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The carotenoid lutein enhances matrix metalloproteinase-9 production and phagocytosis through intracellular ROS generation and ERK1/2, p38 MAPK, and RARβ activation in murine macrophages

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ABSTRACT

Early studies have demonstrated the ability of dietary carotenoids to enhance immune response, but the mechanism underlying their influence on macrophage activity remains unclear. Here, we investigated the effects of carotenoids on macrophage activity. Carotenoids, including lutein and lycopene, enhanced MMP-9 activity in RAW264.7 macrophages. Lutein was chosen as a representative and analyzed further in this study. It increased the synthesis, activity, and release of MMP-9 in murine RAW264.7 and primary-cultured peritoneal macrophages. MMP-9 induction by lutein was through the transcriptional regulation of *mmp-9*. It was blunted by the MAPK inhibitors targeting ERK1/2 and p38 MAPK, the reagents that inhibit free radical signaling, and the inhibitors and siRNA targeting RARβ. Moreover, lutein induced Nox activation and intracellular ROS production at an early stage of treatment. This carotenoid also caused ERK1/2 and p38 MAPK activation, RARβ expression, and RAR interaction with its responsive element in the promoter region. These findings suggest the involvement of ROS, MAPKs, and RARβ activation in lutein-driven MMP-9 expression and release. Interestingly, lutein enhanced the phagocytic activity of macrophages, and the secreted MMP-9 appeared to be involved in this process. In summary, we provide evidence here for the first time that the carotenoid lutein induces intracellular ROS generation and MAPK and RARβ activation in macrophages, leading to an increase in MMP-9 release and macrophage phagocytosis. Our results demonstrate that lutein exerts an immunomodulatory effect on macrophages. *J. Leukoc. Biol.* 93: 723–735; 2013.

Introduction

Macrophages are part of the innate immune system. They are highly efficient phagocytes that play an important role in the recognition and clearance of many potential pathogens. They can also recognize tumor, senescent, and dying cells. Aside from their roles in primary innate immunity, macrophages function as regulator and effector cells in humoral and cell-mediated immune responses [1]. Resting macrophages have limited antimicrobial activity; however, immunologically activated macrophages acquire microbicidal and tumoricidal activities. These macrophages produce proinflammatory and suppressive cytokines, chemokines, growth factors, and proteases. They also produce ROS and reactive nitrogen metabolites.

MMPs participate in much of the turnover and degradation of ECM components and basement membranes, cell migration, and the processing and activation or inactivation of soluble factors. Hitherto, >20 MMPs have been identified [2, 3]. MMPs are involved in a wide range of proteolytic events in fetal development and normal tissue remodeling, as well as wound healing and inflammation [4]. Dysregulation in the levels and control of MMPs can lead to pathological conditions, such as tumor formation, arthritis, and atherosclerosis. Cells of the monocyte/macrophage lineage secrete diverse

Abbreviations: β-gal=β-galactosidase, BCECF/AM=2′,7′ bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein/AM, HPF=high-power field, Luc=luciferase, LXR=liver X receptor, MMP=matrix metalloproteinase, NAC=N-acetylcysteine, Nox=NADPH oxidase, NR=nuclear receptor, PPAR=peroxisome proliferator-activated receptor, RA=retinoic acid, RAR=retinoic acid receptor, RARE=retinoic acid response element, RXR=retinoid X receptor, siRNA=small interference RNA, VSMC=vascular smooth muscle cell

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MMPs in large quantities [5]. Monocyte/macrophage MMP production has been linked to ECM degradation, cell migration, and regulation of cytokines, chemokines, and other soluble proteins during inflammation [2].

Carotenoids are plant pigments responsible for the color observed in many fruits and vegetables, as well as the natural colors seen in certain animal species [6]. Humans—incapable of synthesizing carotenoids—obtain them by eating foods rich in these compounds. The predominant carotenoids found in human plasma are β-carotene, lycopene, and lutein, and their concentrations vary from 0 to 8 μM, depending on dietary intake [7]. These carotenoids share a polyene backbone structure with retinol (vitamin A) and its bioactive metabolite ATRA [8]. Retinol and ATRA have been known to interact with RA-binding transcriptional regulators belonging to the NR superfamily. The RARs (RARα, -β, and -γ isotypes) bind ATRA and 9-cis RA, whereas the RXRs (RXRα, -β, and -γ isotypes) only bind 9-cis RA [9].

The role of vitamin A in immunity has been well established. Early studies have demonstrated that dietary β-carotene prevents organ infection in vitamin A-deficient rats [10] and reduces ear infection in young children [11]. Moreover, vitamin A deficiency compromises the mucosal barriers of several tissues [12]. As β-carotene possesses provitamin A activity, these studies raised the possibility that the action of β-carotene is a result of its prior conversion to vitamin A. Thereafter, several studies have shown the possible immunomodulatory action of nonprovitamin A carotenoids, but these studies focused mostly on the effects of these carotenoids on the functions of T- and B-lymphocytes and splenocytes [13]. The effects of carotenoids on monocytes/macrophages are less understood.

In the present study, we investigated the effects of carotenoids on macrophage activity. We found that lutein and lycopene were able to increase MMP-9 activity. We demonstrate here for the first time that lutein induces intracellular ROS generation and MAPK and RARβ activation in macrophages, leading to an increase in MMP-9 release and macrophage phagocytosis.

MATERIALS AND METHODS

Materials

MMP inhibitor (GM6001) and DPI were from Enzo Life Sciences (Farmingdale, NY, USA). The MMP-9-specific inhibitor (MMP-9 I) was purchased from Calbiochem (EMD Bioscience, San Diego, CA, USA). All RAR/RXR agonists/antagonists were from Tocris Bioscience (Bristol, UK). MTT, LPS, and signaling inhibitors were purchased from Sigma-Aldrich (St. Louis, MO, USA). The MMP-9 antibody was purchased from Abcam (Cambridge, MA, USA). The antibody against phospho-ERK1/2 was from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and the mAb for JNK, phospho-p38, and phospho-JNK were from Cell Signaling Technology (Danvers, MA, USA). The mAb for total p38 and ERK1/2 were from R&D Systems (Minneapolis, MN, USA). Lutein and lycopene were purchased from Extrasynthese (Genay Cedex, France). Lutein was dissolved in DMSO, and lycopene was dissolved in tetrahydrofuran (Sigma-Aldrich).

Cell cultures

Murine RAW 264.7 macrophages and human U937 monocytes were obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). RAW 264.7 macrophages were cultured in 90% DMEM, containing 10% FBS, supplemented with amino acids and antibiotics [14]. Human U937 monocytes were maintained in RPMI-1640 medium. Primary-cultured VSMCs were prepared and maintained as described previously [15]. Primary-cultured peritoneal macrophages were prepared following the procedure described previously [16]. Briefly, ICR mice were untreated or injected i.p. with 1.0 ml of 3% (w/v) thioglycolate (Sigma-Aldrich) in serum-free medium, 5 days prior to cell harvest. ICR mice were sacrificed by CO_2 asphyxiation. The peritoneal macrophages were collected by peritoneal lavage using 10 ml serum-free medium. After several washes, cells were allowed to adhere to culture dishes and were cultured in the same medium used for RAW264.7 before further analysis. Animal experimental procedures were approved by the Laboratory Animal Use and Care Committee of Fu-Jen University.

Gelatin zymography and Western blot analysis of MMP-9 expression and release

For measuring MMP-9 activity in culture media, equal numbers of cells were seeded in 24-well culture plates, to which equal volumes of medium were added. After cells were treated with lutein, equal volumes of culture medium were removed, and MMP-9 activity was analyzed by gelatin zymography under nonreducing conditions [15]. In some instances, MTT assay (see below) of the remaining cells was performed to ensure that equal numbers of cells were seeded. Gelatinolytic activity of MMP-9 was detected as clear bands. Western blot analysis was used to detect MMP-9 expression and release in cell lysates or culture media. Cell lysates were prepared by washing cells with prechilled PBS, followed by lysis in RIPA buffer [20 mM Tris-HCl (pH 7.4), 137 mM NaCl, 2 mM EDTA, 1 mM sodium fluoride, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10% glycerol, 1 mM sodium orthovanadate, 1 mM PMSF, and 1 mg/ml aprotinin and leupeptin (freshly prepared)]. The protein content was quantified using the Pierce protein assay kit (Pierce, Rockford, IL, USA). Equal amounts of samples were analyzed by Western blotting [17]. Zymographs and chemiluminescent immunoblot images were captured using the BioSpectrum imaging system (Ultra-Violet Products, Cambridge, UK). The intensity of each band in the captured images was analyzed using ImageQuant 5.0 software (Molecular Dynamics, Amersham Pharmacia Biotech, Sunnyvale, CA, USA).

