



Notch Signaling Modulates Macrophage Polarization and Phagocytosis Through Direct Suppression of Signal Regulatory Protein α Expression

Yan Lin^{1,2,3†}, Jun-Long Zhao^{3,4*†}, Qi-Jun Zheng⁵, Xun Jiang¹, Jiao Tian¹, Shi-Qian Liang³, Hong-Wei Guo¹, Hong-Yan Qin³, Ying-Min Liang^{2*} and Hua Han^{3,4*}

¹Department of Pediatrics, Tangdu Hospital, Fourth Military Medical University, Xi'an, China, ²Department of Hematology, Tangdu Hospital, Fourth Military Medical University, Xi'an, China, ³Department of Genetics and Developmental Biology, Fourth Military Medical University, Xi'an, China, ⁴Department of Biochemistry and Molecular Biology, Fourth Military Medical University, Xi'an, China, ⁵Department of Cardiac Surgery, Xijing Hospital, Fourth Military Medical University, Xi'an, China

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*Correspondence:

Jun-Long Zhao
bio_junlongzhao@163.com;
Ying-Min Liang
liangym@fmmu.edu.cn;
Hua Han
huahan@fmmu.edu.cn

[†]These authors have contributed
equally to this work.

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The Notch pathway plays critical roles in the development and functional modulation of myeloid cells. Previous studies have demonstrated that Notch activation promotes M1 polarization and phagocytosis of macrophages; however, the downstream molecular mechanisms mediating Notch signal remain elusive. In an attempt to identify Notch downstream targets in bone marrow-derived macrophages (BMDMs) using mass spectrometry, the signal regulatory protein α (SIRP α) appeared to respond to knockout of recombination signal-binding protein Jk (RBP-J), the critical transcription factor of Notch pathway, in macrophages. In this study, we validated that Notch activation could repress SIRP α expression likely *via* the Hes family co-repressors. SIRP α promoted macrophage M2 polarization, which was dependent on the interaction with CD47 and mediated by intracellular signaling through SHP-1. We provided evidence that Notch signal regulated macrophage polarization at least partially through SIRP α . Interestingly, Notch signal regulated macrophage phagocytosis of tumor cells through SIRP α but in a SHP-1-independent way. To access the translational value of our findings, we expressed the extracellular domains of the mouse SIRP α (mSIRP α^{ext}) to block the interaction between CD47 and SIRP α . We demonstrated that the soluble mSIRP α^{ext} polypeptides could promote M1 polarization and increase phagocytosis of tumor cells by macrophages. Taken together, our results provided new insights into the molecular mechanisms of notch-mediated macrophage polarization and further validated SIRP α as a target for tumor therapy through modulating macrophage polarization and phagocytosis.

Keywords: macrophages, notch, signal regulatory protein α , SHP-1, polarization, phagocytosis

INTRODUCTION

The important roles of microenvironmental elements in tumor development and progression have attracted intensive research attentions recently (1). Macrophages are the predominant population of tumor-infiltrating immune cells and participate in immune regulation in a polarized manner in response to microenvironmental stimuli (2). Classically activated macrophages or M1-polarized macrophages are typically induced by bacterial lipopolysaccharide (LPS) and interferon (IFN)- γ and invoke a type 1 response through secreting cytokines such as interleukin (IL)-12 and increasing

antigen presentation capacity (3–5). Moreover, M1-macrophages display a stronger phagocytic activity to “eat” abnormal cells, including dying erythrocytes and mutant cells. On the other hand, M2-polarized macrophages are prototypically activated by IL-4/IL-13 and exhibit phenotypes roughly opposite to M1 macrophages (6). Tumor-associated macrophages (TAMs) share a part of M2 macrophage properties and promote tumor angiogenesis and immune editing through multiple mechanisms (7, 8). Therefore, reprogramming TAMs from M2 into the M1 activation pattern might be a potential therapy for cancers, given that the critical molecular mechanisms controlling M1/M2 polarization are unveiled.

The Notch-RBP-J (recombination signal-binding protein J κ) signaling pathway plays significant roles in macrophage development and activation (9–19). We have recently demonstrated that Notch blockade by RBP-J disruption skews TAMs toward M2 polarization to facilitate tumor progression (10). On the other hand, forced Notch activation by conditional overexpression of Notch intracellular domain (NIC) in macrophages directs TAMs toward an antitumor phenotype by upregulating a few miRNAs (13, 14). Besides, Notch-mediated macrophage polarization also participates in the regulation of phagocytosis and tissue fibrosis (12). However, the downstream molecular mechanisms of Notch signal remain to be elucidated.

Signal regulatory protein (SIRP) α , also known as SIRPA, SHPS-1, p84, and BIT, belongs to the immunoglobulin (Ig) superfamily. SIRP α is abundantly expressed in macrophages, dendritic cells, neutrophils, and neurons (20). Its best characterized ligand is the ubiquitously expressed “don’t-eat-me” signal molecule CD47. When SIRP α binds with CD47 through the Ig-like domains in its N-terminal region, signaling through SHP-1 or SHP-2 is activated in a phosphorylation-dependent manner, followed by a panel of different downstream signaling events (21–23). Kong et al. have demonstrated that SIRP α plays a critical role in regulating innate immune activation (24). Recently, they have further found that SIRP α functions as a significant regulator of TAMs in hepatoma (25).

In this study, we explored the regulatory mechanisms of Notch signal in macrophage polarization. Our results show that Notch signal regulates macrophage polarization at least partly by inhibiting SIRP α . We found that soluble mSIRP α^{ext} polypeptide, a recombinant extracellular fragment of the mouse SIRP α (mSIRP α^{ext}), could promote M1 polarization of macrophages and increase their phagocytic activity to L1210 tumor cells in a CD47-dependent way. Our findings extend the molecular signaling mechanisms downstream to Notch in macrophage polarization and highlight SIRP α as a novel target of Notch-mediated macrophage polarization for tumor therapy.

MATERIALS AND METHODS

Animals

Mice were maintained on the C57BL/6 background in a specific pathogen-free facility. RBP-J-floxed (RBP-J f) mice (12) or ROSA-Stop f -NIC transgenic mice (14) were mated with Lyz2-Cre (#019096, Jackson Laboratory) mice to obtain mice with myeloid-specific Notch blockade (Lyz2-Cre-RBP-J ff , with Lyz2-Cre-RBP-J $^{+/f}$ as a control) or activation (Lyz2-Cre-ROSA-Stop f -NIC,

with ROSA-Stop f -NIC as a control) mice, respectively. Mice were genotyped with tail DNA using polymerase chain reaction (PCR) primers listed in Table S1 in Supplementary Material. All animal experiments were approved by the Animal Experiment Administration Committee of Fourth Military Medical University.

Cell Culture and Transfection

L1210 murine leukemia cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 μ g/mL streptomycin, and 100 U/mL penicillin. Cells were maintained in 5% CO $_2$ to 95% air. To culture bone marrow-derived macrophages (BMDMs), mononuclear cells were isolated from tibias and femurs of C57BL/6 mice. Cells were cultured at a density of 2×10^6 /mL in DMEM containing 10% FBS and 25 ng/mL murine macrophage-colony stimulating factor (M-CSF) (PeproTech, Rocky Hill, NJ, USA) for 7 days. In some experiments, IFN γ (20 ng/mL, PeproTech), LPS (50 ng/mL, Sigma, St. Louis, MO, USA), or IL4 (20 ng/mL, PeproTech) was added and cultured for 24 h before further analyses. Macrophages treated with PBS, LPS + IFN γ , or IL4 were named as M $^{\text{PBS}}$, M $^{\text{LPS}}$, or M $^{\text{IL4}}$, according to Epelmann et al. (5). Cells were transfected siRNA with Lipofectamine LTX (Invitrogen) according to the recommended protocol. Three pairs of siRNA for each target were designed and their knockdown efficiency was determined by qRT-PCR and Western blotting. The siRNA with highest efficiency was chosen for further experiments. Lentivirus was packaged by Cyagen Biosciences with commercial service (Guangzhou, China).

