의학박사 학위논문

CD47-SIRPα 상호작용이 전신 홍반 루푸스의 면역반응에 미치는 영향

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박진균의 박사학위논문을 인준함

2018년 8월

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ABSTRACT

Effect of CD47-SIRPα interaction on immune response in systemic lupus erythematosus

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Background

Systemic lupus erythematosus (SLE) is a chronic autoimmune inflammatory multi-system disease mediated by unbalanced activation of innate and adaptive immune responses; this leads to uncontrolled inflammation and organ damage. Signal regulatory protein alpha (SIRPα), a regulatory receptor expressed by monocytes, interacts with CD47.

Objective

To investigate whether CD47 contributes to altered proinflammatory responses in SLE.

Methods

Expression of CD47 and SIRPα by peripheral blood mononuclear cells (PBMCs) from patients with SLE and healthy controls (HCs) was examined by flow cytometry analysis. Changes in CD47 expression by monocytes after exposure to SLE serum, HC serum, recombinant interferon (IFN)-α, or tumor necrosis factor (TNF)-α were investigated. Human monocytes and THP1 cells were stimulated with lipopolysaccharide (LPS), an anti-CD47 antibody, or both. TNF-α production and mitogen-activated protein kinase (MAPK) and NF-κB signaling were then examined. Sera from HCs and SLE patients were screened by enzyme-linked immunosorbent assay (ELISA) to detect autoantibodies specific for CD47.

Results

Twenty-five patients and sixteen HCs were enrolled. CD47 expression by monocytes from SLE patients was higher than that by those from HCs (mean fluorescence intensity ± SD: 815.9 ± 269.4 *vs.* 511.5 ± 199.4, respectively; $p < 0.001$). CD47 expression by monocytes correlated with SLE disease activity (Spearman's rho = 0.467, $p = 0.019$). CD47 expression was increased by serum from SLE patients or IFN-α but not by TNF-α. Exposing monocytes to an anti-CD47 antibody plus LPS increased TNF-α production by 21.0 ± 10.9-fold (compared with 7.3 ± 5.5-fold for LPS alone). CD47 activation induced MAPK,

but not NF-κB, signaling. In a murine lupus model, expression of CD47 mRNA by tissue-infiltrating macrophages increased during active nephritis. Finally, levels of autoantibodies against CD47 were higher in SLE patients than in HCs (21.4 ± 7.1 ng/mL *vs.* 16.1 ± 3.1 ng/mL, respectively; p = 0.02).

Conclusion

CD47 expression by monocytes is upregulated in SLE and correlates with disease activity. CD47 contributes to augmented inflammatory responses in SLE by activating the MAPK signaling pathway. Targeting CD47 might be a novel treatment for SLE.

Keywords: Systemic lupus erythematosus, CD47, SIRP-alpha, inflammatory response.

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INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic multi-system autoimmune inflammatory disease mediated by abnormal activation of immune cells and autoantibody production [1, 2]. Unbalanced activation of the innate and adaptive immune systems leads to uncontrolled inflammation and subsequent damage to various organs. The balance between the numerous activating and inhibiting signals is critical for appropriate immune responses; this balance might be transiently or permanently disrupted in patients with SLE [3]. Like all immune cells, monocytes express both activating receptors (e.g., toll-like receptors (TLRs)) and regulatory (inhibitory) receptors (e.g., immunoglobulin-like transcripts) [4, 5]. Signal regulatory protein alpha (SIRPα) is an important regulatory receptor [6]. SIRPα interacts with integrin-associated protein cluster of differentiation (CD)-47. Although SIRPα activation by CD47 suppresses monocyte activity, the impact of CD47 activation on monocyte function is unclear [7].

CD47, a 50 kDa membrane receptor that belongs to the immunoglobulin superfamily, binds to thrombospondin-1 and SIRPα. It is expressed ubiquitously by human cells as a "marker of self". The distinction between self and the non-self is a critical step during initiation of immune responses [8, 9]. "Self" cells expressing CD47 transduce a so-called "do not eat me" inhibitory signal to

adjacent macrophages *via* the SIRPα receptor; these cells escape phagocytosis. CD47 expression is upregulated markedly under both physiologic and pathologic conditions. Long-lived memory T cell progenitors show increased CD47 expression. Strikingly, increased CD47 expression by cancer cells helps them evade macrophage-mediated elimination and inhibits anti-tumor immune responses [10].

Bidirectional signaling via CD47-SIRPα exerts biological effects on CD47-expressing cells. CD47 signaling is involved in important cellular functions such as apoptosis, proliferation, adhesion and migration, and cell fusion [8, 11, 12]. During inflammatory responses, activation of CD47 leads to production of interleukin (IL)-1β, and activated cells exhibit increased resistance to cellular stress [13, 14] (**Supplementary** f**igure 1**). In addition, CD47 is involved in regulating chronic inflammation; low expression of CD47 by CD4 effector T cells is required for active resolution of immune responses [15]. Indeed, CD47 deficiency ameliorates autoimmune nephritis in Fas (lpr) murine lupus model [16]. Taken together, the evidence suggests that CD47 activation promotes proinflammatory responses.

Monocytes and macrophages play a crucial role in damage and repair of inflamed tissues [17]. Healthy monocytes and macrophages remove apoptotic cells, which are a source of autoantigens that can trigger autoimmunity in a

susceptible host [18]. During phagocytosis of apoptotic cells, monocytes and macrophages actively inhibit proinflammatory cytokine production through autocrine and paracrine secretion of anti-inflammatory cytokines that lead to induction and maintenance of tolerance to apoptotic "self" cells [19]. However, SLE monocytes show impaired phagocytosis of apoptotic cells; they also produce more proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, when challenged with apoptotic bodies/debris [20-22]. TNF-α can, in turn, increase expression of type 1 interferon (IFN), recruit neutrophils, and activate monocytes, all of which contribute to the damaging inflammatory response in organs such as the kidney [23]. Therefore, monocytes/macrophages in SLE patients might harbor intrinsic defects that alter immune regulation and inflammatory responses. Abnormal CD47-SIRPα interactions might dampen the phagocytic activity of monocytes/macrophages, thereby disrupting clearance of apoptotic materials and heightening proinflammatory responses.

The aim of this study was to investigate whether CD47 contributes to augmented inflammatory responses in SLE patients.

MATERIALS AND METHODS

Study population

Twenty-five patients fulfilling the 1997 revised American College of Rheumatology classification criteria for SLE were recruited at Seoul National University Hospital [24]. Disease activity at the time of blood sampling was determined using the SLE disease activity index 2000 (SLEDAI-2K) [25]. Sixteen healthy individuals without comorbidities were included as healthy controls (HCs). Informed consent was obtained from all participants in accordance with the Declaration of Helsinki. The study was approved by the Institutional Review Board at Seoul National University Hospital (IRB no: 1606-078-771).

Cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized peripheral venous blood by density gradient centrifugation using Ficoll-Paque (GE Healthcare, NJ, USA). Cell viability was assessed by trypan blue dye exclusion. Monocytes were isolated using CD14 Microbeads (MACS, Miltenyi Biotec) in accordance with the manufacturer's instructions. Cell purity was >

90%, as assessed by flow cytometry.

Cell stimulation

To examine cell surface expression of CD47 and SIRPα, healthy PBMCs (10^6 cells/mL) were stimulated at 37°C for 5 h with 100 µL serum from SLE patients or HCs, IFN-α (Peprotech, NJ, USA), or TNF-α (Peprotech, NJ, USA). To measure TNF-α production, PBMCs were treated for 30 min at 37°C with a mouse anti-human CD47 monoclonal antibody (1 µg/mL; eBioscience, CA, USA) or with an isotype control (at 1 µg/mL; BD Biosciences). After treatment with GolgiStop (BD Biosciences), cells were stimulated for 5 h with LPS (at 3 ng/mL; *Escherichia coli*; Invitrogen, CA, USA).

Flow cytometry analysis

PBMCs (10^6 cells/mL) were incubated for 30 min at 4°C in the dark with anti-human CD3, CD19, and CD14 (BD Biosciences, San Jose, CA, USA). For intracellular staining of cytokines, cells were permeabilized and fixed using Perm/Fix solution (BD Biosciences) and then stained with an anti-TNF-α antibody.

Stained cells were analyzed using an LSR Fortessa (BD Biosciences, San Jose, CA, USA) cytometer, and data were analyzed using FlowJo software, version 8.8 (Treestar, Ashland, OR, USA).

