Neutrophil Extracellular Traps May Contribute to the Pathogenesis in Adult-onset Still Disease

Mi-Hyun Ahn, Jae Ho Han, Young-Jun Chwae, Ju-Yang Jung, Chang-Hee Suh, Ji Eun Kwon, and Hyoun-Ah Kim

ABSTRACT. ***Objective.*** Release of neutrophil extracellular traps (NET) has been described as an effector mechanism of polymorphonuclear neutrophils in several inflammatory diseases. Thus, this study was performed to evaluate the role of NET in the pathogenesis of adult-onset Still disease (AOSD).

Methods. We determined the serum levels of NET molecules and investigated their associations with clinical disease activities in patients with AOSD. Further, we analyzed the differences in the NETosis response in AOSD patients compared to healthy controls (HC). To explore the *in vivo* involvement of NET in AOSD, we performed immunohistochemical analysis of skin and lymph node (LN) biopsies for proteins related to NET in patients with active AOSD.

Results. Serum levels of cell-free DNA, myeloperoxidase (MPO)-DNA complex, and α-defensin were significantly increased in patients with AOSD compared to HC. Serum levels of the NET molecules, cell-free DNA, MPO-DNA, and α-defensin were correlated with several disease activity markers for AOSD. In followup of patients with AOSD after treatment with corticosteroid, the levels of cell-free DNA and α-defensin decreased significantly. On immunohistochemistry, neutrophil elastase–positive and MPO-positive inflammatory cells were detected in skin and LN of patients with AOSD, and were expressed in fiber form in the lesions. The serum from patients with active AOSD induced NETosis in neutrophils from HC. NET molecules induced interleukin 1β production in monocytes, representing a novel mechanism in the pathogenesis of AOSD.

Conclusion. The findings presented here suggest that NET may contribute to the inflammatory response and pathogenesis in AOSD. (First Release September 1 2019; J Rheumatol 2019;46:1560–9; doi:10.3899/jrheum.181058)

Key Indexing Terms:
ADULT-ONSET STILL DISEASE | NEUTROPHIL | PATHOGENESIS
NEUTROPHIL EXTRACELLULAR TRAP | DISEASE ACTIVITY

Adult-onset Still disease (AOSD), a systemic inflammatory disorder of unknown etiology, is considered an adult form of systemic juvenile idiopathic arthritis[1,2]. It is characterized by a high spiking fever, evanescent rash, arthritis, and organomegaly[3]. Proinflammatory cytokines, such as interleukin 1β (IL-1β), tumor necrosis factor-α (TNF-α), and IL-18, are elevated in patients with acute AOSD[1,4].

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Recently, the release of neutrophil extracellular traps (NET) has been described as an effector mechanism of neutrophils in many inflammatory diseases and malignancies[5]. NET are characterized by net-like chromatin fibers that can trap and possibly kill bacteria and fungi. NET contain DNA, histones, and several granular and cytoplasmic antimicrobial proteins, such as elastase and myeloperoxidase (MPO). NET formation involves the Raf-MEK-ERK pathway through the activation of NADPH oxidase, and upregulation of antiapoptotic proteins[6]. In this pathway, peptidylarginine deiminase 4–dependent citrullination of histones induces decondensation of DNA, resulting in the release of a mixture of DNA and bactericidal proteins, such as neutrophil elastase (NE), which are originally contained in intracytoplasmic granules[7,8,9]. Further, several stimuli including phorbol 12-myristate 13-acetate (PMA), calcium ionophore, and nigericin, were known to induce NET and NETosis through different signaling pathways[10]. Konig, *et al* suggested that NETosis, an antimicrobial neutrophil cell death, was different from leukotoxic hypercitrullination and defective mitophagy for a primary source of citrullinated autoantigens in several immune diseases[11].

Many studies have demonstrated the interaction between

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NET and sterile inflammation[12,13,14,15]. The activated neutrophils were able to activate antigen-presenting cells, such as dendritic cells, through the release of NET and may drive activation-related disease. AOSD is characterized by leukocytosis with neutrophilia, and some neutrophil-related chemokines were significantly elevated in patients with AOSD[1,16,17]. Moreover, the neutrophils infiltrated into the tissue of patients with AOSD and were correlated with S100A8/A9 levels[18]. However, there have been few studies regarding the role of the neutrophils in the pathogenesis of AOSD.

This study was performed to evaluate the role of NET in the pathogenesis of AOSD. We determined the serum levels of NET molecules and analyzed the response of NETosis in patients with AOSD compared to healthy controls (HC). To explore the involvement of NET in AOSD *in vivo*, we performed immunohistochemical analysis for proteins related to NET in skin and lymph node (LN) biopsies from patients with AOSD.

MATERIALS AND METHODS

Subjects. The study population consisted of 35 patients with AOSD and 20 HC. Patients were diagnosed using Yamaguchi criteria after exclusion of individuals with infectious, neoplastic, and autoimmune disorders[19]. Serum samples were collected from all subjects and stored at –70°C immediately, and followup samples were collected from 9 of the 35 patients 8.6 ± 4.3 months later. HC were recruited from among healthy individuals without a medical history of autoimmune, rheumatic, or any other diseases. AOSD disease activity was scored as described previously by Pouchot, *et al*[20]. We defined active AOSD when the patients had systemic symptoms, such as fever, pericarditis, and lymphadenopathy (systemic score ≥ 4). Inactive AOSD was defined when the patients had no systemic symptoms with systemic score ≤ 2. This study was approved by the Ajou University Hospital Institutional Review Board (IRB no. AJIRB-MED-MDB-17-65), and informed consent was obtained from all subjects in accordance with the Declaration of Helsinki.

