

冠蛋白-1不同表达水平对巨噬细胞iNOS、TLRs表达以及凋亡的影响

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摘要 冠蛋白-1(Coronin-1)在真核细胞中广泛表达, 涉及钙离子稳态、细胞骨架动力学、免疫及炎症反应等多种细胞功能。该研究探讨了冠蛋白-1不同表达水平对巨噬细胞诱导性一氧化氮合酶(inducible nitric oxide synthase, iNOS)、Toll样受体(Toll-like receptors, TLRs)表达和凋亡的影响并探讨其分子机制。根据冠蛋白-1表达水平的差异, 实验细胞分为RAW264.7-Cor.Plus、RAW264.7、RAW264.7-Cor.Minus 3组。各组细胞分别经4 $\mu\text{mol/L}$ 钙调磷酸酶抑制剂环孢素A(cyclosporin A, CsA)处理24 h或2 $\mu\text{mol/L}$ 肌动蛋白聚合抑制剂松胞菌素D(cytochalasin D, cytD)处理48 h, 同时设未处理对照, 再分别用Real-time PCR法检测*iNOS* mRNA水平, Western blot法检测细胞TLR2、TLR4、TLR9及钙调磷酸酶(calcineurin, CaN)的蛋白质水平, 流式细胞术检测细胞凋亡百分率。各组细胞用四甲基异硫氰酸荧光素标记的鬼笔环肽(TRITC-phalloidin)染色后, 计算纤维型肌动蛋白(F-actin)重排率。结果显示, 冠蛋白-1过表达细胞相对于冠蛋白-1正常表达细胞, 其*iNOS* mRNA水平以及TLR2、TLR4、TLR9蛋白表达水平和细胞凋亡率均显著降低($P < 0.05$); CsA作用后, RAW264.7-Cor.Plus的*iNOS* mRNA水平以及TLR2、TLR4、TLR9蛋白质水平和细胞凋亡率均较未处理对照细胞显著增加($P < 0.05$), RAW264.7和RAW264.7-Cor.Plus细胞的CaN水平较未处理对照细胞显著降低($P < 0.05$); cytD作用后, 与未处理对照细胞相比, *iNOS* mRNA水平及细胞凋亡率无显著性差异($P > 0.05$), 但TLRs的表达显著降低($P < 0.05$); 经鬼笔环肽染色后, RAW264.7-Cor.Plus细胞F-actin重排率显著高于RAW264.7细胞($P < 0.05$)。以上结果表明, 冠蛋白-1过表达不仅促进了巨噬细胞F-actin重排, 而且下调了iNOS、TLRs水平并且抑制细胞凋亡, 其分子机制与冠蛋白-1促进钙调磷酸酶调控的Ca²⁺信号通路有关。

关键词 巨噬细胞; 冠蛋白-1; iNOS; TLRs; F-actin; 凋亡; 钙调磷酸酶

The Effects of Coronin-1 with Different Expression Levels on iNOS and TLRs Expression and Apoptosis in RAW264.7 Cells

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Abstract Coronin-1 is widely expressed in eukaryotic cells, which have been reported to be involved in a wide array of cellular functions including calcium homeostasis, cytoskeletal dynamics, immune and inflammatory responses. In order to investigate the effect and molecular mechanisms of different levels of Coronin-1 on the

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protein levels of iNOS and TLRs and apoptosis in RAW264.7 cell line, the experiment was divided into three groups (RAW264.7-Cor.Plus, RAW264.7 and RAW264.7-Cor.Minus) according to the differences of Coronin-1 expression level. Before and after treated with the CsA (a Calcineurin inhibitor) for 24 hours or cytD (an actin polymerization inhibitor) for 48 hours respectively, the level of *iNOS* mRNA was tested by Real-time PCR. The protein levels of TLR2, TLR4, TLR9 and calcineurin were detected by Western blot, and the apoptosis percent was analyzed by flow cytometry. Three groups of cells were stained with TRITC-phalloidin, then the percentage of F-actin rearrangement was calculated. The results were showed that the mRNA level of *iNOS*, the protein level of TLRs and apoptosis percent were significantly decreased in cells with over-expression of Coronin-1 (RAW264.7-Cor.Plus) ($P < 0.05$). After treated with CsA, the mRNA levels of *iNOS*, the protein levels of TLR2, TLR4 and TLR9 and apoptosis rate in RAW264.7-Cor.Plus cells were significantly increased compared with the untreated control cells ($P < 0.05$), but the CaN level of RAW264.7 and RAW264.7-Cor.Plus cells were significantly lower than that of untreated control cells ($P < 0.05$). After treated with cytD, between the treated cells and their untreated control cells, there were no significant difference in the mRNA levels of *iNOS* and apoptosis rate ($P > 0.05$), but the mRNA level of TLRs was significantly decreased ($P < 0.05$). By phalloidin staining, the F-actin rearrangement rate of RAW264.7-Cor.Plus cells was significantly higher than that of RAW264.7 cells ($P < 0.05$). These results indicated that the over-expression of Coronin-1 not only promotes the F-actin rearrangement, but also down-regulates the expression of TLRs and iNOS and inhibits cell apoptosis, of which the molecular mechanism is associated with that Coronin-1 promotes Ca^{2+} signaling pathway regulated by Calcineurin.