Analysis of signaling cascades

THP1 cells (1×10^6 cells/mL) were cultured under a 5% CO_2 atmosphere at 37°C in RPMI-1640 supplemented with 1% penicillin/streptomycin and 0.05 mM β-mercaptoethanol. Cells were treated with an anti-CD47 antibody (1 μg/mL) for 30 min, followed by stimulation with LPS (3 ng/mL) for 30 min. Cells were then lysed and analyzed using a Human Phospho-MAPK Array Kit (cat. no. ARY002B) and a Human Phospho-NF-κB Pathway Array Kit (cat. no. ARY029), according to the manufacturer's recommendations (R&D Systems, USA). Spot intensities were assessed using Image studio software (LI-COR Biosciences, Germany).

Microarray analysis of macrophages isolated from nephritic kidneys

Affymetrix microarray data derived from cDNA obtained from sorted NZB/W

F4/80hi cells from healthy kidneys (n = 6), nephritic kidneys (n = 7), and kidneys exposed to cyclophosphamide, CTLA4-Ig, or an anti-CD145 antibody (n = 4) were analyzed using the software built into GEO Data Sets (https://www.ncbi.nlm.nih.gov/gds/). Expression of mRNA encoding CD47 and SIRPα was examined.

ELISA

To measure anti-CD47 antibodies in serum, an ELISA plate (Nunc MaxiSorp®) was coated with recombinant CD47 (Novoprotein, NJ, USA), blocked and then incubated with serum from SLE patients and HCs. Standard curves were generated using a mouse anti-human CD47 antibody (eBioscience). Rabbit anti-mouse IgG (Abcam) was used as the secondary antibody. A goat anti-human IgG antibody was used (Abcam) to detect anti-CD47 antibodies in serum. All samples were measured in triplicate.

Statistical analysis

The Mann-Whitney U test or a t-test was used to compare continuous variables as appropriate. Correlation analysis was performed using Spearman's method.

All reported p values were two-sided. P values $\leq$ 0.05 were considered significant. All statistical analyses were performed using GraphPad Prism 5.01 (GraphPad Software Inc., La Jolla, CA, USA).

RESULTS

Patient characteristics

The mean age of the 25 SLE patients was 38.7 ± 12.8 years. The majority of patients were female (92.0%). The median disease duration [interquartile range (IQR)] was 4.2 [0.9–14.0] years, and the median SLEDAI-2K was 6 [2–9.5]. The majority of patients were taking glucocorticoids and hydroxychloroquine at the time of blood sampling. Only a few patients were taking additional immunosuppressants such as azathioprine, mycophenolate mofetil, or sulfasalazine (**Table 1**).

CD47 is expressed by circulating blood cells

PBMCs from HCs and SLE patients were examined for surface expression of CD47 and SIRPα. B cells, T cells, and monocytes were identified based on their surface expression of CD19, CD3, and CD14, respectively (**Figure 1A, left panel**). CD19+ B cells, CD3+ T cells, and CD14+ monocytes expressed CD47, although CD47 expression by monocytes was higher than that by B and T cells, which expressed similar levels of CD47 (**Figure 1B, right panel**). By contrast, SIRPα was expressed mainly by CD14+ monocytes but not by B and T cells.

SLE monocytes show increased expression of CD47

Expression of CD47 and SIRPα by PBMCs from SLE patients (n = 25) and HCs (n = 14) was compared. CD47 expression by B and T cells did not differ between HCs and SLE patients; however, CD47 expression by SLE monocytes was higher than that by HC monocytes [mean fluorescence intensity (MFI) ± SD: 815.9 ± 269.4 *vs.* 511.5 ± 199.4, respectively; $p < 0.001$] (**Figure 2A**). SIRPα expression by B cells, T cells, and monocytes from HCs and SLE patients was comparable (**Figure 2B**).

CD47 expression is associated with SLE disease activity

Higher expression of CD47 by SLE monocytes suggests that it might be associated with SLE disease activity. Therefore, the association between the SLE disease activity and CD47 expression by monocytes was investigated. CD47 expression by SLE monocytes correlated with SLE disease activity (Spearman's rho = 0.467, p = 0.019); there was no association between SIRPα expression and disease activity (Spearman's rho = -0.319, p = 0.119) (**Figure 3A and B**). CD47 and SIRPα expression did not correlate with patient age (**Figure 3C and D**).

CD47 expression is upregulated in serum from SLE patients

Healthy PBMCs were incubated with serum from HCs (n = 6) and from SLE patients with low (n = 6) and high disease activity (n = 4). CD47 expression by monocytes was higher in SLE serum than in healthy serum (155.9% ± 17.5% *vs.* 119.8% ± 9.8%, respectively; $p < 0.001$) (**Figure 4A**). Subgroup analysis (according to SLE disease activity) revealed that the -fold increase in CD47 expression by monocytes was higher when cells were exposed to serum from SLE patients with high disease activity (SLEDAI > 12) (MFI: 164.5% ± 21.7%, $p = 0.002$) or serum from those with low disease activity (SLEDAI < 12) (MFI: 150.3% ± 13.1%, $p = 0.001$) than when cells were exposed to healthy serum (**Figure 4B**). Since IFN-α is a key cytokine involved in SLE pathogenesis, and its levels correlate with disease activity, healthy monocytes were stimulated with increasing concentrations of IFN-α. IFN-α increased CD47 expression in a dose-dependent manner (**Figure 4C**), whereas TNF-α did not (**Figure 4D**).

CD47 activation potentiates proinflammatory responses

To investigate the role of CD47 during proinflammatory responses, healthy

PBMCs were incubated with an anti-CD47 monoclonal antibody. The anti-CD47 antibody increased TNF-α production by 1.2 ± 0.4-fold compared with the isotype control antibody (used as a reference). LPS increased TNF-α production by monocytes by 7.3 ± 5.5-fold. Interestingly, treating cells with an anti-CD47 antibody and LPS increased TNF-α production up to 21.0 ± 10.9-fold, suggesting that CD47 might potentiate inflammatory responses by monocytes (**Figure 5**).

CD47 activation induces MAPK, but not NF-κB, signaling

Production of TNF-α involves activation of the NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) and mitogen-activated protein kinase (MAPK) pathways. We used THP1 cells, a monocytic tumor cell line, to examine how ligation of CD47 affects the MAPK and NF-κB signaling pathways. At baseline, THP1 cells showed strong surface expression of CD47 (**Figure 6A**).

THP1 cells were activated by exposure to an anti-CD47 antibody for 30 min and downstream MAPK signaling was investigated using a phospho-MAPK array kit. ERK, JNK, and p38 were activated but AKT was not (**Figure 6B**). By contrast, ligation of CD47 did not affect phosphorylation of NF-κB signaling

components. The phosphorylation status of STAT1 was influenced by CD47 activation (**Figure 6C**). Of note, activating CD47 with soluble recombinant SIRPα elicited an effect similar to that observed for MAPK signaling (**Figure 7**).

To summarize, during NF-κB signaling diverse proinflammatory cytokines (including LPS via TLR4) activate MyD88 and, subsequently, inhibitor of NF-κB (IκB) kinase (IKK), which releases NF-κB (Rel-p50 dimer) from IκB. Free NF-κB translocates to the nucleus and binds to the promotors of genes that upregulate transcription of proinflammatory cytokines. NF-κB signaling is terminated by IκB-mediated relocation of NF-κB to the cytoplasm and/or ubiquitination-dependent degradation of Rel in the Rel-p50 dimer [26].

Next, changes in key components of NF-κB signaling were examined in activated THP-1 cells. Ligation of CD47 with an anti-CD47 antibody did not activate MyD88, an upstream molecule in the NF-κB signaling pathway; however, compared with that in untreated control samples, the MyD88 level changed markedly after stimulation with LPS (**Figure 8A**). Similarly, treatment with an anti-CD47 antibody and LPS did not influence the levels of IKK (the activator of NF-κB) or IκB (the inhibitor of NF-κB). However, LPS treatment increased IKK levels and decreased IκB levels markedly. Strikingly, treatment with the anti-CD47 antibody and LPS reduced IκB levels further (**Figure 8A**).

The nuclear fraction of RelA (phospho-p65) did not increase after treatment with the anti-CD47 antibody and LPS (**Figure 8A and B**).

CD47 expression increases in a murine active nephritis model of SLE

Bethunaickan et al. described the role of monocytes/macrophages in a murine model of lupus nephritis at three different disease stages (i.e., the pre-nephritic, nephritic, and remission phases) [27]. Using the published microarray data (https://www.ncbi.nlm.nih.gov/geo/; GSE27045), expression of CD47 at three different stages of nephritis was examined. In the pre-nephritis kidney, relative expression of CD47 mRNA was low, but rose in kidneys with active nephritis. After treatment, expression of CD47 mRNA fell to baseline levels. SIRPα expression showed a similar pattern (**Figure 9**).

Autoantibodies directed against CD47 are present in SLE.

