neutrophil extracellular traps. By contrast, the circulating levels of α1-antitrypsin (A1AT), an endogenous inhibitor of NSPs, are decreased in T1D patients. These findings support an early role of neutrophil activation and augmented NSPs activities in the pathogenesis of β-cell autoimmunity, and also suggest that circulating NE and PR3 may serve as sensitive biomarkers for diagnosis of T1D.

(194 words)

Key words: Autoimmune diabetes, neutrophil serine proteases, neutrophil elastase, proteinase 3, α1-antitrypsin, neutrophil extracellular traps, NETosis, biomarker
INTRODUCTION

The global incidence of type 1 diabetes (T1D), an autoimmune disease caused by an interactive combination of genetic and environmental factors, has more than doubled in the past two decades (1; 2). Although the triggering factors that are involved in the initiation of T1D remain unclear, it is widely accepted that organ-specific autoimmune destruction of the insulin-producing β-cells in the pancreatic islets of Langerhans is mediated primarily by autoreactive T cells, which is accompanied by the production of different autoantibodies to β-cell antigens, including glutamic acid decarboxylase autoantibody (GADA), insulinoma-associated protein 2 autoantibody (IA2A) and zinc transporter-8 autoantibody (ZnT8A) (3-5). Although these autoantibodies have been proven to be instrumental for prediction and diagnosis of T1D, they are deemed not to be pathogenic (6; 7). A number of other immune cells, including dendritic cells (DCs), macrophages, B cells, neutrophils, are also implicated in the development of insulitis in T1D (8; 9).

Neutrophils, which are the most abundant (40-75%) type of white blood cells, have recently been implicated in both the onset and progression of T1D (10; 11). The primary functions of neutrophils are to eliminate extracellular pathogens by multiple strategies, including phagocytosis, degranulation to release lytic enzymes and neutrophil extracellular traps (NETs), which are formed through a unique cell death process clearly differentiated from both apoptosis and necrosis, termed “NETosis” (12-14). On the other hand, improper activation of neutrophils may lead to tissue damage during autoimmune or exaggerated inflammatory responses (15). Notably, circulating
neutrophil counts are reduced in patients with T1D as well as their non-diabetic first-degree relatives, but not in patients with type 2 diabetes (11). In non-obese diabetic (NOD) mice (a spontaneous model of T1D), neutrophil infiltration and NET formation in the islets were observed as early as two weeks after birth, and blockage of neutrophil activities with an anti-Ly6G antibody reduces the subsequent development of insulitis and diabetes (9).

Neutrophil serine proteases, including neutrophil elastase (NE), proteinase 3 (PR3) and cathepsin G (CG), are the major components of neutrophil azurophilic granules that participate in the elimination of engulfed microorganisms (16). Neutrophil activation and degranulation can result in the release of neutrophil serine proteases into the extracellular medium and circulation, where they not only help to eliminate the invaded pathogens but also serve as the humoral regulators of the immune responses during acute and chronic inflammation, modulating cellular signalling network by processing chemokines and activating specific cell surface receptors (17-19). Abnormal activities of neutrophil serine proteases have been implicated in the pathogenesis of several inflammatory and autoimmune diseases, including chronic obstructive pulmonary disease, cystic fibrosis, Wegener granulomatosis, Papillon-Lefèvre syndrome and small-vessel vasculitis (20). However, their association with T1D has not been explored so far.

In this study, we measured circulating levels of two main types of neutrophil serine proteases (NE and PR3) as well as their enzymatic activities in T1D patients with different disease duration,
together with age- and sex-matched healthy controls. Furthermore, we explored whether altered NET formation and α1-antitrypsin (A1AT, a major endogenous inhibitor of neutrophil serine proteases) are associated with reduced neutrophil counts and markedly increased activities of neutrophil serine proteases in patients with T1D. In addition, we measured the dynamic changes of circulating NE/PR3 activities during the development of autoimmune diabetes in T1D.

RESEARCH DESIGN AND METHODS

Study cohort.

One hundred and forty-nine patients with T1D were randomly selected from children diagnosed at the Diabetes Center, Second Xiangya Hospital of Central South University from October 2000 to October 2013. Patients with T1D were diagnosed according to the criteria of the American Diabetes Association (21). All patients were treated with insulin. The disease duration of T1D was 4.2 (1.7-7.1) years [median (interquartile range)].

A total of 77 age- and sex-matched healthy controls were recruited from children in the community participating in health screening at the Children Health Center of the Second Xiangya Hospital, Central South University, using the following inclusion criteria: (1) fasting plasma glucose less than 5.6 mmol/L and 2-h plasma glucose less than 7.8 mmol/L; (2) no family history of diabetes, and other autoimmune or chronic diseases.
A total of 25 adults with type 2 diabetes (T2D) diagnosed within one year and 25 age- and sex-matched healthy controls were recruited at the Diabetes Center, Second Xiangya Hospital of Central South University, and the inclusion criteria was described in our previous study (22).

The study was approved by the Institutional Review Board of Second Xiangya Hospital of Central South University, and written informed consent was obtained from the patients and healthy controls.

Clinical and biochemical assessments.

