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Macrophage Differentiation and Polarization via Phosphatidylinositol 3-Kinase/Akt–ERK Signaling Pathway Conferred by Serum Amyloid P Component

Weijuan Zhang,* Wei Xu,* and Sidong Xiong*†

Macrophage differentiation and polarization is influenced by, and act on, many processes associated with autoimmunity. However, the molecular mechanisms underlying macrophage polarization in systemic lupus erythematosus (SLE) remain largely debated. We previously demonstrated that macrophage M2b polarization conferred by activated lymphocyte-derived (ALD)-DNA immunization could initiate and propagate murine lupus nephritis. Serum amyloid P component (SAP), a conserved acute-phase protein in mice, has been reported to bind to DNA and modulate immune responses. In this study, murine SAP was shown to promote macrophage-mediated ALD-DNA uptake through binding to ALD-DNA (SAP/ALD-DNA). Moreover, macrophage phenotypic switch from a proinflammatory M2b phenotype induced by ALD-DNA alone to an anti-inflammatory M2a phenotype stimulated with SAP/ALD-DNA were found because of PI3K/Akt–ERK signaling activation. Both in vivo SAP supplements and adoptive transfer of ex vivo programmed M2a macrophages induced by SAP/ALD-DNA into SLE mice could efficiently alleviate lupus nephritis. Importantly, increased IL-10 secretion, accompanied by anti-inflammatory effect exerted by M2a macrophages, was found to predominantly impede macrophage M2b polarization. Furthermore, neutralization of IL-10 notably reduced the suppressive effect of M2a macrophages. Our results demonstrate that binding of SAP to ALD-DNA could switch macrophage phenotypic polarization from proinflammatory M2b to anti-inflammatory M2a via PI3K/Akt–ERK signaling activation, thus exerting protective and therapeutic interventions on murine lupus nephritis. These data provide a possible molecular mechanism responsible for modulation of macrophage polarization in the context of lupus nephritis and open a new potential therapeutic avenue for SLE. The Journal of Immunology, 2011, 187: 1764–1777.

upus nephritis is a major cause of morbidity in patients with systemic lupus erythematosus (SLE), which is generally thought to be triggered by production of various autoantibodies and the subsequent inflammation (1, 2). Anti-dsDNA autoantibody, which is a serological hallmark of SLE, has been proved to be pathogenic and could cause subsequent tissue deposition of immune complexes (ICs) and tissue damage (3, 4). A series of events including polyclonal B lymphocyte activation, molecular mimicry, and disruption of an idiotypic network have been reported to be involved in the process of autoantibody production during the pathogenic development of the disease (5–8). Genetic studies in SLE patients showed that anti-dsDNA autoantibodies, which generally belong to IgG subtype with high-affinity binding to dsDNA, differ from the germline because of somatic mutations (9). Accumulating evidence indicates that the production of anti-dsDNA Abs could be initiated by self-DNA (10, 11).

In our previous studies, by means of immunization with activated lymphocyte-derived (ALD)-DNA, we generated a murine model of SLE that developed high levels of anti-dsDNA Abs, as well as proteinuria and glomerulonephritis (12), indicating that ALD-DNA might serve as an important self-immunogen to trigger the autoimmune responses that eventually lead to the pathogenesis of SLE (13, 14). These findings were consistent with previous reports in which extensive lymphocyte activation and consecutive excessive apoptosis have been widely observed in SLE patients, and a large amount of DNA released from apoptotic lymphocytes might exceed normal clearance ability and comprise the major source of autoantigens in SLE patients (15, 16). During lymphocyte activation induced by infection, stress, and other danger signals, DNA was released from activated lymphocytes, but not always provoking the autoimmunity, indicating that free DNA could be cleared by the intrinsic physiological mechanisms (17).

Previous studies have suggested that increased liberation and disturbed clearance of nuclear DNA–protein complexes after cell apoptosis may initiate and propagate SLE disease (17, 18). Thus, in addition to DNA overload in SLE, insufficiency of DNA clearance represents the other side of the coin. Lack or reduction of DNase1, which is the major nuclease present in serum, was shown to be a crucial factor in the initiation of SLE as verified by the classical symptoms of SLE in DNase1-deficient mice and
reduced serum DNase1 activities in SLE patients (19). More recently, lack of serum amyloid P component (SAP), which is the single normal circulating protein in mice that shows specific calcium-dependent binding to DNA and chromatin in physiological conditions, was found to play a key role in the onset of SLE, which was validated by the spontaneous development of antinuclear autoimmunity and severe glomerulonephritis in SAP-deficient mice and lower binding ability of SAP to DNA in SLE patients (20–23). Moreover, SAP was reported to recognize nuclear autoantigens released from apoptotic cells, opsonize them through interaction with cell-surface FcγR, and thereby activate macrophage-mediated phagocytosis (24–26). In addition to activating phagocytosis through FcγR, SAP could also mediate the immune responses (25, 26), indicating that SAP could modulate nuclear Ag-mediated autoimmune diseases such as SLE.

Macrophages have long been considered to be prodigious phagocytic cells that could serve as potent immune effector cells with well-established roles in tissue homeostasis and primary response to self- or pathogen-derived signals (27). Cellular debris and apoptotic cells could be removed rapidly and efficiently by macrophages through binding with their cell-surface receptors including FcγR, complement receptors, and scavenger receptors (28). In general, such homeostatic clearance processes either actively produce inhibitory signals and/or cytokines or fail to transduce signals that induce cytokine–gene transcription (27). Different local environmental factors shape macrophage properties and activation state, which represents different extremes of a continuum ranging from M1 to M2 (including M2a, M2b, and M2c) (29–32). Unlike M1 macrophages that have enhanced production of pro-inflammatory cytokine (TNF-α, IL-6, IL-12) and increased generation of reactive oxygen species such as NO via activation of inducible NO synthase (iNOS), M2 macrophages are generally characterized by low level of proinflammatory cytokine and high expression of anti-inflammatory cytokines (IL-12, IL-10) and increased generation of reactive oxygen species such as NO via activation of inducible NO synthase (iNOS), M2 macrophages are generally characterized by low level of proinflammatory cytokine and high expression of anti-inflammatory cytokines (IL-12, IL-10) and increased generation of reactive oxygen species such as NO via activation of inducible NO synthase (iNOS), M2 macrophages are generally characterized by low level of proinflammatory cytokine and high expression of anti-inflammatory cytokines (IL-12, IL-10) and increased generation of reactive oxygen species such as NO via activation of inducible NO synthase (iNOS). M2b subset of macrophages is an exception, in that it retains high levels of inflammatory cytokine production with concomitant high IL-10 and low IL-12 (31). Our previous study has demonstrated that ALD-DNA–induced M2b-differentiated macrophages were found to mediate the initiation and progression of SLE disease (14). SAP has been reported to facilitate phagocytosis of DNA and mediate the immune responses (22, 23), whereas it is not clear whether SAP-mediated DNA phagocytosis modulates macrophage differentiation and SLE disease.