Keywords macrophage; Coronin-1; iNOS; TLRs; F-actin; apoptosis; calcineurin

结核分枝杆菌(*Mycobacterium tuberculosis*, Mtb)是一种胞内寄生菌, 感染人体后可逃逸巨噬细胞的免疫杀伤而在细胞中长期存活^[1-2]。Mtb诱导巨噬细胞高表达冠蛋白-1(Coronin-1), 聚集在吞噬体膜上的冠蛋白-1激活钙调磷酸酶信号通路, 抑制吞噬体和溶酶体融合, 是Mtb逃逸免疫杀伤的机制之一^[3-4]。研究表明, 用钙调磷酸酶(calcineurin, CaN)抑制剂——环孢素A(cyclosporin A, CsA)干预Mtb感染的巨噬细胞, 能有效地杀伤和清除胞内滞留的Mtb^[5]。研究表明, 冠蛋白-1与依赖纤维型肌动蛋白(F-actin)的细胞骨架有关, 但是在冠蛋白-1缺陷小鼠的巨噬细胞和沉默冠蛋白-1基因的巨噬细胞中, F-actin细胞骨架和依赖F-actin的细胞活性并没有显著缺失^[6-7]。巨噬细胞表达的诱导性一氧化氮合酶(inducible nitric oxide synthase, iNOS)和Toll样受体(Toll-like receptors, TLRs)等分子以及细胞凋亡效应在巨噬细胞吞噬和清除Mtb中发挥重要作用^[8-10]。冠蛋白-1不同表达水平是否影响iNOS、TLRs(TLR2、TLR4、TLR9)的表达和细胞凋亡, 其机制是否与F-actin和钙调磷酸酶信号通路相关, 尚未见报道。本研究拟利用冠蛋白-1抑制表达、正常表达和过表达的RAW264.7巨噬细胞株, 探讨不同表达水平的冠蛋白-1对iNOS、

TLRs的表达和细胞凋亡的影响及其分子机制, 为进一步阐明冠蛋白-1的效应并研究以冠蛋白-1为靶点的抗结核治疗机制奠定基础。

1 材料与方法

1.1 主要材料和试剂

RAW264.7鼠巨噬细胞株由重庆医科大学分子医学与肿瘤研究中心保存, 过表达冠蛋白-1的RAW264.7-Cor.Plus细胞和抑制表达冠蛋白-1的RAW264.7-Cor.Minus细胞由重庆医科大学分子医学与肿瘤研究中心前期构建^[11]。DMEM高糖培养基、胎牛血清、胰蛋白酶、青霉素-链霉素、环孢素A(CsA)购自北京鼎国昌盛生物技术公司。RIPA裂解液、凝胶试剂盒、BCA蛋白浓度测定试剂盒、4',6-二脒基-2-苯基吲哚(4',6-diamidino-2-phenylindole, DAPI)购自上海碧云天生物技术有限公司。CCK-8细胞增殖-毒性检测试剂盒购自日本同仁公司。PE Annexin V Apoptosis Detection Kit购自BD Biosciences公司。四甲基异硫氰酸标记的鬼笔环肽(TRITC-phalloidin)、松胞菌素D(cytochalasin D, cytD)、兔抗小鼠冠蛋白-1抗体、兔抗小鼠TLR2、TLR4、TLR9抗体、兔抗小鼠 β -actin抗体均购自

美国Sigma公司。兔抗小鼠钙调磷酸酶抗体购自美国Cell Signaling Technology公司。Trizol、Two Step SYBR[®] PrimeScript RT-PCR试剂盒、SYBR[®] Premix Ex Taq[™] II均购自大连TaKaRa公司。HRP标记的山羊抗兔二抗购自美国ABGNT公司。

1.2 细胞培养和分组

1.2.1 细胞培养 将RAW264.7-Cor.Plus、RAW264.7、RAW264.7-Minus这3种细胞置于含10%胎牛血清和1%青霉素-链霉素混合液的DMEM高糖培养基中,然后放置于37℃、含5% CO₂的孵箱中,每隔24~48 h观察细胞状态并换液,必要时以1:2或1:3比例传代,以维持细胞的良好生长状态。

1.2.2 实验分组 (1)CsA处理组:将生长于对数期的3种细胞调整细胞密度为 $7 \times 10^5 \sim 1 \times 10^6$ /mL,接种于6孔板,待细胞完全贴壁且生长状态良好时加入4 μmol/L CsA,作用时间为24 h。(2)cytD处理组:取相同密度的细胞接种于6孔板,待细胞生长状态良好时加入2 μmol/L cytD,作用时间为48 h^[11]。(3)对照组:未加任何药物作用的3种细胞作为未处理细胞对照。

1.3 Real-time PCR法测定细胞的*iNOS* mRNA水平

根据试剂盒说明分别提取CsA作用24 h、cytD作用48 h以及未处理组细胞的总RNA,用紫外分光光度法测定RNA的纯度与浓度。按照逆转录试剂盒操作说明将各组提取的总RNA使用两步法逆转录成cDNA,再用SYBR II嵌合荧光法,测定各组细胞*iNOS* mRNA水平。反应条件为:95℃温育10 s;95℃变性5 s,61.5℃退火20 s,72℃延伸12 s,共45个循环;最后,72℃延伸10 min至PCR反应结束。每个反应做3个复孔。*iNOS*引物和内参*GAPDH*引物由生工生物工程(上海)股份有限公司合成。上游引物*iNOS*-F:5'-GTT CTC AGC CCA ACA ATA CAA GA-3';下游引物*iNOS*-R:5'-GTG GAC GGG TCG ATG TCA C-3'。*GAPDH*-F:5'-AGG TCG GTG TGA ACG GAT TTG -3';*GAPDH*-R:5'-GGG GTC GTT GAT GGC AAC A-3'。扩增完毕后进行熔解曲线分析。熔解曲线在产物T_m处表现为单峰,表明扩增特异性良好。Real-time PCR结果采用2^{-ΔΔCt}法进行相对定量分析,ΔΔCt=对照组ΔCt(目的基因Ct-管家基因Ct)-各组ΔCt(目的基因Ct-管家基因Ct)。