Detection of cell-free DNA in serum of patients with AOSD. Cell-free DNA is a NET remnant that can be detected using the PicoGreen method in serum from patients with AOSD and HC[21]. Briefly, aliquots of 100 μl of serum were incubated with Quant-iT PicoGreen working reagent (Molecular Probes Inc.) and read on a fluorometer with excitation and emission wavelengths of 480 nm and 520 nm, respectively. This assay was based on Hoechst dye to detect dsDNA.

Measurement of MPO-DNA complex and α-defensin by ELISA. To quantify NET release by neutrophils, MPO-DNA complex was measured in serum from patients with AOSD and HC. We used a modified commercially available cell death detection ELISA kit (Roche). The reaction was detected and measured at 450 nm with TMB substrate (Dongin Bio). Previous studies have suggested that α-defensin (human neutrophil peptide-3) was a component of the NET[22,23]. Therefore, human α-defensin was measured using commercial ELISA kits (Hycult Biotech).

Conformation of NET molecules in skin and LN biopsy samples from patients with AOSD. We collected 16 skin and 7 LN tissue samples from 21 patients with AOSD. For comparative analyses, skin biopsy samples were obtained from 5 HC and 5 patients with psoriasis. Immunohistochemistry was performed using a Benchmark XT automated staining system (Ventana Medical Systems Inc.). The primary antibodies were anti-MPO, antihistone H1, and anti-NE antibodies (1:100; Abcam). Immunoreactive proteins were detected using a Ventana Optiview DAB kit (Ventana Medical Systems). As a control for the immunohistochemical evaluation of NE, histone H1, and MPO, we chose a nonspecific reactive hyperplasia of LN, and the lymphoid cells in the lymphoid follicle or paracotical zone and neutrophils in the LN sinus were stained. For NE and MPO, the neutrophils exhibited a granular cytoplasmic staining, and for histone H1, the lymphoid cells exhibited a nuclear staining. The staining patterns of inflammatory cells from the skin and LN biopsies were similar to those of neutrophils and lymphoid cells in the reactive LN (Figure 1 and Supplementary Figure 1, available with the online version of this article). Scores were calculated by the numbers of positive inflammatory cells (neutrophils or histiocytes) from 5 high-power fields. For immunofluorescence (IF) staining, sections were incubated with primary antibodies (1:100) for 60 min at room temperature (RT). Sections were then incubated with tetramethylrhodamine-conjugated host anti-mouse IgG antibody and fluorescein isothiocyanate-conjugated host antirabbit antibody for 60 min at RT. Nuclei were stained with DAPI. Results were analyzed by confocal microscopy (Leica TCS-NP/SP; Leica Microsystems).

Neutrophil isolation and NETosis assay. Neutrophils and serum were isolated from 7 patients with AOSD and 5 HC. Human peripheral blood neutrophils were isolated by density centrifugation using Histopaque-1119 and Histopaque-1077 double gradient reagent (Sigma Aldrich). NET induction and detection were performed with a NETosis Assay kit (Cayman Chemical). Briefly, cells treated with or without 25 nM PMA were incubated at 37°C for 4 h to induce NET formation. After induction of NET, the culture supernatant was collected and NET-associated NE activity was detected at 405 nm. Citrullinated histone H3 (CitH3) was detected from the neutrophil culture supernatant of active or inactive AOSD and HC using commercial ELISA kit (Cayman Chemical).

Expression of NET-associated proteins in neutrophils from HC treated with serum from patients with AOSD. Neutrophils from HC were incubated as described above. The neutrophils were cultured with 5% serum from 10 patients with AOSD or 4 HC in RPMI 1640 medium. The total proteins from neutrophils were extracted with radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich) containing 100× protease inhibitor (Pierce). The blotting membranes were incubated with anti-MPO (1:1000; Bio-Rad), anti-NE (1:1000; Abcam), and antihistone H3 (1:1000; Abcam). The housekeeping proteins, actin or GAPDH, were used as a control in the protein blots. Protein expression was measured using Image J as relative integrated optical density compared with housekeeping protein of untreated neutrophils.

Monocytes with NET protein complex. THP-1 (human monocytic leukemia) cells were cultured in RPMI-1640 medium (Welgene) containing 10% fetal bovine serum and antibiotics (penicillin and streptomycin; Gibco, Thermo Fisher Sci.). Also, peripheral blood mononuclear cells (PBMC) were isolated from 3 patients with AOSD and 3 HC. THP-1 and PBMC from AOSD and HC were stimulated with NET protein complex (supernatant from neutrophils stimulated with PMA). After 1, 2, 4, 12, and 24 h, the total proteins from cell lysates were isolated with RIPA buffer containing protease inhibitor (Pierce) to confirm IL-1β expression on protein blots. The membranes were incubated with anti-IL-1β antibody (1:1000; R&D Systems).

Statistical analysis. All data are presented as the means ± SD. Differences in NET component levels between groups were evaluated using the independent t test or Mann–Whitney U test. Correlations between disease activity marker levels and NET component levels were evaluated using Spearman correlation test. Wilcoxon signed-rank test was also used to compare NET component levels in patients who underwent followup serum sampling. All statistical analyses were performed using SPSS version 23.0 (SPSS Inc.). In all analyses, $p < 0.05$ was taken to indicate statistical significance.

