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Neutrophil extracellular trap (NET) formation contributes to immune defense and is a distinct form of cell death. Excessive NET formation is found in patients with anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV), contributing to disease progression. The clearance of dead cells by macrophages, a process known as efferocytosis, is regulated by the CD47-mediated “don’t eat me” signal. Hence, we hypothesized that pathogenic NETs in AAV escape from efferocytosis via the CD47 signaling pathway, resulting in the development of necrotizing vasculitis. Immunostaining for CD47 in human renal tissues revealed high CD47 expression in crescentic glomerular lesions of patients with AAV. In ex vivo studies, ANCA-induced netting neutrophils increased the expression of CD47 with the reduction of efferocytosis. After efferocytosis, macrophages displayed pro-inflammatory phenotypes. The blockade of CD47 in spontaneous crescentic glomerulonephritis-forming/Kinjoh (SCG/Kj) mice ameliorated renal disease and reduced myeloperoxidase (MPO)-ANCA titers with a reduction in NETs formation. Thus, CD47 blockade would protect against developing glomerulonephritis in AAV via restored efferocytosis of ANCA-induced NETs.

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CD47 blockade ameliorates autoimmune vasculitis via efferocytosis of neutrophil extracellular traps

Satoka Shiratori-Aso¹, Daigo Nakazawa¹, Takashi Kudo¹, Masatoshi Kanda², Yusho Ueda¹, Kanako Watanabe-Kusunoki¹, Saori Nishio¹, Sari Iwasaki³, Takahiro Tsuji³, Sakiko Masuda⁴, Utano Tomaru⁵, Akihiro Ishizu⁴, Tatsuya Atsumi¹

¹Department of Rheumatology, Endocrinology, and Nephrology, Faculty of Medicine and Graduate School of Medicine, Hokkaido University, Sapporo, Japan
²Division of Rheumatology and Clinical Immunology, Sapporo Medical University, Sapporo, Japan
³Department of Pathology, Sapporo City General Hospital, Sapporo, Japan
⁴Department of Medical Laboratory Science, Faculty of Health Sciences, Hokkaido University, Sapporo, Japan
⁵Department of Pathology, Faculty of Medicine and Graduate School of Medicine, Hokkaido University, Sapporo, Japan

Corresponding Author: Daigo Nakazawa
Department of Rheumatology, Nephrology and Endocrinology, Faculty of Medicine, Hokkaido University, Kita 14, Nishi 5, Kita-ku, Sapporo, 060-8648, Japan
Tel: +81-11-706-5915 Fax: +81-11-706-7710. E-mail: daigo-na@med.hokudai.ac.jp

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Abstract

Neutrophil extracellular trap (NET) formation contributes to immune defense and is a distinct form of cell death. Excessive NET formation is found in patients with antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV), contributing to disease progression. The clearance of dead cells by macrophages, a process known as efferocytosis, is regulated by the CD47-mediated “don’t eat me” signal. Hence, we hypothesized that pathogenic NETs in AAV escape from efferocytosis via the CD47 signaling pathway, resulting in the development of necrotizing vasculitis.

Immunostaining for CD47 in human renal tissues revealed high CD47 expression in crescentic glomerular lesions of patients with AAV. In ex vivo studies, ANCA-induced netting neutrophils increased the expression of CD47 with the reduction of efferocytosis. After efferocytosis, macrophages displayed pro-inflammatory phenotypes. The blockade of CD47 in spontaneous crescentic glomerulonephritis-forming/Kinjoh (SCG/Kj) mice ameliorated renal disease and reduced myeloperoxidase (MPO)-ANCA titers with a reduction in NETs formation. Thus, CD47 blockade would protect against developing glomerulonephritis in AAV via restored efferocytosis of ANCA-induced NETs.
Introduction

Necrosis, which is a form of cell death resulting in leakage of cellular contents, induces inflammatory response (1). Dead or dying cells are removed by macrophages through a phagocytic process known as efferocytosis, and CD47 acts as a regulator of this procedure (2). CD47 is a widely expressed cell surface protein and serves as the “don’t eat me” signal or a “marker of self”. The binding of CD47 to signal regulatory protein α (SIRPα) on macrophages inhibits phagocytosis (3). Apoptotic cells are efficiently engulfed by macrophages through the exposure of phosphatidylserine as the “eat me” signal and the downregulation of CD47 as the “don’t eat me” signal (4). Tumor cells and atherosclerotic necrotic cores with increased CD47 expression escape efferocytosis, contributing to disease progression (5, 6). Recently, the involvement of CD47 in autoimmune diseases including systemic lupus erythematosus (SLE) has been described (7).

Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) is a group of disorders associated with systemic small-vessel vasculitis (8, 9). Renal disease in AAV is characterized by necrotizing crescent glomerulonephritis. Crescents are formed by the infiltrating immune and local necrotic cells (10). In particular, ANCA and humoral factors influence neutrophils to induce neutrophil extracellular trap (NET) formation, promoting vascular injury and activation of autoimmunity (11, 12). NETs contribute to immune defense against invading pathogens with neutrophil death as NETosis (13). However, dysregulated NETs cause excessive tissue damage via releasing reactive oxygen species and damage-associated molecular patterns (DAMPs) (1, 12). Moreover, persistent NETs can result in a breakdown of immunotolerance to NET-derived antigens. Therefore, NETs require clearance system. For example, phorbol myristate acetate (PMA)-induced NETs, which is a well-
characterized NETosis model, is known to be properly digested by deoxyribonuclease I and phagocytosis (14-16).

