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N-methyl pyrrolidone (NMP) inhibits lipopolysaccharide-induced inflammation by suppressing NF-κB signalling*

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*Running title: NMP lowers inflammatory response.

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Abstract

OBJECTIVE: *N*-methyl pyrrolidone (NMP), a small bioactive molecule, stimulates bone formation and inhibits osteoclast differentiation and bone resorption. The present study was aimed to evaluate the anti-inflammatory potentials of NMP on the inflammatory process and the underlying molecular mechanisms in RAW264.7 macrophages.

MATERIAL AND METHODS: RAW264.7 macrophages and mouse primary bone marrow macrophages (mBMMs) were used as an *in vitro* model to investigate inflammatory processes. Cells were pre-treated with or without NMP and then stimulated with lipopolysaccharides (LPS). The productions of cytokines and NO were determined by proteome profiler method and nitrite analysis, respectively. The expressions of nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) were measured by Western blotting and/or qPCR. Western blot, ELISA-base reporter assay and immunofluorescence were used to evaluate the activation of MAP kinases and NF-κB.

RESULTS: LPS-induced mRNA expression of TNF-α, IL-1β, IL-6, iNOS and COX2-2 were inhibited by NMP in a dose-dependent manner. NMP also suppressed the LPS-increased productions of iNOS and NO. The proteome profiler array showed that several cytokines and chemokines involved in inflammation and up-regulated by LPS stimulation were significantly down-regulated by NMP. Additionally, this study shows that the effect of NMP is mediated through down-regulation of NFκB pathway.

CONCLUSIONS: Our results show that NMP inhibits the inflammatory mediators in macrophages by an NFκB-dependent mechanism, based on the epigenetical activity of NMP as bromodomain inhibitor. In the light of its action on osteoblast and osteoclast differentiation process and its anti-inflammatory potential, NMP might be used in inflammation-related bone loss.

Keywords: NMP, Inflammatory mediators, Macrophages, LPS, NFκB, MAPK.

Introduction

Chronic inflammation plays an important role in the development of several diseases such as rheumatoid arthritis (RA) and osteoporosis. Rheumatoid arthritis is associated with both increased risk of fractures and systemic bone loss. Moreover, fragility fractures are more prevalent in osteoporosis and other inflammatory diseases, when compared to the healthy population. The link between osteoclast, macrophage colony stimulating factor (M-CSF) and pro-inflammatory cytokines, especially TNF-α and interleukin-1 (IL-1) explains the association between inflammation and osteoporosis [1]. All diseases involving bone loss have a common pattern: osteoclast activity overcomes osteoblast activity. TNF-α has been described as a key cytokine mediating bone loss in osteolysis and other inflammatory bone diseases. Another important role of TNF-α in inflamed tissue is its capacity to induce intercellular adhesion molecule (ICAM-1) in endothelial cells. This molecule binds with circulating leukocytes in vessels, causing the accumulation of lymphocytes which will produce more TNF-α and resulting in a self-feeding loop. Activated T-cells besides expressing receptor activator of nuclear factor-B ligand (RANKL) also adhere to osteoblasts and induce adhesion-dependent osteoclast maturation [2]. Lipopolysaccharide (LPS), a major component of Gram-negative bacteria cell walls, activates a variety of mammalian cell types including monocytes/macrophages and endothelial cells [3], and induces inflammatory responses when used to stimulate cells or administered to animals. In various cells, including macrophages, LPS stimulates toll-like receptor 4 (TLR4) to activate nuclear factor κB (NF-κB) which is an important transcription factor for pro-inflammatory cytokines, iNOS and COX-2 [4]. LPS has also been shown to activate mitogen-activated protein kinases (MAPKs), including extracellular signal-related kinase (ERK)-1/2, p38MAPKs, or c-Jun NH2-terminal kinase (JNK) to enhance iNOS and COX-2 gene expression in macrophages [5]. Recently, we showed that N-methyl pyrrolidone (NMP), a small bioactive molecule, enhances bone formation and inhibits osteoclast differentiation and bone resorption [6, 7]. In the present

study, we investigated the effects of NMP in the inflammatory process by using RAW264.7 macrophages stimulated with lipopolysaccharides (LPS) as an inflammatory process model. We found that NMP reduced the production of nitric oxide (NO) and the expression of iNOS, and inflammatory cytokines (IL-1α, IL-1β, IL-6 and TNF-α) induced by LPS. Moreover, we showed that the effect of NMP is mediated through down-regulation of NFκB activation and thus we provide the molecular mechanism by which NMP might exert its anti-inflammatory function. Based on our previous results and the data presented herein, we suggest that NMP may have a potential usage in ameliorating inflammatory bone damages.

Material and Methods

Reagents and Antibodies

Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were obtained from Life Technologies Inc. (GrandIsland, NY, 99 USA). Primers for RT-PCR were purchased from Microsynth AG (Switzerland). Anti-iNOS (H-174) polyclonal antibody was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Polyclonal anti-phospho-ERK, anti-phospho-p38 and anti-phospho-JNK were obtained from Cell Signaling Technology (CST, Inc, USA). TransAM NFκB/p65 transcription factor kit was from Active Motif (Rixensart, Belgium). The RNA extraction kit (RNeasy kit) was from Qiagen. The BCA kit for protein determination was from Pierce. LPS (Escherichia coli, serotype 0111:B4) and all other chemicals were obtained from Sigma (St. Louis,MO,USA).

Cell cultures

The RAW264.7 macrophage cell line was obtained from ATCC and cultured in DMEM supplemented with 10% FBS and antibiotics (100 units/ml penicillin G and 100 mg/ml streptomycin). The cultures were never allowed to become confluent. Incubations were performed at 37 °C in 5% CO_2 in humidified air. Bone marrow-derived macrophages

(BMMs) were isolated from the long bones of 6-week-old mice and were maintained in α-minimal essential medium as described previously [6, 8].

Cell viability assay

Cell viability was analyzed using WST-1, a colorimetric assay for the nonradioactive quantification of cellular proliferation, viability and cytotoxicity (Roche Diagnostics, Switzerland) according to the manufacturer's instruction. Briefly, RAW264.7 cells and mouse bone marrow-derived macrophages (mBMMs) were plated in 96 well plates for 24h and then incubated with various concentrations of NMP or LPS for 48h. After the stimulation, WST-1 (1/10 of total volume) was added to each well and incubated for 2 hours at 37°C in the dark. Following incubation, absorbance of each well was measured at 450 nm.

