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CTRP9 induces iNOS expression through JAK2/STAT3 pathway in Raw 264.7 and peritoneal macrophages

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ABSTRACT

The C1q tumor necrosis factor (TNF)-related proteins 9 (CTRP9), an adipocyte-derived cytokine, affects a number of physiological processes, including immune function and inflammation. We investigated whether CTRP9 affects the expression of inflammation-related genes in Raw 264.7 and peritoneal macrophages. The CTRP9-induced expression of iNOS increased in a time- and dose-dependent manner. LPS and CTRP9 promote the expression of iNOS jointly in Raw 264.7 and peritoneal macrophages. CTRP9 induced the phosphorylation of JAK2 and STAT3 in Raw 264.7 and peritoneal macrophages. VX509 (JAK2 inhibitor) reduced the CTRP9-induced iNOS protein production. In addition, the CTRP9-induced phosphorylation of JAK2 and STAT3 was dramatically reduced by VX509. Collectively, these results suggest that JAK2/STAT3 signaling is involved in the CTRP9-induced expression of iNOS.

1. Introduction

CTRP9 is a recently discovered highly conserved adipokine and it shares the highest degree of amino acid identity with APN in its globular C1q domain [1,2]. CTRP9 exerts by far the highest expression in the heart and can also be found in serum and adipose tissue [3]. Serum CTRP9 level is associated with arterial stiffness in patients with type 2 diabetes [4]and carotid IMT in diabetic patients without CKD, but not in those with CKD [5]. In addition, it decreases significantly in CAD patients as compared to non-CAD patients [6]. These data show serum CTRP9 is an independent protective factor of CAD.

Many previous studies have demonstrated that CTRP9 protects the heart against I/R injury [7–10] through AMPK pathway [9,10], attenuates adverse cardiac remodeling [1,11], and inhibition of CTRP9 contributes to exacerbated cardiac injury in diabetic mice [12], CTRP9 also regulates lipid metabolism [13] reduces lipid accumulation [1], CTRP9 transgenic mice are protected from diet-induced obesity and metabolic dysfunction [14]. Moreover, CTRP9 attenuates hepatic steatosis [15]. Ameliorates pulmonary arterial hypertension through attenuating inflammation [16], induce vascular relaxation through the adiponectin receptor-1/AMPK/eNOS/Nitric Oxide signaling pathway [17]. CTRP9 can inhibits ox-LDL-induced inflammatory [18] and inhibits cytokine-induced vascular inflammation and leukocyte adhesiveness via AMP-activated protein kinase activation in endothelial cells [19]. However, there are also studies show CTRP9 promotes cardiac hypertrophy and failure through ERK5-GATA4 pathway [3]. These data show CTRP9 exerts its biological functions through multiple signaling pathways.

In the present study, our study aimed to investigate whether CTRP9 could induces iNOS expression and to explore the underlying mechanisms behind this effect. Our results showed that CTRP9 up-regulates iNOS expression through JAK2/STAT3 pathway.

2. Material

2.1. Chemicals and reagents

Recombinant mouse gCTRP9 protein was purchased from

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Aviscera Bioscience (Santa Clara, CA). Antibodies for iNOS was obtained from Abcam, JAK2, phosphorylated-JAK2, STAT3, phosphorylated-STAT3 were bought from CST and ACTIN was bought from Bioworld Technology (Minneapolis, MN). VX509 was obtained from Selleck. Cell culture medium was purchased from HyClone Laboratories (Utah, USA).

2.2. Cell culture

Raw 264.7 macrophages were obtained from the American Type Culture Collection (ATCC), which is cultured in DMEM medium with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 °C and 5% CO2 in a humidified incubator.

