Original Article

Antibody against chromatin assembly factor-1 is a novel autoantibody specifically recognized in systemic lupus erythematosus

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Running Title: Novel SLE-specific autoantibody to CAF-1

Abstract

Autoantibodies to proliferating cell nuclear antigen (PCNA) are specifically, if rarely, present in systemic lupus erythematosus (SLE) patient sera. Even SLE patients lacking PCNA-reactivity often show reaction to PCNA-binding protein. Here, immunoreactivity to chromatin assembly factor-1 (CAF-1), an essential molecule for DNA replication and a PCNA-binding protein, was compared for the sera of SLE patients, normal healthy controls (NHCs) and other disease controls, and in autoimmune sera reactive to standard autoantigens, by enzyme-linked immunosorbent assay (ELISA), indirect immunofluorescence, and immunoblotting. *CAF1* and *IRF1* expression in SLE and NHC peripheral mononuclear cells were compared by quantitative real-time polymerase chain reaction. Serum interferon-γ-inducing protein-10 and anti-double-stranded (ds)-DNA antibody levels were measured by ELISA. Increased CAF-1 autoimmune reactivity was recognized in SLE or serum anti-ds-DNA antibody-positive patients. Significantly greater CNS involvement (aseptic meningitis) and serum anti-ds-DNA antibody titers were present more often in anti-CAF-1 antibody-positive than antibody-

negative SLE patients. IFN-γ positively regulated CAF-1 expression in vitro and was associated with anti-CAF-1 antibody production in SLE. Thus, a novel anti-CAF-1 autoantibody is frequently found in SLE patients and is a useful biomarker for diagnosis, especially in cases with CNS involvement. Aberrant IFN-γ regulation appears to play an important role in anti-CAF-1 antibody production in SLE.

Keywords

autoantibody, autoantigen, chromatin assembly factor-1, systemic lupus erythematosus

Abbreviation list

ANA: antinuclear antibod**y**

CAF-1: chromatin assembly factor-1

CNS: central nervous system

CSF: cerebrospinal fluid

ELISA: enzyme-linked immunosorbent assay

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

IP-10: interferon-γ-inducing protein-10

IRF-1: interferon regulating factor-1

MCTD: mixed connective tissue disease

NHC: normal healthy controls

NPSLE: neuropsychiatric SLE

OD: optical density

PBMCs: peripheral mononuclear cells

PBS: phosphate-buffered saline

PCNA: proliferating cell antigen

PM/DM: polymyositis/dermatomyositis

RA: rheumatoid arthritis

RT-PCR: real-time polymerase chain reaction

SS: Sjögren's syndrome

SLE: systemic lupus erythematosus

SSc: scleroderma

Introduction

Our previous studies showed that proliferating cell nuclear antigen (PCNA) is a 34-kDa intranuclear polypeptide[1, 2] that shows an increase in expression during the late G1 to S phases of the cell cycle, immediately prior to DNA synthesis.[3] PCNA was later identified as an auxiliary protein of DNA polymerase (Pol)-δ, which plays an essential role in DNA replication and repair. Further analysis of the structure and function of PCNA has revealed that PCNA interacts not only with enzymes involved in the mechanics of DNA replication and repair,[4-8] but also with many other proteins involved in DNA methylation,[9] chromatin assembly,[10] cell cycle regulation[11] and ribosomal DNA transcription.[12]

Interestingly, PCNA is known to be an autoantigen that is frequently recognized by antibodies in sera from patients with systemic lupus erythematosus (SLE).[13] Although the prevalence of anti-PCNA antibodies among patients with SLE is relatively rare (< 5%), our previous studies demonstrated that patients with SLE often develop an autoimmune response not only to PCNA itself but also to multiple PCNA-binding

proteins, which constitute the PCNA complex, even in the absence of an immune response to PCNA itself.[14, 15] We have also previously reported that SLE patient sera were more reactive to PCNA-binding proteins than were sera from patients with other connective tissue diseases and that the SLE sera often contained autoantibodies to polypeptides of PCNA-binding proteins.[15] Indeed, we identified glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as being one of the PCNA-binding proteins using 2D-PAGE and ion-pair chromatography, as well as a novel autoantigen specifically recognized by sera from SLE patients.[14] Moreover, we found that the PCNA epitope, to which the serum anti-PCNA antibody reacts, was hidden in patients with SLE when it is bound to other proteins associated with cell proliferation, although it still reacted with a series of anti-PCNA monoclonal antibodies (mAb) raised in our laboratory.[16, 17] Therefore, our findings have suggested that many PCNA-binding proteins can be targeted as autoantigens in SLE, suggesting that “intermolecular and/or intrastructural help” leads to the widening of the autoimmune response from PCNA to other functionally associated proteins.[16-18] These data also suggested that analysis of the

targeted antigens contained within PCNA-binding proteins would shed light on the mechanisms by which autoimmune responses are induced in SLE.

One of these PCNA-binding proteins, chromatin assembly factor 1 (CAF-1), was originally identified by complementation as a factor participating in the assembly of chromatin during Simian virus 40 (SV40) origin-dependent DNA replication in human cell extracts.[19] CAF-1 has been shown to colocalize with PCNA in vivo and to bind directly to PCNA in vitro.[20] Several biochemical and genetic lines of evidence have supported the role of PCNA in CAF-1-mediated nucleosome assembly.[21] CAF-1 is considered to be a chaperone protein that assists in the loading of the H3–H4 tetramer for DNA replication or repair. Further fractionation of nuclear extracts containing this activity led to the isolation of the 3 CAF-1 subunits: p150, p60, and p48.[22] Several studies have shown that CAF-1 associates with PCNA via p150.[10, 23] A yeast two-hybrid screen using human p150 as bait further confirmed this association with PCNA.[23] This data suggested that multiple sites on p150 interact with PCNA.

We conducted the present study to clarify the autoimmune response against

CAF-1, one of the PCNA-binding proteins, in SLE. We observed an increased autoimmune response to CAF-1 in the sera of SLE patients, compared to sera from disease controls and normal healthy controls (NHCs). Anti-CAF-1 antibody was found to be a novel SLE-related autoantibody; thus, this antibody is a useful biomarker for the diagnosis for SLE.

