肺炎支原体MsrB经MAPK/NF-κB通路抑制脂质相关膜蛋白诱生促炎细胞因子

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摘要 蛋氨酸亚砜还原酶B(methionine sulfoxide reductase, MsrB)是一种重要的氧化还原蛋白。该文探究了肺炎支原体(*Mycoplasma pneumoniae*, Mp) MsrB对Mp脂质相关膜蛋白(lipid-associated membrane proteins, LAMPs)刺激的人髓系白血病单核细胞(THP-1细胞)分泌促炎细胞因子肿瘤坏死因子α(TNF-α)和白细胞介素1β(IL-1β)的调节及相关信号通路, 以进一步了解Mp的免疫逃避机制。论文构建了pET28a(+)-*msr*B重组质粒, 诱导表达、鉴定、纯化重组蛋白(rMsrB)并制备了多克隆抗体; Western blot检测MsrB、TLR1、TLR2、TLR6和MyD88蛋白表达水平及NF-κB p65、P38、ERK和JNK的总蛋白和磷酸化蛋白水平; 间接免疫荧光分析NF-κB核转位; ELISA测定TNF-α和IL-1β分泌水平。结果显示MsrB可表达于Mp胞质和胞膜。rMsrB预处理可抑制LAMPs刺激的THP-1细胞合成TNF-α和IL-1β。Mp rMsrB可抑制LAMPs刺激的THP-1细胞表达TLR2、MyD88、p-ERK、p-JNK、p-p38和p-p65, 并抑制NF-κB的核转位。抑制NF-κB、P38、ERK和JNK表达后, rMsrB可进一步抑制LAMPs刺激的THP-1细胞产生TNF-α和IL-1β。综上, Mp MsrB经MAPK/NF-κB信号通路抑制LAMPs刺激的THP-1细胞分泌TNF-α和IL-1β。

关键词 肺炎支原体; 蛋氨酸亚砜还原酶B; 脂质相关膜蛋白; 促炎细胞因子

Mycoplasma pneumoniae MsrB Inhibits the Secretion of Proinflammatory Cytokines by Lipid-Associated Membrane Proteins via the MAPK/NF-κB Pathway

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Abstract MsrB (methionine sulfoxide reductase) is an essential redox protein in prokaryotic and eukaryotic cells. This study aimed to investigate the regulation and signaling pathway of MsrB on the secretion of the proinflammatory cytokines TNF-α (tumor necrosis factor-alpha) and IL-1β (interleukin 1β) in THP-1 cells (human myeloid leukemia monocytes) which were stimulated with LAMPs (lipid-associated membrane proteins) derived from

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Mp (*Mycoplasma pneumoniae*), so as to further understand the immune evade of Mp. In this study, pET28a(+)-*msr*B recombinant plasmid was constructed, the rMsrB (recombinant MsrB protein) was expressed, identified, and purified, and polyclonal antibody was prepared. The expression of MsrB, TLR1, TLR2, TLR6, and MyD88, the total and phosphorylated proteins of NF-κB p65, P38, ERK, and JNK were detected by Western blot. The nuclear translocation of NF-κB was tested by indirect immunofluorescence. The secretion of TNF-α and IL-1β was analyzed using ELISA. Data showed that MsrB was expressed in the cytoplasm and cytosol of Mp. Mp MsrB could reduce the synthesis of TNF-α and IL-1β in LAMPs-stimulated THP-1, and decrease the expression of TLR2, MyD88, p-ERK, p-JNK, p-p38, and p-p65. Moreover, NF-κB nuclear translocation was inhibited by Mp MsrB in LAMPs-stimulated THP-1 cells. Moreover, inhibiting NF-κB, P38, ERK, and JNK further reduced TNF-α and IL-1β production by rMsrB in LAMPs-stimulated THP-1 cells. In summary, Mp MsrB protein inhibits TNF-α and IL-1β secretion via the TLR2/MyD88/MAPK/NF-κB signaling pathway.

Keywords *Mycoplasma pneumoniae*; methionine sulfoxide reductase B; lipid-associated membrane proteins; proinflammatory cytokines

肺炎支原体(*Mycoplasma pneumoniae*, Mp)是一种常见的呼吸道病原体, 可引起支气管炎和社区获得性肺炎[1]。Mp是3~18岁儿童和青少年呼吸道感染首要病原体[2], 其感染率高达10%~30%, 流行期间高达50%[3-5]。2023年10月我国浙江、江苏、广东、湖南省等多省爆发Mp感染, 感染率高达60%。虽然支原体肺炎(Mycoplasma pneumoniae pneumonia, MPP)具有一定自限性, 但逐年增加的数据表明其可继续进展为难治性、严重的MPP, 甚至危及生命[6]。此外, 由于我国Mp临床分离株对首选治疗药物阿奇霉素高度耐药, 且耐药检出率高达65.4%~100%[7-8], 导致MPP的治疗愈发困难。