Here, we hypothesized that ANCA-induced NETs in AAV escape efferocytosis via the upregulation of CD47 and that persistent NETs amplify inflammatory reactions in the disease. We investigated the involvement of CD47 in crescentic glomerulonephritis using human renal tissues from patients with AAV and performed a series of in vitro experiments to examine the effects of CD47 blockade on the efferocytosis of NETs. Finally, we evaluated the therapeutic effects of anti-CD47 antibody in a spontaneous AAV mouse model.

Results

Enhanced expression of CD47 in crescentic glomerulonephritis of patients with AAV.

To assess the expression of CD47 in the kidney, we first performed IHC staining for CD47 in human renal biopsy specimens from patients with AAV in the active stage, lupus nephritis (LN) class IV, LN class V, and minor glomerular abnormalities (MGA) patients as a control. The clinical characteristics of the patients are presented in Supplementary Table S1. CD47 expression was significantly up-regulated in the renal tissues of patients with AAV compared to those of patients with LN and MGA. Immunostaining showed remarkable CD47 positivity in glomerular crescentic lesions, particularly in infiltrated granulocytes and injured epithelium (Figure 1A, 1B).

High expression of CD47 in ANCA-induced netting neutrophils. Next, we assessed the expression of CD47 on human non-stimulated neutrophils, apoptotic neutrophils, PMA-induced NETs, and ANCA-induced NETs. Ex vivo NET formation was induced by human TNF-α and ANCA-IgGs from patients with myeloperoxidase (MPO)-
ANCA positive AAV. TNF-α-primed neutrophils incubated with or without IgGs from healthy volunteers were used as controls. Neutrophils were treated with staurosporine (STS), an inducer of apoptosis, and the TdT-mediated dUTP nick end labeling (TUNEL) assay revealed increased apoptotic neutrophils with condensed nuclei (Supplementary Figure S1A and S1B). Immunofluorescence images revealed that ANCA-IgG or healthy-IgG-treated neutrophils primed with TNF-α, and PMA-induced netting neutrophils expressed high levels of CD47, whereas the CD47 expression levels on non-stimulated, apoptotic, and TNF-α-primed neutrophils were low (Figure 2A and Supplementary Figure S1C, S1D). Increased CD47 expression was observed on the cell membrane of neutrophils with ANCA- and PMA-induced NETs, but not on extracellular DNA fibers (Supplementary Figure S1E and Supplementary Movie S1). FCM analysis showed that healthy-IgG-treated neutrophils primed with TNF-α expressed significantly higher levels of CD47 than non-stimulated neutrophils, and that the expression of CD47 on ANCA-induced netting neutrophils was significantly higher than that on neutrophils treated with healthy-IgGs (Figure 2B and 2C).

Macrophage efferocytosis of ANCA-induced NETs via CD47 signaling. Based on human and ex vivo data, we hypothesized that enhancing the expression of CD47 on injured cells, including ANCA-induced NETs, initiates escape from efferocytosis. 5-chloromethylfluorescein diacetate (CMFDA)-labeled human neutrophils were induced into STS-treated apoptotic neutrophils, TNF-α + healthy-IgG-treated neutrophils, or TNF-α + ANCA-treated NETs. Apoptotic neutrophils, and NET neutrophils were co-cultured with M1 macrophages in the presence of pretreatment with PBS, mouse IgG1 isotype control Ab (CT-Ab), anti-CD47 mAb, anti-CD47 mAb + Fc receptor (FcR)
blocker, or anti-CD47 F(ab’)2 fragments. Phase and fluorescence imaging showed
that after 3 h of co-culture, non-stimulated neutrophils and TNF-α + healthy-IgG-
treated neutrophils escaped efferocytosis. Apoptotic neutrophils were engulfed more
efficiently by macrophages than NETs (efferocytosis rate/apoptotic neutrophils +
macrophages; 20.5 ± 3.8%, NETs pretreated with CT-Ab + macrophages; 7.7 ± 2.2%,
*p<0.05), whereas the efferocytosis rate of NETs was significantly increased by
pretreatment with anti-CD47 mAb (NETs pretreated with anti-CD47 mAb +
macrophages; 19.1 ± 4.2%, p<0.05, compared to NETs pretreated with CT-Ab +
macrophages) (Figure 3A and 3B). The efferocytosis rate of NETs was not recovered
by pretreatment with anti-CD47 mAb and FcR blocker, or anti-CD47 F(ab’)2
fragments. To precisely evaluate cellular dynamics during efferocytosis, time-lapse
imaging was performed using CMFDA-labeled neutrophils (green) and PKH26
labeled macrophages (red). Fluorescent images of co-cultures were captured every 7
min for 1 h from the beginning of the co-culture. NETs were not engulfed by
macrophages, but treatment with anti-CD47 mAb increased the engulfed NETs by
macrophages, similar to the apoptotic neutrophils (Figure 3C, 3D, and Movie S2-S6).
In immunostaining to determine the clearance of NETs by efferocytosis, unengulfed
NETs (citrullinated histone H3 (citH3) +/PKH26+) were reduced by anti-CD47 mAb
treatment (residual rate/NETs + macrophages; 86.7 ± 18.9%, NETs pretreated with
CT-Ab + macrophages; 84.3 ± 11.2%, NETs pretreated with anti-CD47 mAb; 42.4 ±
10.7%, p<0.05) (Supplementary Figure S2A and S2B).
To evaluate the effect of CD47 on ANCA-induced NET formation, NETs were
pretreated with anti-CD47 mAb or CT-Ab. SYTOX Green staining showed that
ANCA-induced NET formation was not affected by CD47 blockade (Supplementary
Figure S2C and S2D), indicating that CD47 blockade initiates the clearance of NETs
via efferocytosis but does not directly affect NET formation. To assess the response of
macrophages during efferocytosis of ANCA-induced NETs, total RNA was extracted
from macrophages after 2 h of exposure to neutrophils. In macrophages, co-incubation
with NETs enhanced the mRNA expression of pro-inflammatory cytokines (\textit{IL1B},
\textit{IL8}, monocyte chemotactic protein-1 \textit{[MCP1]}, and \textit{TNFA}), and treatment with anti-
CD47 mAb further increased these expression levels (Figure 3E).