Plasmid Construction and Recombinant Protein Purification

Genes encoding the extracellular domain of mouse SIRP α (mSIRP α^{ext}) or CD47 (mCD47 $^{\text{ext}}$) was amplified with PCR using a mouse embryo cDNA library as a template. The primers used were listed in Table S1 in Supplementary Material. The amplified fragments were inserted into pET32a(+) plasmid (Novagen, Darmstadt, Germany), and the expressed fusion protein Trx-mSIRP α^{ext} (55.2 kDa) or Trx-mCD47 $^{\text{ext}}$ (32.5 kDa) was purified as described previously in Ref. (26). pEFBOS-NIC was as described in Ref. (14).

Reverse Transcription (RT)-PCR

Total RNA was extracted from cell samples with the Trizol reagent (Invitrogen). cDNA was prepared with a reverse transcription kit (Takara, Dalian, China) following the supplier’s instruction. Quantitative real-time PCR was performed using a kit (SYBR Premix EX Taq, Takara) and the ABI Prism 7500 Real-Time PCR System in triplicates, with β -actin as an internal control. Primers are listed in Table S1 in Supplementary Material.

Western Blot

Cells were harvested and the whole cell lysates were extracted on ice with the RIPA buffer containing a protease inhibitor cocktail (Beyotime, Haimen, China). Lysates were centrifuged and the supernatants were collected. Protein concentration was determined using a BCA protein assay kit (Pierce). Aliquots of protein lysates were separated by SDS-PAGE and blotted onto

polyvinylidene fluoride membrane. Membranes were blocked with bovine serum albumin solution and probed with different primary antibodies and washed, followed by HRP-conjugated secondary antibodies (Table S2 in Supplementary Material). Protein bands were visualized with chemoluminescent reagents (Pierce).

In Vitro Pull-Down Assay

The recombinant Trx-mSIRP α^{ext} protein was cleaved using thrombin (Novagen) in a buffer containing 20 mM Tris-HCl, 150 mM NaCl, and 2.5 mM CaCl₂ (pH8.4) at room temperature away from light for 20 h. The ProBond™ purification system (Invitrogen) was used to purify the recombinant mSIRP α^{ext} proteins according to manufacturer's protocols. The S-tagged mSIRP α^{ext} was mixed with purified Trx-mCD47 $^{\text{ext}}$ protein (ratio 1:1), and the mixture was incubated at 4°C for 2 h. Then anti-His antibodies (Sigma) pre-coupled to the Dynabeads-protein G (Invitrogen) were added and incubated at room temperature for 30 min with rotation. After washing with PBS-0.02% Tween 20, 50 mM glycine eluent was added and incubated at room temperature for 5 min with rotation. Proteins were collected and analyzed using SDS-PAGE with 15% acrylamide, followed by Western blotting with the anti-S tag and anti-His antibodies.

Proteomic Analysis

Four-plex iTRAQ-based quantitative proteomics analysis was carried out using proteins isolated from BMDMs of Lyz2-Cre-RBP-J $^{\text{fl}}$ or control mice. Each sample was labeled using iTRAQ 4-plex kits (AB Sciex Inc., Foster City, CA, USA) according to the manufacturer's instructions. Samples from the control BMDMs were labeled with 114, 115 tags, and samples from the RBP-J knockout BMDMs were labeled with 116, 117 tags, respectively. After labeling, the peptide samples were mixed for further LC-MS/MS analysis. The protein expression level in each sample was quantified and the fold change between control and the RBP-J knockout BMDMs was determined.

Luciferase Assay

The 5' flanking sequence (−2,615 to +123) of the murine SIRP α gene was amplified by PCR with mouse genomic DNA as a template. The fragment was inserted into pGL3-basic to generate pGL3-mSIRP α -promoter. Different truncated fragments of the 5' flanking region, as depicted in **Figure 1F**, were also generated by PCR and inserted into pGL3-basic (pGL3-mS-T1, 2,3, or 4). HeLa cells (2×10^4) were transfected with different reporters, NIC overexpression plasmid, and phRL-TK using Lipofectamine 2000™ (Invitrogen). The luciferase activity was assessed 24 h later using Luminoskan Ascent (Labsystems, Helsinki, Finland) and a Dual-Luciferase Reporter Assay Kit (Promega) according to the manufacturer's protocol. All luciferase activity was normalized to the Renilla luciferase activity.

Chromatin Immunoprecipitation (ChIP)

Chromatin immunoprecipitation assay was performed using a kit (Merck Millipore) according to the manufacturer's instructions. BMDMs were polarized with LPS + IFN γ for 24 h and fixed with formaldehyde. Cross-linked immune complexes were sonicated

and precipitated with anti-Hes1 antibody. DNA was extracted from the collected samples and analyzed by PCR with the primers listed in Table S1 in Supplementary Material.

Flow Cytometry

L1210 cells (3×10^5) were incubated with mSIRP α^{ext} (10 $\mu\text{g}/\text{mL}$) for 30 min on ice in dark. After washing with PBS, cells were stained with FITC-conjugated anti-S tag antibody (Sigma), followed by FACS analysis using a Calibur™ (BD Immunocytometry Systems, San Jose, CA, USA). BMDMs (3×10^5) were cultured in the presence of PBS (M^{PBS}) or LPS + IFN γ (M^{LPS}) for 24 h, and then incubated with mSIRP α^{ext} (10 $\mu\text{g}/\text{mL}$) at 37°C for 2 h. After washing, cells were stained with anti-Ki67 (Sigma) or anti-Annexin V (BD), followed by FACS analysis. Data were analyzed using the Flowjo™ software.

In Vitro Phagocytosis Assay

L1210 cells were labeled with carboxyfluorescein succinimidyl amino ester (CFSE, Dojindo Molecular Technologies, Inc.) according to the recommended protocol, and loaded onto macrophages. In some cases, L1210 cells were pre-incubated with purified recombinant proteins at the concentration of 10 $\mu\text{g}/\text{mL}$ at 37°C for 2 h before coculturing with macrophages. Cells were stained with anti-F4/80, rinsed with PBS, and visualized under a fluorescence microscope (BX51, Olympus). Phagocytosis was quantified by calculating the average number of ingested L1210 cells in macrophages.