Mp的致病机制目前尚不完全清楚, 国内外研究主要集中在Mp诱导炎症反应及其机制。由于Mp无细胞壁, 故不像细菌那样可通过细胞壁的脂多糖、脂磷壁酸和可溶性肽糖等致病。Mp感染所引起的一系列炎症反应很大程度上是由脂质相关膜蛋白(lipid-associated membrane proteins, LAMPs)诱导的[9], 课题组以往的研究发现支原体的LAMPs能与单核巨噬细胞表面的Toll样受体(toll-like receptors, TLRs)(包括TLR2/1和TLR2/6)结合, 然后通过髓样分化蛋白88(myeloid differential protein-88, MyD88)和肿瘤坏死因子受体相关因子6(tumor necrosis factor receptor-associated factor 6, TRAF 6)激活核转录因子κB(nuclear factor kappa B, NF-κB)和激活蛋白-1(activator protein-1, AP-1), 从而诱导单核巨噬细胞合成分泌肿瘤坏死因子-α(tumor necrosis factor-α, TNF-α)、白细胞介素-1β(inleukine-1β, IL-1β)和IL-6等促炎细胞因子和活性氧(reactive oxygen species, ROS), 引起炎症反应[9-12]。

炎症是一个损伤与抗损伤的过程。病原微生物激发的炎症反应既可导致宿主细胞的病理损伤, 又可促进吞噬细胞等对病原菌的清除。而病原菌又可通过一些抗炎机制来减轻炎症反应以逃避宿主对其本身的免疫清除。蛋氨酸亚砜还原酶(methionine sulfoxide reductase, Msr)系统是一个保守的抗氧化相关酶家族, 可以将蛋氨酸亚砜(MetO)还原成蛋氨酸, 进而修复氧化损伤的蛋白质[13-14]。不同生物体和环境产生的氧自由基, 均可进一步导致各种炎症相关疾病发生。因此, 作为抗氧化剂, Msrs也可抑制炎症反应[15]。

Msr系统有3种, MsrA、MsrB和游离蛋氨酸R型亚砜还原酶(fRMsr)[16-17]。目前对Msr的研究主要集中在真核细胞, 对原核细胞型微生物的Msr蛋白亦有报道, 如空肠弯曲菌、金黄色葡萄球菌、耻垢分枝杆菌和粪肠球菌等细菌的MsrA和/或MsrB具有一定的抗氧化和抑炎功能[18-22]。国内外对支原体Msr蛋白的研究非常少, 仅有文献报道生殖支原体的MsrA有抗氧化和保护宿主细胞的功能[23-24]。我们通过生物信息学软件分析发现Mp的基因组中亦有编码MsrA和MsrB蛋白的基因, 分别为*mpn*607(*msr*A)和*mpn*662(*msr*B)。为了解MsrB在Mp激发的炎症反应中的作用, 本文初步探讨了Mp MsrB对LAMPs诱生的促炎细胞因子TNF-α、IL-1β的调节及相关的信号通路。

1 材料和方法

1.1 材料

1.1.1 细胞株和菌株 Mp标准菌株(M129, ATCC29342)保存于南华大学衡阳医学院病原生物学研究所。人髓系白血病单核细胞(THP-1 细胞)购自中国科学院细胞库。

1.1.2 主要试剂 Human TNF-α ELISA Kit、Human IL-1β ELISA Kit均购自湖南艾方生物科技有限公司; SB203580和U0126购自德国Calbiochem公司; SP600125购自美国Selleck公司; BAY 11-7082购自美国Sigma-Adrich公司; 抗TLR1、TLR2、TLR6、MyD88、p65、ERK、JNK、p38、p-p65、p-ERK、p-JNK、p-p38及β-actin的兔源性抗体均购自美国CST公司; Mp兔源性抗体购自美国Novus公司; Cy3偶联羊抗兔IgG(H+L)抗体和HRP偶联羊抗兔IgG(H+L)抗体购自上海优宁维生物科技股份有限公司; SuperKine™ ECL发光液购自中国Abbkine公司。

1.2 方法

1.2.1 Mp MsrB重组蛋白的表达纯化及多克隆抗体制备 NCBI在线数据库检索获取*msr*B(*mpn*662)基因序列后在上海生工生物工程有限公司合成。将合成的基因连接至pET28a(+)载体以构建pET28a(+)-*msr*B重组质粒。将重组质粒转化至*E. coli* BL21(DE3)感受态细胞, 用IPTG诱导表达重组蛋白(rMsrB), Western blot鉴定表达的蛋白为MsrB后, 用Ni^{2+}-NTA树脂的层析柱纯化重组蛋白。收集用rMsrB免疫后的新西兰兔血清, 用亲和柱纯化所制备的多克隆抗体, 于–20 °C保存。