Quantitative Real-time RT-PCR

RNA from RAW264.7 cells was extracted using the RNeasy kit. The mRNA was reverse-transcribed into cDNA. The resultant cDNA was subjected to real-time PCR with gene-specific primers using iQ SYBR Green Supermix and an iCycler real-time PCR machine (both from Bio-Rad) according to the manufacturer's instructions. The primer sequences (forward; reverse) used in this study are as follows:

IL-1α: (CGTCAGGCAGAAGTTTGTCA; TTAGAGTCGTCTCCTCCCGA)

IL-1β: (TGTGAAATGCCACCTTTTGA; TGAGTGATACTGCCTGCCTG)

IL-6: (CCGGAGAGGAGACTTCACAG; CAGAATTGCCATTGCACA)

TNF-α: (GAACTGGCAGAAGAGGCACT; GGTCTGGGCCATAGAACTGA)

COX-2: (TCCATTGACCAGAGCAGAGA; TCTGGACGAGGTTTTTCCAC)

iNOS/NOS2: (CACCTTGGAGTTCACCCAGT; ACCACTCGTACTTGGGATGC)

GAPDH: (GGCATTGCTCTCAATGACAA; TGTGAGGGAGATGCTCAGTG).

Western blot analysis

Treated cells were rapidly frozen in liquid nitrogen and stored at - 80°C until used for analysis as described previously [6]. Proteins were separated on a 4–20% precast polyacrylamide gel

(Bio-Rad), and transferred to PVDF membrane using Trans-Blot Turbo Transfer System (Bio-Rad). The proteins were detected by using the appropriate primary antibodies followed by horseradish peroxidase (HRP)-coupled secondary antibody. The membranes were washed, treated with the ECL reagent and exposed to X-ray films. Filters that were reprobed were stripped according to the manufacturer's protocol.

Nitrite determination

Cells were plated in 24-well plates and then incubated with LPS in the absence or presence of NMP (5 and 10 mM) for 48 h. Nitrite levels in culture media was determined using the Nitrite/Nitrate colorimetric assay kit (Sigma) according to the manufacturer's instruction. After 10 min incubated at room temperature, absorbance at 540 nm was measured in a microplate reader (Synergy HT, BioTek). Fresh culture medium was used as the blank in all experiments. The amount of nitrite in the samples was quantified using the serial dilution standard curve from sodium nitrite.

Cytokine protein array analyses

RAW 264.7 cells were seeded in 75 cm^2 flask and treated with 1μg/mL of LPS in the absence or presence of NMP (10 mM) for 18 h. Cells were lysed at 4°C in lysis buffer and lysates were then cleared by centrifugation at 6000g for 30 min. The protein concentration was determined using the Pierce protein assay reagent according to the manufacture's instruction. Screening for different acute phase proteins, cytokines, and chemokines in cell lysates was performed with a Proteome profiler array (Mouse Cytokine Array Panel A) from R&D Systems. *Chemi reagent* mix provided with the kit (Horseradish peroxidase substrate) was used to detect protein expression and data were captured by exposure to Lumi-Film Chemiluminescent Detection film (Roche, Switzerland). Pixel densities on developed X-ray films were collected and analyzed using image analysis software (Image J). The average background signal was subtracted from the average signal (density) of the pair of duplicate

spots representing each cytokine. The relative change in cytokine levels between samples was determined by comparing corresponding signals on different arrays.

Immunofluorescence Staining for Detection of NF-κB p65.

RAW264.7 cells plated on 15 µ-Chamber 12 weel (Ibidi GmbH, Martinsried, Germany) were treated with LPS in the absence or presence of NMP and then fixed with 4% formaldehyde. The cells were incubated in blocking buffer for 60 min (1X PBS, 5% normal serum and 0.3% Triton X-100). The cells were then incubated overnight at 4°C with anti-NF-κB p65 antibody (Cell signaling) diluted in antibody dilution buffer (1X PBS, 1% BSA and 0.3% Triton X-100), followed by 1h incubation with anti-rabbit IgG antibody labeled with Alexa 488 (Invitrogen) and 10 min incubation with DAPI (Invitrogen). The fluorescence was visualized by a confocal laser scanning microscope (Leica TSC SP5).

NFκB/p65 activation analysis

To detect NFκB activation in RAW264.7 cells, we used ELISA-based TransAM p65 transcription factor kit (Active Motif, Rixensart, Belgium). Cells were plated in Petri dishes for 48h and then pre-incubated 4h in serum-free fresh medium before stimulation. Preparation of nuclear cell extract was done with Nuclear Extract Kit (Active Motif, Rixensart, Belgium) according to the manufacturer's instructions. The active form of p65 in nuclear extracts can be detected using an antibody specific for epitope that is accessible only when the nuclear factor is activated and bound to its DNA target. An HRP-conjugated secondary antibody provides a sensitive colorimetric readout. Absorbance was determined with a microplate reader (SynergyHT, BioTek).

Transient Transfection and Luciferase Reporter Assay

RAW264.7 cells were plated into 96-well plates and cultured for 24 h. The cells were then transfected with pGL4.32[luc2P/NF-κB-RE/Hygro] luciferase reporter plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

After 24 h of incubation, the cells were stimulated with LPS (1µg/ml) in the presence or absence of NMP for 24 h. The luciferase activity was measured in a microplate reader (Synergy HT, BioTek) using the Bright-Glo™ Luciferase Assay System (Promega). Luciferase activity values were normalized to total protein content measured by Commassie protein assay (Thermo Scientific) and reported as the mean ± SD.

Statistical analysis

Experiments were carried out independently at least three times. Results are expressed as the mean ± SD and were compared by Student's *t*-test. Results were considered significantly different for $p<0.05$ (*) or $p<0.01$ (**).

Results

Effects of NMP on cell viability and nitric oxide (NO) production

Macrophages play a crucial role in both the specific and non-specific immune responses. Activation by inflammatory stimuli produces a variety of inflammatory mediators such as nitric oxide (NO), prostaglandin E2 (PGE2) and pro-inflammatory cytokines including TNF-α, IL-1 and IL-6. However, if left uncontrolled, the inflammatory mediators get involved in the pathogenesis of many inflammatory disorders [9, 10].

In order to test the possibility that NMP might modulate the inflammatory response, the mediators of inflammation as well as mitogen-activated protein kinases (MAPK) were evaluated *in vitro*. For this purpose, RAW264.7 macrophage cell line treated with LPS was used as model of inflammatory process. We first investigated the effects of NMP and LPS on cell viability. As shown in **figure 1A**, neither NMP nor LPS had an effect on mouse bone marrow-derived macrophages (mBMMs) or RAW264.7 cell viability. Nitric oxide (NO) production is a characteristic feature of activated macrophages. To study the effect of NMP on

NO production, RAW264.7 cells and mBMMs were stimulated with LPS (1μg/ml) in the absence or presence of NMP and the accumulation of NO was measured using *Griess* reagent (**Fig.1B**). In both types of cells, production of NO was increased by LPS stimulation and NMP treatment suppressed NO production. To investigate the effect of NMP on iNOS and Cox-2, enzymes responsible for NO and prostaglandins production respectively, changes of mRNA expression was examined using quantitative real-time PCR (**Fig.2A**). The stimulation of RAW264.7 cells by LPS increases iNOS and Cox-2 mRNA expression. NMP treatment significantly suppressed iNOS and Cox-2 mRNA expression induced by LPS in a dose-dependent manner. Furthermore, the effects of NMP on iNOS expression following LPS stimulation was examined (**Fig.2B**). iNOS expression was undetectable in untreated cells. However, after LPS treatment, iNOS expression was markedly and dose dependently increased. NMP treatment inhibited iNOS expression induced by LPS in a dose-dependent manner.