RT-PCR analysis of MMP-9, RAR, and RXR mRNA levels

Oligonucleotide PCR primers, targeting murine RARα/β/γ, RXRα/β/γ, and MMP-9, were synthesized (MDBio, Taipei, Taiwan; **Table 1**). Macrophage total RNA was extracted using Trizol reagents (Invitrogen, Carlsbad, CA, USA). RT reaction was performed with aliquots of 1–2 μg total RNA and random hexaprimers using the Superscript III First-Strand Synthesis System (Invitrogen), according to the manufacturer's instructions. PCR was performed with 30 cycles of denaturation at 94°C for 1 min, annealing at 51–56°C for 1 min, and elongation at 72°C for 1.5 min using the ABI 7200 Thermal Cycler (Applied Biosystems, Foster City, CA, USA). The amplification products were analyzed by electrophoresis in 2% agarose gels.

MTT assay

MTT assay for cell viability (growth) was performed as described previously [18]. Cells were incubated with 0.5 mg/ml MTT for 2 h at 37°C. Formazan crystals resulting from MTT reduction were dissolved by adding DMSO. The absorbance of the supernatant was then measured spectrophotometrically by using an ELISA reader at 550 nm.

Measurement of intracellular ROS production

Intracellular ROS production was determined by flow cytometry or fluorometry. Treated cells were washed with serum-free medium and loaded with a ROS-specific dye, 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (5 μM; Invitrogen), at 37°C for 15 min. After a brief wash with PBS, cells were analyzed immediately by a CyFlow ML

TABLE 1. Primers for RT-PCR Analysis

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
MMP-9	TTCTGCCCTACCCGAGTGGA	CATAGTGGGAGGTGCTGTCGG
GAPDH	ATGGTGAAGGTCGGTGTGAACG	GTTGTCATGGATGATCTTGGCC
RARα	TGAGGGCTGTAAGGGCTTC	GTGCATCTGAGTCCGGTTC
RARβ	TCCTCCTCGGGTGTACAAG	TCTGGTCTGCGATGGTCAG
RARγ	GCCCGACAGCTATGAACTG	AGCTTGTCCACCTTCTCGG
RXRα	GCCGAGACAACAAGGACTG	CAGCTCTGTTAGCACCCTG
RXRβ	CAACTCCACAGTGTCGCTC	TCTGCTCCACAGCAAGCTC
RXRγ	TGTCTCATCGACAAGCGCC	CTCTCGAAGAGTCTCCACC

flow cytometer (Partech GmBH, Munster, Germany). Fluorescence signals from 10,000 cells were collected to calculate the mean fluorescence intensity of a single cell. Alternatively, fluorescence signals from cells on plates were measured by fluorometry using a fluorescence microplate reader (Wallac 1420 Victor^3 multilabel counter; PerkinElmer, Turku, Finland).

Analysis of p47 phox Nox level

Levels of p47 phox Nox in cytosolic and membrane fractions were measured by Western blotting, as described previously [19]. RAW264.7 macrophages cultured in 10-cm culture dishes were treated with DMSO or lutein. They were washed once with ice-cold PBS and collected with a rubber policeman after adding 300 μl homogenization buffer A [20 mM Tris-HCl (pH 8.0), 10 mM EGTA, 2 mM EDTA, 2 mM DTT, 1 mM PMSF, 25 μg/ml aprotinin, and 10 μg/ml leupeptin] to each dish. The suspension was sonicated for 10 s at Output 4 with a sonicator and centrifuged at 8000 rpm for 15 min at 4°C. The resultant pellet was the nuclear fraction. The supernatant was centrifuged at 15,000 rpm for 60 min at 4°C to yield a pellet (membrane fraction) and a supernatant (cytosolic fraction). The membrane fraction was resuspended in 60 μl Buffer A with Triton X-100 (Sigma-Aldrich). Samples from these supernatant fractions were denatured, subjected to analysis by SDS-PAGE, and transferred to PVDF membranes. The translocation of p47 phox was identified by Western blot analysis using an anti-p47 phox antibody (Cell Signaling Technology). The levels of p47 phox, α-tubulin (Calbiochem, EMD Bioscience), and PDGFRβ (Santa Cruz Biotechnology) in the cytosolic or membrane fractions were analyzed by the BioSpectrum imaging system and ImageQuant 5.0 software.

Transient transfection and Luc reporter assay

For the MMP-9-Luc reporter assay, cells were transfected with pGL-MMP-9-Luc, which was a reporter construct with a murine WT MMP-9 promoter fragment spanning nucleotides −1340 to +1. The promoter region was amplified from the genomic DNA of RAW264.7 macrophages using primers with linkers and restriction enzyme sites for cloning into the pGL3-Luc reporter plasmid. The primers were designed according to the promoter region of the *Mus musculus* pro-MMP-9 (*mmp-9*) gene (Accession No: AF403768) [20]. They were as follows: 5′-CCGGGTACCTGTAGAGAGCGTATCACAACGTCC-3′ (forward) and 5′-CCGGAGCTCATGGTGAGGACCGCAGCTTCTGGC-3′ (reverse). The accuracy of the resulting MMP-9 sequence was confirmed by DNA sequencing. For the pRAR-Luc reporter assay, cells were transfected with pRAR-Luc [a reporter construct containing three copies of RARE (Signosis, Sunnyvale, CA, USA)]. Reporter transfection was performed by seeding cells (2.5×10^5 cells/ml) in 12-well culture plates (Costar, Corning, NY, USA); the cells were then transfected with 0.75 μg total DNA using PolyJet in vitro DNA transfection reagent (SignaGen Lab, Rockville, MD, USA) for 18 h in medium, according to the manufacturer's protocol. All DNAs were prepared using endotoxin-free plasmid preparation kits (Qiagen, Valencia, CA, USA) with 0.75 μg reporter construct or pSV-β-gal control vector (Promega, Madison, WI, USA). Following transfection, cells were washed once with endotoxin-free medium and then allowed to grow for 24 h in complete medium containing antibiotics. The reporter firefly Luc values were obtained by analyzing 1 ml purified cell extract, according to standard instructions provided by the Luc kit (Promega) in a Wallac 1420 Victor^3 multilabel counter (PerkinElmer). β-Gal activity was determined using o-nitrophenyl-β-D-galactopyranoside as a substrate [21]. The relative Luc activities of cell extracts were typically represented as firefly Luc/β-gal values.

siRNA transfection

ON-TARGET plus SMARTpool RARβ (Gene ID: 218772) and negative control siRNA were purchased from Dharmacon RNAi Technologies (Thermo Fisher Scientific, Waltham, MA, USA). Transfection was performed according to the manufacturer's protocol with some modifications. Macrophages were seeded in 24-well plates for incubation overnight in complete medium. Cell cultures were transfected with control siRNA or RARβ siRNA stock (100 μM) using the DharmaFECT transfection reagent for 72 h. After treatment with vehicle (DMSO) or lutein, the culture media and cell lysates were prepared for further analysis.

Phagocytosis assay

BCECF/AM (Invitrogen) is the most widely used fluorescent indicator of intracellular pH, cell adhesion, and migration. The phagocytosis of BCECF/AM-loaded yeast cells by leukocytes has been described previously [22]. Macrophages were incubated with vehicle or lutein for 16 h. The yeasts were then labeled with BCECF/AM (10 μg/ml) for 30 min at 37°C, and an equal number (1×10^7/well) were added directly into macrophage serum-free medium. After incubation for 1 h, cells were washed thrice with medium, followed by fixation with 4% paraformaldehyde. Phagocytosis was examined and photographed under an Eclipse Ti-S inverted fluorescence microscope (Nikon, Japan), equipped with a high-resolution camera. The phagocytic index was measured by: (1) the direct counting of ingested yeasts (fluorescent spots) in nine randomly selected regions at a magnification of 200× (HPF); (2) the number of macrophages associated with ingested yeasts/total macrophages; and (3) the number of ingested yeasts/one macrophage.

Statistical analysis

Data were expressed as mean ± SEM. The means of two groups of data were compared using the unpaired, two-tailed Student's *t*-test.