1.2.2 MsrB的定位 将Mp接种于PPLO液体培养基, 置于37 ℃温箱培养5~7天。将45 mL Mp经4 ℃、10 000 ×*g*离心15 min后收集菌体沉淀, 用Triton X-114分离法按照文献分别提取Mp胞质和胞膜蛋白[25]。将分离的胞质与胞膜蛋白分别与上样缓冲液混合, 行SDS-PAGE蛋白胶电泳分离后, 转印至硝酸纤维素膜上。加入兔抗MsrB多克隆抗体(1:1 000稀释)在4 °C孵育过夜, 然后加入HRP标记的羊抗兔IgG抗体(1:2 000稀释)在37 °C孵育1.5 h, 最后用全自动化学发光图像分析系统检测Mp胞质和胞膜中MsrB蛋白的表达情况。

1.2.3 LAMPs的提取 参照HU等[9]方法抽提Mp LAMPs。将1 L Mp经4 ℃、10 000 ×*g*离心15 min后收集沉淀, 用预冷PBS洗涤后再用5 mL TBSE溶液重悬。而后加入1/100体积Trition X-114, 振荡混匀, 冰浴静置 1 h, 37 ℃水浴5 min; 8 000 ×*g*室温离心3 min, 弃上层水相, 加入等体积的TBSE缓冲液, 重复上述水相分离操作1次。第2次水相分离结束后弃去水相, 再加入5 mL TBSE溶液使之恢复至最初体积, 在整个体系中加入12.5 mL无水乙醇, 置于–20 °C冰箱过夜(>12 h)。次日于4 °C、8 000 ×*g*离心20 min, 收集沉淀, 而后用5 mL预冷PBS重悬, 低温超声破碎混合液后, BCA试剂盒检测蛋白浓度, –80 °C保存备用。

1.2.4 THP-1细胞的培养及处理 THP-1细胞按常规方法接种于含10%胎牛血清的RPMI 1640培养基中, 置于37 °C、5% CO_2培养箱内培养。将THP-1细胞按照1×10^6/mL接种至6孔板内, 每孔加入1 μL 100 ng/μL的佛波酯, 24 h后待悬浮细胞均贴壁后, 用20 μg/mL rMsrB预处理2 h(及先用10 μmol/L NF-κB、30 μmol/L P38、30 μmol/L ERK或30 μmol/L JNK抑制剂分别作用30 min, 用rMsrB预处理)后, 再用5 μg/mL LAMPs刺激24 h。设置阴性对照(PBS、20 μg/mL rMsrB)及阳性对照(5 μg/mL LAMPs处理组)。收集细胞和培养上清。

1.2.5 ELISA检测TNF-α和IL-1β 收集处理后的细胞培养上清, 根据试剂盒说明, ELISA检测TNF-α和IL-1β。

1.2.6 Western blot检测NF-κB通路相关信号分子的激活情况 收集处理后的THP-1细胞, 提取总蛋白, BCA法测定浓度。在80 μg总蛋白中加入等体积2× SDS上样缓冲液, 煮沸10 min后进行SDS-PAGE, 再将蛋白转印至PVDF膜上。用含5%脱脂牛奶的TBST将膜室温封闭2 h, 随后加入1:1 000稀释的一抗(抗TLR1、TLR2、TLR6、MyD88、p65、ERK、JNK、p38、p-p65、p-ERK、p-JNK、p-p38及β-actin的兔源性抗体)于4 °C孵育过夜。用TBST洗涤后加入HRP标记的羊抗兔IgG (1:2 000稀释)于37 °C孵育1 h。最后用全自动化学发光图像分析系统检测相应蛋白。

1.2.7 间接免疫荧光检测THP-1细胞NF-κB核转位 在6孔板中加入盖玻片爬片, 细胞处理同前。处理后的细胞用4%多聚甲醛室温固定30 min后用0.3%的Triton X-100 4 °C透化20 min, 再用RPMI-1640完全培养基于37 °C封闭2 h。用兔抗NF-κB p65抗体(1:200稀释) 37 °C避光孵育2 h, 加入Cy3标记山羊抗兔NF-κB p65抗体(1:200)和DAPI, 室温避光孵育1 h, PBS

洗涤3次。镊子夹取爬片倒置于含荧光淬灭剂玻片，指甲油封闭爬片，激光共聚焦显微镜观察并拍照记录。

1.3 统计学分析

所有数据均运用GraphPad Prism 8.0.2软件分析，采用两独立样本*t*检验和One-Way ANOVA等方法对所得数据进行统计学分析。$P<0.05$认为具有统计学意义。