\textit{Efferocytosis of injured endothelium via CD47 signaling.} Injured vascular
endothelium is a part of crescentic glomerular lesions in AAV. To investigate the role
of efferocytosis of necrotic endothelial cells, we performed an efferocytosis assay
using CMFDA labeled human umbilical vein endothelial cells (HUEhT) (green) and
PKH26 labeled macrophages (red). Reactive oxygen species released from NETs
result in injury of vascular endothelial cells. Thus, HUEhT cells were treated with 500
µM hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) to induce necrosis. The necrosis was confirmed by
propidium iodide (PI) staining (Supplementary Figure S3A and S3B). Time-lapse
imaging showed that necrotic HUEhT escaped macrophage efferocytosis, but necrotic
HUEhT pretreated with anti-CD47 mAb were engulfed by macrophages
(Supplementary Figure S3C, S3D, and Movie S7-S10).

\textit{Blockade of CD47 improves renal involvement in Spontaneous crescentic
glomerulonephritis-forming/Kinjoh (SCG/Kj) mice via the restored efferocytosis of
NETs.} Having shown that CD47 blockade ex vivo contributes to the clearance of
NETs and injured endothelium via efferocytosis, we tested whether renal disease in
AAV is abrogated by the systemic administration of anti-CD47 antibody. SCG/Kj
mice develop systemic necrotizing glomerulonephritis with ANCA production (17).
Eight-week-old mice were treated with intraperitoneal injections of either anti-CD47 mAb or rat IgG2a isotype control Ab (CT-Ab) every five days for two weeks, and ten-week-old mice were analyzed. CT-Ab-treated SCG/Kj mice developed glomerulonephritis with renal dysfunction. Treatment with anti-CD47 mAb improved renal failure (serum creatinine: anti-CD47 mAb; 0.96 ± 0.30 vs. CT-Ab; 0.61 ± 0.32 mg/dL, p<0.05) and attenuated kidney injury in histopathological examination (glomerular score: anti-CD47 mAb; 2.0 ± 0.63 vs. CT-Ab; 2.8 ± 0.41, p<0.05) (Figure 4A). Ly6B/citH3 double positive NETs were detected in glomeruli of untreated SCG/Kj mice (Supplementary Figure S4A), and the area of MPO and citH3 double positive NETs was decreased in glomeruli of anti-CD47 mAb-treated mice, compared to that of CT-Ab-treated mice (Figure 4B).

Moreover, anti-CD47 mAb reduced the mRNA expression of pro-inflammatory genes, including Ifna, Ifng, Mcp1, Prf1, and Il1b in renal tissue (Figure 4C).

We performed RNA-seq to assess the transcriptome changes in the renal tissue of SCG/Kj mice. Canonical pathway analysis using Ingenuity Pathway Analysis (IPA) software revealed the involvement of immunological responses including ‘Communication between innate and adaptive immune cells’ in CD47 blockade-treated mice (Supplementary Figure S4B). Next, to elucidate the mechanism of the protective effect of CD47 blockade, we tested whether anti-CD47 antibody binds to injured glomerular cells by immunostaining with anti-rat IgG, which is a host species of anti-CD47 mAb. Binding of anti-CD47 mAb was observed in the glomerulus of anti-CD47 mAb-treated SCG/Kj mice, whereas that of anti-CD47 mAb-treated C57BL/6 mice was not detected (Supplementary Figure S4C).

*The immune cell profiles in kidney of SCG/Kj mice treated with CD47 blockade.*
Considering ex vivo findings that macrophages engulf necrotic cells through the effects of CD47 blockade, but macrophages show pro-inflammatory properties, we questioned the mechanism how CD47 blockade influences the dynamics of immune cells in the kidneys of SCG/Kj mice. Immunostaining showed that the number of glomerular infiltrating CD68-positive macrophages in SCG/Kj mouse kidneys was not influenced by CD47 blockade (Figure 5A). Moreover, Nos2 (M1 macrophage marker) and Cd206 (M2 macrophage marker) mRNA expression in kidneys treated with CD47 blockade was similar to the expression in those treated with CT-Ab (Figure 5B). In mice treated with CD47 blockade, the glomerular infiltrating Ly6B-positive neutrophils showed an increasing trend (Figure 5C), implying that activated macrophages exhibiting efferocytosis may promote the recruitment of immune cells beyond those needed for focal cellular waste disposal. Furthermore, we performed deconvolution of our bulk kidney RNA-seq data using a reference single-cell RNA-seq data (18) to estimate the immune cell ratio in our data. The deconvolution revealed that CD47 blockade did not alter the profiles of immune cells, including neutrophils, macrophages, and lymphocytes in the kidney (Supplementary Figure S4D), which was consistent with the histological findings.