RESULTS

Carotenoids increase MMP activity in murine macrophages

To investigate whether carotenoids increased MMP activity in murine macrophages, gelatin zymography was performed to analyze the macrophage culture medium. **Figure 1A** shows that lutein and lycopene increased MMP activity in the culture medium of RAW264.7 macrophages. The activities of a major MMP migrating at ~80 kDa and a minor MMP migrating at

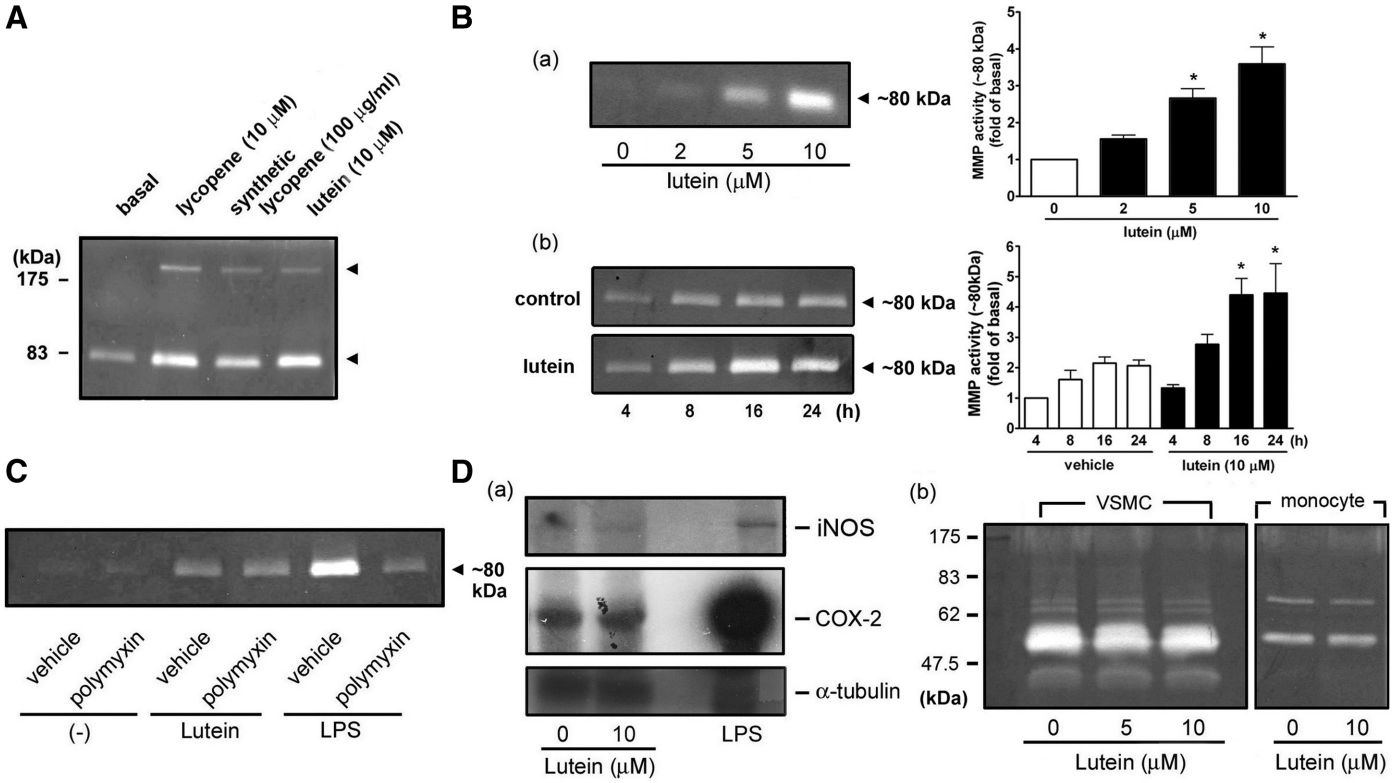

Figure 1. Effect of carotenoids on MMP activity. (A) RAW264.7 macrophages were treated with vehicle or carotenoids for 16 h. (B) RAW264.7 macrophages were treated with lutein (a) for 16 h or (b) for the indicated time intervals. MMP activity was assayed by gelatin zymography (n=3–4). *$P < 0.05$ versus vehicle treatment alone. (C) Effect of polymyxin B on lutein- and LPS-induced MMP activity. RAW264.7 macrophages were pretreated with DMSO or polymyxin B (10 μg/ml) for 30 min, followed by stimulation with lutein (10 μM) or LPS (100 ng/ml) for 8 h. The culture media were analyzed by gelatin zymography. (D) Effect of lutein on (a) iNOS and COX-2 expression in RAW264.7 macrophages and (b) MMP activity in VSMCs and U937 monocytes (n=2–3). Cells treated with lutein (10 μM) for 16 h were analyzed by (a) Western blotting or (b) gelatin zymography (n=2–3).

~200 kDa were found to be increased. Because of its solubility and stability in DMSO, lutein was chosen as a representative and further analyzed in this study. Figure 1B shows the concentration- and time-dependent effect of lutein in increasing the activity of the MMP migrating at ~80 kDa in the macrophage culture medium.

Polymyxin B, an antibiotic that binds to the lipid A portion of the LPS molecule and interferes with LPS function [23], was used to exclude the possibility of LPS contamination. Figure 1C shows that polymyxin B did not affect MMP activity induced by lutein; however, it inhibited LPS-induced MMP activity. Furthermore, lutein did not enhance the expression of COX-2 and iNOS in macrophages, whereas LPS did (Fig. 1D, a). The induction of MMP-9 activity by lutein was not observed in primary-cultured VSMCs and U937 monocytes (Fig. 1D, b).

The released MMP is MMP-9

As the major MMP induced by lutein migrated near 80 kDa (Fig. 1A), it was highly suspected to be MMP-9. Western blotting was performed to verify its identity. The result revealed that the protein that migrated at ~80 kDa was MMP-9, and its release into culture media was apparently enhanced by lutein in a concentration-dependent manner (**Fig. 2A**, **a**). The MMP-9 antibody also recognized the protein that migrated at ~200 kDa (data not shown), which could be a dimer of MMP-9. Lutein increased the MMP-9 protein (~80 kDa) level in cell lysates in a concentration-dependent manner (Fig. 2A, b), suggesting an inductive synthesis of intracellular MMP-9 protein on lutein treatment. Next, we examined whether lutein had a similar effect on primary-cultured peritoneal macrophages. As shown in Fig. 2B, lutein also induced MMP-9 release in resident and recruited peritoneal macrophages in a concentration-dependent manner. The lutein-induced increase of MMP-9 activity and expression in murine macrophages was not a result of uneven loading of samples, as the cells seeded were demonstrated to be of approximately equal numbers by the MTT assay (data not shown).

DNA transcription and protein synthesis are essential for lutein-induced MMP-9 production in murine macrophages

The effect of lutein on MMP-9 mRNA expression in macrophages was evaluated by RT-PCR. As shown in **Fig. 3A**, MMP-9 mRNA was up-regulated significantly by lutein, suggesting that lutein induced MMP-9 expression in macrophages through a transcriptional regulation. This was supported by the following

