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Preliminary report

NecroX-5 ameliorates inflammation by skewing macrophages to the M2 phenotype

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ABSTRACT

This study aimed to evaluate the role of NecroX-5, a powerful anti-inflammatory agent, on the functional plasticity of macrophages and the possible underlying mechanism using RAW264.7 cells, thioglycollate-elicited peritoneal macrophages from C57BL/6 mice, and a murine model of dextran sodium sulfate (DSS)-induced colitis. The change in cell morphology was examined by scanning electron microscopy. The expression of CD206, arginase (Arg)-1, and inducible nitric oxide synthase (iNOS) were examined by western blotting. The production of inflammatory cytokines was detected by enzyme-linked immunosorbent assays and statistical comparisons were made. The results showed that treatment of RAW264.7 cells with NecroX-5 caused an elongated shape in comparison to non-treated cells. The expression levels of macrophage mannose receptor CD206 and Arg-1, specific markers of M2 cells, were significantly upregulated by NecroX-5 treatment, while those of iNOS (M1 macrophages) was decreased. In addition, NecroX-5 significantly reduced the secretion of inflammatory cytokines, while interleukin (IL)-4 and IL-13 secretion in the supernatant was significantly enhanced. Treatment with NecroX-5 considerably ameliorated the progression of DSS-induced colitis and significantly inhibited the mRNA expression of pro-inflammatory cytokines, including tumor necrosis factor-α and IL-1β. Taken together, our findings demonstrated that NecroX-5 might dampen inflammation by switching the M1 phenotype to the M2 phenotype due to IL-4 and IL-13 induction.

1. Introduction

Macrophages are functionally plastic cells that could polarize toward a spectrum of phenotypes depending on the local tissue microenvironment [1,2]. Polarized macrophages can display two functionally opposite forms including classically activated or pro-inflammatory macrophages (M1), and alternatively activated or anti-inflammatory (M2) phenotypes [3,4]. M1 macrophages can be activated by tumor necrosis factor (TNF)-α or toll-like receptor ligands including lipopolysaccharides (LPS) and/or interferon (IFN)-γ. Such M1-polarized macrophages display the capacity to trigger Th1 and Th17 reactions by secreting excess levels of reactive oxygen, pro-inflammatory cytokines, and nitrogen species [5]. In contrast, wound healing M2 macrophages can be alternatively activated in response to interleukin (IL)-10, IL-4, IL-13, immune complexes, as well as apoptotic cells [6], and can be characterized by several markers such as IL-10, CD206, CD163, and CD209. Their primary function is to promote the Th2 response, and exhibit anti-inflammatory properties by upregulating the immunosuppressive cytokine IL-10, CD206 antigen, and arginase 1 (Arg-1) [1,7]. In addition, the M2 macrophages participate in wound healing through matrix production by endogenous fibroblasts. Therefore, it has been hypothesized that editing macrophage activation to attenuate the inflammatory M1 phenotype and induce the pro-healing M2 phenotype is a power strategy to alleviate inflammatory responses.

Cyclopentylamino carboxymethylthiazolylindole (NecroX; Fig. 1) compounds have been recently found as inhibitors of necrosis, which accumulate mostly in the mitochondria [8]. NecroX-5 ($C_{25}H_{31}N_{3O}3S{\cdot}2CH_4O_3S$) with a molecular weight of 453.61 is a derivative of the NecroX series of compounds. Previous studies have highlighted its pharmacological activities against various injury factors, such as neomycin, nitroprusside, oxidative stress, and gentamicin [9,10]. The purpose of this study was to examine the effects of NecroX-5 on macrophage polarization and the underlying regulatory mechanism in the RAW264.7 macrophage cell line, thioglycollate (TG)-elicited peritoneal macrophages from C57BL/6 mice, and a murine model of dextran sodium sulfate (DSS)-induced colitis.

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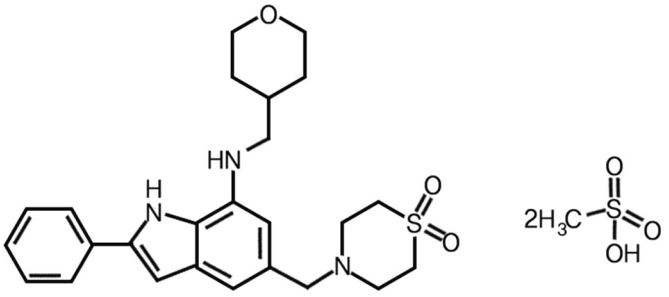

Fig. 1. Chemical structure of NecroX-5.

2. Materials and methods

2.1. Cell culture and treatment

RAW 264.7 cells were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA) and were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco, Carlsbad, CA) in a 37 °C humidified incubator with 5% CO_2. Cells were treated with 5, 10, or 20 μM of NecroX-5 (LG Life Sciences, Daejeon, Korea) for 1 h prior to stimulation with 1 μg/ml of LPS and incubated at 37 °C for 24 h. For the inhibition assay, NecroX-5 was treated with 3 μg/ml of anti-IL-4 and/or anti-IL-13 together for 1 h prior to stimulation with LPS.