2 结果

2.1 Mp rMsrB的表达纯化与鉴定

含pET28a(+)-Mp *msr*B重组质粒的BL21菌株用IPTG诱导后，表达出分子量约为17 kDa的可溶性重组蛋白，Western blot鉴定该可溶性重组蛋白为rMsrB。

2.2 Mp MsrB的定位

利用Western blot分别检测Mp的胞质蛋白和胞膜蛋白，结果显示MsrB在Mp胞质和胞膜中均有表达(图4)。

2.3 rMsrB抑制LAMPs刺激的THP-1细胞分泌TNF-α和IL-1β

结果显示，单独使用LAMPs刺激的THP-1细胞上清中IL-1β和TNF-α的表达水平较PBS对照组显著升高($P<0.05$)；而用rMsrB预处理后再用LAMPs刺激，THP-1细胞上清中IL-1β和TNF-α表达水平较LAMPs单独刺激组均显著降低($P<0.05$)(图5)。

2.4 rMsrB抑制LAMPs刺激的THP-1细胞NF-κB核转位和p65磷酸化

间接免疫荧光检测结果显示，PBS处理的THP-1细胞NF-κB主要定位于胞质，LAMPs刺激后NF-κB定位于THP-1细胞胞质和胞核，但用rMsrB预处理后再用LAMPs刺激，THP-1细胞核内NF-κB较LAMPs处理组细胞减少，即rMsrB可抑制LAMPs刺激的THP-1细胞NF-κB的核转位(图6)。此外，Mp LAMPs刺激THP-1细胞后，NF-κB p-p65的表达水平较PBS处理显著升高($P<0.05$)；而经rMsrB预处理可显著降低LAMPs刺激的THP-1细胞p65的磷酸化水平

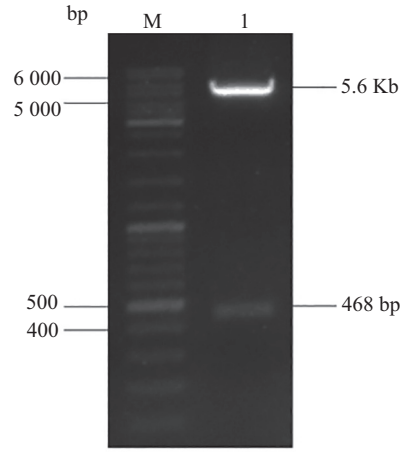
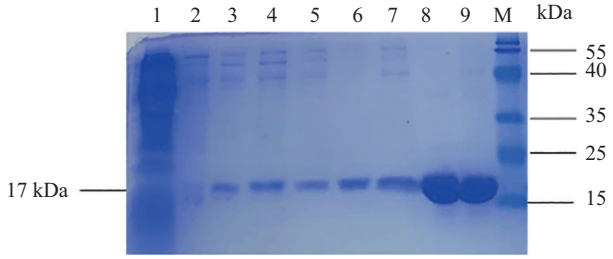


图1 琼脂糖凝胶电泳分析pET28a(+)-Mp *msr*B重组质粒双酶切结果

Fig.1 Double enzymes digestion of pET28a(+)-Mp *msr*B by electrophoretic analysis

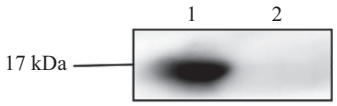


泳道1至9: rMsrB在咪唑浓度为40、50、60、70、80、90、100、150、200 mmol/L时的洗脱情况; 泳道M: 标准分子量。

Lanes 1 to 9: rMsrB elution at imidazole concentrations of 40, 50, 60, 70, 80, 90, 100, 150, 200 mmol/L; lane M: molecular weight marker.

图2 SDS-PAGE分析Mp rMsrB的表达和纯化

Fig.2 SDS-PAGE analysis of the expression and purification of Mp rMsrB

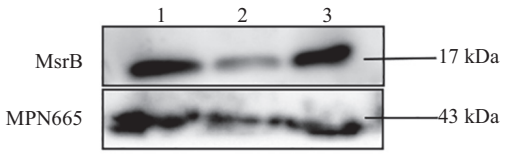


泳道1: pET28a(+)-*msr*B/BL21 蛋白表达的鉴定; 泳道2: 空白 pET28a(+) 载体蛋白表达的鉴定。

Lane 1: identification the proteins expression of pET28a(+)-*msr*B/BL21; Lane 2: identification the proteins expression of blank pET28a(+).

