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Enhanced formation and disordered regulation of NETs in MPO-ANCA-associated microscopic polyangiitis

Running title: NETs-ANCA vicious cycle in MPA

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Abstract

Microscopic polyangiitis (MPA) is an antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis that affects small vessels especially renal glomeruli. We recently demonstrated that the abnormal formation and impaired degradation of neutrophil extracellular traps (NETs) could be crucially involved in the generation of myeloperoxidase (MPO)-ANCA and subsequent development of MPA. The aim of this study is to elucidate the enhanced formation and disordered regulation of NETs in patients with MPO-ANCA-associated MPA. Peripheral blood samples were obtained from 38 MPO-ANCA-associated MPA patients, 23 systemic lupus erythematosus (SLE) patients, and 8 healthy controls. First, IgG eluted from MPO-ANCA-associated MPA sera demonstrated high ability for NETs induction compared to healthy controls, and the ability correlated to the disease activity and was parallel to the ANCA affinity to MPO. Moreover, the NETs induction ability was absorbed by addition of recombinant human MPO in the IgG samples. These findings indicated the serum factor that induced NETs as MPO-ANCA. Next, low ability of MPO-ANCA-associated MPA serum for NETs degradation was determined. The ability was partially recovered by depletion of IgG from the sera; thus, the presence of serum factors that precluded NETs degradation besides IgG was suggested. Correspondingly, activity of DNase I, an important regulator of NETs, was generally low in MPO-ANCA-associated MPA similar to SLE. Furthermore, the presence of anti-NETs antibodies, which could interfere with the DNase I activity, was demonstrated in some MPO-ANCA-associated MPA sera. The collective evidence suggests that a vicious cycle through NETs and MPO-ANCA could be critically involved in the pathogenesis of MPO-ANCA-associated MPA.

Introduction

Microscopic polyangiitis (MPA) is a systemic necrotizing vasculitis that affects small vessels predominantly in the kidney.¹ Anti-myeloperoxidase (MPO) autoantibody, which is detected as peri-nuclear antineutrophil cytoplasmic antibody (ANCA) by immunofluorescent staining (IF), is frequently present in the serum. Although MPO is an intra-cytoplasmic granule mainly in neutrophils, it can be released through the plasma membrane when these cells are activated by pro-inflammatory cytokines, such as TNF- α . It is considered that pathogenic ANCA can bind to the cell surface MPO of the pro-inflammatory cytokine-primed neutrophils, which leads to excessive activation of neutrophils and subsequent destruction of small vasculature.² This concept called “ANCA-cytokine sequence theory” is commonly accepted as the critical part in the pathogenesis of MPO-ANCA-associated MPA.³ However, it remains unrevealed why such pathogenic MPO-ANCA is produced.

Some patients under the antithyroid drug regimen, propylthiouracil (PTU), are known to develop MPA with production of MPO-ANCA.⁴ Recently, we have demonstrated that the process of abnormal formation and impaired degradation of neutrophil extracellular traps (NETs) induced by PTU was critically involved in the generation of MPO-ANCA and subsequent development of MPA.⁵⁻⁷ NETs represent the unique death of neutrophils, in which there is extracellular release of chromatin fibers and antibacterial cytoplasmic proteins, including MPO.⁸ The process is an innate defense mechanism to trap and kill invading microbes.⁹ Interestingly, NETs have been detected in glomerular crescents in patients diagnosed with MPA regardless of the absence of infectious agents.¹⁰ Based on the collective evidence, we hypothesize that the persistent MPO in the PTU-induced protracted NETs could be recognized as an autoantigen by the immune system.⁵ This hypothesis corresponds to the finding that

NETs mediate the transfer of MPO to myeloid dendritic cells toward ANCA induction.¹¹ However, the majority of MPA patients develop the disease without administration of PTU or related drugs. Thus, we consider that there are mechanisms unrelated to PTU but with influence on the formation and regulation of NETs in MPA.¹²

In this study, we first focused on the ability of serum IgG to induce NETs and then demonstrated the high ability of MPO-ANCA-associated MPA IgG for NETs induction. The ability for NETs induction was correlated to the disease activity and parallel to the ANCA affinity to MPO. Furthermore, the NETs induction ability was absorbed by addition of recombinant human MPO in the IgG samples. These findings indicated that the NETs induction factor in MPO-ANCA-associated MPA serum was MPO-ANCA. Next, we examined the NETs degradation ability of MPO-ANCA-associated MPA serum. NETs degradation ability of serum was generally low in MPO-ANCA-associated MPA, and it was partially but not completely recovered by depletion of IgG from the sera. This finding suggested the presence of serum factors that precluded NETs degradation in MPO-ANCA-associated MPA in addition to IgG. Correspondingly, low activity of DNase I, an important regulator of NETs in the serum, was determined in MPO-ANCA-associated MPA. Furthermore, the presence of anti-NETs antibodies, which could possibly interfere with the degradation of NETs by DNase I, was demonstrated in some patients with MPO-ANCA-associated MPA. These findings demonstrated the high ability for NETs induction and low ability for NETs degradation of serum in MPO-ANCA-associated MPA patients. The collective evidence suggests that a vicious cycle through NETs and MPO-ANCA could be involved in the pathogenesis of MPO-ANCA-associated MPA.

