Int J Clin Exp Med 2011;4(1):67-73
www.ijcem.com /ISSN:1940-5901/IJCEM1101002

Original Article

Cell type-specific release of matrix-metallo-proteinase-9 by bacterial chemoattractant in human blood phagocytic leukocytes

Astrid M. Doerner[1], Ling-Yu Chen[1], Richard D. Ye[2], Jiang Yong[3], Shuang Huang[4], Zhixing K. Pan[1,4]

[1]Department of Medical Microbiology and Immunology, Medical University of Ohio, Toledo, OH 43614, USA; [2]Department of Pharmacology, University of Illinois at Chicago, Chicago, IL 60612, USA; [3]Department of Pathophysiology and Key Laboratory of Proteomics of Guangdong Province, Southern Medical University, Guangzhou, P.R. China; [4]Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, CA 92037, USA.

Received January 11, 2011; accepted January 28, 2011; Epub February 6, 2011; published February 28, 2011

Abstract: Stimulation of phagocytic leukocytes with bacterial chemoattractant resulted in the release of matrix metalloproteinases (MMPs). Little is known about the mechanisms of bacterial chemoattractant regulation of MMP in phagocytic leukocytes. We report here that the mechanisms of the bacterial chemotactic peptide fMLP-induced MMP -9 release in monocytes appeared to be different from fMLP-stimulated MMP-9 release in neutrophils. In freshly prepared peripheral blood monocytes, fMLP induces MMP-9 release, starting at 8 h after stimulation. These functions of fMLP is accompanied by an increase in TNFα expression, and mediated through the phosphorylation of ERK1/2 in monocytes. However, neutrophil preparations that responded to fMLP with MMP-9 release did not require activation of ERK1/2 and TNFα expression. These results suggest a different role of fMLP in MMP-9 expression in neutrophils and monocytes, and the signal molecules involved in mediating this effect in human blood monocytes stimulated by bacterial chemoattractant.

Keywords: Bacterial chemoattractant, inflammation, phagocytic leukocytes, matrix-metallo-proteinase

Introduction

Leukocytes constitute the first line of host defense against invading microorganisms and are a major cellular component of the inflammatory reaction. In their extravasation from the vascular system to the site of inflammation they have to migrate through the extracellular matrix, a complex structure of insoluble molecules. The recruitment of leukocytes to the site of inflammation and infection is coordinated by a gradient of chemotactic factors/chemoattractants. The formylated peptide f-Met-Leu-Phe (fMLP), derived from bacteria, was classically described as chemoattractant and induces the chemotaxis of phagocytic leukocytes [1]. FMLP functions as an agonist for the N-formyl-peptide receptor (FPR), which belongs to the seven-transmembrane G-protein coupled receptor (GPCR) family and serves as chemoattractant receptor on phagocytic leukocytes such as neutrophils and monocytes [2]. It has been shown in PBMC that FPR is coupled to downstream signaling events in a pertussis sensitive manner suggesting that Gαi containing G-proteins may mediate the process [2-4]. Recent studies from our laboratory have evaluated signaling molecules downstream from G-protein coupled chemoattractant receptors [1, 5-7].

Leukocyte extravasation requires the secretion of matrix-degrading enzymes, in particular matrix metalloproteinases (MMP), which allow leukocytes to penetrate through the basement membrane and into the tissue stroma. MMPs are a family of zinc-dependent proteinases that digest specific extracellular matrix (ECM) components. They are released from the cells as inactive pro-enzymes (zymogens) and activated extracellularly by other proteinases such as the uPA/plasmin system [8, 9]. It has also been shown, that MMP-9 is able to shed TNFα from

the membrane [10]. Furthermore stimulation of PBMC with TNFα is known to induce the release of MMP-9 from macrophages and monocytes [11]. Little is known about the mechanisms of chemoattractant regulation of MMP in phagocytic leukocytes. In the present study we demonstrate that fMLP stimulation of monocytes induces MMP-9 transcription and release. The fMLP-induced MMP-9 release was accompanied by an increase in TNFα expression. These functions of fMLP appeared to be mediated through the phosphorylation of ERK1/2, as ERK inhibitors, U0126 and PD98059, markedly abolished fMLP-stimulated TNFα expression as well as activation of MMP-9. However, neutrophil preparations that responded to fMLP with MMP-9 release did not require transcription activation. These results suggest a potential different role of fMLP in MMP-9 expression in neutrophils and monocytes, and that the signal pathways involved in mediating this effect in human blood phagocytic leukocytes stimulated by bacterial chemoattractant.