Materials and Methods

Patients and sera

Sera with human connective tissue diseases were obtained from a laboratory serum bank of Juntendo University Hospital, with institutional ethics committee approval. Sera of patients with SLE (n = 100), polymyositis/dermatomyositis (PM/DM; n = 100), scleroderma (SSc; n = 100), Sjögren's syndrome (SS; n = 100), mixed connective tissue disease (MCTD; n = 100), rheumatoid arthritis (RA; n = 100), and NHCs (n = 116) were assessed in the study. Patients with SS fulfilled the American–European Group Criteria.[24] Patients with RA, SLE, and SSc fulfilled the criteria of the

American College of Rheumatology.[25-27] Patients with MCTD fulfilled the classification criteria described by Alarcon-Segovia and Villarreal.[28] Patients with PM/DM fulfilled Bohan's criteria.[29] Autoimmune sera reacting with standard autoantigens (dsDNA; n = 98, SS-A/Ro; n = 100, U1-RNP; n = 97, and centromere; n = 58) were also obtained from the serum bank of Juntendo University Hospital. Neuropsychiatric SLE (NPSLE) was diagnosed according to standardized nomenclature system standardized by ACR.[30]

Enzyme-linked immunosorbent assay (ELISA)

An ELISA protocol described previously[31] was used with some modifications. In brief, recombinant p150 protein (ABNOVA, Taipei, Taiwan) was diluted in phosphate-buffered saline (PBS) to a final concentration of 100 ng/ml and then 100 μl of the dilution was used to coat the wells (10 ng/well) of Immunolon2 microtiter plates (Dynatech Laboratories, Alexandria, VA, USA), overnight at 4°C. Human sera were diluted 1:100 in PBS and then incubated in the antigen-coated wells. Horseradish peroxidase-conjugated goat anti-human IgG (CALTAG Laboratories, San Francisco, CA,

USA) was used at a 1:5000 dilution and the substrate 2,2′ -azinobis (3-ethylbenzthiazoline) sulfonic acid was added as the detection reagent.

Samples were analyzed in duplicate: after substrate development for the appropriate time, the average optical density (OD) at 405 nm was determined. The cut-off value designating a positive reaction was the mean OD of normal healthy controls + 3 standard deviations (SDs).

The serum levels of IP-10 and anti-double strand DNA (ds-DNA) antibody in SLE (n = 80), PM/DM (n = 28), SSc (n = 21), and NHC (n = 60) sera were also measured by ELISA (R&D systems Minneapolis, MN, USA; and Bio-Rad, Hercules, CA, USA, respectively) according to the manufacturer's instructions.

Immunoblotting

PCNA-binding proteins, including CAF-1, were purified in our laboratory, as described previously, using an anti-PCNA monoclonal antibody.[14, 15] Concentrations of the purified proteins were determined using the Protein DC Assay kit (Bio-Rad,

Hercules, CA, USA) to ensure the equal loading of proteins in each 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel lane. The purified proteins were mixed with 2 × gel sample buffer containing 6% sodium dodecyl sulfate, 20% glycerol, 10% β-mercaptoethanol, 0.02% bromophenol blue and complete protease inhibitor cocktail (Roche). The equivalent of 20 μg total protein was loaded into each gel lane, separated by electrophoresis and then transferred to nitrocellulose membranes using a Semi-Dry Trans-Blot apparatus (Bio-Rad). After blocking, primary antibodies, including a mouse anti-CAF-1 monoclonal antibody (ABNOVA) and a rabbit anti-PCNA monoclonal antibody (Abcam, Cambridge, UK), at a 1:500 dilution, or human sera, at a 1:100 dilution, were incubated with the blots at room temperature for 1h. The detection of bound antibodies was achieved using a horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Invitrogen, Eugene, OR, USA), anti-rabbit IgG, and anti-human IgG antibody at a 1:2000 dilution (Jackson Immuno Research, Baltimore, MA, USA) in combination with enhanced chemiluminescence (Super Signal west pico; PIERCE Products, Rockford, IL, USA).

Indirect immunofluorescence microscopy

Immunofluorescence staining was performed using rabbit anti-PCNA monoclonal antibody (Abcam) and mouse anti-CAF-1 monoclonal antibody (ABNOVA) at a 1:200 dilution for 1 h. The bound antibodies on HEp-2 cells substrate (MBL, Nagoya, Japan) were labeled with Alexa-488-conjugated donkey anti-rabbit IgG antibody or Alexa-594-conjugated donkey anti-mouse IgG antibody (Invitrogen, Grand Island, NY, USA), at a 1:500 dilution for 1 h. After washing with PBS, cells were counterstained with 4′ ,6-diamidino-2-phenylindole (DAPI) to visualize nuclei. Fluorescent images were captured with a fluorescence microscope (KEYENCE, BIOREVO, BZ-9000, Osaka, Japan) at 200 × magnification.

Real-time polymerase chain reaction (RT-PCR)

Peripheral blood mononuclear cells (PBMCs) derived from SLE patients and NHCs were separated using Ficoll-Plaque Plus (GE Healthcare, Little Chalfont, UK).

MOLT4 cells (human $CD4^+$ T cells) were purchased from the American Type Culture Collection (Manassas, VA, USA) and were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma; St. Louis, MO, USA) containing 10% fetal bovine serum (FBS) under standard conditions. MOLT4 cells were treated with or without 100 U/ml interferon (IFN)-γ (R&D Systems) for 24 h. Total RNA was isolated from the PBMCs and the MOLT4 cells treated with IFN-γ using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). cDNA was synthesized using a PrimeScript™ RT Reagent kit (Takara Bio Inc, Shiga, JAPAN), and then RT-PCR was performed using SYBR Premix Ex Taq™ (Takara Bio Inc.) and an ABI Prism 7900HT Sequence Detection System (Perkin Elmer/Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions (Takara Bio Inc). The primers used for amplification were: 5′ -GCTCCTGGACTATGAGGTGGACA-3′ (forward) and 5′ -CACACCTTCGTCCTCAGACAGGTA-3′ (reverse) for CAF-1, 5′ -GGCTGGGACATCAACAAGGA-3′ (forward) and 5′ GAGTTCATGGCACAGCGAAAC-3′ (reverse) for IRF-1, 5′ -

TGGTGCTCATCTTAATGGCCTA-3′ (forward) and 5′ -

CTTTGATTGCAAACATGGGTTC-3′ (reverse) for Fas, 5′ -

GCTCCTGGACTATGAGGTGGACA-3′ (forward) and 5′ -

CACACCTTCGTCCTCAGACAGGTA-3′ (reverse) for CAF-1, and 5′ -

TGGCACCCAGCACAATGAA-3′ (forward) and 5′ -

CTAAGTCATAGTCCGCCTAGAAGCA-3′ (reverse) for beta actin. The amplification cycles consisted of 95°C for 10 s as the 1st step (1 cycle), 95°C for 5 s and 65°C for 30 s as the 2nd step (40 cycles), then 95°C for 15 s and 60°C for 60 s and 95°C for 15 s as the 3rd step (1 cycle), according to the protocol described in the manufacturer's instructions (Takara Bio Inc.). To determine the quantitative expression levels of the transcripts, sample loading was monitored and normalized to the expression of β-actin transcripts.