2.3. Preparation and culture of mouse peritoneal macrophages

Mouse peritoneal macrophages were elicited by 6% starch. Briefly, C57BL/6j mice were injected with 6% starch into the peritoneal cavity. Peritoneal exudates were obtained 72 h after injection by flushing the peritoneal cavity with ice-cold serum-free DMEM medium using a 22 G needle. Peritoneal lavage was pooled and centrifuged in a 50 ml conical centrifuge tube at 800 rpm for 5 min at 4 °C, and the pellet was resuspended in DMEM medium supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin. Monocytes were allowed to adhere to tissue culture wells for 1 h prior to removal of other cells by washing with serum-free DMEM.

2.4. CCK8 assay

After treating macrophage with different concentrations of CTRP9 in a 96-well plate for 24 h, each well was added with 10 μl of CCK-8 solution and were incubated for 2 h under a temperature of 37 °C. Subsequently, the color intensity was measured at an absorbance of 450 nm to access cell viability. Three separated experiments were conducted 3 times.

2.5. Western blotting

Cell lysates were prepared from Raw 264.7 cells or peritoneal macrophages (5 × 106 cells) in lysis buffer according to the manufacturer's protocol and boiled for 10 min. The samples (20 g) were diluted with 1 × lysis buffer and were separated by electrophoresis and transferred onto polyvinylidene fluoride membranes (PVDF). After blocking with 5% skimmed milk. The membranes were reacted with primary antibodies against iNOS(1:1000 dilution), p-JAK2 (1:1000 dilution), JAK2 (1:1000 dilution), p-STAT3 (1:1000 dilution), STAT3 (1:1000 dilution), and β-actin (1:1000 dilution) at 4 °C overnight, followed by incubation with peroxidase conjugated secondary antibodies at room temperature for 2 h. The blots were