Results

High ability of serum IgG from MPO-ANCA-associated MPA patients for NETs induction

First, we confirmed the high ability of MPO-ANCA-associated MPA IgG for NETs induction which was reported previously.¹⁰ NETs induction was quantified by 2 independent methods, including IF for citrullinated histone 3 (Cit H3)-positive neutrophils (Figure 1, A-G) and ELISA for MPO-DNA complexes in culture supernatants (Figure 1H). Since deimination of histones is an essential process for the generation of NETs, the detection of Cit H3-positive neutrophils is regarded as an accurate method to identify NETs.^{13,14} The MPO-DNA complexes in the serum and culture supernatants were shown to reflect the amounts of NETs in the tissue¹⁰ and dish,⁵ respectively. There was a significant correlation between the values of IF and ELISA ($R^2=0.2485$, $p<0.01$); thus, the results indicated the reliability of these 2 methods (Supplemental Figure 1). In this experiment, neutrophils from healthy volunteers were pre-treated with TNF- α . When the TNF- α -primed neutrophils were incubated with serum IgG from MPO-ANCA-associated MPA patients, the rate of Cit H3-positive neutrophils was 16.6 ± 9.7 % (Figure 1G). This value was significantly higher than when the neutrophils were incubated with serum IgG from healthy controls (3.2 ± 1.4 %). Correspondingly, the amounts of MPO-DNA complexes in the supernatants were significantly higher when the TNF- α -primed neutrophils were incubated with serum IgG from MPO-ANCA-associated MPA patients (O.D.: 0.29 ± 0.21) than from healthy controls (O.D.: 0.13 ± 0.03) (Figure 1H). Although the NETs induction ability was also present in serum IgG from patients with systemic lupus erythematosus (SLE), the ability of SLE IgG was not so high (7.0 ± 3.5 % in IF, O.D.: 0.25 ± 0.10 in ELISA) compared to MPO-ANCA-associated MPA IgG (Figure 1, G and H).

MPO-ANCA as NETs induction factor in MPO-ANCA-associated MPA serum

In order to determine that the NETs induction ability of MPO-ANCA-associated MPA IgG could be caused by MPO-ANCA, recombinant human MPO was added to the IgG samples. When neutrophils were incubated with the anti-human MPO monoclonal antibody, NETs were induced (Figure 2A). On the other hand, the NETs induction was inhibited by addition of recombinant MPO dose-dependently (Figure 2, B and C). Similarly, the NETs induction ability of MPO-ANCA-associated MPA IgG was absorbed by the addition of MPO (Figure 2, D-G). These findings indicated that the NETs induction factor in MPO-ANCA-associated MPA serum was MPO-ANCA.

Association of NETs induction ability with vasculitis activity and MPO-ANCA affinity

Next, we determined that the NETs induction ability of MPO-ANCA-associated MPA IgG was correlated to the disease activity as represented by the Birmingham Vasculitis Activity Scores (BVAS) (Figure 3A). The result was in contrast to the finding in SLE patients that the NETs induction ability did not show a significant correlation to the disease activity as represented by the SLE disease activity index (SLEDAI) (Supplemental Figure 2). Although MPO-ANCA is a useful diagnostic marker of MPA, it is known that the disease activity sometimes dissociates from the ANCA titer.¹⁵ Correspondingly, the NETs induction ability of MPO-ANCA-associated MPA IgG did not show a significant correlation to the MPO-ANCA titer in our patients (Figure 3B). On the other hand, good association between MPO-ANCA affinity and disease activity was reported with clinical evidence.¹⁶ According to this finding, we determined if the NETs induction ability of MPO-ANCA-associated MPA IgG would be correlated to the MPO-ANCA affinity, which was measured by the competitive inhibitory ELISA as previously described.¹⁶ Among 9 patients with MPO-ANCA-associated MPA included in this study, 4 patients had high affinity ANCA; whereas, 5 patients had low affinity

ANCA (Supplemental Figure 3A). In consistence with the earlier report,¹⁶ BVAS were significantly higher in patients with high affinity MPO-ANCA than with low affinity MPO-ANCA (Supplemental Figure 3B). As expected, NETs induction ability of serum IgG from patients with high affinity MPO-ANCA was significantly higher than that from patients with low affinity MPO-ANCA (Figure 3C).

Low ability of MPO-ANCA-associated MPA serum for NETs degradation

It was reported that sera from healthy individuals could digest NETs effectively, but the sera from SLE patients had low ability for NETs degradation.¹⁷ We determined if the sera from MPO-ANCA-associated MPA patients would be impotent to digest NETs similar to SLE. For this purpose, 10 % serum was added to NETs, which were induced on healthy donor neutrophils by phorbol myristate acetate (PMA) (Supplemental Figure 4). Sera from healthy controls could digest the PMA-induced NETs almost completely (NETs degradation rates more than 80 %). In contrast, most MPO-ANCA-associated MPA and SLE sera examined in this study showed impaired degradation of the PMA-induced NETs (NETs degradation rates less than 80 %) (Figure 4, A and B). The NETs degradation rates of MPO-ANCA-associated MPA and SLE sera were 47.4 ± 24.2 % and 48.8 ± 21.7 %, respectively. Both rates were significantly lower than that of healthy controls (91.5 ± 5.1 %) (Figure 4B).

Low DNase I activity in MPO-ANCA-associated MPA serum

In order to eliminate the NETs induction effects by MPO-ANCA-associated MPA IgG on NETs digestion, NETs degradation ability of IgG-depleted serum was examined. Results indicated that the NETs degradation ability was recovered in some samples by IgG-depletion (6 out of 15 samples) (Figure 5A, Group 1). At this time, although the mean value of NETs degradation ability tended to increase by IgG-depletion, there was

no significant difference between the sera with and without IgG-depletion (Figure 5B). These findings suggested the presence of other serum factors that precluded NETs degradation in MPO-ANCA-associated MPA besides IgG. In particular, NETs are known to be digested by serum DNase I physiologically. It has been reported that DNase I activity in the serum was generally low in SLE,¹⁸ and that the low DNase I activity could induce the abnormal regulation of apoptotic cells in SLE.¹⁹ Similar to SLE, the DNase I activity in sera from MPO-ANCA-associated MPA patients was significantly lower than that in healthy controls (Controls: 52.6 ± 12.1 %, MPO-ANCA-associated MPA: 28.3 ± 7.8 %, SLE: 26.7 ± 14.3 %) (Figure 6A). Interestingly, the DNase I activity in MPO-ANCA-associated MPA serum was not correlated to the disease activity as represented by BVAS (Figure 6B), a feature which is similar to SLE (Supplemental Figure 5). In order to demonstrate that the low activity of DNase I in MPO-ANCA-associated MPA serum contributed to the low ability for NETs degradation, we determined if addition of DNase I to the MPO-ANCA-associated MPA sera with depletion of IgG would recover the NETs degradation ability. As shown in Figure 5A, all samples (N=15, including 9 samples in Group 2) recovered the NETs degradation ability by the combination of IgG-depletion and addition of DNase I. Correspondingly, the mean value of NETs degradation ability in MPO-ANCA-associated MPA serum was significantly increased by the combination of IgG-depletion and DNase I-addition (Figure 5B).