Materials and methods

Preparation of human peripheral blood leukocytes

Human peripheral blood leukocytes were fractionated using Percoll based on the method of Ulmer and Flad [1]. The monocytic cell line THP-1 was stably infected with the retroviral vector pMSCV-VIG-GFP encoding for the human N-formyl-peptide receptor (FPR).

Reverse transcription of RNA and quantitative real-time PCR

Total RNA was extracted from cells using the RNeasy Mini kit (Qiagen), then reversed transcribed following the manufacturer's instruction.

Gelatin Zymography

Conditioned media (15 μl) were loaded with non -reducing sample buffer (BioRad) onto 10 % SDS-Ready-Gels containing 0.1 % Gelatin (BioRad) and subject to electrophoresis. Bands of enzymatic activity appear as clear bands in blue background.

Immunoblotting

Total protein (20 μg) of the cell lysate were separated on a 10 % SDS-PAGE and transferred on Nitrocellulose membrane (Osmonics Inc.). Blots were incubated overnight with primary phospho-ERK-antibody (1:2000) at 4ºC followed by incubation with secondary peroxi-conjugated goat-anti-mouse antibody (1:2000) for 1 h at RT and analyzed with enhanced chemiluminescenece reagents (Supersignal, Pierce).

Detection of immunoreactive MMP-9

Monocytes were stimulated with fMLP at a concentration 100 nM for various times. The conditioned media were collected and secreted MMP-9 measured by enzyme linked immunosorbent assay (ELISA) using a commercially available kit (Amersham Biosciences Corp. Piscataway, NJ) using the manufacturer's recommended protocol. Quantitation of secreted MMP-9 was accomplished by normalization of the ELISA data with a standard MMP-9 dose curve.

Results

MMP-9 release by monocytes, and neutrophils stimulated by fMLP

To assess the relationship between fMLP stimulation and synthesis of MMPs, we examined the effects of fMLP on MMP-9 release in human blood neutrophils and monocytes. MMP-9, a 92 kDa type IV collagenase, can digest extracellular matrix (ECM) components, which allow leukocytes to penetrate through the basement membrane and into the tissue stroma. Unstimulated human peripheral blood monocytes and neutrophils produced little MMP-9. Addition of fMLP (100 nM) resulted in a time-dependent release of MMP-9 as measured by ELISA (**Figure 1**). A notable increase in the release of MMP-9 was detected 5 min after fMLP stimulation and continued for at least 30 min in neutrophils (**Figure 1A**). However, in freshly prepared peripheral blood monocytes, fMLP (100 nM) induces MMP-9 release, starting at 8 h after stimulation (**Figure 1B**).

fMLP-stimulates de novo production of MMP-9 in monocytes but not in neutrophils

The results presented above demonstrate that fMLP stimulates MMP-9 release in both human blood neutrophils and monocytes, however, the time course of fMLP-induced MMP-9 release in monocytes is different from fMLP-stimulated neutrophils. To determine whether fMLP-