Systemic immune responses in SCG/Kj mice by CD47 blockade. As CD47 blockade promoted immune cell infiltration in the kidneys of SCG/Kj mice, we next evaluated the effect of CD47 blockade on systemic immunity. Treatment with CD47 blockade did not affect total IgG (Figure 5D) but significantly reduced MPO-ANCA (Figure 5E) in the serum of SCG/Kj mice. FCM analysis revealed that there were no significant differences in the number and proportion of splenic cells (neutrophils, monocytes, macrophages, dendritic cells, T cells and B cells) between anti-CD47
mAb-treated and CT-Ab-treated mice (Supplementary Figure S5A and S5B).

Discussion

Necrotizing vasculitis in AAV is formed by the necrosis of immune cells and blood vessels (10). We showed efferocytosis as a novel therapeutic target for AAV to treat necrotic lesions. Hence, we demonstrated several novel findings. Crescentic glomerular lesions in the kidneys of AAV patients and ex vivo ANCA-induced NETs increased the expression of CD47, an anti-efferocytotic signal. These results indicate that increased expression of CD47 in crescentic glomerulonephritis and ANCA-induced NETs is involved in AAV pathogenesis. In the efferocytosis assay, ANCA-NETs and injured endothelium escaped efferocytosis by macrophages, and the blockade of CD47 restored the efferocytosis of these cells. In vivo, blockade of CD47 by anti-CD47 mAb ameliorated renal injury and inflammation in a spontaneous AAV mouse model via the restored efferocytosis of NETs.

Neutrophils without stimulation store the majority of CD47 in intracellular-specific granules, and chemoattractant-stimulated neutrophils express CD47 to the cell membrane with granular pattern (19, 20). Apoptotic cells are removed by macrophage-mediated efferocytosis through the downregulation of CD47 as a “don’t eat me” signal (2, 4). Tumor and atherosclerotic necrotic cores increase the expression of CD47 and prevent efferocytosis, thereby causing disease progression. Blockade of CD47 prevents tumor growth (3) and atherosclerosis (6). Moreover, treatment with a CD47 blocking antibody ameliorates renal ischemia-reperfusion injury in mice (21). Thus, targeting CD47 is now in the spotlight as a therapy for various diseases involving impaired efferocytosis. Here, blockade of CD47 restored the efferocytosis of NETs and necrotic endothelium in an ex vivo model and protected against renal...
injury in AAV mice. NETs and necrotic debris themselves serve as DAMPs, initiating surrounding cell injury and amplifying the loop between cell death and inflammation (22); thus, the removal of necrotic contents through efferocytosis might prevent renal disease in AAV. In addition, CD47 blockade reduced pathogenic MPO-ANCA levels in spontaneous AAV mice with a decrease of NETs in glomeruli. The number of splenocytes and total IgG levels were not affected by the CD47 blockade; thus, blockade of CD47 in AAV does not influence systemic immunity but specifically might contribute to the reduction of MPO-ANCA via the restored efferocytosis of NETs containing MPO as an auto-antigen. Meanwhile, the migration of neutrophils into the kidneys of AAV mice was enhanced by the CD47 blockade. In vitro, macrophages exhibited a pro-inflammatory phenotype during efferocytosis of ANCA-NETs via CD47 blockade. In cancer cells, blockade of CD47 actively modulates the immune response and efferocytosis to initiate anti-tumor cytotoxicity (23, 24). In lymphocytic choriomeningitis virus-infected mice, antibody-mediated blockade of CD47 enhances antigen-presenting cell function and CD8+ T cell responses (25). Thus, it is important to understand the mechanism of immune response under the blockade of CD47, particularly in autoimmune diseases. CD47-knockout in \( \text{Fas}^{lpr} \) lupus mice reduces autoantibodies and protects mice against lupus (26). In experimental autoimmune encephalomyelitis (EAE) mouse models, pharmacological and genetic inhibition of CD47 in the induction phase reduces disease severity (27). In contrast, blocking with anti-CD47 mAb at the peak of disease worsens lupus (26) and EAE (27). The therapeutic effect may be influenced by the timing of CD47 inhibition in the development of autoimmune diseases. In our mouse study, anti-CD47 mAb was administered in the phase of disease development (17); as a result, it prevented renal disease with a reduction in pro-inflammatory gene expression, whereas it increased
infiltrating neutrophils with macrophage activation. Our study revealed the
differences in gene expression of pro-inflammatory cytokines under treatment with
CD47 blockade between macrophage co-incubated with NETs in vitro (Figure 3E)
and the whole kidneys of SCG/Kj mice (Figure 4C). This discrepancy is likely due to
several causes; in vitro condition, enforced efferocytosis against NETs initiates
inflammatory signals in macrophages, but in injured kidney, necrotic cells are
processed by restored efferocytosis, which might ameliorate surrounding tissues
damage and reduce the inflammatory gene expressions. These findings indicate that
the renoprotective effects of efferocytosis of NETs and glomerular necrotic cells
might exceed the activated immune response via CD47 blockade (Supplementary
Figure S6). In addition, blockade of CD47 in combination with immunosuppression,
including rituximab, is known to be more effective in patients with non-Hodgkin’s
lymphoma (28). Previous reports have shown that CD47 blockade induces
efferocytosis of lymphoma cells via Fc-FcR interactions (29, 30), and efferocytosis
assay of ANCA-induced NETs using FcR blocker or anti-CD47 F(ab’)2 in this study
showed that the effects of anti-CD47 mAb on efferocytosis were FcR dependent.
Thus, regulating immune response during efferocytosis might boost the effectiveness
of CD47 blocking therapy. Further studies are needed to understand the immune
mechanisms of AAV under CD47 inhibition. There are other limitations to our study.
In histological analysis of human renal biopsy, patients with biopsy-proven MGA
clinically presented with hematuria or proteinuria; therefore, they might be in
subclinical phage of kidney disease, which could influence CD47 expression. Second,
the effects of the anti-CD47 mAb on the active phase of AAV were not evaluated.
Considering that contradictory effects of CD47 blockade, presumably due to the
macrophage activation, have been reported in lupus and EAE mouse models, further
studies are needed to assess the therapeutic effects of anti-CD47 mAb in AAV mouse models with active disease.

