Master of Science

Functional regulation of macrophages phenotype by SLIT3

SLIT3 에 의한 대식세포 표현형의 기능적 조절

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Functional regulation of macrophage phenotypes by SLIT3

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of macrophage phenotypes by SLIT3

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Abstract

Slit homolog 3 protein (SLIT3)/roundabout (ROBO) receptor axis plays a critical role in nerve cell induction, vascular necrosis, embryonic development, bone remodeling, and tumor microenvironment. However, the functional role of this axis in regulation of macrophage phenotypes has not been elucidated yet. In the current study, lipopolysaccharides (LPS)-stimulated enhanced SLIT3 expression in macrophages. We found that LPS stimulated SLIT3 expression in bone marrow macrophages (BMMs) and in turn elevated SLIT3 led to enhancement of phagocytic capacity of these cells *in vitro*. On the other hand, SLIT3 deficiency skewed towards macrophage 1 (M1) profile accompanied by increase in the expression of pro-inflammatory cytokines. This phenotype is linked to increased NLR family pyrin domain containing 3 (NLRP3) and adaptor molecule apoptosis-associated speck-like protein containing a CARD (ASC) in macrophages primed with LPS. Moreover, SLIT3-deficient mice displayed increased M1 population in peritoneal cells compared with that of wild-type mic followed by LPS administration *in vivo*. These findings suggest that the induction of SLIT3 may play an important role in the macrophage-associated inflammation, and thereby provide the potential treatment strategy for the excessive inflammation or sustained hyperinflammatory response.

Keywords: SLIT3, LPS, inflammation, phagocytosis, Macrophage

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Introduction

The secreted slit homolog (SLIT) glycoproteins including SLIT1-3, mediate their functions by binding to the transmembrane receptors known as roundabout (ROBO) receptors[1]. SLIT family was first identified as regulators of axon induction and cell migration in Drosophila and has been suggested to regulate tumor proliferation, apoptosis, cell migration, and angiogenesis[2-9]. The SLIT/ROBO pathway reduces the detrimental aspects of the host response to pathogen-induced cytokine pathways10). SLIT2 in humans inhibits the function of lymphocytes and leukocytes by triggering chemokines[11]. SLIT3 has recently been found to be associated with bone absorption and formation[12]. In addition, SLIT3 functions as an inflammatory mediator involved in monocyte migration[13,14]. With respect to inflammation however, the functional role of SLIT3 in the regulation of macrophage phenotypes has not been elucidated.

Lipopolysaccharide (LPS) is a major component of the gram-negative bacterial outer membrane and is a pathogenic substance that activates macrophages[15]. LPS binds to Toll-like receptor 4 (TLR4) expressed on the surface of macrophages and induces the secretion of inflammatory cytokines and other factors that induce the inflammatory responses needed to protect against infection or tissue injury[16,17]. In the innate immune system, macrophages, neutrophils, and dendritic cells recognize pathogen invasion or pattern recognition receptors (PRRs) such as LPSs and trigger various immune response including septic inflammation[18,19].

Macrophages are immune cells of a hematopoietic origin and are important innate immune defense cells that play roles in development, homeostasis, and in the repair mechanisms that follow the phagocytosis of pathogens20). Macrophages are present in all organs of the body, such as the spleen, liver, lung, kidney, brain and even bone[21,22], and can differentiate into two phenotypes, M1 (classically activated macrophage, pro-inflammatory) and M2 (alternatively activated macrophages, anti-inflammatory)[23]. LPS molecules and

interferon-γ (IFN-γ) activate TLRs on macrophages and induce their differentiation into the M1 phenotype that secretes pro-inflammatory cytokines such as interleukin-1ß (IL-1ß), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α)24,25). Interleukin-4 (IL-4) and interleukin-10 (IL-10) induce M2 macrophages to secrete anti-inflammatory cytokines such as IL-10 and immunosuppressive factors[24)].

In this present study, we aimed to elucidate the functional role of SLIT3 in the regulation of macrophage phenotypes. We found from our analysis that that LPS induces SLIT3 expression in bone marrow-derived macrophages (BMMs) and that this upregulated SLIT3 enhances the phagocytic capacity of these cells. Furthermore, a SLIT3 depletion skewed the differentiation of macrophages to an M1 profile, accompanied by an increase in the expression of pro-inflammatory cytokines. Taken together, our current data suggest that SLIT3 potentially regulates the polarization of macrophages and thereby plays a role in macrophage-associated inflammation.

Materials and Methods

Reagents

Lipopolysaccharide (LPS) from Escherichia coli O11:B4, PD98059, SB203580, SP60025, ATP, cytochalasin D (Cyto D) and nigericin were purchased from Sigma-Aldrich (St. Louis, MO). Fluorescein-labeled E. coli (K-12 core type) bioparticles were purchased from Molecular Probes (Eugene, OR). Recombinant SLIT3 (rSLIT3) was purchased from R&D Systems (Minneapolis, MN, USA).

Mice

Slit3 deficient ($SLIT3^{-/-}$) mice (030759-Mu) and WT littermates were purchased from the Mutant Mouse Regional Resource Center (MMRRC) at the University of Missouri (Columbia, MO).

Cell cultures

Six or 8-week-old female C57BL/6J (Wild type; WT) and $TRL4^{LPS\text{-}del}$ ($TLR4^{-/-}$) mice were purchased from The Jackson Laboratory (Maine, USA) and bone marrow-derived macrophages (BMMs) obtained from those mice were isolated from bones of mice as previously described (Chang et al., 2008) and cultured in α-MEM containing 10% heat-inactivated fetal bovine serum in the presence of M-CSF (30ng/ml). BMMs (3×10^5 cells) were cultured overnight on a 6-wells plate and then stimulated with 10 ng/ml LPS from Escherichia coli strain O26:B6 (Sigma, St. Louis, MO).