Although the respective roles of SAP in opsonization by macrophages and dysfunctional macrophage differentiation in SLE development have been studied previously, the contribution of SAP in abnormal macrophage differentiation during the pathogenesis of SLE remains poorly understood. In this work, we tested the hypothesis whether SAP could inhibit the development of SLE syndrome through reversing pathogenic macrophage differentiation. We found that SAP binding to ALD-DNA could induce macrophage phenotypic switch from pathogenic M2b phenotype to IL-10–dependent inhibitory M2a phenotype via PI3K/Akt–ERK signaling, which contribute to alleviating SLE syndrome in lupus mice. These results indicated that in vivo SAP supplements could complement insufficient DNA phagocytosis ability and reverse pathogenic M2b macrophage differentiation, which reveal the possible molecular mechanisms for the modulation of macrophage differentiation and the pathogenesis of SLE disease.

Materials and Methods

Mice

Six- to 8-wk-old female BALB/c mice were purchased from the Experimental Animal Center of Chinese Academy of Sciences (Shanghai, P. R. China) and maintained in pathogen-free housing. All animal procedures were performed according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication, 86-23 revised 1985).

Reagents and pharmacological inhibitor treatment

Recombinant mouse IL-4, IL-10, and IL-13 were purchased from PeproTech (Rocky Hill, NJ) and were reconstituted in sterile H2O according to the manufacturer’s instructions. IL-10 neutralization Ab and relative iso-type IgG were purchased from R&D Systems (Minneapolis, MN). The pharmacological reagents were obtained from Calbiochem (San Diego, CA) and were reconstituted in sterile DMSO and used at the following concentrations: PI3K inhibitor LY294002 (50 μM), MEK1/2 inhibitor U0126 (10 μM), p38 MAPK inhibitor SB203580 (20 μM), and JNK inhibitor SP600125 (50 μM). DMSO at 0.1% concentration was used as the vehicle control. In all experiments with inhibitors, a tested concentration was used after careful titration experiments assessing the viability of the macrophages, and the chosen concentrations are in agreement with published reports (34). In addition, when a given inhibitor was tested, its efficacy in terms of inhibition of phosphorylation of the intended signaling molecule, as well as a nonintended signaling molecule, was also tested. In the experiments with inhibitors, the macrophages were treated with a given inhibitor for 1 h before SAP/ALD-DNA stimulation.

Cell culture

RAW264.7 cells were cultured in DMEM (Invitrogen Life Technologies, Gaithersburg, MD) supplemented with 10% FBS (Invitrogen Life Technologies) in a 5% CO2 incubator at 37°C. For generation of bone marrow–derived macrophages (BMDMs), bone marrow (BM) cells were harvested from uninfected, normal BALB/c mice and filtered through nylon mesh. BM cells were cultured in 1,929 cell-conditioned medium at a density of 3 × 105 cells/ml medium and maintained in a 5% CO2 incubator at 37°C as described previously (35, 36). Six days after initial BM cell culture, the medium was changed and the purity of F4/80+ cells was >90%, as determined by flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA).

Transient transfection of Akt plasmids

RAW264.7 cells were transiently transfected with a dominant-negative (DN) mutant of Akt plasmid (DN-Akt), a constitutive-active mutant of Akt plasmid (CA-Akt), or empty vector control plasmid (VC), which were kindly provided by Dr. Mien-Chie Hung (MD Anderson Cancer Center, Houston, TX), using Lipofectamine LTX with Plus Reagent (Invitrogen Life Technologies), following the manufacturer’s instructions. On the next day, flow cytometry analysis was performed with anti-Akt and anti–phospho-Akt (Ser473) mouse Abs (Cell Signaling Technology, Beverly, MA) to detect the phosphorylation levels of Akt. Twenty-four hours posttransfection, cells were treated with SAP/ALD-DNA (50 μg/ml) for functional analysis.

Small interfering RNA transfection

RAW264.7 cells were transiently transfected with 100 nM control small interfering RNA (siRNA) or IL-10 siRNA (Santa Cruz Biotechnology, Santa Cruz, CA) using a Lipofectamine 2000 transfection reagent (Invitrogen Life Technologies) at 2:1 (lipid/siRNA) ratio following the manufacturer’s instructions for siRNA transfection. The transfection mixture was added to cells in serum-free DMEM and incubated at 37°C for 6 h before the medium was replaced with DMEM with 10% FBS. Twenty-four hours later, the cells were treated with SAP/ALD-DNA, and total RNA was harvested 24 h posttransfection. Real-time PCR was performed to determine the knockdown effect of IL-10, and the expression of IL-10 was normalized to that of GAPDH. No cytoxic effect of siRNA was observed on RAW264.7 cells, as determined by trypan blue exclusion assay.