2.2. Animal colitis model and NecroX-5 treatment

All animal experimental procedures were approved by the Animal Care and Use Committee of the Seoul National University Bundang Hospital (permit number: BA1809-256/077-01). Male C57BL/6 mice about 21–30 g used in this study were maintained under pathogen-free conditions, 21 ± 2 °C, controlled humidity of 50 ± 15%, and 12:12 light-dark cycle. During the 7 days of experimental period, individual body weights were evaluated daily over an initial condition. During experiments, mice were allowed access to autoclaved tap water and food for libitum and drinking. Animals were randomly allocated into the following 4 groups with 5 each; negative control, DSS, DSS + NecroX-5 (10 mg/kg), and DSS + dexamethasone (Dexa; 1.5 mg/kg). The DSS, DSS + NecroX-5, and DSS + Dexa groups were received 4% DSS (MP biomedicals, Solon, OH, USA) dissolved in drinking water for 12 days ad libitum in drinking water starting from day 0. Control mice received water ad libitum, NecroX-5 group was dissolved in distilled water and administered to the DSS + NecroX-5 group using oral gavage at concentration 10 mg/kg of mouse per day from day 3 through for 10 days [11,12].

2.3. Peritoneal macrophages culture

Peritoneal macrophages were isolated after an intra-peritoneally injection of 2.5 ml 4% thioglycolate, as mentioned previously [13]. Peritoneal lavage was washed using 8 m of DMEM. The cells were divided into 24-well tissue culture plates (3×10^5 cells/well) in DMEM together with 10% heat-inactivated FBS. Incubation of cells is done for 3 h at 37 °C in a 5% CO_2 atmosphere, washed 3 times with DMEM to eliminate non-adherent cells, and balanced with DMEM containing 10% FBS.

2.4. Electron microscopy

We imaged the morphology of the RAW 264.7 cells with scanning electron microscopy (FE SEM II; S-4300SE Hitach). Fixation was performed with 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer for 20 min. After washing three times with PBS, dehydration was carried out by immersing in 70%, 80%, 90%, and 100% ethanol sequentially for 5 min, dried in hexamethyldisilazane, and was sputter-coated with platinum. Samples were observed with the JEOL 5200 SEM.

2.5. Immunofluorescence staining

For immunofluorescence staining, cells were washed with PBS (pH 7.4) three times for 5 min each. Next, the slide was treated with blocking solution (0.2% Triton X-100, 1% BSA in PBS) for 1 h to block nonspecific antigen binding. The slide was then incubated overnight with diluted primary antibodies. The next day, after washing 3 times with PBS, the plate was incubated at room temperature for 1 h with secondary antibodies diluted 1:2000. Then, the slide was washed thoroughly with PBS and finally stained with DAPI (VECTASHIELD, Vector Laboratories, USA) to stain cell nuclei. It was then photographed using a z-stack with a confocal microscope.

2.6. Western blot analysis

After 24 h of LPS and NecroX-5 treatment, RAW 264.7 cells were detached with cold phosphate-buffered saline (PBS) and homogenized on ice using cell lysis buffer (Cell Signaling Technology, Danvers, MA, USA). Samples were heated at 95 °C for 5 min and briefly cooled on ice, and 30 μg of protein were loaded onto 10% SDS-PAGE polyacrylamide gels. After gel electrophoresis, gels were transferred to nitrocellulose membranes (GE Healthcare, Piscataway, NJ, USA). Membranes were blocked with 5% BSA in PBS for 2 h at room temperature and incubated with primary antibodies against iNOS (Abcam, Cambridge, UK), Arg-1 and GAPDH from Santa Cruz Biotechnology (Santa. Cruz, CA, USA) 4 °C overnight. GAPDH was used as control. Following four times washing with PBS-T (pH 7.4), membranes were incubated with HRP-conjugated anti-mouse or anti-rabbit IgG secondary antibodies (Santa Cruz Biotechnology, Santa. Cruz, CA, USA) at a 1:2000 dilution for 2 h at room temperature. Next, membranes were washed with PBS-T four times. A western blotting detection kit (EZ-Western Lumi pico, Dogen, Korea) was used for protein detection. Finally, quantify the proteins on the blot, a densitometric analysis of the blot was performed on Image J (National Institutes of Health, USA), and the relative quantities were calculated by normalizing to levels of GAPDH. These experiments were performed in duplicate.

2.7. Reverse transcription-polymerase chain reaction (RT-PCR)

RNA from the RAW 264.7 cells was extracted according to the manufacturer's instructions for easy-BLUE RNA extraction kit (iNtRON Biotechnology, Kyunggi-do, Korea). RNA was quantified with a spectrometer (Nanodrop 1000, Wilmington, DE). Starting from 2 μg of RNA, 20 μl of cDNA was synthesized using AccuPower® RT PreMix (Bioneeer Corporation, Daejeon, Republic of Korea) according to the manufacturer's instructions. Primers are shown in Table 1. The annealing temperature was 62 °C for mouse IL-6 and GAPDH, 60 °C for mouse TNF-α, and 50 °C for mouse IL-1β, respectively, Products were electrophoresed on a 2% agarose gel and visualized by staining with ethidium bromide.

2.8. Quantitative real-time PCR analysis

Quantitative real-time PCR was performed using a SYBR Green master Mix and the detection of mRNA was analyzed using an ABI StepOne Real-time PCR System (Applied Biosystems, foster City, CA, USA). Primer sequences for the reference gene GAPDH and the genes of interest are shown in Table 1. Typical profile times were the initial step, 95 °C for 10 min followed by a second step at 95 °C for 15 s and 60 °C for 30 s for 40 cycles with a melting curve analysis. The level of target mRNA was normalized to the level of the GAPDH and compared with the control. Data were analyzed using the ΔΔCT method.