Presence of anti-NETs antibodies in MPO-ANCA-associated MPA

The NETs degradation ability in sera was increased by depletion of IgG in some MPO-ANCA-associated MPA patients (Figure 5A, Group 1). Although this could be interpreted by the removal effect of the NETs-inducible MPO-ANCA, another possibility is the presence of inhibitory antibodies for NETs degradation in

MPO-ANCA-associated MPA serum. Based on this consideration, we determined if anti-NETs antibodies would be present in MPO-ANCA-associated MPA serum. As a result, the presence of anti-NETs antibodies was demonstrated in some MPO-ANCA-associated MPA patients in Group 1 but not in Group 2 and in healthy controls (Figure 7A). The amount of anti-NETs antibodies was not correlated to the titer of MPO-ANCA in the serum (Figure 7B). In addition, the intervention of anti-MPO antibody did not inhibit the NETs degradation by DNase I (Figure 7C).

Discussion

Although NETosis is an essential defense mechanism, its disorder could be related to autoimmune diseases, including SLE^{20,21} and rheumatoid arthritis.²² In the present study, we demonstrated 3 diverse disorders in MPO-ANCA-associated MPA serum, which were related to the formation or regulation of NETs.

Firstly, the high ability of serum IgG for NETs induction in MPO-ANCA-associated MPA was shown. Although the feature of MPA IgG has been reported previously,¹⁰ as far as we know, this is the first evidence that the NETs induction ability of MPO-ANCA-associated MPA IgG is correlated to the disease activity and reflects the ANCA affinity to MPO. In addition, the NETs induction ability of MPO-ANCA-associated MPA IgG was absorbed by addition of recombinant human MPO in the IgG samples. These findings indicate that the NETs induction factor in MPO-ANCA-associated MPA serum is MPO-ANCA. On the other hand, it has not been revealed what factors contribute to the NETs induction in SLE serum. Although one or more autoantibodies seem likely to play a role in the NETs induction, further studies are needed to identify the factors.

Secondly, as reported in SLE,¹⁷ NETs degradation ability in the serum was significantly lower in MPO-ANCA-associated MPA patients than healthy controls. The serum DNase I activity is generally low in SLE,¹⁸ and the low levels of DNase I activity in the serum could cause the disordered elimination of apoptotic cells in SLE.¹⁹ Further investigation is needed to clarify if the disorder of scavenger system for apoptotic cells is involved in the pathogenesis of MPO-ANCA-associated MPA, as well as in SLE. Although we should take into account the possibility that the low level of DNase I activity in the sera of these patients could be caused by the consumption of DNase I in order to eliminate NETs, it seems unlikely relevant because the serum DNase I activity

in these patients is not correlated to the disease activity. It is rather considered that the low level DNase I activity in the serum, which may be defined genetically, is a common feature between MPO-ANCA-associated MPA and SLE. In addition, the low levels of serum DNase I activity in both SLE and MPO-ANCA-associated MPA patients could induce impaired degradation of NETs and consequent breakdown of tolerance against NETs components, which result in the production of anti-DNA antibodies¹⁷ and anti-MPO antibodies (MPO-ANCA),^{5,12} respectively.

Thirdly, the presence of anti-NETs antibodies was demonstrated in some patients with MPO-ANCA-associated MPA. The amount of anti-NETs antibodies was not correlated to the MPO-ANCA titer, and the intervention of the anti-MPO antibody did not inhibit the DNase I activity. In addition, most MPA patients did not produce anti-DNA antibodies. These findings suggested that the anti-NETs antibodies in MPO-ANCA-associated MPA were regarded as autoantibodies other than MPO-ANCA or anti-DNA antibodies. In SLE, the presence of anti-NETs antibodies, including anti-DNA antibody, was shown, and that these anti-NETs antibodies could interfere with DNase I, the important regulator of NETs.¹⁷ Similar to SLE, the anti-NETs antibodies could possibly contribute to the low ability for NETs degradation in MPO-ANCA-associated MPA. The components of anti-NETs antibodies in MPO-ANCA-associated MPA IgG should be revealed in future studies.

Although these 3 mechanisms have individual variations, the comprehensive feature of MPO-ANCA-associated MPA serum could cause the excessive formation and persistence of NETs. Since such dysregulation of NETs could induce NETs producers, including MPO-ANCA, and inhibitors for NETs regulators, including anti-NETs antibodies, it is considered that a vicious cycle through NETs and MPO-ANCA, namely “NETs-ANCA vicious cycle,” is critically involved in the pathogenesis of MPO-ANCA-associated MPA.

Based on the involvement of the “NETs-ANCA vicious cycle” in the pathogenesis of MPO-ANCA-associated MPA, novel therapies can be considered. It is known that nicotinamide adenine dinucleotide phosphate (NADPH) oxidase²³ and peptidylarginine deiminase 4 (PAD4)¹³ are essential for the generation of NETs. Blockades for NETs generation, such as NADPH oxidase inhibitors²⁴ and PAD4 inhibitors,²⁵ could be candidates to cause interference in the “NETs-ANCA vicious cycle” in the pathogenesis of MPA. Although further examinations using animal models and prospective clinical trials are needed to establish novel therapeutic strategies for the treatment of MPO-ANCA-associated MPA, we have a foresight for future studies.