Effects of NMP on pro-inflammatory cytokine production

Macrophages are a major source of many cytokines involved in immune response, hematopoiesis, inflammation, and other homeostatic processes. Upon stimulation by LPS, mRNA expression of IL-1α, IL-1β, IL-6 and TNF-α was markedly induced. NMP treatment suppresses LPS-induced pro-inflammatory cytokines mRNA expression (**Fig. 3A).**

As shown in the proteome profiler array, cytokines such as GM-CSF, IL-1α, IL-1β, and IL-6 were up-regulated after LPS stimulation. However, the expression of these cytokines was significantly down-regulated by NMP (**Fig.3B**). In parallel to this inhibitory effect, the data shown by this experiment revealed that NMP increases the expression of CXCL11 and TIMP-1, two proteins involved in the inflammatory process.

Effects of NMP on MAP Kinases activation

Phosphorylation and activation of MAPK are crucial for LPS-induced inflammatory mediators. In order to investigate whether the MAPK pathways are involved in the inhibition

of inflammatory mediators by NMP, we examined the effect of NMP on the LPS-induced MAPK phosphorylation. As shown in **figure 4A**, LPS significantly induced the phosphorylation of p38, JNK and ERK compared to unstimulated cells. The presence of NMP has no effect on p38 and JNK phosphorylation; however NMP improved ERK phosphorylation induced by LPS. These data indicate that the inhibition of inflammatory mediators by NMP occurs probably through the activation of ERK in LPS-stimulated RAW264.7 cells. This was confirmed by using U0126, a pharmacological inhibitor of ERK, and high serum (30 % FBS) usually used as an activator of ERK pathway. Indeed, ERK inhibitor increases NO production induced by LPS treatment (**Fig.4B**), whereas pretreatment with a high percentage of serum decreases LPS-induced NO production compared to the standard condition (10% FBS). Similar result was obtained from PMA treatment, which is known to activate ERK pathway (data not shown). U0126 and FBS effects are also reflected on iNOS protein expression. In fact, ERK inhibition is accompanied by an increase in iNOS, while ERK stimulation results in a decrease of iNOS protein expression (**Fig.4C**).

Effects of NMP on NFκB pathway

Nuclear factor-κB (NFκB) is an important regulation factor involved in the transcription of cytokine genes for inflammatory mediators. The critical step in NFκB activation is the IκBα phosphorylation by IκB kinase complex (IKK) [11]. **Figure 5A** shows that LPS induces IκBα phosphorylation and the pre-treatment with NMP significantly reduced this phosphorylation, suggesting that NMP might inhibit the LPS-induced NFκB activation by blocking the phosphorylation and degradation of IκBα. IκBα phosphorylation is specific to canonical NFκB pathway and the activation of this pathway is generally associated with inflammatory exposure, whereas activation of the non-canonical pathway is mostly related to developmental cues. The non-canonical pathway is based on the inducible phosphorylation and proteasome-mediated partial degradation of NFκB2 p100 to p52. In order to examine if the non-canonical NFκB pathway is also involved in the effect of NMP, we performed western-blot using

phospho- NFκB2 (p100/p52) antibody. **Figure 5B** shows that LPS induces a partial degradation of NFκB2 p100 to p52. NMP treatment induces no change in the LPS-induced NFκB2 p100 degradation suggesting that NMP does not affect the non-canonical pathway.

To confirm the involvement of the canonical NFκB pathway, we examined whether NMP could suppress the nuclear translocation of p65 which is associated with the activation of the NFκB. Using a DNA-binding ELISA method we showed that the nuclear translocation of p65 increased when cells were exposed to LPS (**Fig.5C**). The presence of NMP suppressed LPS-induced p65 translocation, and thus inhibits LPS-induced NFκB activation. The specificity of the assay was confirmed by using wild-type (WT) and mutant (MUT) oligonucleotides.

Additionally, to explore the effect of NMP on NFκB activation, we performed a new experiment using pGL4.32, a plasmid containing luciferase gene under the control of 5x binding sites of NFκB. Indeed, RAW264.7 cells were transfected with the pGL4.32 plasmid and then stimulated 18h with LPS in the presence or not of NMP. As shown in the **figure 5D**, LPS induced luciferase activity. In the presence of NMP, luciferase activity is almost reduced to unstimulated level suggesting that NMP inhibits the transcription driven by NFκB.

To further explore the effect of NMP on NFκB activation, we analyzed by immunofluorescence the cellular location changes of NFκB/p65 after LPS stimulation with or without NMP pre-treatment (**Fig.5E**). In unstimulated and NMP-stimulated cells NFκB/p65 was located in the cytoplasm. In LPS-treated cells NFκB/p65 translocate into the nucleus and pre-treatment with NMP significantly suppressed the LPS-induced p65 translocation.

Discussion

Macrophages play an important role during the inflammation process. A large amount of cytokines and inflammatory mediators are released after macrophage activation, including IL-1, IL-6, TNF-α, nitric oxide (NO) and prostaglandin E2 (PGE2). The inhibition of the production of these inflammatory mediators is an important target in the treatment of

inflammatory diseases. Hence, LPS-induced macrophages have usually been used as a model for evaluating the anti-inflammatory effects of various agents [12, 13].

We recently reported that NMP inhibited the osteoclast differentiation induced by RANKL [6]. Since macrophages and osteoclasts are both monocytes derived cells, we hypothesized that NMP might also affect macrophage activation and their inflammatory responses. To test this hypothesis, we investigated the effect of NMP on inflammatory mediators in LPS-stimulated RAW264.7 cells. NMP pre-treatment significantly inhibited the production of NO induced by LPS. In macrophages, NO is primarily produced by inducible nitric oxide synthase (iNOS) and high levels of this free radical might cause damage to a target tissue during an infection. We also found that NMP suppressed LPS-stimulated iNOS expression. Therefore, the regulation of NO release via NMP-induced iNOS inhibition is helpful to reduce the inflammatory destruction.

Prostaglandin E2 (PGE2), produced by COX-2 from arachidonic acid, exerts an important role during inflammation. In the present study, we found that NMP reduces LPS-induced COX-2 mRNA expression. Means to modulate NO and PGE2 are crucial for the control of an inflammatory process. Thus, NMP capable of suppressing the expression of pro-inflammatory mediators could possess anti-inflammatory potential.

Pro-inflammatory cytokines, such as TNF-α, IL-1, IL-6, GM-CSF are known to be important mediators of the inflammatory response to pathologic stimuli. The results of this study showed that NMP significantly and dose-dependently decreased the expression of the pro-inflammatory cytokines. It has been show that inhibition of the differentiation of monocytes into osteoclasts by IFN-β is mediated by an autocrine action of CXCL11 [14].The increased expression of CXCL11in this study needs to be put in perspective with our previous study showing the inhibitory effect of NMP on osteoclast differentiation [6]. Activated macrophages are known to produce MMPs to achieve matrix destruction and cell infiltration [15]. MMP activity can be reduced by TIMPs by forming 1:1 enzyme-inhibitor complexes.