Table 1
List of primers used for the real-time PCR and RT-PCR.

Real-Time PCR primer sequence (5′-3′)	
Primers	Sequence
TNF-α forward	5′- CAG ACC CTC ACA CTC AGA TCA TCT -3′
TNF-α reverse	5′- CCT CCA CTT GGT GGT TTG CTA -3′
IL-1β forward	5′- GGA CAG AAT ATC AAC CAA CAA GTG ATA -3′
IL-1β reverse	5′- GTG TGC CCGTCTT TCA TTA CAC AG -3′
IL-6 forward	5′- CCA GAA ACC GCT ATG AAG TTC CT -3′
IL-6 reverse	5′- CAC CAG CAT CAG TCC CAA GA -3′
GAPDH forward	5′- GGC AAA TTC AAC GGC ACA -3′
GAPDH reverse	5′- GTT AGT GGG GTC TCG CTC CTG -3′

RT-PCR primer sequence (5′-3′)	
Primers	Sequence
TNF-α forward	5′- ATG AGA ACA GAA AGC ATG ATC -3′
TNF-α reverse	5′- TAC AGG CTT GTC ACT CGA ATT -3′
IL-1β forward	5′- AGG CCA CAG GTA TTT TGT CG -3′
IL-1β reverse	5′- GCC CAT CCT CTG TGA CTC AT -3′
IL-6 forward	5′- CGG GAT CCA TGT TCC CTA CTT CAC AA -3′
IL-6 reverse	5′- CCC AAG CTT CTA CGT TTG CCG ATG AGA -3′
GAPDH forward	5′- GGC ATG GAC TGT GGT CAT GA -3′
GAPDH reverse	5′- TTC ACC ACC ATG GAG AAG GC -3′

2.9. Enzyme-linked immunosorbent assay (ELISA)

An ELISA was used to quantify the levels of IL-13 and IL-4 in RAW 264.7 cells culture supernatants. The media were collected for the assay after the cells were treated for 24 h with NecroX-5 (20 μM), LPS (1 μg/ml), or combinations of NecroX-5 (5, 10, 20 μM) and LPS (1 μg/ml). The captured antibody was diluted with PBS and used to coat 96 well plates at room temperature for 24 h. The plates were then washed twice with PBS and blocked with 10% FBS with PBS for 2 h. After the sample was added, the reaction was carried out at room temperature for 2 h. After the second antibody treatment, the substrate reagent was reacted and read at 405 nm wavelength in an ELISA reader (EPOCH2, BioTek).

2.10. MTT assay

After treating the RAW 264.7 cells with the drug for 24 h, the medium was carefully removed and the cells were washed once with PBS. MTT at a final concentration of 0.5 mg/mL in DMEM medium was added to each well, and then the cells were incubated at 37 °C for 4 h. The MTT solution was then carefully removed. Finally, formazan crystals were dissolved in DMSO and the absorbance was read at 560 nm in a microplate reader (EPOCH2, BioTek).

