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在线全文

·论 著·

*Pp2cm*基因沉默对小鼠巨噬细胞通过TLR通路抵抗金黄色葡萄球菌感染的影响*

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【摘要】 目的 探究蛋白磷酸酶2Cm（protein phosphatase 2Cm, PP2Cm）的基因*Pp2cm*沉默对感染金黄色葡萄球菌（*Staphylococcus aureus*, *S. aureus*）后巨噬细胞炎症因子表达的影响及作用机制。**方法** 通过腺病毒（adenovirus, Ad）转染Raw264.7小鼠巨噬细胞系分析了*Pp2cm*基因敲低对巨噬细胞炎症因子、细胞增殖凋亡和Toll样受体（Toll-like receptor, TLR）信号通路的影响。细胞处理分为4组，包括Ad-Ctrl组、Ad-*Pp2cm*组、Ad-Ctrl+*S. aureus*组和Ad-*Pp2cm*+*S. aureus*组。分别在细胞中加入对照腺病毒（Ad-Ctrl）或针对*Pp2cm*基因的腺病毒（Ad-*Pp2cm*），使用或不使用金黄色葡萄球菌进行炎症诱导。实时荧光定量聚合酶链式反应（real-time fluorescent quantitative polymerase chain reaction, RT-qPCR）检测肿瘤坏死因子α（tumor necrosis factor-alpha, *TNF-α*）、白细胞介素1β（interleukin 1 beta, *IL-1β*）、*TLR2*、*TLR4*、Toll样受体衔接蛋白（Toll-like receptor adaptor protein, *Tirap*）和髓样分化因子88（myeloid differentiation factor 88, *Myd88*）基因表达，蛋白印迹法（Western blot）技术检测PP2Cm蛋白表达，Cell Counting Kit-8（CCK-8）法测定细胞增殖，流式细胞术检测细胞凋亡。**结果** Ad-*Pp2cm*组巨噬细胞*Pp2cm* mRNA和PP2Cm蛋白表达水平均低于Ad-Ctrl组，差异有统计学意义（$P<0.05$）。与Ad-Ctrl+*S. aureus*组相比，Ad-*Pp2cm*+*S. aureus*组巨噬细胞中*TNF-α*与*IL-1β*基因表达水平升高，差异有统计学意义（$P<0.01$）。与Ad-Ctrl组巨噬细胞相比，Ad-*Pp2cm*组巨噬细胞中*TLR2*、*TLR4*、*Tirap* 和*Myd88*基因表达水平升高，差异有统计学意义（$P<0.05$）。Ad-Ctrl组与Ad-*Pp2cm*组巨噬细胞的细胞凋亡和细胞增殖差异无统计学意义。**结论** *Pp2cm*基因沉默促进巨噬细胞对金黄色葡萄球菌感染的炎症响应。TLR通路在巨噬细胞炎症激活过程中发挥重要作用。

【关键词】 金黄色葡萄球菌 蛋白磷酸酶2Cm Toll样受体

Effect of *Pp2cm* Gene Silencing on Mouse Macrophage Resistance Against *Staphylococcus aureus* Infection via TLR Pathway

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【Abstract】 Objective To investigate the effect of silencing protein phosphatase 2cm (*Pp2cm*) gene on the expression of inflammatory factors in macrophages infected with *Staphylococcus aureus* (*S. aureus*) and the mechanisms involved. **Methods** The effects of *Pp2cm* knockdown on inflammatory factors, proliferation, apoptosis, and Toll-like receptor (TLR) signaling were analyzed in RAW 264.7 cells, a murine macrophage cell line, transfected with adenovirus (Ad). The cells were divided into four groups, including Ad-Ctrl group, Ad-*Pp2cm* group, Ad-Ctrl+*S. aureus* group and Ad-*Pp2cm*+*S. aureus* group. Cell transfection was achieved by separately introducing control adenovirus (Ad-Ctrl) or adenovirus targeting the *Pp2cm* gene (Ad-*Pp2cm*) and inflammation or the absence of inflammation was induced by applying or not applying *S. aureus*. The expression of tumor necrosis factor-alpha (*TNF-α*), interleukin-1β (*IL-1β*), *TLR2*, *TLR4*, Toll-like receptor adaptor protein (*Tirap*) and myeloid differentiation factor 88 (*Myd88*) was determined by real-time fluorescent quantitative polymerase chain reaction (RT-qPCR). PP2Cm protein expression was determined by Western blot. Cell proliferation was determined by cell counting kit-8 (CCK-8) assay and cell apoptosis was measured by flow cytometry. **Results** The expression of *Pp2cm* gene and PP2Cm protein was downregulated in the Ad-*Pp2cm* group when compared to the Ad-Ctrl group, with the diference showing statistical significance ($P<0.05$). When compared to those of the Ad-Ctrl+*S. aureus* group, macrophages in the Ad-*Pp2cm*+*S. aureus* group showed significantly increase in the *TNF-α* and *IL-1β* gene levels ($P<0.01$). Furthermore, the Ad-*Pp2cm* group demonstrated elevated gene expression levels of