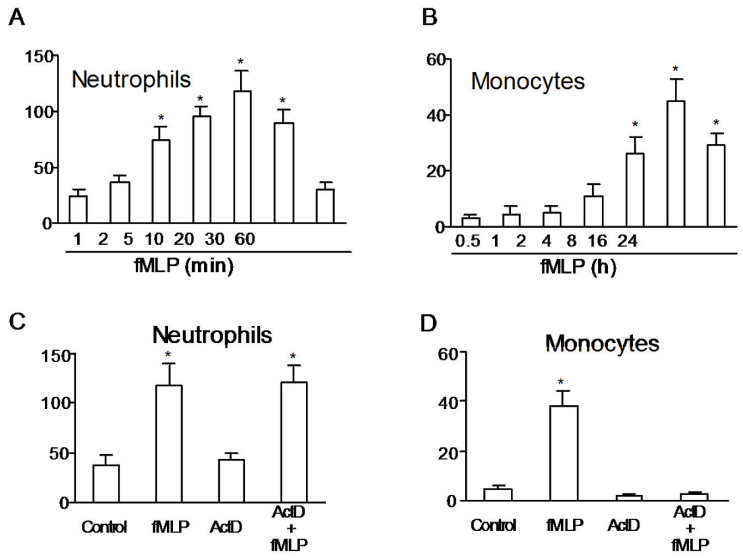

Figure 1. MMP-9 release by monocytes, and neutrophils stimulated with fMLP. The ability of fMLP to stimulate MMP-9 release in human peripheral blood neutrophils (**A**) and monocytes (**B**) was determined using enzyme-linked immunosorbent assay as described under "Experimental Procedures". Approximately 2 5 10^6 cells/ml were stimulated with medium (Control) or 100 nM fMLP for the indicated time points. Neutrophils (**C**) and monocytes (**D**) were stimulated with 100 nM fMLP with or without prior treatment of actinomycin D (1μg/ml, 60 min). The cells were removed from the medium, and immunoreactive MMP-9 in the supernatant was measured. The readings ± S.E. from three independent experiments are shown. Significance ($p<0.05$), indicated by *, compared with control.

induced release of MMP-9 represented new synthesis, monocytes and neutrophils were treated with the transcription inhibitor actinomycin D (ActD, 1 μg/ml) before fMLP stimulation. Pretreatment with ActD completely inhibited fMLP-induced MMP-9 release in monocytes (**Figure 1D**), but not in neutrophils (**Figure 1C**). These results indicate that fMLP stimulates *de novo* MMP-9 protein synthesis in monocytes.

fMLP stimulates upregulation of pro-MMP-9 and MMP-9 mRNA in THP1 cells

To overcome variability among donors, we examined THP-1, a human monocytic cell line, for MMP-9 production. The release of the pro-MMP -9 protein was further examined by Gelatin zymography analysis from human THP-1 cells stimulated with fMLP. A 92 kDa band was found in the zymogram gel stimulated with fMLP, which we identified as MMP-9 by ELISA using a specific antibody set, but not in unstimulated THP-1 cells. Although the base-line level of MMP -9 in THP1 cells was very low, it was released after stimulation with fMLP, starting at 10 h, and was maintained for at least 24 h (**Figure 2A**). FMLP induced MMP-9 release in a dose-dependent manner in THP1 cells, with a detectable release of MMP-9 at approximately 10 nM fMLP (**Figure 2B**). Semi-quantitative analysis of the MMP-9 bands in the Zymogram-gels shows a bell-shaped dose response curve for the MMP-9 release upon fMLP stimulation (data not shown) typically seen for chemokine action on cell migration [12]. MMP-9 protein purified from human neutrophils served as control and appeared at a seize corresponding to 86 kDa for the activated form of MMP-9. THP-1 cells have been reported to release only pro-MMP-9 [13]. Cleavage of pro-MMP-9 might depend on proteases released *in vivo*, which are lacking in the cell line system here. We also could observe a band for the constitutive MMP-2 protein in THP-1 cells.

Considering the rather late protein release we further investigated upregulation of MMP-9 on the mRNA level using quantitative real-time RT-PCR. Increased levels of MMP-9 mRNA were observed after 6 h with a maximum of 60-fold induction at 12 h after fMLP stimulation (**Figure 2C**). At 16 to 24 hours of stimulation MMP-9 mRNA levels decreased to about 10 fold over the level of unstimulated control cells. Pre-incubated THP1 cells with ActD also completely inhibited fMLP-induced MMP-9 mRNA upregulation (**Figure 2D**).

Phosphorylation of ERK1/2 is required for MMP -9 release in fMLP-stimulated monocytic cells

The mechanisms of fMLP-induced MMP-9 release in monocytes are not fully understood. We used the antibody, which strictly recognizes Thr/Tyr-phophorylated ERK1/2 MAPK, to detect the activity of ERK1/2 MAPK in monocytes and