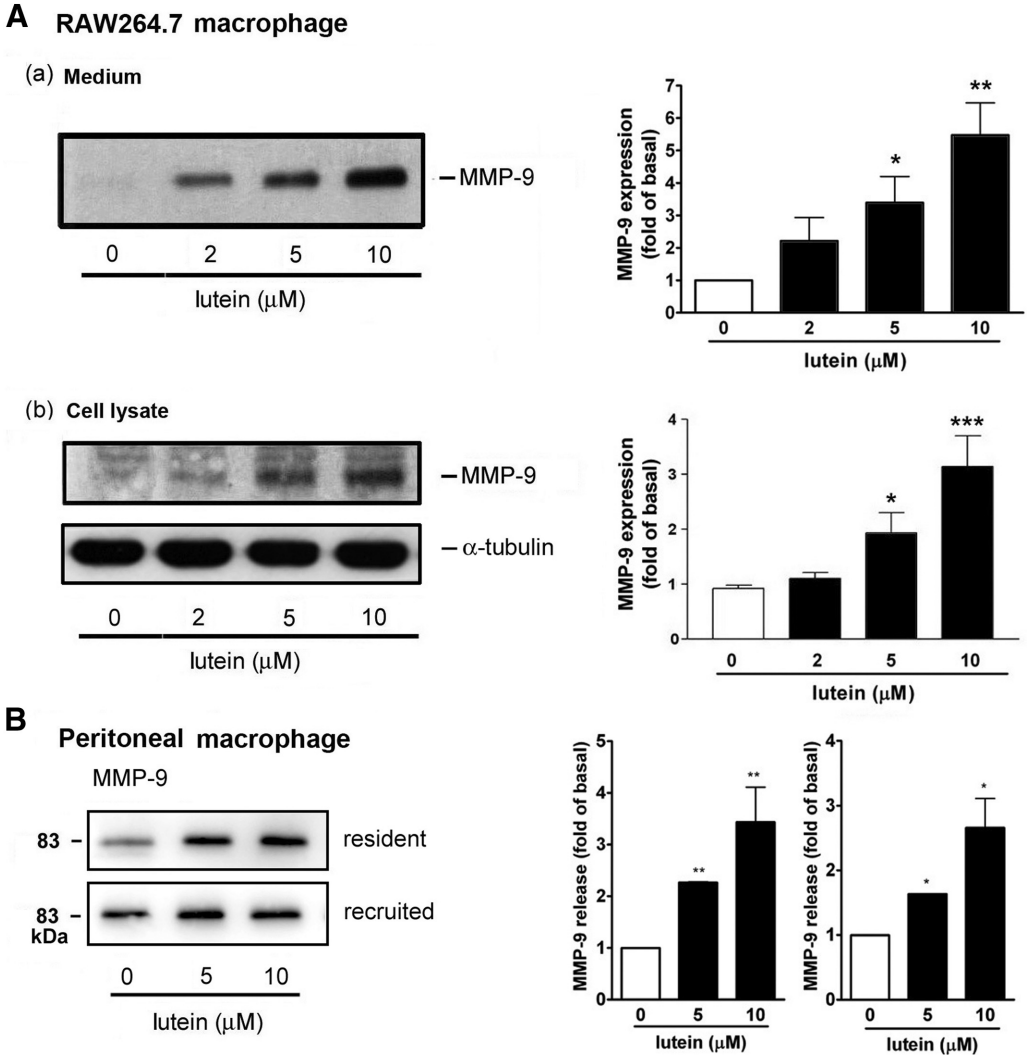

Figure 2. The released MMP is MMP-9. (A) RAW264.7 or (B) primary-cultured peritoneal macrophages were treated with lutein for 16 h. The culture media or cell lysates were analyzed by Western blotting using an antibody specific for MMP-9 or α-tubulin (as a loading control). Data were quantified by densitometry (n=3–5). *P < 0.05; **P < 0.01; ***P < 0.001 versus basal level.

observations. Firstly, the transcription inhibitor (actinomycin D) and protein synthesis inhibitor (cycloheximide) reduced lutein-induced MMP-9 release but did not affect cell viability (Fig. 3B). Secondly, a Luc reporter assay using the construct with a murine MMP-9 promoter region demonstrated that lutein could enhance Luc activity (Fig. 3C).

Effect of signaling inhibitors on lutein-induced MMP-9 expression in macrophages

It has been reported that retinoids mediate transcription through PKA, PKC, and MAPKs [24]. To investigate the possible signaling pathways involved in the induction of MMP-9 by lutein, various inhibitors targeting signaling kinases were used. The gelatin zymography assay results indicated that the MAPK inhibitors targeting ERK1/2 and p38 MAPK (PD98059 and SB202190, respectively) slightly affected basal MMP-9 activity but markedly inhibited lutein-induced MMP-9 activity. In contrast, the inhibitors for JNK (SP600125), PI3K (LY294002), PKA (H89), PKC (GF109203X), and PPARs (GW9662) did not affect MMP-9 activity (**Fig. 4A**, **a** and **b, upper**). The inhibitory effect was not a result of decreased cell viability (as shown in Fig. 4A, b, lower). Further analysis by Western blotting and RT-PCR showed that PD98059 and SB202190 attenuated the MMP-9 mRNA level and MMP-9 release (Fig. 4B and C). In parallel, a similar inhibitory effect of the MAPK inhibitors on MMP-9 production was also observed in peritoneal macrophages and RAW264.7 macrophages treated with lutein and lycopene, respectively (Supplemental Fig. 1).

Several studies have demonstrated the role of ROS in the activation of signaling pathways, such as those of MAPKs [25, 26]. Therefore, NAC, a ROS scavenger, and DPI, a Nox inhibitor, were used to investigate the role of ROS in lutein-induced MMP-9 expression. Figure 4D shows that NAC slightly affected MMP-9 basal levels; however, NAC and DPI markedly inhibited lutein-induced MMP-9 release.

Lutein induces MAPK activation and intracellular ROS generation in murine macrophages

Subsequently, we tried to establish whether lutein could activate signaling pathways directly in macrophages. **Figure 5A** shows that lutein induced ERK1/2 and p38 MAPK activation, but not JNK activation, in macrophages. Surprisingly, lutein

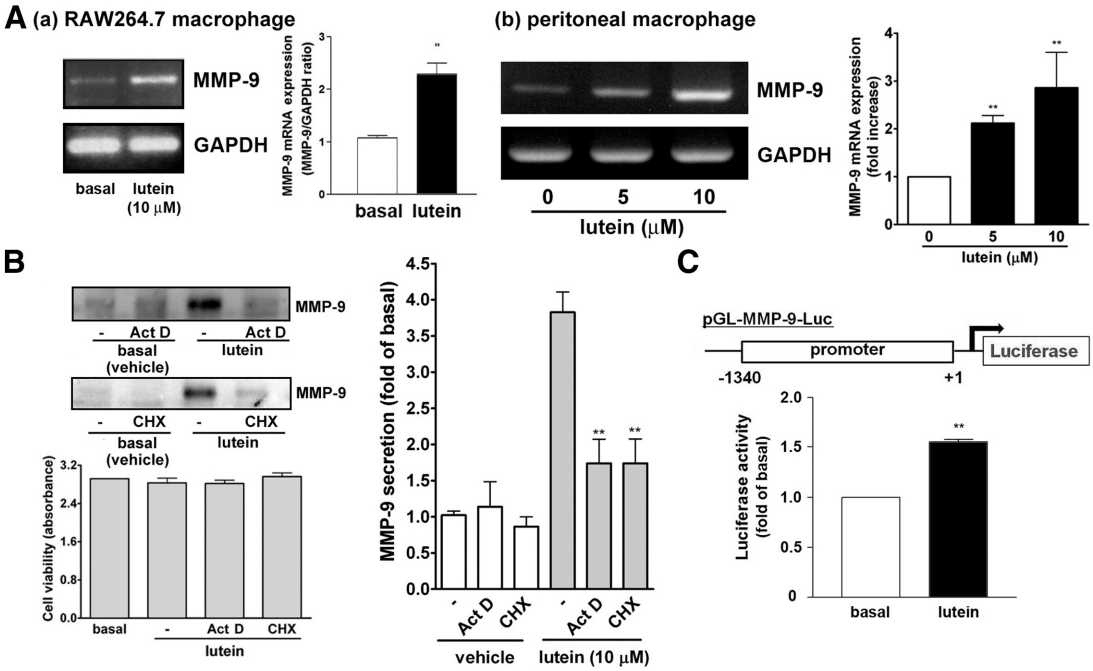

Figure 3. Lutein transcriptionally regulates MMP-9 expression. (A) RT-PCR analysis of MMP-9 mRNA expression in macrophages. (a) RAW264.7 and (b) recruited primary-cultured macrophages were treated with lutein for 6 h and 16 h, respectively. Total RNA was prepared and analyzed by RT-PCR (n=3–4). (B) Effect of actinomycin D (Act D; 1 μM) and cycloheximide (CHX; 2 μM) on lutein-induced MMP-9 release, which was analyzed by Western blotting (left upper and right panels), whereas the viability of treated cells was determined by MTT assay (left, lower panel; n=3–4). (C) Effect of lutein on MMP-9 reporter Luc activity (n=3). The MMP-9 reporter construct was transiently transfected into RAW 264.7 macrophages for 18 h, followed by growth for 24 h in endotoxin-free medium. Cells were treated with vehicle (basal control) or lutein (10 μM) for 12 h. Cells were then lysed, and Luc activity was determined by luminometry (n=3). $^{**}P <$ 0.01 versus control.

also caused intracellular ROS production, as assayed by flow cytometry. ROS production increased within 60 min after lutein treatment and declined thereafter (Fig. 5B). The Nox family proteins are enzymes that play major roles in producing O_2^- and/or H_2O_2 [27]. We explored whether lutein affected cytosolic and membrane levels of p47 phox, a regulatory cytosolic subunit of Nox. As shown in Fig. 5C, a, we verified the fractionation procedure by examining the distribution of PDGFRβ and α-tubulin in the fractionations. PDGFRβ was found to locate mainly at the membrane, whereas α-tubulin was at the cytosolic fraction. Next, the p47 phox level in the fractionations was determined. In Fig. 5C, b, the membrane and cytosolic p47 phox levels were increased and decreased, respectively, by lutein; however, α-tubulin and PDGFRβ expression was not changed (left panel). Validation of p47 phox level with the α-tubulin and PDGFRβ level (as loading controls) indicated that lutein slightly affected the p47 phox level at 5 min and changed the p47 phox level significantly in the membrane and cytosol at 15 min (right panel).

Role of RAR and RXR in lutein-induced MMP-9 production in macrophages

To investigate whether lutein induced MMP-9 production through the activation of NRs, specific inhibitors targeting RAR or RXR were used for pharmacological intervention. **Figure 6A** shows that CD2665, a RARβ/γ antagonist, markedly reduced lutein-induced MMP-9 release (left panel). The role of RARβ/γ was confirmed by the observation that MMP-9 release was induced strongly by adapalene (a RARβ/γ agonist; middle panel). On the other hand, the RXR antagonist UVI3003 did not have any inhibitory effect on lutein-induced MMP-9 release (right panel). It was also found that under the same conditions, LE135 (a specific RARβ antagonist) prevented lutein-induced MMP-9 release, whereas MM11253 (a specific RARγ antagonist) did not (Fig. 6B), demonstrating the role of RARβ in lutein-induced MMP-9 release.