2.11. Histological examination

In PBS containing 10% neutral-buffered formalin, colon tissue

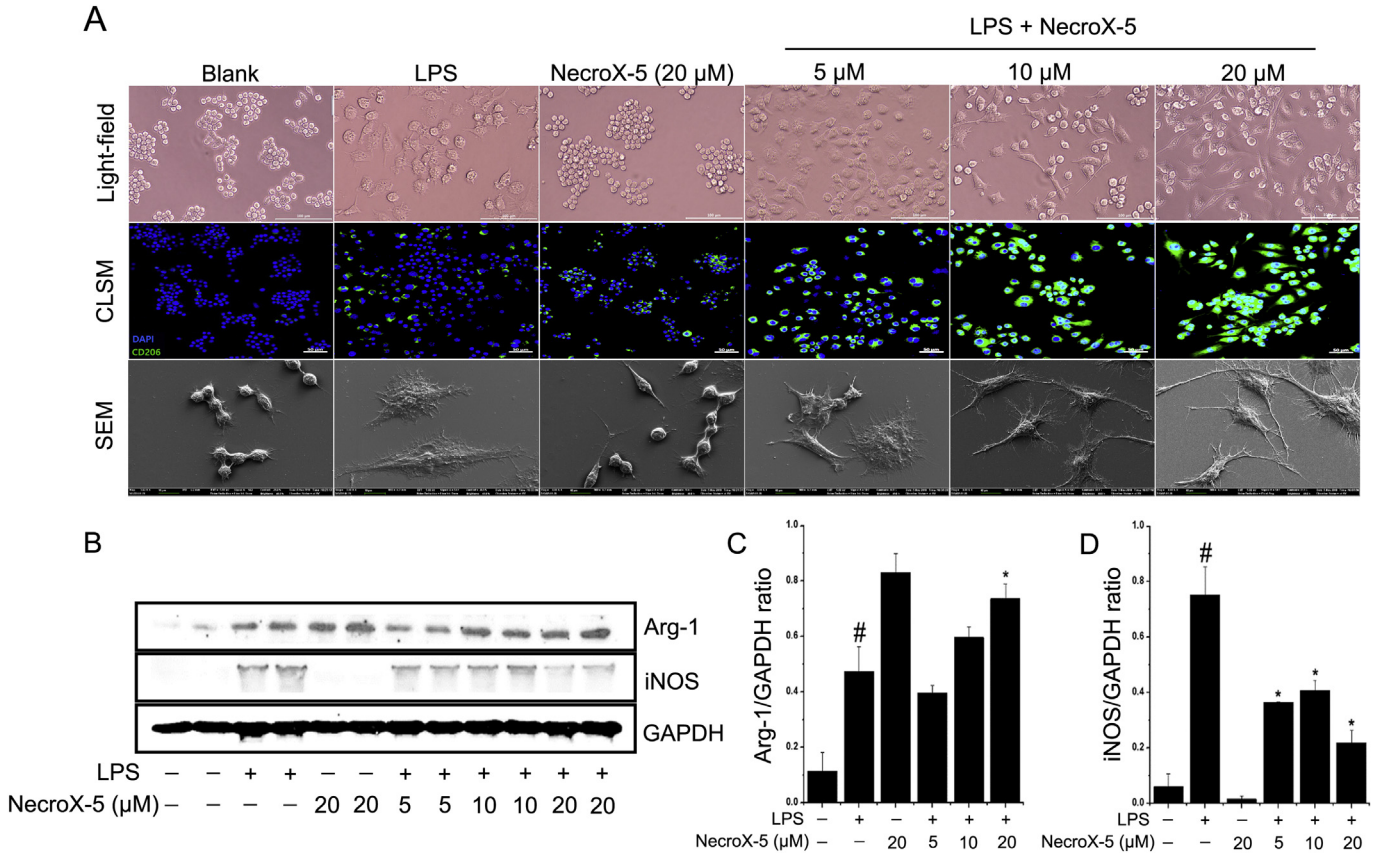

Fig. 2. Effect of NecroX-5 on the modulation of macrophage phonotype. RAW264.7 cells (1×10^5) were treated with NecroX-5 (5, 10, and 20 μM) for 1 h and then stimulated with LPS for 24 h. (A) Phase contrast images of RAW264.7 cells left untreated or treated with LPS or NecroX-5 (5, 10, and 20 μM). (Scale bar: 50 μm), fluorescence images of cells immunostained for CD206 (green) and hoechst nuclear counterstain (blue) of control, LPS-treated, and NecroX-5 (5, 10, and 20 μM)-treated cells (Scale bar: 50 μm), and SEM images of RAW264.7 cells left untreated or treated with LPS or NecroX-5 (5, 10, and 20 μM) (B) Representative immunoblots of iNOS (M1 marker) and Arg-1 (M2 marker) in RAW264.7 cells were treated with NecroX-5 at the different doses for 24 h. (C) iNOS and (D) Arg-1 expression relative to the GAPDH level. Each datum represents the ± S.E.M. of three independent experiments. $^{*}P < 0.05$; significantly different from the unstimulated cells. $^{\#}P < 0.05$; significantly different from the LPS-stimulated cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