Since SLE is a characterized by production of various autoantibodies, serum of SLE patients (n = 13) and HC (n = 13) were screened for the presence of autoantibodies directed against recombinant CD47 using an ELISA. Anti-CD47

antibody levels were significantly higher in SLE patients than in HCs (21.4 ± 7.1 ng/mL *vs.* 16.1 ± 3.1 ng/mL, respectively; p = 0.02) (**Figure 10**). Antibody levels did not correlate with the SLEDAI (Spearman's rho = -0.11, p = 0.759).

Model of enhanced proinflammatory response by CD47 activation.

Based on the current findings, a following model of CD47 activation in SLE was postulated. Immune and non-immune cells respond to stimulatory nucleic acids derived from apoptotic cell debris by producing type 1 IFN, which upregulates CD47 expression by circulating monocytes and tissue-resident macrophages (e.g., in the kidney). Circulating SIRPα, thrombospondin, and/or autoantibodies directed against CD47 bind to CD47 and activate inflammatory MAPK signaling (e.g., via ERK, JNK, and p38). Activation of monocytes by a TLR agonist such as LPS activates NF-κB signaling and induces transcription of TNF-α mRNA. Activation of ERK, JNK, and p38 by CD47 promotes transcription of mRNA and stabilizes it, which ultimately leads to an increase in total TNF-α production (**Supplementary figure 3**).

Table 1. Baseline characteristics of the 25 SLE patients and 16 heathy controls.

	SLE patients (n = 25)	HCs (n = 16)
Age, years	38.7 ± 12.8	28.8 ± 4.2
Female, n (%)	23 (92.0)	12 (75)
SLE duration, years	4.2 [0.9–14.0]	
White blood cell, × 10^3/μL	5.7 ± 2.9	
Hematocrit, %	38.6 [34.5–39.9]	
Platelet, × 10^3/μL	190.7 ± 71.0	
ESR, mm/hour	19 [14.5–33.0]	
Anti-dsDNA, IU/mL (ref: 0–10 IU/mL)	16.7 [7.7–40.5]	
C3, mg/dL (ref: 83–193 mg/dL)	86.0 [57.5–98.7]	
C4, mg/dL (ref: 15–57 mg/dL)	14.5 [7.0–16.7]	
SLEDAI-2K	6 [2–9.5]	
Treatment		
Corticosteroids	20 (80.0)	
Prednisolone equivalent, mg/day	5 [0–125]	

Hydroxychloroquine	16 (64.0)
Azathioprine	2 (8.0)
Mycophenolate mofetil	1 (4.0)
Sulfasalazine	1 (4.0)
NSAIDs	6 (24.0)

Data are expressed as the mean ± SD, median [IQR], or n (%).

ESR, erythrocyte sedimentation rate; HC, heathy control; IQR, interquartile range; NSAIDs, nonsteroidal anti-inflammatory drugs; ref, reference range; SLE, systemic lupus erythematosus; SLEDAI-2K, SLE disease activity index 2000.

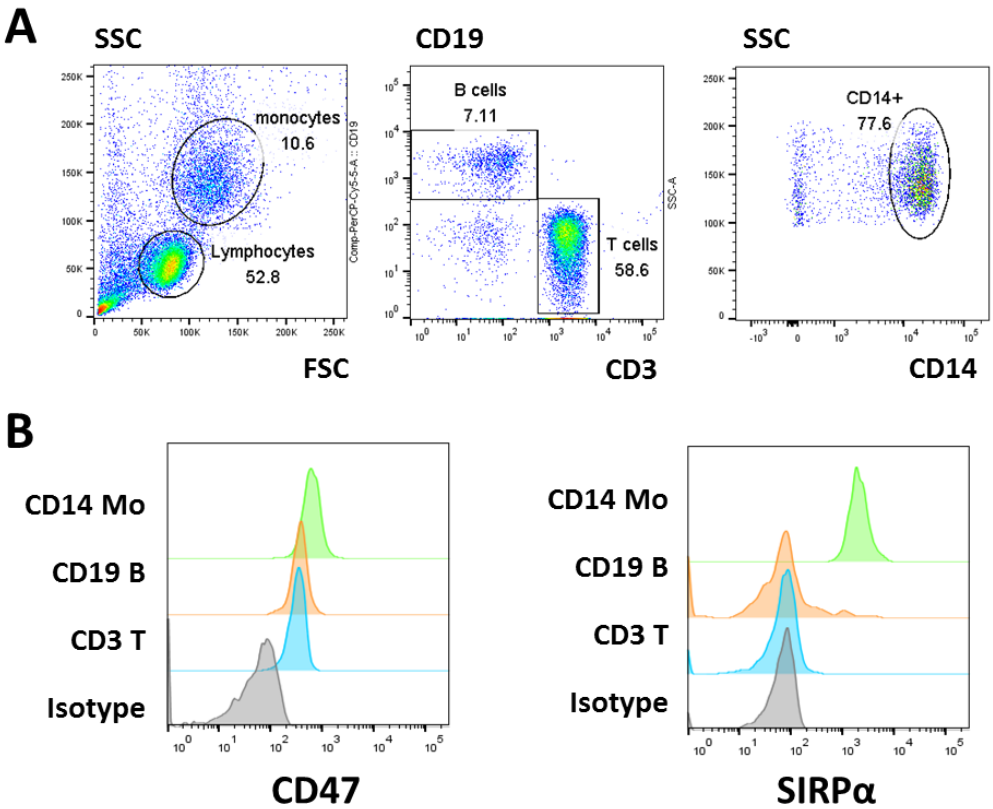


Figure 1. Expression of CD47 and SIRPα by circulating blood cells.

PBMCs from healthy controls were examined with respect to CD47 and SIRPα expression. (A) CD19+ B cells, CD3+ T cells, and CD14+ monocytes were gated. (B) All three cell types showed strong expression of CD47 on the surface. CD47 expression was highest on monocytes; B and T cells expressed similar levels of CD47 (histogram). SIRPα was expressed by monocytes, but not by B and T cells.

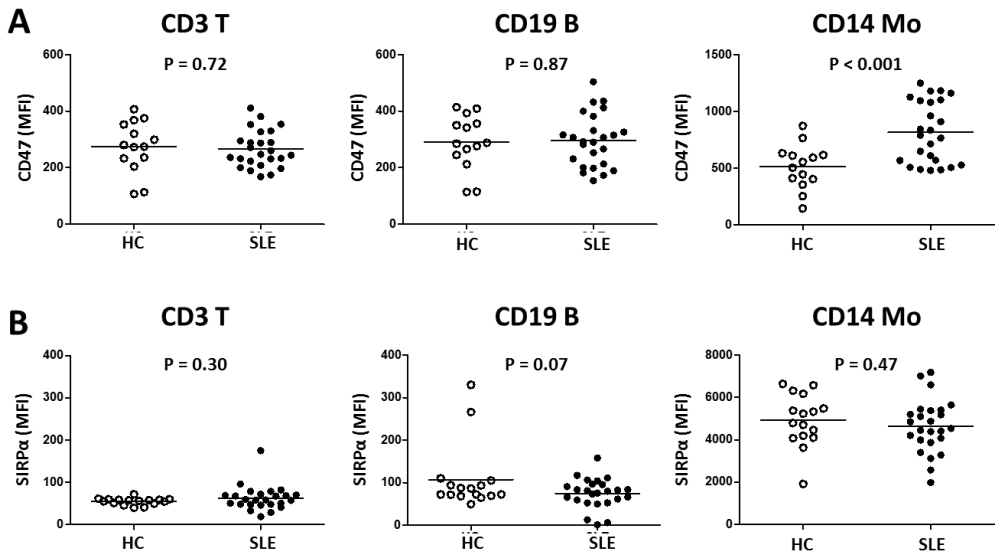


Figure 2. CD47 expression by SLE monocytes is increased.

Expression of CD47 and SIRPα by PBMCs from 25 SLE patients and 14 healthy controls (HCs). (A) CD47 expression by T and B cells did not differ between SLE patients and HCs. However, SLE monocytes expressed higher levels of CD47 than HC monocytes. (B) SIRPα expression did not differ between SLE patients and HCs. P values were generated using t-tests. MFI, mean fluorescence intensity; Mo, monocytes.