Enzyme-linked immunosorbent assay for SLIT3, IL-1β and IL-18

The levels of secreted SLIT3 from conditioned medium, cell lysates in vitro or peritoneal lavages were measured using a mouse SLIT3 ELISA kit (antibody-online Inc.; Atlanta, GA) in accordance with the manufacturer's instructions.

For IL-1β detection, its secreted levels were also determined by ELISA. ELISA plates were coated with 5 μg/ml human anti-IL-1β in PBS overnight at RT and then blocked for 1 h at room temperature with PBS containing 1% BSA. All supernatants or lysates from the tested cell cultures were incubated in triplicate wells for 3 h at room temperature. Biotinylated anti-mouse SLIT3 or IL-1β detection antibodies at a 0.5 μg/ml concentration were then added for 1 h at room temperature. Tetramethyl benzidine substrates were added for 30 min and the OD values were determined at 450 nm. The average absorbance of the blank wells was subtracted. The IL-18 levels were then detected using a commercially available ELISA kit (R&D Systems, Minneapolis, MN, USA).

Western blotting

For western blotting analysis, cells were lysed in RIPA buffer Thermo Scientific, Waltham, MA) in the presence of phosphatase and protease inhibitors. The resulting protein lysates were then resolved using SDS–PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. Each membrane was blocked in Tris buffered saline with Tween 20 (TBST) including 5% bovine serum albumin and incubated overnight at 4°C with the indicated primary antibodies against adaptor molecule apoptosis-associated speck-like protein containing a CARD (ASC) (Santa Cruz, CA), NLR family pyrin domain containing 3 (NLRP3) (Adipogen, San Diego, CA, USA), and β-actin (Sigma-Aldrich).

Gene silencing and transfection

A combination of selected sequences of small interfering RNA (siRNA) oligonucleotides, a SMARTpool siRNA against SLIT3, and a negative control non-specific siRNA were purchased from Thermo Scientific Dharmacon (Lafayette, CO). BMMs were transfected with siRNAs using the transfection reagent RNAiMAX (Invitrogen, Carlsbad, CA).

Reverse transcription–polymerase chain reaction (RT PCR) and quantitative PCR (qPCR) analysis

For RT-PCR and qPCR assays, total RNAs were first isolated from the selected tissues using Qiazol reagent (Qiagen, Germany), and reverse transcribed to cDNA using the RevertiAid First strand cDNA Synthesis kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). Quantitative real-time PCR was then performed using a LightCycler480 SYBR Green I Master (Roche, Penzberg, Germany) in accordance with the manufacturer's instructions. The sequences of the primers used were as follows: GAPDH (forward 5′ AGC CAC ATC GCT CAG ACA 3′ and reverse 5′ GCC CAA TAC GAC CAA ATC C 3), IL-1β (forward 5′ AAA TAC CTG TGG CCT TG 3′ and reverse 5′ TTA-GGA-AGA-CAC-GGA-TTC 3), Slit3 (forward 5′ -CTA-AAC-CAG-ACC-CTG-AAC-CTG-GTG-GTA-GA-3′ and reverse 5′ -AAG-GTA-GAG-GGG-GCT-GTT-GCT-GCC-CAC-T-3′), Ym1 (forward 5′ CAG-GTC-TGG-CAA-TTC-TTC-TGA-A 3′ and reverse 5′ GTC-TTG-CTC-ATG-TGT-GTA-AGT-GA 3). Primers for mouse iNOS, IL-12β, Arg1 and Fizz1 were used in this assay, and these sequences were retrieved from the PrimerBank database. The amplification cycles comprised a denaturation step for 30 s at 94°C, annealing for 30 s at 60–68°C, and extension for 45 s at 72°C.

Phagocytosis Assay

BMMs were cultured overnight at 37°C in α-MEM containing 10% FBS at a density of 2 x 10^5 cells/well in 6-well plates. If necessary, control or SLIT3 siRNAs were transfected. The cells were then further incubated for 24 h and assayed. Briefly, FITC-labeled E. coli bioparticles were added and all plates were incubated for 1 h at 37°C to allow their phagocytic uptake into the cells. The E. coli bioparticle suspension was then aspirated, and extracellular fluorescence was quenched with Trypan blue suspension. The excess Trypan blue was removed with three PBS washes, and the cells were detached from the

dish with PBS. The level of phagocytosis was assayed by measuring the fluorescence intensity of the bioparticles in the cell lysates.

Fluorescence-activated cell sorting (FACS)

For FACS analysis. BMMs or peritoneal cells were first washed with 4% FBS, 0.1% BSA and 0.01% NaN_3 at 4°C and then incubated with the following primary antibodies for 2 h (all purchased from Biolegend, San Diego, CA): CD4-PE, CD8-FITC, CD11b-Pacific blue, Ly6C-APC, CD206-PE-Cy7, MHCⅡ-APC-Cy7, Gr-1-Pacific Blue and F4/80-PE. The cells were then further washed and evaluated by flow cytometry using a FACStarPLUS analyzer (BD Biosciences, San Jose, CA). Data were expressed as the mean fluorescence intensity.

Statistical analysis

All quantitative results were obtained from least three independent experiments and the data were expressed as a mean with standard deviation. Statistical significance was determined by comparing the mean values of the groups using a paired two-tailed t-test. A P value of less than 0.05 was considered statistically significant. Values of $p<0.05$, $p<0.005$ and $p<0.0005$ are designated by *, **and ***, respectively.