Statistical analysis

The experimental data were compared using the Mann–Whitney *U*-test or Chi-

square test, and differences with P-values < 0.05 were considered to be statistically significant.

Results

Increased immunoreactivity to CAF-1 in SLE

To confirm whether an autoimmune reaction to CAF-1 specifically occurred in patients with SLE, we evaluated the immunoreactivity to CAF-1 in sera of SLE patients compared to that in sera of disease controls and NHCs, using a recombinant CAF-1 protein in an ELISA. As shown in Figure 1(a), the mean anti-CAF-1 antibody titer was significantly higher in SLE sera than in those of the disease controls and NHCs. We also investigated immunoreactivity to CAF-1 in autoimmune sera that react to standard autoantigens, which consisted of ds-DNA, SS-A/Ro, U1-RNP and centromere. As expected, autoimmune sera reacting with ds-DNA revealed significantly higher immunoreactivity to CAF-1 as compared to sera that reacted with other standard autoantigens, as shown in Figure 1(b).

For further confirmation, we investigated immunoreactivity to CAF-1 using a native form of CAF-1 protein instead of the recombinant protein. As shown in Figure 2, immunoblotting analysis revealed that the anti-PCNA monoclonal antibody reacted with proteins of 3 different molecular sizes (lane 1). PCNA is known to have dimeric and trimeric forms, in addition to the native PCNA.[32] Our previous study has shown that purified PCNA-binding proteins contain multiple forms of PCNA.[31] We therefore deduced that the anti-PCNA monoclonal antibody reacted with native PCNA (34 kDa), dimeric PCNA (68 kDa), and trimeric PCNA (110 kDa). The molecular weight of CAF-1 was originally reported as 105 kDa.[33] As expected, anti-CAF-1 (p150) monoclonal antibody reacted with a 105-kDa protein (lane 2). Moreover, SLE sera that had reacted with the CAF-1 recombinant protein in ELISA (lanes 3–7) exhibited a positive reaction against the 105-kDa CAF-1 protein among the purified PCNA-binding proteins.

Next, we performed indirect immunofluorescence staining using an anti-PCNA monoclonal antibody and an anti-CAF-1 monoclonal antibody. The cellular distribution of CAF-1 was compared to that of PCNA. Anti-PCNA antibody strongly reacted to G1-

S phase cells (Figure 3 upper panels, dotted box) and weakly reacted to metaphase cells (Figure 3 upper panels, box), as previously reported. In comparison with the findings for PCNA, the anti-CAF-1 antibody reacted strongly to non-metaphase cells (Figure 3 lower panels, arrow heads), with enhanced staining in the periphery of the nucleus, and reacted weakly to metaphase cells (Figure 3 lower panels, arrows). The staining pattern of the anti-CAF-1 antibody on HEP-2 substrates was similar to that of the anti-PCNA antibody, suggesting that CAF-1 is enriched during the G1-S phase of the cell, similar to PCNA.

Subsequently, we investigated the prevalence of anti-CAF-1 antibody in the positive sera. As shown in Table 1, the frequency of anti-CAF-1 antibody-positive sera was 33% in SLE patients, 3% in PM/DM patients, 2% in SSc patients, 3% in SS patients, 6% in MCTD patients, 4% in RA patients, and 0% in NHCs. The prevalence of anti-CAF-1 antibody-positive sera was significantly higher in SLE compared to the disease controls and NHCs ($P < 0.0001$). To our knowledge, this is the first report in which a profound autoimmune reaction against CAF-1 was observed in patients with

SLE. The anti-CAF-1 antibody was considered to be a novel SLE-related autoantibody.

Comparison of clinical profile in patients with SLE with or without anti-CAF-1 antibody

A comparison of the clinical profile of SLE patients with or without the anti-CAF-1 antibody is summarized in Table 2. Anti-CAF-1 antibody-positive patients exhibited a significantly higher incidence of NPSLE ($P = 0.0243$).

Interestingly, all SLE patients who tested positive for the anti-CAF-1 antibody developed aseptic meningitis. Patients lacking the anti-CAF-1 antibody developed other NPSLE manifestations (e.g., anxiety disorder, mood disorder, and seizure disorders). Our data suggested that aseptic meningitis, an NPSLE manifestation, is a unique characteristic of SLE patients carrying the anti-CAF-1 antibody.

Moreover, SLE patients with the anti-CAF-1 antibody had a significantly higher level of serum anti-ds-DNA antibody than did SLE patients lacking the anti-CAF-1 antibody (Figure 4).

Possible aberrant IFN-γ regulation involved in anti-CAF-1 antibody production in SLE

We have shown that IFN-γ also plays an important role in autoantibody production [34]. Therefore, we next investigated whether IFN-γ contributed to the mechanism underlying anti-CAF-1 antibody production. Initially, we evaluated whether CAF-1 expression was affected by IFN-γ. *CAF1* expression was evaluated in human lymphocytes (MOLT4 cells) stimulated with or without IFN-γ. As expected, *CAF1* expression was significantly upregulated by IFN-γ, as compared to non-treated controls in vitro (Figure 5a). To verify that increased IFN- γ regulation occurred in SLE patients in vivo, *CAF1* expression in PBMCs from SLE patients was evaluated by quantitative RT-PCR. As shown in Figure 5(b), significant upregulation of expression of *CAF1* was observed in SLE patients, as compared to that in NHCs. For further confirmation, we investigated changes in production of IFN-γ-related molecules, such as interferon regulating factor 1 (IRF1), which is known to be a transcription factor that is specifically upregulated by IFN-γ. As expected, significantly increased *IRF1* expression was observed in PBMCs from SLE patients (Figure 5c).

Next, we investigated another possibility of IFN- γ involvement in anti-CAF-1 antibody production. Several studies have indicated that IFN- γ increases susceptibility of lymphocytes to apoptosis by introducing FAS/CD95 expression.[35-37] Therefore, we investigated *FAS* expression in PBMCs. As expected, we found increased *FAS* expression in PBMCs from SLE patients (Figure 5d). We assumed that IFN- γ is associated with anti-CAF-1 autoantibody production by increasing both *CAF1* and *FAS* expression in PBMCs from SLE patients. Intracellular nuclear antigens can be released into the extracellular space by aberrantly increased apoptosis and may be recognized by the immune system as autoantigens.

Finally, we investigated whether the IFN- γ participation in anti-CAF-1 autoantibody production is a unique characteristic of SLE. IP-10/CXCL10 belongs to the C-X-C motif chemokine family. IP-10 is released from T cells and is produced in response to stimulation by IFN-γ. Therefore, we measured the IP-10 serum concentration in SLE by ELISA, other connective tissue diseases (PM/DM and SSc), and NHCs. We confirmed that serum IP-10 concentration was significantly higher in

SLE patients than in patients with other connective tissue diseases and in NHCs, suggesting that IFN- γ plays a significant role in anti-CAF-1 antibody production in SLE (Figure 6).