In conclusion, ANCA-induced NETs in AAV escape efferocytosis via the upregulation of CD47. Blockade of CD47 restores efferocytosis of NETs and injured vascular endothelium, resulting in the improvement of renal disease in AAV. Therefore, CD47 blocking may be a novel therapeutic strategy for AAV.

**Methods**

*IHC for CD47 in human renal tissues.* Formalin-fixed, paraffin-embedded renal biopsy tissues from patients with AAV, LN class IV, LN class V, and controls (MGA) were sliced, deparaffinized, and rehydrated with lemosol and ethanol. Antigen retrieval was performed in sodium citrate buffer (10 mM sodium citrate, pH 6) in a microwave. Endogenous peroxidase was blocked with 3% H₂O₂ in methanol, and samples were covered with 10% goat serum at room temperature for 1 h. Slides were incubated at 4°C overnight with anti-CD47 antibody (1:700; HPA044659, Sigma-Aldrich). Biotin-labeled anti-rabbit polyclonal antibodies (Nichirei) were added for 1 h at room temperature, followed by peroxidase-conjugated streptavidin (Nichirei) and Histofine DAB Substrate Kit (Nichirei). The positive area was quantified using the ImageJ/Fiji software (NIH).

*Induction of apoptosis and NETs in human neutrophils.* Neutrophils were isolated from healthy human donors by density centrifugation using Polymorphprep (Axis- Shield), and were suspended in RPMI 1640 (Sigma-Aldrich). Apoptosis was induced using 0.05 µM STS (FUJIFILM Wako) for 4 h. Cell apoptosis was assessed by fluorescence microscopy using TUNEL staining kit (MBL). For ANCA-NET
induction, neutrophils were primed with human TNF-α (5 ng/mL; Merck) for 15 min
and then exposed to 400 µg IgGs eluted from the serum of healthy volunteers or
patients with MPO-ANCA positive AAV for 4 h (2 h for the efferocytosis assay). For
PMA-NET induction, neutrophils were exposed to PMA (50 nmol/L; Merck) for 2 h.
NET formation was quantified based on the SYTOX Green-positive area of
immunostaining as the mean luminance value using ImageJ software. To assess the
effects of CD47 blockade on NET induction, neutrophils were exposed to PBS, mouse
IgG1 isotype control Ab (CT-Ab) (10 µg/mL; MOPC-21, BioXcell), or anti-CD47
mAb (10 µg/mL; B6H12, BioXcell) for 30 min before NET induction.

Immunofluorescent staining for CD47 of human neutrophils. Extracted neutrophils
were seeded into 8-well chamber slides and stimulated to induce NET formation or
apoptosis. Neutrophils were fixed with 4% paraformaldehyde (PFA), covered with 1%
BSA at room temperature for 1 h, and exposed to phycoerythrin (PE)-anti-CD47
antibody (1:100; BioLegend) for 30 min. Slides were mounted using VECTASHIELD
with DAPI (Vector Laboratories).

Flow cytometric detection of CD47-positive neutrophils. To measure the cell surface
expression of CD47, neutrophils were stained with PE-anti-CD47 antibody. After
filtering out the debris with a mesh, the percolated cells were analyzed using a BD
FACS Verse.

Induction of necrosis in HUEhT cells. HUEhT cells, obtained from the Japanese
Collection of Research Bioresources (JCRB) cell bank (Osaka, Japan), were
suspended in HuMedia-EG2 (Kurabo) and seeded into plates at a density of
1.0×10^5/mL. After overnight culture, HuMedia-EG2 medium was replaced with serum-free DMEM (Sigma-Aldrich). Then, 500 µM H_2O_2 was added to induce necrosis for 4 h. Cell necrosis was assessed by immunofluorescence staining with PI (BD Biosciences). The PI-positive area of immunostaining was quantified as the mean luminance value using ImageJ software.

**Preparation of F(ab’)2 fragments.** CD47 F(ab’)2 fragments were produced from ficin digest of IgG using Pierce™ Mouse IgG1 Fab and F(ab’)2 Micro Preparation Kit, (Thermo Scientific) according to the manufacture’s recommendations. The purified anti-CD47 F(ab’)2 fragments were collected for the efferocytosis assay.

**Isolation and phenotype induction of human monocyte-derived macrophages.** PBMCs were isolated from healthy human donors by density centrifugation using Ficoll-Paque Plus (GE Healthcare). Monocytes were isolated from PBMCs using CD14 MicroBeads (Miltenyi Biotech) and magnetic columns, according to the manufacturer’s instructions. After magnetic separation, purified CD14^+ monocytes were suspended in ImmunoCult™-SF Macrophage Medium (STEMCELL Technologies) with human recombinant macrophage colony stimulating factor (M-CSF; 5 µg/mL; PEPROTECH) and seeded into plates at a density of 1.0×10^6/mL. LPS (10 ng/mL; Sigma-Aldrich) and IFN-γ (50 ng/mL; PEPROTECH) were added on days 4 or 6 for M1 activation, and differentiated macrophages were detached using accutase (STEMCELL Technologies) on days 6 or 8, respectively.