Result

LPS induces SLIT3 expression in primary macrophages

It was demonstrated previously that SLIT3 regulates the differentiation of osteoclasts, which are cells of macrophage origin[27]. To elucidate in our current study whether SLIT3 is associated with the macrophage phenotype, the functionality of these cells, we first evaluated SLIT3 expression upon exposure to LPS from an inflammatory E. Coli pathogen that is known to stimulate immune responses in BMMs. This LPS treatment augmented the SLIT3 mRNA (Fig. 1A) and protein (Fig. 1B) expression levels in BMMs transfected with a control siRNA, as revealed by qPCR and enzyme-linked immunosorbent assay (ELISA), respectively. However, this effect was completely abolished by a SLIT3 knockdown using a SLT3-specific siRNA (Fig. 1A and 1B). These results indicated that LPS induces SLIT3 expression in macrophages.

SLIT3 augments the phagocytic capacity of macrophages *in vitro*.

To next determine whether LPS-stimulated SLIT3 activation is attributed to phagocytosis, we exposed BMMs transfected with control or SLIT3 siRNAs to the E. coli bioparticles in the presence or absence of LPS, and then compared the phagocytic capacity of these cells. We observed that LPS administration significantly enhanced the phagocytic activity in BMMs transfected with control siRNA but that this effect was completely abolished by the SLIT3 knockdown (Fig. 2A). Toll-like receptor 4 (TLR4) is one of the pathogen-recognition receptors activated by LPS and is thus linked to pro-inflammatory responses28). To confirm that the increased phagocytosis caused by LPS stimulation is dependent on TLR4, we utilized TLR4 deficient ($TLR4^{-/-}$) primary macrophages and compared their phagocytic potential with the wild type (WT) cells. Indeed, $TLR4^{-/-}$ BMMs showed no increase in phagocytic activity upon LPS stimulation (Fig. 2B).

We next examined whether recombinant SLIT3 protein (rSLIT3) augmented the phagocytosis index in BMMs and found that the protein itself increased phagocytic activity in these cells (Fig. 2C). To confirm this SLIT3-mediated induction of phagocytosis, we tested whether $SLIT3^{-/-}$ primary macrophages displayed a lower phagocytic activity level compared to WT macrophages. Indeed, the phagocytosis index in $SLIT3^{-/-}$ primary macrophages was significantly reduced compared to the WT macrophages (Fig. 2D). Taken together, these results indicate that SLIT3 enhances the phagocytic capacity of macrophages.

SLIT3 augments the phagocytic capacity of macrophages *in vivo*

To elucidate whether SLIT3 protein enhances the uptake of bacterial particles in peritoneal cells *in vivo*, $SLIT3^{-/-}$ mice was administered with buffer (PBS) or rSLIT3 (50ug/mouse) by intraperitoneal (IP) injection. After 1hr IP injection of rSLIT3, FITC-conjugated E. coli bioparticles (0.1mg/mouse) were injected to detect the phagocytosis and mice were euthanized 5 hr later. Importantly, rSLIT3 administration increased the uptake of FITC-conjugated *E. coli* bioparticles compared to FITC-conjugated *E. coli* bioparticles alone (Fig. 3A, Fig. 3B). To specify the cell subpopulation responsible for SLIT3-mediatd induction of bacterial particles *in vivo*, we determine the counts of neutrophils ($Gr\text{-}1^{+}$) or macrophages ($F4/80^{+}$) positive for FITC-conjugated *E. coli* bioparticles after injection of bacterial particle and/or rSLIT3. rSLIT3 treatment led to increase in the number of cells positive for FITC-conjugated *E. coli* bioparticles in both neutrophils or macrophages (Fig. 3C, Fig. 3D). Uncleared bacterial particles circulate throughout the multiple organs in mice. Therefore, we compared these circulating *E. coli* bioparticles in spleen and observed that SLIT3 treatment increases the clearance of the *E. coli* bioparticles (Fig. 3E). Taken together, SLIT3 may play an important role in the pathogen-induced phagocytic activity in neutrophils and macrophages.

A SLIT3 deficiency enhances M1 polarization and inflammasome activation under inflammation condition

We next examined whether SLIT3 modulates the M1 or M2 polarization of macrophages. Following the differentiation of macrophages into M1 (LPS/IFN-γ stimulation) or M2 (IL-4 stimulation) populations, the expression of M1 markers including iNOS, IL-1β, and IL-12β in SLIT3$^{-/-}$ mice macrophages were significantly increased when compared with those in WT mice [24), 25)] (Fig. 4A). By contrast, the expressions of M2 markers such as Fizz1, Ym1, and Arg1 were reduced in SLIT3$^{-/-}$ mice macrophages[24)] (Fig. 4A), indicating that SLIT3 may skew BMMs differentiation towards a less inflammatory form of macrophage. Given the observed SLIT3 deficiency-mediated increase in M1 polarization, we next evaluated the expression of M1-related proinflammatory cytokines such as IL-1β and IL-18 after LPS-primed WT or SLIT3$^{-/-}$ BMMs that were then stimulated with activators of the NLRP3 inflammasome such as ATP, monosodium urate (MSU), and nigericin. The SLIT3$^{-/-}$ significantly increased the secretion of IL-1β and IL-18 proteins, as determined by ELISA[32)] (Fig. 4B).). In addition, we analyzed the expression levels of inflammasome complex components, including NLRP3, ASC, and procaspase-1 protein, by immunoblotting. The expression of NLRP3 and ASC was found to be increased after LPS priming in SLIT3$^{-/-}$ mouse macrophages compared to WT (Fig. 4C). These results indicated that SLIT3 may induce NALP3 inflammasome-mediated IL-1β and IL-18 secretion.

A SLIT3 deficiency increases the M1 macrophage population *in vivo*.

