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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, Papillion-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

Services, the University of Hong Kong). 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 χ^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 ($p<0.001$)

for PR3.

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 retrospectively 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

design. In addition, since our samples were collected from Chinese only, whether or not the findings are replicable in other ethnic group 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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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.

REFERENCES

1. Hummel K, McFann KK, Realsen J, Messer LH, Klingensmith GJ, Chase HP: The increasing onset of type 1 diabetes in children. The Journal of pediatrics 2012;161:652-657
2. Tuomilehto J: The emerging global epidemic of type 1 diabetes. Current diabetes reports 2013;13:795-804
3. van Belle TL, Coppieters KT, von Herrath MG: Type 1 diabetes: etiology, immunology, and therapeutic strategies. Physiological reviews 2011;91:79-118
4. Skyler Js SJM: The evolution of type 1 diabetes. JAMA 2013;309:2491-2492
5. Gan MJ, Albanese-O'Neill A, Haller MJ: Type 1 diabetes: current concepts in epidemiology, pathophysiology, clinical care, and research. Current problems in pediatric and adolescent health care 2012;42:269-291
6. Pihoker C, Gilliam LK, Hampe CS, Lernmark Å: Autoantibodies in Diabetes. Diabetes 2005;54:S52-S61
7. Miao D, Yu L, Eisenbarth GS: Role of autoantibodies in type 1 diabetes. Frontiers in bioscience : a journal and virtual library 2007;12:1889-1898
8. Richardson SJ, Willcox A, Bone AJ, Morgan NG, Foulis AK: Immunopathology of the human pancreas in type-I diabetes. Semin Immunopathol 2011;33:9-21
9. Diana J, Simoni Y, Furio L, Beaudoin L, Agerberth B, Barrat F, Lehuen A: Crosstalk between neutrophils, B-1a cells and plasmacytoid dendritic cells initiates autoimmune diabetes. Nat Med 2013;19:65-73
10. Harsunen MH, Puff R, D'Orlando O, Giannopoulou E, Lachmann L, Beyerlein A, von Meyer A, Ziegler AG: Reduced blood leukocyte and neutrophil numbers in the pathogenesis of type 1 diabetes. Horm Metab Res 2013;45:467-470
11. Valle A, Giamporcaro GM, Scavini M, Stabilini A, Grogan P, Bianconi E, Sebastiani G, Masini M, Maugeri N, Porretti L, Bonfanti R, Meschi F, De Pellegrin M, Lesma A, Rossini S, Piemonti L, Marchetti P, Dotta F, Bosi E, Battaglia M: Reduction of circulating neutrophils precedes and accompanies type 1 diabetes. Diabetes 2013;62:2072-2077
12. Mestas J, Hughes CC: Of mice and not men: differences between mouse and human immunology. Journal of immunology 2004;172:2731-2738
13. Kolaczkowska E, Kubes P: Neutrophil recruitment and function in health and inflammation. Nat Rev Immunol 2013;13:159-175
14. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, Weinrauch Y, Zychlinsky A: Neutrophil extracellular traps kill bacteria. Science 2004;303:1532-1535
15. Mocsai A: Diverse novel functions of neutrophils in immunity, inflammation, and beyond. J Exp Med 2013;210:1283-1299
16. Korkmaz B, Moreau T, Gauthier F: Neutrophil elastase, proteinase 3 and cathepsin G: Physicochemical properties, activity and physiopathological functions. Biochimie 2008;90:227-242
17. Wiedow O, Meyer-Hoffert U: Neutrophil serine proteases: potential key regulators of cell signalling during inflammation. Journal of Internal Medicine 2005;257:319-328
18. Pham CT: Neutrophil serine proteases fine-tune the inflammatory response. Int J Biochem Cell Biol 2008;40:1317-1333