1.4 Western blot法检测冠蛋白-1、Toll2、Toll4、Toll9及CaN蛋白质水平表达

将表达不同水平Coronin-1的3种细胞及其CsA

处理、cytD处理组巨噬细胞,分别用RIPA裂解液裂解提取细胞总蛋白,BCA蛋白定量试剂盒测定各组样品蛋白浓度后加入蛋白上样缓冲液煮沸备用。细胞的总蛋白经120 g/L SDS-PAGE分离后,转膜至PVDF膜上;用50 g/L脱脂奶粉室温封闭2 h后,与一抗4℃孵育过夜,一抗浓度:冠蛋白-1(1:800)、Toll2(1:1 000)、Toll4(1:1 200)、Toll9(1:1 200)、CaN(1:1 200)。TBST洗涤后,加HRP标记的山羊抗兔二抗(1:3 000),室温孵育2 h;洗涤后,采用化学发光试剂盒在凝胶成像系统上成像显影,使用Image Lab分析条带吸光度值。

1.5 流式细胞术(flow cytometry, FCM)检测细胞凋亡率

将表达不同水平冠蛋白-1的3种细胞及其CsA处理、cytD处理组巨噬细胞,经胰酶收集、冰PBS轻轻洗涤后,加入400 μL 1×Bing Buffer悬浮细胞,调整细胞浓度为 1×10^6 /mL,并加入5 μL Annexin V-PE,于冰上孵育约15 min后加入10 μL 7-ADD,避光染色5 min,FCM检测细胞凋亡率。

1.6 荧光显微镜下观察TRITC-phalloidin染色细胞的F-actin重排情况

置盖玻片于24孔板中,分别种板RAW264.7-Cor.Minus、RAW264.7和RAW264.7-Cor.Plus组细胞,爬片生长24 h,使其密度达到50%的汇合度。37℃预热的PBS清洗细胞2次,40 g/L的多聚甲醛室温固定10 min;PBS洗涤3次,200 μL 100 nmol/L TRITC-phalloidin室温避光染色40 min;PBS洗3次,DAPI染色30 s;PBS洗涤4次,荧光显微镜下计数100个细胞,镜下显示典型F-actin聚集结构者计1分,非典型者计0.5分,没有发生聚集者计0分,F-actin重排率=总计分/100^[12-13]。

1.7 统计学分析

所有实验均重复3次,实验结果的计量资料以均值±标准差($\bar{x} \pm s$)来表示。采用SPSS 16.0软件进行正态性检验及One-Way ANOVA(单因素方差分析)比较各组内及组间差异, $P < 0.05$ 为差异具有统计学意义。

2 结果

2.1 冠蛋白-1过表达下调巨噬细胞*iNOS* mRNA水平

Real-time PCR结果显示,冠蛋白-1过表达的RAW264.7-Cor.Plus细胞的*iNOS* mRNA水平显著低于冠蛋白-1正常表达的RAW264.7细胞和冠蛋白-1抑

制表达的RAW264.7-Cor.Minus细胞($P<0.05$), 但是RAW264.7细胞与RAW264.7-Cor.Minus细胞相比较, 其*iNOS* mRNA水平无显著性差异($P>0.05$)(图1A)。CsA处理细胞后, RAW264.7-Cor.Plus细胞的*iNOS* mRNA水平显著高于相应的未处理细胞($P<0.05$), 但是RAW264.7细胞在CsA处理前后, 其*iNOS* mRNA水平无显著性差异($P>0.05$)(图1B)。cytD处理前后, RAW264.7-Cor.Plus细胞及RAW264.7细胞的*iNOS* mRNA水平没有呈现显著差异($P>0.05$)(图1B)。此结果表明, 冠蛋白-1过表达抑制了巨噬细胞*iNOS* mRNA的转录水平, 但是钙调磷酸酶抑制剂CsA可以部分解除或抵消冠蛋白-1对*iNOS*的抑制效应。

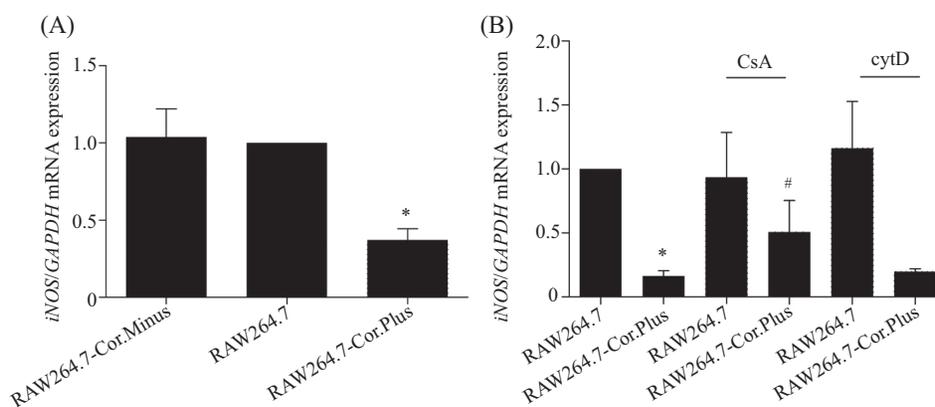
2.2 冠蛋白-1过表达抑制巨噬细胞Toll2、Toll4及Toll9蛋白质表达