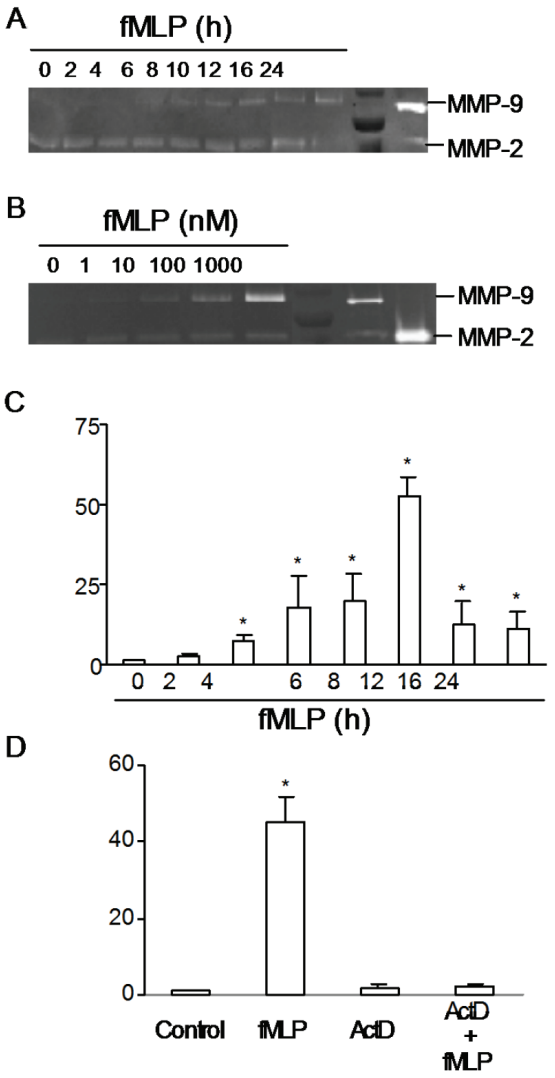

Figure 2. fMLP stimulates upregulation of pro-MMP-9 and MMP-9 mRNA in THP1 cells. (**A**) Zymogram showing effect of increasing dose of fMLP on pro-MMP-9 production in THP1 cells. Supernatants of stimulated cells (2 5 10^6/ml) were collected at the indicated time points and 15 µl per time point were used for the gelatin zymography. (**B**) Time course of MMP-9 release from THP-1 cells stimulated with 100 nM fMLP. Data shown are representative of three separate experiments. (**C**) THP-1 cells were stimulated with 100 nM fMLP for the indicated time points. Total-RNA was isolated and analyzed by quantitative real-time PCR for the expression of MMP-9 and the housekeeping gene GAPDH. (**D**) THP1 cells were stimulated with 100 nM fMLP with or without prior treatment of actinomycin D (1µg/ml, 60 min). The cells were removed from the medium, and total-RNA was isolated and analyzed by quantitative real-time PCR for the expression of MMP-9 as described above. Results shown are the mean of three independent experiments. *$p < 0.05$ compared with control.

monocytic cells. The fMLP-stimulated a time-dependent increase in phosphorylation of ERK1/2 within 2 min of stimulation and peaked at 15-20 min (**Figure 3A** and **C**). Pretreated monocytes or THP1 cells with MEK-1 inhibitors, U0126 and PD98059 [14], completely inhibited fMLP-induced MMP-9 release (**Figure 3B** and **D**). These results suggest that ERK activity is required for MMP-9 release in human blood monocytes and monocytec cells stimulated with fMLP.

Effects of TNFα neutralizing antibodies on fMLP-induced MMP-9 expression

We then assessed a potential role of TNFα in the fMLP stimulated MMP-9 release. Unstimulated human peripheral blood monocytes (**Figure 4A**) and THP1 cells (**Figure 4B**) produced little TNFα. Addition of fMLP (100 nM) resulted in a time-dependent production of TNFα as measured by enzyme-linked immunosorbent assays (ELISA) and real-time RT-PCR.

To block TNFα release from the cell and assess its role in induction of MMP-9 release we used neutralizing antibodies inhibiting the interaction of TNFα protein with the TNFα receptors. Human blood monocytes or THP-1 cells were stimulated for 16 h with 100 nM fMLP in the presence of 5-20 µg/ml neutralizing TNFα antibody and MMP-9 protein release was analyzed by ELISA (**Figure 4B**) or Zymography (**Figure 4D**). Anti-TNFα antibodies at 5-10 µg/ml completely blocked MMP-9 protein release in fMLP-stimulated cells to basal levels. These results suggest that TNFα expression is involved in MMP-9 release in fMLP-stimulated human blood monocytes.