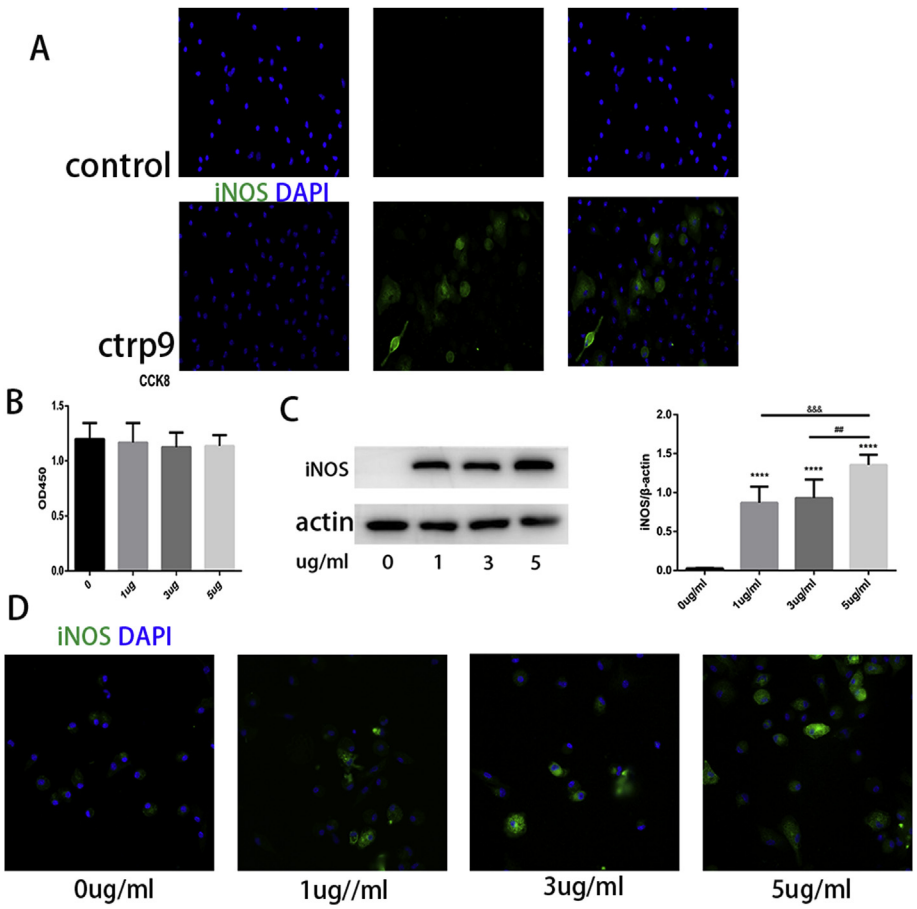

Fig. 1. CTRP9 induces iNOS expression in peritoneal macrophages with dose-dependent effect. (A)Peritoneal macrophages were treated with 1 μg/ml CTRP9 for 24 h and observed under a fluorescence microscope. (B) Peritoneal macrophages undertook a 24-h culture with increasing doses of CTRP9 (0–5 μg/mL). The CCK-8 assay was adopted to detect the cell viability and the levels of iNOS were measured by Western blotting(C). (D) Representative images of iNOS (green) expression induced by CTRP9 (0–5 μg/ml) for 24 h taken by fluorescence microscope (Scale bar is 100 μm). All the results were expressed as mean ± SD (n = 3). ****, $p < 0.0001$, (other groups vs control); &&&, $p < 0.001$; ##, $p < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

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visualized with enhanced chemiluminescence detection reagent. β-Actin was used as internal controls for total protein. Bands were quantified by densitometry using Quantity One software (Bio-Rad, Hercules, CA).

2.6. Real-time PCR

Total RNA was extracted using the TRIzol reagent (Invitrogen), and then reverse transcribed into cDNA using Reverse Transcription kit (Takara Biotechnology) according to the manufacturer's instructions. All gene transcripts were measured by quantitative PCR with the SYRB Premix Ex TaqTM kit (TaKaRa). Results were calculated by 2-DDCt general method, and β-actin was used as an internal control. The sequences of the primers used were as follows: 5′-AGTGAAGCAAAGCCCAACAA-3' (forward) and 5′-TGGACGG GTCGATGTCA-3' (reverse) for iNOS; 5′-GGCTGTATTCCCCTCCATCG-3' (forward) and 5′-CCAGTTGGTAACAATGCCATGT-3' (reverse) for β-actin.

2.7. Immunofluorescence staining

Cells were washed three times with PBS and fixed in 4% paraformaldehyde for 10 min, washed again, then treated with 0.1% Triton X-100 for 20 min to increase antigen accessibility. After blocking with 1% BSA for 30 min, and incubating with the indicated antibodies overnight at 4 °C, we then stained with the appropriate secondary antibodies for 1 h at 37 °C in the dark. Finally, DAPI was used to stain the cell nuclear. Coverslips were mounted on slides and immunofluorescent staining was evaluated under a confocal microscope or fluorescence microscope.

2.8. Statistical analysis

SPSS software 22.0 was used for statistical analysis. Data were presented as mean ± SD of at least 3 independent experiments. The differences were determined by one-way ANOVA with LSD post hoc test. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. iNOS expression shows a dose-dependent up-regulation by CTRP9 in peritoneal macrophages

First, we found CTRP9 promoted iNOS expression in peritoneal macrophage through immunofluorescence image (Fig. 1A). Then different concentrations of CTRP9 (0, 1, 3, and 5 μg/mL) were added to macrophages for 24 h to assess cytotoxicity. As shown in Fig. 1B, cell viability was not affected. To determine the effect of CTRP9 on iNOS expression, peritoneal macrophages were incubated with 0–5 μg/ml CTRP9 for 24 h, and the iNOS protein expression was measured by Western blot. The iNOS protein expression increased in a dose-dependent manner, initially increasing at 1 μg/ml after 24 h (Fig. 1C). The immunofluorescence signal further confirmed that iNOS protein expression was increased in the CTRP9-treated group compared to the control group and showed that CTRP9-induced iNOS expression was in a dose-dependent manner (Fig. 1D).