Western blot检测3种细胞冠蛋白-1蛋白质水平, 冠蛋白-1相对水平分别为: RAW264.7-Cor.Minus(0.29 ± 0.06)<RAW264.7(0.91 ± 0.21)<RAW264.7-Cor.Plus(1.54 ± 0.34); 各组间比较, 差异具有统计学意义($P<0.05$), 符合siRNA沉默和过表达预期结果(图2A)。Western blot检测各组巨噬细胞Toll2、Toll4、Toll9蛋白质水平, 通过与内参 β -actin比较, 过表达冠蛋白-1的RAW264.7-Cor.Plus细胞, 其Toll2、Toll4、Toll9蛋白质水平显著低于正常表达冠蛋白-1的RAW264.7细胞($P<0.05$), 其相对水平分别为Toll2: RAW264.7(1.18 ± 0.35)>RAW264.7-Cor.Plus(0.39 ± 0.08); Toll4: RAW264.7(1.39 ± 0.69)>RAW264.7-Cor.Plus(0.30 ± 0.09); Toll9: RAW264.7(1.22 ± 0.41)>RAW264.7-

Cor.Plus(0.21 ± 0.07)(图2B)。抑制表达冠蛋白-1的RAW264.7-Cor.Minus细胞与正常表达冠蛋白-1的RAW264.7细胞相比, 其TLRs差异无显著性($P>0.05$)(图2B)。CsA处理细胞后, Coronin-1过表达的RAW264.7-Cor.Plus细胞相对于CsA处理前, 其Toll2、Toll4、Toll9蛋白质水平显著增加($P<0.05$), 相对水平分别为: Toll2(0.65 ± 0.06)、Toll4(0.72 ± 0.10)、Toll9(0.56 ± 0.19); cytD显著下调了RAW264.7-Cor.Plus细胞及RAW264.7细胞的Toll2、Toll4、Toll9蛋白质水平表达, 故该抑制效应与冠蛋白-1水平无关(图2C和图2D)。此结果表明, 冠蛋白-1过表达下调了巨噬细胞TLRs的水平, 但是钙调磷酸酶抑制剂CsA可以部分解除或抵消冠蛋白-1对TLRs的下调效应。

2.3 冠蛋白-1过表达抑制巨噬细胞的凋亡

Annexin-V-PE/7-ADD双染后, FCM检测CsA和cytD处理前后细胞的凋亡率。如图3A所示, 冠蛋白-1过表达的RAW264.7-Cor.Plus细胞的凋亡率显著低于冠蛋白-1正常表达的RAW264.7细胞, 其凋亡率分别为: RAW264.7-Cor.Plus($3.69\%\pm 0.02\%$)<RAW264.7($11.59\%\pm 1.58\%$)($P<0.05$); 冠蛋白-1抑制表达的RAW264.7-Cor.Minus细胞的凋亡率与RAW264.7细胞相比, 差异无显著性($P>0.05$)。CsA处理后, 冠蛋白-1过表达的RAW264.7-Cor.Plus细胞相对于CsA处理前, 其凋亡率显著增加为($8.22\%\pm 0.68\%$)($P<0.05$); cytD处理前后, RAW264.7-Cor.Plus细胞及RAW264.7细胞的凋亡率没有呈现显著差异($P>0.05$)(图3A和图3B)。结果表明, Coronin-1过表