Isolation and purification of mouse SAP protein

Isolation and purification of SAP protein from mouse serum was performed as described previously by Pepsy (37). In brief, fresh mouse serum was applied to a Sepharose 4B (GE Healthcare, Chalfont St Giles, U.K.) column and equilibrated with a Tris/saline/calcium buffer (0.14 M NaCl/0.01 M Tris/0.002 M CaCl2 at pH 8.0). After standing overnight at 4°C, the Sepharose was washed with Tris/saline/calcium buffer until no further material absorbing at 280 nm was eluted. The Sepharose was then packed and equilibrated with the same buffer in a chromatographic column before elution with Tris/saline/EDTA (0.14 M NaCl/0.01 M Tris/0.01 M EDTA at pH 8.0). A single protein peak was recovered at the column volume and
concentrated by ultrafiltration on an Amicon PM30 membrane (Amicon, High Wycombe, Bucks, U.K.). This protein, >90% of which was SAP, was gel filtered on Ultrogel AcA44 (LKB Instruments, London, U.K.), equilibrated with the same EDTA buffer in a Pharmacia K26/100 column (volume, 500 ml; GE Healthcare). The single major eluted peak was pooled conservatively, concentrated by filtration on an Amicon PM30 membrane, and stored in liquid nitrogen. To exclude contaminations with LPS, we used sterile endotoxin-free plastic ware and reagents for SAP preparation. SAP samples were monitored for low level of endotoxin by Limulus amebocyte lysate assay (BioWhittaker, Walkersville, MD) according to the manufacturer’s instructions. Electron micrographs revealed the single annular pentameric molecules (diameter, 11 nm) in the SAP preparations. The purity of the SAP isolate was also determined by SDS-PAGE and subsequent Coomassie brilliant blue staining, and levels of SAP protein were further confirmed by Western blot analysis with rabbit anti-mouse SAP (Calbiochem). The SAP concentration was determined by ELISA assay as previously described (38). SAP was oxidatively iodinated using N-bromosuccinimide (Sigma), and functional integrity of the labeled protein was demonstrated by full retention of its capacity for calcium-dependent binding to phosphophanolamine covalently immobilized on Sepharose (GE Healthcare), as previously described (39, 40). The binding ability of purified mouse SAP protein to DNA was detected by dot blot analysis with rabbit anti-mouse SAP (Calbiochem), as previously described (Supplemental Fig. 1, Supplemental Table I) (41).

DNA preparation

ALD-DNA and unactivated lymphocyte-derived (UnALD)-DNA were prepared with murine splenocytes that were generated from surgically resected spleens of 6- to 8-week-old female BALB/c mice and cultured with or without Con A (Sigma-Aldrich, St. Louis, MO) in vitro, as previously described (14). In brief, for generation of ALD-DNA, splenocytes were seeded at 2 × 10⁶ cells/ml in 75-cm² cell culture flask and cultured in the presence of Con A (5 μg/ml) for 6 d to induce apoptosis. The apoptotic cells were stained with FITC-labeled Annexin V (BD Biosciences) and propidium iodide (Sigma-Aldrich), and sorted using a FACSARia (BD Biosciences). Genomic DNAs from syngeneic apoptotic splenocytes were extracted using the same methods. To exclude contaminations with LPS, we used sterile endotoxin-free plastic ware and reagents for DNA preparation. DNA samples were also monitored for low level of endotoxin by the Limulus amebocyte lysate assay (BioWhittaker) according to the manufacturer’s instructions. The concentration of DNA was determined by detection of the absorbance at 260 nm. The apoptotic DNA ladder of ALD-DNA was confirmed by agarose gel electrophoresis.

Generation of SLE murine model

To generate the SLE murine model, we divided 6- to 8-wk-old syngeneic female BALB/c mice into several groups of 8–10 mice; mice were s.c. injected on the back with 0.2 ml of an emulsion containing ALD-DNA (50 μg/mouse) in PBS plus equal volume of CFA (Sigma-Aldrich) at week 0, followed by two booster immunizations of ALD-DNA (50 μg/mouse) emulsified with IFA (Sigma-Aldrich) at weeks 2 and 4 for a total of 3 times, as previously described (12, 13). Mice receiving an equal volume of PBS plus CFA or IFA, or UnALD-DNA (50 μg/mouse) plus CFA or IFA were used as controls. Mice were bled from retro-orbital sinus before immunization and at 2-wk intervals until 3 mo after the initial immunization. Eight or 12 wk later, mice were sacrificed, and surgically resected spleens and kidneys were collected for further analysis.

Binding ability of SAP to ALD-DNA

ELISA analysis was performed to detect the binding ability of SAP to ALD-DNA as previously described (41). In brief, ELISA plates (Costar) were pretreated with 0.5% protamine sulfate (Sigma-Aldrich) and then coated with ALD-DNA (1 μg/ml). The wells were blocked with 1% BSA and incubated with indicated mouse SAP protein. After incubating with anti-SAP Abs, the binding ability of SAP to ALD-DNA was detected with the HRP-conjugated goat anti-mouse IgG (Southern Biotech). Tetramethylbenzidine substrate (eBioscience, San Diego, CA) was used to develop colors, and absorbance at 450 nm was measured on a microplate reader (Bio-Tek ELX800).

ELISA assay

To assess protein levels of TNF-α, IL-1β, IL-6, IL-10, IL-12, IL-23, MCP-1, and TGF-β in the cell culture supernatants, we performed ELISA assays with cytokine ELISA kits (eBioscience) according to the manufacturer’s instructions. A standard curve was generated using known amounts of the respective purified recombinant mouse cytokines or chemokines.

Real-time PCR analysis

Total RNA was extracted from cultured cells or mouse renal macrophages with TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer’s instructions. The cDNA was synthesized with PrimeScript RT reagent kit (Takara Bio). The expression of the genes encoding arginine

![Figure 1](http://www.jimmunol.org/)

**Figure 1.** SAP binds to ALD-DNA and promotes the uptake of ALD-DNA by macrophages. A. The binding ability of SAP to ALD-DNA was detected by ELISA assay. Data are representative of results obtained in three independent experiments. B. The phagocytosis of AF488-ALD-DNA by macrophages treated with chloroquine was detected by flow cytometry. Data are representative of results obtained in three independent experiments. C. The levels of RNA uptake in B were represented as mean fluorescent intensity (MFI). Data are means ± SD of three independent experiments. **p < 0.001. D. AF488-ALD-DNA (1 μg/ml) was incubated with indicated mouse SAP for 2 h; then SAP/ALD-DNA complexes were exposed to chloroquine-treated BMDMs. The levels of DNA uptake were assessed by flow cytometry and represented as MFI. Data are means ± SD of three independent experiments.
Arginase assay

Arginase assay was performed as previously described (43). In brief, macrophages were lysed with 0.1% Triton X-100. Lysates were combined with 12.5 mM Tris-HCl and 1 mM MnCl₂. Arginase was activated by heating for 10 min at 56°C, and L-arginine substrate was added to 250 mM final concentration. Reactions were incubated at 37°C for 30 min and stopped by the addition of H₂SO₄/H₃PO₄. After addition of α-isonitroso-propiophenone and heating for 30 min at 95°C, urea production was measured by absorbance at 540 nm and normalized to cell counts.