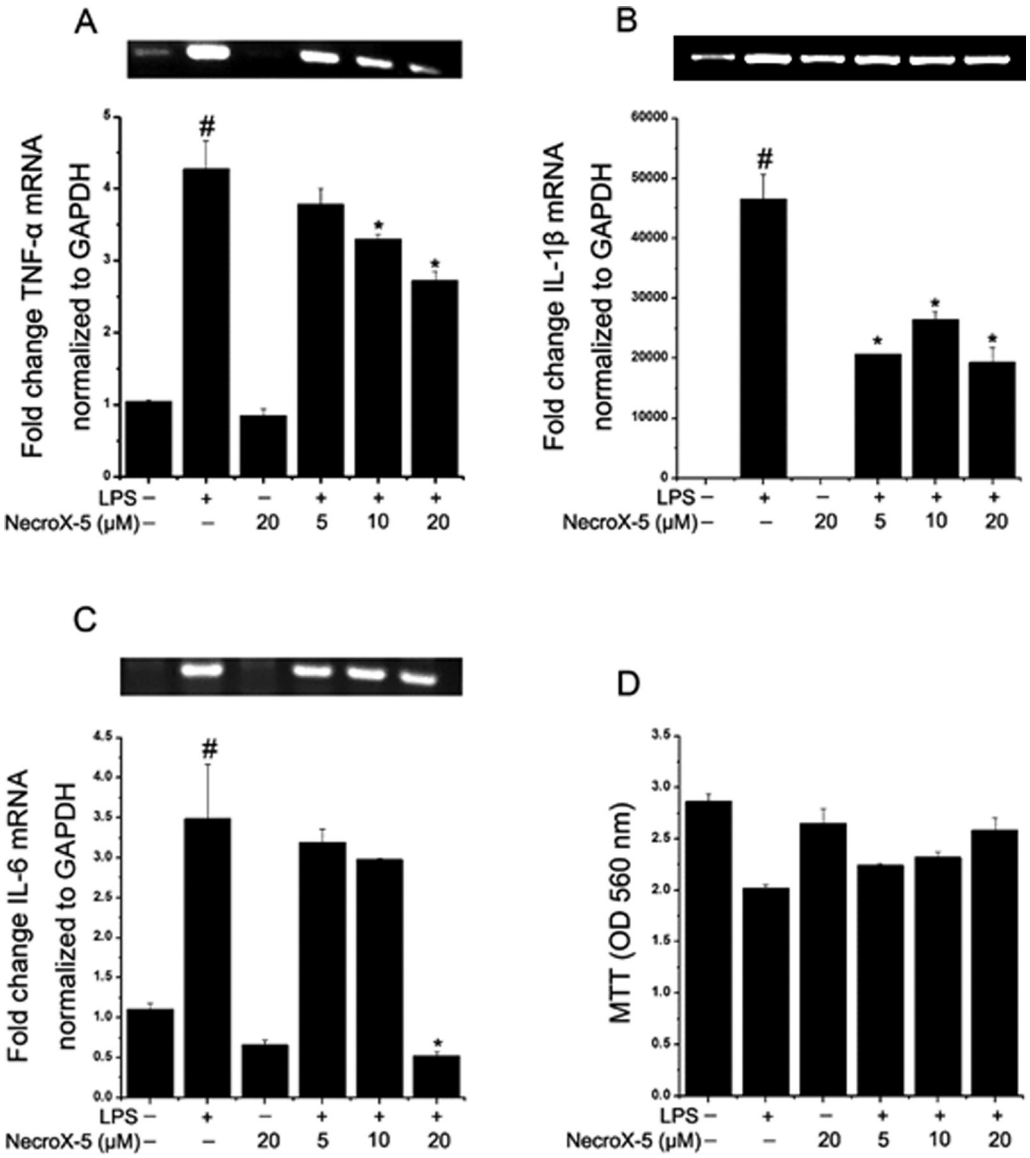

Fig. 3. NecroX-5 inhibited levels of mRNA for proinflammatory cytokines. RAW264.7 cells (3×10^6) were treated with NecroX-5 (5, 10, and 20 μM) for 1 h and then stimulated by LPS for 5 h. (A-C) TNF-α, IL-1β, and IL-6 mRNA expression levels were analyzed by real-time PCR (lower panel) and RT-PCR (upper panel). (D) MTT assay. Each datum represents the ± S.E.M. of three independent experiments. $^{*}P < 0.05$; significantly different from the unstimulated cells. $^{\#}P < 0.05$; significantly different from the LPS-stimulated cells.

samples were immediately fixed for 24 h and were embedded in paraffin. Sections (5 μm) of colon tissue samples were stained with hematoxylin and eosin. Histology scoring of hematoxylin and eosin (H&E)-stained sections were randomly analyzed in a blinded fashion.

2.12. Statistical analysis

Data are presented as means ± standard error of the mean (SEM). One-way ANOVA for multigroup comparisons after Tukey's test and independent *t*-test was used. Power analysis was applied to determine the difference of the control and the treatment groups. $P < 0.05$ was considered significant.

3. Results

3.1. Effect of NecroX-5 on the modulation of macrophage phonotype

The M1/M2 phenotypes in vitro display changes in cell morphologies [14]. M1 macrophages exhibited a round, pancake-like shape, whereas M2 macrophages exhibited an elongated shape [14]. We hypothesized that NecroX-5 might be attributable to changes in macrophage phenotypes via transforming LPS-induced M1-like toward M2-like phenotypes. To test our hypothesis, we first determined the morphology of macrophages in LPS-triggered RAW264.7 cells after incubation with or without NecroX-5. Our results showed that macrophages stimulated in culture with LPS were spread out and adhered to a flat, round, and pancake shape on dish surfaces as can be seen in Fig. 2A. By contrast, the addition of NecroX-5 markedly led to cellular elongation compared with either cells stimulated with LPS alone or unstimulated cells (Fig. 2A). Subsequently, to analyze the morphological changes in greater detail, we performed a scanning electron microscopy assessment. LPS-triggered RAW264.7 cells displayed flatten, round, and pancake-like morphology, while cells treated with NecroX-5 showed more elongation compared with unstimulated cells or M1 cells (Fig. 2A). Unstimulated cells retained their native round morphology.

CD206 is a widely accepted marker for M2 [15]. To confirm the macrophage polarization state, the expression of CD206 was also studied immunofluorescence staining. Intriguingly, the