To elucidate whether a SLIT3 deficiency has impacts on distinct subsets of immune cells, we analyzed the profiles of the CD4+ T cell, CD8+ T cell, Ly6C+ and CD11b+ populations in peritoneal lavage preparations after injection of the mice with LPS. Although the CD4+ and CD8+ T cell populations in the peritoneal lavage fluids were not significantly different between WT and SLIT3-/- mice, the macrophage population, presenting as CD11b^{high} Ly6C- cells, was

dramatically increased by the suppression of SLIT3 (Fig. 5A). We further analyzed these cells and found that M1 but not M2 population in SLIT3$^{-/-}$ mice is significantly increased compared to that of WT mice (Fig. 5B). We further observed that rSLIT3 administration blocked the LPS-mediated increase in the expression of IL-1β, an M1-related proinflammatory cytokine (Fig. 5C). Taken together, these findings imply that a SLIT3 dysfunction *in vivo* may impact the immune response through an increased M1 macrophage population.

Discussion

SLIT3 plays critical physiological roles in multiple organs including the kidneys, liver, spleen, bone, and embryogenesis[29),30)]. Recently reports are mainly suggested that the effect of SLIT3 contribute to tumor immune microenvironment, angiogenesis and bone modeling and remodeling system[31)]. In addition, SLIT3 has been found to regulate macrophage motility through Rac/Cdc42 GTPase activity, which mediates the functions of cytokines following LPS stimulation[13)]. Notably however, little has been reported to date on the association of SLIT3 with macrophage polarization. In our present study, we hypothesized that LPS stimulation of immune cells modulates SLIT3 and thereby has functional effects on phagocytosis.

We identified and characterized the interactions between SLIT3 and macrophages as we considered that this would yield important new biological insights into inflammation. We demonstrate from our current analyses that LPS-stimulated primary macrophages have upregulated SLIT3 expression (Fig. 1). Primary macrophages were found to be mediated by LPS stimulation. In addition, a knockdown of SLIT3 in mouse BMMs in vivo caused a clear suppression of phagocytosis. We further found that the LPS induction of SLIT3 in mouse macrophages was part of the host defense mechanism against bacteria such as *E. coli*.

We conducted in vitro experiments to confirm that the expression of SLIT3 in BMMs is regulated by LPS and that this mediates the phagocytic capacity of these cells, a key component of the innate immune response. This phenotype was validated in vivo, in which phagocytosis was confirmed to be SILT3 dependent by exposing mice to rSLIT3 and *E. coli* (Fig. 3). When phagocytic cells could not clear *E. Coli* particles, they circulated and accumulated in various mouse organs including the spleen. The maintenance of these particle levels in the spleen were also markedly reduced by treatment of the mice with rSLIT3 (Fig. 3E). These data suggest that SLIT3 promotes inflammation *in vivo*.

Increased levels of phagocytosis suggest an increase in the M1 population, i.e. in the levels of pro-inflammatory macrophages, which represents the initial defense mechanism during an inflammatory response. Over time, principally to avoid tissue damage, the macrophage polarization mechanisms increase the M2 population, which reduces the inflammatory response level and induces tissue regeneration[23-25]. We demonstrated from our current analyses however that M1 polarization, and hence the expression of pro-inflammatory cytokines, was increased and that of M2 was decreased in SLIT3$^{-/-}$ macrophages (Fig. 4A). In addition, the populations of CD4+ and CD8+ T cells did not differ significantly between SLIT3$^{-/-}$ and WT mice upon LPS injections in vivo, but the M1 population was clearly increased by the suppression of SLIT3 (Fig. 5). It can be concluded therefore that a SLIT3 deficiency inhibits the M1 to M2 transition. We further observed in the SLIT3$^{-/-}$ mice, where the M2 macrophage population is low, that M1-related cell death accumulates to above normal levels and contributes to post-apoptotic necrosis. It will be necessary in the future to study the impacts of a SLIT3$^{-/-}$ on inflammation activity in the mouse over the long term.

We additionally show from our present analyses that SLIT3 may control NLRP3 and ASC in primary BMMs (Fig. 4C). We confirmed in vivo that the secretion of IL-1ß increases following LPS exposure in SLIT3$^{-/-}$ mice and decreases to the control level when rSLIT3 is co-injected into these animals. Through these analyses, we have observed that when SLIT3 is deficient, the differentiation of macrophages into their M2 form is disrupted. Further research is necessary to evaluate whether SLIT3 affects the signaling pathways involved in the polarization to M2.

In summary, the LPS inflammation response induces SLIT3 upregulation which then potentially regulates phagocytosis and the polarization of macrophages. Excessive inflammation is induced by a loss of SLIT3 through the disrupted polarization of macrophages to a predominantly M1 form, which will likely lead to a chronic inflammatory state.

국문요약

Roundabout (ROBO) 수용체에 작용하는 Slit homolog 3 (SLIT3) 단백질은 신경 세포 유도, 혈관 괴사, 배아 발달, 뼈 리모델링 및 종양 미세 환경에서 중요한 역할을 담당하고 있다. 이러한 SLIT3는 대식세포에서 지질다당류 (LPS)에 의해 증가하는 것으로 보고되어 있으나 어떠한 역할을 담당하는지는 아직 알려져 있지 않다.

본 연구에서는 대식세포에 작용하는 SLIT3의 역할을 규명하기 위해 대식세포의 식균 작용과 표현형 조절에 관여하는지를 확인하였다. 골수 대식세포 (BMM)에서 LPS에 의해 SLIT3 발현이 증가하여 대식세포의 식균 작용을 억제하였다. SLIT3 유전자가 결손된 BMM에서는 식균 작용이 촉진되고 전염증성 사이토카인 (pro-inflammatory cytokines)의 발현은 증가하였으며, M1 대식세포군으로의 전환이 촉진됨을 관찰하였다. 이러한 현상은 NLRP3와 ASC 단백질의 발현 증가와 연관이 있음을 확인하였다. 또한 SLIT3 결핍 마우스에 LPS 투여한 결과, 복막에서 M1 대식세포군의 유입이 정상 마우스에 비해 유의하게 증가하는 것을 확인하였다.