Therefore, the increase of TIMP-1 by NMP, as demonstrated in our study, could decrease MMP activity and then reduce the infiltration of monocytes.

Several studies have shown that MAP kinases play an important role in the signal transduction pathways that appears to be critical in inflammatory processes [16, 17]. This increased expression of ERK phosphorylation by NMP in this study is in line with a recently published study [18, 19] where it was shown that anti-inflammatory mechanism of wedelolactone and Cucurbitacin E is accompanied by an increase of ERK phosphorylation induced by LPS. Since phosphorylation of JNK and p38 MAPKs are not affected, the effect of NMP is possibly independent and downstream of the JNK and p38 MAPK pathways. In our previous study we showed that NMP inhibits ERK activation induced by RANKL [6], whereas in the present study NMP enhances ERK activation induced by LPS. These two ligands, RANKL and LPS, act through two different receptors RANK and TLR4 (Toll-like Receptor 4) respectively. It is therefore possible that the effect of NMP on ERK activation is dependent on the activated receptor and the related intracellular pathway involved.

The transcription of many genes involved in inflammatory and immune response is controlled by NFκB [20, 21]. It is known that the activation of NFκB dimers is due to IKK (IκB kinase)-mediated phosphorylation-induced proteasomal degradation of IκB inhibitor, enabling the active NFκB to translocate to the nucleus [22]. Active NFκB induces target gene expression, among them IκB, which leads to sequester NFκB subunits and terminates transcription activity. Activation of the canonical NFκB pathway is generally associated with inflammatory exposure, while activation of the non-canonical pathway is typically related to developmental cues. Several studies have provided evidence that inter-connections between these two pathways exist. In our study, we found that NMP prevents LPS-induced inflammatory process by inhibiting canonical NFκB pathway. However, we still cannot exclude a possible inter-

connection between the canonical and non-canonical NFκB pathways or involvement of the inhibition of other signal-transduction factor.

The fact that NMP increases ERK phosphorylation induced by LPS is in agreement with the data which showed that the constitutive active MEK/ERK pathways negatively regulate NFκB-driven transcription [23]. Moreover, it has been shown that ERK inhibitor potentiates the binding of NFκB and consequently its pro-inflammatory effect [24]. Other studies have reached the same conclusion; Jones et al., [25] shows that PMA (phorbol-12-myristate-13-acetate), which is known to activate ERK pathway, significantly attenuated LPS-induced NO production in RAW264.7 cells. Another group demonstrated that hyper activation of the ERK pathway contributes to the negative regulation of LPS-induced IL-12 p40 production in mouse peritoneal macrophages [26]. In our study, we found that ERK inhibition (by U0126) and ERK activation (by serum and PMA) potentiates and inhibits LPS-induced NO production respectively, which is in line with iNOS expression. Taken together our results suggest that NMP inhibits LPS-induced inflammatory processes by a mechanism involving ERK and NFκB pathways.

Recently, we showed that NMP acts as a low affinity bromodomain inhibitor and its possible role as clinically applicable candidate for the application of epigenetics as new mechanism for osteoporosis treatment [27]. Epigenetics refers to transmissible changes in gene expression that does not involve changes to the underlying DNA sequence. BRD/BET (bromodomain and extraterminal domain) proteins are a group of epigenetic regulators. Inhibitors of BRD and BET, which prevent bromodomain binding to acetyl-modified histone tails, have shown therapeutic promise in several diseases including inflammation related ones [28]. This result is in agreement with recently published data. In fact, Shortt at al. showed that NMP is a functional lysine mimetic molecule [29]. Moreover, JQ1 a potent inhibitor of BRD2, BRD3, BRD4 and BET, was shown to suppress inflammation by a reduction of the inflammatory

cytokine release and bone destruction by the inhibition of osteoclast maturation [30]. In this study, the authors showed that JQ1 neutralized BRD4 enrichment at several gene promoter regions, including NF-κB, TNF-α, c-Fos, and NFATc1. Furthermore, the binding of Brd4 to acetylated lysine-310 is essential for the recruitment of Brd4 to the promoters of NF-κB target genes and to coactivate NF-κB [31]. Recently we showed that NMP prevents BRD4 from binding acetylated lysine [27]. Here we showed that NMP inhibits NF-κB pathway, most likely by preventing BRD4 binding. Apparently NMP has a similar mode of action as JQ1 since both inhibit bone destruction and reduce inflammatory response.

In summary, our findings demonstrate that NMP is an effective inhibitor of LPS-induced pro-inflammatory mediators in RAW264.7 macrophages through the inhibition of NFκB pathway. Our result is in line with recently published paper showing that NMP possess an immunomodulatory activity [29].

Nevertheless, the role and underlying mechanism of NMP-induced activation of ERK MAPK in LPS-stimulated cells remain to be further clarified.

Molecules, like NMP which enhance bone regeneration and suppress osteoclast differentiation [6, 7] and inhibit inflammatory mediators as demonstrated in this study strengthens its potential for treating RA, osteoporosis [27] and other inflammatory-related bone diseases.

The major focus of the present work is the early inflammatory events that have been shown to contribute to the exacerbation of damage. However, it has also been suggested that inflammation is a necessary part of the healing process; inflammation is important in recovery and repair at later time points. We recognize that further studies are needed to investigate this point and to verify the effectiveness of NMP *in vivo*.