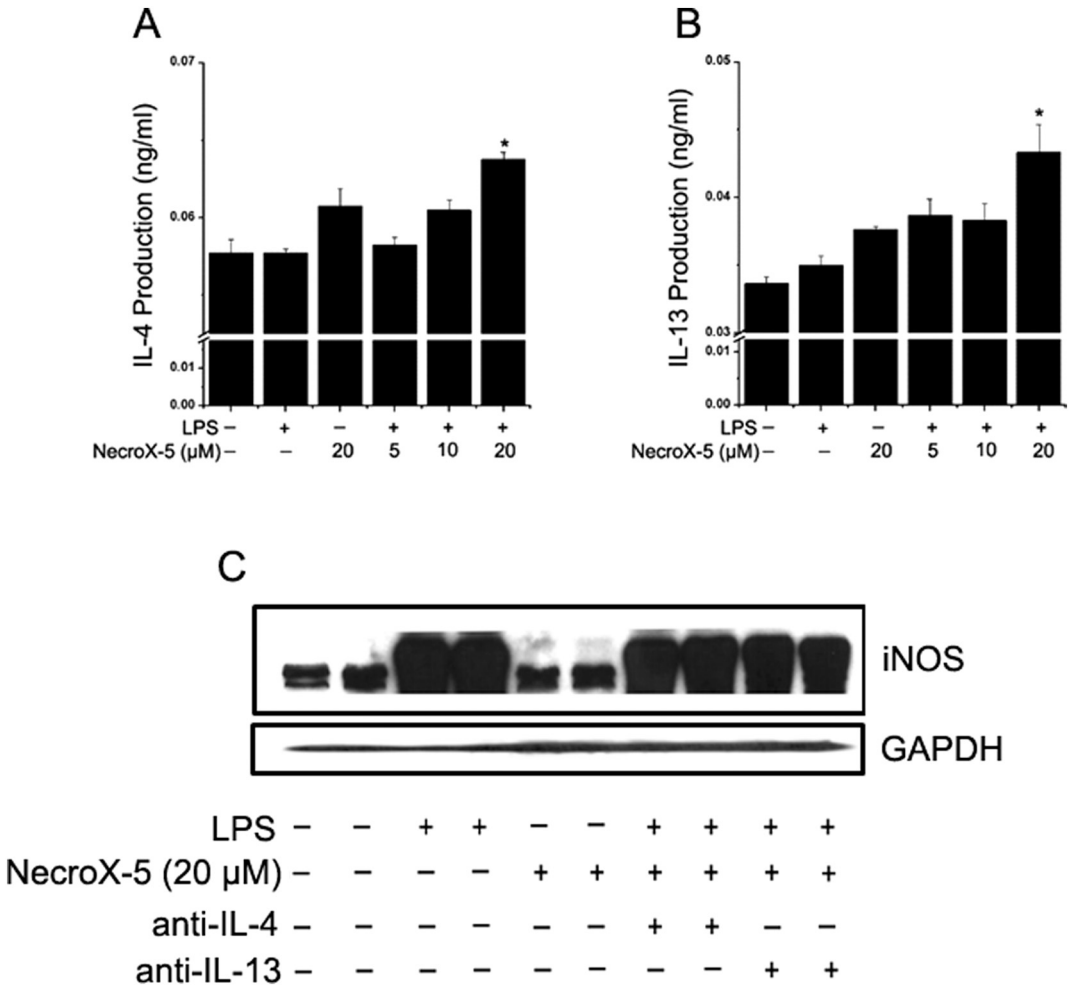

Fig. 4. Effect of NecroX-5 on the production of IL-4 and IL-13. (A) ELISA for IL-4 and IL-13 in supernatant of RAW264.7 cells were treated with the indicated concentrations of NecroX-5 for 1 h and then treated with LPS for 24 h. (B) Representative immunoblots of iNOS protein in RAW264.7 cells treated by 20 μM NecroX-5 with or without IL-4 and/or IL-13 neutralizing antibody for 24 h. (C) The phosphorylated STAT6 was determined by Western blot analysis. $^{\#}P < 0.05$; significantly different from unstimulated cells. $^{*}P < 0.05$; significantly different from LPS-stimulated cells.

immunofluorescence labeling study revealed a sharp and clear detection of CD206 along the surface of the cells after being cultured with NecroX-5, with modest expression of CD206 after LPS treatment (Fig. 2A).

The expression of iNOS is typically used as a marker for the M1 phenotype, while Arg-1 is mainly expressed in M2 macrophages. LPS treatment enhanced the expression of iNOS to some extent, but decreased Arg-1 protein expression in RAW264.7 cells. On the other hand, NecroX-5 significantly suppressed LPS-stimulated iNOS protein levels and elevated Arg-1 protein levels in a dose-dependent manner in comparison to the LPS group (Fig. 2B-2D). Overall these results indicated that NecroX-5 was able to shifts cells from the M1 to M2 phenotype.

3.2. NecroX-5 inhibited levels of mRNA for proinflammatory cytokines

To determine the anti-inflammatory effect of NecroX-5 by ameliorating proinflammatory M1 macrophages, the mRNA expression of IL-6, IL-1β, and TNF-α were assessed with quantitative RT-PCR. Results showed that the mRNA expression of IL-6, IL-1β, and TNF-α were up-regulated by LPS (3.5-fold for IL-6, 46,535.9-fold for IL-1β, 4.3-fold for TNF-α, respectively), whereas the amount of these cytokines were all significantly abolished by the presence of NecroX-5 as detected by quantitative real time-PCR (Fig. 3A–C; lower panel) and RT-PCR (Fig. 3A–C; upper panel) ($P < 0.05$). Inhibition rates at 20 μM were about 124.5% for IL-6, 44% for IL-β, and 33% for TNF-α. The mRNA levels of IL-6, IL-1β, and TNF-α were faintly visible in the culture medium of unstimulated RAW264.7 cells. No obvious changes were observed when cells were exposed with NecroX-5 alone. Exposure of RAW264.7 cells to LPS had no statistical impact on cell survival at 24 h (Fig. 3D).

3.3. Effect of NecroX-5 on the production of IL-4 and IL-13

IL-4 and IL-13 has been demonstrated to convert resident macrophages into a population of cells that are programmed to promote M2 phenotype, which is considered to serve a role in promoting anti-inflammation and tissue repair [16]. To investigate the role of IL-4 and IL-13 on the regulation of the macrophage phenotype by NecroX-5, we determined the protein levels of IL-4 and IL-13 via sandwich ELISA. The concentrations of IL-4 and IL-13 were significantly elevated in response to NecroX-5 in a dose-dependent manner, relative to those of either LPS- or unstimulated cells for 24 h ($P < 0.05$, Fig. 4A–3B).