Flow cytometry analysis and cell sorting

Renal macrophages were sorted from nephritic single-cell suspensions using a FACSaria (BD Biosciences) with FITC-labeled anti-F4/80 (eBioscience) and PE-labeled anti-CD11b (BD Biosciences). Macrophages were stained with the following Abs for functional analysis: FITC- or PE-labeled anti-F4/80 (eBioscience), PE-labeled anti-mannose receptor (anti-MR; Santa Cruz Biotechnology), anti-MGL (Abcam, Cambridge, U.K.), and FITC-labeled anti-IgG (eBioscience). To assess iNOS expression, we stained macrophages with FITC-labeled anti-F4/80, then resuspended them in fixation/permeabilization solution (BD Cytofix/Cytperm Kit; BD Biosciences) before staining with PE-labeled anti-iNOS (Santa Cruz Biotechnology). To detect the expression and phosphorylation levels of Akt, ERK1/2, JNK, and p38 MAPK in macrophages, we performed flow cytometry analysis with anti-Akt, anti–phospho-Akt (Ser473), anti-ERK1/2, anti–phospho-p44/42 MAPK/ERK (Thr202/Tyr204), anti-JNK, anti-phospho-SAPK/JNK (Thr183/Tyr185), anti-p38 MAPK, anti–phospho-p38 MAPK (Thr180/Tyr182) mouse Abs (Cell Signaling Technology), and then FITC-labeled anti-IgG, (eBioscience) as described previously (44). All flow cytometry data were acquired on a BD FACSCalibur (BD Biosciences) and analyzed by FlowJo software (Tree Star, Ashland, OR).

DNA uptake in vitro

ALD-DNA was labeled with Alexa Fluor 488 (AF488-ALD-DNA; Invitrogen) according to the manufacturer’s instructions. AF488-ALD-DNA was purified using Bio-Rad Micro Bio-Spin P-30 column (Bio-Rad, Hercules, CA) and then incubated with purified murine SAP protein (SAP/ALD-DNA) at 37°C for 2 h. BMDMs were treated with chloroquine (100 FIGURE 2. SAP binding to ALD-DNA induces a phenotypic switch of macrophage from M2b to M2a. SAP was incubated with ALD-DNA (SAP/ALD-DNA) for 2 h. BMDMs were treated with PBS, SAP (50 µg/ml), ALD-DNA (50 µg/ml), or SAP/ALD-DNA (50 µg/ml). A, Twelve hours later, levels of IL-10, IL-12, IL-23, TNF-α, IL-6, IL-1β, MCP-1, and TGF-β in the supernatants of BMDMs were measured by ELISA. Data are means ± SD of three independent experiments. **p < 0.01, ***p < 0.001. B, Twelve hours later, levels of Arg1, Mrc (MR), Mgl, Ym1, Fizz1, IL-10, TNF-α, and Nos2 (iNOS) in BMDMs were analyzed by real-time PCR. Data are means ± SD of three independent experiments. **p < 0.01. ***p < 0.001. C, Forty-eight hours later, arginase activity was assessed by an assay of urea production from arginine substrate and was normalized to cell counts. Data are means ± SD of three independent experiments. **p < 0.01. D, Twenty-four hours later, levels of MR, MGL, and iNOS in BMDMs were determined by flow cytometry. Data are representative of results obtained in three independent experiments.
DNA by macrophages, we performed flow cytometry to determine the macrophage-mediated phagocytosis of ALD-DNA or SAP/ALD-DNA complex. It was found that SAP promoted the uptake of ALD-DNA by macrophages (Fig. 1B–D). These data indicate that SAP binding to ALD-DNA could efficiently promote ALD-DNA uptake by macrophages.

### Anti-dsDNA Ab examination and proteinuria examination

Serum samples were collected periodically and determined by ELISA for the presence of anti-dsDNA Abs using ELISA kit (Alpha Diagnostic International, San Antonio, TX) according to the manufacturer’s instructions (46). Proteinuria of mice was measured with the BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer’s instructions.

### Adoptive transfer of macrophages

BMDMs were treated with 50 μg/ml SAP/ALD-DNA or 50 μg/ml ALD-DNA alone for 48 h to induce macrophage M2a and M2b differentiation, respectively. Macrophages were detected by real-time PCR for IL-10, Arg1 (M2a macrophages), TNF-α, and iNOS (M2b macrophages) expression. M2a or M2b macrophages were then injected i.v. into ALD-DNA–immunized mice (2.5 × 10⁷ cells/mouse) at weeks 0, 2, and 4 after the initial immunization for a total of three times.

### Pathological analysis

For histological analysis, murine kidney tissues were stained with H&E according to the manufacturer’s instructions. Fluorescent staining of cryosections was used for IC deposition analysis in the glomeruli. Sections were fixed in acetone for 10 min and incubated with FITC-conjugated goat anti-mouse IgG (H+L chain-specific) Abs (Sigma-Aldrich) for 30 min. Pictures were acquired with Nikon SCLIPSS TE2000-S microscope (Nikon, Melville, NY) equipped with ACT-1 software (Nikon; original magnification ×200).

### Statistical analysis

All data are expressed as means ± SD of three independent experiments or from a representative experiment of three independent experiments. The statistical significance of the differences in the experimental data was valued by Student t test. The statistical significance level was set as *p < 0.05, **p < 0.01, and ***p < 0.001.