RESULTS

Detection of NET markers in patients with AOSD and HC. Supplementary Table 1 (available with the online version of this article) summarizes the clinical characteristics of the 35 patients with AOSD and HC. The cell-free DNA levels, indicators of neutrophil cell lysis, were significantly

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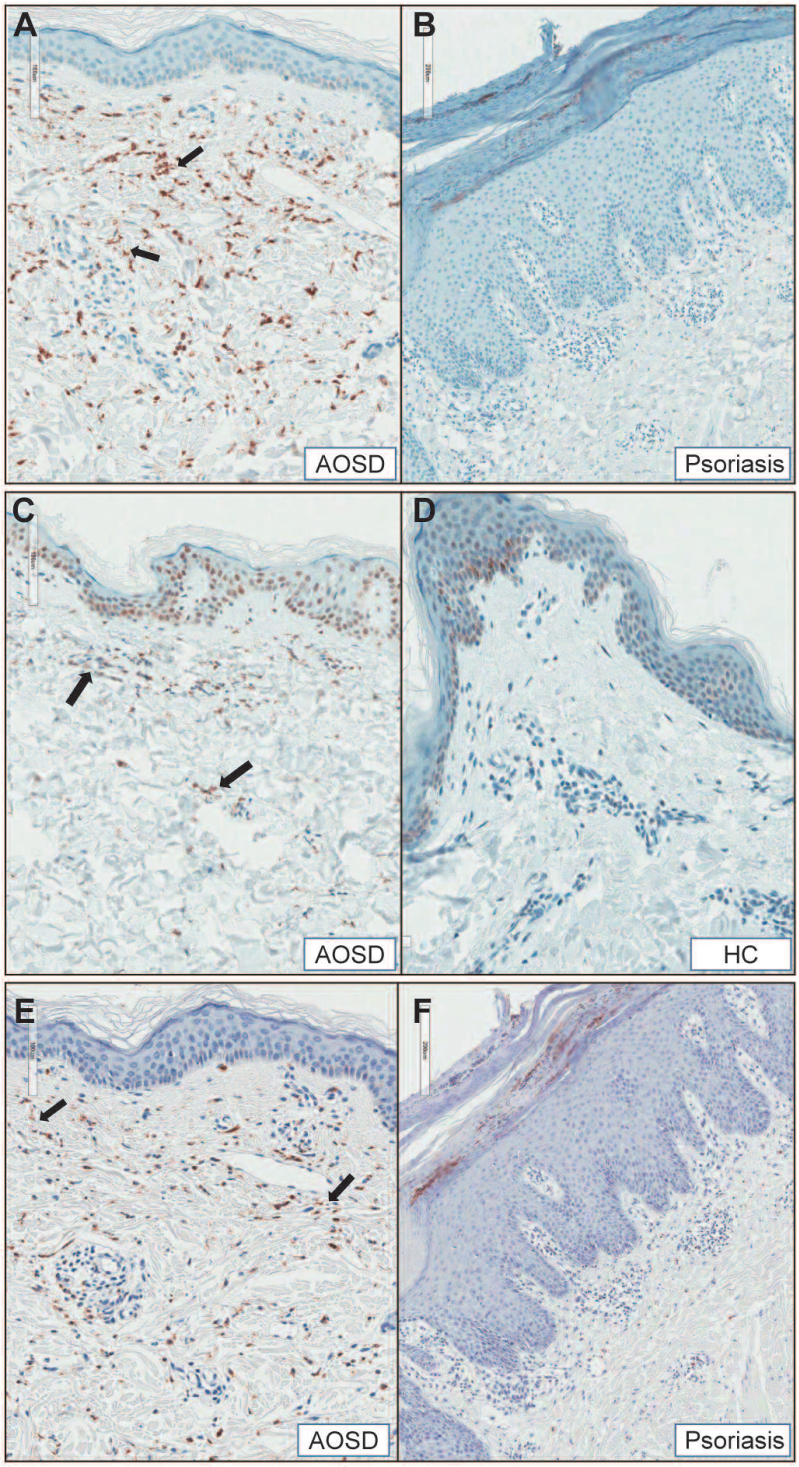

Figure 1. Neutrophil elastase (NE; panels A–B), histone H1 (panels C–D) and myeloperoxidase (MPO; panels E–F) expression in inflammatory cells of skin biopsy from patients with AOSD, psoriasis, and HC. Original magnifications ×100 (B, F) and ×200 (A, C, D, E). Note that neutrophils and nuclear dust were positive for markers of NE, histone H1, or MPO. The fiber formation was occasionally seen outside of cells (arrows). In contrast, the infiltrating lymphocytes were mostly negative to above markers in skin biopsy from AOSD, psoriasis patients, and HC. AOSD: adult-onset Still disease; HC: healthy controls.

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increased in patients with AOSD (594.1 ± 483.6 ng/ml) compared with HC (305.5 ± 106.1 ng/ml, p = 0.002; Figure 2A). In particular, the MPO-DNA complex, which is a specific marker of NET, was increased in patients with AOSD (111.3 ± 101.9%) compared to HC (69.0 ± 38.1%, p = 0.037; Figure 2B). The level of the nuclear protein, α-defensin, was also increased in AOSD (1221.3 ± 767.3 pg/ml) compared to HC (734.9 ± 338.7 pg/ml, p = 0.014; Figure 2C). Correlations between the levels of disease activity markers and the NET markers are shown in Table 1, and correlations between the neutrophil counts and the NET markers are shown in Supplementary Figure 2. Cell-free DNA levels were correlated with systemic score, neutrophil count, and C-reactive protein (CRP). Serum α-defensin levels were correlated with leukocyte count, neutrophil count, CRP, and bilirubin, as well as systemic score. Serum MPO-DNA levels were correlated with systemic score and bilirubin.

Followup serum samples were collected from 9 patients with AOSD 8.6 ± 4.3 months after the initial sampling (Table 2). The mean levels of cell-free DNA, α-defensin, and MPO-DNA complex were 315.9 ± 44.5 ng/ml, 725.9 ± 851.2 pg/ml, and 50.6 ± 32.0%, respectively (Figure 2D–F). The levels of cell-free DNA and α-defensin, but not those of MPO-DNA complex, were significantly decreased compared with initial samples.