19. Meyer-Hoffert U, Wiedow O: Neutrophil serine proteases: mediators of innate immune responses. Current opinion in hematology 2011;18:19-24
20. Korkmaz B, Horwitz MS, Jenne DE, Gauthier F: Neutrophil Elastase, Proteinase 3, and Cathepsin G as Therapeutic Targets in Human Diseases. Pharmacological Reviews 2010;62:726-759
21. American Diabetes Association: Diagnosis and classification of diabetes mellitus. Diabetes care 2013;36 Suppl 1:S67-74
22. Xiao Y, Xu A, Law LS, Chen C, Li H, Li X, Yang L, Liu S, Zhou Z, Lam KS: Distinct changes in serum fibroblast growth factor 21 levels in different subtypes of diabetes. The Journal of clinical endocrinology and metabolism 2012;97:E54-58
23. Yang L, Luo S, Huang G, Peng J, Li X, Yan X, Lin J, Wenzlau JM, Davidson HW, Hutton JC, Zhou Z: The diagnostic value of zinc transporter 8 autoantibody (ZnT8A) for type 1 diabetes in Chinese. Diabetes/metabolism research and reviews 2010;26:579-584
24. Wiesner O, Litwiller RD, Hummel AM, Viss MA, McDonald CJ, Jenne DE, Fass DN, Specks U: Differences between human proteinase 3 and neutrophil elastase and their murine homologues are relevant for murine model experiments. FEBS Lett 2005;579:5305-5312
25. Labow RS, Erfle DJ, Santerre JP: Elastase-induced hydrolysis of synthetic solid substrates: poly(ester-urea-urethane) and poly(ether-urea-urethane). Biomaterials 1996;17:2381-2388
26. Kessenbrock K, Krumbholz M, Schonermarck U, Back W, Gross WL, Werb Z, Grone HJ, Brinkmann V, Jenne DE: Netting neutrophils in autoimmune small-vessel vasculitis. Nat Med 2009;15:623-625
27. Villanueva E, Yalavarthi S, Berthier CC, Hodgin JB, Khandpur R, Lin AM, Rubin CJ, Zhao W, Olsen SH, Klinker M, Shealy D, Denny MF, Plumas J, Chaperot L, Kretzler M, Bruce AT, Kaplan MJ: Netting neutrophils induce endothelial damage, infiltrate tissues, and expose immunostimulatory molecules in systemic lupus erythematosus. Journal of immunology 2011;187:538-552
28. Naegele M, Tillack K, Reinhardt S, Schippling S, Martin R, Sospedra M: Neutrophils in multiple sclerosis are characterized by a primed phenotype. Journal of neuroimmunology 2012;242:60-71
29. Lande R, Ganguly D, Facchinetti V, Frasca L, Conrad C, Gregorio J, Meller S, Chamilos G, Sebasigari R, Riccieri V, Bassett R, Amuro H, Fukuhara S, Ito T, Liu YJ, Gilliet M: Neutrophils activate plasmacytoid dendritic cells by releasing self-DNA-peptide complexes in systemic lupus erythematosus. Science translational medicine 2011;3:73ra19
30. Garcia-Romo GS, Caielli S, Vega B, Connolly J, Allantaz F, Xu Z, Punaro M, Baisch J, Guiducci C, Coffman RL, Barrat FJ, Banchereau J, Pascual V: Netting neutrophils are major inducers of type I IFN production in pediatric systemic lupus erythematosus. Science translational medicine 2011;3:73ra20
31. Zhang S, Zhong J, Yang P, Gong F, Wang CY: HMGB1, an innate alarmin, in the pathogenesis of type 1 diabetes. International journal of clinical and experimental pathology 2009;3:24-38
32. Knight JS, Carmona-Rivera C, Kaplan MJ: Proteins derived from neutrophil extracellular traps