### Results

**SAP binds to ALD-DNA and promotes macrophage-mediated ALD-DNA uptake**

Accumulating data indicate that SAP has the capacity of binding to DNA under physiological conditions (22). To examine the binding ability of SAP to ALD-DNA, we performed ELISA assay and found that SAP could bind to ALD-DNA with high capacity in a dose-dependent manner (Fig. 1A). To investigate whether the binding of SAP to ALD-DNA had any effects on uptake of ALD-DNA by macrophages, we performed flow cytometry to determine

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**Phenotype**

| M1 | M2a | M2b | M2c | M2a | M2b |

Blank cells indicate no detection.

−, No expression; +, low expression; ++, middle expression; ++++, high expression; TLRL, TLR ligand.
FIGURE 3. Macrophage phenotypic switch induced by SAP/ALD-DNA is associated with increased PI3K/Akt–ERK signaling. SAP was incubated with ALD-DNA (SAP/ALD-DNA) for 2 h. A, BMDMs were stimulated with ALD-DNA (50 μg/ml), SAP/ALD-DNA (50 μg/ml), or PBS for 1 h. Expression and phosphorylation levels of Akt, ERK1/2, JNK, and p38 in macrophages were determined by flow cytometry. Data are representative of results obtained in three independent experiments. B and C, BMDMs were pretreated with LY294002 (50 μM), U0126 (10 μM), SP600125 (50 μM), SB203580 (20 μM), or DMSO (0.1%) for 1 h and then exposed to SAP/ALD-DNA (50 μg/ml) or PBS for 12 h. B, Levels of IL-10 in the supernatants of macrophages were measured by ELISA. C, Levels of Arg1 in BMDMs were analyzed by real-time PCR. Data are means ± SD of three independent experiments. B and C, ***p < 0.001. D, BMDMs were pretreated with LY294002 (50 μM), U0126 (10 μM), or DMSO (0.1%) for 1 h and then exposed to SAP/ALD-DNA (50 μg/ml) or PBS for 1 h. Phosphorylation levels of ERK1/2 and Akt in macrophages were determined by flow cytometry. Data are representative of results obtained in three independent experiments. E and F, RAW264.7 cells were transfected with a DN-Akt, a CA-Akt, or empty VC, and then stimulated with SAP/ALD-DNA (50 μg/ml) for 12 h. E, Levels of IL-10 in the supernatants of macrophages were measured by ELISA. F, Levels of Arg1 in macrophages were analyzed by real-time PCR. E and F, Data are means ± SD of three independent experiments. **p < 0.01, ***p < 0.001. G and H, RAW264.7 cells were transfected with CA-Akt or VC. CA-Akt–transfected RAW264.7 cells were treated with U0126 (10 μM) or DMSO (0.1%) for 1 h, then exposed to
whereas MEK1/2 inhibitor U0126 abrogated the enhanced level of phospho-ERK but had no significant effect on expression of phospho-Akt (Fig. 3D), which raised the possibility that SAP/ALD-DNA treatment increased IL-10 and Arg1 production of macrophages via upregulating PI3K/Akt–ERK signaling pathway. Macrophages were transiently transfected with a DN-Akt, a CA-Akt, or empty VC, and the effects were studied to further test this possibility. As presented in Fig. 3E–H, levels of IL-10 and Arg1 were higher in CA-Akt–transfected macrophages than in other treated cells (Fig. 3E,3F), whereas U0126 treatment abrogated this effect (Fig. 3G,3H), suggesting that ERK is downstream of PI3K/Akt in SAP/ALD-DNA–triggered signaling pathway in macrophages (Fig. 3I). These results demonstrate that macrophage phenotypic switch induced by SAP/ALD-DNA is associated with increased PI3K/Akt–ERK signaling as compared with ALD-DNA treatment.

**FIGURE 4.** SAP treatment alleviates ALD-DNA–induced SLE disease accompanied by macrophage M2a differentiation in lupus mice. BALB/c mice were divided into four groups and immunized with PBS, UnALD-DNA (50 µg), ALD-DNA (50 µg), or SAP/ALD-DNA (50 µg SAP were preincubated with 50 µg ALD-DNA for 2 h to form SAP/ALD-DNA complex). A, mRNA levels of IL-10, Arg1, Mrc, Mgl, Nos2 (iNOS), and TNF-α in renal macrophages purified from mice were evaluated by real-time PCR. Data are means ± SD of three independent experiments. **p < 0.01, ***p < 0.001. n = 6. B, Arginase activity in renal macrophages purified from mice was assessed by an assay of urea production from arginine substrate and was normalized to cell counts. Data are means ± SD from 10 mice in each group. ***p < 0.001. C and D, Serum anti-dsDNA IgG levels every 2 wk (C) and at week 12 (D) were measured by ELISA. ***p < 0.001. n = 10. E and F, Urine protein levels of mice were assessed every 2 wk (E) and at week 12 (F). ***p < 0.001. n = 10. G, Twelve weeks after initial immunization, glomerular immune deposition was detected by direct immunofluorescence for IgG in frozen kidney sections of mice. Representative images (original magnification ×200) of 10 mice are shown for each group. H, Twelve weeks after initial immunization, nephritic pathological changes were shown by H&E staining of renal tissues resected from mice. Representative images (original magnification ×200) of 10 mice are shown for each group. I, Kidney score was assessed using paraffin sections stained with H&E. ***p < 0.001. n = 10.