Our data suggested that PBMCs in SLE patients undergo aberrant regulation of IFN-γ, resulting in increased CAF-1 expression and apoptosis. In SLE, enriched intracellular CAF-1 may be released into the extracellular space along with PCNA by enhanced apoptosis, thus presenting a source of the autoantigens to the immune system.

Discussion

We have previously reported that the autoimmune response to PCNA-binding proteins is a unique mechanism of autoantibody production that is related to SLE[14,15]. We conducted this study to clarify whether an autoimmune response to CAF-1 occurs in patients with SLE, as CAF-1 is one of the PCNA-binding proteins. In the present study, we found the following interest findings. (I) The autoimmune response to CAF-1 was significantly increased in SLE compared to the disease controls and NHCs (Table 1,

Figures 1 and 2); (II) significantly more CNS involvement (aseptic meningitis) was seen in the anti-CAF-1 antibody-positive SLE patients in comparison with anti-CAF-1 antibody-negative SLE patients (Table 2); (III) SLE patients with anti-CAF-1 antibody had higher serum levels of anti-ds-DNA antibody than did SLE patients without anti-CAF-1 antibody (Figure 4); (IV) increased IFN-γ regulation is possibly involved in anti-CAF-1 antibody production in SLE (Figure 5(a–d), Figure 6).

CAF-1 has been reported to colocalize with PCNA in vivo and binds directly to PCNA in vitro.[10, 20, 21, 38] Although the prevalence of anti-PCNA antibody among SLE patients is relatively rare, we have previously reported, after analysis of the reaction of SLE sera with PCNA-binding proteins, that more than 35% of the serum samples reacted with at least 1 PCNA-binding proteins.[18] Most of these sera also contained antibodies to several other components of this complex.[18] These findings can be explained by the concept that epitopes recognized by the serum autoantibody to PCNA in antibody-positive sera are hidden when PCNA binds to other proteins.[17] Anti-PCNA antibody may be produced when PCNA is separated from the PCNA-binding proteins,

due to the unmasking of functional epitopes of PCNA. Additionally, we have previously found that the serum levels of free PCNA-binding proteins was specifically elevated in SLE.[14, 15] Therefore, PCNA-binding proteins tend to elicit an autoimmune response more frequently in SLE than in other diseases. Moreover, we have reported that a widening of the immune response to elements of the PCNA-binding proteins was observed in anti-PCNA antibody-negative SLE patients when reactivity to the PCNA-binding proteins was analyzed sequentially over time.[18] These results strongly suggested the importance of "intermolecular–intrastructural help" in the extent of the autoimmune response to PCNA-binding proteins[39]

Although CNS involvement in SLE is a severe complication during the course of SLE and contributes to significant patient morbidity and mortality, no accurate indicator of NPSLE has been identified to date. In the present study, we found significantly more CNS involvement in the anti-CAF-1 antibody-positive SLE patients in comparison with anti-CAF-1 antibody-negative SLE patients (Table 2). Moreover, we also found that increased IFN-γ regulation, as indicated by elevated serum level of IP-10,

is possibly involved in anti-CAF-1 antibody production in SLE (Figure 6). It has not been elucidated whether anti-CAF-1 antibody plays a pathogenic role in CNS involvement in SLE; however, our data suggested that anti-CAF-1 antibody can be used as a possible biomarker of NPSLE.

Svenungsson and co-workers investigated the levels of IFN- γ and other cytokines in the peripheral blood and cerebrospinal fluid (CSF) of 30 NPSLE patients and found significantly elevated levels of *IFNG* mRNA levels in peripheral blood lymphocytes and in the CSF of patients with NPSLE[40]. Moreover, several reports have shown that levels of IP-10 are elevated in the CSF of NPSLE patients, compared to non-NPSLE patients.[41-44] Although not only IFN-γ and IP-10, but also many other cytokines, have been reported to be involved as causative factors in NPSLE, certain SLE patients may require increased IFN-γ regulation, leading to onset of NPSLE; this may lead to production of anti-CAF-1 antibody in these NPSLE patients.

In the present study, we found that IFN-γ could upregulate *CAF1* expression in vitro (Figure 5a) and that aberrant regulation of IFN-γ occurred in PBMCs of SLE

patients in vivo (Figures 5b–d). It is commonly accepted that IFN-γ can promote Th1 polarization, facilitate specific cytotoxicity by increasing the expression of MHC class-I and -II molecules, and boost antigen processing and immunoglobulin switching. Furthermore, there is some evidence that IFN-γ may play a role specifically in the initiation of antinuclear antibody (ANA) production. For example, the emergence of SLE, with de novo anti-dsDNA antibody production, has been described in some patients receiving systemic treatment with IFN-γ, suggesting that excess amounts of this cytokine in susceptible individuals can trigger an autoimmune responses relevant to SLE.[12] Harigai and co-workers reported enhanced levels of IFN-γ in SLE patients, which may contribute to SLE pathogenesis by inducing BAFF production.[45] Yanaba and co-workers reported that PBMCs from SLE patients may produce large amounts of IFN-γ in response to costimulation with anti-CD3 and monoclonal antibody against CD28.[46] Moreover, the essential role of IFN-γ in the pathogenesis of immune complex-mediated diseases has also been established in several experimental models.[47] This contention is further supported by results from transgenic mice expressing IFN-γ, which

develop a lupus-like syndrome.[48] Interestingly, IFN-γ has also been shown to be a potent stimulus of apoptosis.[49] IFN-γ has been reported to induce a number of other proapoptotic molecules, including FAS and the TNF-α receptor.[50] In the present study, we showed that IFN-γ-related molecules, such as IRF-1, were upregulated in patients with SLE (Figure 5b). IRF-1 is a well-known IFN-γ-induced molecule, similar to IP-10,[51] and has been shown to play roles in the immune response or in the regulation of apoptosis. Several reports have shown that induction of apoptosis by death signals, such as DNA damage, requires IRF-1.[52,53] Indeed, in the present study, we found that upregulation of proapoptotic genes occurred in PBMCs of patients with SLE (data not shown). IRF-1 may be a deciding factor in IFN-γ induced apoptosis.[54,55] Taken together, IFN-γ facilitates apoptosis in PBMCs through IRF-1 expression, resulting in aberrant regulation of apoptosis in patients with SLE. Intracellular proteins involving PCNA-binding proteins can be released into the extracellular space due to this increased apoptosis, subsequently resulting in initiation of an autoimmune response against CAF-1 by the “antigen-driven” system. Moreover, the autoimmune response is probably

amplified by several mechanisms, including “intermolecular–intrastructural help” in patients with SLE. Further analysis of autoimmune responses to other PCNA-binding proteins may shed light on the pathogenesis and mechanisms underlying autoantibody production in patients with SLE.