图3 rMsrB的Western blot鉴定

Fig.3 Identification of rMsrB by Western blot

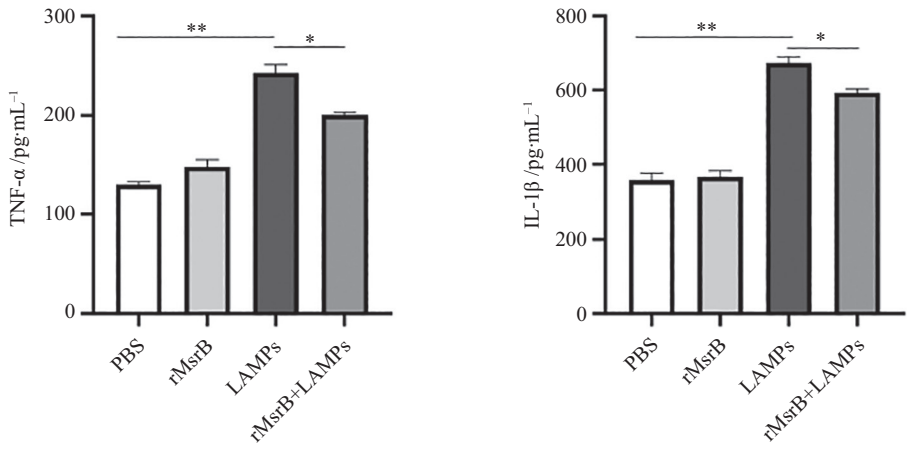


泳道1: Mp全蛋白; 泳道2: Mp胞膜蛋白; 泳道3: Mp胞质蛋白。

Lane 1: total proteins expressed in Mp; lane 2: cytosolic protein expressed in Mp; lane 3: cytoplasmic protein expressed in Mp.

图4 Mp MsrB的胞质胞膜定位

Fig.4 The location of Mp MsrB



*P<0.05, **P<0.01.

图5 rMsrB抑制LAMPs刺激的THP-1细胞分泌TNF-α和IL-1β

Fig.5 rMsrB inhibited TNF-α and IL-1β secretion by LAMPs-stimulated THP-1 cells

(P<0.05)(图7)。

2.5 rMsrB抑制LAMPs刺激的THP-1细胞MAPK通路活性

使用LAMPs刺激THP-1细胞后, ERK、JNK、p38的磷酸化水平较PBS处理组显著升高(P<0.05); 而用rMsrB预处理后再用LAMPs刺激, THP-1细胞中ERK、JNK和p38的磷酸化水平较单独LAMPs处理组显著降低(P<0.05)(图8)。

2.6 rMsrB抑制LAMPs诱导的THP-1细胞TLRs和MyD88的激活

单独使用LAMPs刺激后, THP-1细胞中TLR2、TLR6和MyD88的蛋白表达水平较PBS对照组显著升高(P<0.05), TLR1的表达水平相较于PBS对照组差异无显著性(P>0.05); 而用rMsrB预处理后, THP-1细胞中TLR2及MyD88的表达水平较LAMPs单独处理组均显著降低(P<0.05), 但TLR1和TLR6的表达水平相较于LAMPs组差异无显著性(P>0.05)(图9)。

2.7 rMsrB经MAPK/NF-κB通路抑制LAMPs刺激的THP-1细胞分泌TNF-α和IL-1β

如图10所示, 预先用P38、ERK、JNK和NF-κB的抑制剂预处理细胞, 能显著抑制rMsrB和LAMPs刺激的THP-1细胞分泌TNF-α(P<0.05)和IL-1β(P<0.05)。因此, rMsrB可能通过MAPK/NF-κB通路抑制LAMPs刺激的THP-1细胞分泌促炎细胞因子。