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References

1. Huang H, Zhao N, Xu X, Xu Y, Li S, Zhang J, et al. Dose-specific effects of tumor necrosis factor alpha on osteogenic differentiation of mesenchymal stem cells. Cell Prolif 2011; 44:420-7.
2. Tanaka Y, Nakayamada S, Okada Y. Osteoblasts and osteoclasts in bone remodeling and inflammation. Curr Drug Targets Inflamm Allergy 2005; 4:325-8.
3. Guha M, Mackman N. LPS induction of gene expression in human monocytes. Cell Signal 2001; 13:85-94.
4. Kim JB, Han AR, Park EY, Kim JY, Cho W, Lee J, et al. Inhibition of LPS-induced iNOS, COX-2 and cytokines expression by poncirin through the NF-kappaB inactivation in RAW 264.7 macrophage cells. Biol Pharm Bull 2007; 30:2345-51.
5. Chan ED, Riches DW. IFN-gamma + LPS induction of iNOS is modulated by ERK, JNK/SAPK, and p38(mapk) in a mouse macrophage cell line. Am J Physiol Cell Physiol 2001; 280:C441-50.
6. Ghayor C, Correro RM, Lange K, Karfeld-Sulzer LS, Gratz KW, Weber FE. Inhibition of osteoclast differentiation and bone resorption by N-methylpyrrolidone. J Biol Chem 2011; 286:24458-66.
7. Miguel BS, Ghayor C, Ehrbar M, Jung RE, Zwahlen RA, Hortschansky P, et al. N-methyl pyrrolidone as a potent bone morphogenetic protein enhancer for bone tissue regeneration. Tissue Eng Part A 2009; 15:2955-63.
8. Takahashi N, Udagawa N, Kobayashi Y, Suda T. Generation of osteoclasts in vitro, and assay of osteoclast activity. Methods Mol Med 2007; 135:285-301.
9. Gao HM, Hong JS. Why neurodegenerative diseases are progressive: uncontrolled inflammation drives disease progression. Trends Immunol 2008; 29:357-65.
10. Ritchlin CT, Haas-Smith SA, Li P, Hicks DG, Schwarz EM. Mechanisms of TNF-alpha- and RANKL-mediated osteoclastogenesis and bone resorption in psoriatic arthritis. J Clin Invest 2003; 111:821-31.
11. Boyer L, Travaglione S, Falzano L, Gauthier NC, Popoff MR, Lemichez E, et al. Rac GTPase instructs nuclear factor-kappaB activation by conveying the SCF complex and IkBalpha to the ruffling membranes. Mol Biol Cell 2004; 15:1124-33.
12. Ci X, Ren R, Xu K, Li H, Yu Q, Song Y, et al. Schisantherin A exhibits anti-inflammatory properties by down-regulating NF-kappaB and MAPK signaling pathways in lipopolysaccharide-treated RAW 264.7 cells. Inflammation 2010; 33:126-36.
13. Rhule A, Navarro S, Smith JR, Shepherd DM. Panax notoginseng attenuates LPS-induced pro-inflammatory mediators in RAW264.7 cells. J Ethnopharmacol 2006; 106:121-8.
14. Coelho LF, Magno de Freitas Almeida G, Mennechet FJ, Blangy A, Uze G. Interferon-alpha and -beta differentially regulate osteoclastogenesis: role of differential induction of chemokine CXCL11 expression. Proc Natl Acad Sci U S A 2005; 102:11917-22.
15. Ye S. Influence of matrix metalloproteinase genotype on cardiovascular disease susceptibility and outcome. Cardiovasc Res 2006; 69:636-45.
16. Amir M, Somakala K, Ali S. P38 MAP Kinase Inhibitors as Anti inflammatory Agents. Mini Rev Med Chem 2013.
17. Wysk M, Yang DD, Lu HT, Flavell RA, Davis RJ. Requirement of mitogen-activated protein kinase kinase 3 (MKK3) for tumor necrosis factor-induced cytokine expression. Proc Natl Acad Sci U S A 1999; 96:3763-8.
18. Yuan F, Chen J, Sun PP, Guan S, Xu J. Wedelolactone inhibits LPS-induced pro-inflammation via NF-kappaB Pathway in RAW 264.7 cells. J Biomed Sci 2013; 20:84.

19. Qiao J, Xu LH, He J, Ouyang DY, He XH. Cucurbitacin E exhibits anti-inflammatory effect in RAW 264.7 cells via suppression of NF-kappaB nuclear translocation. Inflamm Res 2013; 62:461-9.
20. Caamano J, Hunter CA. NF-kappaB family of transcription factors: central regulators of innate and adaptive immune functions. Clin Microbiol Rev 2002; 15:414-29.
21. McKay LI, Cidlowski JA. Molecular control of immune/inflammatory responses: interactions between nuclear factor-kappa B and steroid receptor-signaling pathways. Endocr Rev 1999; 20:435-59.
22. Vallabhapurapu S, Karin M. Regulation and function of NF-kappaB transcription factors in the immune system. Annu Rev Immunol 2009; 27:693-733.
23. Carter AB, Hunninghake GW. A constitutive active MEK --> ERK pathway negatively regulates NF-kappa B-dependent gene expression by modulating TATA-binding protein phosphorylation. J Biol Chem 2000; 275:27858-64.
24. Puig-Kroger A, Relloso M, Fernandez-Capetillo O, Zubiaga A, Silva A, Bernabeu C, et al. Extracellular signal-regulated protein kinase signaling pathway negatively regulates the phenotypic and functional maturation of monocyte-derived human dendritic cells. Blood 2001; 98:2175-82.
25. Jones E, Adcock IM, Ahmed BY, Punchard NA. Modulation of LPS stimulated NF-kappaB mediated Nitric Oxide production by PKCepsilon and JAK2 in RAW macrophages. J Inflamm (Lond) 2007; 4:23.
26. Saito S, Matsuura M, Hirai Y. Regulation of lipopolysaccharide-induced interleukin-12 production by activation of repressor element GA-12 through hyperactivation of the ERK pathway. Clin Vaccine Immunol 2006; 13:876-83.
27. Gjoksi B, Ghayor C, Siegenthaler B, Roungsawasdi N, Zenobi-Wong M, Weber FE. The epigenetically active small chemical N-Methyl Pyrrolidone (NMP) prevents estrogen depletion induced osteoporosis. Bone 2015; DOI: 10.1016/j.bone.2015.05.004.
28. Mirguet O, Lamotte Y, Chung CW, Bamborough P, Delannee D, Bouillot A, et al. Naphthyridines as novel BET family bromodomain inhibitors. ChemMedChem 2014; 9:580-9.
29. Shortt J, Hsu AK, Martin BP, Doggett K, Matthews GM, Doyle MA, et al. The drug vehicle and solvent N-methylpyrrolidone is an immunomodulator and antimyeloma compound. Cell Rep 2014; 7:1009-19.
30. Meng S, Zhang L, Tang Y, Tu Q, Zheng L, Yu L, et al. BET Inhibitor JQ1 Blocks Inflammation and Bone Destruction. Journal of dental research 2014; 93:657-662.
31. Huang B, Yang XD, Zhou MM, Ozato K, Chen LF. Brd4 coactivates transcriptional activation of NF-kappaB via specific binding to acetylated RelA. Molecular and cellular biology 2009; 29:1375-87.