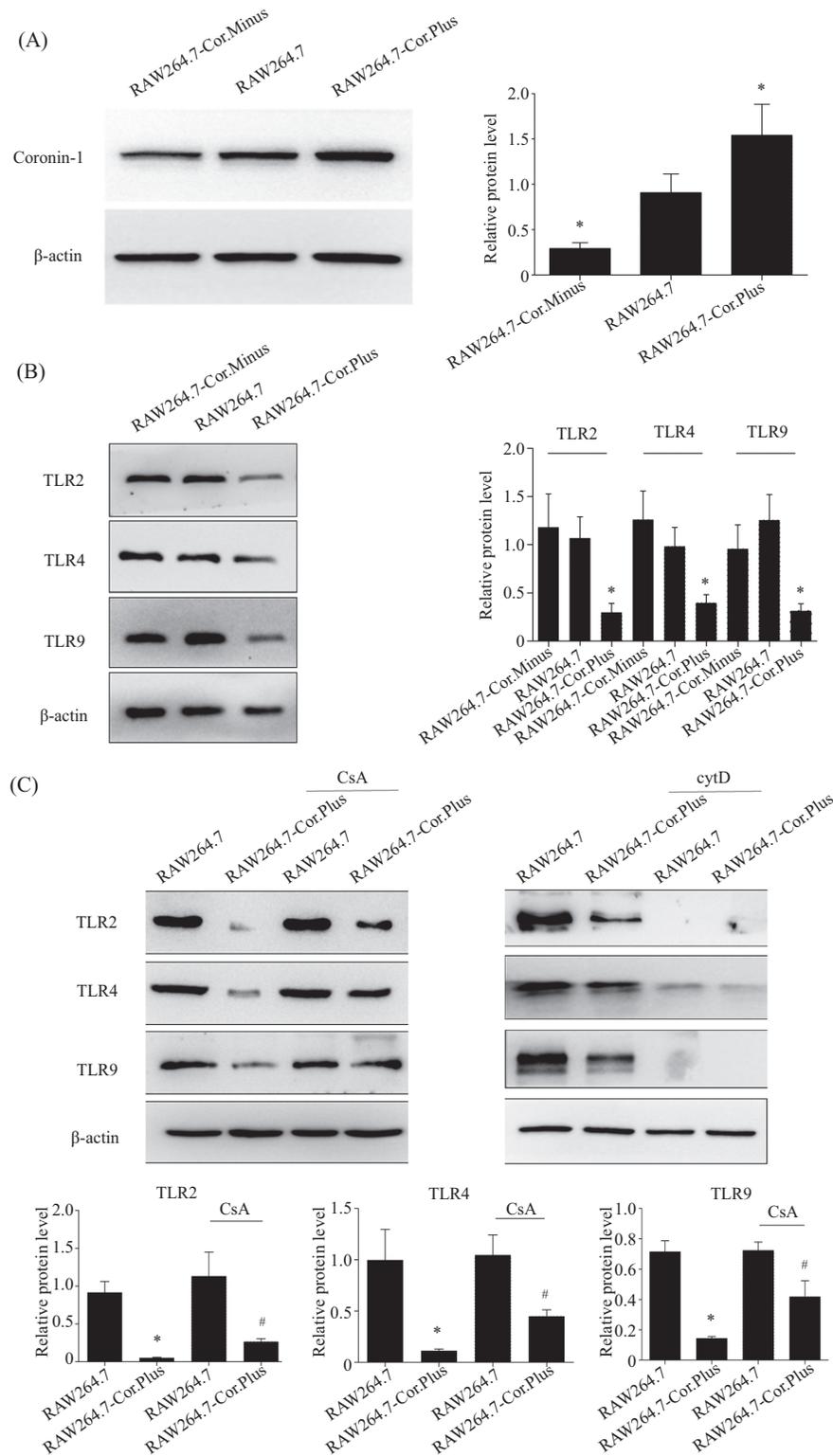


A: 表达不同水平冠蛋白-1的3组细胞的*iNOS* mRNA水平比较; B: CsA和cytD对*iNOS* mRNA水平的影响。* $P<0.05$, 与正常组RAW264.7相比较; # $P<0.05$, 与未处理组RAW264.7-Cor.Plus相比较。

A: the level of *iNOS* mRNA in cells with different levels of Coronin-1; B: effect of CsA or cytD on *iNOS* mRNA level. * $P<0.05$ compared with the control group RAW264.7; # $P<0.05$ compared with the untreated group RAW264.7-Cor.Plus.

图1 Real-time PCR检测*iNOS* mRNA水平

Fig.1 mRNA level of *iNOS* detected by Real-time PCR



A: RAW264.7-Cor.Minus、RAW264.7和RAW264.7-Cor.Plus这三种细胞Coronin-1的蛋白表达水平; B: 表达不同水平冠蛋白-1的3组细胞TLR2、TLR4和TLR9蛋白质水平; C: CsA和cytD对TLR2、TLR4和TLR9蛋白质水平的影响; D: CsA作用前后RAW264.7和RAW264.7-Cor.Plus细胞TLR2、TLR4和TLR9的相对水平。* $P < 0.05$, 与正常组RAW264.7相比较; # $P < 0.05$, 与未处理组RAW264.7-Cor.Plus相比较。

A: the relative protein levels of Coronin-1 in RAW264.7-Cor.Minus, RAW264.7 and RAW264.7-Cor.Plus cells; B: the protein levels of TLR2, TLR4 and TLR9 in cells with different levels of Coronin-1; C: effect of CsA or cytD on the expression of TLR2 and TLR4 and TLR9; D: relative gray scale of RAW264.7 and RAW264.7-Cor.Plus cells before and after treated with CsA. * $P < 0.05$ compared with the control RAW264.7; # $P < 0.05$ compared with the untreated group RAW264.7-Cor.Plus.