Concise Methods

Patients and blood samples

Patients enrolled in this study included 38 MPA and 23 SLE patients diagnosed and treated in Hokkaido University Hospital between January 2008 and May 2012. Among the 38 MPA patients, there were 31 females and 7 males (age, mean \pm standard deviation: 66.7 ± 13.3 years; range: 38 to 82 years). All MPA patients enrolled in this study were positive for MPO-ANCA but negative for proteinase 3 (PR3)-ANCA. After acquirement of written informed consent, peripheral blood samples were obtained without anticoagulants, and sera were stored at -80 °C until use. At the time of blood sampling, BVAS and SLEDAI were recorded concerning MPA patients and SLE patients, respectively. For controls, peripheral blood samples were obtained from 8 healthy volunteers.

NETs induction by IgG eluted from serum

IgG was eluted from sera using an immunoabsorbent column (Protein G HP SpinTrap, GE healthcare, Tokyo, Japan). Contamination of endotoxin in the IgG samples was ruled out using the Limulus test kit (Wako Pure Chemical, Osaka, Japan). Neutrophils were extracted from peripheral blood samples of healthy volunteers as described.⁵ The neutrophils were seeded on chamber slides (1×10^6 /ml), pre-treated with 5 ng/ml TNF- α for 15 min at 37 °C, and then treated with 250 μ g/ml of eluted IgG from MPO-ANCA-associated MPA patients (N=38), SLE patients (N=23), and healthy controls (N=8). After incubation for 3 hours at 37 °C, the supernatants were collected and the remaining samples on the slides were fixed with 4 % paraformaldehyde (PFA).

IF for Cit H3-positive neutrophils

After fixation with 4 % PFA, the samples were made to react with 1:100 dilution of anti-Cit H3 antibody (rabbit IgG) (Abcam, Tokyo, Japan) for 60 min at room temperature. After removal of unbound antibody by washing with PBS, the samples were next allowed to react with 1:500 dilution of Alexa Fluor 594-conjugated goat anti-rabbit IgG (Invitrogen, Tokyo, Japan) for 60 min at room temperature. After washing with PBS, DNA was stained using DAPI (Sigma-Aldrich, St. Louis, MO), which was contained in mounting solution. The Cit H3 and DAPI-positive NETs were counted, and then the data were standardized by the number of DAPI-positive neutrophils.

ELISA for MPO-DNA complexes

The MPO-DNA complexes in supernatants were measured using the capture ELISA system as previously described.¹⁰

Absorption of MPO-ANCA by recombinant MPO

Recombinant human MPO (1 µg/ml or 100µg/ml) (Creative BioMart, Shirley, NY) was added to the IgG samples obtained from MPO-ANCA-associated MPA patients, and then the NETs induction assay was performed as described above. For control, mouse anti-human MPO monoclonal antibody (AbD Serotec, Düsseldorf, Germany) was used instead of the IgG samples.

Measurement of MPO-ANCA affinity

The MPO-ANCA affinity was determined by the competitive inhibition assay in the ELISA system as previously described.¹⁶

NETs degradation by serum

Peripheral blood neutrophils obtained from healthy volunteers were seeded on chamber slides (1×10^6 /ml), and then treated with 100 nM PMA for 3 hours at 37 °C. After washing with PBS, the samples were incubated in 10 % serum for 6 hours at 37 °C. For negative control, PBS was used instead of sera. In order to stop the serum nuclease activity, 2 mM EDTA was added, and then the samples were fixed with 4 % PFA followed by mounting with the solution containing DAPI. The DAPI-positive residual NETs were quantified using Image J software. NETs degradation rate (%) was calculated as follows; $\{(\text{residual NETs incubated with PBS}) - (\text{residual NETs incubated with serum}) / (\text{residual NETs incubated with PBS})\} \times 100$.

Measurement of DNase I activity

Serum DNase I activity was measured using the ELISA kit (Orgentec GmbH, Mainz, Germany) according to the instruction of the manufacturer. Briefly, sera were allowed to react with the specific substrate coated on the ELISA plate. After incubation for 1 hour at 37 °C, horseradish peroxidase-conjugated antibodies to the residual DNase I substrate were added. Then, the developing color of the tetramethylbenzidine substrate, which showed a negative correlation to the amount of active DNase I in the serum, was measured using a spectrophotometer.

Interference with DNase I by anti-MPO antibody

NETs were induced by PMA as described above. The NETs were treated by 1 U/ml of DNase I with 10 µg/ml or 100 µg/ml of the anti-human MPO antibody (AbD Serotec) at room temperature. Before and 10, 20, and 30 min after the treatment, the residual NETs were determined using Image J software.

Statistics

Student *t*-test was applied for comparison of mean values between 2 groups. Pearson test was applied to evaluate the correlation of values between 2 groups. A *p*-value below 0.05 was regarded statistically significant.

Study approval

This study was permitted by the Institutional Clinical Research Committee in Hokkaido University Hospital (No. 12-0423).

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Disclosures

None.

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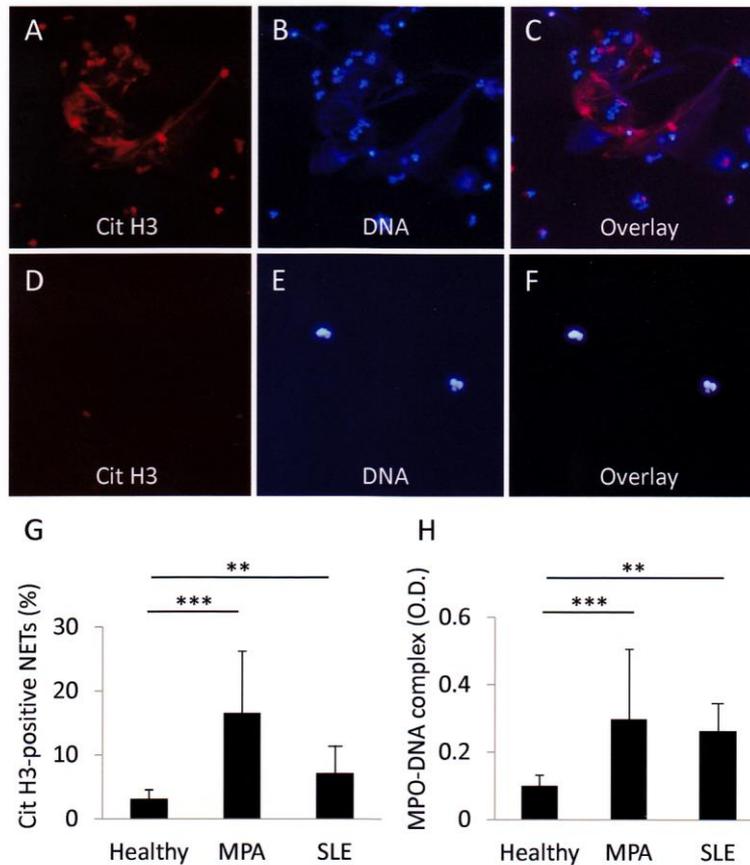