본 연구 결과를 통해 SLIT3는 염증반응에 의해 유도되어 대식세포의 기능을 음성적으로 조절하는 중요 인자임을 확인할 수 있었다.

중심단어: SLIT3, 지질다당류, 염증, 식균 작용, 대식세포

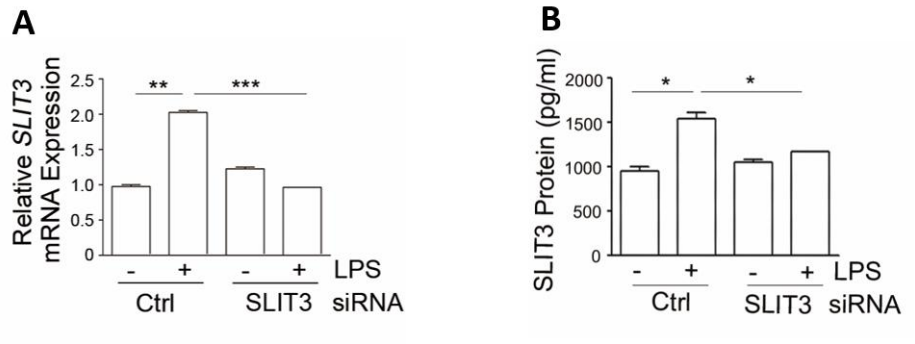

FIGURE 1. SLIT3 is increased in primary macrophages upon LPS stimulation

(A, B) The bone marrow-derived macrophages (BMMs) were transfected with control siRNA (siCtrl) or SLIT3 siRNA (siSLIT3). After transfection, these cells were incubated with receptor activator of nuclear factor kappa-B ligand (RANKL) (100 ng/ml) and LPS (10 ng/ml) for 24hrs and SLIT3 mRNA and protein expression levels were assessed by qPCR (A) and by ELISA (B), respectively. Data are means ± SD of three independent experiments; *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$. vs control.

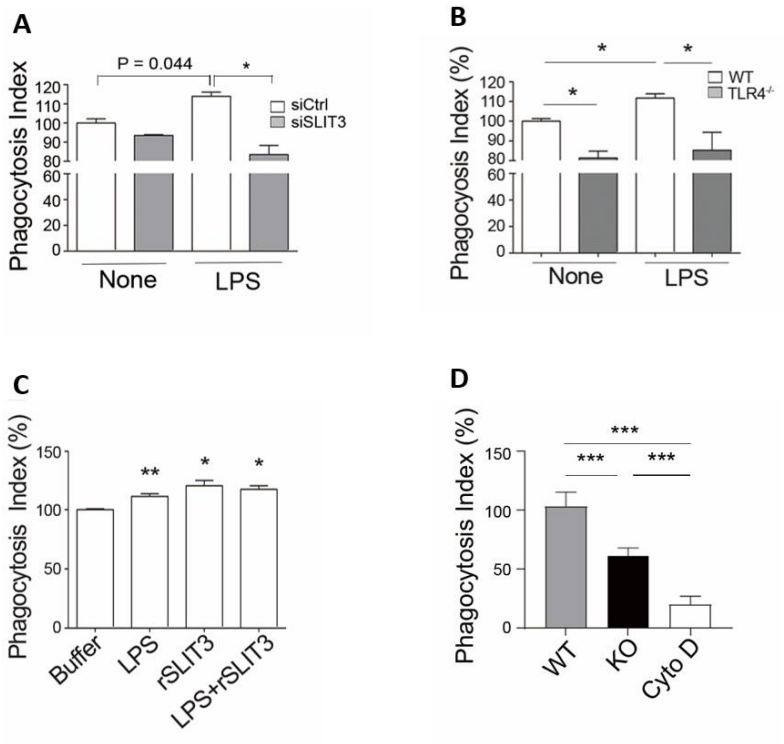

FIGURE 2. SLIT3 regulates phagocytosis activity of macrophages

(A) BMMs were transfected with siCtrl or siSLIT3 and then incubated with RANKL (100 ng/ml) and LPS (10 ng/ml) for 4 h. E. coli bioparticles were then added and the phagocytosis index was evaluated 40 mins later. (B) BMMs were isolated from wild type (WT) or TLR4 deficient ($TLR4^{-/-}$) mice and cultured with RANKL (100 ng/ml) and LPS (10 ng/ml) for 4 h. E. coli bioparticles were then added and the phagocytosis index was evaluated 40 mins later. (C) BMMs was pretreated with PBS, LPS (10 ng/ml), recombinant mouse SLIT3 (rSLIT3) protein (100 ng/ml), or a combination of both proteins, respectively, and the phagocytosis index was determined after the addition of E. coli bioparticles. (D) WT, $SLIT3^{-/-}$ (KO), and WT Cyto D-treated mouse macrophages were treated with RANKL (100 ng/ml) and LPS (10 ng/ml) for 4 h, followed by the addition of E. coli bioparticles. The phagocytosis index was then measured by flow cytometry 40 mins later. The data shown are means ± SD of three independent experiments; *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$. vs control.