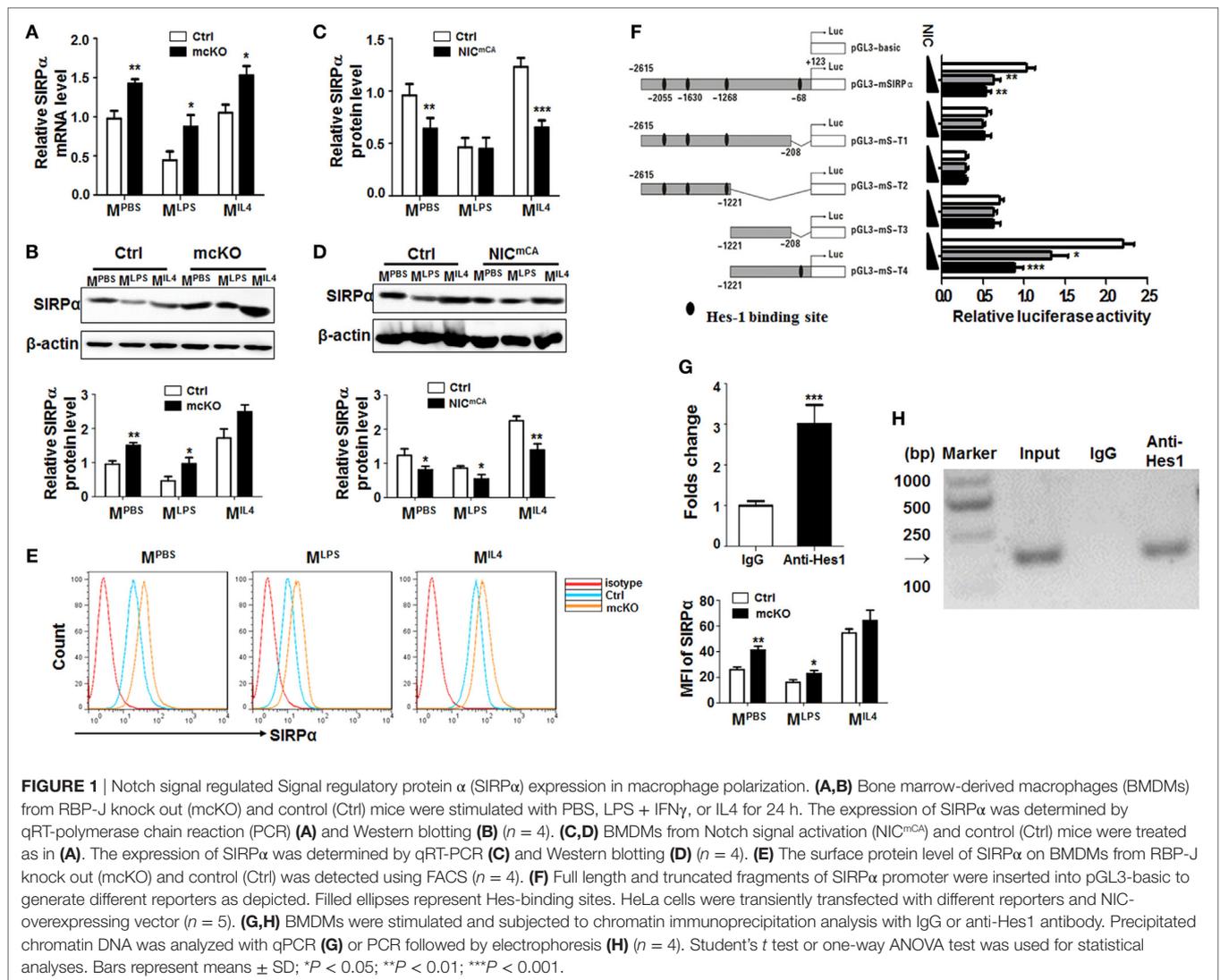
Statistics

Statistical analysis was performed with the Graph Pad Prism 5 software. Student's *t*-test or one-way ANOVA test was used for statistical analyses. Data were expressed as means \pm SD. $P < 0.05$ was considered statistically significant.

RESULTS

SIRP α Was Involved in Notch Signal-Mediated Macrophage Polarization

In an attempt to identify Notch downstream molecules involved in macrophage activation, we employed mice with myeloid-specific Notch blockade (Lyz2-Cre-RBP-J $^{\text{fl}}$ or RBP-J mcKO, with Lyz2-Cre-RBP-J $^{\text{fl}}$ as a control) or activation (Lyz2-Cre-ROSA-Stop $^{\text{f}}$ -NIC or NIC $^{\text{mCA}}$, with ROSA-Stop $^{\text{f}}$ -NIC as a control). In an initial proteomic analysis of RBP-J deficient and control BMDMs using mass spectrometry, we found that the protein level of SIRP α was upregulated in RBP-J-deficient BMDMs (Figure S1A in Supplementary Material). To validate this finding, RBP-J mcKO, NIC $^{\text{mCA}}$, and control BMDMs were stimulated with PBS (M^{PBS}), LPS + IFN γ (M^{LPS}), or IL4 (M^{IL4}) (5), and the expression of polarization markers, Hes1 and SIRP α was examined by qRT-PCR or Western blotting. These treatments induced the expression of various macrophage polarization markers (Figure S1B in Supplementary Material). Meanwhile, Hes1 and Hey1 were upregulated in M^{LPS} and downregulated in M^{IL4} macrophages (Figure S1C in Supplementary Material). SIRP α expression was downregulated in M^{LPS} and upregulated in M^{IL4}



BMDMs, as well as in RAW264.7 cells treated in the same way (Figure S1D in Supplementary Material). Moreover, we found that RBP-J deficiency led to upregulated SIRP α expression under different stimuli (Figures 1A,B). On contrary, constitutive Notch activation resulted in a tendency of SIRP α downregulation in macrophages (Figures 1C,D). Flow cytometry confirmed that the expression of SIRP α was upregulated in RBP-J deficiency BMDMs (Figure 1E). These data suggested that Notch signal repressed SIRP α expression in macrophages.

There are four Hes recognition sites ($-2,615$, $-1,630$, $-1,268$, and -68 bp) in the murine SIRP α promoter, suggesting that Notch signal might regulate SIRP α expression through Hes family co-repressors that are downstream to Notch receptors. We, therefore, constructed reporter genes with different truncated fragments of the SIRP α promoter, which were inserted into pGL3-basic (Figure 1F). Reporter assay in the presence of NIC overexpression showed that Notch activation could significantly repress luciferase expression driven by SIRP α promoter fragments, and the Hes-binding site at -68 bp was required for

Notch activation-mediated repression of the SIRP α promoter (Figure 1F). Indeed, a ChIP assay indicated that anti-Hes1 antibody could significantly pull-down the SIRP α promoter fragment surrounding the Hes1 binding site at -68 bp in M^{LPS} (Figures 1G,H). These data indicated that Notch signal directly inhibited expression of SIRP α through Hes proteins.

Notch Signal Negatively Regulated SHP-1 Phosphorylation Triggered by SIRP α -CD47 Interaction

Signal regulatory protein α is enriched in myeloid cells, while its ligand CD47 is universally expressed. Interaction between CD47 and SIRP α induces SHP1/SHP2 activation through tyrosine phosphorylation in the SH2 domains (27, 28), leading to inhibited macrophage phagocytosis and pro-inflammatory response. BMDMs from normal mice were transfected with SIRP α siRNA (si-SIRP α) or control oligonucleotide (NC), which were proven to inhibit SIRP α expression efficiently (Figures

S2A,B in Supplementary Material), and stimulated with PBS or LPS + IFN γ for 24 h. Western blotting showed that knock-down of SIRP α by siRNA (si-SIRP α) significantly reduced SHP1 phosphorylation in M^{PBS} and M^{LPS} macrophages (Figures 2A,B). On the other hand, SIRP α overexpression obviously increased SHP1 phosphorylation in macrophages (Figure S2C in Supplementary Material; Figures 2C,D). We then examined the effect of Notch signal on SIRP α signaling using BMDMs isolated from RBP-J mcKO and control mice and stimulated with PBS, LPS + IFN γ , or IL4. The result showed that Notch signal deficiency significantly increased SHP1 phosphorylation both in M^{PBS} and M^{LPS} macrophages (Figures 2E,F). When the expression of SIRP α was knocked down with siRNA, the RBP-J deficiency-induced increase of SHP1 phosphorylation was canceled (Figures 2E,F). On the other hand, forced Notch activation by NIC overexpression reduced SHP-1 phosphorylation, which was reversed by SIRP α overexpression mediated by a lentivirus (Figures 2G,H). These results indicated that Notch signal could repress SHP-1 activation in macrophages, likely through blocking SIRP α expression.