**Efferocytosis assay of human neutrophils and HUEhT in macrophages.** 20,000 macrophages per well were seeded in a 96-well plate. After overnight culture,
complete DMEM was replaced with serum-free DMEM before the introduction of neutrophils or HUEhT. Neutrophils isolated from healthy human donors and HUEhT were labeled with 10 μM CMFDA. Non-stimulated neutrophils, apoptotic neutrophils, TNF-α + healthy-IgG-treated neutrophils, ANCA-induced NETs, non-stimulated HUEhT, and necrotic HUEhT were washed, and NETs and necrotic HUEhT were exposed to PBS, CT-Ab (10 µg/mL), or anti-CD47 mAb (10 µg/mL) for 30 min. To address the role of FcR in efferocytosis of ANCA-induced NETs, macrophages were pretreated with FcR blocker (10 μg/mL; Invitrogen) or NETs were pretreated with anti-CD47 F(ab’)2 fragments for 30 min. After incubation, 100,000 neutrophils or 20,000 HUEhT detached using accutase were added to the macrophages and co-incubated. Efferocytosis was assessed using still or time-lapse imaging. The percentage of CMFDA-labeled macrophages after 3 h of co-incubation and removal of the supernatant in the number of macrophages before introducing neutrophils was defined as the efferocytosis rate for still images. For time-lapse imaging, macrophages were labeled using PKH26 Red Fluorescent Cell Linker Kit (Sigma-Aldrich) before seeding, and the number of CMFDA-labeled cells in PKH26 labeled macrophages was evaluated. Images were captured every 7 min for 1 h from the beginning of co-culture. For assessing residual rate of NETs, 30,000 macrophages per well were seeded into 8-well chamber slides. PKH26-labeled NETs were exposed to PBS, CT-Ab, or anti-CD47 mAb for 30 min and then co-incubated with macrophages for 3 h. Slides were fixed with 4% PFA, covered with 1% BSA at room temperature for 1 h, and exposed to anti-citH3 antibody (1:150; ab5103, Abcam). The sections were washed, incubated with Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen), and mounted with VECTASHIELD with DAPI. The percentage of remaining NETs (PKH26+/citH3+/DAPI+ cells with one nucleus) in the number of engulfed NETs
(PKH26+/citH3+/DAPI+ cells with multiple nuclei) was defined as residual rate of NETs.

Quantitative real-time RT-PCR (qRT-PCR) for human macrophages. 1,000,000 human non-stimulated neutrophils or NETs treated with CT-Ab or anti-CD47 mAb and 200,000 macrophages were co-incubated for 2 h as described above. After removing the supernatant, total RNA was extracted using the RNeasy Mini Kit (Qiagen), according to the manufacturer’s instructions. A fixed amount of RNA was reverse-transcribed into first-strand cDNA using SuperScript III (Invitrogen). qRT-PCR was performed using Fast SYBR Green Master Mix (Thermo Fisher Scientific), according to the manufacturer’s instructions. The amount of transcripts was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

The primers used were: 
- **TNFA**, F: CTCTTCTGCTGCTGCACTTTG, R: ATGGGCTACAGGCTTGTCACTC;
- **IL8**, F: GAGAGTGATTGAGAGTGGACCAC, R: CACAACCCTCTGCACCCAGTTT;
- **MCP1**, F: GCCTCCAGCATGAAAGTCTC, R: AGATCTCCTTGGCCACAATG;
- **IL1B**, F: CCACAGACCTTCCAGGAGAATG, R: GTGCAGTTCAGTGATCGTACAGG;
- **GAPDH** F: GGGAAGCTTGTCATCAATGGA, R: TCTGGCTCCTGGAAGATGGT.

Animal models and treatment protocol. SCG/Kj mice were purchased from the National Institute of Biomedical Innovation, Health, and Nutrition (Osaka, Japan). Eight-week-old mice were treated with intraperitoneal injections of either 200 μg of anti-CD47 mAb (MIAP301, BioXcell) or rat IgG2a isotype control Ab (CT-Ab) (2A3, BioXcell) every 5 days for 2 weeks (n=6 for each). Ten-week-old mice were then analyzed.
Disease activity and severity in SCG/Kj mice. For assessment of laboratory data, serum creatinine levels were determined using Creatinine kit (Serotec). Serum IgG levels were measured using IgG (total) Mouse Uncoated ELISA Kit (Thermo Fisher Scientific). The MPO-ANCA titer was determined by ELISA at the A-CLIP Institute (Chiba, Japan).

For histopathologic examination, fixed kidneys were embedded in paraffin and stained with hematoxylin-eosin and periodic acid-Schiff (PAS). The sections for IHC staining were deparaffinized and rehydrated, followed by antigen retrieval using sodium citrate buffer (pH 6). Slides were probed with rat anti-mouse Ly6B antibody (1:100; MCA771G, Bio-Rad). For immunofluorescence staining, antigen retrieval was performed in sodium citrate buffer (pH 6) for CD68 staining. To confirm the presence of NETs, frozen kidney tissues were fixed with 4% PFA for 4 h, dehydrated with 30% sucrose overnight at 4°C, frozen in OCT compound (Sakura), and cryosectioned (5 μm thick). The MPO and citH3 double-positive NETs were quantified using ImageJ software. Rat anti-mouse CD68 antibody (1:50; MCA1957GA, Bio-Rad), rabbit anti-citH3 antibody (1:100; ab5103, Abcam), or goat anti-MPO antibody (15 μg/ml; AF3667, R&D Systems) were used as the primary antibodies. Secondary antibodies included Alexa Fluor 488 goat anti-rat IgG (Invitrogen), Alexa Fluor 594 donkey anti-rabbit IgG (Invitrogen), and Alexa Fluor 488 donkey anti-goat IgG (Invitrogen). To assess the deposition of anti-CD47 mAb in glomeruli, specimens were immediately frozen in liquid nitrogen and then embedded in OCT compound. C57BL/6 mice treated with an intraperitoneal injection of 200 μg anti-CD47 mAb were used as controls. Specimens were sliced into 5-μm sections using a cryostat and stained with
Alexa Fluor 488 goat anti-rat IgG. To evaluate pathological changes in the kidney, glomerular activity (crescent formation, glomerular proliferation, and inflammatory cell infiltration) was calculated as previously reported (31). Sections were scored using a 0–3 scale for glomerular activity as follows: 0, no lesions, 1 = lesions in <25% of glomeruli, 2 = lesions in 25–50% of glomeruli; and 3, lesions in >50% of glomeruli.