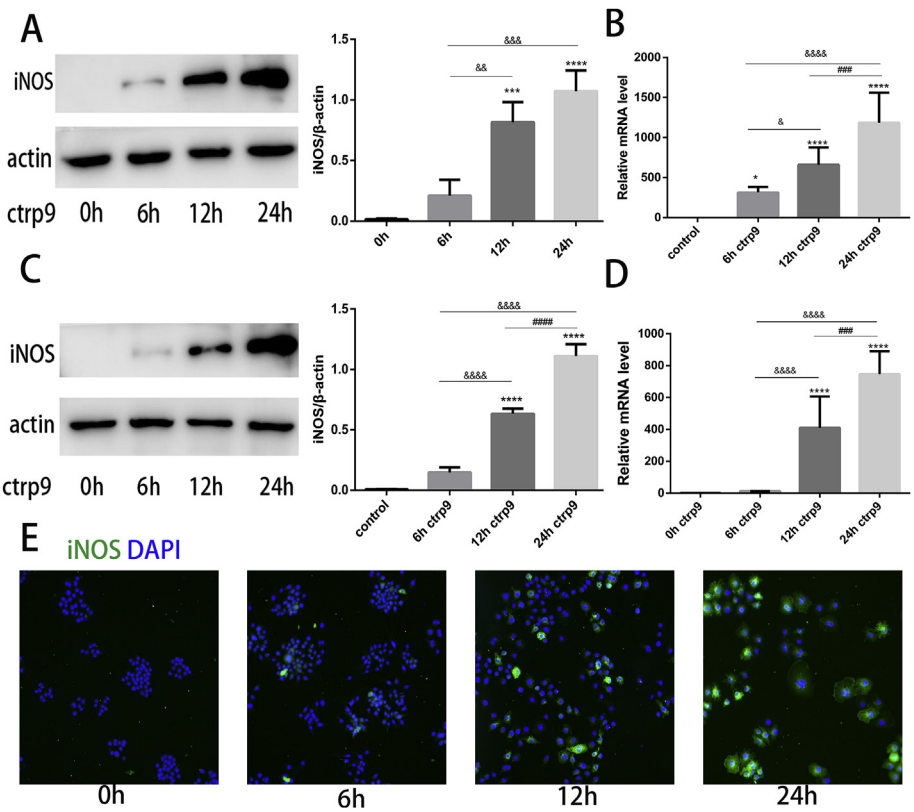

Fig. 2. iNOS expression shows a time-dependent up-regulation by CTRP9 in Raw 264.7 and peritoneal macrophages. (A) Raw 264.7 and peritoneal macrophages(C) were stimulated with 1 μg/ml CTRP9 for 0–24 h. The protein level of iNOS were measured by Western blotting. (B) The mRNA levels of iNOS induced by CTRP9 for different hours in Raw 264.7 macrophages. And peritoneal macrophage(D). (E) Representative images of iNOS (green) expression induced by CTRP9 for different time at high magnification taken by fluorescence microscope in Raw264.7 macrophages. (Scale bar is 100 μm). All the results were expressed as mean ± SD (n = 3). ***, $p < 0.001$; (other groups vs control); &&, $p < 0.01$; ###, $p < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

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3.2. iNOS expression shows a time-dependent up-regulation by CTRP9 in Raw 264.7 and peritoneal macrophages

To examine the time-effect of CTRP9 on iNOS expression, Raw 264.7 and peritoneal macrophages were incubated for 0–24 h with 1 μg/ml CTRP9. Western blot was used to assess the protein expression levels of iNOS, a time-dependent rise in iNOS expression was observed, with the iNOS level initially rising when incubation with CTRP9 for 6 h (Fig. 2A,C). Fig. 2B and D also showed that CTRP9 significantly induce iNOS mRNA expression in Raw 264.7 and peritoneal macrophages as early as 6 h of incubation. The immunofluorescence staining results further showed that CTRP9 incubation triggered iNOS protein expression in time-dependent manners (Fig. 2E).