SAP REGULATES MACROPHAGES M2b-TO-M2a SWITCH IN SLE DISEASE

In vivo SAP supplements mitigate SLE syndrome accompanied by macrophage phenotypic switch

The dynamics of SAP levels and circulating DNA levels in serum of the lupus mice were assayed next. Slightly increased serum SAP levels accompanied with remarkably enhanced circulating DNA levels were found in the ALD-DNA–immunized lupus mice as compared with those in controls (Supplemental Fig. 2A,2B). Pearson correlation analysis showed that the serum SAP levels were closely correlated with the circulating DNA levels (Supplemental Fig. 2C). However, the ratios of SAP to DNA were lower in lupus mice than in controls, indicating that SAP protein were relatively insufficient in lupus mice (Supplemental Fig. 2D). Notably, the ratios of SAP to DNA were negatively correlated with the levels of anti-dsDNA Abs in SLE mice (Supplemental Fig. 2E). These results indicate that SAP was relatively insufficient in lupus mice, suggesting that SAP supplements in vivo could be
a potential therapeutic approach for SLE. In this study, female BALB/c mice were immunized with SAP/ALD-DNA, ALD-DNA, UnALD-DNA, or PBS. Slightly increased serum SAP levels, but remarkably decreased circulating DNA levels, were found in SAP/ALD-DNA–treated mice as compared with those in ALD-DNA–immunized lupus mice (Supplemental Fig. 3A, 3B). However, the ratios of SAP to DNA were significantly increased in SAP/ALD-DNA–treated mice than in lupus mice, which suggested that SAP supplement could promote DNA clearance in lupus mice (Supplemental Fig. 3C).

Meanwhile, the effect of SAP supplements on macrophage differentiation and SLE development in lupus murine model was studied. Consistent with the earlier results in vitro, renal macrophages from ALD-DNA–immunized mice exhibited M2b phenotype with enhanced levels of TNF-α, IL-10, and Nos2 (Fig. 4A), whereas those from SAP/ALD-DNA–treated mice showed M2a phenotype with increased expression levels of IL-10, Arg1, Mrc, and Mgl (Fig. 4A, 4B), indicating that SAP supplements induce a phenotypic switch of macrophage polarization from proinflammatory M2b to anti-inflammatory M2a differentiation in the lupus murine model. When the SLE pathology was assessed,

**FIGURE 5.** Adoptive transfer of SAP/ALD-DNA–programmed M2a macrophages reduces lupus nephritis. Macrophages were differentiated into M2a macrophages (induced by 50 μg/ml SAP/ALD-DNA for 48 h) or M2b macrophages (induced by 50 μg/ml ALD-DNA for 48 h) in vitro. Then cells were retrieved and 2.5 × 10⁶ injected (i.v.) into mice at weeks 0, 2, and 4 after the initial ALD-DNA immunization. Mice immunized with SAP/ALD-DNA (50 μg/mice) were used as control. A and B, The serum anti-dsDNA IgG levels every 2 wk (A) and at week 12 (B) were measured by ELISA. **p < 0.01, n = 10. C and D, Urine protein levels of mice were assessed every 2 wk (C) and at week 12 (D). **p < 0.01, n = 10. E, Twelve weeks after initial immunization, glomerular immune deposition was detected by direct immunofluorescence for IgG in frozen kidney section of mice. Representative images (original magnification ×200) of 10 mice are shown for each group. F, Twelve weeks after initial immunization, nephritic pathological changes were shown by H&E staining of renal tissues resected from mice. Representative images (original magnification ×200) of 10 mice are shown for each group. G, Kidney score was assessed using paraffin sections stained with H&E. ***p < 0.001, n = 10.
remarkably decreased anti-dsDNA IgG levels (Fig. 4C, 4D), reduced urine protein levels (Fig. 4E, 4F), diminished autoantibody deposition (Fig. 4G), and alleviated glomerulonephritis (Fig. 4H, 4I) were found in SAP/ALD-DNA–immunized mice compared with those of ALD-DNA–immunized SLE mice. These data suggest that SAP supplements alleviate SLE syndrome accompanied by the phenotypic switch of macrophage differentiation from proinflammatory M2b to anti-inflammatory M2a phenotype in the lupus murine model.

Adoptive transfer of ex vivo programmed M2a macrophages inhibits lupus nephritis

To determine whether the protective effect of SAP was exerted through modulation of macrophage differentiation, we injected ex vivo programmed M2a macrophages (induced by SAP/ALD-DNA) or M2b (induced by ALD-DNA) into lupus mice. ALD-DNA–induced lupus nephritis was unaffected by administration of M2b macrophages (M2b/ALD-DNA groups; Fig. 5). However, remarkably decreased anti-dsDNA IgG levels (Fig. 5A, 5B), reduced urine protein levels (Fig. 5C, 5D), decreased autoantibody deposition (Fig. 5E), and alleviated glomerulonephritis (Fig. 5F, 5G) were found in lupus mice receiving M2a macrophages (M2a/ALD-DNA groups) as compared with lupus mice receiving M2b macrophages (M2b/ALD-DNA mice) and lupus mice immunized with ALD-DNA alone (ALD-DNA mice). These results indicated that administration of ex vivo programmed M2a macrophages could alleviate lupus nephritis, which further suggest that in vivo SAP supplements could alleviate SLE syndrome via inducing macrophage M2a differentiation.

M2a macrophages induced by SAP/ALD-DNA impends ALD-DNA–stimulated macrophage M2b differentiation

Because M2a macrophages have been found to play protective roles in many inflammatory disorders (47–49), we predicted that SAP/ALD-DNA–induced M2a macrophages might exert anti-inflammatory functions. Increased expression levels of TNF-α, IL-6, and MCP-1 in macrophages stimulated with ALD-DNA were found to be inhibited by coculture with M2a macrophages (Fig. 6A). In addition, no M2b macrophages were differentiated when BMDMs (low chamber) were cultured with M2a macrophages (upper chamber) in a dual-chamber transwell supplemented with ALD-DNA (Fig. 6B). Moreover, expression levels of TNF-α, IL-6, and MCP-1 in macrophages drastically decreased when the cultivation was performed with M2a culture supernatant (Culture-sup) even in the presence of ALD-DNA (Fig. 6C). Similar results were obtained when IL-4/IL-13–induced M2a macrophages or the culture supernatants of these macrophages were subjected to the same test (Fig. 6B, 6C). These data suggest