Immunohistochemical data and conformation of NET in skin lesions of patients with AOSD. The levels of NET-related proteins were increased in the tissues from patients with AOSD. We examined inflammatory cells positive for various NET-related proteins in skin and LN biopsies from patients with active AOSD. The mean numbers of inflammatory cells expressing NE, histone H1, and MPO per 5 high-power fields in skin lesions from patients with AOSD were 57.3 ± 14.0, 11.2 ± 11.0, and 10.1 ± 12.6, respectively (Figure 1A, 1C, 1E). The skin lesions of patients with psoriasis and HC rarely contained cells expressing these markers (Figure 1B, 1D, 1F), and these NET molecules were more frequently expressed in inflammatory cells from patients with AOSD than in those from HC or patients with psoriasis ($p < 0.05$; Supplementary Figure 3, available with the online version of this article). Both neutrophils and nuclear dust stained positive for NE, histone H1, or MPO, and fiber formation was occasionally seen outside of cells. These values in LN lesions from patients with AOSD were 248.9 ± 100.4, 7.4 ± 6.3, and 17.7 ± 30.1, respectively (Supplementary Figure 1). Sixteen skin samples from patients with AOSD were used for confirmation of NE and histone H3. Using a slide scanner, we confirmed and compared the protein expression on H&E-stained and IF-stained slides (Supplementary Figure 4A). The NE and histone H3 were

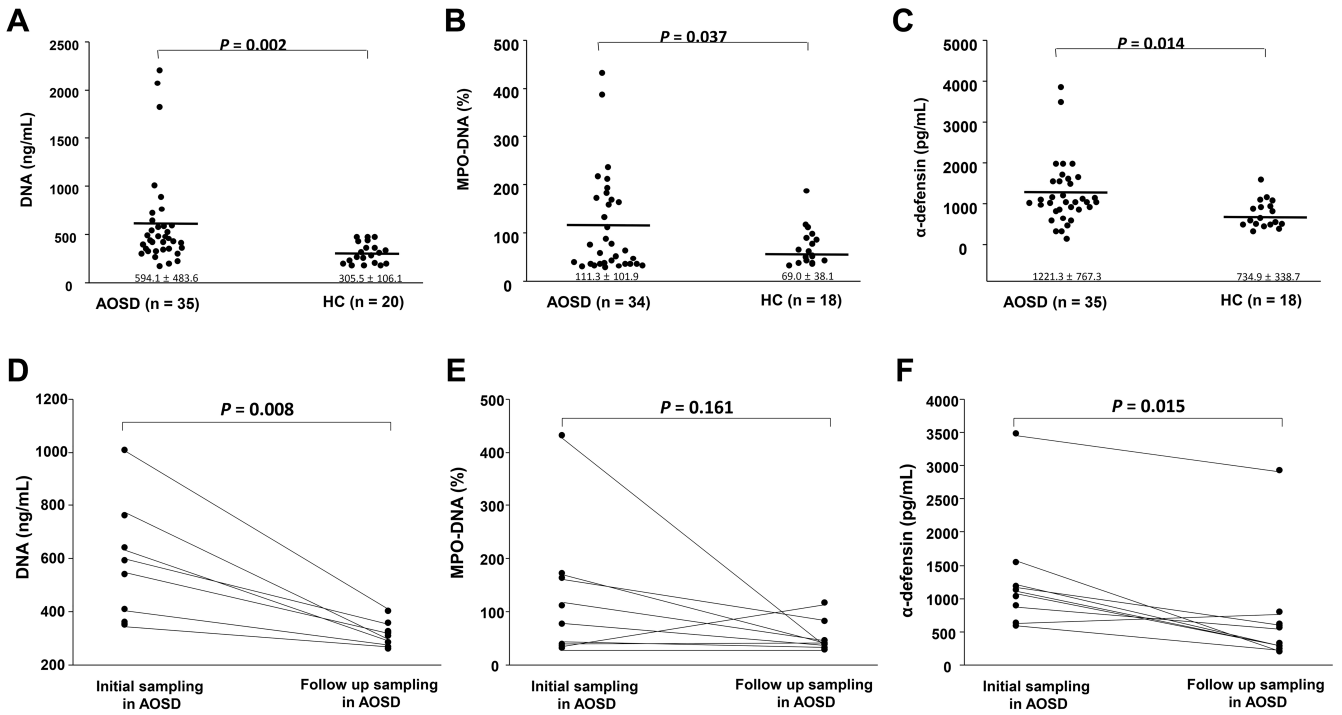

Figure 2. Levels of neutrophil extracellular trap (NET) markers in 35 patients with adult-onset Still disease (AOSD) and 20 healthy controls (HC). Cell-free DNA was detected using the PicoGreen method and the levels of MPO-DNA complex and α-defensin were measured by ELISA in serum from patients with AOSD and HC. The levels of (A) cell-free DNA, (B) MPO-DNA complex, and (C) α-defensin of patients with AOSD and HC are shown. The levels of NET markers in 9 patients with AOSD at followup are shown in panels D–F. Data are expressed as means ± SD. The independent t test and Wilcoxon signed-rank test were used for statistical analyses. MPO: myeloperoxidase.

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Table 1. Correlations of cell-free DNA, α-defensin, and myeloperoxidase (MPO)-DNA levels with disease activity markers and between each other in 35 patients with adult-onset Still disease.