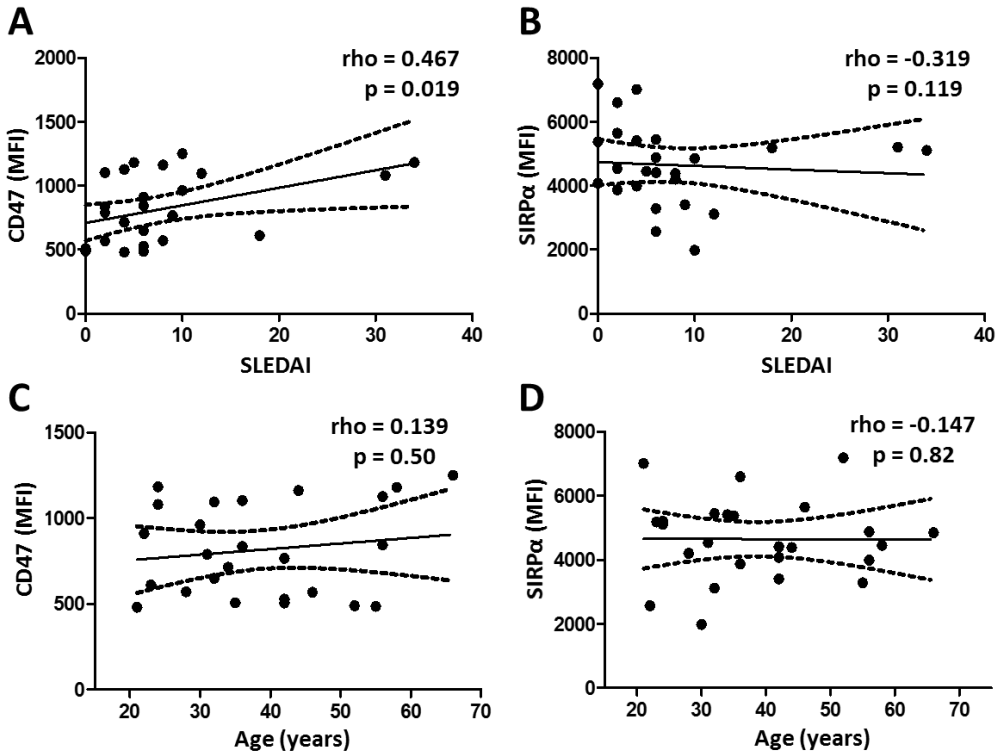


Figure 3. Expression of CD47, but not SIRPα, by monocytes correlates with SLE disease activity.

(A) CD47 expression by monocytes correlated with disease activity. (B) SIRPα expression did not correlate with disease activity. CD47 (C) and SIRPα (D) expression did not correlate with patient age. P values were generated by Spearman's correlation analysis. MFI, mean fluorescence intensity; SLE, systemic lupus erythematosus; SLEDAI, SLE disease activity index 2000.

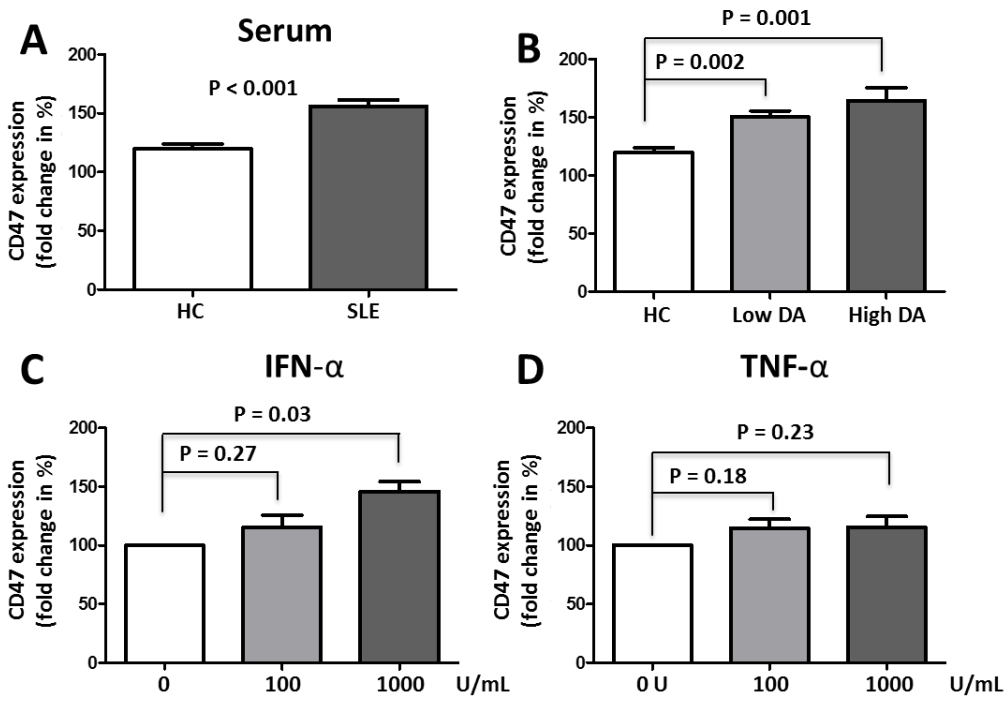


Figure 4. Upregulation of CD47 by SLE serum or inflammatory cytokines.

Healthy PBMCs were incubated with serum from healthy controls (n = 6) and SLE patients (n = 10), and -fold changes in CD47 expression by monocytes were investigated by flow cytometry analysis. (A) SLE serum increased CD47 expression. (B) Upregulation of CD47 increased significantly when cells were activated by serum from patients with low (n = 6) and high (n = 4) disease activity. No significant difference in CD47 induction was detected between low and high disease activity samples. (C and D) Healthy PBMCs (n = 3) were incubated with increasing concentrations of interferon-alpha (IFN-α) and tumor necrosis factor-alpha (TNF-α). CD47 expression tended to increase with

increasing IFN-α concentration, whereas treatment with TNF-α had no significant effect. Untreated samples served as a reference (i.e., 100%). P values were generated using t-tests. DA, disease activity.

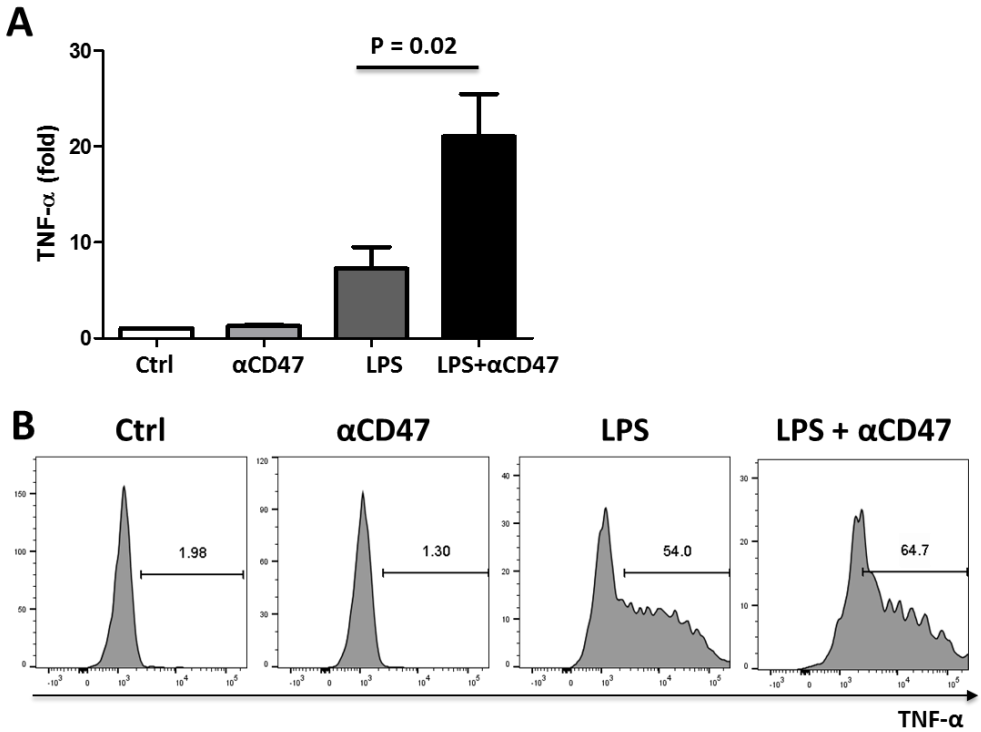
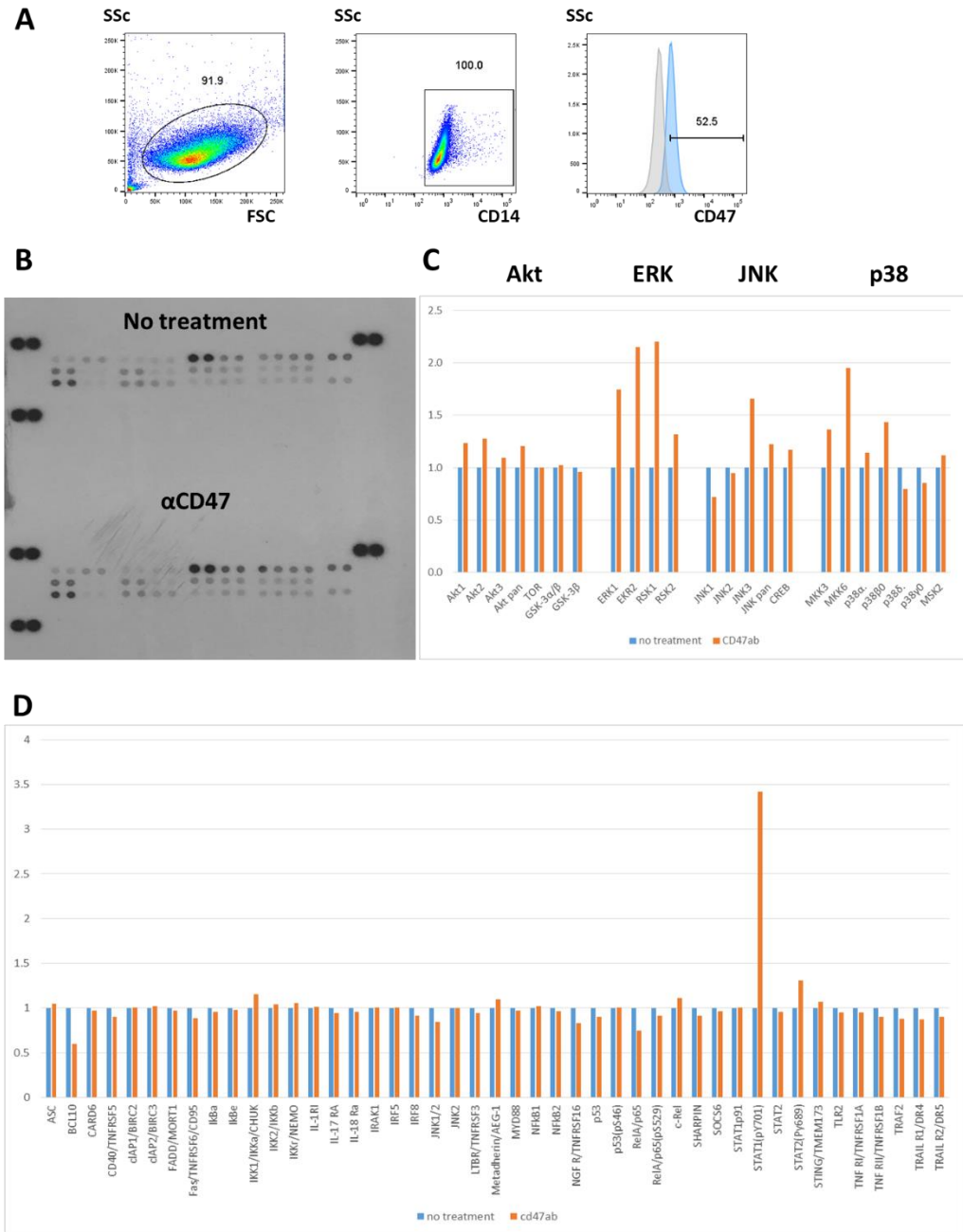