may serve as self-antigens and mediate organ damage in autoimmune diseases. Frontiers in immunology 2012;3:380. doi: 310.3389/fimmu.2012.00380
33. Dwivedi N, Radic M: Citrullination of autoantigens implicates NETosis in the induction of autoimmunity. Annals of the rheumatic diseases 2014;73:483-491
34. Ziegler AG, Hummel M, Schenker M, Bonifacio E: Autoantibody appearance and risk for development of childhood diabetes in offspring of parents with type 1 diabetes: the 2-year analysis of the German BABYDIAB Study. Diabetes 1999;48:460-468
35. Lebastchi J, Herold KC: Immunologic and metabolic biomarkers of beta-cell destruction in the diagnosis of type 1 diabetes. Cold Spring Harb Perspect Med 2012;2:a007708
36. Atkinson MA: The pathogenesis and natural history of type 1 diabetes. Cold Spring Harb Perspect Med 2012;2:a007641
37. Pham CT: Neutrophil serine proteases: specific regulators of inflammation. Nat Rev Immunol 2006;6:541-550
38. Meyer-Hoffert U: Neutrophil-derived serine proteases modulate innate immune responses. Front Biosci (Landmark Ed) 2009;14:3409-3418
39. Coeshott C, Ohnemus C, Pilyavskaya A, Ross S, Wieczorek M, Kroona H, Leimer AH, Cheronis J: Converting enzyme-independent release of tumor necrosis factor alpha and IL-1beta from a stimulated human monocytic cell line in the presence of activated neutrophils or purified proteinase 3. Proceedings of the National Academy of Sciences of the United States of America 1999;96:6261-6266
40. Sugawara S, Uehara A, Nochi T, Yamaguchi T, Ueda H, Sugiyama A, Hanzawa K, Kumagai K, Okamura H, Takada H: Neutrophil proteinase 3-mediated induction of bioactive IL-18 secretion by human oral epithelial cells. Journal of immunology 2001;167:6568-6575
41. Walsh DE, Greene CM, Carroll TP, Taggart CC, Gallagher PM, O'Neill SJ, McElvaney NG: Interleukin-8 up-regulation by neutrophil elastase is mediated by MyD88/IRAK/TRAF-6 in human bronchial epithelium. J Biol Chem 2001;276:35494-35499
42. Devaney JM, Greene CM, Taggart CC, Carroll TP, O'Neill SJ, McElvaney NG: Neutrophil elastase up-regulates interleukin-8 via toll-like receptor 4. FEBS Lett 2003;544:129-132
43. Grieco FA, Vendrame F, Spagnuolo I, Dotta F: Innate immunity and the pathogenesis of type 1 diabetes. Semin Immunopathol 2011;33:57-66
44. Padgett LE, Broniowska KA, Hansen PA, Corbett JA, Tse HM: The role of reactive oxygen species and proinflammatory cytokines in type 1 diabetes pathogenesis. Annals of the New York Academy of Sciences 2013;1281:16-35
45. Talukdar S, Oh da Y, Bandyopadhyay G, Li D, Xu J, McNelis J, Lu M, Li P, Yan Q, Zhu Y, Ofrecio J, Lin M, Brenner MB, Olefsky JM: Neutrophils mediate insulin resistance in mice fed a high-fat diet through secreted elastase. Nat Med 2012;18:1407-1412
46. Bae S, Choi J, Hong J, Jhun H, Hong K, Kang T, Song K, Jeong S, Yum H, Kim S: Neutrophil proteinase 3 induces diabetes in a mouse model of glucose tolerance. Endocr Res 2012;37:35-45
47. Song S, Goudy K, Campbell-Thompson M, Wasserfall C, Scott-Jorgensen M, Wang J, Tang Q, Crawford JM, Ellis TM, Atkinson MA, Flotte TR: Recombinant adeno-associated virus-mediated

alpha-1 antitrypsin gene therapy prevents type I diabetes in NOD mice. Gene therapy 2004;11:181-186

48. Lu Y, Tang M, Wasserfall C, Kou Z, Campbell-Thompson M, Gardemann T, Crawford J, Atkinson M, Song S: Alpha1-antitrypsin gene therapy modulates cellular immunity and efficiently prevents type 1 diabetes in nonobese diabetic mice. Human gene therapy 2006;17:625-634

49. Janciauskiene SM, Bals R, Koczulla R, Vogelmeier C, Kohnlein T, Welte T: The discovery of alpha1-antitrypsin and its role in health and disease. Respiratory medicine 2011;105:1129-1139

50. Stoller JK, Aboussouan LS: Alpha1-antitrypsin deficiency. Lancet 2005;365:2225-2236

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

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

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, $p < 0.05$.