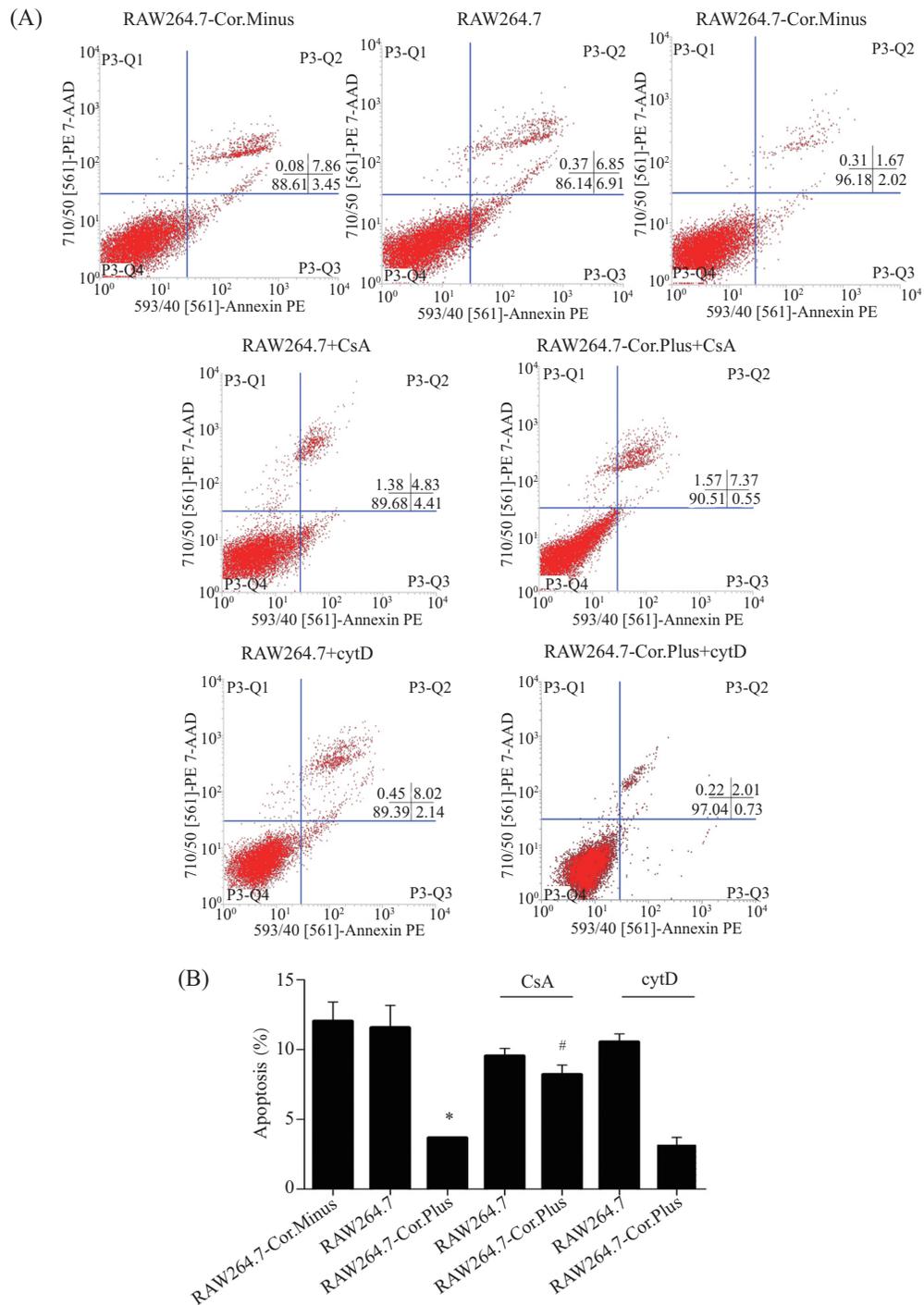
图2 Western blot检测巨噬细胞TLR2、TLR4和TLR9蛋白质水平

Fig.2 Protein levels of TLR2, TLR4 and TLR9 of macrophages detected by Western blot

达抑制了巨噬细胞的凋亡, 但是钙调磷酸酶抑制剂 CsA 可以部分消除冠蛋白-1 对细胞凋亡的抑制效应。

2.4 冠蛋白-1 过表达促进巨噬细胞 CaN 的表达

Western bolt 法检测表达不同水平冠蛋白-1 的 3 组细胞的 CaN 蛋白质水平。结果显示, 钙调磷酸酶



A: 表达不同水平冠蛋白-1 的 3 种细胞及 CsA、cytD 处理后 RAW264.7 和 RAW264.7-Cor.Plus 细胞的凋亡情况; B: 表达不同水平冠蛋白-1 的 3 种细胞及 CsA 和 cytD 处理后 RAW264.7 和 RAW264.7-Cor.Plus 细胞凋亡率。* $P < 0.05$, 与正常组 RAW264.7 相比较; # $P < 0.05$, 与未处理组 RAW264.7-Cor.Plus 相比较。

A: apoptosis of cells with different levels of Coronin-1 and RAW264.7 and RAW264.7-Cor.Plus cells before and after treated with CsA or cytD; B: the apoptosis rate of RAW264.7 and RAW264.7-Cor.Plus cells before and after treated with CsA or cytD. * $P < 0.05$ compared with the control group RAW264.7; # $P < 0.05$ compared with the untreated group RAW264.7-Cor.Plus.

图3 流式细胞术检测巨噬细胞的凋亡率

Fig.3 Apoptosis rate of macrophages detected by flow cytometry

水平与细胞冠蛋白-1的表达呈正相关,冠蛋白-1水平最高的RAW264.7-Cor.Plus细胞,其CaN表达量也最高,冠蛋白-1表达水平最低的RAW264.7-Cor.Minus细胞,其CaN水平也最低,相对水平分别为:RAW264.7-Cor.Plus(1.02 ± 0.15)>RAW264.7(0.69 ± 0.07)>RAW264.7-Minus(0.38 ± 0.09)($P < 0.05$)(图4)。钙调磷酸酶抑制剂CsA处理RAW264.7和RAW264.7-Cor.Plus细胞后,CaN水平较未处理组显著降低($P < 0.05$)(图4)。结果表明,冠蛋白-1过表达可上调巨噬细胞CaN的水平,CsA可下调巨噬细胞CaN的水平。