SIRP α Signaling Modulated Macrophage Polarization

Signal regulatory protein α was differentially expressed in macrophages under different polarization stimuli (Figure S1D in Supplementary Material), suggesting that SIRP α -mediated signaling might be involved in macrophage polarization. To validate this, BMDMs from normal mice were transduced with SIRP α -overexpressing lentivirus and stimulated with PBS, LPS + IFN γ , or IL4. qRT-PCR was carried out to access the expression of polarization markers in BMDMs. The result showed that SIRP α overexpression downregulated the expression of M^{LPS} markers TNF- α and IL12 and upregulated M2 markers IL10 and MR under LPS + IFN γ stimulation (Figure 3A). Moreover, BMDMs from normal mice were transfected with SIRP α siRNA or NC oligonucleotides and stimulated as above. The result showed that the M^{LPS} markers were upregulated while the M^{IL4} markers were downregulated upon SIRP α knockdown (Figure 3B). Knockdown of SHP-1 with siRNA exhibited similar effects on macrophage polarization under various stimuli (Figures S2D,E in Supplementary Material; Figure 3C). Furthermore, SIRP α

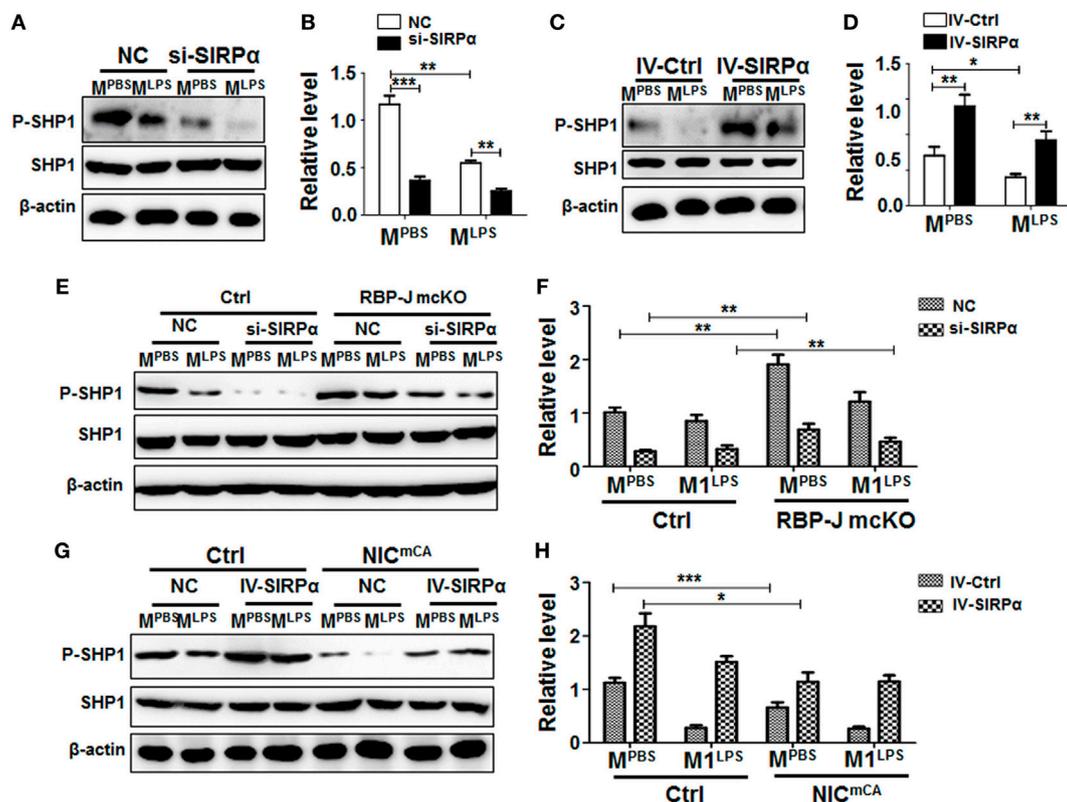


FIGURE 2 | Notch signal regulated signal regulatory protein α (SIRP α) expression and SHP-1 activation. (A–D) Bone marrow-derived macrophages (BMDMs) with SIRP α knockdown (A,B) or overexpression (C,D) were stimulated with PBS or lipopolysaccharide (LPS) + IFN γ for 24 h. Western blotting was carried out to analyze SHP-1 phosphorylation ($n = 4$). (E,F) BMDMs from RBP-J deficient (mcKO) and control (Ctrl) mice were transfected with SIRP α siRNA or NC and stimulated with PBS or LPS + IFN γ for 24 h. The phosphorylation of SHP-1 was determined by Western blotting ($n = 4$). (G,H) BMDMs from Notch signal activation (NIC^{mCA}) and control (Ctrl) mice were infected with SIRP α -overexpressing lentivirus and stimulated with PBS or LPS + IFN γ for 24 h. The phosphorylation of SHP-1 was determined by Western blotting ($n = 4$). One-way ANOVA test was used for statistical analyses. Bars represent means \pm SD; * or #, $P < 0.05$; ** or ##, $P < 0.01$; *** $P < 0.001$.