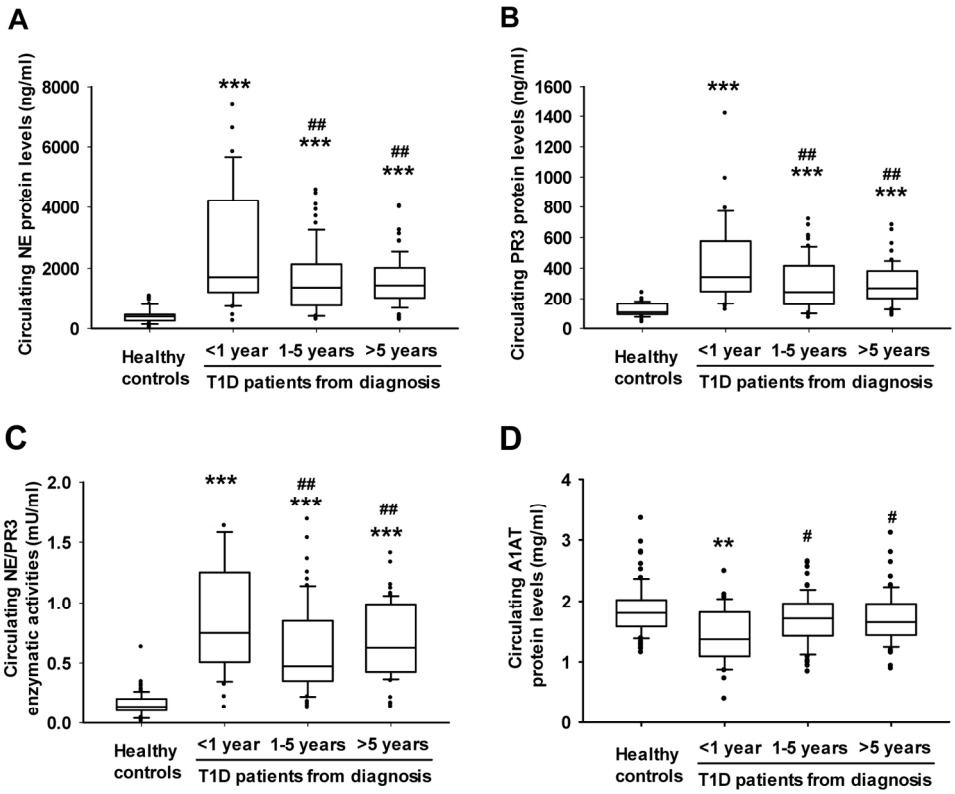

FIG.1. Circulating protein levels of NE (A) and PR3 (B), NE/PR3 enzymatic activities (C), and A1AT protein levels (D) in healthy controls (n=77), T1D patients within 1 year from diagnosis (n=28), with a disease duration >1 and <5 years from diagnosis (n=59) and with duration >5 years (n=62) from diagnosis 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.
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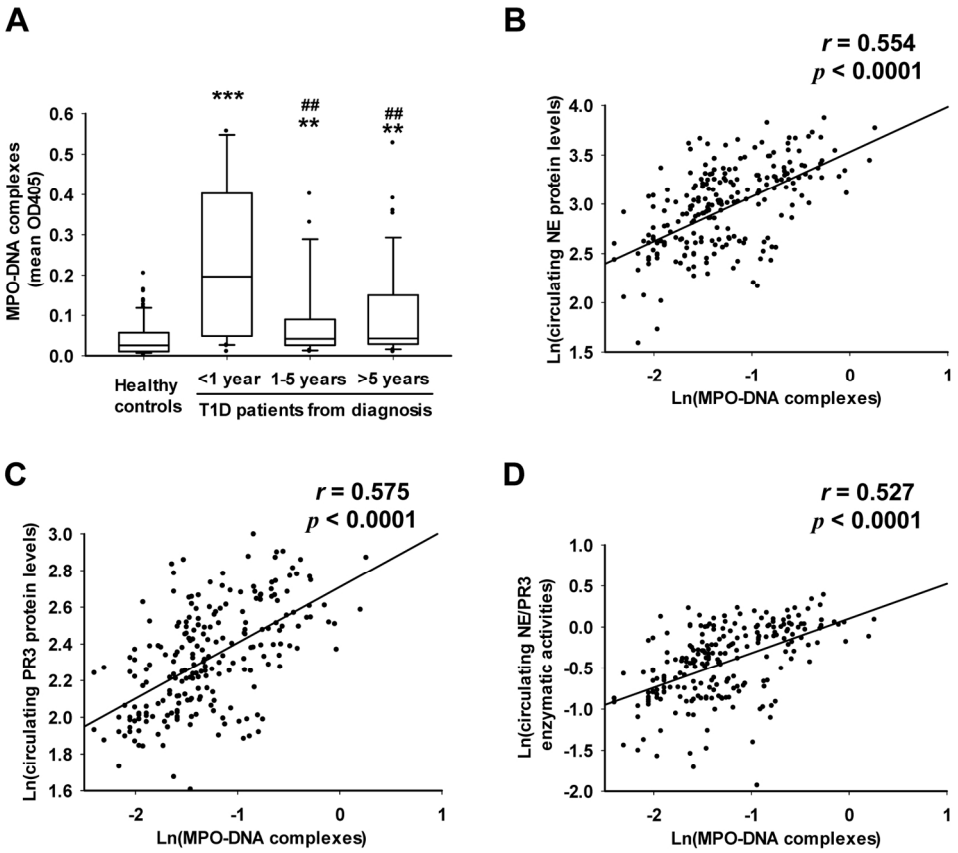