3.3. Effects of LPS and CTRP9 on iNOS expression

To investigate the effects of LPS and CTRP9 on iNOS expression in Raw264.7 and peritoneal macrophages, we first analyzed the mRNA levels of the iNOS after CTRP9 treatment for 2 h and LPS for another 24 h without moving the CTRP9. The results show both CTRP9 and LPS increased iNOS expression and they show synergistic effect both in Raw264.7 and peritoneal macrophage (Fig. 3A and C). Then we observed same finding in inducing expression of iNOS protein in peritoneal macrophage (Fig. 3D) while there is no synergistic effect in Raw264.7 (Fig. 3B). Collectively, these findings indicate that LPS and CTRP9 promote the expression of iNOS jointly in Raw 264.7 and peritoneal macrophages. As show in Fig. 3E and F, the induction of iNOS secretion was abrogated by the use of CTRP9 with proteinase K, indicating that the effect was not attributable to contamination of the recombinant protein by LPS.

3.4. CTRP9 and LPS induce iNOS expression through JAK2-STAT3 pathway

To further study the mechanism of inducing iNOS expression by LPS and CTRP9, we tested phosphorylation of JAK2, STAT3 and iNOS with LPS stimulates and the results show increased phosphorylation of JAK2, STAT3 and up-regulated iNOS in peritoneal macrophages (Fig. 3G). Inhibiting JAK2/STAT3 pathway by VX509, an inhibition of JAK2, could down-regulate p-jak2, p-stat3, and iNOS expression in peritoneal macrophages (Fig. 3H).

Next, we observed phosphorylation of JAK2 and STAT3 after 3 h and 6 h with CTRP9 stimulates and the results show increased phosphorylation of JAK2, STAT3 in Raw 264.7 macrophages (Fig. 4A). VX509 could down-regulate P-JAK2, P-STAT3, and iNOS expression both in Raw 264.7 (Fig. 4B) and peritoneal macrophages (Fig. 4C). The immunofluorescence staining results also showed that CTRP9 incubation triggered P-JAK2 (Fig. 4D). As show in Fig. 4C, peritoneal macrophages have more obvious iNOS down-expression when performed VX509 compared with Raw264.7 macrophages. Similarly, the results of confocal microscopy in Fig. 4E also show the same results.

4. Discussion

The most important observation of this report is that CTRP9 could up-regulate iNOS expression in a dose-and time-dependent way through JAK2-STAT3 pathway, and it has synergetic effect with LPS in inducing iNOS expression. Inducible nitric oxide synthase