In conclusion, our previous reports have shown that an autoimmune response to PCNA-binding proteins is unique to autoantibody production in SLE. We conducted this study to clarify the autoimmune response to CAF-1, one of the PCNA-binding proteins, in SLE patients. A significantly increased autoimmune response to CAF-1 was seen in SLE patients compared to disease controls and NHCs. Moreover, the presence of anti-CAF-1 antibody was correlated with CNS involvement in SLE patients. This is the first report in which anti-CAF-1 antibody was identified as an SLE-related autoantigen. Anti-CAF-1 antibody is useful as a biomarker for the diagnosis for SLE. Moreover, our results also suggested that increased apoptosis and aberrant regulation of IFN-γ in PBMCs plays an important role in anti-CAF-1 antibody production in SLE.

Conflict of Interest Statement

The authors declare that there is no conflict of interest.

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Figure Legends

Figure 1. Immunoreactivity to CAF-1 in connective tissue diseases and autoimmune sera reacting with standard autoantigens.

Immunoreactivity to CAF-1 was evaluated by ELISA using the recombinant protein in sera from patients with SLE (n = 100), PM/DM (n = 100), SSc (n = 100), SS (n = 100), MCTD (n = 100), RA (n = 100), and from NHCs (n = 116) (a). Immunoreactivity against CAF-1 in autoimmune sera reacting standard autoantigens (dsDNA, SS-A/Ro, U1-RNP, and centromere) is also indicated (b). Bars indicate the mean serum anti-CAF-1 antibody titer (OD_{405}) in each group. Statistical analysis was performed using the Mann-Whitney *U*-test against SLE, and P-values < 0.05 (*) were considered as statistically significant.

Figure 2. Immunoblotting analysis of purified PCNA-binding proteins.

PCNA-binding proteins, purified by an anti-PCNA monoclonal antibody, were used as source of the native CAF-1 antigen for immunoblotting analysis. Anti-PCNA

monoclonal antibody (lane 1) reacted with native PCNA (34 kDa), dimeric PCNA (58 kDa), and trimeric PCNA trimer (110 kDa). Anti-CAF-1 monoclonal antibody (lane 2) and SLE patients' sera that had reacted with the CAF-1 recombinant protein in ELISA (lanes 3–7), reacted positively with the native form of CAF-1 antigen (predicted molecular weight: 105 kDa). NHCs' sera that had not reacted with the CAF-1 recombinant protein did not exhibit a positive reaction with the native form of CAF-1 antigen (lane 8).

Figure 3. Staining pattern of anti-CAF-1 antibody upon indirect immunofluorescence microscopy.

The staining pattern of anti-CAF-1 antibody was investigated by indirect immunofluorescence and compared to those of the anti-PCNA monoclonal antibody. Indirect immunofluorescence staining was performed using the anti-PCNA monoclonal antibody (upper panels) and anti-CAF-1 monoclonal antibody (lower panels) on HEp-2 substrates. The cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) to

visualize nuclei. Metaphase cells and G1-S phase cells, in the indicated area (box: M phase cell, dotted box: G1-S phase cell), are shown at higher magnification for comparison.

Figure 4. Increased serum level of anti-ds DNA antibody in SLE patients with anti-CAF-1 antibody.

Serum level of anti-ds DNA antibody was evaluated by ELISA in SLE patients with anti-CAF-1 antibody, SLE patients without anti-CAF-1 antibody, and NHCs. Bars indicate SD. Statistical analysis was performed using the Mann-Whitney *U*-test against SLE, and P-values < 0.05 (*) were considered as statistically significant.

Figure 5. Increased IFN-γ regulation may be involved in anti-CAF-1 antibody production in SLE.

The effect of IFN-γ on regulation of *CAF1* mRNA expression *in vitro* was evaluated in human lymphocytes (MOLT4 cells; a human $CD4^+$ T cell line), treated with

or without IFN-γ, using RT-PCR (a). Gene expression of *CAF1*, *IRF1*, and *FAS* was evaluated by RT-PCR in PBMCs derived from patients with SLE and NHCs (b–d). Statistical analysis was performed using the Mann-Whitney *U*-test, against SLE. Bars in (a–d) indicate SD. P-values < 0.05 (*) were considered as statistically significant.

Figure 6. Increased serum levels of IP-10 in patients with SLE.

Serum levels of IP-10 protein were evaluated by ELISA in patients with SLE (n = 80), PM/DM (n = 28), SSc (n = 21), and in NHCs (n = 60). Statistical analysis was performed using the Mann-Whitney *U*-test, against NHCs. Bars in indicate SD. P-values < 0.05 (*) were considered as statistically significant.

Table1. Prevalence of anti-CAF-1 antibody in connective tissue diseases

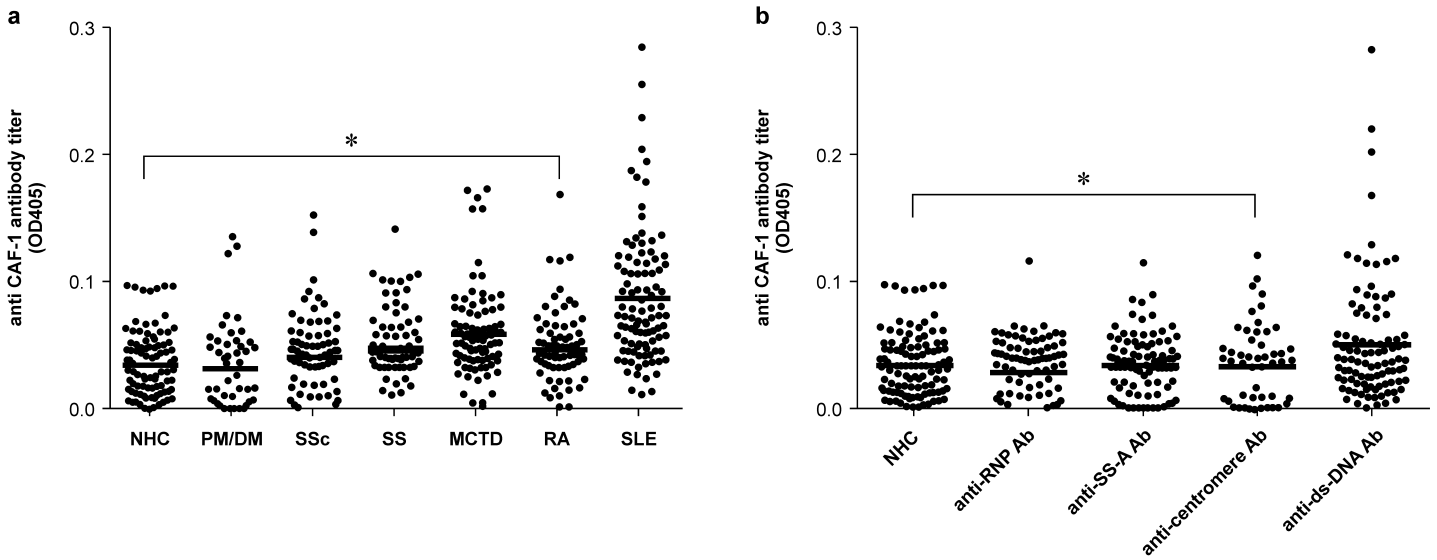
	NHC	SLE	PM/DM	SSc	SS	MCTD	RA
Nuber of patients	116	100	100	100	100	100	100
Number of anti-CAF-1 antibody	0	33	3	2	3	6	4
Statistical analysis (P value vs SLE)	<0.0001		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