FIG. 2. Circulating levels of MPO-DNA complexes in healthy controls (n=77), T1D patients within 1 year from diagnosis (n=28), with a disease duration >1 and <5 years (n=59) and with duration >5 years (n=62) (A). Circulating MPO-DNA complexes were significantly correlated with circulating NE (B) and PR3 (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.
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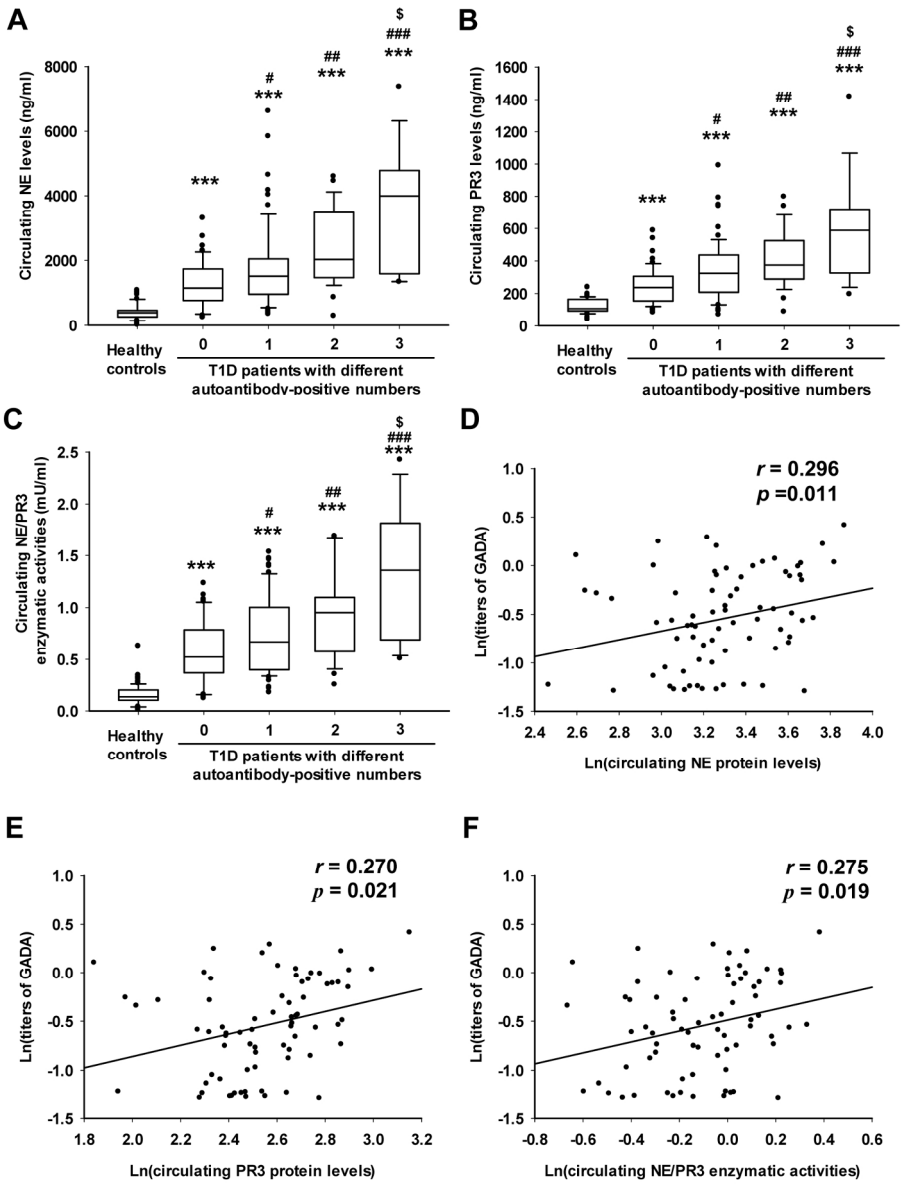