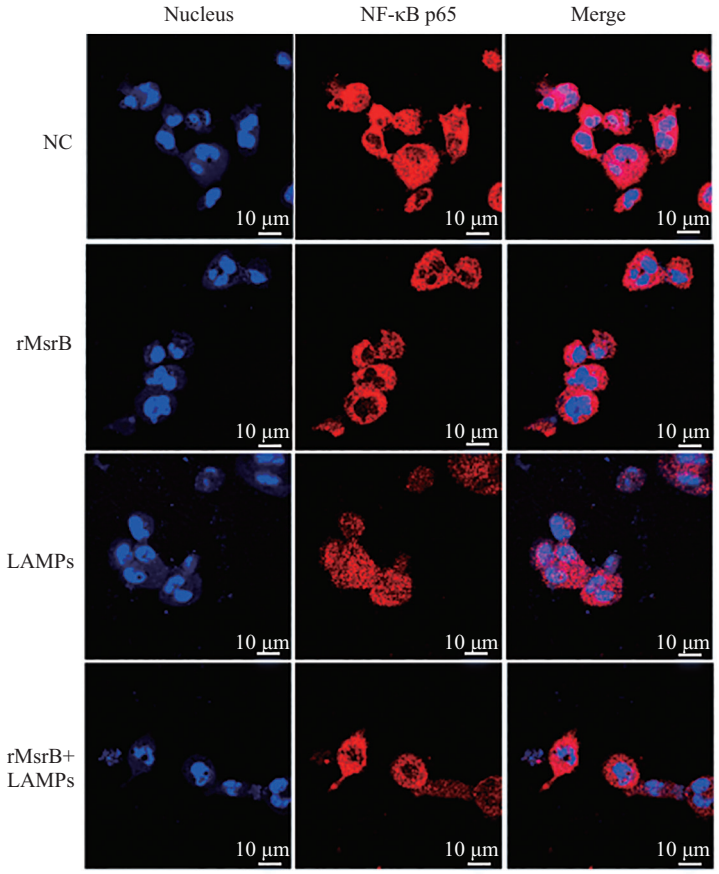
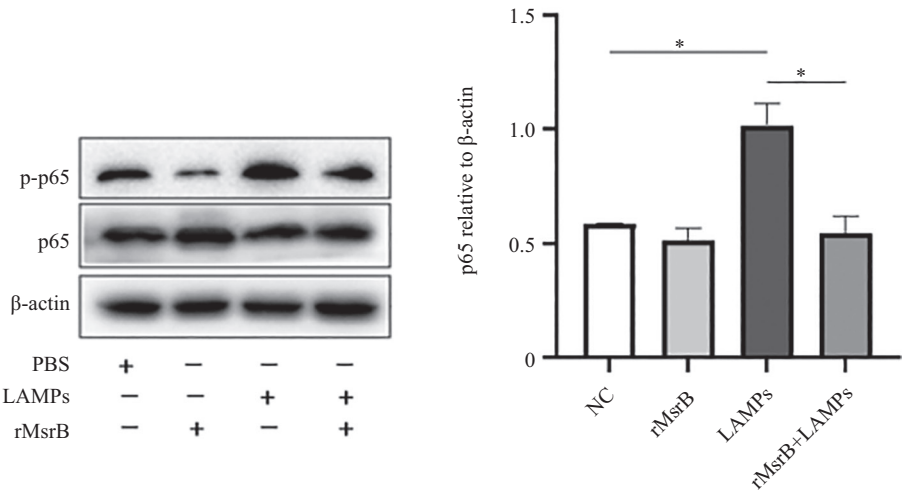


图6 Mp rMsrB抑制LAMPs刺激的THP-1细胞NF-κB核转位

Fig.6 Mp rMsrB inhibited NF-κB p65 translocation in LAMPs-stimulated THP-1 cells



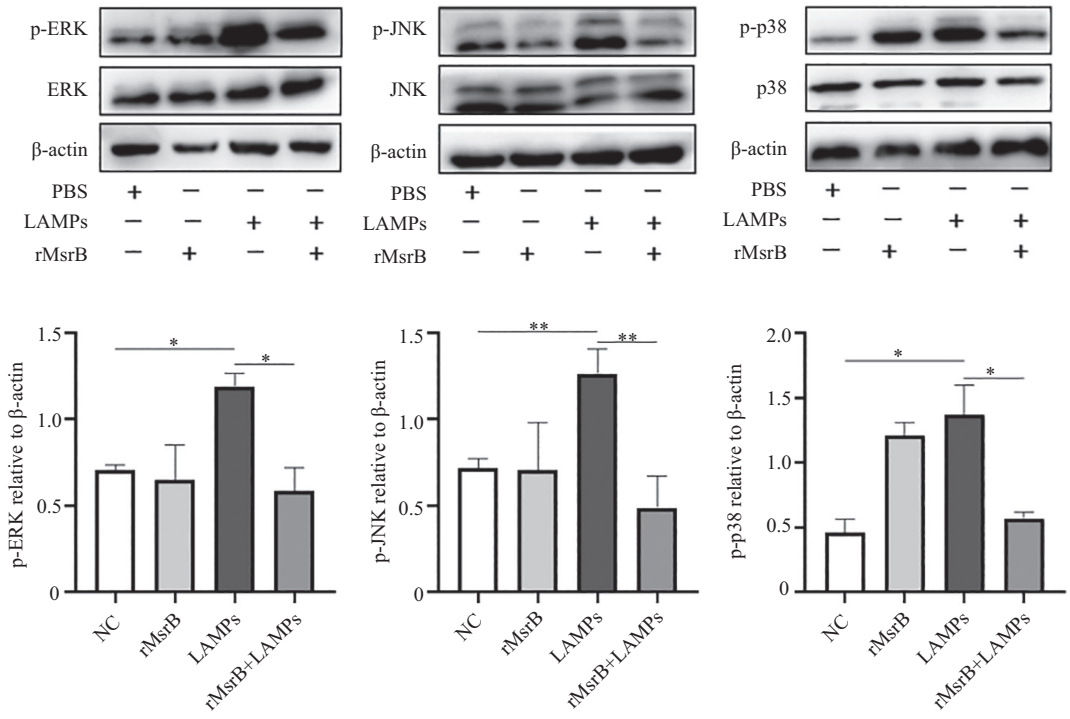
*P<0.05.