After overnight fasting, a venous blood specimen was collected in the morning (around 0800 am) for analysis of various biochemical parameters. Plasma glucose was measured enzymatically on a Hitachi 7170 analyzer (Boehringer Mannheim, Mannheim, Germany). HbA1c was measured by automated liquid chromatography (Bio-Rad VARIANT II Hemoglobin Testing System, Hercules, CA, USA). Serum levels of C-peptide and C-reactive protein were quantified using a chemiluminescence immunoassay on a Bayer 180SE Automated Chemiluminescence Systems (BayerAG Leverkusen, Germany), and an immunoturbidometric assay (Orion Diagnostica, Espoo, Finland), respectively. The titers of GADA, IA2A and ZnT8A were determined by in-house radioligand assays as previously described (22; 23).

Circulating protein levels of NE, PR3 and A1AT were measured using the enzyme-linked immunosorbent assay (ELISA) kits established in our laboratory (Antibody and Immunoassay
The limits of detection for NE, PR3 and A1AT ELISA kits were 0.156 ng/ml. No cross reactivity among these proteins or with other proteins were detected. The intra- and inter-assay variations were 4.5% and 5.1%, respectively for NE ELISA kit; 3.9% and 4.3%, respectively for PR3 ELISA kit; and 4.9% and 5.3%, respectively for A1AT ELISA kit.

The combined enzymatic activities of PR3 and NE in serum were determined with a chromogen-based assay using N-methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide (Sigma-Aldrich, St. Louis, MO, USA) as the substrate, which has a catalytic constant $K_{cat}/K_m$ of 33915 M$^{-1}$s$^{-1}$ for NE and 499 M$^{-1}$s$^{-1}$ for PR3 (24). Briefly, 20 µl of serum was incubated with 180 µl of 0.1 M Tris-HCl buffer (pH 8.0) containing 0.5M NaCl and 1 mM substrate at 37°C for 24 hours. The amount of p-nitroaniline released was measured spectrophotometrically at 405 nm. The enzymatic activities of PR3 and NE were calculated according to the delta OD values before and after 24-hour incubation with substrate and expressed as mU/ml serum, where one unit was defined as the amount of PR3 and NE that hydrolyze the substrate to yield 1 µmol of p-nitroaniline per minute at 37°C (25).

The levels of neutrophil NETosis were measured by quantifying the amount of circulating myeloperoxidase (MPO)-DNA complexes, a well-established marker of NET formation as previously described (26). Briefly, 5 µg/ml of mouse anti-MPO monoclonal antibody (ABD Serotec, Germany) was coated to 96-well microtiter plates overnight at 4°C. After blocking with 1% BSA, serum samples were added per well in combination with the peroxidase-labeled anti-DNA
monoclonal antibody (component No.2 of the Cell Death Detection ELISA PLUS kit, Roche Diagnostics, USA) according to the manufacturer’s instructions. After two hours of incubation at room temperature on a shaking device (300 rpm), the wells were washed three times and then incubated with the peroxidase substrate at 37°C for 60 minutes. The optical density (OD) at wavelength of 405 nm was measured using a µQuant microplate reader (Biotek Instruments, USA).

Animal studies.

NOD/ShiLtJ breeder mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA).

BALB/c and C57BL/6N mice were obtained from the Animal Unit of the University of Hong Kong.

All mice were housed in a room under specific pathogen–free conditions and 12-hour light-dark cycles at 22 to 24°C, with *ad libitum* access to water and standard chow (PicoLab Rodent Diet 20, LabDiet). Blood was collected weekly from female mice from 2 to 30 weeks of age. Blood glucose was monitored using an Accu-Chek Advantage glucose meter (Roche Diagnostics, USA) and diabetes was defined as two consecutive readings above 11.1 mmol/L (9). Circulating NE/PR3 enzymatic activities were measured as described above. All experimental procedures were approved by the Committee on the Use of Live Animals for Teaching and Research of the University of Hong Kong and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals.

Statistical analysis.
All analyses were performed with Statistical Package for Social Sciences version 16.0 (SPSS, Chicago, IL). Normality was tested using the Kolmogorov-Smirnov test. Data that were not normally distributed were logarithmically transformed before analysis. Differences between groups were assessed by $\chi^2$ or unpaired $t$ test. Comparisons among groups were performed using one-way ANOVA and independent $t$-tests. Correlations were analyzed using Pearson correlation or partial correlation as appropriate. Data were expressed as mean ± SD or median with interquartile range as appropriate. In all statistical comparisons, a $p$ value < 0.05 was used to indicate a statistically significant difference.

RESULTS

Subject characteristics.

The clinical characteristics of T1D patients and their healthy controls were described in Table 1. T1D patients were further divided into three groups based on their disease duration, including patients within 1 year from diagnosis (n=28), with a disease duration >1 and <5 years (n=59) and with duration >5 years (n=62). Compared with healthy subjects, T1D patients had higher fasting glucose and HbA1c, but lower fasting C-peptide levels. No significant differences in C-reactive protein were found among these groups (Table 1). Consistent with the previous reports (10; 11), the circulating neutrophils were moderately reduced in T1D patients diagnosed within 1 year compared with the healthy controls [median (interquartile range) (x 10$^6$/ml), 2.27 (1.80-3.52) vs 3.63 (3.02-4.15), $p$<0.05], but not in T1D patients with a disease duration >1 and <5 years, or with...
duration $>5$ years (Table 1).

Circulating protein levels and enzymatic activities of NE and PR3 are dramatically increased in T1D patients.