FIGURE LEGENDS

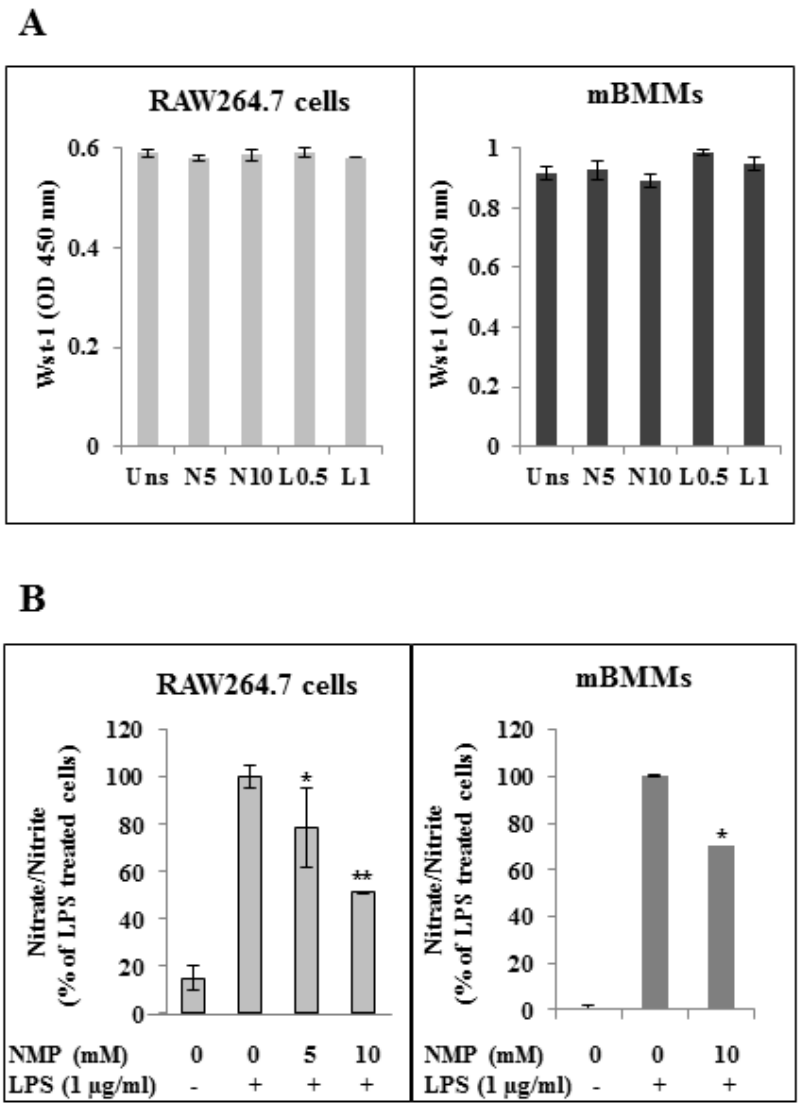

Figure 1 *Effect of NMP on cell viability/cytotoxicity and NO production.* **A:** Cell viability assay. RAW 264.7 cells and mouse Bone Marrow-derived Macrophages (mBMMs) were seeded on a 96-well plate, treated for 48h as indicated in the figure. Cell viability/cytotoxicity was measured using Wst-1 reagent as described in Methods. Data are expressed as mean S.D. (n=3) from a representative experiment. **B:** *Effect of NMP on NO production.* RAW264.7 cells and mouse Bone Marrow-derived Macrophages (mBMMs) were incubated with 1µg/ml LPS with or without NMP for 24h. The amount of NO in the supernatants was measured by Griess reagent and the results are expressed as percentage in comparison with LPS-treated samples. Data are expressed as mean S.D. (n=3) from a representative experiment.

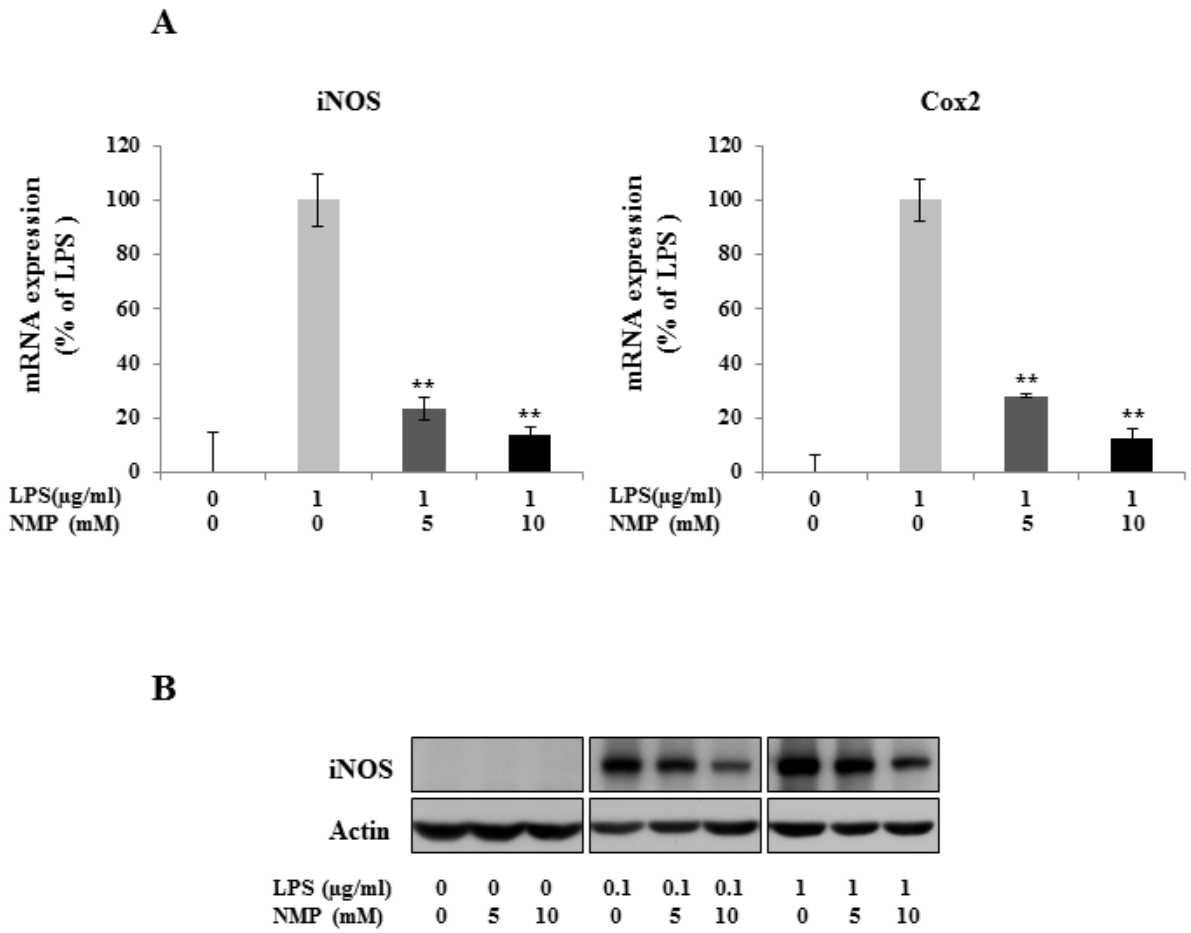

Figure 2 *Effect of NMP on iNOS and COX-2 expression*. **A:** RAW 264.7 cells were treated with LPS (1μg/ml) in the absence or presence of NMP, and total RNA was isolated 6h after treatment. iNOS and COX-2 mRNA levels were determined by real-time PCR and normalized to GAPDH level and expressed as percentage in comparison with LPS-treated samples. Data are expressed as mean S.D. (n=3) from a representative experiment. **B:** RAW264.7 cells were treated for 48h as indicated in the figure, and whole cell lysates were subjected to Western-blot analysis with antibody against iNOS. Actin antibody was used as loading control.