Figure 1

High ability of MPO-ANCA-associated MPA IgG for NETs induction. Neutrophils (1×10^6) from healthy volunteers were pre-treated with 5 ng/ml of TNF- α for 15 min at 37 °C. The TNF- α -primed neutrophils were incubated with 250 μ g/ml of MPO-ANCA-associated MPA IgG (A-C) and control IgG (D-F) for 3 hours at 37 °C. Representative figures are shown. A and D: Cit H3, B and E: DNA, C and F: Overlay. Original magnification: $\times 400$. (G) NETs induction rate was evaluated by calculating the proportion of Cit H3-positive neutrophils in IF. (H) MPO-DNA complexes in the supernatants were measured by ELISA. Healthy: N=8, MPO-ANCA-associated MPA: N=38, SLE: N=23. ** $p < 0.01$, *** $p < 0.001$.

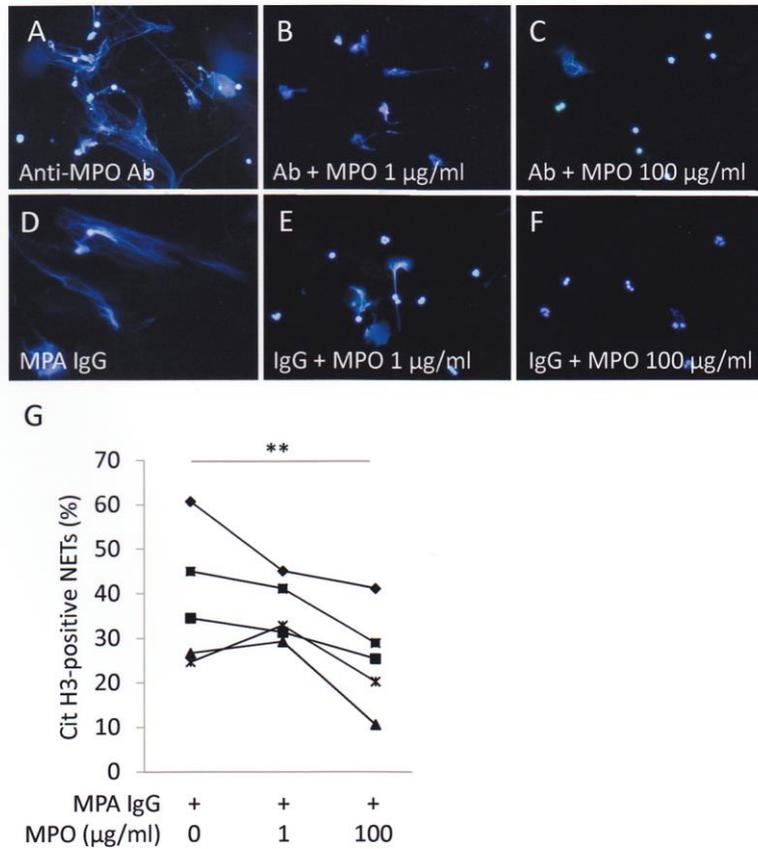


Figure 2

MPO-ANCA is the factor that certainly induced NETs in MPO-ANCA-associated MPA serum. Neutrophils (1×10^6) from healthy volunteers were pre-treated with 5 ng/ml of TNF- α for 15 min at 37 °C. The TNF- α -primed neutrophils were incubated with 250 μ g/ml of mouse anti-human MPO monoclonal antibody (A-C) and MPO-ANCA-associated MPA IgG (D-F) with or without recombinant human MPO for 3 hours at 37 °C. The concentrations of recombinant human MPO were 0 μ g/ml (A and D), 1 μ g/ml (B and E), and 100 μ g/ml (C and F). NETs formation was evaluated as DAPI-positive extracellular chromatin fibers. Representative figures are shown. Original magnification: $\times 400$. (G) The NETs induction ability of MPO-ANCA-associated MPA serum (N=5) was significantly decreased by addition of 100 μ g/ml recombinant human MPO. ** $p < 0.01$.

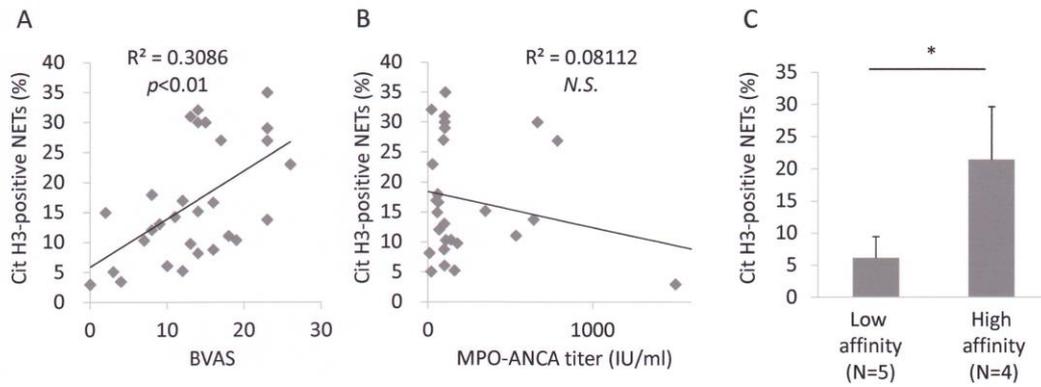


Figure 3

Association of NETs induction ability with BVAS and MPO-ANCA affinity. (A) Correlation between NETs induction ability and BVAS. (B) No correlation between NETs induction ability and MPO-ANCA titer. (C) Comparison of NETs induction ability between patients with low affinity MPO-ANCA (N=5) and with high affinity MPO-ANCA (N=4). * $p < 0.05$. *N.S.*: not significant.