In contrast to the mild reduction of peripheral neutrophils, we found that the circulating protein levels of both NE and PR3 were dramatically increased in T1D patients compared to the healthy controls [NE: 1594.7 (988.4-2284.6) vs 397.0 (262.2-468.8) ng/ml, $p<0.001$; PR3: 295.3 (206.0-430.4) vs 107.4 (92.5-165.0) ng/ml, $p<0.001$]. Notably, the magnitude of increases in protein levels of NE and PR3 was significantly higher in T1D patients diagnosed within 1 year as compared to the other two groups of patients with longer disease duration (all $p<0.01$, Fig. 1A and 1B). There were no differences in circulating protein levels of NE and PR3 between men and women in patients or controls.

To further confirm the above findings, we measured the combined enzymatic activities of NE and PR3 using a common substrate, N-methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide (24). The results showed that circulating NE/PR3 enzymatic activities in T1D patients were also substantially higher than those in healthy individuals [0.69 (0.41-1.03) vs 0.14 (0.10-0.21) mU/ml, $p<0.001$]. Likewise, the most significant increase in NE/PR3 enzymatic activities was observed in T1D patients within 1 year from diagnosis (Fig. 1C). The correlation coefficient between NE/PR3 enzymatic activities and circulating protein levels was 0.915 ($p<0.001$) for NE and 0.874 ($p<0.001$)
Circulating A1AT levels are decreased in T1D patients.

The activities of plasma NE and PR3 are tightly controlled by their associated endogenous inhibitors, especially A1AT, an archetype member of the serine protease inhibitor (SERPIN) superfamily. Since our data showed that the amplitude of increases in NE/PR3 enzymatic activities was higher than that of the circulating protein levels, we next investigated whether dysregulated A1AT contributed to the increased enzymatic activities of NE and PR3 in T1D. In contrast to elevated NE and PR3 levels, the circulating concentrations of A1AT in T1D patients diagnosed within one year were significantly decreased compared to healthy subjects [1.37 (1.07-1.83) vs 1.80 (1.57-2.07) mg/ml, \( p<0.01 \)], whereas the decline in patients with disease duration for 1-5 years or with disease duration >5 years did not reach statistical significance (Fig. 1D).

Neutrophil NETosis is increased in T1D patients

To explore the underlying mechanism responsible for the markedly elevated circulating NE and PR3 levels, we examined the levels of neutrophil NETosis by quantifying the amount of circulating MPO-DNA complexes, a well-established marker of NET formation (26). In line with the increased NE and PR3 levels, a significant elevation of circulating MPO-DNA complexes was observed in T1D patients, especially in T1D patients with the disease duration of less than one year, compared to the healthy individuals [0.197 (0.049-0.412) vs 0.026 (0.011-0.058) (mean OD405), \( p<0.001 \)].
(Fig. 2A). Furthermore, the amount of MPO-DNA complexes in serum was significantly correlated with the circulating protein levels of NE ($r=0.554$, $p<0.001$) and PR3 ($r=0.575$, $p<0.001$), as well as NE/PR3 enzymatic activities ($r=0.527$, $p<0.001$) (Fig 2B-2D), suggesting that the increased circulating NE and PR3 protein levels in T1D patients are at least in part attributed to enhanced neutrophil NETosis.

Circulating NE and PR3 are associated with the numbers and titers of autoantibodies in T1D patients

We next explored the relationship between circulating neutrophil serine proteases and the three autoantibodies associated with β-cell autoimmunity in T1D patients, including GADA, IA2A and ZnT8A. Among 149 T1D patients, 54 (36%) were autoantibody-negative, 61 (41%), 24 (16%), and 10 (7%) had one, two and three autoantibodies-positive, respectively. Notably, circulating levels of both NE and PR3 proteins as well as their enzymatic activities were increased progressively with increased numbers of the autoantibodies detected in these patients (Figure 3A-3C). Even for the autoantibody-negative T1D patients, the circulating protein levels and enzymatic activities of both NE and PR3 were much higher than those in healthy controls [protein levels: NE: 1154.90 (770.8-1749.5) vs 397.0 (262.2-468.8) ng/ml, $p<0.0001$; PR3: 237.4 (154.3-307.1) vs 107.4 (92.5-165.0) ng/ml, $p<0.0001$; enzymatic activities: 0.53 (0.37-0.79) vs 0.14 (0.10-0.21) mU/ml, $p<0.001$] (Fig. 3A-3C). Furthermore, a strong correlation between the titers of GADA and the circulating protein levels of NE ($r=0.296$, $p=0.011$) and PR3 ($r=0.270$, $p=0.021$) as well as NE/PR3
enzymatic activities ($r=0.275$, $p=0.019$) were detected in T1D patients with GADA-positive (n=73) (Fig. 3D-3F). Likewise, the titers of IA2A in T1D patients with IA2A-positive (n=44) were also positively associated with the protein levels of NE, PR3 and their enzymatic activities (supplementary Fig.1A-1C). After adjustment for disease duration, the circulating protein levels and enzymatic activities of both NE and PR3 were still significantly correlated with the numbers and titers of these autoantibodies (all $p<0.05$, supplementary Table 1). On the contrary, no significant correlation between fasting blood glucose and circulating protein levels of NE ($r=-0.103$, $p=0.211$) or PR3 ($r=-0.097$, $p=0.237$) or NE/PR3 enzymatic activities ($r=-0.078$, $p=0.342$) was observed in the present study cohort. We further measured and compared the circulating protein levels and enzymatic activities of NE and PR3 in 25 T2D patients within 1 year from diagnosis and 25 age- and sex-matched healthy controls (supplementary Table 2). The results showed that there was no significant difference in either protein levels or enzymatic activities of NE and PR3 or NETosis between the two groups (supplementary Table 2). Taken together, these data suggested that elevated NE and PR3 may be closely associated with β-cell autoimmunity, but not glycemic status in T1D patients.