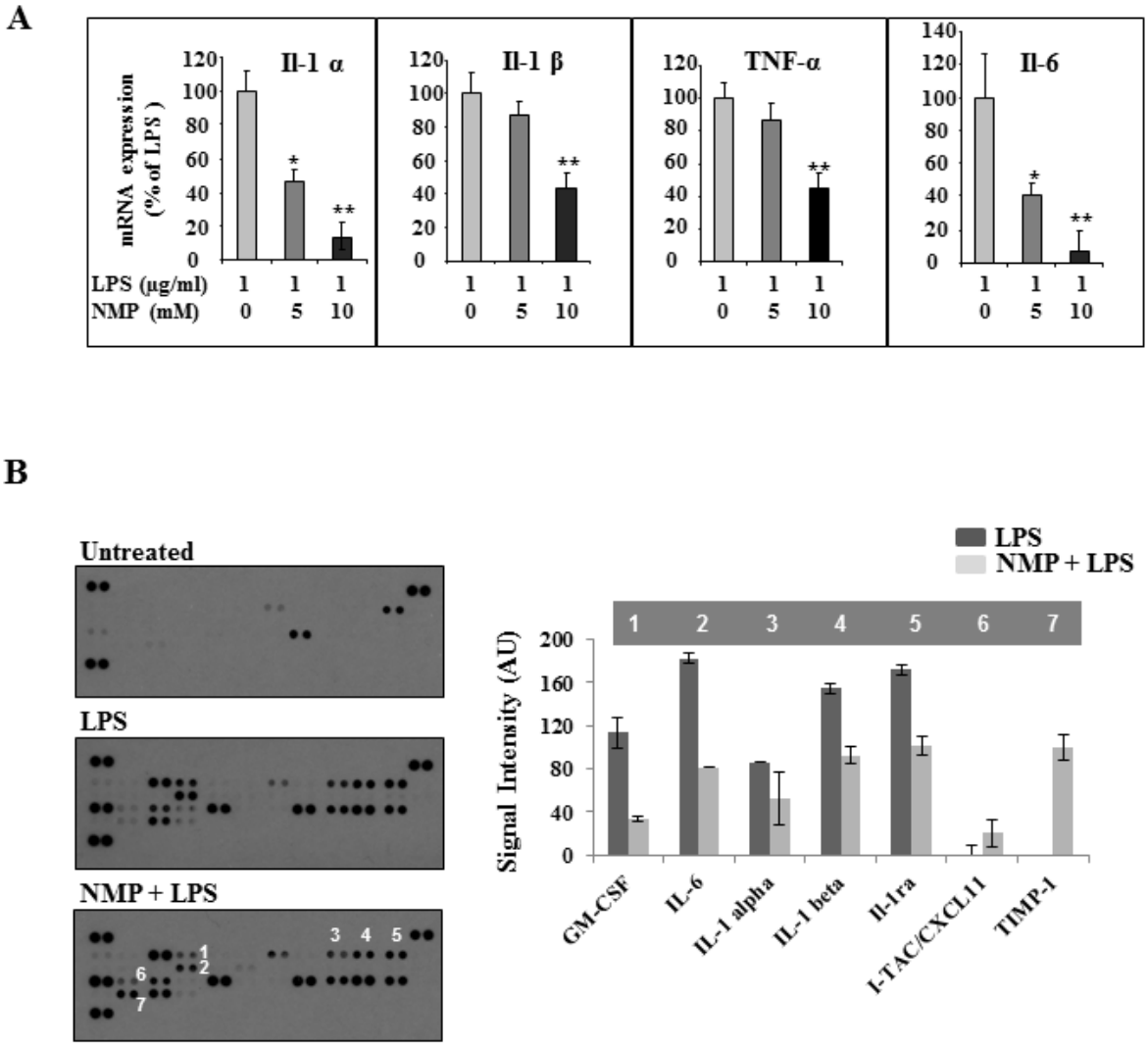

Figure 3 *Effect of NMP on LPS-induced pro-inflammatory cytokines*. **A:** RAW 264.7 cells were treated with LPS (1µg/ml) in the absence or presence of NMP, and total RNA was isolated 6h after treatment. Il-1α, IL-β, TNF-α and IL-6 mRNA levels were determined by real-time PCR and normalized to GAPDH level. Results are expressed as percentage in comparison with LPS-treated samples. Data are expressed as mean S.D. (n=3) from a representative experiment. **B:** RAW 264.7 cells were treated with 1µg/mL LPS in the absence or presence of NMP (10 mM) for 18 h. Proteome Profiler system (mouse cytokine array panel A, R&D) was used to screen different acute phase proteins, cytokines, and chemokines involved in the inflammatory process. Numbers on membranes mark the following targets: "**1**" GM-CSF; "**2**" IL-6; "**3**" IL-1α; "**4**" IL-1β; "**5**" IL-1ra; "**6**" I-TAC/CXCL11; "**7**" TIMP-1.

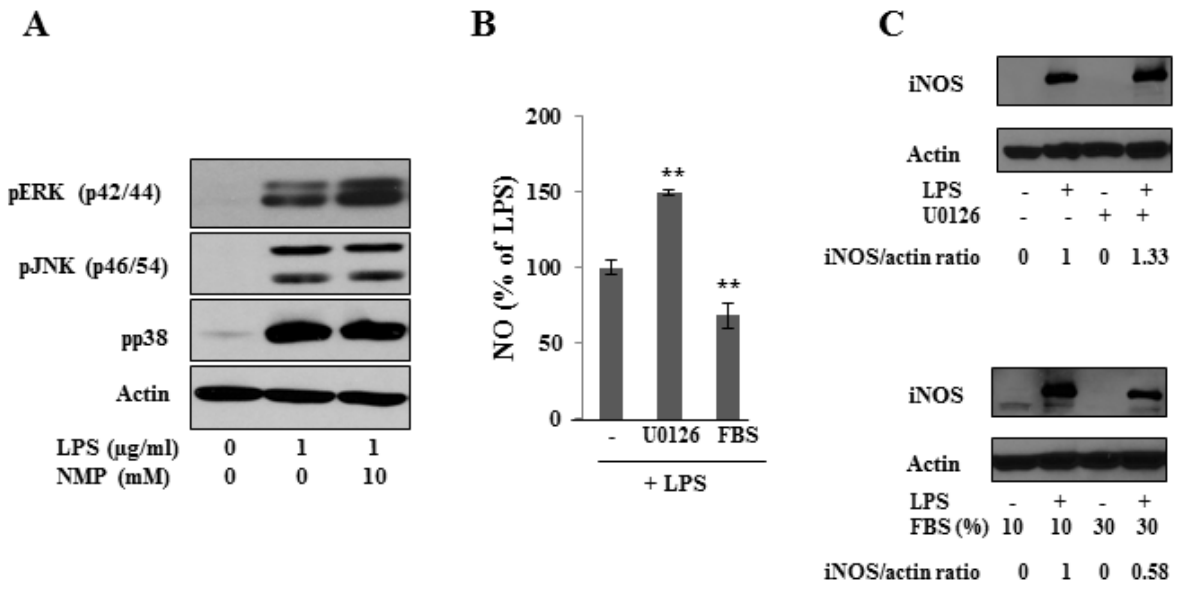

Figure 4 *Effect of NMP on LPS-induced MAP kinases phosphorylation.* **A:** RAW 264.7 cells were treated with LPS (1 µg/ml) or LPS and NMP (10 mM) for 30 min. Cells were lysed and total proteins were subjected to SDS-PAGE and blotted onto PVDF membrane. Phospho-p38, phospho-ERK and phospho-JNK were immunodetected using specific rabbit polyclonal antibodies. Actin was used as a loading control. **B:** RAW 264.7 cells were incubated 1h with ERK inhibitor (10 µM U0126) or 30% of FBS and then treated with 1µg/ml LPS for 24h. The amount of NO in the supernatants was measured by Griess reagent. Data are expressed as mean S.D. (n=3) from a representative experiment. **C:** RAW 264.7 cells were pre-treated 1h with ERK inhibitor (10 µM U0126) or with serum (30% FBS) as indicated in the figure and then stimulated 48 h with LPS. After stimulation whole cell lysates were subjected to Western-blot analysis with iNOS antibody. Actin antibody was used as loading control.