Disease Activity Markers	Correlation Coefficient, r (p value) DNA	α-defensin	MPO-DNA
Systemic score	0.335 (0.049)	0.525 (0.001)	0.363 (0.038)
Leukocyte	0.281 (0.102)	0.431 (0.010)	–0.115 (0.525)
Neutrophil	0.342 (0.044)	0.409 (0.015)	–0.078 (0.668)
Platelet	–0.085 (0.627)	0.045 (0.796)	–0.111 (0.537)
ESR	0.312 (0.068)	0.387 (0.022)	0.055 (0.763)
CRP	0.345 (0.043)	0.443 (0.008)	0.111 (0.537)
Ferritin	0.082 (0.639)	0.296 (0.084)	0.053 (0.771)
Albumin	0.002 (0.993)	–0.259 (0.133)	–0.043 (0.814)
Bilirubin	0.130 (0.456)	–0.367 (0.030)	–0.355 (0.042)
AST	0.073 (0.675)	0.062 (0.725)	–0.203 (0.258)
ALT	–0.031 (0.859)	0.222 (0.200)	0.027 (0.882)
α-defensin	0.350 (0.039)		0.506 (0.003)
MPO-DNA	–0.333 (0.058)	0.506 (0.003)	

The systemic scoring system of Pouchot, *et al* assigns a score from 0 to 12, with 1 point for each of the following manifestations: fever, typical rash, pleuritis, pneumonia, pericarditis, hepatomegaly or abnormal liver function test data, splenomegaly, lymphadenopathy, leukocytosis ≥ 15,000/mm^2, sore throat, myalgia, and abdominal pain[20]. Spearman rank correlation coefficients were calculated. ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; AST: aspartate transaminase; ALT: alanine transaminase.

Table 2. Cell-free DNA, α-defensin, and myeloperoxidase (MPO)-DNA levels of active and inactive followup conditions in 9 patients with adult-onset Still disease (AOSD).

Variables	Active AOSD, n = 9	Followup Inactive AOSD, n = 9	p
DNA, ng/ml	556.9 ± 223.3	315.9 ± 44.5	0.008
α-defensin, pg/ml	1279.2 ± 872.6	725.9 ± 851.2	0.015
MPO-DNA, %	121.4 ± 129.2	50.6 ± 32.0	0.161
Leukocyte, /μl	11,944.4 ± 4144.0	8244.4 ± 2281.0	0.066
Neutrophil, /μl	10,257.4 ± 4127.8	5605.7 ± 2051.2	0.028
Ferritin, ng/ml	10,520.4 ± 18,045.2	257.2 ± 357.4	0.008
ESR, mm/h	48.0 ± 21.5	17.6 ± 12.9	0.008
CRP, mg/dl	6.44 ± 2.89	0.66 ± 1.25	0.008
LDH, mg/dl	642.9 ± 796.6	212.7 ± 57.7	0.015
AST, mg/dl	65.2 ± 48.9	32.6 ± 25.8	0.012
ALT, mg/dl	37.0 ± 25.8	28.0 ± 17.9	0.141
Systemic score	5.33 ± 2.00	0.63 ± 1.41	0.012

The systemic scoring system of Pouchot, *et al* assigns a score from 0 to 12, with 1 point for each of the following manifestations: fever, typical rash, pleuritis, pneumonia, pericarditis, hepatomegaly or abnormal liver function test data, splenomegaly, lymphadenopathy, leukocytosis ≥ 15,000/mm^2, sore throat, myalgia, and abdominal pain[20]. Data are expressed as means ± SD and were analyzed using a Wilcoxon signed-rank test. ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; LDH: lactate dehydrogenase; AST: aspartate transaminase; ALT: alanine transaminase.

expressed at high levels in fiber form within the lesions (Supplementary Figure 4B).

Comparison of NETosis activity among active or inactive AOSD and HC. The NETosis activity was determined by measuring NET-related protein expression by Western blotting and ELISA. The neutrophils were isolated from 2 active AOSD, 2 inactive AOSD, and 1 HC, and stimulated with PMA. NE expression in the cell lysate was significantly increased in patients with active AOSD compared to inactive AOSD or HC. In addition, NE was expressed weakly with PMA stimulation compared to the level without PMA stimulation (Figure 3A). This pattern was also observed on immunoblotting for MPO. NE and CitH3 were detected by ELISA in the supernatants of PMA-stimulated neutrophils from 4 patients with active AOSD, 3 patients with inactive AOSD, and 5 HC. The NE level in neutrophil culture supernatants was significantly increased in patients with active AOSD compared to inactive AOSD ($p < 0.05$) and HC ($p < 0.05$), even without PMA stimulation (Figure 3B). NE in patients with active AOSD with PMA stimulation showed a further significant increase compared to inactive AOSD patients with PMA ($p < 0.01$) and HC with PMA ($p < 0.01$). The patients with inactive AOSD showed patterns similar to those with active disease compared to HC ($p < 0.05$). CitH3 was significantly increased in active AOSD patients compared to inactive AOSD ($p < 0.05$) and HC ($p < 0.05$) without PMA. With PMA stimulation, the CitH3 level was significantly higher in patients with active AOSD or inactive disease compared to HC ($p < 0.05$; Figure 3C). With PMA stimulation, the NE levels of patients with AOSD were correlated with systemic scores ($r = 0.847$, $p = 0.016$), but CitH3 levels were not (Figure 3D-E).

NET effect on neutrophils from HC treated with serum from patients with active AOSD. The serum from patients with AOSD contained numerous possible triggers including NET protein complexes. However, it was not clear that the several molecules contained in serum from patients could trigger NETosis. Therefore, we cultured neutrophils from HC with 5% serum of 10 patients with active AOSD and 4 HC. Supplementary Table 1 (available with the online version of this article) summarizes the clinical characteristics of the 10 patients with AOSD. The NET proteins, such as MPO and NE, were expressed in the neutrophils treated with serum from 10 AOSD but were rarely expressed in those with serum from HC (Figure 4A). The biological morphology of the HC neutrophils treated with AOSD serum was changed to NETosis, similar to the HC neutrophils treated with PMA (Supplementary Figure 5).