For flow cytometry (FCM) of splenocytes, the splenic cells were filtered through 100-μm cell strainers, washed, and filtered again through 70-μm cell strainers. Red blood cell lysis was performed with RBC lysis buffer (Thermo Fisher Scientific). Aliquots of 5×10^5 cells/tube were stained with the following antibody combinations: A) Ly6C peridinin chlorophyll protein (PerCP)/Ly6G FITC/CD11b allophycocyanin (APC); B) CD11c FITC/MHC-II APC/F4/80 PE; C) CD3 PE; and D) B220 FITC.

For qRT-PCR for mouse kidneys, renal tissue samples were stored in RNAlater solution (Invitrogen) until RNA extraction was performed. Total RNA was extracted, and first-strand cDNA was synthesized using 1 μg of total RNA, as described above. qRT-PCR was performed using Fast SYBR Green Master Mix. The amount of transcript was normalized to that of 18S ribosomal RNA. The primers used were: *Mcp1*, F: TTAAAAACCTGGATCGGAACCAA, R: GCATTAGCTTCAGATTACGGGT; *Ifng*, F: CAGCAACAGCGAAAAAGG, R: TTTCCGCTTCCTGAGGCTGGAT; *Prf1*, F: GATGTGAACCCTAGGCCAGA, R: GGTTTTTGTACCAGGCGAAA; *Iifna*, F: CCTGAGAGAGAAACACAGCC, R: TTCTGCTCTGACCACCTCCC; *Il1b*, F: TGGACCTTCCAGGATGAGGACA, R: GTTCATCTCGGAGCCTGTAGTG;
Nos2, F: GAGACAGGGAAGTCTGAAGCAC, R:
CCAGCAGTAGGTGCTCTCTTC; Cd206, F:
GTTCACCTGGAGTGGTTCTC, R: AGGACATGCCAGGGTCACCTTT; and
I8s, F: GCAATTATTCCCCATGAA, R: AGGGCCTCACTAAACCAT.

For RNA-seq and data analysis, the quality of total kidney RNA was measured using a
Bioanalyzer 2100 (Agilent). Libraries were generated using the NEBNext rRNA
Depletion Kit (#E6310; New England Biolabs) and NEBNext Ultra RNA Library Prep
Kit for Illumina (#E7530; New England Biolabs) according to the manufacturer’s
instructions. The libraries were sequenced on a NovaSeq 6000 system (Illumina) by
2x150 bp paired-end settings. Raw sequencing reads with low quality and adapter
sequences were removed or trimmed using Cutadapt v 2.10 (32). The trimmed reads
were mapped to mm10 (Ensembl version 93) using STAR aligner v 2.5.1 (33).
Differentially expressed genes (DEGs) were identified based on differences in
expression levels (|log2 fold-change| > 1 and adjusted p-value < 0.05) between sample
groups after removing genes with zero read count and non-protein-coding genes using
DESeq2 v 1.28.1 (34). The Benjamini-Hochberg method was used to adjust the p-
value for multiple hypothesis testing. Canonical pathway analysis of DEGs was
performed using Ingenuity Pathway Analysis v 51963813 (QIAGEN Inc., Redwood
City, CA, USA, https://www.qiagenbioinformatics.com/products/ingenuity-pathway-
analysis/). BisqueRNA was used for bulk RNA-seq deconvolution to estimate cell-
type proportions (35). A single-cell RNA-seq dataset from the kidneys of C57BL/6
mice (18) was used as a reference. Although RNA-seq was performed on whole
kidney RNA from three mice each, one RNA sample from anti-CD47 mAb-treated
mice was excluded because of its poor RNA quality.
Statistics. The results obtained from in vitro and animal experiments are expressed as mean ± SEM and mean ± SD, respectively. Groups were compared using the two-tailed Student’s t-test for Gaussian distributions, Mann-Whitney U test for non-Gaussian distributions. Differences between greater than two groups were analyzed by one-way ANOVA followed by Dunnett’s multiple comparisons test. A statistical $p$-value < 0.05 was considered significant. Statistical analysis was performed using JMP Pro, version 14 (SAS Institute Inc.) and GraphPad Prism version 8.0.2 for Windows (GraphPad Software).

Study approval. Experiments using human materials were approved by the Sapporo City General Hospital Clinical Research Committee (approval number: R02-059-726). Animal experiments were approved by the Hokkaido University Animal Experiment Committee (approval number: 17-0016).

Data availability. The data generated are available from the corresponding author on reasonable request. RNA-Seq data were deposited in DDBJ (DRA014078). The supporting data values file is provided.