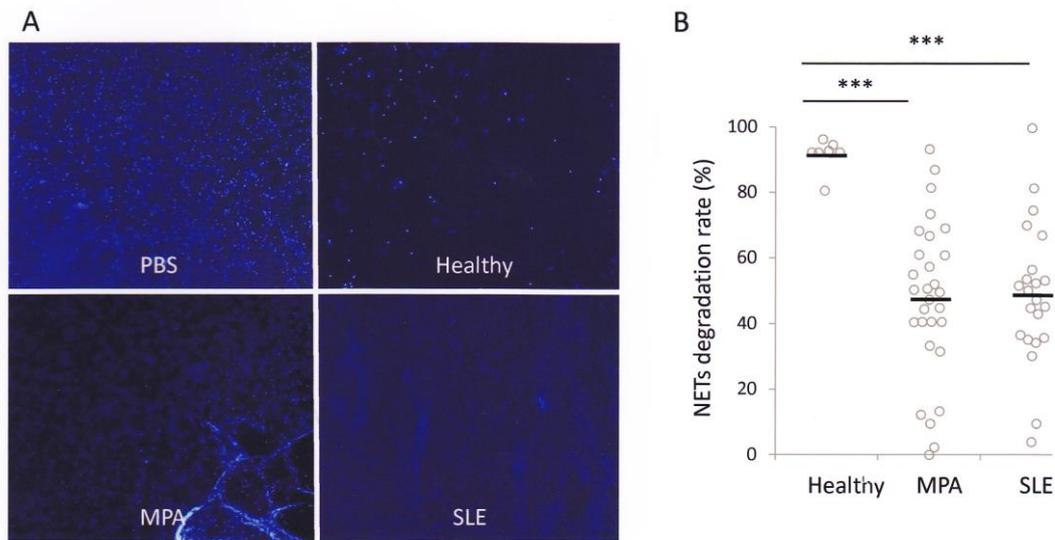


Figure 4

Low ability of MPO-ANCA-associated MPA serum for NETs degradation. (A) Neutrophils (1×10^6) from healthy volunteers were treated with 100 nM PMA for 3 hours at 37 °C. The PMA-induced NETs were incubated with 10 % serum from healthy controls, MPO-ANCA-associated MPA patients, and SLE patients, or with PBS for 6 hours at 37 °C. The residual NETs were recognized as DAPI-positive extracellular chromatin fibers. Representative figures are shown. Original magnification: $\times 40$. (B) Comparison of NETs degradation ability of serum among healthy controls (N=8), MPO-ANCA-associated MPA patients (N=38), and SLE patients (N=23). *** $p < 0.001$.

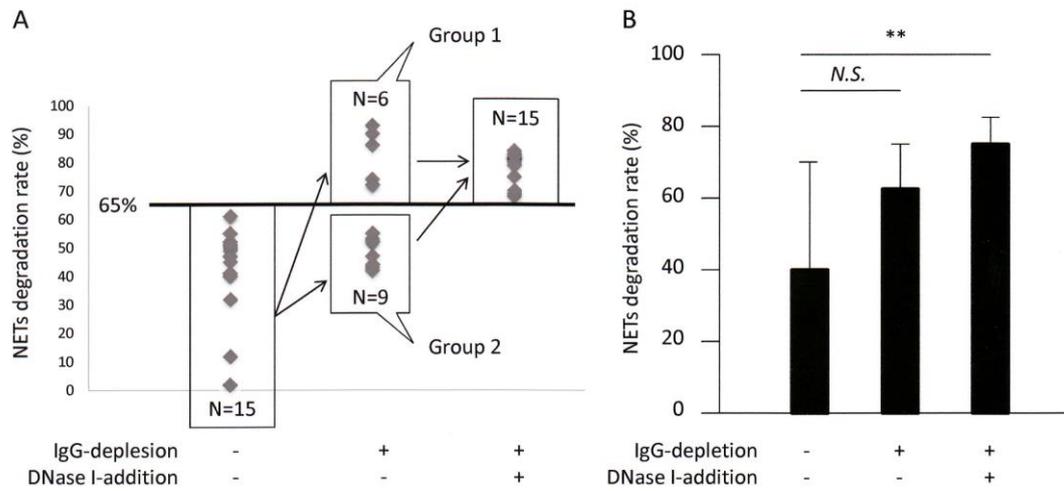


Figure 5

Recovery of NETs degradation ability of MPO-ANCA-associated MPA serum by IgG-depletion and addition of DNase I. (A) In this study, 15 MPO-ANCA-associated MPA serum samples that showed low ability for NETs degradation (NETs degradation rates less than 65 %) were included. IgG was removed from the sera using the immunoadsorbent column. Thereafter, 10 U/ml of DNase I was added to the IgG-depleted serum. After these preparations, NETs degradation ability of each sample was evaluated. Fifteen samples were classified into 2 groups; Group 1- ability for NETs degradation was recovered by IgG-depletion and Group 2- ability for NETs degradation was not recovered by IgG-depletion. (B) The NETs degradation ability of MPO-ANCA-associated MPA serum (N=15) was significantly increased by the combination of IgG-depletion and DNase I-addition but not by the IgG-depletion alone. $**p < 0.01$. *N.S.*: not significant.