![FIGURE 6. SAP/ALD-DNA–programmed M2a macrophages hamper ALD-DNA–induced macrophage M2b differentiation. To induce M2a macrophages, we treated BMDMs (1 × 10⁶ cells/ml) with SAP/ALD-DNA (50 μg/ml) or IL-4/IL-13 (10 ng/ml) for 24 h. A, Coculture. In the presence of ALD-DNA, BMDMs were cocultured with SAP/ALD-DNA–programmed M2a macrophages. Levels of TNF-α, IL-6, and MCP-1 in the culture supernatants were measured by ELISA. Data are means ± SD of three independent experiments. **p < 0.01. B, Transwell cultures. In the presence of ALD-DNA, BMDMs were cultured with complete medium supplemented with the culture supernatants from BMDMs (BMDM Culture-sup), SAP/ALD-DNA–induced M2a macrophages (SAP/ALD-DNA M2a Culture-sup), or IL-4/IL-13–induced M2a macrophages (IL-4/IL-13 M2a Culture-sup) at a ratio of 15% (v/v) for 24 h. The cells harvested were washed and cultured for an additional 24 h. Levels of TNF-α, IL-6, and MCP-1 in the culture supernatants were measured by ELISA. Data are means ± SD of three independent experiments. **p < 0.01, ***p < 0.001. C, M2a Culture-sup. In the presence of ALD-DNA (50 μg/ml), BMDMs were cultured with complete medium supplemented with the culture supernatants from BMDMs (BMDM Culture-sup), SAP/ALD-DNA–induced M2a macrophages (SAP/ALD-DNA M2a Culture-sup), or IL-4/IL-13–induced M2a macrophages (IL-4/IL-13 M2a Culture-sup) at a ratio of 15% (v/v) for 24 h. The cells harvested were washed and cultured for an additional 24 h. Levels of TNF-α, IL-6, and MCP-1 in the culture supernatants were measured by ELISA. Data are means ± SD of three independent experiments. **p < 0.05, ***p < 0.01.](http://www.jimmunol.org/)
that M2a macrophages induced by SAP/ALD-DNA could blunt ALD-DNA–induced macrophage M2b differentiation.

IL-10 secreted by M2a macrophages hampers ALD-DNA–induced macrophage M2b differentiation

To further clarify the molecular mechanism involved in the inhibitory effect of M2a macrophages induced by SAP/ALD-DNA, we screened cytokine profiles in the culture supernatants of SAP/ALD-DNA–induced M2a macrophages, and a dramatically increased IL-10 level was noticed (Fig. 7A, 7B). As one of the major inhibitory cytokines, IL-10 has been reported to be critical in ensuring cellular homeostasis and suppression of autoimmunity (50). Further experiments of treating macrophages with anti–IL-10 neutralizing Abs or knockdown of IL-10 in macrophages by IL-10–specific siRNA abrogated the inhibitory action of M2a macrophages, which revealed that IL-10 enabled SAP/ALD-DNA–induced M2a macrophages to inhibit ALD-DNA–induced macrophage M2b differentiation (Fig. 7C, 7D). Moreover, increased levels of TNF-α, IL-6, and MCP-1 in ALD-DNA–induced macrophages were found to be efficiently inhibited by administration.
with exogenous IL-10 (Fig. 7E). Taken together, these data suggest that IL-10 secreted by SAP/ALD-DNA–induced M2a macrophages could inhibit ALD-DNA–induced macrophage M2b differentiation.

Neutralization of IL-10 impairs the ability of SAP to alleviate SLE syndrome

To further confirm the critical role of IL-10 in the SAP-mediated inhibitory effect, we treated mice immunized with SAP/ALD-DNA with anti–IL-10 neutralizing Abs. Autoantibody production (Fig. 8A, 8B), proteinuria (Fig. 8C, 8D), IC deposition (Fig. 8E), lupus nephritis (Fig. 8F), and kidney score (Fig. 8G) in the lupus mice receiving SAP/ALD-DNA plus anti–IL-10 Abs were significantly severe compared with those in mice receiving only SAP/ALD-DNA (Fig. 8). These data suggest that neutralization of IL-10 impairs the ability of SAP to alleviate SLE syndrome, demonstrating the indispensable role of IL-10 in the SAP-mediated inhibitory effect on the ALD-DNA–induced SLE disease (Fig. 9).

Discussion

The data presented in this study provide compelling evidence that SAP supplements could switch macrophage polarization from pathogenic M2b to inhibitory M2a phenotype, resulting in the alleviation of lupus nephritis in SLE murine model (Fig. 9), which
reveals that SAP might be a new candidate for modulating macrophage differentiation and could open a new avenue for developing novel therapeutic approaches for SLE patients. Furthermore, we have demonstrated for the first time, to our knowledge, that SAP insufficiency for DNA excess could promote abnormal M2b macrophage differentiation, which aggravates pathological syndrome in SLE murine model. This finding also provides a molecular mechanism for our previous established SLE murine model in which the female BALB/c mice were immunized with ALD-DNA, highly anti-dsDNA Abs and lupus nephritis were induced, accompanied by renal macrophage M2b polarization, which resembles human SLE disease (14).

Because of various factors including genetic susceptibility and environmental factors, exorbitant cell apoptosis and prominent tissue damage have been found to occur frequently in SLE patients and lupus mice (1, 51). During the pathogenesis of SLE, the excessive apoptotic and necrotic cells were reported to serve as strong candidates for sources of the autoantigens that drive autoimmune-mediated pathogenic immune response, which accounts predominantly for tissue damage and pathological sequelae (52, 53). Autoantigens including self-dsDNA released from these excessive apoptotic and necrotic cells could potently activate pathological immune responses, mainly autoantibody-mediated immunoreactions, and then lead to IC-mediated tissue damage, thus aggravating autoantigen release and autoantibody production in the progress of SLE (11, 54). Previous reports indicated that individuals who are prone to the multisystem autoimmunity that is seen in SLE might have uncharacterized defects in the ability of macrophages to clear apoptotic cells (55). Chromatin fragments including self-dsDNA that escaped from noningested apoptotic cells could be directly immunogenic (56, 57). Anti-dsDNA autoimmunity and SLE-like glomerulonephritis could be observed in mice that are deficient in SAP, which binds chromatin and DNA, and masks them from the immune system, thus driving DNA immunological privilege (17, 52). Because ALD-DNA immunization mimics apoptotic DNA overload, which comprises one pathogenic factor in the pathogenesis of SLE, deficient dsDNA clearance caused by insufficient SAP might constitute the other causative factor during this pathological process in our lupus murine model. However, previous studies doubted whether SAP deficiency or strain combination contributed to the pathogenesis of autoimmune disease in SAP-deficient mice, and also raised a new issue that avoiding the effect of linked genes would be crucial to elucidate the role of SAP in autoimmunity (20, 58–60). Therefore, study of the role for SAP in SLE pathogenesis in a mouse model with clear genetic background would be very critical and should be a prerequisite. In this study, we addressed this question by adopting a lupus murine model via immunizing normal female BALB/c mice with ALD-DNA, which avoided the confused effect of SAP linked genes. It was found that SAP was relatively insufficient in lupus mice. Also, a negative correlation of SAP/DNA ratio with the pathogenic anti-dsDNA level was confirmed in ALD-DNA–induced lupus mice, demonstrating that SAP insufficiency might play important pathogenic roles in lupus mice.