Author Contributions

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Supplemental Material

Supplementary Table S1. Patients’ characteristics of AAV patients for renal histology

Supplementary Figure S1. Apoptosis of neutrophils induced by STS

Supplementary Figure S2. The effect of CD47 blockade on NET formation

Supplementary Figure S3. Efferocytosis of injured endothelium via CD47 signaling

Supplementary Figure S4. Effects of CD47 blockade on the kidneys of SCG/Kj mice

Supplementary Figure S5. FCM plots of splenic immune cells from SCG/Kj mice

Supplementary Figure S6. Working model for the role of CD47 in AAV

Supplementary Movie S1. 3D projection of Z-stack image of ANCA-induced NETs. Red: CD47, blue: DAPI staining.

Supplementary Movie S2. Efferocytosis of non-stimulated neutrophils

Supplementary Movie S3. Efferocytosis of apoptotic neutrophils

Supplementary Movie S4. Efferocytosis of ANCA-induced NETs

Supplementary Movie S5. Efferocytosis of ANCA-induced NETs pretreated with CT-Ab

Supplementary Movie S6. Efferocytosis of ANCA-induced NETs pretreated with anti-CD47 mAb

Supplementary Movie S7. Efferocytosis of non-stimulated HUEhT

Supplementary Movie S8. Efferocytosis of necrotic HUEhT
Supplementary Movie S9. Efferocytosis of necrotic HUEhT pretreated with CT-Ab
Supplementary Movie S10. Efferocytosis of necrotic HUEhT pretreated with anti-CD47 mAb

References


Figure 1. CD47 expression on human renal tissue. (A) Representative IHC images for CD47 in MGA, AAV, LN class IV, and LN class V patients. Scale bars: 50 μm. (B) Quantification of CD47-positive area of renal biopsy sections from patients with MGA (n=8), AAV (n=7), LN class IV (n=4), and LN class V (n=4) as a percentage of glomerular area. Error bars represent SD *p<0.05, **p<0.01 (one-way ANOVA with post hoc Dunnett’s multiple comparison test).
Figure 2. CD47 expression on human neutrophils. Non-stimulated, TNF-α-primed neutrophils incubated with healthy-IgGs or ANCA-IgGs, and PMA-induced netting neutrophils were examined. (A) Representative images of CD47 and DAPI staining of neutrophils. Red: CD47; blue: DAPI staining. Scale bars: 10 μm. (B) Representative FCM plots of the gating strategy and histogram of CD47 expression on neutrophils. (C) Mean fluorescence intensity of CD47 in B. Error bars represent SEM. ***p<0.001 (one-way ANOVA with post hoc Dunnett’s multiple comparison test).
**Figure 3. Efferocytosis of human neutrophils via CD47 signaling.** (A) Representative images of macrophages after 3 h incubation with CMFDA labeled non-stimulated, apoptotic neutrophils, neutrophils incubated with TNF-α + healthy IgGs, ANCA-induced NETs, and NETs treated with CT-Ab, anti-CD47 mAb, anti-CD47-mAb + FcR blocker, or anti-CD47 F(ab’)2 fragments. Co-cultures were rinsed before imaging. Green: neutrophils. Scale bars: 50 μm. (B) Efferocytosis rate (the percentage of CMFDA-positive macrophages) in A. (C) Representative time-lapse images of efferocytosis assay of neutrophils. Green: neutrophils, Red: macrophages. Scale bars: 50 μm. (D) Quantification of the number of engulfed non-stimulated (n=3), apoptotic neutrophils (n=3), NETs (n=2), and NETs treated with CT-Ab (n=3) or anti-CD47 mAb (n=3) every 7 min. Statistical analysis was performed using one-way ANOVA, followed by Dunnett’s multiple comparisons compared to NETs treated with CT-Ab. (E) mRNA expressions of IL1B, IL8, MCP1, and TNFA by efferocytosis. Total RNA was extracted from macrophages after 2 h of their exposure to non-stimulated neutrophils, NETs treated with CT-Ab or anti-CD47 mAb, and mono-cultured macrophages (n=4 for each). Error bars represent SEM. *p<0.05, **p<0.01, ***p<0.001, ns: not significant (one-way ANOVA with post hoc Dunnett’s multiple comparison test).
Figure 4. Blockade of CD47 protected mice from spontaneous development of vasculitis. Eight-week-old SCG/Kj mice were intraperitoneally injected with CT-Ab or anti-CD47 mAb every 5 days for 2 weeks (n=6 for each). (A) Results of serological tests (as assessed by Mann-Whitney U-test) and histopathology. The glomerular activity score was assessed. Scale bars: 100 μm. (B) Representative images of MPO/citH3 staining and quantitative analysis of glomeruli for MPO/citH3+ area/glomerulus (%). (C) mRNA expression of Ifna, Ifng, Mcp1, Prf1 and Il1b in the whole kidney. Error bars represent SD. *p<0.05, **p<0.01 (Student's unpaired t-test).
Figure 5. The immune cell profiles of the kidneys and systemic immune responses in SCG/Kj mice treated with CD47 blockade. (A) Representative images and quantitative analysis of glomeruli by immunostaining for macrophages. White dotted lines and yellow arrowheads indicated glomeruli and CD68-positive cells, respectively. Scale bars: 50 μm. (B) mRNA expressions of Nos2 and Cd206 in whole kidney. (C) Representative images and quantitative analysis of glomeruli by immunostaining for neutrophils. Scale bars: 50 μm. (D) The results of serum IgG. (E) The results of serum MPO-ANCA titer. Error bars represent SD. *p<0.05, ns: not significant (Student’s unpaired t-test).