Discussion

In the extravasation of leukocytes from the vascular system to the site of infection, they have to migrate through the extracellular matrix, a complex structure of insoluble molecules. MMPs thought to be required for human neutrophils, monocytes and other leukocytes to migrate through the basement membrane [9]. Stimulation of neutrophils with fMLP has been shown to induce MMP-9 release from their granules [15]. Our data showed that fMLP stimulated a rapid release in MMP-9 in human neutrophils. Little is known about chemoattractant regulation of MMP in monocytes. Recent studies have been shown that human monocytes did

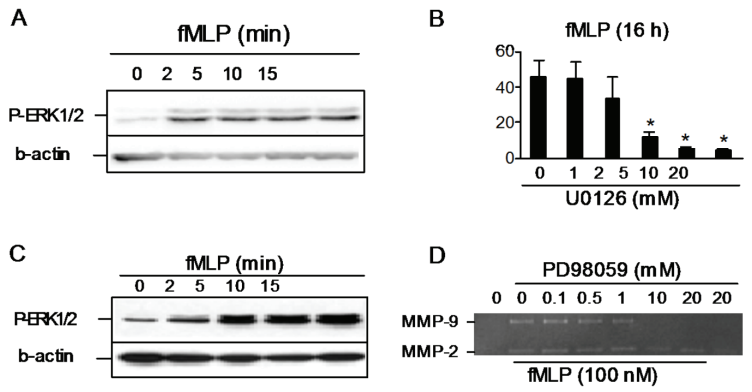

Figure 3. fMLP stimulated phosphorylation of ERK1/2 is required for the release of MMP-9 in human blood monocytes. Human blood monocytes (**A** and **B**) and THP-1 cells (C and D) (2 5 10^6/ml) were stimulated for the indicated times with 100 nM fMLP. Total protein lysate (20 µg) were subject to immunoblotting as described in Experimental Procedures using a phospho-ERK specific antibody. The membrane was stripped and rehybridized with a control antibody for β-actin to verify equal loading (A and C). Cells were stimulated with 100 nM fMLP in the presence or absence of ERK inhibitors, U0126 and PD98059 at the indicated concentrations, and immunoreactive MMP-9 in the supernatant was measured by ELISA (**B**). The supernatants (15 µl) were subject to gelatin zymography as described (**D**). Significance ($p<0.05$), indicated by *, is U0126 treated cells versus U0126 untreated cells.

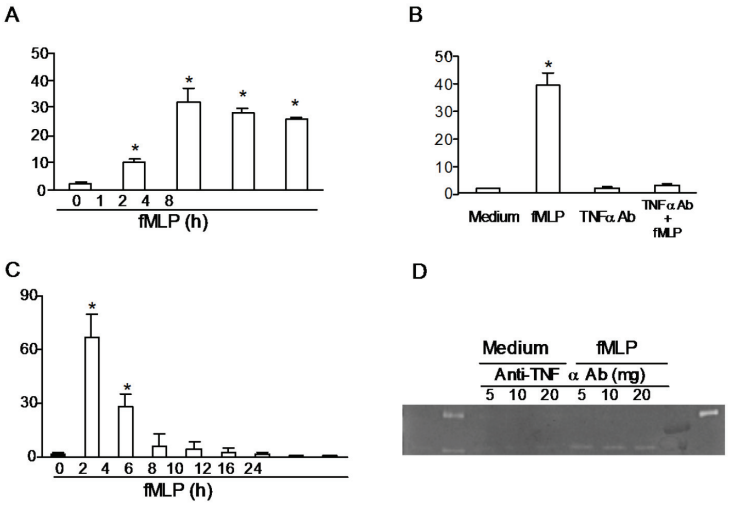

Figure 4. fMLP stimulated TNFα synthesis and release is involved in MMP-9 expression. (**A**) Human blood monocytes were stimulated with fMLP (100 nM) for different times. The supernatants were collected and the secreted TNFα was measured by ELISA as described in methods. Results shown are mean SEM from three separate measurements. (**B**) Human blood monocytes were stimulated with fMLP (100 nM) for 24 h in the presence or absence of 10 µg/ml TNFα neutralizing antibodies. The supernatants were collected and the secreted MMP-9 was measured by ELISA as described in methods. Results shown are mean SEM from three separate measurements. (**C**) THP-1 cells were stimulated with 100 nM fMLP for the indicated time points. Total-RNA was isolated and analyzed by quantitative real-time PCR for the expression of TNFα and the housekeeping gene GAPDH. RNA levels are normalized to levels of the housekeeping gene GAPDH and calculated as the mean of induction compared with control untreated cells. Results shown are the mean of three independent experiments. (**D**) THP-1 cells (2 5 10^6/ml) were stimulated for 24 h with 100 nM fMLP in the presence of 10 µg/ml TNFα neutralizing antibodies. The supernatants (15 µl) were subject to gelatin zymography. Results shown are representative of three separate experiments. *$p < 0.05$ compared with control.