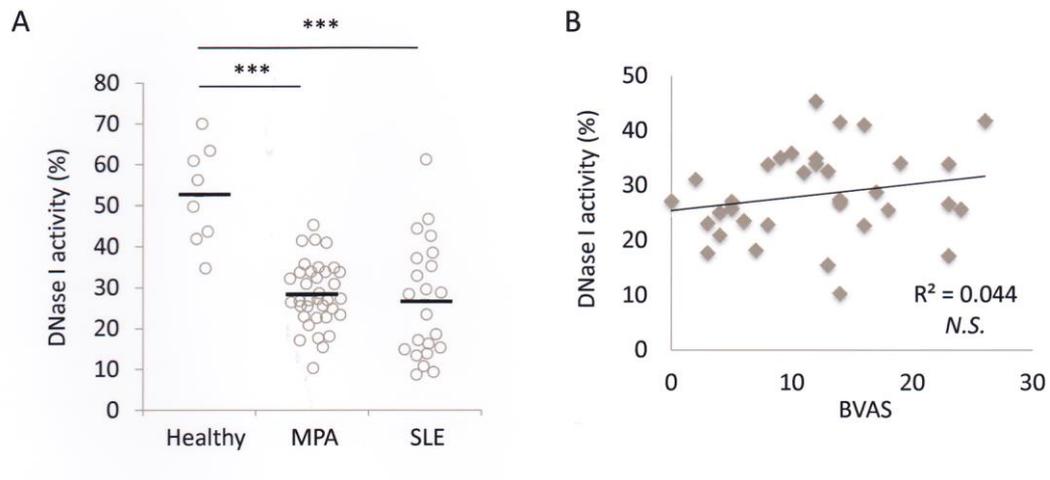


Figure 6

Low DNase I activity in MPO-ANCA-associated MPA serum. (A) Comparison of serum DNase I activity among healthy controls (N=8), MPO-ANCA-associated MPA patients (N=38), and SLE patients (N=23). (B) No correlation between DNase I activity and BVAS in MPO-ANCA-associated MPA. *** $p < 0.001$. N.S: not significant.

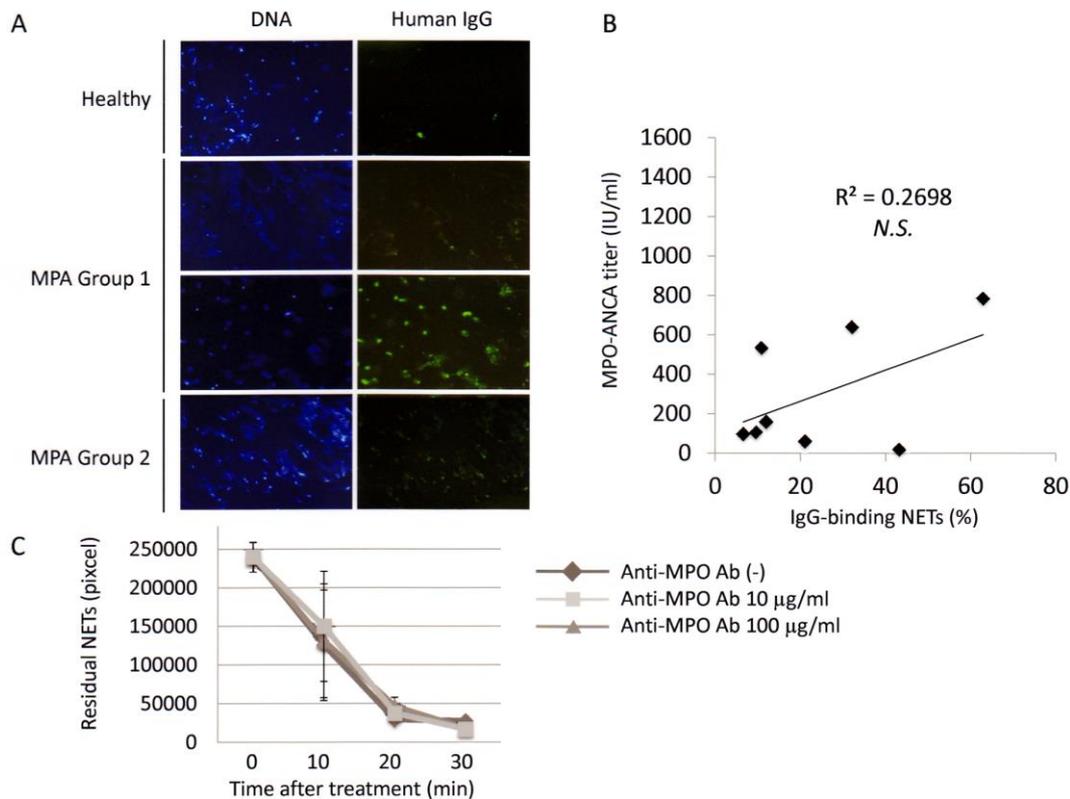
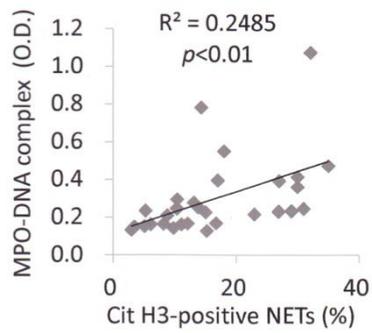