FIG. 3. Circulating protein levels of NE (A) and PR3 (B), enzymatic activities of both NE and PR3 (C) in healthy controls (n=77), T1D patients with autoantibody negative (n=54), one autoantibody-positive of GADA, IA2A or ZnT8A (n=61), two autoantibodies-positive of GADA, IA2A or ZnT8A (n=24), or three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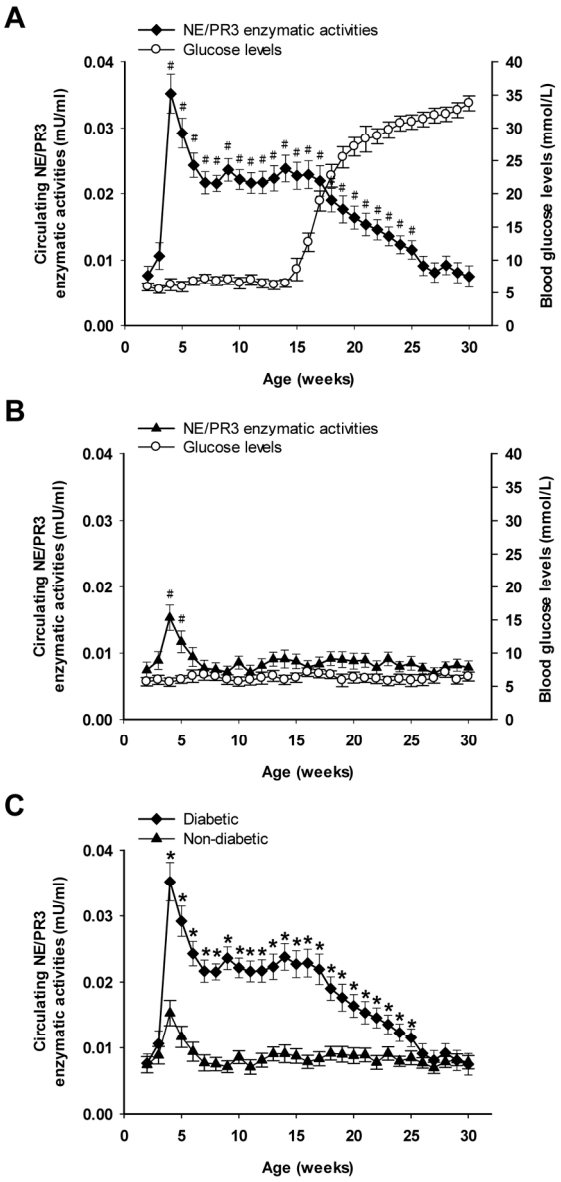

FIG. 4. Dynamic changes in enzymatic activities of circulating NE/PR3 in NOD female mice. Blood samples were collected weekly from 30 NOD female mice from 2 to 30 weeks after birth. Circulating NE/PR3 enzymatic activities along with their blood glucose levels were measured in mice that developed diabetes (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.
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Supplementary Table 1

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

	Circulating NE protein levels		Circulating NE protein levels (Diabetes duration-adjusted)	
	r	*p*	*r*	*p*
Diabetes duration (years)	**-0.203**	**0.017**	—	—
Fasting glucose (mmol/L)*	-0.103	0.211	-0.106	0.200
HbA1c (%)*	0.083	0.315	0.069	0.405
Fasting C-peptide (pmol/L)*	0.151	0.066	0.125	0.130
Neutrophils (x 10^6/ml)*	0.108	0.189	0.101	0.213
Numbers of autoantibodies-positive	**0.427**	**<0.001**	**0.415**	**<0.001**
GADA tirers*[a]	**0.296**	**0.011**	**0.313**	**0.007**
IA2A titers*[b]	**0.422**	**0.004**	**0.403**	**0.007**
ZnT8A titers*[c]	0.205	0.360	0.201	0.380