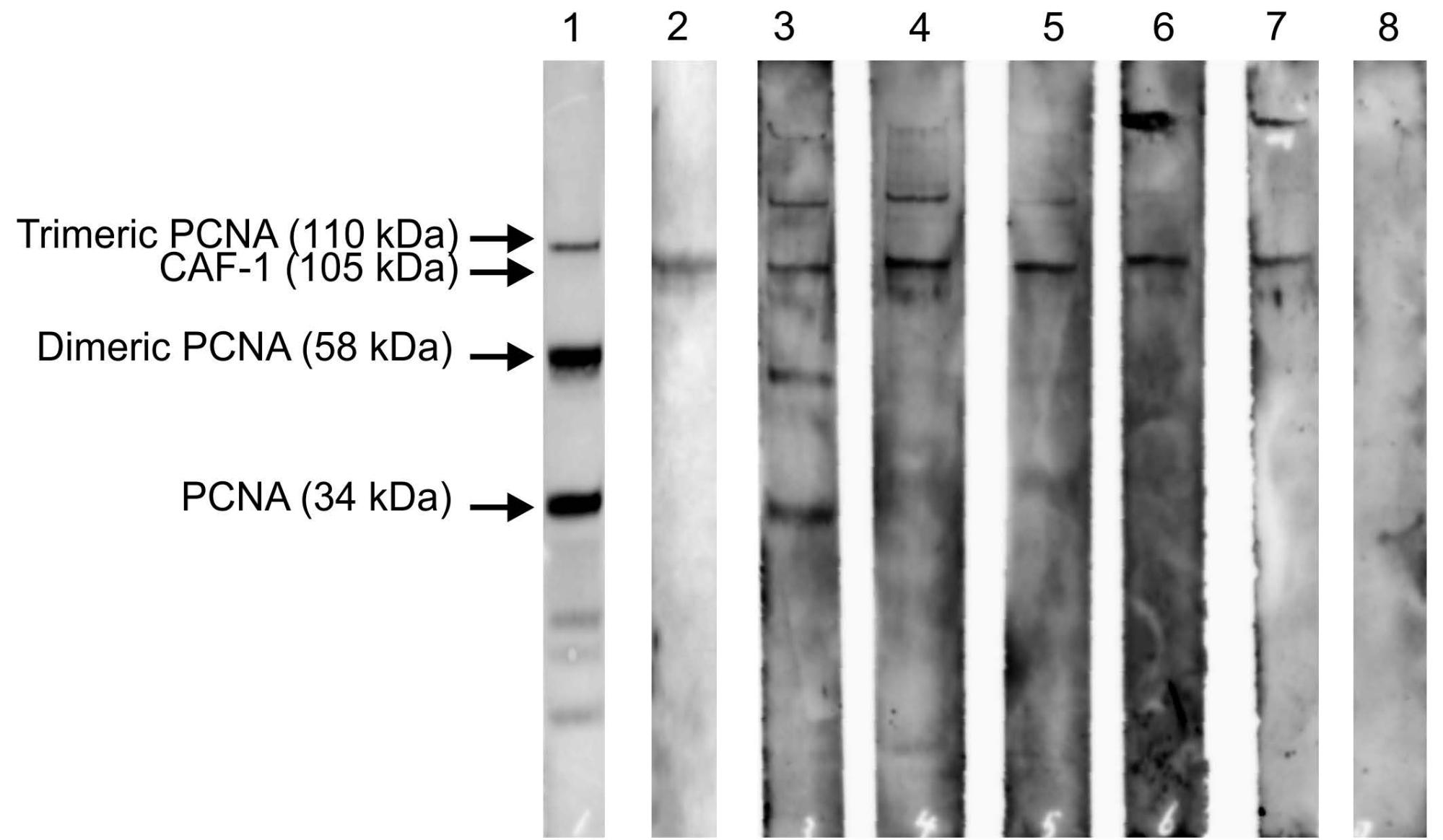
Cut off point was designed as the mean value of NHC + 3SD and sera with values above the cut off point were considered as anti-CAF-1 positive sera. Statistical analysis was performed using the qui square test for SLE, and p-values less than 0.05 were considered as statistically significant.

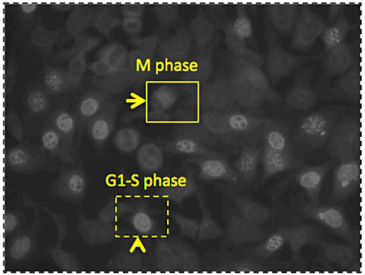
NHC, normal healthy control; SLE, systemic lupus erythematosus; PD/DM, polymyositis/dermatomyositis; SSc, scleroderma; SS, Sjögren's syndrome; MCTD, mixed connective tissue disease; RA, rheumatoid arthritis

Table2. Comparison of clinical profile in SLE patients with or without anti-CAF-1 antibody

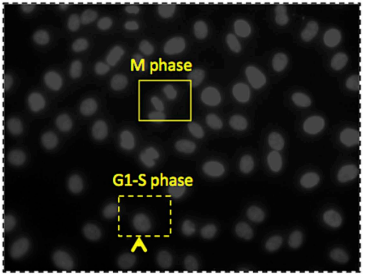
	Anti-CAF-1 antibody(+)	Anti-CAF-1 antibody(-)	Statistical analysis
Number of patients	33	67	
Gender			
Female	27	61	
Male	6	6	P=0.1819
Age(mean age)	24.9±10.2	30.2±14.0	P=0.0662
Rash(%)	21(63.6%)	40(59.7%)	P=0.7044
Nephritis(%)	23(69.7%)	35(52.2%)	P=0.0963
Lymphopenia(%)	29(87.9%)	60(85.7%)	P=0.8014
Hemolytic anemia(%)	3(9.1%)	6(9.00%)	P=0.9822
Thrombocytopenia(%)	11(33.3%)	29(43.3%)	P=0.3396
Arthritis(%)	20(60.6%)	47(70.1%)	P=0.3399
Serositis(%)	5(15.2%)	8(11.9%)	P=0.6534
CNS involvement(%)	6(18.2%)	3(4.5%)	P=0.0243*


a
anti CAF-1 antibody titer (OD405)
0.3
0.2
0.1
0.0
*
NHC
PM/DM
SSc
SS
MCTD
RA
SLE
b
anti CAF-1 antibody titer (OD405)
0.3
0.2
0.1
0.0
*
NHC
anti-RNP Ab
anti-SS-A Ab
anti-centromere Ab
anti-ds-DNA Ab