not show any spontaneously induced MMP-9 release in early time frame [16]. These earlier findings prompted us to investigate whether MMP-9 release from fMLP-stimulated monocyte is due to *de novo* synthesis. If so, what is the signal transduction pathways that lead to MMP-9 expression in fMLP-stimulated monocytes. Our data demonstrated that fMLP induces *de novo* synthesis and release of MMP-9 in monocytes. Our results also indicated that MMP-9 release from fMLP-stimulated cells is due to *de novo* synthesis. These results suggest that stimulation at the transcriptional level contribute to fMLP-induced up-regulation of the MMP-9 message in human blood monocytes.

Several studies have identified signal transduction pathways that are involved in the expression of MMP-9 in endothelial cells [17], keratinocytes [18], and tumor cell lines [19]. However, the mechanisms of chemoattractant-induced MMP-9 release in leukocytes are not fully understood. In our experiments we showed that the fMLP-stimulates a time-dependent increase in phosphorylation of ERK1/2 and that inhibition of MAP kinase almost completely abrogated MMP-9 protein release, suggesting that

ERK1/2 is a major factor in the regulation of MMP-9 expression in fMLP-stimulated monocytes.

MMPs are considered to be critical to facilitate migration of monocytes and other leukocytes through the basement membrane. Studies on the chemotaxis of eosinophils towards PAF or IL-5 and U937 cells towards TNFα or IL-1α through matrigel coated inserts also showed the necessity of MMP-9 protein activity for this process since blocking MMP-9 protein by antibodies inhibited the migration [20]. Conversely Mackarel et al found that fMLP induced neutrophil migration through a HPAEC cell bilayer and collagen IV matrix was not inhibited by serine proteinase nor MMP inhibitors [21]. These results suggest that the importance of MMPs for the process of extravasation varies in different cell types and other mechanisms might facilitate the cells to overcome the extracellular matrix barrier.

In summary, we have shown that stimulation of the N-formyl-peptide receptor with bacterial chemotactic peptide fMLP induces MMP-9 release in both neutrophils and monocytes, and the mechanisms of MMP-9 *de novo* synthesis in the human monocytes. We further demonstrated that phosphorylation of ERK1/2 plays an important role in the induction of MMP-9 protein release since inhibition of the upstream Kinase MEK1 almost completely abrogated MMP-9 release. By blocking TNFα release from the membrane we then showed that the MMP-9 transcription requires TNFα synthesis and release from fMLP-stimulated cells. Our results suggest a different role of fMLP in MMP-9 expression in neutrophils and monocytes, and the signal molecules involved in mediating this effect in human blood monocytes stimulated by chemoattractant. The specificity of this response also suggests a novel and potentially important mechanism through which fMLP not only attracts leukocytes but may also contribute directly to infection.

Acknowledgements

This work was supported by USPHS Grants AI43524.

Please address correspondence to: Zhixing K. Pan, MD, PhD, Department of Medical Microbiology and Immunology, Medical University of Ohio, 3035 Arlington Avenue, Toledo, OH 43614, Tel: (419) 383-5466, Fax: (419) 383-3002, E-mail: kevin.pan@utoledo.edu

References

[1] Pan ZK, Chen L-Y, Cochrane CG, Zuraw B. fMet-Leu-Phe stimulates proinflammatory cytokine gene expression in human peripheral blood monocytes: The role of phosphatidylinositol 3-kinase. J. Immunol. 2000, 164:404-411.

[2] Murphy PM. The molecular biology of leukocyte chemoattractant receptors. Annu. Rev. Immunol. 1994, 12:593-633.

[3] Yang M, Sang H, Rahman A, Wu D, Malik AB, Ye RD. G alpha 16 couples chemoattractant receptors to NF-kappa B activation. J. Immunol. 2001, 166 (11):6885-6892.

[4] Tsu RC, Lai HW, Allen RA, Wong YH. Differential coupling of the formyl peptide receptor to adenylate cyclase and phospholipase C by the pertussis toxin-insensitive Gz protein. Biochem. J. 1995 309:331-339.