Elevated NE/PR3 enzymatic activity is closely associated with the development of diabetes in NOD mice.

To further explore the relationship between neutrophil serine proteases and the development of T1D, we determined the dynamic changes of PR3 and NE in NOD mice (n=30), a well-established
animal model for autoimmune diabetes from 2 to 30 weeks of age. These mice were then retroactively assigned to two groups: those that eventually developed diabetes (n=22, called “diabetic”) and those that did not (n=8, called “non-diabetic”). In diabetic mice, the circulating NE/PR3 enzymatic activities were markedly elevated by 4 folds as early as 4 weeks after birth compared to those in 2-week-old mice, and such an elevation sustained for over 10 weeks before the onset of diabetes. Afterwards, the NE/PR3 activities in diabetic mice were gradually decreased to the baseline levels, presumably due to the termination of autoimmune responses as a result of complete β-cell destruction (Fig. 4A). In non-diabetic mice, although there was a transient and modest increase of circulating NE/PR3 activities between 4 and 5 weeks after birth (Fig. 4B), the magnitude and duration of NE/PR3 elevation was substantially lower than in age-matched diabetic mice (Fig. 4C). In BALB/c and C57BL/6N mice which do not develop insulitis and autoimmune diabetes, circulating NE/PR3 activities remained little changed throughout the 30-week observation period (Supplementary Fig. 2A-2B).

DISCUSSION

In this study, we demonstrated that a modest reduction of neutrophil counts in patients with T1D at onset is accompanied by a marked elevation of both protein levels and enzymatic activities of the two major neutrophil serine proteases NE and PR3. Furthermore, these changes in T1D patients are closely associated with increased neutrophil NETosis, as determined by quantification of MPO-DNA complexes in the circulation. These findings suggest that the reduction of neutrophil
counts in T1D patients is attributed in part to augmented NETosis, which in turn leads to increased
NET formation and release of NE and PR3 into the blood stream.

We showed that the amplitude of elevation in circulating NE/PR3 enzymatic activities and NET
formation in patients with the disease duration of less than one year is substantially higher than
those with disease duration of more than one year. A significant reduction in neutrophil counts is
observed only in T1D patients with disease duration of less than one year. Consistent with our
findings, a previous study in Italy also found that neutrophil reduction is greatest in individuals with
the highest risk of developing T1D (11). After the disease onset, mild neutropenia persists for a few
years and then resolves at 5 years after diagnosis (as determined by a longitudinal analysis). In
NOD mice with spontaneous development of autoimmune diabetes, neutrophil infiltration and NET
formation in the islets are detected as early as two weeks after birth, well before the onset of overt
diabetes (9). Furthermore, neutrophil depletion at the early stage reduces subsequent development
of diabetes in NOD mice (9). Taken together, these data support an early role of neutrophil NETosis,
NET formation and augmented release of neutrophil serine proteases in the onset of β-cell
autoimmunity in T1D. Indeed, increased neutrophil NETosis and NET formation have been
implicated in a number of autoimmune diseases, including small vessel vasculitis (SVV), systemic
lupus erythematosus (SLE), and multiple sclerosis (26-28).

In SLE, NETs has been demonstrated to stimulate plasmacytoid DCs (pDCs) for releasing IFN-α.
which in turn augments the autoreactivity of both antigen-presenting and antibody-producing cells
(29; 30). NETosis leads to the release of intracellular proteins, including histones and high mobility

group protein B1, the latter of which is implicated in initiation and/or perpetuation of autoimmunity
in several types of autoimmune disorders, including T1D (30; 31). Furthermore, NETs is associated
with altered patterns of epigenetic and posttranslational modifications, such as methylation,
acetylation and citrullination, which may represent an important source of autoantigens promoting
the generation of autoantibodies (32). In particular, a growing body of evidence supports a
pathogenic role for citrullinated autoantigens in triggering autoimmune responses in SLE,
rheumatoid arthritis and multiple sclerosis (33). However, the pathophysiological roles of NETosis
and its associated changes in T1D remain elusive.