NET effect on monocyte activation. Activated monocytes release IL-1β, which is a major cytokine involved in the pathogenesis of AOSD. IL-1β was expressed in the THP-1 cell lysate with the maximum level at 12 h after NET induction, which was stimulated by PMA and secreted from HC neutrophils (Figure 4B). The IL-1β was expressed in the PBMC lysate from AOSD with the maximum level at 4 h, and expressed in those from HC with the maximum level at 12 h after NET induction. However, IL-1β was not expressed

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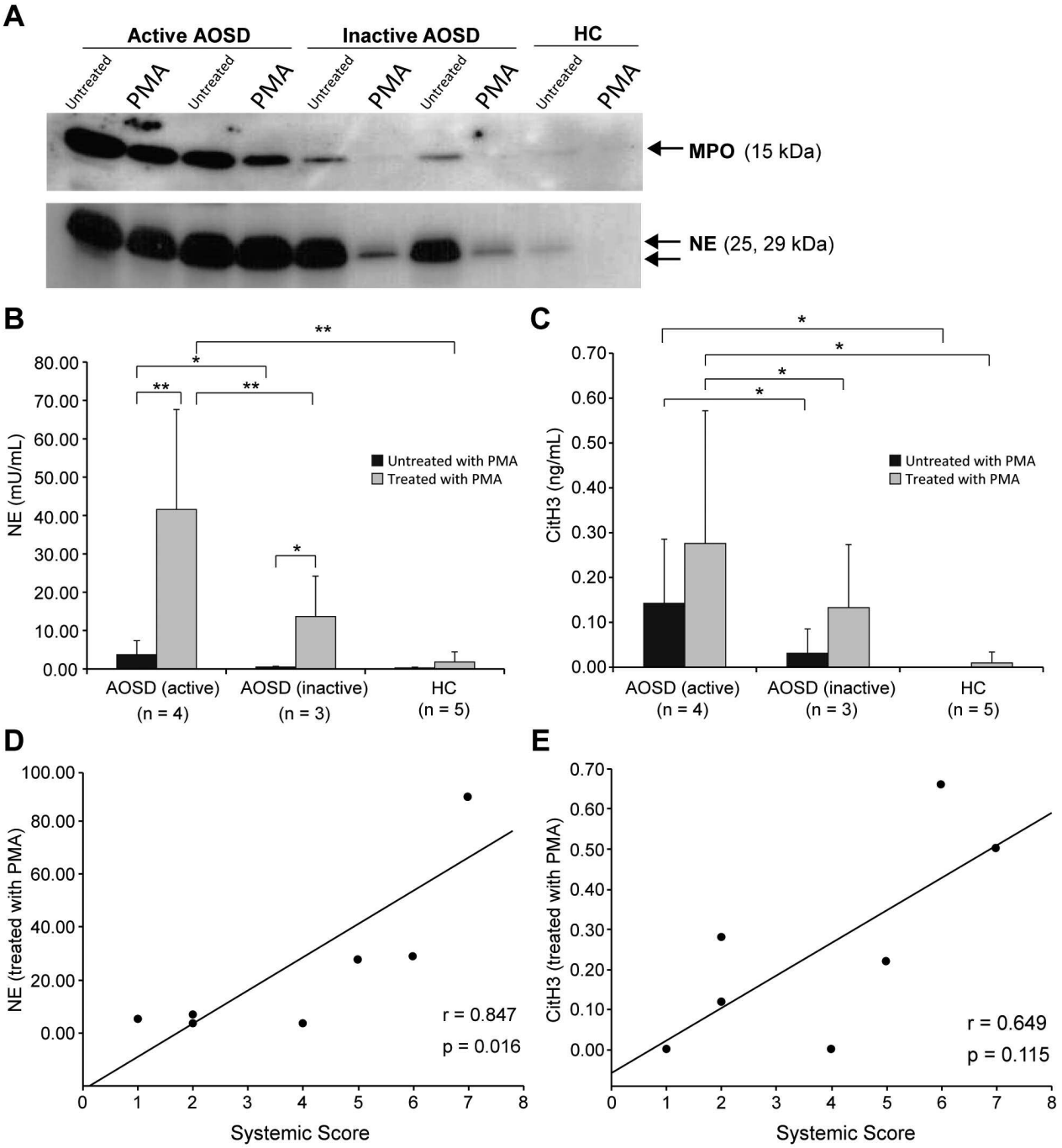

Figure 3. Differences in neutrophil extracellular trap (NET) activity between active AOSD, inactive AOSD, and HC. A. The cell lysates showed differences in NET protein levels in 2 active, 2 inactive, and 1 HC with or without 25 nM phorbol 12-myristate 13-acetate (PMA) stimulation by Western blot. B and C. The response to PMA stimulation of neutrophils from patients with AOSD and HC. The levels of neutrophil elastase (NE) and citrullinated histone H3 (CitH3) in cell-cultured supernatant were measured by ELISA in patients with 4 active AOSD or 3 inactive disease and 5 HC. * $P < 0.05$. ** $P < 0.01$. The (D) NE levels were correlated with systemic scores ($r = 0.847$, $p = 0.016$), but the (E) CitH3 levels were not ($r = 0.649$, $p = 0.115$). AOSD: adult-onset Still disease; HC: healthy controls; MPO: myeloperoxidase.

in the THP-1 cell lysate, which was treated only with PMA (data not shown).