	Circulating PR3 protein levels		Circulating PR3 protein levels (Diabetes duration-adjusted)	
	r	*p*	*r*	*p*
Duration of diabetes (years)	**-0.212**	**0.010**	—	—
Fasting glucose (mmol/L)*	-0.097	0.237	-0.102	0.214
HbA1c (%)*	0.140	0.090	0.120	0.145
Fasting C-peptide (pmol/L)*	0.158	0.052	0.120	0.145
Neutrophils (x 10^6/ml)*	0.074	0.368	0.065	0.470
Numbers of autoantibodies-positive	**0.428**	**<0.001**	**0.411**	**<0.001**
GADA tirers*[a]	**0.270**	**0.021**	**0.279**	**0.018**
IA2A titers*[b]	**0.486**	**0.001**	**0.450**	**0.002**
ZnT8A titers*[c]	0.288	0.194	0.285	0.209

	NE/PR3 enzymatic activities		NE/PR3 enzymatic activities (Diabetes duration-adjusted)	
	r	*p*	*r*	*p*
Duration of diabetes (years)	**-0.196**	**0.021**	—	—
Fasting glucose (mmol/L)*	-0.078	0.342	-0.080	0.334
HbA1c (%)*	0.101	0.219	0.093	0.260
Fasting C-peptide (pmol/L)*	0.151	0.066	0.137	0.098
Neutrophils (x 10^6/ml)*	0.128	0.119	0.107	0.198
Numbers of autoantibodies-positive	**0.403**	**<0.001**	**0.397**	**<0.001**
GADA tirers*[a]	**0.275**	**0.019**	**0.280**	**0.017**
IA2A titers*[b]	**0.389**	**0.009**	**0.386**	**0.011**
ZnT8A titers*[c]	0.193	0.390	0.190	0.409