The current etiopathological diagnosis of autoimmune T1D heavily relies on the detection of the
autoantibodies against several β-cell antigens. However, in children these autoantibodies are rarely
detectable before six months of age (34). Moreover, the diagnostic sensitivity of the single
autoantibody measurement in T1D patients is as low as 59%-67% (35). To capture the therapeutic
window for this disease, it is critically important to identify new biomarkers for detection of early
immunological events that affect human islets. Our current study demonstrated approximately
4-fold increase of circulating protein levels and more than 5-fold elevation of enzymatic activities
of NE and PR3 in T1D patients. Furthermore, elevated NE and PR3 are significantly associated
with the positive numbers and titers of the autoantibodies detected in T1D patients. Even in those
autoantibody-negative patients, the circulating enzymatic activities of NE and PR3 are still substantially higher than healthy controls. Using the animal model of T1D, we found that elevated circulating NE/PR3 activities occur well before the onset of hyperglycemia and diabetes, and their activities gradually decline after the development of overt diabetes. Taken together, our data suggest that circulating NE and PR3 may serve as sensitive biomarkers for early detection of those individuals with high risk of developing T1D. On the other hand, we found no significant association between increased levels of NE and PR3 and the severity of hyperglycemia in T1D patients. In fact, while hyperglycemia becomes more severe with the progression of T1D, circulating levels of NE and PR3 exhibit opposite changes, suggesting that increased neutrophil NETosis and augmented release of NE and PR3 are not the consequence of impaired glycemic controls, but are related to β-cell autoimmunity. Indeed, our observation that NETosis and NE/PR3 levels in T1D patients with longer disease duration are much lower than newly-onset patients (<1 year) may be attributed to the gradual attenuation of β-cell autoimmunity with the progression of diabetes to an advanced stage. This is also in line with the fact that the number of autoantibodies in newly-onset T1D patients was much higher than those with longer disease duration (36). In addition to its classical roles for host defence against infection, neutrophil serine proteases are important regulator of inflammation and innate immunity (17; 19; 37; 38). Both NE and PR3 are involved in maturation and release of pro-inflammatory cytokines such as TNFα, IL-1β and IL-18, and also induces expression and activation of Toll-like receptors (39-42), all of which are important
mediator of insulitis and β cell destruction (43; 44). Furthermore, NE and PR3 play an
indispensable role in recruiting neutrophils to the site of inflammation. Notably, neutrophil serine
proteases have recently been implicated in high fat diet-induced obesity, inflammation and
macrophage infiltration in adipose tissues in mice (45). Injection of recombinant PR3 alone is
sufficient to induce hyperglycemia in mice (46). By contrast, treatment with A1AT, a major
endogenous inhibitor of NE and PR3, decreases lymphocyte infiltration in the islets, and prevents β
cell loss and diabetes in rodent models of T1D (47; 48). These animal studies, in conjunction with
our clinical findings, suggest that elevated NE and PR3 may be the direct contributors to the
pathogenesis of autoimmune diabetes by early involvement of autoimmune inflammatory responses
in pancreatic islets.

A1AT, the most abundant circulating serpin secreted from hepatocytes, inhibits neutrophil serine
proteases by covalent binding to the enzymes (49). Deficiency of A1AT has been implicated in a
number of inflammatory disorders such as chronic obstructive pulmonary disease (50). Our present
study observed a modest, but significant reduction of circulating A1AT in patients with T1D,
suggesting that augmented circulating NE and PR3 activities may result from a combination of
increased release of these two enzymes from neutrophil NETosis and decreased production of their
endogenous inhibitor A1AT.

Our study has several limitations, including the relatively small sample size and the cross-sectional
In addition, since our samples were collected from Chinese only, whether or not the findings are replicable in other ethnic groups remains to be determined. Further large scale, longitudinal studies on different ethnic groups are mandatory to clarify the roles of NE and PR3 in the initiation and progression of β-cell autoimmunity, and to evaluate their clinical value for prediction and early diagnosis of T1D.

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No potential conflicts of interest relevant to this article were reported.

Y.W, Y.X, and L.Z conducted the experiments, analyzed data and wrote the manuscript. D.Y, J.Z and Y.T conducted the experiments. S.R.B and K.S.L.L were involved in data analysis and edited the manuscript. A.X and Z.Z contributed to experimental design, analyzed data, and wrote the manuscript. A.X and Z.Z are the guarantors of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Figure Legends

FIG. 1. Circulating protein levels of NE (A) and PR3 (B), NE/PR3 enzymatic activities (C), and A1AT protein levels (D) in healthy controls (n=77), T1D patients within 1 year from diagnosis (n=28), with a disease duration >1 and <5 years from diagnosis (n=59) and with duration >5 years (n=62) from diagnosis are shown as box plots. The horizontal line in the middle of each box indicates the median value; the top and bottom borders of the boxes represent the 75th and 25th percentiles, respectively; the whiskers represent the 10th and 90th percentiles, and the dots represent the outliers. ** p<0.01, *** p<0.001 vs Healthy controls; # p<0.05, ## p<0.01 vs T1D patients within 1 year from diagnosis.

FIG. 2. Circulating levels of MPO-DNA complexes in healthy controls (n=77), T1D patients within 1 year from diagnosis (n=28), with a disease duration >1 and <5 years (n=59) and with duration >5 years (n=62) (A). Circulating MPO-DNA complexes were significantly correlated with circulating NE (B) and PR3 (C) protein levels, and enzymatic activities of both NE and PR3 (D). ** p<0.01, *** p<0.001 vs Healthy controls; ## p<0.01 vs T1D patients within 1 year from diagnosis.

FIG. 3. Circulating protein levels of NE (A) and PR3 (B), enzymatic activities of both NE and PR3 (C) in healthy controls (n=77), T1D patients with autoantibody negative (n=54), one autoantibody-positive of GADA, IA2A or ZnT8A (n=61), two autoantibodies-positive of GADA, IA2A or ZnT8A (n=24), or three autoantibodies-positive of GADA, IA2A and ZnT8A (n=10).
Circulating protein levels of NE (D) and PR3 (E), and enzymatic activities of both NE and PR3 (F) were significantly correlated with the titers of GADA in T1D patients with GADA-positive (n=73).

*** $p<0.001$ vs Healthy controls; $^\# p<0.05, ^\## p<0.01, ^\### p<0.001$ vs T1D patients with autoantibody negative; $^\$ p<0.05$ vs T1D patients with one autoantibody-positive.