SAP is the normal circulating protein that shows specific calcium-dependent binding to DNA and chromatin in physiological conditions in mice, thus inhibiting the formation of pathogenic autoantibodies against chromatin and DNA with uncharacterized mechanisms (20). Importantly, SAP could compete with IgG for the same binding site of FcyR, thus inhibiting IC-mediated phagocytosis followed by pathological proinflammatory immune responses (26). In SLE patients, excessive amounts of self-DNA released from unremoved apoptotic cells, partly because of SAP insufficiency or low binding ability, could not be eliminated efficiently by macrophages (21, 61). The accumulating self-DNA could bind to anti-dsDNA Abs and form the IC, which might be phagocytized by macrophages through FcγR and/or complement receptors (25). However, this pathogenic clearance of dsDNA and IC could induce macrophage M2b differentiation and then drive Th2 immune responses, thus inducing more anti-dsDNA autoantibody production, all of which may result in IC deposition and tissue damage (31). However, because SAP and IgG Ab have the same binding sites on FcγR, SAP could block Ab-mediated phagocytosis and consecutive pathogenic immune responses through competing for the overlapping binding sites on FcγR of macrophages, which indicated that SAP could be used for treating Ab-mediated autoimmune disorders such as SLE (25). In this sense, SAP-mediated DNA phagocytosis may transform the effect of DNA phagocytic removal from the proinflammatory response to anti-inflammatory response.

During the regulation of the balance between the proinflammatory and anti-inflammatory effects of phagocytic removal of apoptotic cells in the pathogenesis of autoimmune disorders, macrophage activation could play critical roles through providing initiative immune signals (52, 62). In this study, we found that although human SAP could bind with ALD-DNA with higher ability than murine SAP (data not shown), mouse SAP could bind with ALD-DNA with high affinity and efficiently promote the uptake of ALD-DNA by macrophages, possibly because of hypermethylation of ALD-DNA. Furthermore, we demonstrated that macrophages stimulated with ALD-DNA displayed the proinflammatory M2b differentiated phenotype, which could drive Th2 responses and consequent autoantibody production, thus contributing to the pathogenesis of SLE (14, 63). However, treatment with SAP/ALD-DNA could switch the phenotype of macrophage differentiation from M2b to M2a, which have been found to play protective roles in many inflammatory disorders such as obesity, myocarditis, and experimental autoimmune encephalitis (43, 47, 64). In this study, in vivo SAP supplements and M2a macrophage transfusion experiments confirmed that SAP treatment relieved lupus nephritis via inducing macrophage phenotypic switch from M2b to M2a. Whether SAP plays the same protective roles in SLE patients and whether human SAP have the similar regulatory roles in macrophage-mediated DNA
phagocytosis and DNA-induced immune response remains to be elucidated.

A previous study reported that Notch1 signaling facilitates ALD-DNA–induced M2b differentiation through PI3K/Akt–ERK and p38 MAPK pathways (14). ERK activation after macrophage FcyR ligation led to enhanced IL-10 production (65, 66). We detected PI3K and MAPK pathways, and found that SAP/ALD-DNA–induced IL-10 and Arg1 production and macrophage M2a differentiation were associated with enhanced PI3K/Akt and ERK1/2 MAPK signaling. Further inhibition of PI3K or MEK1/2 transfection of CA-Akt plasmids confirmed that SAP/ALD-DNA induced macrophage IL-10 production and M2a differentiation via PI3K/Akt–ERK1/2 signaling pathway. Many factors can govern macrophage M2a differentiation. Apart from this signaling pathway, whether SAP/ALD-DNA modulates other events such as cytokine production, signaling pathway cross-talk, or chromatin remodeling remains to be investigated. Moreover, we found in this study that anti-inflammatory cytokine IL-10, which was overexpressed in M2a macrophages induced by SAP/ALD-DNA, inhibited ALD-DNA–triggered macrophage M2b differentiation, thus facilitating the protective role of SAP in SLE. In this sense, SAP might exert IL-10–dependent anti-inflammatory and immunosuppressive effects in the pathogenesis of SLE through regulating the balance between pathogenic proinflammatory M2b macrophage differentiation and protective anti-inflammatory M2a macrophage differentiation. In vivo IL-10 neutralizing Ab treatment and knockdown of IL-10 by siRNA further confirmed that SAP played a protective role via promoting macrophage M2a differentiation and IL-10 production. However, the molecular mechanism responsible for inhibitor effect of IL-10 produced by M2a macrophages remains to be elucidated.

In addition to providing that ALD-DNA–triggered pathogenic M2b differentiation of macrophages caused by SAP insufficiency constituted a specific molecular mechanism for the pathogenesis of SLE, our results demonstrated that SAP supplement could inhibit lupus nephritis via switching macrophage polarization from pathogenic proinflammatory M2b to anti-inflammatory M2a phenotype. These results supply a putative molecular mechanism involved in modulating macrophage differentiation in the pathogenesis of SLE and imply a potential therapeutic strategy for treating SLE and other autoimmune diseases.

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Disclosures

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