With the use of a RARE Luc reporter system (pRAR-Luc), we then showed that RAR activation was involved in the induction of MMP-9 activity by lutein in macrophages. Cells with a pRAR-Luc reporter treated with lutein exhibited markedly increased RARE-mediated Luc activity (2.2-fold; **Fig. 7A**). In line with this observation, RARβ knockdown by siRNA caused a decrease in MMP-9 release and MMP-9 mRNA level (Fig. 7B), and lutein slightly up-regulated RARβ levels (Fig. 7C). Possible signaling pathways involving lutein in the macrophages were investigated. Figure 7D shows that ROS production was reduced by DPI but not by CD2665 (a RARβ/γ antagonist; left panel). Moreover, DPI reduced lutein-induced RAR interaction with RARE, as determined by the RARE Luc reporter assay (right panel), indicating that ROS generation was essential for lutein-induced RAR activation.

Lutein enhances phagocytosis in macrophages

To test whether lutein affected the biological function of macrophages, we performed a phagocytic assay by fluorescence microscopy. **Figure 8A** shows that only some fluorescent spots, representing the ingested yeasts, were observed in control RAW264.7 macrophages; however, many fluorescent spots were found in lutein-treated macrophages (Fig. 8A, a). A magnifica-

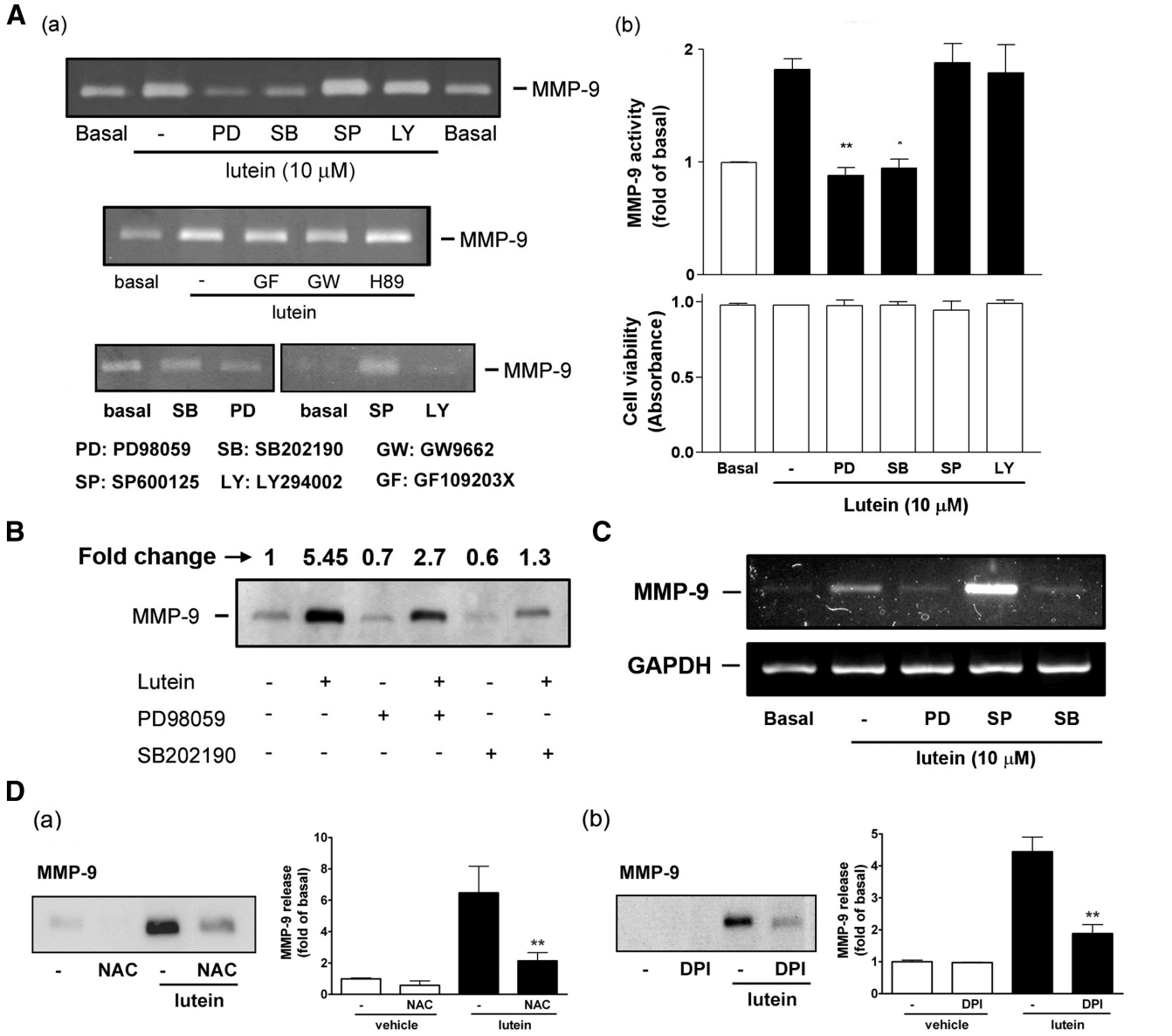

Figure 4. Effect of signaling inhibitors on lutein-induced MMP-9 production. (A) RAW264.7 macrophages were pretreated with DMSO or signaling inhibitors (10 μM, except for GF109203X; concentration: 1 μM) for 30 min, followed by the addition of vehicle (basal) or lutein (10 μM) for 16 h. The culture media were analyzed by (A) gelatin zymography, (B) Western blotting, and (C) RT-PCR. Cell viability was analyzed by MTT assay (A, b), and quantitation of data was performed by densitometry (n=3). (D) Effect of ROS inhibitors on lutein-induced MMP-9 release (n=3). RAW264.7 macrophages were pretreated with (a) NAC (10 mM) or (b) DPI (10 nM) for 1 h and then incubated with lutein (10 μM) for 16 h. The culture media were collected and analyzed by Western blotting. *$P < 0.05$; **$P < 0.01$ versus control.

tion of a randomly selected region (white frames in Fig. 8A, a) and some macrophages with yeasts indicated that these yeasts were not adhered to but had been ingested by RAW264.7 macrophages (Fig. 8A, b). Direct counting of the fluorescent spots under HPF revealed a significant increase in ingested yeasts in lutein-treated macrophages (Fig. 8A, c, left). Moreover, the number of macrophages containing ingested yeasts and the number of ingested yeasts/macrophage were increased significantly in lutein-treated macrophages (middle and right panels). This was supported by the observation that lutein enhanced clearance of the microorganisms by macrophages (Supplemental Fig. 2). Next, we examined whether lutein had a similar effect on primary-cultured macrophages. As seen in Fig. 8B, lutein also enhanced MMP-9 release (Fig. 8B, a) and phagocytosis in primary-cultured peritoneal macrophages (Fig. 8B, b).

The role of MMP-9 in macrophage phagocytosis was then investigated further. Figure 8C and D shows that the GM6001 (a broad-spectrum MMP inhibitor) and MMP-9-specific inhibitor (MMP-9 I) significantly reduced lutein-induced phagocytosis by macrophages. Zymography indicated that MMP-9 I reduced medial MMP-9 activity (Fig. 8D, left panel, inset). The