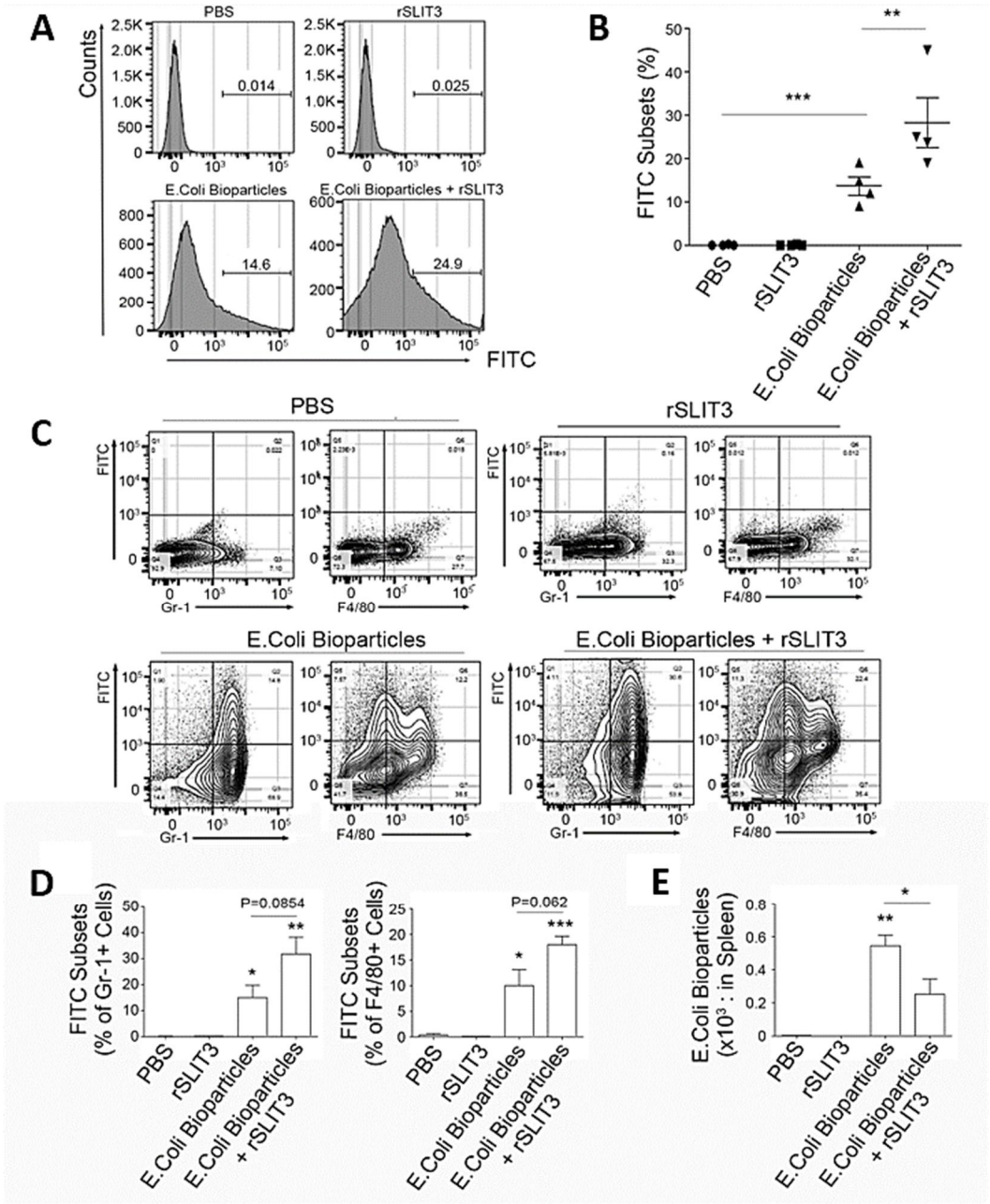

FIGURE 3. Phagocytic capacity of macrophage is upregulated by exogenous SLIT3 *in vivo*.

(A-B) PBS, rSLIT3 (50 μg/mouse), or FITC fluorescent *E. coli* bioparticles (0.1 mg/mouse) were administered to SLIT3$^{-/-}$ mice by intraperitoneal injection. The phagocytic capacity of the mouse macrophages from a peritoneal lavage was

assessed by flow cytometry (A) and the percentage of FITC-positive cells was determined (B). (C, D) The *E. coli* bioparticle counts in Gr-1+- neutrophil or F4/80+- macrophages (C) and the percentage of FITC-positive cells (D) were determined by flow cytometry. (E) The numbers of bacterial bioparticle-positive cells in the mouse spleen were counted by flow cytometry. All groups, n=4. The data shown are the means ± SD of three independent experiments; *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$. vs control.