To demonstrate the effector functions of IL-4 and/or IL-13 in NecroX-5-induced macrophage polarization toward the M2 phenotype, cells were treated with anti-IL-4 and/or anti-IL-13 antibodies, respectively. As expected, the treatment of anti-IL-4 or anti-IL-13 antibodies strongly induced iNOS expression (Fig. 4C). Collectively, this indicated that NecroX-5 induced macrophage polarization toward the M2 phenotype by inducing IL-4 and/or IL-13 secretion.

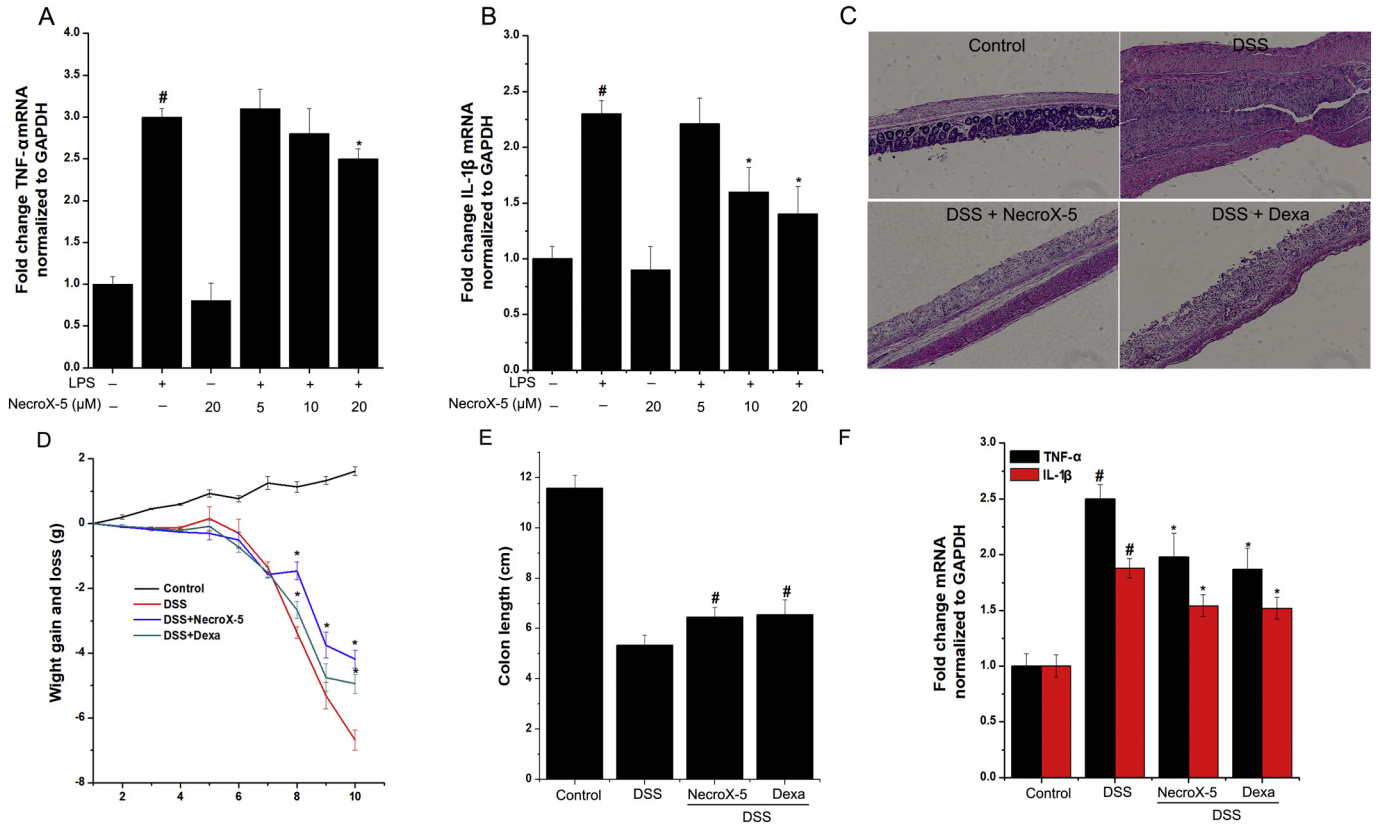

Fig. 5. The anti-inflammatory effect of NecroX-5 in LPS-stimulated PMCs and DSS-induced colitis murine model. PMCs (3×10^6) were treated with NecroX-5 (5, 10, and 20 μM) for 1 h and then stimulated by LPS for 5 h. (A-B) TNF-α and IL-1β mRNA expression levels were analyzed by real-time PCR. C57BL/6 mice were orally administrated with 4% DSS in drinking water and mice were treated with NecroX-5 (10 mg/kg) or Dexa (1.5 mg/kg). (C) Histological appearance of colons (D) relative body weight change (E) colon length (F) quantitation of the colonic TNF-α and IL-1β mRNA expression. Each datum represents the ± S.E.M. of three independent experiments. $^{*}P < 0.05$; significantly different from the unstimulated cells or DSS-administered mice $^{\#}P < 0.05$; significantly different from the LPS-stimulated cells or control mice.