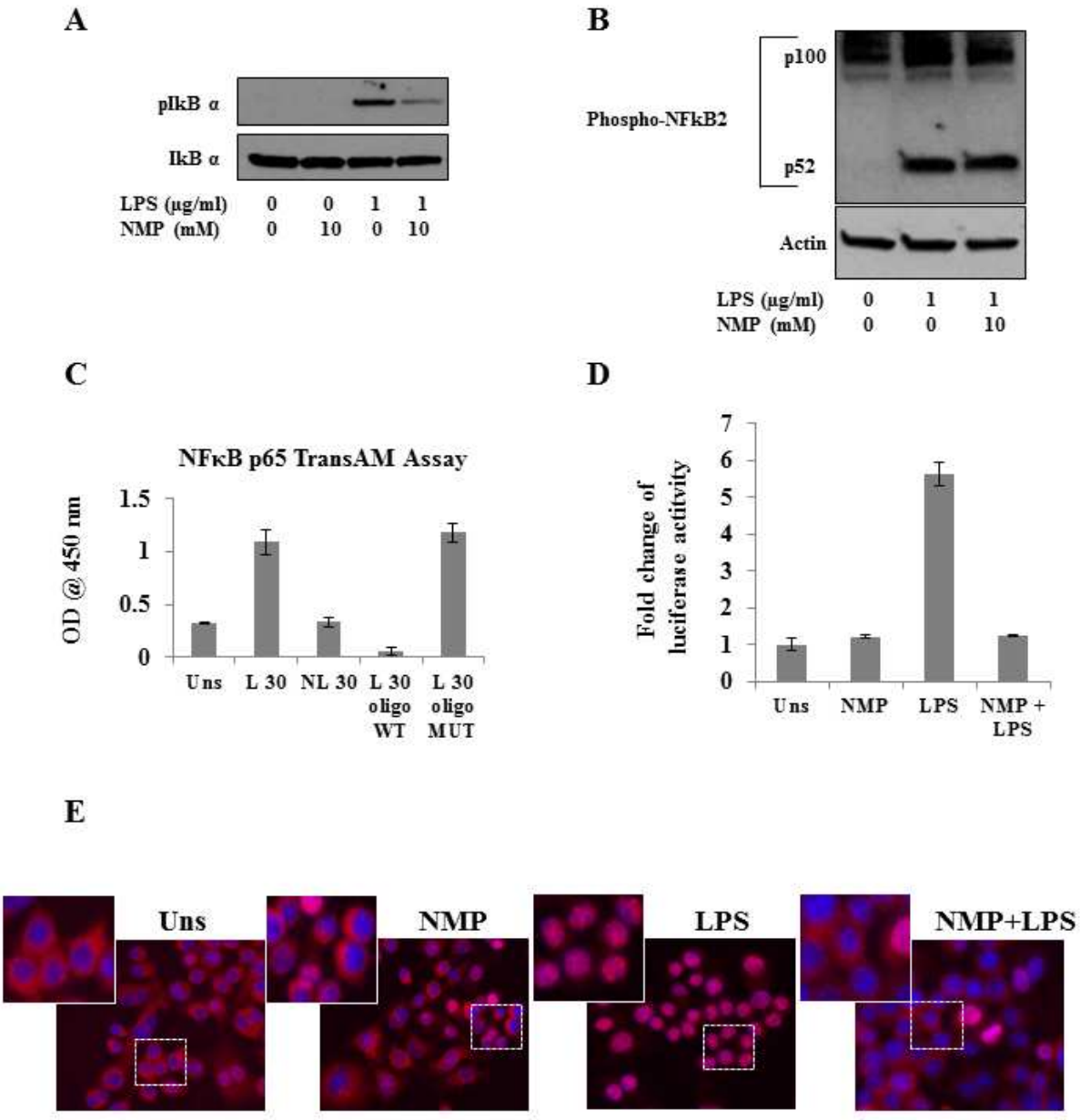

Figure 5 *Effect of NMP on NF-kB* activation. **A:** RAW 264.7 cells were pre-treated or not with NMP for 1h and then stimulated with LPS for 5 minutes. After stimulation whole cell lysates were subjected to Western-blot analysis with antibody against phosphorylated and non-phosphorylated forms of IkBα. **B:** RAW 264.7 cells were pre-treated or not with NMP for 1h and then stimulated with LPS for 5 minutes. After stimulation whole cell lysates were subjected to Western-blot analysis with antibody against phospho- NFκB2 (p100/p52). Actin was used as a loading control. **C:** RAW 264.7 cells were stimulated 30 minutes with LPS (**L30**) or incubated 1h with NMP and then stimulated with LPS for 30 minutes (**NL30**).

Nuclear extracts from unstimulated (Uns) and stimulated cells were used to detect NF-kB activation using an ELISA-based method (TransAM NF-kB p65, Active Motif, Rixensart, Belgium) as indicated in Methods. The specificity of the assay was monitored by stimulating cells with LPS (**L30**) and using free wild-type (**WT**) or mutated (**MUT**) oligonucleotides according to the manufacturer's instructions. Data are expressed as mean S.D. (n=3) from two different experiments. **D:** RAW264.7 cells were transfected with pGL4.32 vector as indicated. An NFκB assay was performed as described in **MATERIALS AND METHODS**. After transfection, the cells were treated with or without NMP before 24-h treatment with 1 μg/ml LPS. Values are means ± SEM; representative data from 1 of 3 independent experiments performed in triplicate are shown. **E:** RAW 264.7cells were grown on 15 μ-Chamber 12 well (Ibidi, Martinsried, Germany), stimulated with NMP, LPS and LPS after NMP pre-treatment (NMP+LPS). After 30 min stimulation, cells were fixed in 4% paraformaldehyde for 15min at room temperature and the subcellular localization of the NF-kB p65 subunit was shown by immunofluorescence staining. The fixed cells were blocked for 1h with 5% normal goat serum in PBS and incubated with a diluted solution of the primary antibody (1:100; CST, USA) overnight at 4°C. Cells were then washed 3 times in PBS and incubated for 1 h with the Alexa Fluor 488 F(ab')2 fragment of the anti-rabbit antibody (1:1000; CST, USA). Nuclei were counterstained with Hoechst (Sigma- Aldrich). Preparations were then observed with a fluorescent microscope and images were recorded.