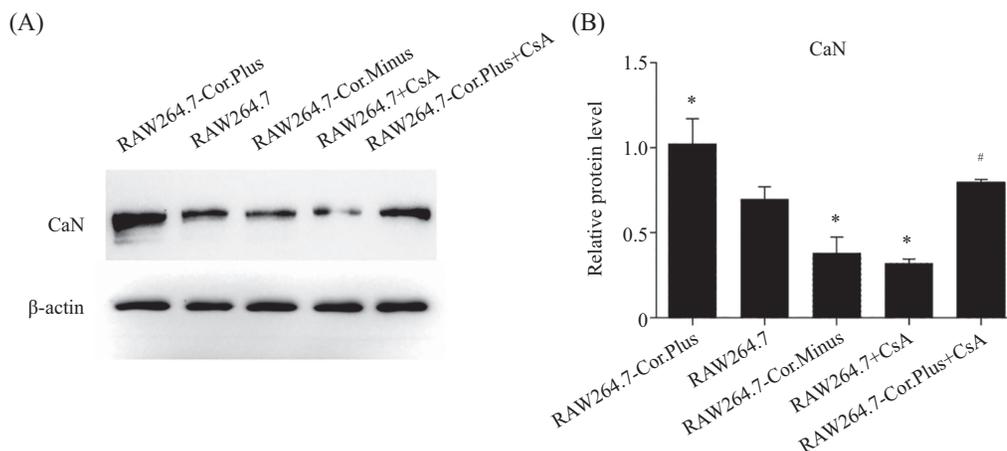
2.5 冠蛋白-1过表达促进巨噬细胞F-actin重排

四甲基异硫氰酸荧光素标记的鬼笔环肽(TRITC-phalloidin)可特异性结合微丝骨架应力纤维主要成分F-actin,且带有荧光标记,常用于研究F-actin的分布。荧光结果显示,冠蛋白-1过表达的细胞RAW264.7-Cor.Plus细胞的F-actin重排率显著高于冠蛋白-1正常表达的RAW264.7细胞:RAW264.7-Cor.Plus(0.81 ± 0.03)>RAW264.7(0.66 ± 0.02)($P < 0.05$)(图5)。当RAW264.7细胞经肌动蛋白聚合抑制剂cytD处理后,相对于cytD处理前,其F-actin重排率显著降低($P < 0.05$)(图5)。结果表明,冠蛋白-1过表达能促进巨噬细胞F-actin重排,肌动蛋白聚合抑制剂cytD对细胞F-actin重排具有抑制效应,但与冠蛋白-1表达水平无关。

3 讨论

大多数病原微生物通过巨噬细胞吞噬、溶酶体降解后能被有效地清除,结核分枝杆菌(*Mycobacterium tuberculosis*, Mtb)却能够逃逸巨噬细胞的免疫杀伤而在细胞中长期存活。研究表明,冠蛋白-1缺乏时,感染的巨噬细胞能有效地杀伤Mtb,说明冠蛋白-1在Mtb感染宿主细胞导致胞内滞留过程中至关重要^[14-15]。本研究构建并筛选了稳定高表达冠蛋白-1的RAW264.7-Cor.Plus细胞和低表达冠蛋白-1的RAW264.7-Cor.Minus细胞,再结合RAW264.7对照细胞,研究高、中、低3种冠蛋白-1表达水平对巨噬细胞iNOS、TLRs表达以及细胞凋亡的影响。结果显示,冠蛋白-1过表达下调了iNOS、TLR2、TLR4和TLR9的水平,同时抑制了细胞凋亡。

iNOS、TLRs和细胞凋亡在宿主细胞清除胞内病原体过程中发挥重要作用。iNOS是一种关键的调节酶,是反应性氮中间产物的主要促发因子。研究表明,iNOS基因缺陷小鼠,其对Mtb的免疫杀伤能力明显减弱。由于TLR2能够识别脂磷壁酸、二酰半胱氨酸等病原体相关模式分子脂肽;TLR4能够识别革兰氏阴性菌的主要致病成分脂多糖(lipopolysaccharides, LPS)和坏死细胞的热休克蛋白(heat shock proteins, HSPs);TLR9能够识别细菌的CpG-DNA,激活B细胞和抗原提呈细胞。据此,本研