**FIG. 4.** Dynamic changes in enzymatic activities of circulating NE/PR3 in NOD female mice. Blood samples were collected weekly from 30 NOD female mice from 2 to 30 weeks after birth. Circulating NE/PR3 enzymatic activities along with their blood glucose levels were measured in mice that developed diabetes (n=22, A) and that remained non-diabetic (n=8, B) until 30 weeks after birth. (C) Comparisons of NE/PR3 enzymatic activities between diabetic mice and non-diabetic mice throughout the observation period. $^\# p<0.05$ vs the NE/PR3 enzymatic activities at 2 weeks of age; $^* p<0.05$ vs age-matched non-diabetic mice.
**TABLE 1**
Characteristics of healthy controls and T1D patients recruited for this study

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Healthy controls</th>
<th>T1D patients from diagnosis</th>
<th>&lt; 1 year</th>
<th>1 - 5 years</th>
<th>&gt; 5 years</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>n</strong></td>
<td>77</td>
<td>28</td>
<td>59</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
<td>13.3 ± 5.3</td>
<td>15.4 ± 6.9</td>
<td>12.9 ± 4.3</td>
<td>14.9 ± 3.8</td>
<td></td>
</tr>
<tr>
<td><strong>Sex (men/women)</strong></td>
<td>43/34</td>
<td>12/16</td>
<td>21/38</td>
<td>24/38</td>
<td></td>
</tr>
<tr>
<td><strong>BMI (kg/m^2)</strong></td>
<td>18.35 ± 2.70</td>
<td>17.56 ± 3.16</td>
<td>17.83 ± 3.77</td>
<td>18.46 ± 3.05</td>
<td></td>
</tr>
<tr>
<td><strong>Duration of diabetes (years)</strong></td>
<td>N/A</td>
<td>0.4 (0.2-0.7)</td>
<td>2.8 (1.9-3.9)</td>
<td>7.6 (6.4-9.3)</td>
<td></td>
</tr>
<tr>
<td><strong>Fasting glucose (mmol/L)</strong></td>
<td>4.69 (4.41-4.89)</td>
<td>7.85 (6.20-11.93)^a</td>
<td>8.4 (6.60-14.20)^a</td>
<td>7.80 (5.68-11.83)^a</td>
<td></td>
</tr>
<tr>
<td><strong>HbA1c (%)</strong></td>
<td>5.00 (4.80-5.15)</td>
<td>8.05 (6.03-11.15)^a</td>
<td>7.50 (6.70-10.10)^a</td>
<td>7.40 (6.48-8.43)^a</td>
<td></td>
</tr>
<tr>
<td><strong>(mmol/mol)</strong></td>
<td>31 (29-33)</td>
<td>64 (42-99)^a</td>
<td>58 (50-87)^a</td>
<td>57 (47-69)^a</td>
<td></td>
</tr>
<tr>
<td><strong>Fasting C-peptide (pmol/L)</strong></td>
<td>445.4 (362.1-678.2)</td>
<td>55.35 (16.92-146.73)^a</td>
<td>22.80 (5.50-92.95)^a,b</td>
<td>5.50 (4.20-28.43)^a,b,c</td>
<td></td>
</tr>
<tr>
<td><strong>C reactive protein (mg/L)</strong></td>
<td>0.23 (0.13-0.61)</td>
<td>0.24 (0.11-0.51)</td>
<td>0.27 (0.13-0.75)</td>
<td>0.28 (0.15-1.12)</td>
<td></td>
</tr>
<tr>
<td><strong>Blood cell counts</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erythrocytes (x 10^6/ml)^</td>
<td>4.66 (4.30-4.94)</td>
<td>4.52 (4.06-5.12)</td>
<td>4.83 (4.54-5.17)</td>
<td>4.82 (4.50-5.03)</td>
<td></td>
</tr>
<tr>
<td>White blood cells (x 10^6/ml)</td>
<td>6.85 (5.80-7.78)</td>
<td>4.70 (3.75-7.35)^a</td>
<td>5.70 (4.95-6.90)</td>
<td>6.40 (5.20-6.97)</td>
<td></td>
</tr>
<tr>
<td>Lymphocytes (x 10^6/ml)</td>
<td>2.18 (1.55-2.62)</td>
<td>1.74 (1.57-2.08)</td>
<td>1.98 (1.54-2.43)</td>
<td>1.84 (1.64-2.38)</td>
<td></td>
</tr>
<tr>
<td>Monocytes (x 10^6/ml)</td>
<td>0.42 (0.34-0.49)</td>
<td>0.29 (0.17-0.37)^a</td>
<td>0.29 (0.22-0.38)^a</td>
<td>0.31 (0.23-0.39)^a</td>
<td></td>
</tr>
<tr>
<td>Neutrophils (x 10^6/ml)</td>
<td>3.63 (3.02-4.15)</td>
<td>2.27 (1.80-3.52)^a</td>
<td>3.29 (2.75-4.15)</td>
<td>3.49 (3.02-4.14)</td>
<td></td>
</tr>
<tr>
<td>Eosinophils (x 10^6/ml)</td>
<td>0.20 (0.12-0.34)</td>
<td>0.13 (0.08-0.20)^a</td>
<td>0.15 (0.12-0.23)</td>
<td>0.15 (0.11-0.21)</td>
<td></td>
</tr>
<tr>
<td>Basophils (x 10^6/ml)</td>
<td>0.05 (0.03-0.06)</td>
<td>0.03 (0.02-0.07)</td>
<td>0.07 (0.05-0.10)^a,b</td>
<td>0.08 (0.06-0.14)^a,b</td>
<td></td>
</tr>
<tr>
<td>Platelets (x 10^6/ml)</td>
<td>256 (223-315)</td>
<td>244 (202-285)^a</td>
<td>219 (193-265)^a</td>
<td>250 (199-280)</td>
<td></td>
</tr>
</tbody>
</table>

Date are expressed as mean ± SD or median (interquartile range) as appropriate.