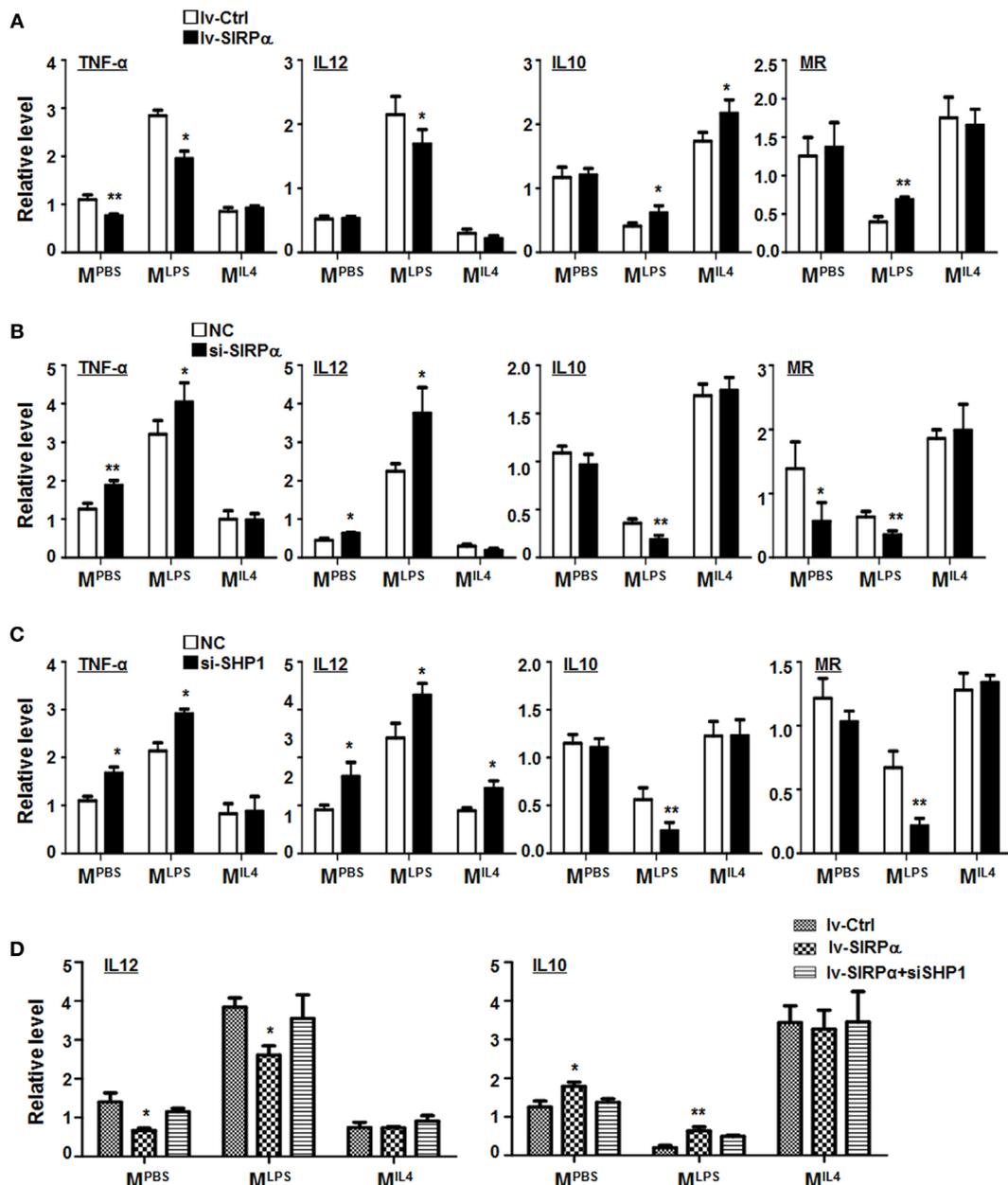


FIGURE 3 | CD47-SIRP α -SHP-1 signal suppressed M1 and promoted M2 polarization. **(A)** Bone marrow-derived macrophages (BMDMs) were transfected with SIRP α or control lentivirus and then cultured with PBS, lipopolysaccharide (LPS) + interferon (IFN) γ , or IL4 for 24 h. The expressions of TNF- α , IL12, IL10, and MR were determined by reverse transcription-polymerase chain reaction (PCR) ($n = 3$). **(B,C)** BMDMs were transfected with siRNA for SIRP α **(B)** or SHP-1 **(C)** followed by stimulation with PBS, LPS + IFN γ , or IL4. The expression of polarization makers was determined by qRT-PCR ($n = 3$). **(D)** BMDMs were transfected with SIRP α or control lentivirus and transfected with SHP-1 siRNA, followed by stimulation with PBS, LPS + IFN γ , or IL4. The expression of IL12 and IL10 was detected by qRT-PCR ($n = 3$). One-way ANOVA test was used for statistical analyses. Bars represent means \pm SD; * $P < 0.05$; ** $P < 0.01$.

was overexpressed in normal BMDMs using lentivirus and transfected with SHP1 siRNA simultaneously, followed by polarization stimulation as above. qRT-PCR showed that knockdown of SHP1 abrogated the effect of SIRP α overexpression on macrophage polarization (Figure 3D). These data suggested that the SIRP α -SHP1 signaling repressed M^{LPS} polarization upon LPS + IFN γ stimulation.

SIRP α Partially Mediated the Effect of Notch Signal on Macrophage Polarization

To access the functional significance of SIRP α downstream to Notch signal in regulating macrophage polarization, we cultured BMDMs from RBP-J mcKO mice and treated cells with PBS, LPS + IFN γ , or IL4 in the presence or absence of SIRP α siRNA. qRT-PCR and ELISA were performed to determine the expression

of polarization markers and the production of cytokines. The result showed that RBP-J knockout reduced M^{LPS} and increased M^{IL4} marker expression obviously, while knockdown of SIRP α could partially cancel the effects of Notch signal deficiency (Figure 4A). ELISA also showed that blockade of Notch signal suppressed secretion of inflammatory cytokines, such as IL12 and TNF- α , and promoted the production of anti-inflammatory cytokine IL10 (Figure 4B). Similarly, inhibiting SIRP α recovered the cytokine production modulated by RBP-J mcKO (Figure 4B). These data suggested that SIRP α as a downstream molecule of Notch signal could at least partially mediate the effect of Notch signal on macrophage polarization.

Interaction Between Notch Signal and SIRP α in Regulating Phagocytosis of Macrophages

To look at the role of SIRP α in macrophage phagocytosis, the L1210 murine leukemia cells were labeled with CFSE, and incubated with PBS-, LPS + IFN γ -, or IL4-stimulated BMDMs for 2 h. The result showed that M^{LPS} BMDMs exhibited increased ability to ingest tumor cells as compared with M^{PBS} or M^{IL4} BMDMs (Figures S3A,B in Supplementary Material). Consistent with previous reports, overexpression of SIRP α in BMDMs using lentivirus attenuated tumor cells engulfment (Figure 5A), and knockdown of SIRP α with siRNA enhanced BMDM phagocytosis (Figure 5B). Consistently, CD47 expressed on L1210 was inhibitory to SIRP α -mediated phagocytosis, because repression of CD47 in L1210 cells significantly increased phagocytosis (Figures S3C,D in Supplementary Material; Figure 5C). Compared with untransfected L1210 cells, incubation with CD47-compromised L1210 cells promoted M^{LPS}-like phenotype

of BMDMs (Figure 5D), which was dependent on SIRP α or SHP1 expression (Figure 5E). These results suggested that interaction of CD47 on tumor cells with SIRP α on BMDMs regulated both phagocytosis and polarized activation of macrophages.

RBP-J deficiency or treatment with GSI, an inhibitor of Notch signaling, reduced phagocytosis by BMDMs significantly (Figures 5E,G), and this effect of Notch signal blockade was reversed by SIRP α siRNA (Figure 5G). These data suggested that Notch signal increased phagocytosis by macrophages likely through repressing SIRP α .

Soluble Extracellular Domain of Mouse SIRP α Could Interrupt the CD47–SIRP α Interaction

It has been shown that blocking CD47–SIRP α interaction could facilitate phagocytosis of tumor cells and promote antitumor immune response. We have also shown that soluble human CD47 could serve as an antagonist to block CD47–SIRP α interaction (26). Expression vector of the extracellular domain of mSIRP α (mSIRP α ^{ext}) was constructed and expressed in *E. coli* together with the mCD47^{ext} (Figure 6A). SDS-PAGE analysis of cell lysates revealed that the mSIRP α ^{ext} and the Trx-mCD47^{ext} proteins were successfully expressed with predicted molecular weights of 36 and 32.5 kDa, respectively (Figure 6B). Pull-down assay followed by Western blotting showed that the mSIRP α ^{ext} protein could be pulled down by the Trx-mCD47^{ext} protein (Figure 6C) but not by Trx (not shown), indicating that mSIRP α ^{ext} could bind to mCD47^{ext}. To examine whether the mSIRP α ^{ext} protein could bind to L1210 cells, we incubated the mSIRP α ^{ext} fusion protein with L1210 cells, and analyzed the cells using flow cytometry after staining with a FITC-conjugated anti-S tag antibody. The