Figure 5. CD47 activation potentiates inflammatory responses.

(A) PBMCs (n = 6) were stimulated for 5 h with a mouse anti-human CD47 antibody, LPS (3 ng/mL), or both, and TNF-α production was measured by flow cytometry. Treatment with an anti-human CD47 antibody and LPS induced a significantly greater increase in TNF-α production than treatment with LPS alone. (B) Representative flow cytometry plots are depicted. P values were generated using t-tests. αCD47, anti-CD47 mouse monoclonal antibody; Ctrl, control; LPS, lipopolysaccharide; TNF, tumor necrosis factor.


A
SSc
91.9
FSC
SSc
100.0
CD14
SSc
52.5
CD47
B
No treatment
αCD47
C
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JNK
p38
Akt1
Akt2
Akt3
Akt pan
TOR
GSK-3α/β
GSK-3β
ERK1
ERK2
RSK1
RSK2
JNK1
JNK2
JNK3
JNK pan
CREB
MKK3
MKK6
p38α
p38β0
p38δ
p38γ0
MSK2
no treatment
CD47ab
D
ASC
BCL10
CARD6
CD40/TNFRSF5
cIAP1/BIRC2
cIAP2/BIRC3
FADD/MORT1
Fas/TNFRSF6/CD95
IκBa
IκBe
IKK1/IKKa/CHUK
IKK2/IKKb
IKKγ/NEMO
IL-1RI
IL-17 RA
IL-18 Ra
IRAK1
IRF5
IRF8
JNK1/2
JNK2
LTBR/TNFRSF3
Metadherin/AEG-1
MYD88
NFκB1
NFκB2
NGF R/TNFRSF16
p53
p53(pS46)
RelA/p65
RelA/p65(pS529)
c-Rel
SHARPIN
SOCS6
STAT1p91
STAT1(pY701)
STAT2
STAT2(Pγ689)
STING/TMEM173
TLR2
TNF RI/TNFRSF1A
TNF RII/TNFRSF1B
TRAF2
TRAIL R1/DR4
TRAIL R2/DR5
no treatment
cd47ab

Figure 6. Impact of CD47 activation by an anti-CD47 antibody on MAPK and NF-κB signaling pathways.

(A) THP1 cells express CD47 on the surface. (B–D) THP1 cells were treated with an anti-CD47 antibody and Phospho-MAPK and Phospho-NF-κB arrays were performed 30 min later. (B) A representative phospho-array blot is shown. (C) ERK, JNK, and p38 components of the MAPK signaling pathway were activated by the anti-CD47 antibody, while AKT was not. (D) NF-κB signaling components remained unaffected by activation of CD47.

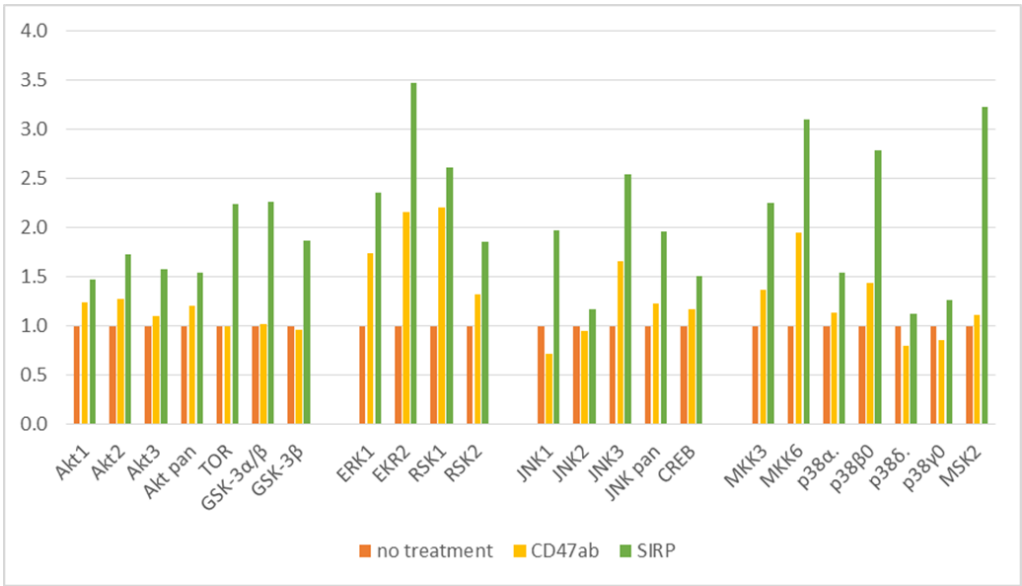


Figure 7. The effects of SIRPα on MAPK signaling are similar to those of the anti-CD47 antibody.

THP1 cells were treated with soluble recombinant SIRPα or an anti-CD47 antibody and -fold changes in MAPK components (relative to untreated samples) were measured. Treatment with SIRPα induced changes in MAPK signaling components similar to those induced by the anti-CD47 antibody.