^ Log transformed before analysis. \(^a\)Compared with healthy controls, \(p < 0.05\); \(^b\)compared with T1D patients from diagnosis < 1 years, \(p < 0.05\); \(^c\)compared with T1D patients from diagnosis 1 - 5 years, \(p < 0.05\).
FIG. 1. Circulating protein levels of NE (A) and PR3 (B), NE/PR3 enzymatic activities (C), and A1AT protein levels (D) in healthy controls (n=77), T1D patients within 1 year from diagnosis (n=28), with a disease duration >1 and <5 years from diagnosis (n=59) and with duration >5 years (n=62) from diagnosis are shown as box plots. The horizontal line in the middle of each box indicates the median value; the top and bottom borders of the boxes represent the 75th and 25th percentiles, respectively; the whiskers represent the 10th and 90th percentiles, and the dots represent the outliers. ** p<0.01, *** p<0.001 vs Healthy controls; # p<0.05, ## p<0.01 vs T1D patients within 1 year from diagnosis.
FIG. 2. Circulating levels of MPO-DNA complexes in healthy controls (n=77), T1D patients within 1 year from diagnosis (n=28), with a disease duration >1 and <5 years (n=59) and with duration >5 years (n=62) (A). Circulating MPO-DNA complexes were significantly correlated with circulating NE (B) and PR3 (C) protein levels, and enzymatic activities of both NE and PR3 (D). ** p<0.01, *** p<0.001 vs Healthy controls; ## p<0.01 vs T1D patients within 1 year from diagnosis.
FIG. 3. Circulating protein levels of NE (A) and PR3 (B), enzymatic activities of both NE and PR3 (C) in healthy controls (n=77), T1D patients with autoantibody negative (n=54), one autoantibody-positive of GADA, IA2A or ZnT8A (n=61), two autoantibodies-positive of GADA, IA2A or ZnT8A (n=24), or three autoantibodies-positive of GADA, IA2A and ZnT8A (n=10). Circulating protein levels of NE (D) and PR3 (E), and enzymatic activities of both NE and PR3 (F) were significantly correlated with the titers of GADA in T1D patients with GADA-positive (n=73). *** p<0.001 vs Healthy controls; # p<0.05, ## p<0.01, ### p<0.001 vs T1D patients with one autoantibody-positive. $ p<0.05 vs T1D patients with autoantibody negative.
FIG. 4. Dynamic changes in enzymatic activities of circulating NE/PR3 in NOD female mice. Blood samples were collected weekly from 30 NOD female mice from 2 to 30 weeks after birth. Circulating NE/PR3 enzymatic activities along with their blood glucose levels were measured in mice that developed diabetes (n=22, A) and that remained non-diabetic (n=8, B) until 30 weeks after birth. (C) Comparisons of NE/PR3 enzymatic activities between diabetic mice and non-diabetic mice throughout the observation period. # p<0.05 vs the NE/PR3 enzymatic activities at 2 weeks of age; * p<0.05 vs age-matched non-diabetic mice.
**Supplementary Table 1**

Correlations of circulating protein levels and enzymatic activities of NE and PR3 with clinical parameters in T1D patients

<table>
<thead>
<tr>
<th></th>
<th>Circulating NE protein levels</th>
<th>Circulating NE protein levels (Diabetes duration-adjusted)</th>
<th>Circulating PR3 protein levels</th>
<th>Circulating PR3 protein levels (Diabetes duration-adjusted)</th>
<th>NE/PR3 enzymatic activities</th>
<th>NE/PR3 enzymatic activities (Diabetes duration-adjusted)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>$r$            $p$</td>
<td>$r$            $p$</td>
<td>$r$            $p$</td>
<td>$r$            $p$</td>
<td>$r$            $p$</td>
</tr>
<tr>
<td>Diabetes duration (years)</td>
<td>-0.203</td>
<td>0.017</td>
<td>-0.106</td>
<td>0.200</td>
<td>-0.212</td>
<td>0.010</td>
</tr>
<tr>
<td>Fasting glucose (mmol/L)*</td>
<td>-0.103</td>
<td>0.211</td>
<td>-0.106</td>
<td>0.200</td>
<td>-0.097</td>
<td>0.237</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>0.083</td>
<td>0.315</td>
<td>0.069</td>
<td>0.405</td>
<td>0.140</td>
<td>0.090</td>
</tr>
<tr>
<td>Fasting C-peptide (pmol/L)*</td>
<td>0.151</td>
<td>0.066</td>
<td>0.125</td>
<td>0.130</td>
<td>0.158</td>
<td>0.052</td>
</tr>
<tr>
<td>Neutrophils ( x 10⁶/ml)*</td>
<td>0.108</td>
<td>0.189</td>
<td>0.101</td>
<td>0.213</td>
<td>0.074</td>
<td>0.368</td>
</tr>
<tr>
<td>Numbers of autoantibodies-positive</td>
<td>0.427</td>
<td>&lt;0.001</td>
<td>0.415</td>
<td>&lt;0.001</td>
<td>0.428</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>GADA titers*ₐ</td>
<td>0.296</td>
<td>0.011</td>
<td>0.313</td>
<td>0.007</td>
<td>0.270</td>
<td>0.021</td>
</tr>
<tr>
<td>IA2A titers*₇b</td>
<td>0.422</td>
<td>0.004</td>
<td>0.403</td>
<td>0.007</td>
<td>0.486</td>
<td>0.001</td>
</tr>
<tr>
<td>ZnT8A titers*₇c</td>
<td>0.205</td>
<td>0.360</td>
<td>0.201</td>
<td>0.380</td>
<td>0.288</td>
<td>0.194</td>
</tr>
</tbody>
</table>