DISCUSSION

NET formation is a potent neutrophil antimicrobial mechanism of action, and the importance of NET in host defense was previously demonstrated. After neutrophil activation, the cells become highly phagocytic and eventually undergo morphological changes that lead to NET formation[24]. Although NET function as a protective

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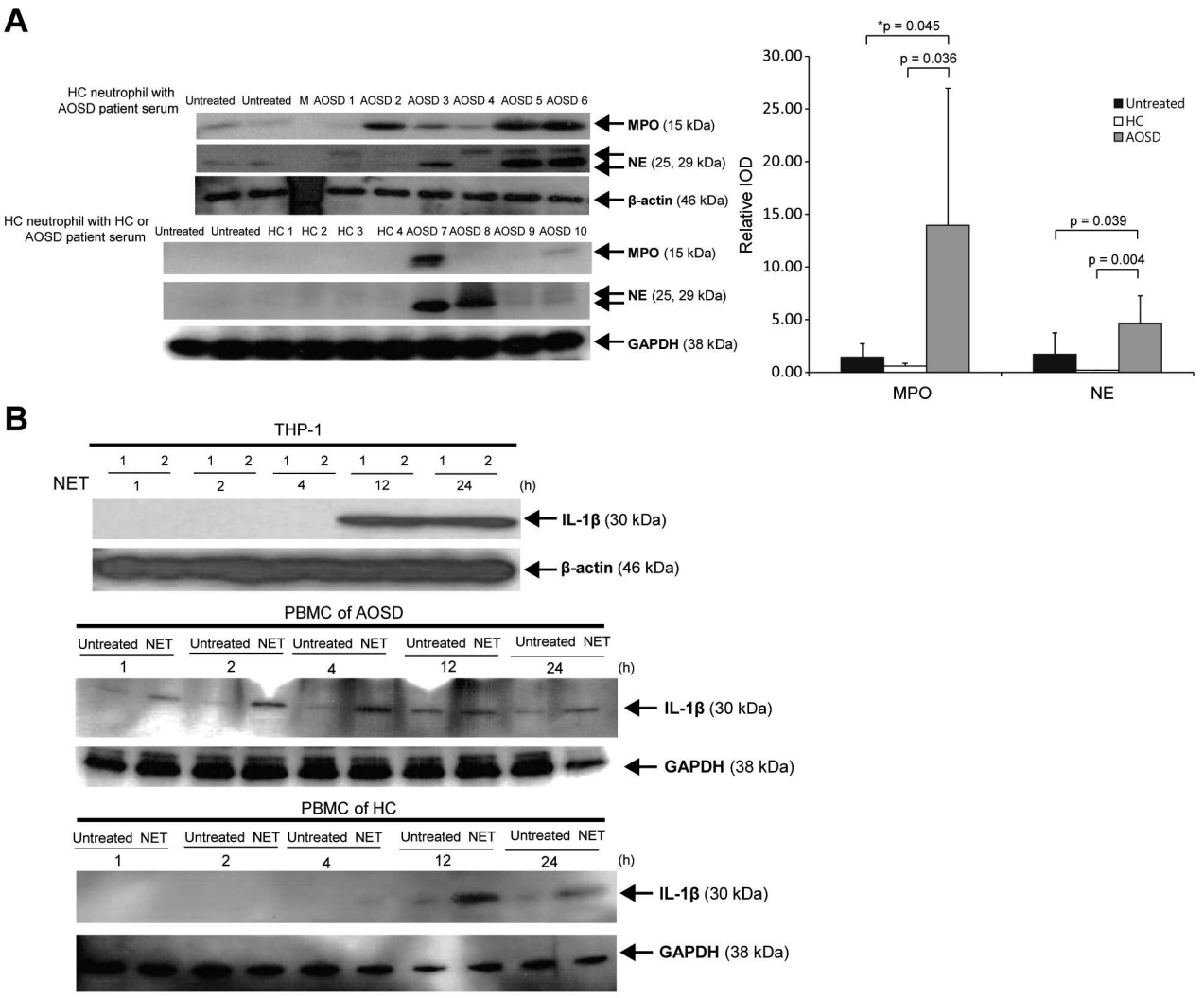

Figure 4. Effects of neutrophil extracellular trap (NET) on normal neutrophils and monocytes. A. The serum of 10 patients with AOSD induced NETosis in normal neutrophils, but the serum of 4 HC did not induce NETosis in normal neutrophils. MPO and NE are NET components. Relative IOD: each protein/GAPDH or β-actin. B. The expression of IL-1β in the monocyte cell line, THP-1 and peripheral blood mononuclear cells (PBMC) from AOSD and HC by NET stimulation. IL-1β was expressed in THP-1 cells and PBMC from AOSD and HC after NET stimulation. AOSD: adult-onset Still disease; HC: healthy controls; MPO: myeloperoxidase; NE: neutrophil elastase; IL-1β: interleukin 1β; THP-1: human monocytic leukemia; M: protein size marker.

mechanism against microbial infection, they are thought to affect the severity of immune diseases. It was also believed that this process mediated the effects of NET as a damage-associated molecular pattern (DAMP) on proinflammatory cytokines, such as IL-1β[25].