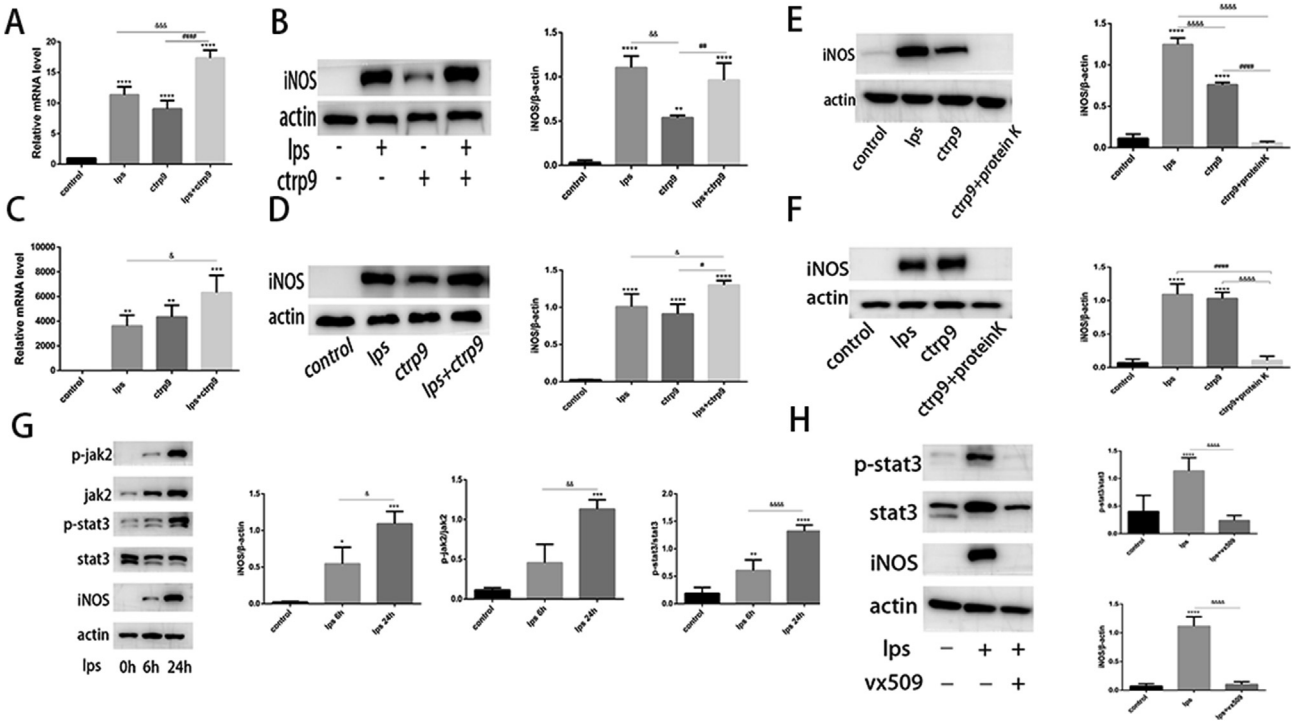

Fig. 3. Effect of LPS and CTRP9 on iNOS expression in Raw 264.7 and peritoneal macrophages (A) Raw 264.7 and peritoneal macrophages (C) were stimulated with 100 ng/ml LPS, 1 μg/ml CTRP9, 1 μg/ml CTRP9 pretreated for 2 h plus LPS 100 ng/ml for another 24 h. The iNOS mRNA expression was then measured by real-time PCR. (B) The protein of iNOS was analyzed by Western blot in Raw 264.7 and peritoneal macrophages(D). (E and F) Raw 264.7 and peritoneal macrophages were stimulated with LPS, CTRP9, CTRP9 plus proteinase K for 24 h. The iNOS was then determined by Western blot. (G) Peritoneal macrophages were stimulated with LPS for 6 h and 24 h and the phosphorylation of JAK2, STAT3 and iNOS were assessed via Western blotting. (H) Peritoneal macrophages were pre-treated with 15 μM JAK inhibitor VX509 for 60 min, and then stimulated with LPS for 24 h. The phosphorylation of STAT3 and iNOS were examined via Western blotting. All the results were expressed as mean ± SD (n = 3). *, $p < 0.05$; **, $p < 0.01$; (other groups vs control); &&&&, $p < 0.0001$; ####, $p < 0.0001$.

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(iNOS) is a kind of enzyme that is absent in normal condition, it expressed only under the stimulation of cell stimulation by immunological or microbial stimuli [20], such as LPS and TNF-α [21,22]. We found here that CTRP9 induces iNOS in Raw 264.7 and peritoneal macrophages.

It is well established that LPS could induce the expression of iNOS in macrophage [23–25]. It is possible that the ability of recombinant CTRP9 to induce iNOS secretion is the result of contamination by LPS. However, iNOS induction was not detected following exposure to heat-digested CTRP9 and proteinase K (Fig. 3E and F). This suggests that the induction of iNOS secretion is not attributable to LPS contamination.