图7 Mp rMsrB抑制LAMPs刺激的THP-1细胞NF-κB p65的磷酸化

Fig.7 Mp rMsrB inhibited the phosphorylation of NF-κB p65 in LAMPs-stimulated THP-1 cells

3 讨论

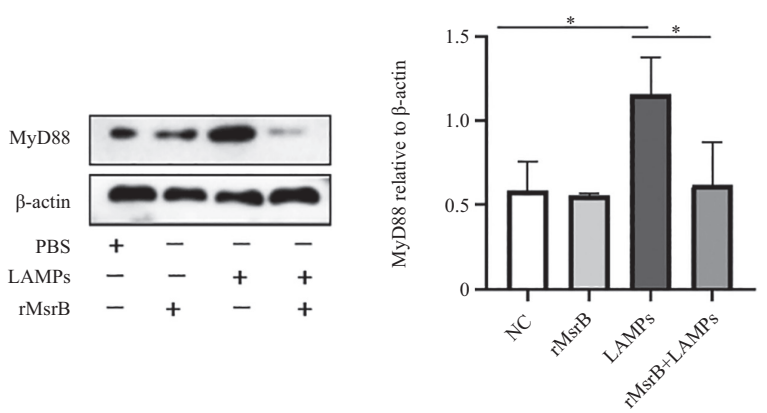
目前国内外关于Mp的抑炎机制研究较少。但有研究报道Mp的硫氧还蛋白可降解ROS[26], Mpn491可水解中性粒细胞诱捕网[27]。我们以往的研究发现渗透压诱导蛋白C超家族成员MPN625和MPN668可降解过氧化氢和过氧叔丁醇[28-29]。ROS的降解和中性粒细胞诱捕网的水解均有助于Mp逃避氧化应激, 从而减轻炎症损伤。

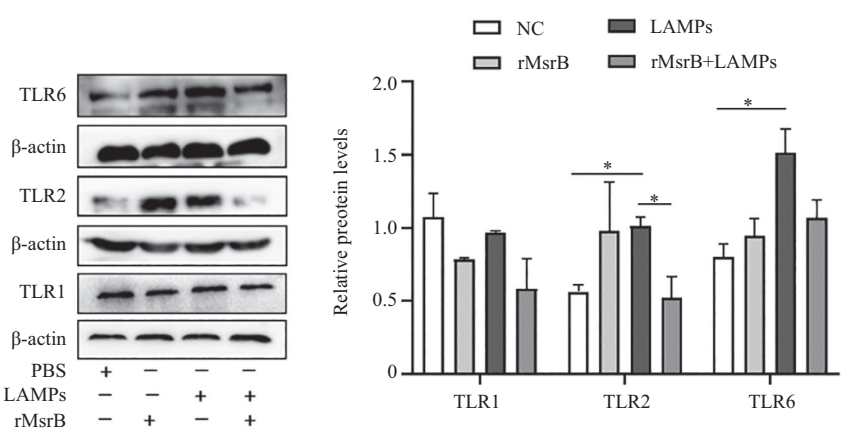


*P<0.05, **P<0.01.

图8 Mp rMsrB抑制LAMPs诱导的THP-1细胞ERK、JNK和p38的磷酸化

Fig.8 The phosphorylation of ERK, JNK and p38 were suppressed by Mp rMsrB in LAMPs-stimulated THP-1 cells

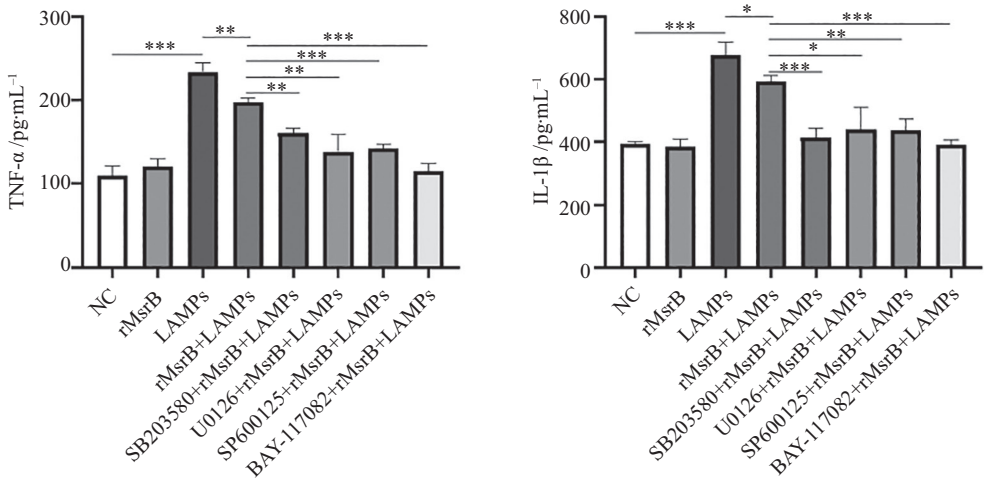




*P<0.05.