[5] Chen LY, Zuraw BL, Ye RD, Pan ZK. A Rho exchange factor mediates fMet-Leu-Phe-induced NF-kappaB activation in human peripheral blood monocytes. J. Biol. Chem. 2004, 279:7208-7212.

[6] Huang S, Chen L.-Y, Zuraw BL., Ye RD., Pan ZK. Chemoattractant-stimulated NF-□B activation is dependent on the low molecular weight GTPase RhoA. J. Biol. Chem. 2001, 276:40977-40981.

[7] Browing DD, Pan ZK., Prossnitz ER., Ye RD. Cell type- and developmental stage-specific activation of NF-kappaB by fMet-Leu-Phe in myeloid cells. J. Biol. Chem. 1997, 272:7995-8001.

[8] Goetzl EJ, Banda MJ, Leppert D. Matrix metalloproteinases in Immunity. J. Immunol. 1996, 156:1-4.

[9] Vaday GG, Lider O. Extracellular matrix moieties, cytokines, and enzymes: dynamic effects on immune cell behavior and inflammation. J. Leukocyte Biol. 2000, 67:149-159.

[10] Beckett G, Christodoulou AJ, Churchill M, Clements JM, Crimmin M, Davidson AH, Drummond, AH, Galloway WA, Gilbert R. Matrix metalloproteinases and processing of pro-TNF-alpha. J. Leukoc. Biol. 1995, 57:774-777.

[11] Zhang Y, McCluskey K, Fujii K, Wahl LM. Differential regulation of monocytes matrix metalloproteinase and TIMP-1 production by TNF-α, granulocyte-macrophage CSF, and IL-1b through prostaglandin-dependent and -independent mechanism. J. Immunol. 1998, 161:3071-3076.

[12] Robinson SC, Scott KA, Balkwill FR. Chemokine stimulation of monocyte matrix metalloproteinase-9 requires endogenous TNF-α. Eur. J. Immunol. 2002, 32:404-412.

[13] Leber T, Blankwill FR. Regulation of monocytes MMP-9 production by TNF-alpha and a tumor-derived soluble factor (mmpsf). Br. J. Cancer

1998, 78:724-732.
[14] Means TK, Pavlovich RP, Roca D, Vermeulen MW, Fenton MJ. Activation of TNF-alpha transcription utilizes distinct MAP kinase pathways in different macrophage populations. J Leukoc Biol. 2000, 67(6):885-893.
[15] Pugin J, Widmer M-C, Kossodo S, Liang C-M, PreasII HL, Suffredini AF. Human neutrophil secrete gelatinase B in vitro and in vivo in response to endotoxin and proinflammatory mediators. Am. J. Respir. Cell. Mol. Biol. 1999, 20:458-464.
[16] Takafuji S, Ishida A, Miyakuni Y, Nakagawa T. Matrix Metalloproteinase-9 release from human leukocytes. J. Invest. Allergol. Clin. Immunol. 2003, 13(1):50-55.
[17] Genersch E, Hayess K, Neuenfeld Y, Haller H. Sustained ERK phosphorylation is necessary but not sufficient for MMP-9 regulation in endothelial cells: involvement of RAS-dependent and –independent pathways. J. Cell Science 2000, 113:4319-4330.
[18] McCawley LJ, O'Brien P, Hudson LG. Epidermal growth factor (EGF)- and scatter factor/hepatocyte growth factor (SF/HGF)- mediated keratinocyte migration is coincident with induction of matrix metalloproteinase (MMP)-9. J Cell Physiol. 1998, 176(2):255-265.
[19] Simon C, Goepfert H, Boyd D. Inhibition of the p38 mitogen-activated kinase by SB 203580 blocks PMA-induced Mr 92,000 type IV collagenase secretion and in vitro invasion. Cancer Res. 1998, 58(6):1135-1139.
[20] Watanabe H, Nakanishi I, Yamashita K, Hayakawa T, Okada Y, Matrix metalloproteinase-9 (92kDa gelatinase/type IV collagenase) from U937 monoblastoid cells: correlation with cellular invasion. J. Cell Sci. 1993, 104:991-999.
[21] Mackarel JA, Cottell DC, Russell KJ, FitzGerald MX, O'Connor CM, Migration of neutrophils across human pulmonary endothelial cells is not blocked by matrix metalloproteinase or serine protease inhibitors. Am. J. Respir.Cell. Mol. Biol. 1999, 20:1209-1219.