A recent study demonstrated that CTRP9 down-regulates LPS-induced iNOS expression [26] in rat peritoneal macrophage which show reverse effects of CTRP9 with our results, our findings substantiate the synergistic effects of LPS and CTRP9 in inducing iNOS expression. These may be because different cell types activate different even opposite pathways under the same stimulus [24]. Adiponectin, a highly homologous to CTRP9, has been reported to induce apoptosis by ROS/NO in Raw264.7 [27], Many studies have implied iNOS plays a detrimental role in cardiovascular diseases (CVD) [28–30] and diabetes mellitus (DM) [31]. Nevertheless, the role of iNOS in diseases still remains conflicting. So further study is needed to clarify the function of iNOS protein.

In the present study, CTRP9 stimulation activates phosphorylation of JAK2 and STAT3 (Fig. 4) and that inhibiting JAK activity with VX509 reduces CTRP9-induced iNOS expression in Raw 264.7 and peritoneal macrophages. These data suggest that CTRP9 induces iNOS expression via the JAK2/STAT3 pathway. JAK/STAT signaling pathway is involved in various physiological processes, including immune function, cell growth, differentiation and death [32] and it described as an important signaling axis in macrophage biology

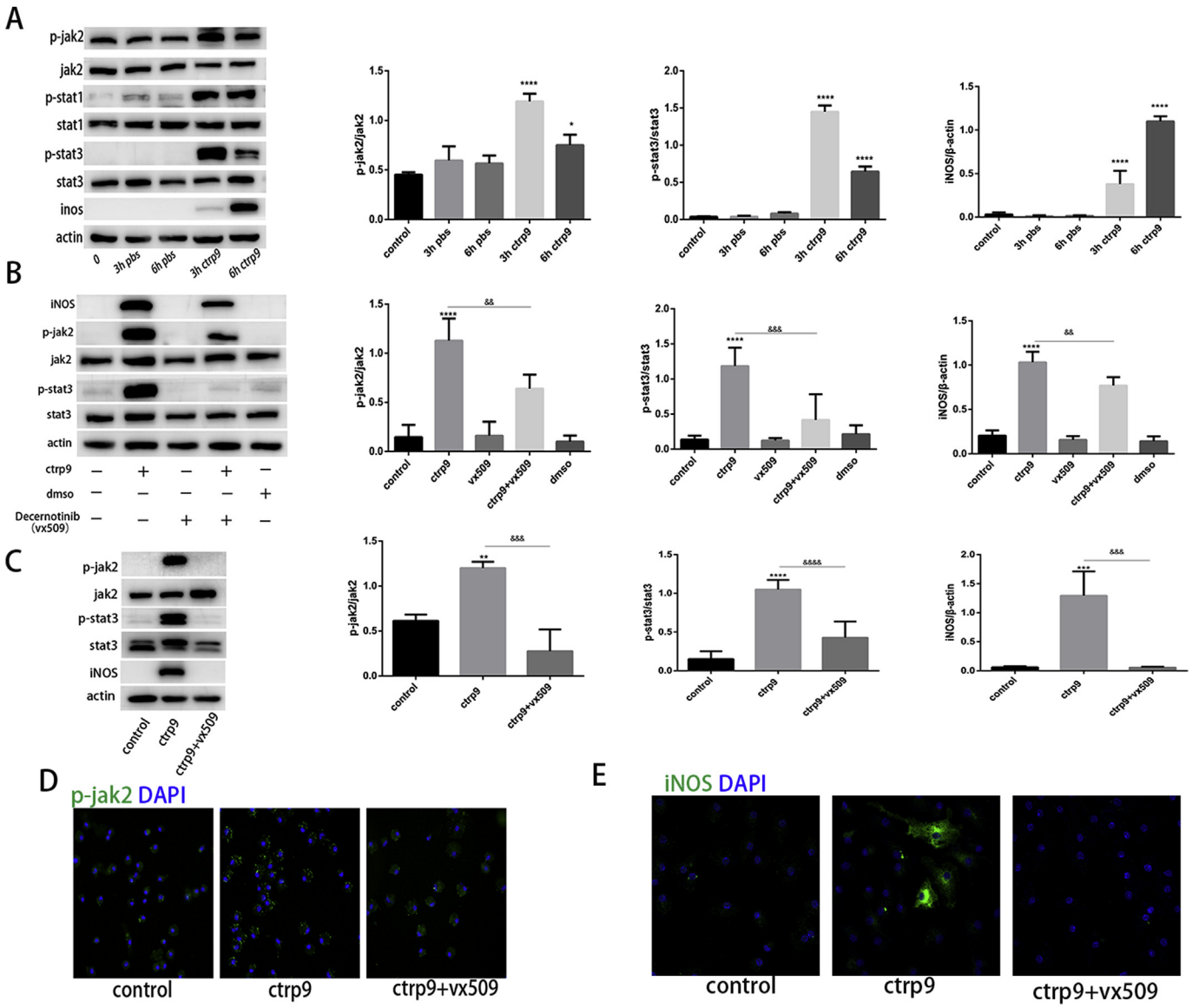

Fig. 4. (A) Raw264.7 were incubation for 3 h and 6 h with pbs and CTRP9 separately. JAK2, STAT3 phosphorylation and iNOS were examined via Western blotting. (B) Raw264.7 were pre-treated with 15 μM JAK inhibitor VX509 for 60 min and then stimulated with CTRP9 for 24 h, the expression of iNOS was examined via Western blotting. (C) Peritoneal macrophages were treated in the same manner as panel A and the expression of P-JAK2, P-STAT3 and iNOS was examined via Western blotting. (D, E) Representative images of P-JAK2, iNOS (green) expression were taken by confocal microscopy (Scale bar is 50 μm). All the results were expressed as mean ± SD (n = 3). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