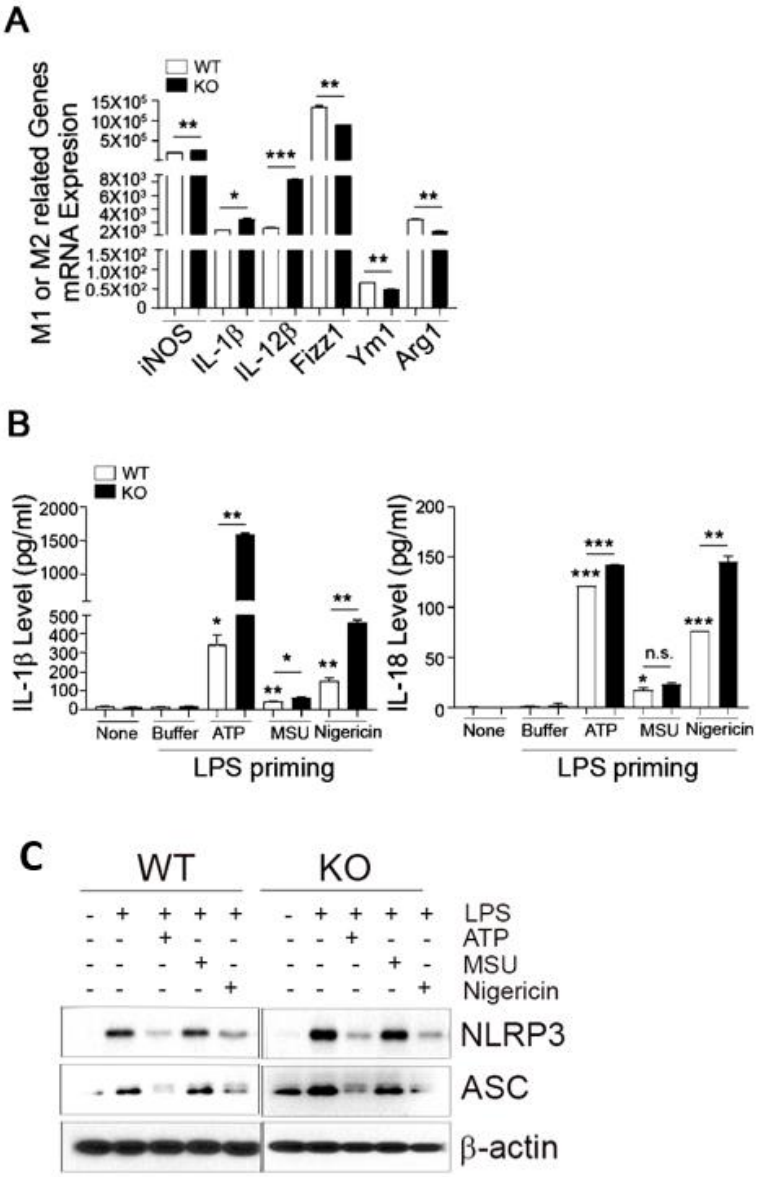

FIGURE 4. SLIT3 attenuates M2 polarization *in vivo* under inflammation conditions

(A) Macrophages isolated from WT and SLIT3$^{-/-}$ mice (KO) were cultured with IFN-γ (25 ng/ml) and LPS (100 ng/ml) (for M1 polarization) or IL-4 (10ng/ml) (for M2 polarization). After 24 h, the mRNA expression levels of iNOS, IL-1β, IL-12β, Fizz1, YM1 and Arg1 were assessed by qPCR. (B) Macrophages from WT and SLIT3$^{-/-}$ mice (KO) were untreated, treated with buffer or LPS (10 ng/ml) for 4 h, LPS for 4 h and ATP (2.5 mM) for 40 mins, LPS for 4 h and MSU (100 μM) for 1 h, or LPS for 4 h and nigericin (5 μM) for 1 h. The secreted IL-1β

and IL-18 protein levels were then measured by ELISA. (C) The protein levels of NLRP3, ASC, and β-actin were evaluated in the indicated mouse macrophages by western blotting. The data shown are the means ± SD of three independent experiments; *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$. vs control.

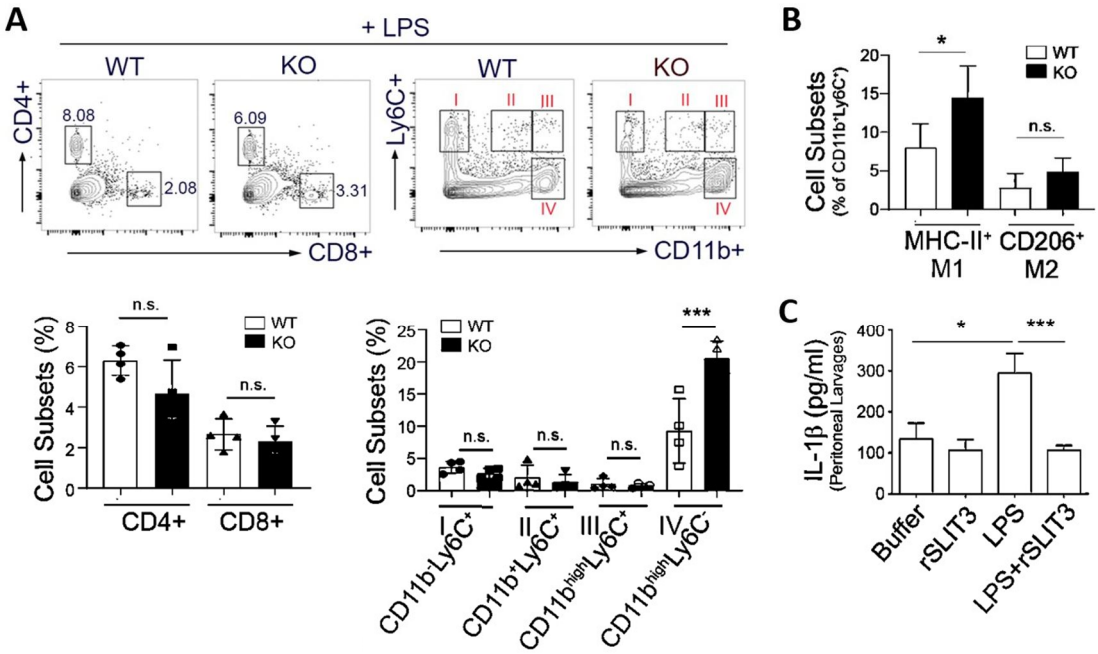

FIGURE 5. Effects of SLIT3 on immune cells population *in vivo*.

(A-B) LPS was administered via the intraperitoneal injection of SLIT3$^{-/-}$ (KO) and wild type (WT) mice. After 6 h, the mice were sacrificed and the peritoneal cells were harvested and analyzed for CD4-, CD8-, CD11b-, and Ly6C-positive cells (A) and MHC-Ⅱ- and CD206-positive cells (B) by flow cytometry. (C) The IL-1β protein levels in peritoneal lavages from the mice were assessed by EILSA. The data shown are the means ± SD of three independent experiments; *p < 0.05, **p < 0.005, ***p < 0.001. vs control.