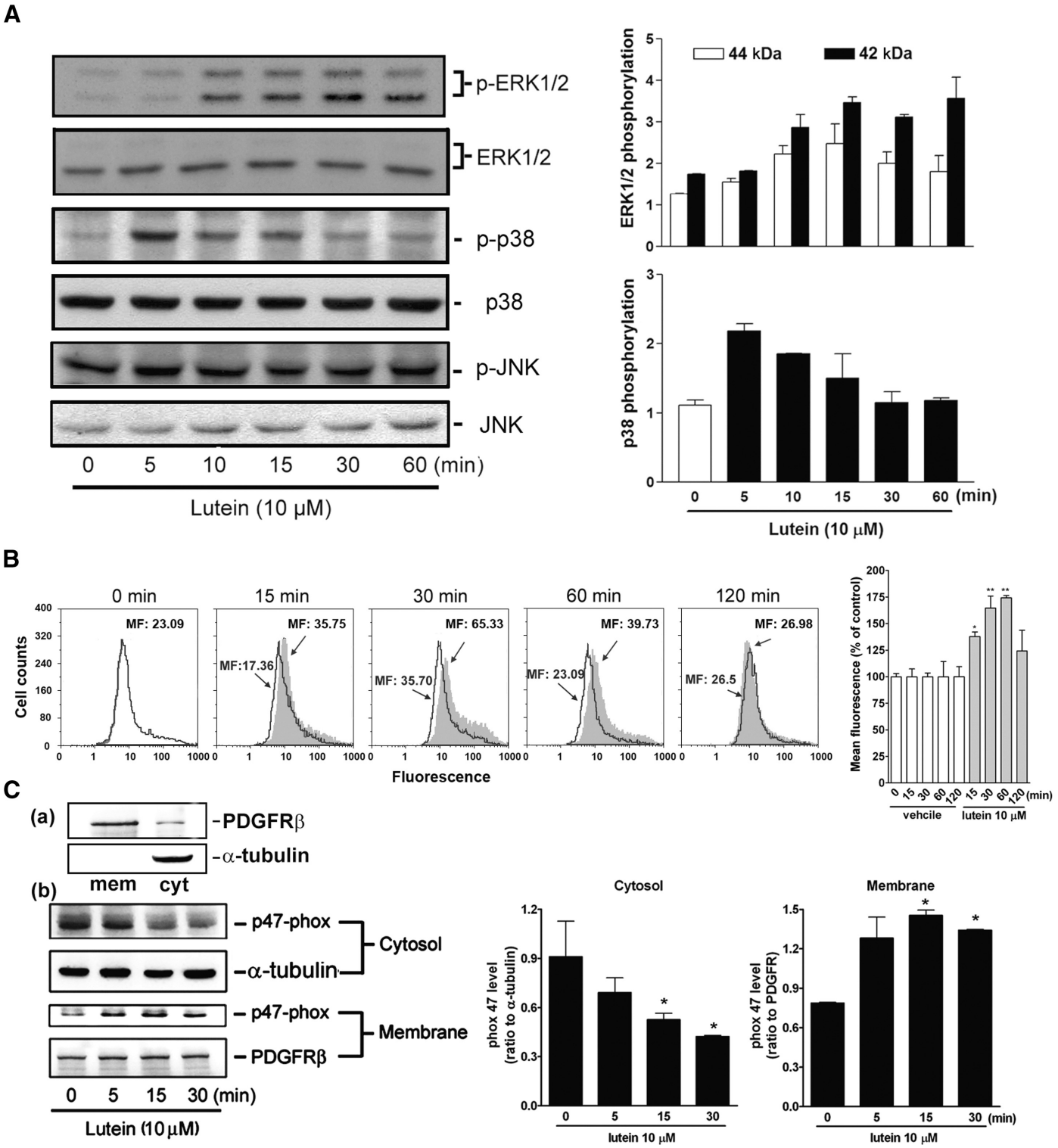

Figure 5. Effects of lutein on MAPK activation, intracellular ROS generation, and p47 phox Nox. RAW264.7 macrophages were treated with lutein. (A) MAPK activation was analyzed by Western blotting and densitometry (n=3). p, Phosphorylation. (B) Cells treated with vehicle (control; solid line) or lutein (gray region) for the indicated time intervals were loaded with the ROS-specific dye, and intracellular ROS production was measured immediately by flow cytometry. The representative histograms are shown. Quantitative analysis of mean fluorescence intensity (MF) from similar results are shown on the right (n=5). (C, a) Western blot analysis of the fractionation procedure by examining PDGFRβ and α-tubulin expression in the membrane (mem) and cytosolic (cyt) fractions (n=2). (b) The levels of p47 phox Nox, α-tubulin, or PDGFRβ in both fractions were analyzed by Western blotting. Data were quantitated by densitometry (n=4). *$P < 0.05$; **$P < 0.01$ versus basal level.

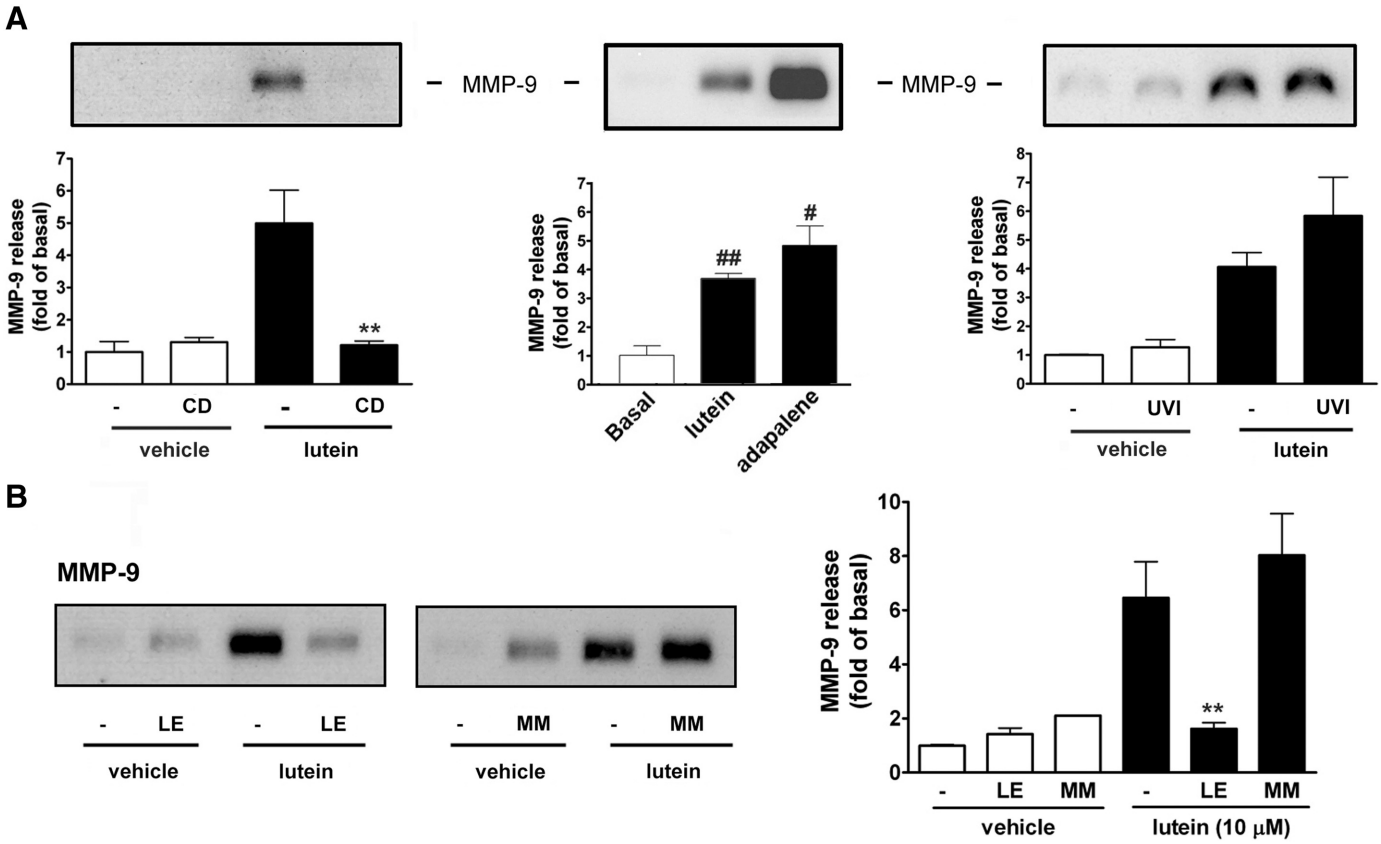

Figure 6. Effect of RAR/RXR antagonists/agonists on lutein-induced MMP-9 production. (A and B) RAW264.7 macrophages were pretreated with vehicle or the indicated antagonists (1 μM each) for 1 h and then incubated with vehicle, lutein (10 μM), or adapalene (1 μM) for 16 h. MMP-9 release in the medium was analyzed by Western blotting (n=3). CD, CD2665; UVI, UVI3003; LE, LE135; and MM, MM11253. $^{**}P < 0.01$ versus lutein treatment alone; $\#P < 0.05$ and $\#\#P < 0.01$ versus basal (vehicle alone).

Nox inhibitor DPI also markedly suppressed lutein-induced phagocytosis (Fig. 8D, right panel).

DISCUSSION

In this study, we demonstrated for the first time that the carotenoid lutein induced MMP-9 release in murine RAW264.7 and primary-cultured peritoneal macrophages (Figs. 1–3). In addition, lycopene and ATRA, a bioactive metabolite of vitamin A, exhibited a similar effect (Fig. 1A and our unpublished data). Most importantly, the increased MMP-9 production significantly contributed to enhance the phagocytic ability of RAW264.7 and peritoneal macrophages (Fig. 8), revealing a novel, functional role of MMP-9 in macrophage phagocytosis in response to carotenoids.