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[33]. Recent studies have revealed that the JAK2/STAT3 signal pathway is hyper-activated in cellular and animal models of inflammation, suggesting a key role of this signaling pathway in regulating inflammation responses [34,35].

Indeed, it has been found that LPS induced inflammation may directly dependent on JAK2 and STAT3 activation in Raw264.7 macrophage [36–38]. Our results in Fig. 4 have the same conclusion that LPS up-regulated iNOS expression in mouse peritoneal macrophage through JAK/STAT3 pathway. Also, inhibiting JAK2-STAT3 pathway by the JAK2 inhibitor AG490 ameliorated RA [39] and the anti-atherogenesis effect was partially attributed to the inhibition of the JAK2/STAT3 pathways [40]. Accordingly, the modulation of the JAK2/STAT3 signaling pathway may provide an effective therapeutic strategy in the treatment of inflammation-associated diseases [41,42]. However, previous results demonstrated inhibition of iNOS is based on activating JAK2-STAT3 pathway [43,44]. For example, The effect of NBE in enhancing the anti-inflammation profiles via upregulation of JAK–STAT expression in hyper-cholesterol diet rabbits have been established [45]. The upstream ligands activating STAT3 influence the resulting transcriptional and functional effects, just like IL-6 and IL-10, two cytokines relying on STAT3 as their effector, have opposite effects on inflammatory processes [46]. These conflicting findings concerning the physiological/pathogenic role of JAK2/STAT3 may be caused by a tissue-specific signal transduction pathway.

In summary, in this study we provided the first evidence that CTRP9 increases iNOS expression in Raw 264.7 macrophage and peritoneal macrophage and we demonstrated that intracellular signaling through the activation of JAK2/STAT3 is involved in CTRP9-induced iNOS expression. iNOS is generally considered to be one of the markers of M1 macrophage, In the present study, further study is needed to address details about polarized macrophages.

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Transparency document

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