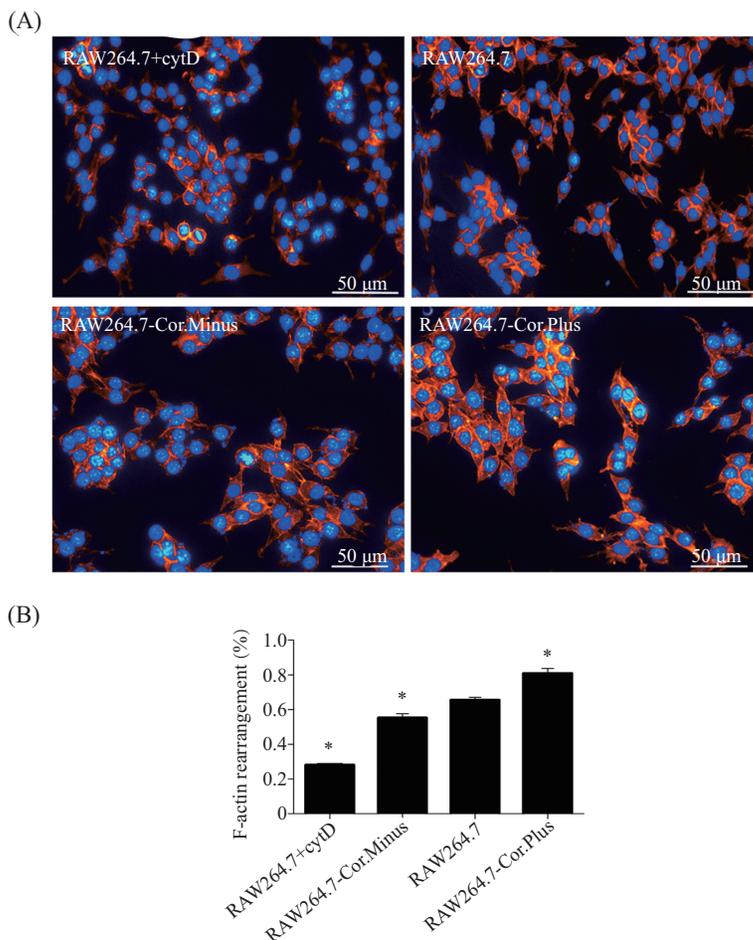


A: 表达不同水平冠蛋白-1的3组细胞及CsA处理后RAW264.7和RAW264.7-Cor.Plus细胞的CaN的Western blot检测结果; B: 表达不同水平冠蛋白-1的3组细胞及CsA处理后RAW264.7和RAW264.7-Cor.Plus细胞的CaN相对水平。* $P < 0.05$,与正常组RAW264.7相比较; # $P < 0.05$,与未处理组RAW264.7-Cor.Plus相比较。

A: protein levels of CaN in cells with different levels of Coronin-1 and RAW264.7 and RAW264.7-Cor.Plus cells treated with CsA; B: the relative protein level of CaN in cells with different levels of Coronin-1 and RAW264.7 and RAW264.7-Cor.Plus cells treated with CsA. * $P < 0.05$ compared with the control group RAW264.7; # $P < 0.05$ compared with the untreated group RAW264.7-Cor.Plus.

图4 Western blot检测巨噬细胞CaN蛋白质水平

Fig.4 Protein levels of CaN of macrophages detected by Western blot



A: 表达不同水平冠蛋白-1的3组细胞及cytD处理的RAW264.7细胞的F-actin重排情况; B: 表达不同水平冠蛋白-1的3组细胞及cytD处理的RAW264.7细胞的F-actin重排率。* $P < 0.05$, 与正常组RAW264.7相比较。

A: the rearrangement of F-actin in cells with different levels of Coronin-1 and RAW264.7 cells treated with cytD; B: the rearrangement rate of F-actin in cells with different levels of Coronin-1 and RAW264.7 cells treated with cytD. * $P < 0.05$ compared with the control group RAW264.7.

图5 TRITC-phalloidin染色分析F-actin的重排情况

Fig.5 The rearrangement of F-actin analyzed by TRITC-phalloidin staining

究选择这3种在巨噬细胞固有免疫过程中相对重要的Toll样受体来研究冠蛋白-1的不同表达水平对巨噬细胞TLRs的影响。此外,小鼠缺乏髓样分化因子88(myeloid differentiation factor 88, MyD88)蛋白时,在感染Mtb后迅速死亡^[16],TLR配体蛋白MyD88和p38能够调节吞噬体的成熟^[17],这些都揭示了TLR信号通路在控制Mtb感染过程中的重要性。

钙离子运动和钙调磷酸酶活性是典型的促生存信号,冠蛋白-1缺陷的T细胞,半胱冬肽酶3(caspase 3)表达增加,BCL-2表达降低,这可能与冠蛋白-1通过激活钙调磷酸酶信号通路来调节细胞凋亡相关。有研究表明,冠蛋白-1基因缺乏或突变的T细胞出现细胞骨架缺陷是由于依赖F-actin调节过程的紊乱^[18-20]。因此,冠蛋白-1与iNOS、TLRs和凋亡之间可能存在某种调节机制,其涉及细胞骨架之间

的相互作用以及钙调磷酸酶信号通路。

本研究筛选出了钙调磷酸酶抑制剂CsA和F-actin解聚剂cytD的最佳作用浓度和时间,以此研究冠蛋白-1的不同表达水平对iNOS、TLRs水平及细胞凋亡的影响及其相关的分子机制。本研究结果表明,巨噬细胞过表达的冠蛋白-1显著下调了iNOS、TLRs水平以及细胞凋亡,冠蛋白-1过表达对iNOS、TLRs水平和细胞凋亡的抑制作用可以被钙调磷酸酶抑制剂CsA部分消除,但是F-actin解聚剂cytD并没有消除冠蛋白-1的这种抑制效应,再结合CsA对冠蛋白-1正常表达细胞无显著影响、冠蛋白-1促进CaN的表达等结果,可以推论:巨噬细胞过表达的冠蛋白-1下调iNOS、TLRs的水平及细胞凋亡,其分子机制与冠蛋白-1促进钙调磷酸酶调控的 Ca^{2+} 信号通路有关。本研究还发现,F-actin解聚剂cytD处理

细胞的TLRs的水平被显著下调,但这种效应与冠蛋白-1的表达水平无关;冠蛋白-1的过表达虽可促进F-actin的重排,但是其解聚剂cytD并不能阻止或解除冠蛋白-1对iNOS、TLRs和细胞凋亡的抑制作用。由此说明,冠蛋白-1可能通过激活钙调磷酸酶信号通路,下调巨噬细胞iNOS、TLRs的水平和细胞凋亡,这一过程并不依赖于F-actin调节。

本研究发现,冠蛋白-1过表达具有下调iNOS、TLRs水平和抑制细胞凋亡的显著效应,该效应有利于Mtb的免疫逃逸,与Mtb利用宿主细胞冠蛋白-1逃逸免疫杀伤的论断相佐证。本研究结果可为进一步阐释冠蛋白-1在固有免疫中的功能及其分子机制、研究以冠蛋白-1为靶点的抗结核机制奠定基础。

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