图9 rMsrB抑制LAMPs刺激的THP-1细胞TLR2和MyD88的表达

Fig.9 Mp rMsrB suppressed the expression of TLR2 and MyD88 in LAMPs-stimulated THP-1 cells



*P<0.05, **P<0.01, ***P<0.001.

图10 rMsrB抑制LAMPs刺激的THP-1细胞TNF-α和IL-1β的表达

Fig.10 rMsrB inhibited the expression of TNF-α and IL-1β in LAMPs-stimulated THP-1 cells

MsrB是一种基于硫醇的氧化还原酶, 以半胱氨酸为酶活性中心催化还原R型蛋氨酸亚砜为游离态和蛋白质结合态的蛋氨酸。MsrB在细菌、酵母、果蝇和哺乳动物等生物体中参与调控蛋白质的结构、功能并影响相关的信号通路[18,30]。为了解Mp MsrB在Mp激发的炎症反应中的作用, 该论文研究了MsrB调控LAMPs激活的THP-1细胞分泌促炎细胞因子的作用及信号通路。THP-1是一种人类白血病单核细胞系细胞, 用PMA处理后THP-1细胞可分化成巨噬细胞样细胞, 已被广泛用作研究单核细胞和单核细胞衍生巨噬细胞免疫反应能力的模型[31]。在本论文中, 我们先用rMsrB预处理THP-1细胞, 再用LAMPs刺激该细胞, 发现促炎细胞因子TNF-α和IL-1β的表达水平明显降低, 表明Mp MsrB能够抑制LAMPs刺激的THP-1细胞激发的炎症反应。

MAPK/NF-κB通路参与许多生理和病理过程, 如炎症分子的调节、细胞凋亡、应激反应和肿瘤生长抑制[32]。已有研究报道, Mp LAMPs与单核巨噬细胞表面的TLRs(包括TLR2/1和TLR2/6)结合, 通过MyD88和肿瘤坏死因子受体相关因子6激活NF-κB和激活蛋白-1, 从而诱导单核巨噬细胞合成分泌TNF-α、IL-1β和IL-6等炎症细胞因子和活性氧等炎性物质的合成[33-35]。为了解Mp MsrB是否通过MAPK/NF-κB通路抑制LAMPs诱生促炎细胞因子, 我们对Mp MsrB进行了定位分析并检测了相关信号分子的表达水平, 结果显示MsrB在Mp细胞质和细胞膜中均有表达。Mp LAMPs刺激后的THP-1细胞TLR2、TLR6和MyD88的表达水平升高, 但rMsrB预处理可以降低THP-1来源的巨噬细胞TLR2和MyD88的表达水平; 然而在本研究中TLR1似乎并没有参与LAMPs诱导的THP-1细胞的炎症反应, 这可能是因为LAMPs中主要含有二酰化脂蛋白, 但也有少量三酰基脂蛋白, 前者是TLR2/6异源二聚体的病原相关模式分子, 而后者可被TLR2/1识别[36]。另外本实验中使用的LAMPs浓度较低, 其中三酰基脂蛋白的浓度可能没有达到激活TLR2/1的临界浓度, 或者LAMPs虽然激活了TLR1, 但由于激活水平低, TLR1表达未显著增加, 导致Western blot未能检出。此外, 实验结果表明, rMsrB预处理可显著降低LAMPs刺激的THP-1细胞ERK、JNK、p38和NF-κB p65的磷酸化水平并抑制NF-κB的核转位, ERK、JNK、p38和NF-κB的抑制剂可进一步抑制rMsrB和LAMPs作用的THP-1细胞合成TNF-α和IL-1β, 这些结果表明rMsrB可能通过抑制MAPK/NF-κB信号通路抑制LAMPs刺激的THP-1细胞产生促炎细胞因子。

综上, 本研究通过体外细胞实验发现Mp MsrB可表达于细胞质与细胞膜, Mp细胞膜上的MsrB可能通过TLR2/MyD88/MAPK/NF-κB信号通路抑制Mp LAMPs刺激的单核巨噬细胞产生TNF-α和IL-1β, 从而发挥抑炎功能。本研究可丰富Mp的抗炎系统, 为了解Mp的免疫逃避机制及其与宿主细胞的相互作用提供了实验依据。但要明确Mp MsrB的抗炎作用

以及相关机制, 还需通过体内动物实验进一步验证。

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