MMP-9 induction by lutein appears to be through a transcriptional regulation (Fig. 3). The increased MMP-9 activity, production, and mRNA level induced by lutein and lycopene were reduced by PD98059 and SB202190 but not by other inhibitors (Fig. 4 and Supplemental Fig. 1). In parallel, ERK1/2 and p38 MAPK, but not JNK, were activated by lutein (Fig. 5). As lutein induced intracellular ROS production, this suggests an orchestration/coordination of MAPKs and intracellular ROS in increasing MMP-9 production during this process. Retinol has also been reported to induce ERK1/2 activation and MMP-2 activity through ROS production in Sertoli cells [28, 29]. It is surprising that lutein caused ROS production in macrophages, as carotenoids are generally associated with their radical scavenging properties and their exceptional singlet oxygen-quenching abilities [8]. However, recently, some experiments have shown that carotenoids may have pro-oxidant effects at higher concentrations, possibly as a result of the direct interaction of carotenoids with other free radicals, alterations in membrane structure, and cause of secondary lipoperoxidation [8, 30–33]. Our results showed that macrophages alone, and not other cells, responded to lutein to induce MMP-9 expression (Fig. 1). Macrophages are specialized cells and efficient phagocytes that respond to many stimuli and are capable of generating certain substances and free radicals during activation. Therefore, it is possible that lutein stimulates ROS signaling, as it reacts with macrophage cellular components to change their chemical properties or alters cell membrane structure by direct incorporation owing to its hydrophobicity. The activation of Nox by lutein was responsible for ROS generation. Nox and ROS generation were increased in parallel at the early stage of lutein treatment (Fig. 5). MAPKs, such as

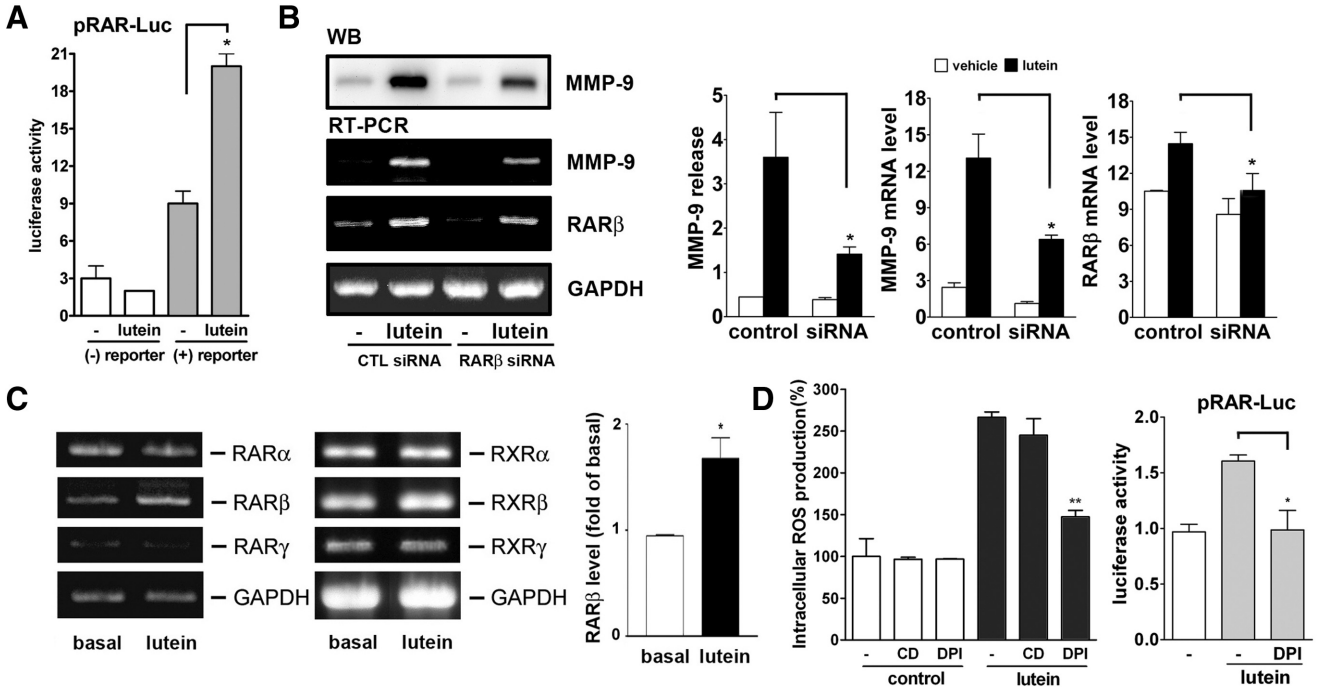

Figure 7. Role of RARβ activation in lutein-induced MMP-9 production. (A) Effect of lutein on RARE-mediated Luc activity. RAW264.7 macrophages were transfected with/without RARE Luc reporter for 72 h and then treated with vehicle (denoted as "−") or lutein (10 μM) for 12 h. Cells were then lysed, and Luc activity was determined by luminometry (n=3). (B) Effect of siRNA knockdown of RARβ on lutein-induced MMP-9 release and expression (n=3). RAW264.7 macrophages were transfected with RARβ siRNA for 72 h, followed by vehicle or lutein treatment for 16 h. The expression of RARβ, MMP-9, and GAPDH was determined by RT-PCR, and MMP-9 release was examined by Western blotting (WB; n=3). CTL, Control. (C) Effect of lutein on the level of RAR/RXR isoforms. RAW264.7 macrophages were treated with lutein (10 μM) for 6 h; RAR/RXR isoform level was determined by RT-PCR (n=4). (D) Effect of ROS production on RAR–RARE interaction. (Left) RAW264.7 macrophages were treated with CD2665 (1 μM) or DPI (10 nM) for 1 h, followed by lutein incubation for an additional 30 min. ROS production was determined by fluorometry (n=3–4) and was expressed as a percentage of basal (vehicle treatment alone). (Right) The effect of DPI on RARE-mediated Luc activity (n=2). *$P < 0.05$; **$P < 0.01$ versus control.

ERK1/2, are known to be activated in a variety of situations, where the cell redox state is modified by pro-oxidant agents [34]. We found that NAC (a ROS scavenger) reduced lutein-induced ERK1/2 phosphorylation in RAW264.7 macrophages (data not shown). Therefore, we speculated that the elevated ROS level acts as a signaling messenger in causing downstream MAPK activation, which is essential for MMP-9 induction. It has been reported that RARβ and -γ can be phosphorylated by MAPKs [24]; MAPK activation by lutein may lead to RAR phosphorylation and subsequent activation of MMP-9 transcription machinery. Interestingly, it was noted that the JNK inhibitor (SP600125) enhanced basal and lutein-induced MMP-9 expression and activity (Fig. 4). Although the underlying mechanism is not clear, we have observed a similar inducing effect by SP600125 in thrombin-induced COX-2 expression but not in iNOS expression in RAW264.7 macrophages [14]. Therefore, it is possible that SP600125 alone can induce the expression of certain genes in murine macrophages.

RARs and RXRs function as heterodimers. However, RXR also serves as a heterodimeric partner for several other NRs, such as PPARs and LXR. It is unlikely that LXR and PPAR activation are responsible for the action of lutein, as an LXR agonist (T0901317) did not cause MMP-9 activation (data not shown), and an inhibitor (GW9662) for PPARs did not inhibit lutein-induced MMP-9 activity (Fig. 4A). A previous study also showed that LXR decreases MMP-9 expression in macrophages [35]. According to the current model of gene regulation by retinoids, RAR binding to RAREs can maintain chromatin in a repressed state in the absence of a ligand; however, it initiates the recruitment of the coregulator complexes, leading to the alteration of chromatin structure for the recruitment of transcription machinery upon ligand binding [36]. In this study, the antagonists and siRNA knockdown of RARβ repressed lutein-induced MMP-9 production (Figs. 6 and 7), demonstrating a critical role of RARβ in MMP-9 induction. A previous study has also shown that β-cryptoxanthin and lutein serve as ligands for the activation of RAR [37].