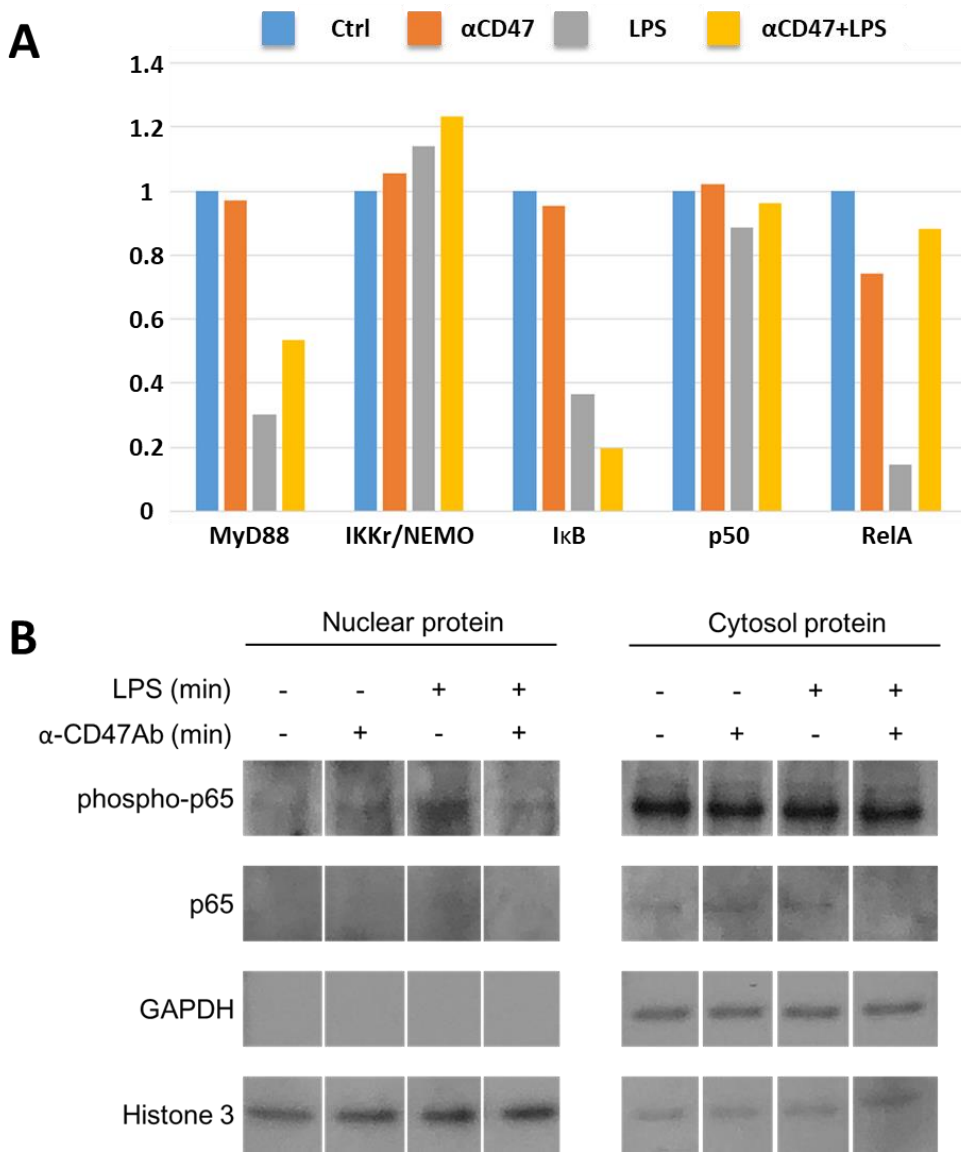


Figure 8. Effect of CD47 and LPS on NF-κB.

(A) THP1 cells were treated with an anti-CD47 antibody and LPS. Fold changes in key NF-κB signaling components (relative to untreated samples) are shown. The effects of an anti-CD47 antibody plus LPS were similar to those of LPS

alone. (B) Nuclear translocation of NF-κB was not enhanced by the anti-CD47 antibody. The level of phospho-p65 in the nucleus was not increased by treatment with the anti-CD47 antibody plus LPS. GAPDH (a cytosolic protein) and Histone 3 (a nuclear protein) served as controls for separation of nuclear from cytosolic material. αCD47, anti-CD47 antibody; IκB, inhibitor of NF-κB; IKK; IκB kinase; LPS, lipopolysaccharide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

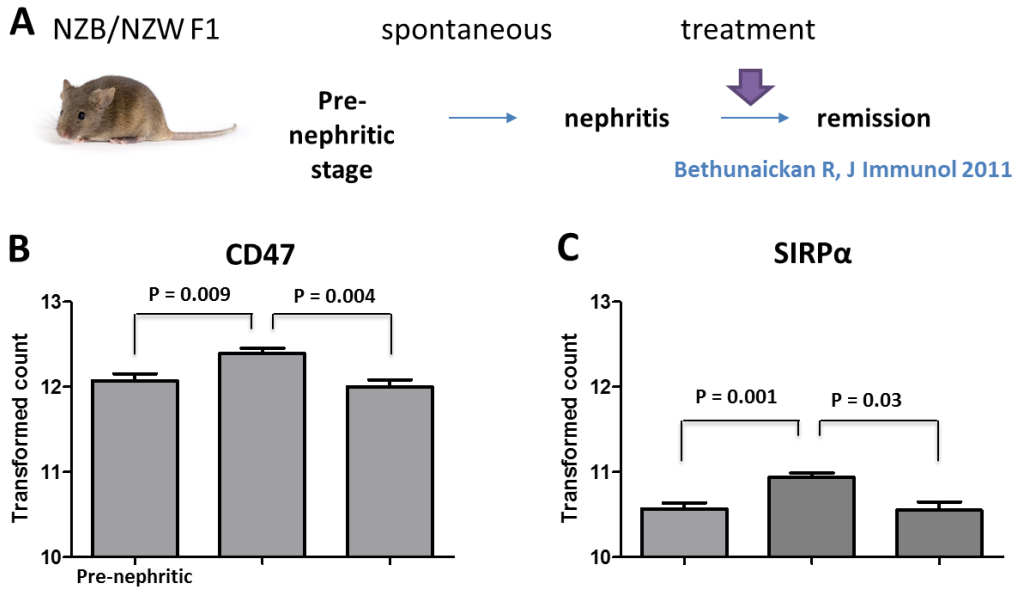


Figure 9. CD47 expression in the nephritic mouse kidney.

(A) NZB/NZW F1 mice develop nephritis spontaneously. Nephritis was treated with a single dose of cyclophosphamide and six doses of CTLA4 Ig plus anti-CD154 antibodies. (B and C) Expression of mRNA encoding CD47 (B) and SIRPα (C) by macrophages in pre-nephritic (n = 6) and nephritic (n = 7) kidneys, and in kidneys in remission (n = 4), was examined. MRNA encoding CD47 or SIRPα increased in nephritis kidneys but fell to baseline levels during remission. GEO DataSets (https://www.ncbi.nlm.nih.gov/gds/). P values were generated using t-tests.

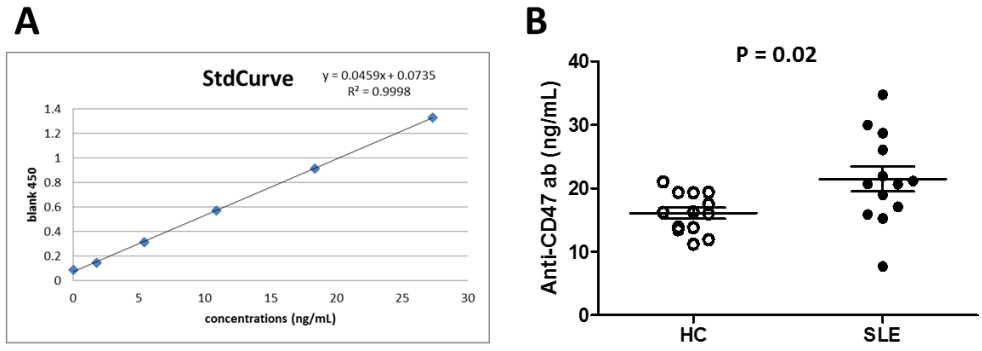
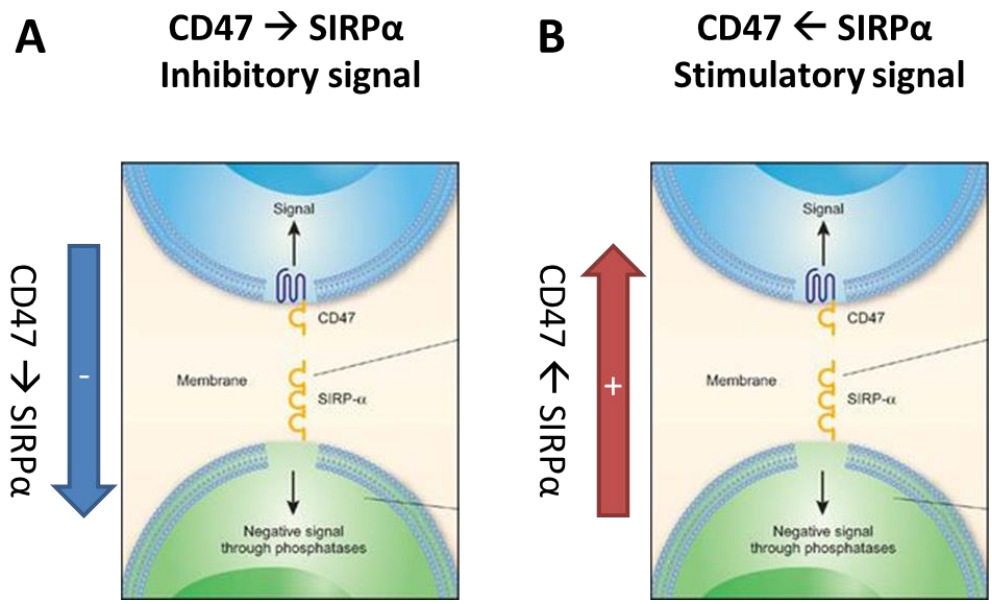