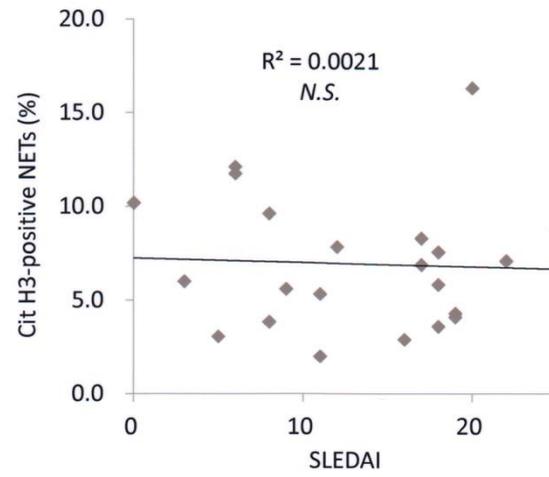
Figure 7

Identification of anti-NETs antibodies in MPO-ANCA-associated MPA IgG. (A) Neutrophils from healthy volunteers were seeded on chamber slides (1×10^6 /ml) and then treated with 20 nM PMA for 2 hours at 37 °C. After fixation with 4 % PFA, the samples were washed with PBS, and then incubated with 250 µg/ml of IgG eluted from MPO-ANCA-associated MPA sera or healthy control sera for 1 hour at 37 °C. After removal of unbound IgG, the samples were next allowed to react with 1:5000 dilution of FITC-conjugated anti-human IgG antibodies (Santa Cruz Biotechnology, Dallas, TX) for 1 hour at 37 °C. In some patients in Group 1 but not in Group 2 or healthy controls, human IgG bound to the NETs was seen. Representative figures are shown. Original magnification: $\times 100$. (B) The amount of anti-NETs antibodies was represented as the rate of IgG-binding area in NETs. No significant correlation between the amount of anti-NETs antibodies and the MPO-ANCA titer. *N.S.*: not significant. (C) The PMA-induced NETs were treated by 1 U/ml of DNase I with 10 µg/ml or 100 µg/ml of the anti-human MPO antibody at room temperature. Before and 10, 20, and 30 min after the treatment, the residual NETs were determined using Image J software. No inhibitory effect of the anti-MPO antibody on the DNase I activity was seen.



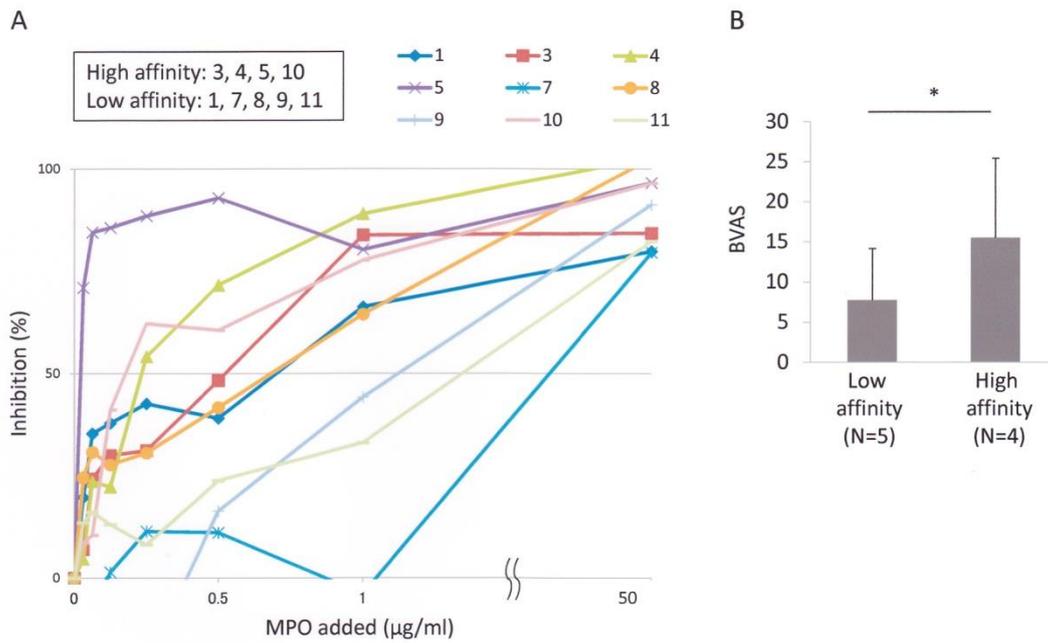
Supplemental Figure 1

Correlation between the 2 methods for NETs detection: IF for Cit H3-positive neutrophils and ELISA for MPO-DNA complexes in supernatants.



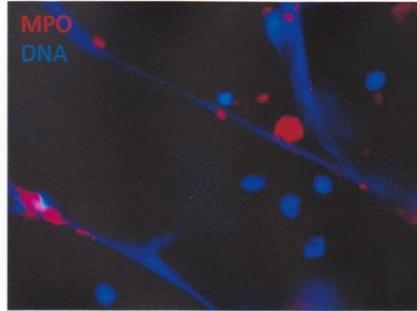
Supplemental Figure 2

No correlation between NETs induction ability and disease activity represented by SLEDAI in SLE patients.



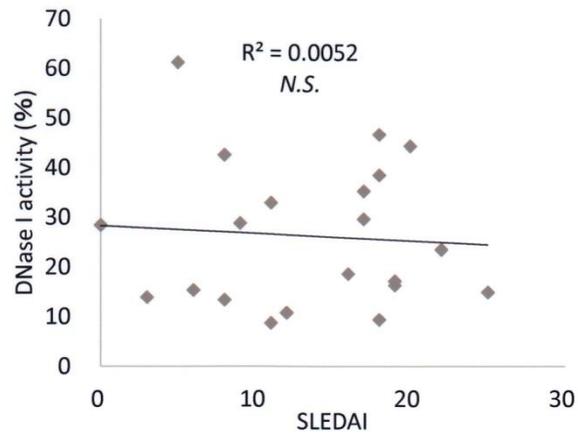
Supplemental Figure 3

MPO-ANCA affinity and disease activity. (A) MPO-ANCA affinity was determined by the competitive inhibition assay in the ELISA system. In this study, 9 MPA patients were included. They were classified into 2 groups, those with high affinity ANCA (Patient No. 3, 4, 5, 10) and those with low affinity ANCA (Patient No. 1, 7, 8, 9, 11). (B) Comparison of BVAS between the patients with high affinity ANCA and with low affinity ANCA. * $p < 0.05$.



Supplemental Figure 4

Representative figure of PMA-induced NETs. Red: MPO, blue: DNA. Original magnification: $\times 400$.



Supplemental Figure 5

No correlation between DNase I activity and disease activity represented by SLEDAI in SLE patients.