References

1. Tong, Mingfu, et al. The role of the Slit/Robo signaling pathway. Journal of Cancer 10.12 (2019): 2694.
2. Battye, Robin, Adrienne Stevens, and J. Roger Jacobs. Axon repulsion from the midline of the Drosophila CNS requires slit function. *Development* 126.11 (1999): 2475-2481.
3. Brose, Katja, and Marc Tessier-Lavigne. Slit proteins: key regulators of axon guidance, axonal branching, and cell migration. Current opinion in neurobiology 10.1 (2000): 95-102.
4. Ng, Lui, et al. Suppression of Slit3 induces tumor proliferation and chemoresistance in hepatocellular carcinoma through activation of GSK3β/β-catenin pathway. BMC cancer 18.1 (2018): 621.
5. Kuriyama, Sei, and Roberto Mayor. Molecular analysis of neural crest migration. Philosophical Transactions of the Royal Society B: Biological Sciences 363.1495 (2008): 1349-1362.
6. Geutskens, Sacha B., Peter L. Hordijk, and Paula B. van Hennik. The chemorepellent Slit3 promotes monocyte migration. *The Journal of Immunology* 185.12 (2010): 7691-7698.
7. Zhang, Bing, et al. Repulsive axon guidance molecule Slit3 is a novel angiogenic factor. *Blood* 114.19 (2009): 4300-4309.
8. Borrell, Víctor, et al. Slit/Robo signaling modulates the proliferation of central nervous system progenitors. Neuron 76.2 (2012): 338-352.
9. Dickinson, Rachel E., and W. Colin Duncan. The SLIT–ROBO pathway: a regulator of cell function with implications for the eproductive system. Reproduction 139.4 (2010): 697-704.
10. London, Nyall R., et al. Targeting Robo4-dependent Slit signaling to

survive the cytokine storm in sepsis and influenza. Science translational medicine 2.23 (2010): 23ra19-23ra19.

11. Guan, Hongbing, et al. Neuronal repellent Slit2 inhibits dendritic cell migration and the development of immune responses. *The Journal of Immunology* 171.12 (2003): 6519-6526.

12. Kim, Beom-Jun, and Jung-Min Koh. Coupling factors involved in preserving bone balance. Cellular and Molecular Life Sciences 76.7 (2019): 1243-1253.

13. Toshihiko, et al. Slit3 regulates cell motility through Rac/Cdc42 activation in lipopolysaccharide-stimulated macrophages. FEBS letters 581.5 (2007): 1022-1026.

14. Geutskens, Sacha B., Peter L. Hordijk, and Paula B. van Hennik. The chemorepellent Slit3 promotes monocyte migration. The Journal of Immunology 185.12 (2010): 7691-7698.

15. Medzhitov, Ruslan. Approaching the asymptote: 20 years later. Immunity 30.6 (2009): 766-775.

16. Underhill, David M., and Helen S. Goodridge. Information processing during phagocytosis. Nature Reviews Immunology 12.7 (2012): 492-502.

17. Liu, Yu, et al. TLR2 and TLR4 in autoimmune diseases: a comprehensive review. Clinical reviews in allergy & immunology 47.2 (2014): 136-147.

18. O'Neill, Luke AJ, Clare E. Bryant, and Sarah L. Doyle. Therapeutic targeting of Toll-like receptors for infectious and inflammatory diseases and cancer. Pharmacological reviews 61.2 (2009): 177-197.

19. Newton, Kim, and Vishva M. Dixit. Signaling in innate immunity and inflammation. Cold Spring Harbor perspectives in biology 4.3 (2012): a006049.

20. Wynn, Thomas A., Ajay Chawla, and Jeffrey W. Pollard. Macrophage biology in development, homeostasis and disease. Nature 496.7446

(2013): 445-455.

21. Gordon, Siamon, and Annette Plűddemann. Tissue macrophage heterogeneity: issues and prospects. Seminars in immunopathology. Vol. 35. No. 5. Springer Berlin Heidelberg, 2013.

22. Rogers, Natasha M., et al. Dendritic cells and macrophages in the kidney: a spectrum of good and evil. Nature Reviews Nephrology 10.11 (2014): 625.

23. Murray, Peter J. Macrophage polarization. Annual review of physiology 79 (2017): 541-566.

24. Nathan, Carl F., et al. Identification of interferon-gamma as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. The Journal of experimental medicine 158.3 (1983): 670-689.

25. Wang, Youhan, et al. M1 and M2 macrophage polarization and potentially therapeutic naturally occurring compounds. International immunopharmacology 70 (2019): 459-466.

26. Gordon, Siamon, and Fernando O. Martinez. Alternative activation of macrophages: mechanism and functions. Immunity 32.5 (2010): 593-604.

27. Kim, Beom-Jun, et al. Osteoclast-secreted SLIT3 coordinates bone resorption and formation. The Journal of clinical investigation 128.4 (2018): 1429-1441.

28. Hagar, Jon A., et al. Cytoplasmic LPS activates caspase-11: implications in TLR4-independent endotoxic shock. Science 341.6151 (2013): 1250-1253.

29. Little, M. H. et al. Dual trafficking of Slit3 to mitochondria and cell surface demonstrates novel localization for Slit protein. Am J Physiol Cell Physiol 281, (2001): C486-495.

30. Liu, J. et al. Congenital diaphragmatic hernia, kidney agenesis and

cardiac defects associated with Slit3-deficiency in mice. Mech Dev 120, (2003): 1059-1070.

31. Gara, R. K. et al. Slit/Robo pathway: a promising therapeutic target for cancer. Drug Discov Today 20, (2015): 156-164.

32. H. Guo, J.B. Callaway, J.P. Ting. Inflammasomes: mechanism of action, role in disease, and therapeutics. Nat Med, 21 (2015): 677-687.