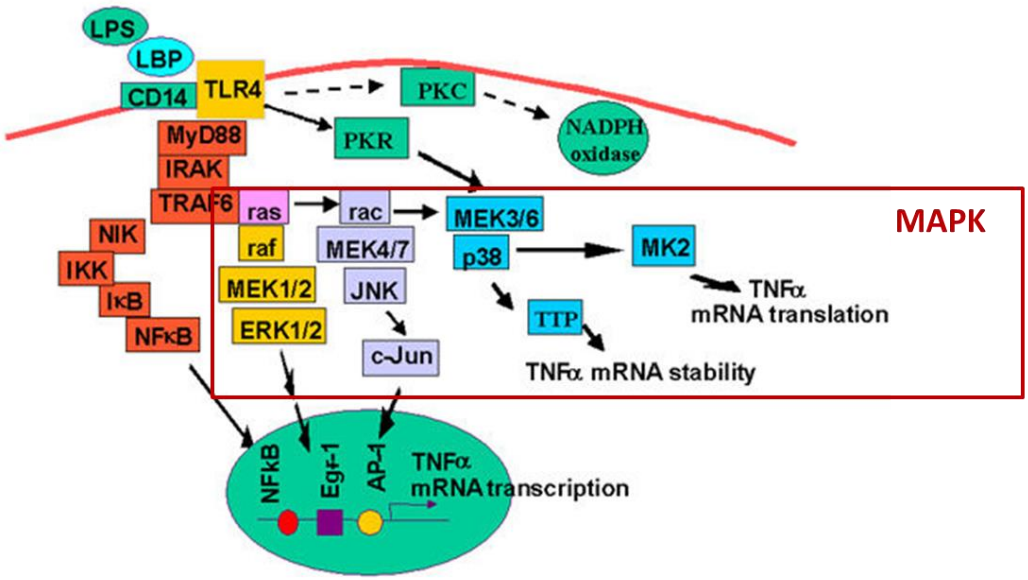
Figure 10. Detection of anti-CD47 antibodies in serum from SLE patients.

(A) A standard curve for the anti-CD47 antibody was generated using recombinant CD47 protein and a monoclonal mouse-human anti-CD47 antibody. (B) Serum from 13 SLE patients and 13 healthy controls (HCs) was screened for anti-CD47 antibodies using ELISA. Serum anti-CD47 antibody levels were significantly higher in SLE patients than in HCs. P value was generated using a t-test.



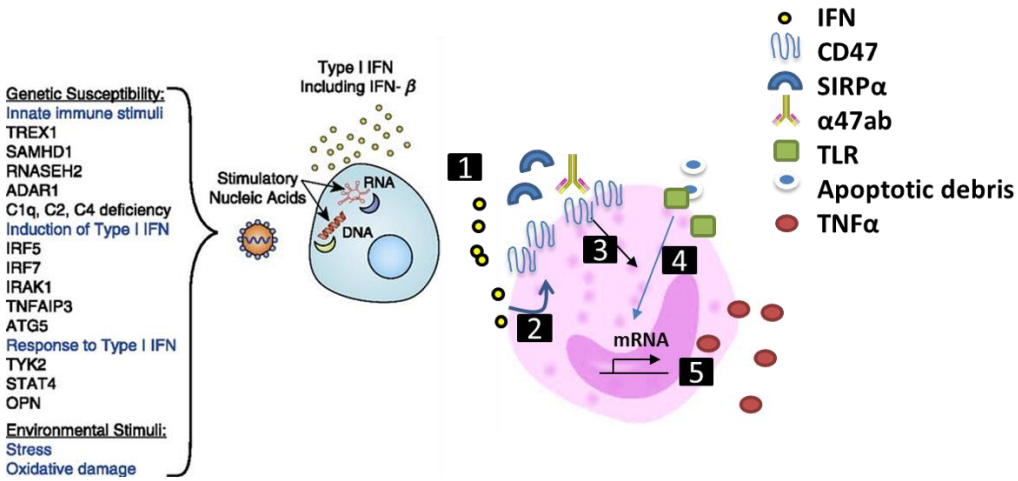
Supplementary figure 1. Bidirectional CD47-SIRPα interaction.

(A) Inhibitory signaling. CD47 transduces a so-called "do not eat me" inhibitory signal to adjacent macrophages via the SIRPα receptor, which suppresses macrophage functions, including phagocytosis. (B) Stimulatory signaling. SIRPα transduces a stimulatory signal to adjacent cells via CD47. In response to CD47 ligation, cells produce cytokines and exhibit increased resistance to cellular stress. Modified from Takizawa et al. Nat Immunol 2007.



Supplementary figure 2. Signaling pathways leading to TNF-α production.

LPS induces TNF-α production by macrophages *via* NF-κB and MAPK. ERK, JNK, and p38 increase transcription of (and stabilize) TNF-α mRNA and promote its translation. LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase. Modified from Kishore et al. Comp Hepatol 2004.



Supplementary figure 3. Proposed role for CD47 in SLE.

(1) Immune and non-immune cells produce type 1 IFN when they encounter stimulatory nucleic acids released from apoptotic debris. (2) Type 1 IFN increases CD47 expression by monocytes. (3) CD47 is activated by circulating autoantibodies; it then activates MAPK signaling components (including ERK, JNK, and p38). (4) LPS or other ligands bind to TLRs and activate NF-κB signaling. (5) Activated MAPK promotes transcription (and stability) and translation of TNF-α mRNA, leading to increased production of TNF-α protein. Modified from Mary K. Crow J Immunol 2014.

DISCUSSION

To the best of our knowledge, this is the first study to suggest that CD47 is involved in pathogenesis of SLE: (1) CD47 expression by monocytes correlates with SLE disease activity, with higher expression being observed in those with active SLE (2) tissue-infiltrating macrophages show higher expression of CD47 and (3) activation of CD47 with an anti-CD47 antibody potentiates production of proinflammatory cytokines by monocytes in response to LPS.

CD47 plays an important role in survival of activated immune cells by providing a “do not eat me” signal to neighboring macrophages [8, 9]. This may explain why activated immune cells are not immediately eliminated by adjacent activated macrophages during a proper immune response. However, prolonged survival of activated immune cells upon upregulation of CD47 might diminish/delay active resolution of inflammatory responses, leading to collateral damage to organs due to prolonged and chronic inflammation [28]. Here, CD47 expression by monocytes correlates with SLE disease activity and is upregulated by sera from SLE (**Figure 3**). A potential candidate factor in SLE serum for CD47 induction is IFN-α, which as a key cytokine in SLE increases during active lupus flare [29]. In the current study, exposure to IFN-α increased CD47 expression by healthy monocytes in a dose-dependent manner (**Figure 4**). Interestingly, TNF-α, a major cytokine produced during inflammatory responses

to bacterial infections and rheumatoid arthritis, did not upregulate CD47 expression significantly. Therefore, it is possible that CD47 upregulation is not a general feature of immune responses. Rather, it might be specific to diseases such as SLE in which type 1 IFN plays a key role. It is possible that other soluble factors, such as circulating immune complexes, might increase expression of CD47 by monocytes.

This suggests that the CD47-SIRPα interaction during active SLE is regulated mainly by CD47 expression. This is crucial, since changes in inhibitory SIRPα expression by monocytes and macrophages would affect the overall interaction with non-immune cells, which also express CD47 as a marker of "self". Therefore, preferential upregulation of CD47 by activated immune cells might help them escape premature elimination by phagocytosis during acute immune responses; this is consistent with a prior finding that high expression of CD47 by CD4 cells is associated with their longevity [30]. Accordingly, high CD47 expression might also lead to accumulation of (autoimmune) cells during chronic autoimmune disease. One might also speculate that low CD47 expression by B and T cells relative to monocytes might contribute to lymphopenia during active SLE, particularly when lymphocytes are additionally targeted by anti-lymphocyte antibodies to induce phagocytosis by activated monocytes and macrophages [31-33]. Further studies are needed to examine

whether sustained CD47 expression contributes to disrupted clearance of apoptotic cells in SLE [29, 34].