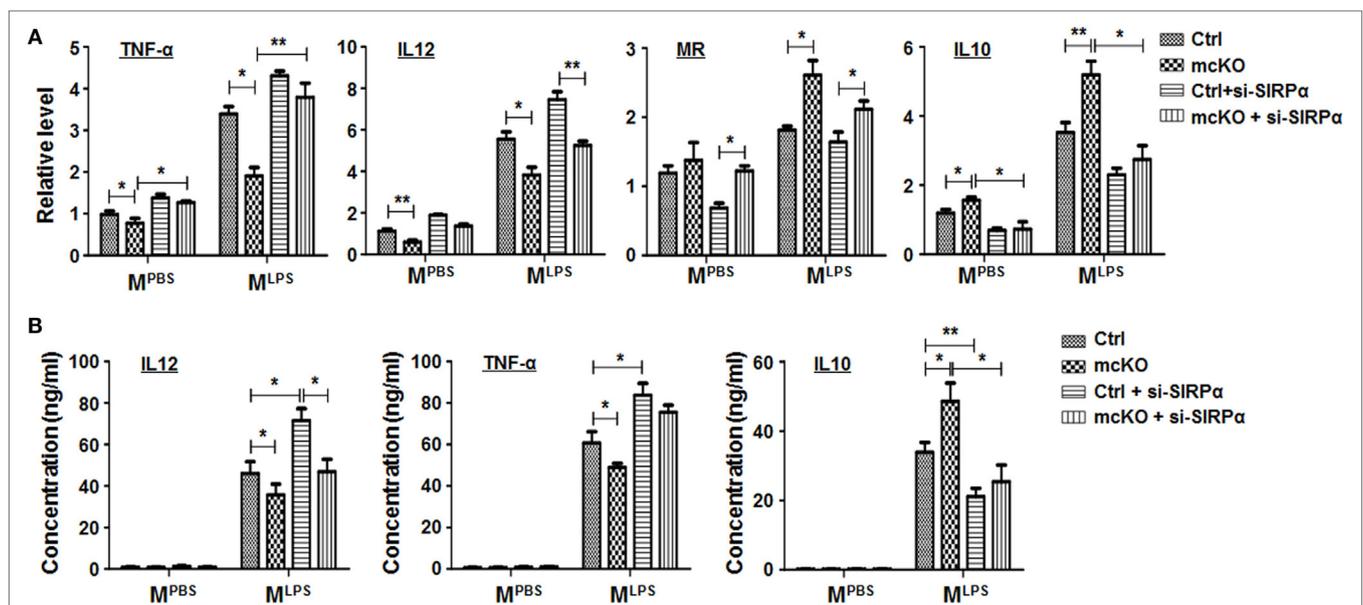
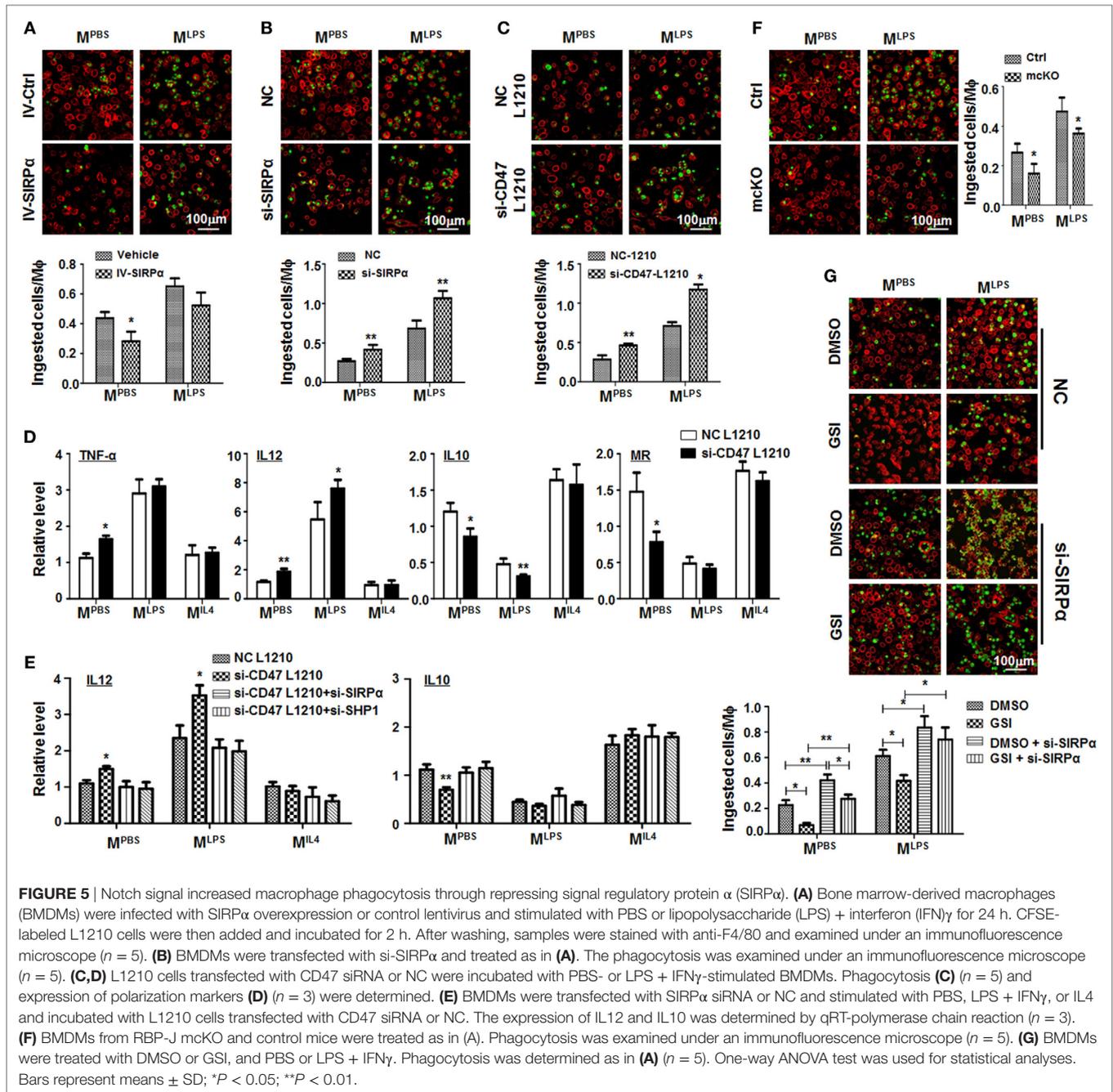


FIGURE 4 | Notch signal regulated macrophage polarization partially through signal regulatory protein α (SIRP α). (A,B) Bone marrow-derived macrophages from RBP-J mcKO and control mice were transfected with siRNA for SIRP α and then cultured in the presence of PBS or lipopolysaccharide + interferon γ for 24 h. The expression of polarization markers (A) and the production of cytokines (B) were detected ($n = 4$). One-way ANOVA test was used for statistical analyses. Bars represent means \pm SD; * $P < 0.05$; ** $P < 0.01$.