*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

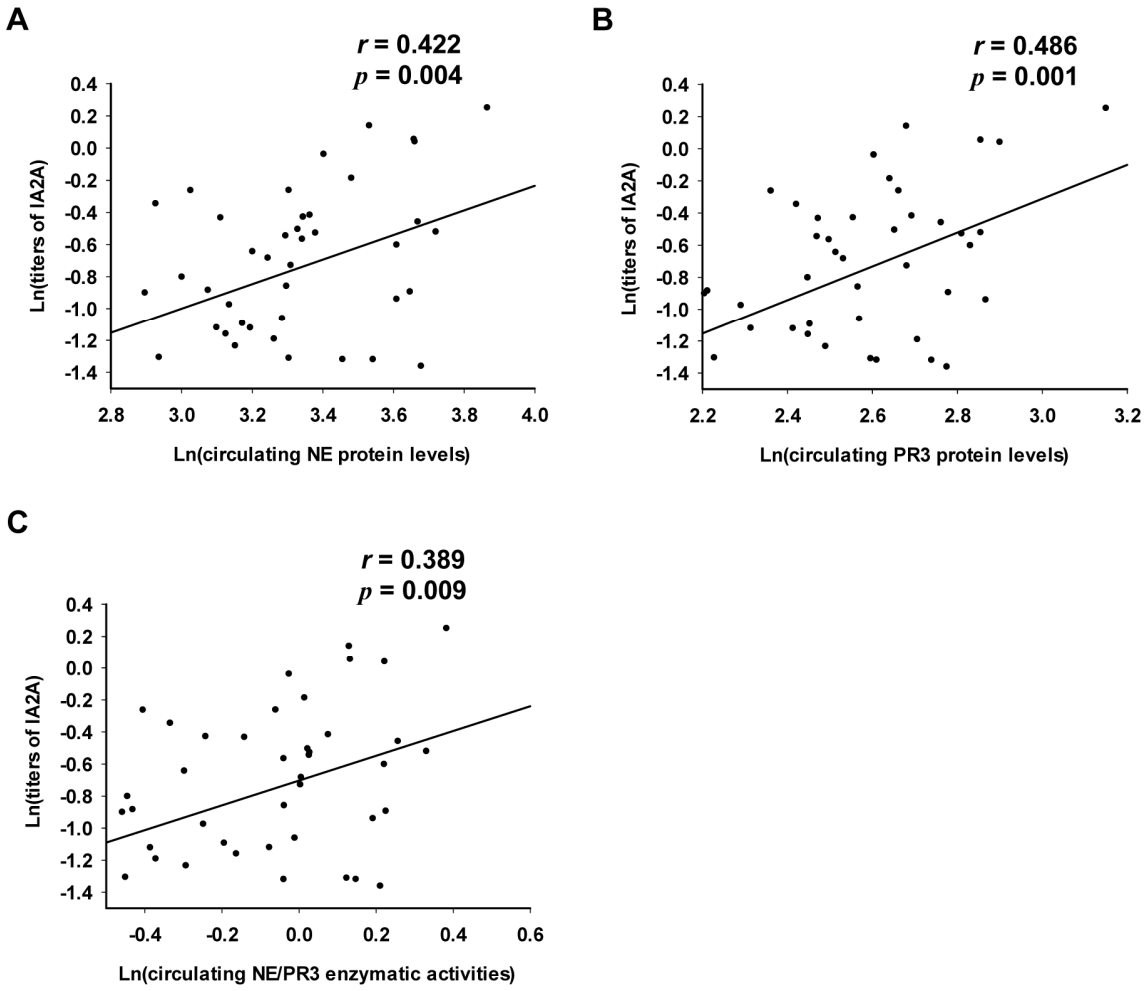
	Healthy controls	T2D patients	*p* value
n	25	25	-
Age (years)	47.8 ± 5.7	48.0 ± 6.0	0.815
Sex (men/women)	12/13	12/13	1.000
BMI (kg/m^2)	20.45 ± 2.57	26.37 ± 3.45[a]	<0.001
Duration of diabetes (years)	N/A	0.4 (0.1-0.7)	-
Fasting glucose (mmol/L)[§]	4.86 (4.59-5.36)	7.62 (6.29-9.35)[a]	<0.001
HbA1c (%)[§]	5.24 (5.02-5.51)	7.45 (6.46-8.70)[a]	<0.001
(mmol/mol)[§]	34 (31-37)	58 (48-72)[a]	<0.001
C reactive protein (mg/L)[§]	0.47 (0.24-0.86)	1.82 (1.03-4.37)[a]	<0.001
Blood cell counts			
Erythrocytes (x 10^6/ml)[§]	4.72 (4.38-5.43)	4.79 (4.45-5.66)	0.877
White blood cells (x 10^6/ml)[§]	5.91 (5.23-7.46)	6.68 (4.62-7.93)	0.014
Lymphocytes (x 10^6/ml)[§]	2.11 (1.59-2.66)	3.08 (1.59-3.92)[a]	0.017
Monocytes (x 10^6/ml)[§]	0.38 (0.24-0.49)	0.39 (0.19-0.51)	0.796
Neutrophils (x 10^6/ml)[§]	3.42 (2.94-4.71)	3.54 (3.07-4.83)	0.487
Eosinophils (x 10^6/ml)[§]	0.15 (0.08-0.21)	0.16 (0.10-0.22)	0.864
Basophils (x 10^6/ml)[§]	0.03 (0.01-0.07)	0.04 (0.02-0.0.07)	0.763
Platelets (x 10^6/ml)[§]	225 (176-279)	228 (187-295)	0.824
Circulating NE protein levels (ng/ml)	403.7 (255.4-482.1)	411.5 (268.3-512.7)	0.377
Circulating PR3 protein levels (ng/ml)	109.3 (87.4-174.5)	113.7 (76.4-201.3)	0.413
Circulating NE/PR3 enzymatic activities (mU/ml)	0.16 (0.11-0.34)	0.18 (0.11-0.40)	0.428
Circulating A1AT protein levels (mg/ml)	1.70 (1.42-2.11)	1.63 (1.37-2.05)	0.275
MPO-DNA complexes (mean OD405)	0.030 (0.011-0.057)	0.033 (0.015-0.064)	0.439

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.