It is striking that CD47 ligation induced proinflammatory responses (**Figure 5**). When monocytes were treated with an anti-CD47 antibody, they produced more TNF-α in response to LPS. This synergistic effect of CD47 can be explained, at least in part, by the indirect stimulatory effect of CD47 ligation on monocytes, since treatment of monocytes with the anti-CD47 antibody alone did not induce TNF-α production. Alternatively, the inhibitory CD47-SIRPα interaction may be blocked by treatment with an anti-CD47 antibody; disinhibition of SIRPα-expressing monocytes might lead to increased production of inflammatory cytokines in response to LPS [35]. However, since treatment of monocytes with recombinant soluble SIRPα elicited a similar synergistic effect to anti-CD47 antibody on MAPK, CD47 activation by the anti-CD47 antibody seems to be crucial for induction of inflammatory responses. Further investigations are needed to determine whether surface SIRPα, a physiologic ligand of CD47, has a proinflammatory effect on CD47-expressing cells and augments proinflammatory responses *in vivo*. This synergistic effect might explain the findings of a previous report showing that SLE monocytes produce more TNF-α in response to apoptotic cells [20].

To further investigate the mechanism by which CD47 activation increases TNF-

α production in response to LPS stimulation, key components of the inflammatory MAPK and NF-κB signaling pathway were examined. CD47 activation with an anti-CD47 antibody had little effect on NF-κB signaling. However, CD47 activation with an anti-CD47 antibody or SIRPα activated the inflammatory components of the MAPK signaling pathway (ERK, JNK, and p38), which increased transcription and stabilization of mRNA encoding TNF-α, resulting in efficient translation [36] (**Supplementary figure 2**). Therefore, the effect of CD47 on TNF-α production is mediated indirectly by MAPK.

CD47 is involved in development of autoimmune diseases. Blockade of CD47 ameliorates encephalitis by suppressing IL-1-mediated infiltration of Th17 cells; indeed, CD47 knockout mice are refractory to development of experimental autoimmune encephalomyelitis [37, 38]. In addition, CD47 deficiency improves autoimmune nephritis in a murine model by suppressing IgG autoantibody production [16]. Expression of CD47 appeared to be increased in the tissue infiltrating monocytes/macrophages during active lupus nephritis. Further research is needed to examine whether CD47 activation with an anti-CD47 antibody worsens, or whether specific blockade of CD47 ameliorates, SLE disease activity in a pristane-induced murine lupus model, which is characterized by IFN production by activated immature monocytes [39, 40].

SLE is a prototype autoimmune disease of the multiple autoantibody production

[1], suggesting that anti-CD47 autoantibodies can interact with CD47 in SLE. In this preliminary study, SLE patients showed higher antibodies levels than HCs. Their biologic effect on CD47 in vivo remains unclear. Anti-CD47 antibodies in SLE patients might have inhibitory or stimulatory functions depending on the situation. Therefore, the functional effects of anti-CD47 autoantibodies on the CD47-SIRPα interaction need to be determined. Furthermore, these antibodies might be an innocent bystander, similar to anti-nuclear antibodies, the titers of which do not correlate with disease activity. Rather, the proinflammatory effects of CD47 might be mediated by direct cell-to-cell contact or by soluble ligands (such as soluble SIRPα). Taken together, the data suggest that CD47 likely contributes to SLE pathogenesis by increasing inflammatory responses; therefore, inhibiting CD47 might offer a new therapeutic target.

It is critical to emphasize that the CD47-SIRPα interaction is bidirectional. While CD47-expressing monocytes receive stimulatory signals, SIRPα-expressing monocytes are inhibited. Since monocytes express both CD47 and SIRPα, the sum or balance of both signals might determine the final impact of the CD47-SIRPα interaction on cellular responses. Net suppression of the CD47-SIRPα interaction might be associated with immune deficiency, leading to increased susceptibility of SLE patients to infection and cancer [41-43]. All this highlights the complexity of immune regulation and importance of the fine

balance between stimulatory and inhibitory signals in immune regulation.

Limitations

This study has several limitations. The exact mechanism by which CD47 expression by monocytes is regulated by type 1 IFN in SLE needs further investigation. Also, it is not clear whether CD47-expressing monocytes contribute directly to tissue damage *in vivo* or whether they are innocent bystanders. The protein that links CD47 to MAPK also needs to be identified.

Conclusions

Monocytes from SLE patients show upregulated expression of CD47, which correlates with disease activity. CD47 activation might contribute to augmented proinflammatory responses in SLE, including TNF-α production. Targeting CD47 might offer a novel therapeutic opportunity in patients with SLE.

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LIST OF ABBREVIATIONS

ANA	Anti-nuclear antibody
CD	Cluster of differentiation
CS	Corticosteroids
DA	Disease activity
ESR	Erythrocyte sedimentation rate
HCs	Healthy controls
ICQ	Interquartile range
IFN	Interferon
IKK	Inhibitor of NFκB (IκB) kinase
IL	Interleukin
IκB	Inhibitor of NFκB
MAPK	Mitogen-activated protein kinase
MFI	Mean fluorescence intensity
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NSAID	Nonsteroidal anti-inflammatory drugs
PBMC	Peripheral blood mononuclear cells
SIRPα	Signal regulatory protein alpha
SLE	Systemic lupus erythematosus
SLEDAI-2K	SLE disease activity index 2000

TLR	Toll-like-receptors
TNF	tumor necrosis factor

국문초록

CD47-SIRPα 상호작용이 전신 홍반 루푸스의 면역반응에 미치는 영향

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박 진 균

배경

전신 홍반 루푸스(Systemic lupus erythematosus, SLE)는 면역세포의 비정상적인 활성화로 인한 만성 염증반응 및 장기 손상을 초래하는 자가면역질환이다. CD47 단백질과 그 리간드인 signal regulatory protein alpha (SIRPα) 간의 상호작용은 단핵구의 활성화의 조절에 중요한 역할을 한다. 본 연구는 CD47 이 루푸스에서 비정상적인 염증 반응에 기여하는지 규명하고자 하였다.

방법

루푸스 환자와 건강 대조군 (HC)의 말초 혈액 단핵 세포 (PBMC)에서 CD47 및 SIRPα 의 발현 정도를 유세포분석(flow cytometry)으로 측정하였다. 루푸스 환자 혈청, 건강인 혈청, 인터페론 (IFN)-α 및 종양 괴사 인자 (TNF)-α 로 자극 후 단핵구에서 CD47 발현을 정량하였다. 단핵구 및 THP1 세포를 항 CD47 항체 또는 lipopolysaccharide (LPS)로 자극한 후 tumor necrosis factor (TNF)-α 생성과 mitogen-activated protein kinase (MAPK) 및 NFκB signaling 전달체의 변화를 분석하였다. 루푸스 환자 및 건강 대조군의 혈청에서 CD47 에 대한 자가항체의 존재 여부를 ELISA 를 이용하여 확인하였다.

결과

단핵구 세포에서의 CD47 발현 정도는 건강 대조군(n = 16)에 비해 루푸스 환자(n = 25)에서 증가하였다(mean fluorescence intensity: 511.5 ± 199.4 vs 815.9 ± 269.4 $p < 0.001$). 단핵구의 CD47 발현 정도는 루푸스 환자의 질병 활성도와 상관관계를 (rho = 0.467, p = 0.019) 보였다. 환자의 혈청 또는 IFN-α 로 단핵구를 자극 할 때 CD47 세포표면 발현이 증가하였지만, TNF-α 자극에 의해 CD47 발현 정도가 변화하지 않았다. 단핵구의 TNF-α 생성이 LPS 자극 후 7.3 배로 증가하였지만, 항-CD47 항체로 전처리 후 LPS 자극 시 TNF-α 생성이 20.1 배로 상승되었다. CD47 자극 후 MAPK

signaling 는 활성화 되었지만 NFκB signaling 은 영향 받지 않았다. 쥐 루푸스 모델의 신염 조직에 침윤하는 대식세포에서 CD47 mRNA 발현 양도는 신염의 정도와 연관되었다. 항 CD47 자가항체는 루푸스 환자 혈청에서 유의하게 증가되었다.

결론

단핵세포에서 CD47 발현은 루푸스 질환 활성도와 상관관계가 있으며, CD47이 MAPK-signaling 을 통해 염증 반응을 증폭시킨다. CD47-SIPRα 상호 작용은 새로운 루푸스 치료제의 표적으로 기대할 수 있을 것으로 사료된다.

주요어: 정신 홍반 루푸스, CD47, SIRP-alpha, 염증

학번: 2015-30715