Proinflammatory cytokines, such as IL-1β, are amplified by endogenous DAMP. Our previous study showed that IL-1β level was significantly increased in patients with AOSD compared to HC, and suggested that IL-1β was amplified by S100A8/A9 as an endogenous DAMP in AOSD[18]. However, candidates for increasing IL-1β in sterile inflammatory conditions of AOSD are unclear, and neutrophils may be important effector cells in the amplified condition. Therefore, we postulated that NET could be a candidate for upregulating IL-1β in the inflammatory condition of AOSD. Indeed, we found that the levels of cell-free DNA, α-defensin, and MPO-DNA complex were significantly increased in the serum of patients with AOSD compared to HC, and the NE and MPO fibers affected skin from patients with AOSD. The serum from the patients with active AOSD induced NETosis in neutrophils from HC. Monocytes are activated from neutrophils during inflammatory reactions. We postulated that this reaction may be due to NETosis. Therefore, we cultured PBMC from HC or patients with AOSD and THP-1 with NET components from PMA-stimulated neutrophil culture supernatant, as reported previously[26]. These experiments showed that IL-1β was expressed and its level decreased in a time-dependent manner in monocytes cultured with NET molecules, thus confirming that NET induce activation of mononuclear cells to produce

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IL-1β. These results suggested that NET may represent a strategy for upregulating inflammation as DAMP in AOSD associated with overwhelming activation of the immune system.

Another study examined the mechanism underlying stress-induced inflammatory attacks with neutrophil activation and release of IL-1β–bearing NET in patients with familial Mediterranean fever (FMF)[27]. The authors reported that NET expressing IL-1β were detected in patients with AOSD, and suggested that AOSD shared similar pathways known as regulated in development and DNA damage response-1 (REDD1) to FMF, indicating IL-1β autoinflammation. Our results were consistent with this previous study, indicating increased levels of NET molecules in AOSD. Further, we showed that NET molecules could be related to IL-1β inflammation in monocytes as DAMP. A recent case report showed the dysregulation of inflammasomes associated with TNF α-induced protein 3 polymorphisms in a patient with AOSD[28]. Further, REDD1-mediated autophagy was suggested as a regulator for IL-1β–driven inflammation by both autophagy-mediated NET release and IL-1β maturation in AOSD[27]. These pathways could interact with one another to sustain inflammation, but further study of the association of NET, inflammasome, and IL-1β maturation are required to understand their involvement with the pathogenesis of AOSD.

In this study, the MPO and NE protein levels were decreased in PMA-stimulated cell lysates. However, the NE and CitH3 levels in the culture supernatants of neutrophils from active AOSD patients were significantly increased depending on disease activity. These observations indicated that NET proteins were secreted from neutrophils within 4 h of stimulation with PMA[14,29,30]. This was likely to be because the proteins inside the cell are released to the extracellular milieu during NETosis. In addition, we confirmed that serum from patients with AOSD induced NETosis of neutrophils from HC. A previous study on neutrophils indicated the induction of NETosis in neutrophils by treatment with serum from patients with AOSD containing NET components[31]. Most previous studies observed NET formation only in human cells[29,32], while our current study observed the changes directly in tissue from patients. The results confirmed that the fiber shape was maintained in the tissues.

Several studies showed that the cell-free DNA and MPO-DNA levels were related to NETosis activity[32,33]. Many groups have examined whether these NET markers reflect disease activity or severity in several inflammatory diseases[34,35,36]. One study showed that elevated plasma cell-free DNA levels were related to lupus nephritis[35]. They reported that cell-free DNA was correlated with urinary protein, albumin, and endogenous creatinine clearance rate. A previous study evaluated NET molecules as disease activity markers in active antineutrophil cytoplasmic antibody–associated vasculitis (AAV)[34]. Although serum levels of NET in patients with active AAV were significantly elevated compared to those in HC, there were no correlations between MPO-DNA complex or CitH3-DNA complex levels and CRP. In addition, there were no correlations between NET level and initial serum creatinine, estimated glomerular filtration rate, or Birmingham Vasculitis Activity Score. Therefore, in evaluating as a biomarker, NET were associated with disease activity in some diseases, but not in others. In the present study, the levels of cell-free DNA, α-defensin, and MPO-DNA complex were significantly increased in AOSD compared with HC. The levels of cell-free DNA and α-defensin in the blood of patients with AOSD were significantly decreased with normalization of neutrophil count after improvement of disease activity. Further, these NET-related molecules correlated with the levels of disease activity markers and systemic scores in patients with AOSD. These results suggest that NET molecules play a significant role in the pathogenesis of AOSD and can serve as additive markers for monitoring disease activity.

The limitations of this study included the lack of a positive control, such as AAV, for comparison with the NET molecules from patients with AOSD, and followup skin and LN biopsies in AOSD for comparison of NET molecule expression. In addition, the sample size was small for comparison with followup samples and subgroup analysis for prognosis and disease patterns. Also, we did not define the NET molecules resulting from vital, suicidal, or mitochondrial NETosis, reported previously[37,38,39,40]. To define them, further molecular pathways with several NET inducers should be studied in AOSD. In this study, we used only PMA as an inducer of NET in patients with AOSD. Further studies including larger sample sizes and control groups consisting of other disorders related to NET are required to assess the usefulness of NET molecules in patients with AOSD. We showed cell-free DNA or α-defensin levels previously reported in other conditions and compared those with our data in Supplementary Table 2 (available with the online version of this article).

We found significantly higher levels of cell-free DNA, MPO-DNA, and α-defensin in serum from patients with AOSD. We confirmed expression of these molecules in rash and lymphadenopathy material from patients with AOSD. Followup samples from patients with AOSD showed reduced cell-free DNA and α-defensin levels after improvement of disease activity. NET protein complexes from patients with AOSD induced NETosis in neutrophils from HC. After induction of NET, the neutrophils released NET molecules to the external milieu. In addition, the NET-induced IL-1β production by monocytes was a mechanism underlying the pathogenesis of AOSD. These data suggest that NET may contribute to the pathogenesis in AOSD.

ONLINE SUPPLEMENT

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