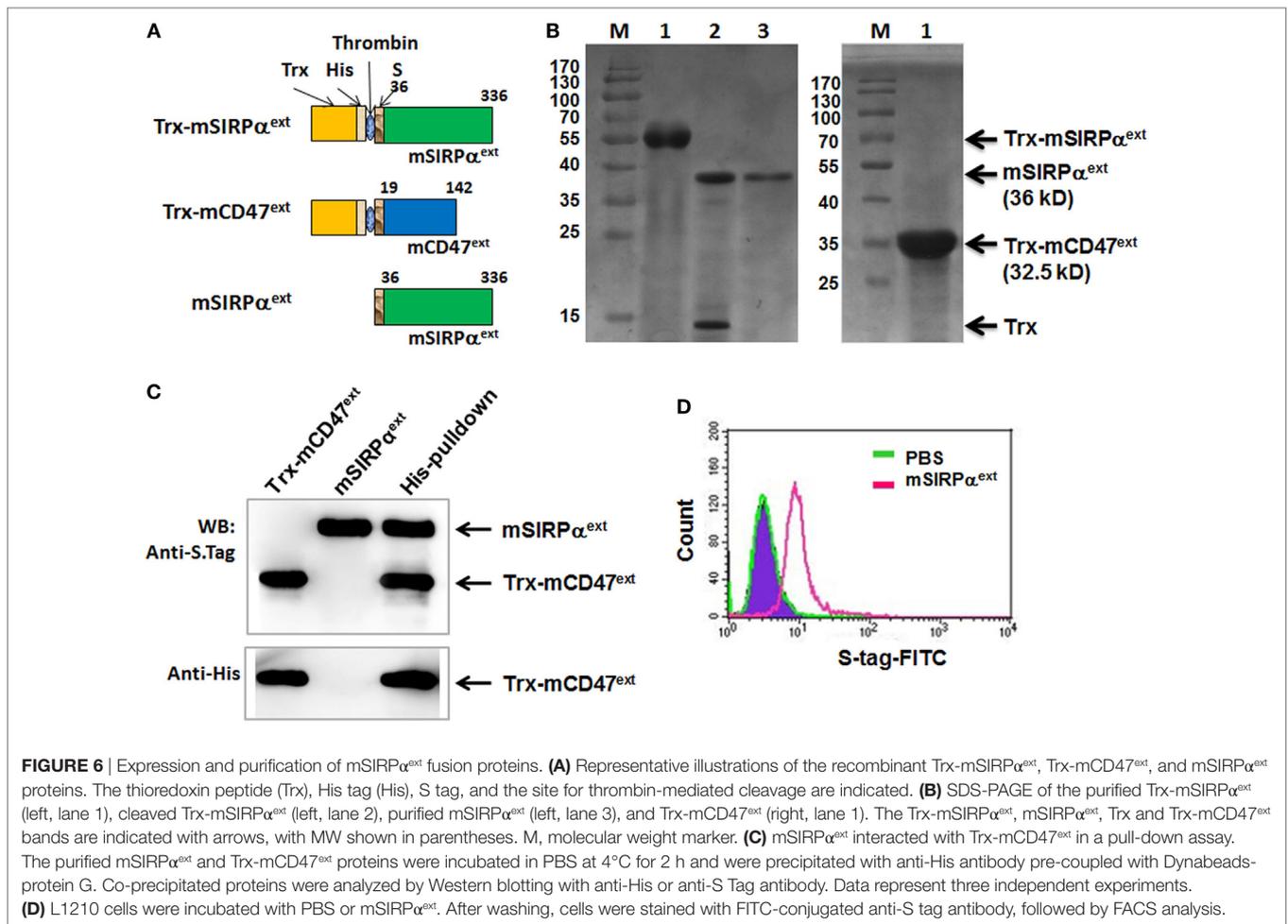


result showed that cells incubated with mSIRP α^{ext} exhibited significantly higher fluorescence intensity as compared with the control cells (**Figure 6D**), suggesting that the mSIRP α^{ext} fusion protein could bind to L1210 tumor cells most likely through the interaction with CD47.

mSIRP α^{ext} Polypeptide Could Promote M1 Polarization and Enhance the Phagocytosis of Macrophages

To verify whether mSIRP α^{ext} could block the interaction between CD47 and SIRP α and attenuate SIRP α signaling in macrophages,

we incubated BMDMs with mSIRP α^{ext} *in vitro*. This treatment did not affect the proliferation or apoptosis of macrophages (Figures S4A,B in Supplementary Material). However, qRT-PCR showed that mSIRP α^{ext} could upregulate the expression of M^{LPS} markers and downregulate that of M^{IL4} marker IL10 (**Figure 7A**). Moreover, the upregulation of M^{LPS} and downregulation of M^{IL4} markers by mSIRP α^{ext} was canceled by forced overexpression of SIRP α through lentivirus-mediated transfection (**Figure 7B**). We also examined the phagocytosis of L1210 cells by macrophages in the presence of mSIRP α^{ext} . CFSE-labeled L1210 cells were incubated with mSIRP α^{ext} in advanced, and then cocultured with M^{PBS}- or M^{LPS}-stimulated BMDMs in the presence of mSIRP α^{ext} .



The result showed that mSIRP α^{ext} could enhance the phagocytosis of macrophages, and overexpression of SIRP α abrogated this effect (**Figure 7C**). These results suggested that mSIRP α^{ext} fusion protein could block the SIRP α signaling to modulate macrophage activation and phagocytosis, likely *via* competitive interaction with CD47.

DISCUSSION

The Notch signaling pathway modulates various cell fate determination events during development. Moreover, Notch signal is involved in differentiation and plasticity of hematopoietic cells under both physiological and pathological conditions (29, 30). Indeed, there are four types of Notch receptors in mammals, all of which could induce RBP-J-mediated Notch signal activation. We have shown that Notch1 is the most abundantly expressed Notch receptor in BMDMs (14). Several groups have reported that Notch signal regulates myeloid development and macrophage polarization through multiple downstream molecules, such as SOCS3, cylindromatosis, interferon regulatory factor 8, miR-125a, and miR-148a (9–19). Although these studies have pointed to that Notch signal was required for macrophages M1 polarization, Foldi et al. have demonstrated that Notch signal

promotes M2 activation of peritoneal macrophages in an *in vivo* model of chitin-induced M2 polarization (31). This probably suggested that the activation modes of macrophages modulated by Notch signal are context-dependent and influenced by specific pathological processes. Therefore, it should be of significance to further identify other potential downstream molecules of Notch signal. To understand the molecular network modulating macrophage activation downstream to Notch signal, we compared protein expression profiles between wild type and RBP-J deficient BMDMs using iTRAQ and found that SIRP α expression increased in the absence of Notch signaling. qRT-PCR and Western blotting validated the conclusion that SIRP α was downregulated by Notch signal activation or M^{LPS} stimulation in macrophages, while M^{IL4} stimulation or Notch blockade upregulated SIRP α . Further experiments showed that Notch activation repressed SIRP α transcription directly through Hes1-binding sites in its promoter region. Functionally, our data suggested that Notch signal modulated macrophage polarization at least partially through regulating the expression of SIRP α .