*Log transformed before analysis;

a. include only subjects with GADA-positive (n = 73); b. include only subjects with IA2A-positive (n = 44); c. include only subjects with ZnT8A-positive (n = 22).
### Supplementary Table 2

Circulating protein levels and enzymatic activities of NE and PR3, A1AT protein levels, and MPO-DNA complexes in T2D patients and their healthy controls

<table>
<thead>
<tr>
<th></th>
<th>Healthy controls</th>
<th>T2D patients</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>25</td>
<td>25</td>
<td>-</td>
</tr>
<tr>
<td>Age (years)</td>
<td>47.8 ± 5.7</td>
<td>48.0 ± 6.0</td>
<td>0.815</td>
</tr>
<tr>
<td>Sex (men/women)</td>
<td>12/13</td>
<td>12/13</td>
<td>1.000</td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td>20.45 ± 2.57</td>
<td>26.37 ± 3.45(a)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Duration of diabetes (years)</td>
<td>N/A</td>
<td>0.4 (0.1-0.7)</td>
<td>-</td>
</tr>
<tr>
<td>Fasting glucose (mmol/L)(§)</td>
<td>4.86 (4.59-5.36)</td>
<td>7.62 (6.29-9.35)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HbA1c (%)(§)</td>
<td>5.24 (5.02-5.51)</td>
<td>7.45 (6.46-8.70)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>(mmol/mol)(§)</td>
<td>34 (31-37)</td>
<td>58 (48-72)(a)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>C reactive protein (mg/L)(§)</td>
<td>0.47 (0.24-0.86)</td>
<td>1.82 (1.03-4.37)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Blood cell counts</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erythrocytes ( x 10(^6)/ml)(§)</td>
<td>4.72 (4.38-5.43)</td>
<td>4.79 (4.45-5.66)</td>
<td>0.877</td>
</tr>
<tr>
<td>White blood cells ( x 10(^6)/ml)(§)</td>
<td>5.91 (5.23-7.46)</td>
<td>6.68 (4.62-7.93)</td>
<td>0.014</td>
</tr>
<tr>
<td>Lymphocytes ( x 10(^6)/ml)(§)</td>
<td>2.11 (1.59-2.66)</td>
<td>3.08 (1.59-3.92)</td>
<td>0.017</td>
</tr>
<tr>
<td>Monocytes ( x 10(^6)/ml)(§)</td>
<td>0.38 (0.24-0.49)</td>
<td>0.39 (0.19-0.51)</td>
<td>0.796</td>
</tr>
<tr>
<td>Neutrophils ( x 10(^6)/ml)(§)</td>
<td>3.42 (2.94-4.71)</td>
<td>3.54 (3.07-4.83)</td>
<td>0.487</td>
</tr>
<tr>
<td>Eosinophils ( x 10(^6)/ml)(§)</td>
<td>0.15 (0.08-0.21)</td>
<td>0.16 (0.10-0.22)</td>
<td>0.864</td>
</tr>
<tr>
<td>Basophils ( x 10(^6)/ml)(§)</td>
<td>0.03 (0.01-0.07)</td>
<td>0.04 (0.02-0.07)</td>
<td>0.763</td>
</tr>
<tr>
<td>Platelets ( x 10(^9)/ml)(§)</td>
<td>225 (176-279)</td>
<td>228 (187-295)</td>
<td>0.824</td>
</tr>
<tr>
<td>Circulating NE protein levels (ng/ml)</td>
<td>403.7 (255.4-482.1)</td>
<td>411.5 (268.3-512.7)</td>
<td>0.377</td>
</tr>
<tr>
<td>Circulating PR3 protein levels (ng/ml)</td>
<td>109.3 (87.4-174.5)</td>
<td>113.7 (76.4-201.3)</td>
<td>0.413</td>
</tr>
<tr>
<td>Circulating NE/PR3 enzymatic activities (mU/ml)</td>
<td>0.16 (0.11-0.34)</td>
<td>0.18 (0.11-0.40)</td>
<td>0.428</td>
</tr>
<tr>
<td>Circulating A1AT protein levels (mg/ml)</td>
<td>1.70 (1.42-2.11)</td>
<td>1.63 (1.37-2.05)</td>
<td>0.275</td>
</tr>
<tr>
<td>MPO-DNA complexes (mean OD405)</td>
<td>0.030 (0.011-0.057)</td>
<td>0.033 (0.015-0.064)</td>
<td>0.439</td>
</tr>
</tbody>
</table>

Date are expressed as mean ± SD or median (interquartile range) as appropriate.
\(§\)Log transformed before analysis.
Supplementary Figure 1.

Circulating protein levels of NE (A) and PR3 (B), and NE/PR3 enzymatic activities (C) were significantly correlated with the titers of IA2A in T1D patients with IA2A-positive (n=44).
Supplementary Figure. 2.

Dynamic changes of circulating NE/PR3 enzymatic activities along with their blood glucose levels in BALB/c (n=10, A) and C57BL/6 mice (n=10, B) from 2 to 30 weeks of age.