It has been reported that the promoter region upstream of the murine *mmp-9* gene does not contain a canonical consensus RARE [38]. Nevertheless, a transcription element search using the Transcription Element Search System revealed that the RARβ/γ- and RXRα/β-binding sequences were located within the murine promoter region, spanning from −1340 to +1 (data not shown). ATRA has been shown to increase MMP-9 production in myeloid DCs, and the authors suggested that it acts through a nonclassical (epigenetic) transcriptional

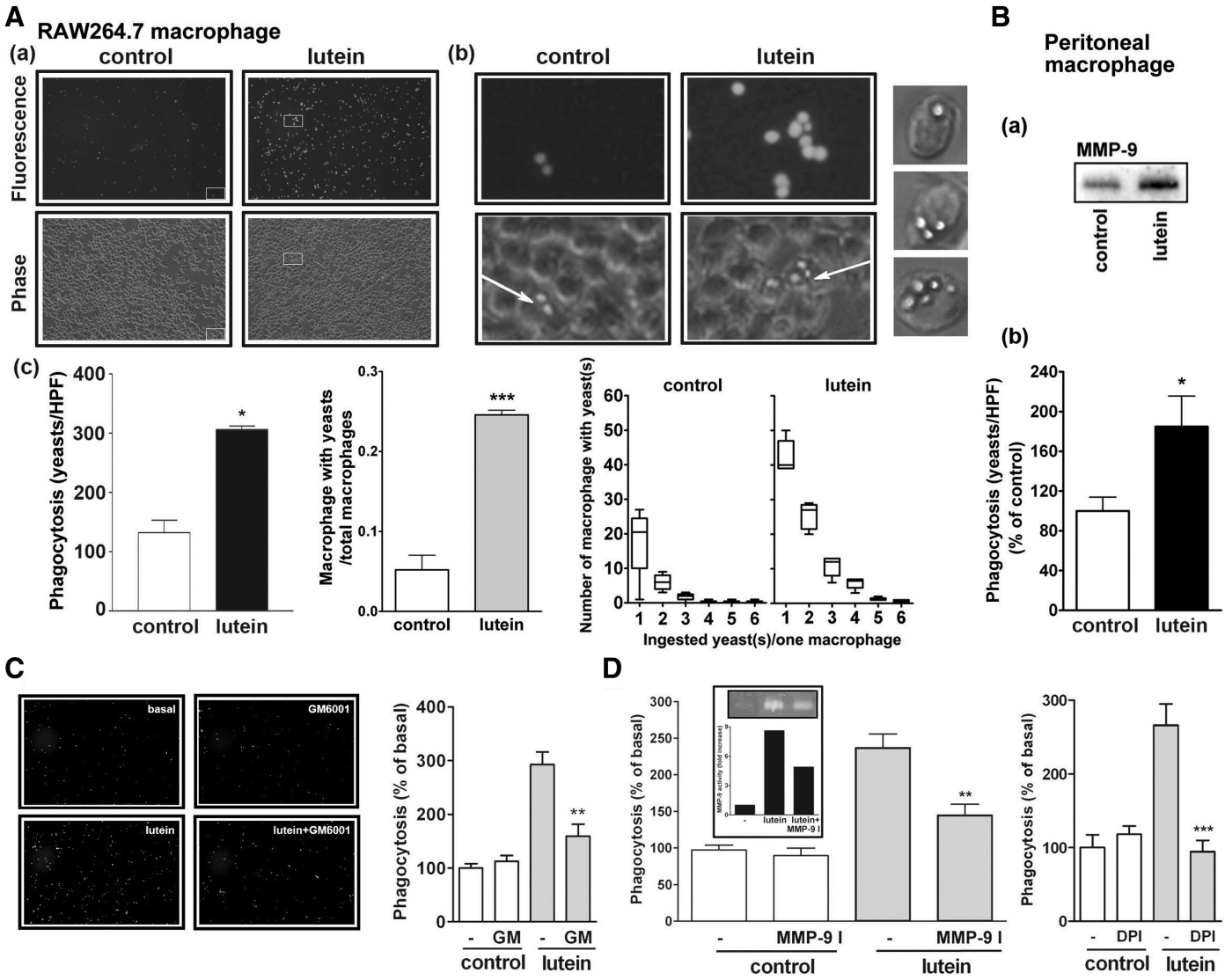

Figure 8. Effect of lutein on phagocytosis. (A and B) Lutein-enhanced macrophage phagocytosis. (A) RAW264.7 or (B) recruited peritoneal macrophages were primed with DMSO (control) or lutein (10 μM) for 16 h, followed by incubation with fluorescent dye-loaded yeasts for 1 h. At the end of incubation, phagocytosis was analyzed by fluorescence microscopy. (A, a) Full view; (b) enlarged view: a random-selected region from a was enlarged to show the ingested yeasts within macrophages. (c) Phagocytic index, described in Materials and Methods, was determined (n=3). (B, a) MMP-9 release and (b) phagocytosis in peritoneal macrophages. (C and D) MMP-9 inhibitors and DPI affected lutein-induced phagocytosis. RAW264.7 macrophages were primed with lutein and incubated with yeasts as described in A, except that they were performed in the presence of (C) GM6001 (GM; 10 μM) or (D) MMP-9 inhibitor (MMP-9 I; 10 nM) or DPI (10 nM). (Inset) Effect of MMP-9 I on medial MMP-9 activity (n=3–5). Data were expressed as a percentage of the control basal level. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ versus control.

mechanism, which involves RARα promoter binding and the recruitment of coregulators [39, 40]. These findings are at odds with our results, which showed that lutein enhanced RARE-mediated Luc activity (Fig. 7A). Moreover, lutein increased the RARβ level (Fig. 7C), possibly owing to the presence of classical RAREs (DR5 elements) in the RARβ gene promoter [41]. The RARE Luc reporter assay showed that DPI reduced lutein-induced RAR interaction with RARE (Fig. 7D). Therefore, we suggest that lutein-activated RARβ interacts with the murine *mmp-9* promoter, which is otherwise dependent on ROS production prior to this process. The interaction by lutein could be achieved by binding directly with the promoter or by acting indirectly within a coregulatory protein complex that associates with the *mmp-9* promoter.

Interestingly, lutein enhanced the phagocytic ability of macrophages, which could be a result of MMP-9 induction (Fig. 8 and Supplemental Fig. 2). Pathogen destruction and phagocytosis by neutrophils and macrophages are mainly mediated by NO, ROS, and (non-) opsonic receptors. Previous studies have shown that certain enzymes secreted by neutrophils or pathogens can also activate the complement system [42]. Furthermore, in this study, DPI and MMP-9 inhibitors reduced lutein-

induced phagocytosis (Fig. 8D), indicating that ROS and MMP-9 may participate in phagocytosis. MMPs secreted by macrophages, such as collagenase and elastase, are known to participate in matrix catabolism [42]. The secreted MMP-9 may promote other factors for enhancing pathogen clearance or may cause ECM degradation to compromise the pathogen's adhesion to ECM. Recently, it was also reported that MMP-25-induced vimentin cleavage is required for the phagocytic activity of macrophages [43], and MMP-9 is required for forming multinucleated giant cells, which are believed to enhance the defensive capacity of macrophages [44]. In this study, it appears unlikely that the secreted MMP-9 participates in opsonic activity, as phagocytosis occurred under serum-free conditions. The detailed mechanisms underlying the influence of secreted MMP-9 on macrophage phagocytic activity merit further study.

In contrast to our findings, two recent studies showed that astaxanthin (a carotenoid) suppresses MMP activity in phorbol ester-stimulated (differentiated) THP-1 monocytes [45], and β-carotene and β-cryptoxanthin, but not lutein, evoke redox and immune changes in macrophages [46]. A major difference between these studies and ours is that they used stimulators to activate monocytes/macrophages in their studies. Moreover, in the latter study, the authors measured the redox status of lutein in macrophages after a 24-h treatment, in which the Nox and ROS generation appear to decline to basal levels, according to our observations (Fig. 5B and C). Such conflicting findings can also be observed for ATRA or vitamin A, which up-regulates MMP-9 expression in some cell types [47, 48] but down-regulates it in other cell types [49].

In conclusion, we provide here the first evidence showing that the carotenoid lutein can induce MMP-9 production in murine macrophages through intracellular ROS generation and ERK1/2, p38 MAPK, and RARβ activation, and this induction contributes, at least in part, to the enhancement of the macrophage phagocytic ability (schematic diagram in Supplemental Fig. 3). Although most studies have linked monocyte/macrophage MMP production to inflammation and diseases [50–52], an appropriate activation of macrophage immune responses also leads to immunomodulation, anti-tumor activity, wound-healing, and other therapeutic effects [53]. The results presented here demonstrate that lutein exerts a modulatory influence on murine macrophages, implying that the daily intake of lutein or lutein supplements may alter macrophage activity and affect their function.

AUTHORSHIP

H-M.L. designed the experiments and performed some Western blot experiments. C-L.C. constructed the murine MMP-9-Luc reporter and performed the Luc assay. C-M.Y. performed ROS production and Nox activation. P-H.W. performed zymographic analysis and evaluated phagocytosis. C-J.T. and K-W.C. performed data analysis. W-B.W. designed experiments, provided supervision, and wrote the paper.

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DISCLOSURES

The authors declare that they have no competing interests.

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KEY WORDS:
lycopene · MMP · Nox · p47 phox · retinoic acid receptor · nuclear receptor