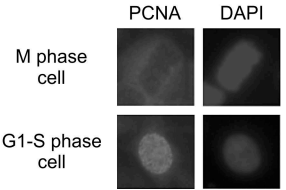

1
2
3
4
5
6
7
8
Trimeric PCNA (110 kDa)
CAF-1 (105 kDa)
Dimeric PCNA (58 kDa)
PCNA (34 kDa)

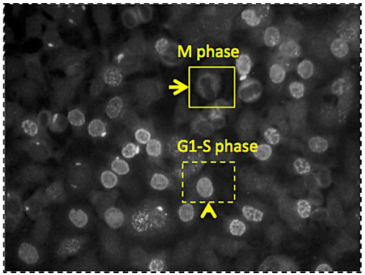


Anti-PCNA
monoclonal Antibody

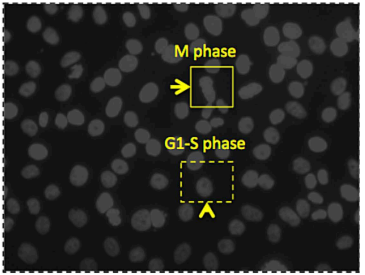


DAPI

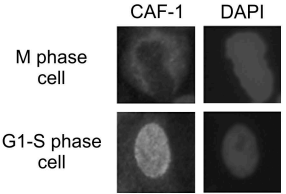


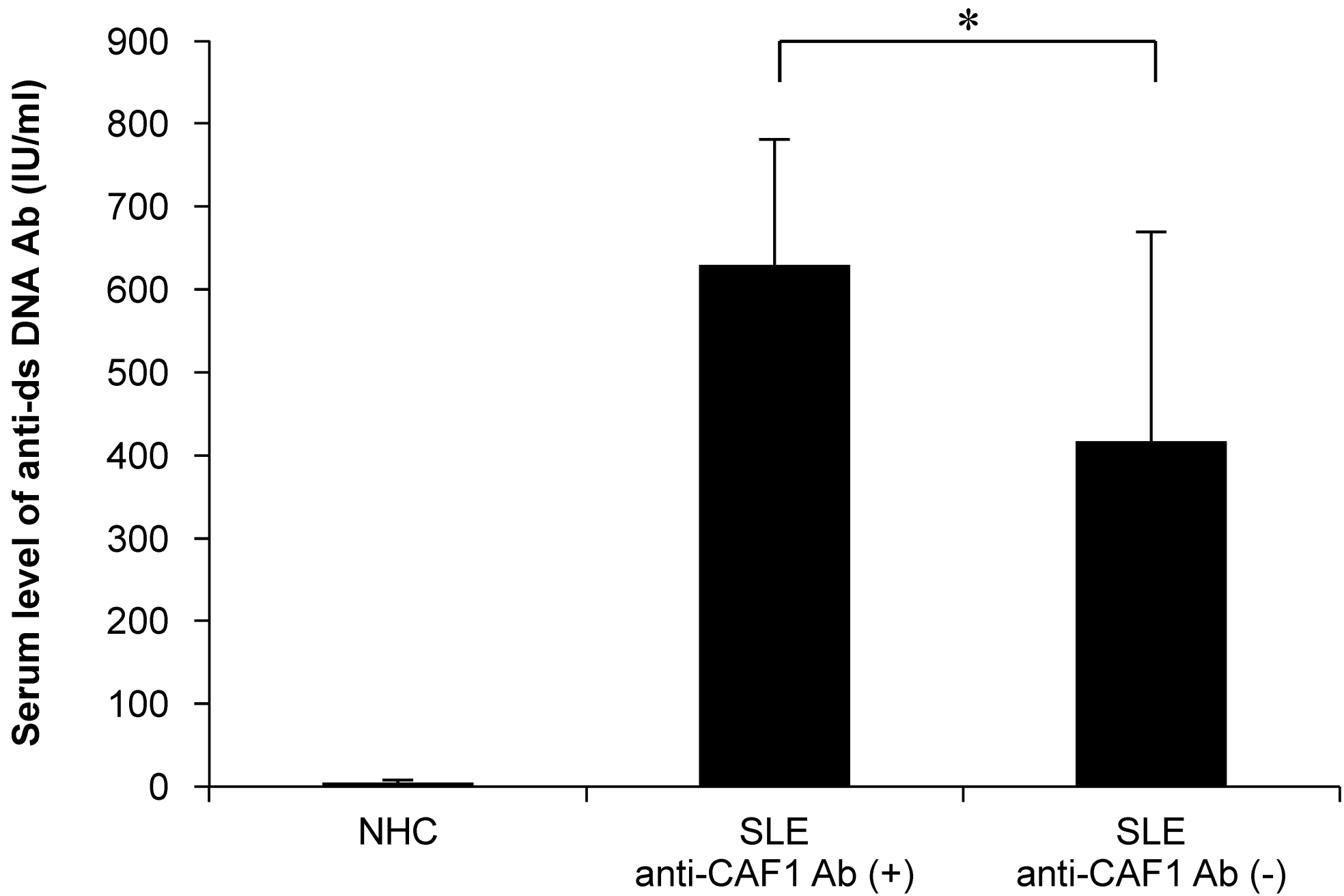


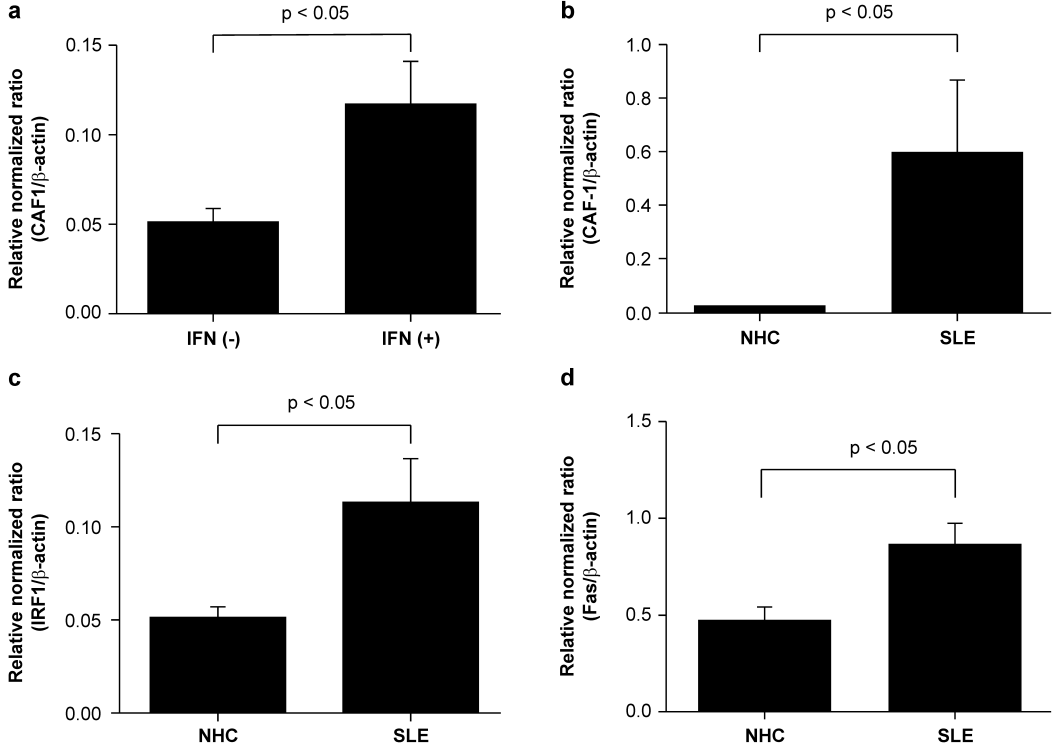
Anti-CAF-1
monoclonal Antibody

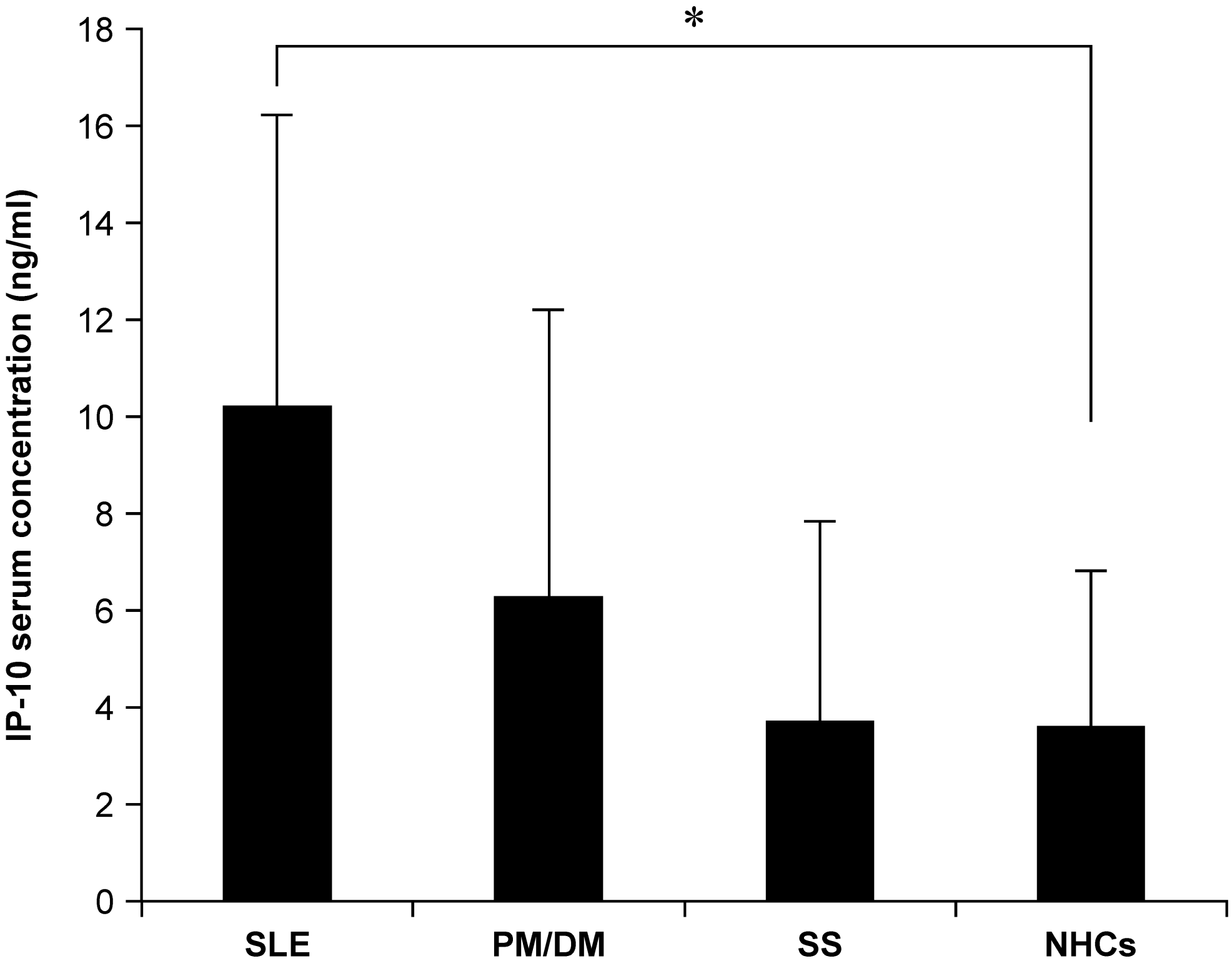


DAPI




Serum level of anti-ds DNA Ab (IU/ml)
900
800
700
600
500
400
300
200
100
0
*
NHC
SLE
anti-CAF1 Ab (+)
SLE
anti-CAF1 Ab (-)


a
p < 0.05
0.15
0.10
0.05
0.00
Relative normalized ratio
(CAF1/β-actin)
IFN (-)
IFN (+)
b
p < 0.05
1.0
0.8
0.6
0.4
0.2
0.0
Relative normalized ratio
(CAF-1/β-actin)
NHC
SLE
c
p < 0.05
0.15
0.10
0.05
0.00
Relative normalized ratio
(IRF1/β-actin)
NHC
SLE
d
p < 0.05
1.5
1.0
0.5
0.0
Relative normalized ratio
(Fas/β-actin)
NHC
SLE


*
IP-10 serum concentration (ng/ml)
18
16
14
12
10
8
6
4
2
0
SLE
PM/DM
SS
NHCs