3.4. Effect of NecroX-5 on DSS-induced colitis

RAW264.7 cell has some macrophage characters but originally is a leukemic cell. Thus, we evaluated the therapeutic potential NecroX-5 in real macrophages both in vitro and murine model of DSS-induced colitis. Results showed that the mRNA expression of TNF-α and IL-1β were upregulated by LPS (3.1-fold for TNF-α and 2.2-fold for IL-1β) in PMCs, whereas the amount of these cytokines were all significantly abolished by the presence of NecroX-5 (Fig. 5A–B) ($P < 0.05$). NecroX-5 treated mice exhibited crypt structure and preserved epithelial layer compared to DSS control group were shown in histological analysis (Fig. 5C). In addition, the NecroX-5 group significantly increased body weight from day 8 to day 10 ($P < 0.05$) compared to DSS control group and they showed a significant improvement in colon length when compared to DSS control group ($P < 0.05$) (Fig. 5D–E). To determine the anti-inflammatory effect of NecroX-5 by ameliorating proinflammatory M1 macrophages, the mRNA expression of TNF-α and IL-1β were assessed with quantitative RT-PCR. Results revealed that the mRNA expression of TNF-α and IL-1β were upregulated by DSS administration, whereas the amount of these cytokines was all significantly decreased by the administration of NecroX-5 ($P < 0.05$) (Fig. 5F).

4. Discussion

Here we demonstrated for the first time that NecroX-5 was able to regulate a phenotypic transformation of macrophages from the M1 to the M2 subtypes and restricted inflammatory reactions. The regulatory effect of NecroX-5 on macrophage phenotypic plasticity was mainly associated with elevated IL-4 and IL-13 production.

Macrophages account for the innate immune response and are responsible for a wide variety of immune functions, such as wound healing and host defense. To fulfill their multiple functions, macrophages polarize toward two distinct subgroups termed M1 and M2. The balance of M1/M2 macrophage polarization exerts potential effects on the regulation of immune response. Soluble factors such as cytokines or chemokines influence macrophage polarization, and some recent studies have suggested that the morphology of cells is a key determinant for the phenotypic transformation of macrophages.

In vitro studies have revealed strong changes in cell morphology by M1- or M2-inducing cytokines: M1 cells exposed to LPS and IFN-γ display a round, pancake-like appearance, while M2 cells exposed to IL-4 and IL-13 showed higher levels of cellular elongation in comparison to M1 cells [14]. The morphological change is correlated with macrophage phenotype change. The elongation of macrophage led to higher levels of arginase-1, CD206, and YM-1, which are markers of M2 polarization. Although elongation had no effect on the expression of iNOS, a marker of M1 polarization, the secretion of proinflammatory cytokines was reduced [14]. These results suggested that cell shape itself polarize macrophages toward an M2 phenotype. In the present study, we observed that RAW264.7 cells exposed to LPS displayed a round, pancake-like shape, while NecroX-5 treatment resulted in cellular elongation. These results indicate that NecroX-5 plays a key role in transforming macrophage cell shape into the M2-like phenotype.

The M2-type macrophages are characterized by their high expression of CD206, are generally activated by Th2 lymphocyte cytokines (IL-4 and IL-13), and by the production of specific cytokines and chemokines such as IL-10 and macrophage-derived chemokines (MDC or

CCL-22), pro-fibrotic metalloproteases, and TGF-β1 [4]. In addition, Arg-1 is a marker of M2 cells. Arg-1 is an enzyme of the urea cycle, which uses the amino acid L-arginine as a substrate and produces L-ornithine and urea. Initial studies on the function of macrophage arginase-1 have demonstrated that L-ornithine may enter the polyamine and collagen biosynthesis pathways, eventually promoting tissue regeneration and wound healing [17]. In the present study, we found that NecroX-5 had a marked influence on the M2 macrophage phenotype, and this compound potently upregulated Th2 cytokines such as IL-4 and IL-13. Moreover, treatment of M1 macrophages with NecroX-5 resulted in upregulation of CD206 and Arg-1, which are typical M2 macrophage markers. Thus, we conclude that NecroX-5 exerts its anti-inflammatory function by inducing the M2 phenotype from the M1 phenotype in macrophages.

The M1-type macrophages accumulated at an early time point from 24 to 48 h after implant transplantation, and secrete large amounts of pro-inflammatory mediators such as IL-1β, IL-6, and TNF-α [3]. However, excessive or unresolved M1 macrophage activation can cause chronic inflammation and tissue damage. Additionally, the M1-type macrophages specifically affect the expression of inducible enzyme cyclooxygenase-2 and iNOS, and can be characterized by high expression of CD80 and CD86 [18]. In the current study, we found that NecroX-5 downregulated proinflammatory cytokines such as IL-1β, IL-6, and TNF-α. Moreover, treatment of M1 macrophages with NecroX-5 resulted in decreased expression of iNOS, a typical M1 cell surface marker.

Macrophage polarization plays a key role in the development of inflammatory bowel disease, including Crohn's disease and ulcerative colitis. M1 cells secret inflammatory mediators, including reactive oxygen species, proinflammatory cytokines and nitric oxide, whereas M2 cells regulate wound repair by activating extracellular matrix protein synthesis [19]. Earlier reports showed that the ratio of M2:M1 are decreased in colitis [19,20]. In the present study, we showed that NecroX-5 ameliorated DSS-induced colitis via the inhibition of M1 cells.

Taken together, our findings clearly demonstrated that NecroX-5 resulting in inflammation resolution by inducing a change in the infiltrated macrophages from the M1 to M2 phenotype. NecroX-5 treatment regiments targeting the balance of M1-M2 macrophages might provide novel therapeutic strategies in the treatment of a variety of inflammatory disorders.

Conflict of interest

None.

Acknowledgements

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