Signal regulatory protein α is a myeloid-specific receptor of CD47, which is broadly expressed on many types of somatic cells including tumor cells. SIRP α binds with CD47 and delivers a “don’t eat me” signal for phagocytic cells to help tumor cells escape

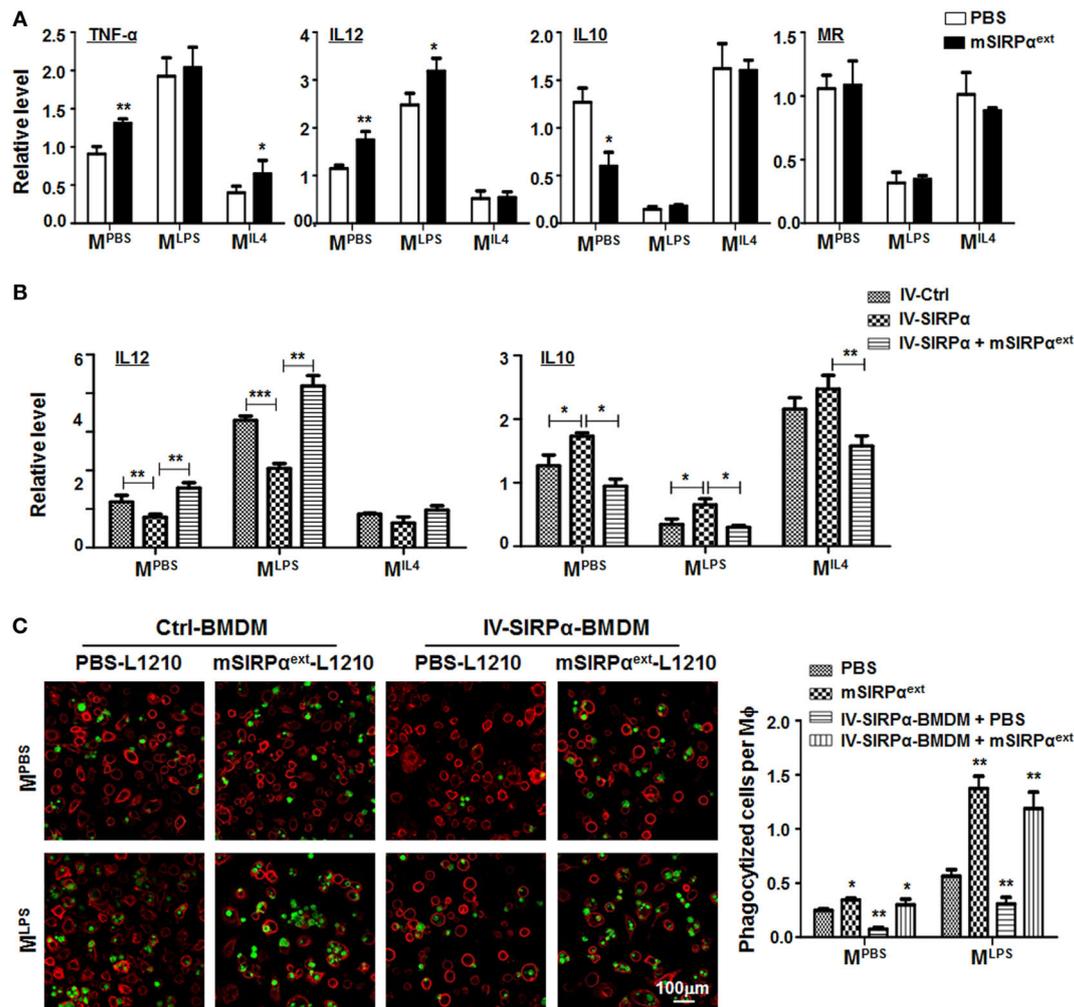


FIGURE 7 | Recombinant mSIRP α^{ext} promoted M1 polarization and enhanced phagocytosis of M1 macrophages *in vitro*. **(A)** Differentially polarized bone marrow-derived macrophages (BMDMs) were incubated with PBS or mSIRP α^{ext} for 6 h. The expression of TNF- α , IL12, IL10, and MR was determined by qRT-polymerase chain reaction (PCR) ($n = 3$). **(B)** BMDMs overexpressing SIRP α were incubated with mSIRP α^{ext} for competitive interaction and stimulated with PBS or lipopolysaccharide (LPS) + IFN γ . The expression of IL12 and IL10 was determined by qRT-PCR ($n = 3$). **(C)** BMDMs were stimulated with PBS or LPS + IFN γ in the presence of mSIRP α^{ext} . CFSE-labeled L1210 cells were then loaded and incubated for 2 h, and examined under an immunofluorescence microscope ($n = 5$). One-way ANOVA test was used for statistical analyses. Bars represent means \pm SD, $n = 3$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

from immune clearance (32–36). Being activated by signal from adjacent cells *via* cell contact, SIRP α could induce intracellular downstream signaling by a cascade of phosphorylation modifications. The predominant pathway of SIRP α is mediated by SHP-1 activation, which could further influence NF- κ B and Akt signaling and then regulate macrophage immune suppression (37, 38). Recent studies have mainly focused on CD47 expression on tumor cells and have developed cancer immunotherapy targeting CD47 (39–41), but the regulation of SIRP α expression and its role in macrophages in tumor microenvironment have been elusive. The present study suggests that SIRP α functions as an important modulator of macrophage polarization. The expression of SIRP α is significantly different in differentially polarized macrophages. Overexpression of SIRP α in BMDMs promoted M2 polarization, while SIRP α knockdown promoted M1 polarization. Interference

of CD47 or SHP-1 of SIRP α signaling in BMDMs also promoted M1 polarization, suggesting that SIRP α regulates macrophage polarization dependent on interaction with CD47 and intracellular SHP-1 signaling.

Macrophages have remarkable potential as mediators of anti-cancer therapies based on their robust ability to ingest tumor cells and modulate tumor microenvironment. The CD47–SIRP α interaction between tumor cells and immune cells represents a critical intercellular communication that inhibits the activation of macrophage-mediated phagocytosis of tumors and thereby acts as a myeloid-specific immune checkpoint (20–23). In this study, we observed that SIRP α overexpression in BMDMs decreased phagocytosis of L1210 leukemia cells, while SIRP α knockdown in BMDMs and CD47 knockdown in L1210 cells both increased phagocytosis by macrophages. Meanwhile,

knock down of SHP-1, which modulates M^{LPs} polarization in BMDMs, did not show significant effect on macrophage phagocytosis (data not shown). These findings might suggest that SIRP α -mediated polarization and phagocytosis could be mediated by different mechanisms. Molecular events downstream to these important processes need further studies. In our study, activation of Notch signaling could suppress SIRP α expression and inhibit M^{IL4} macrophage polarization as well as phagocytosis, which might have important implications in tumor microenvironment.

Blocking CD47 triggers the elimination of cancer cells. Nevertheless, the ubiquitous expression of CD47 on normal cells, such as red blood cells, might create a large antigen sink and unintended binding. The unwanted interaction of CD47 on normal cells could be minimized by reducing the binding strength for CD47 (42). On the other hand, high-affinity SIRP α monomers (~14 kDa) are not sufficient to induce macrophage phagocytosis, instead, act as adjuvant to lower the threshold for phagocytosis in the presence of a separate, tumor-binding antibody. Agents that block SIRP α directly, such as anti-SIRP α antibodies, also act as adjuvants for tumor-binding antibodies (43, 44). To overcome these limitations, we here validated a soluble extracellular domain of mouse SIRP α (36 KD) and confirmed that mSIRP α^{ext} could not only promote M1 polarization but also increase phagocytosis of L1210 leukemia cells by macrophages. Therefore, mSIRP α^{ext} might have a promising potential for the clinical application as a new therapeutic reagent. More studies are needed to assess its future pharmacological and clinical values.

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ETHICS STATEMENT

All animal experiments were approved by the Animal Experiment Administration Committee of the Fourth Military Medical University and in accordance with the recommendations of 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).

AUTHOR CONTRIBUTIONS

HH, Y-ML, and J-LZ designed the research and wrote the manuscript; YL, J-LZ, Q-JZ, XJ, and JT performed experiments; YL, J-LZ, S-QL, H-WG, and H-YQ analyzed data. All authors reviewed and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <https://www.frontiersin.org/articles/10.3389/fimmu.2018.01744/full#supplementary-material>.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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