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TLR2, *TLR4*, *Tirap* and *Myd88* in macrophages when compared to the Ad-Ctrl group, with the difference showing statistical significance (P<0.05). There were no statistically significant differences in cell apoptosis and proliferation between the Ad-Ctrl and Ad-*Pp2cm* groups. **Conclusions** Silencing *Pp2cm* gene promotes the inflammatory response of macrophages to *S. aureus* infection. Moreover, the TLR pathway plays an important role in the inflammatory activation of macrophages.

【Key words】 *Staphylococcus aureus* Protein phosphatase 2Cm Toll-like receptor

2型糖尿病(type 2 diabetes mellitus, T2DM)患者金黄色葡萄球菌(*Staphylococcus aureus*, *S. aureus*)感染率高于非糖尿病患者,严重时可发展为脓毒血症,危及患者生命,预后较差[1-3]。蛋白磷酸酶2Cm(protein phosphatase 2Cm, PP2Cm)参与支链氨基酸(branched-chain amino acids, BCAA)代谢[4]。糖尿病常发生BCAA代谢障碍。有文献报道PP2Cm与糖尿病关系密切,PP2Cm在糖尿病患者中常突变[5-7]。同时文献报道BCAA代谢障碍会引起炎症[8],提示糖尿病患者金黄色葡萄球菌损伤加重可能与BCAA代谢关键酶PP2Cm有关。

巨噬细胞可分泌肿瘤坏死因子α(tumor necrosis factor-alpha, TNF-α)和白细胞介素1β(interleukin 1 beta, IL-1β)等细胞因子[9]。巨噬细胞通过Toll样受体(Toll-like receptor, TLR)等识别细菌成分,识别金黄色葡萄球菌主要依靠的是TLR2受体[10],也有研究报道TLR4参与巨噬细胞对金黄色葡萄球菌的免疫反应[11-12]。

TLR受体及下游部分共同参与炎症,称为TLR信号通路,构成TLR受体的除了TLR分子还有髓样分化因子(myeloid differentiation factor 88, MyD88)和Toll受体衔接蛋白(Toll-like receptor adaptor protein, TIRAP),可激活一系列的信号级联反应向胞内传递信息[13-15]。PP2Cm与金葡菌感染损伤加重以及TLR受体的关系尚无相关报道。本研究采用小鼠单核巨噬细胞白血病细胞系Raw264.7作为研究对象,通过金黄色葡萄球菌刺激构建炎症模型,使用腺病毒(adenovirus, Ad)敲低*Pp2cm*表达,探讨PP2Cm沉默对巨噬细胞抵抗金黄色葡萄球菌感染的影响及相关机制。

1 材料与方法

1.1 细胞培养及分组

在LB培养液中培养金黄色葡萄球菌(ATCC,美国),新鲜扩增的细菌溶液在3 000 r/min离心5 min以去除培养基。使用多聚甲醛脱水固定细菌,离心去除多聚甲醛后,使用PBS重悬细菌后再次离心去除上清,之后再用PBS重悬待用。取Raw264.7小鼠巨噬细胞株(ATCC,美国),在含10%FBS、1%~2%青链霉素的DMEM中培养,在温度设定37 ℃、体积分数5%二氧化碳的恒温孵箱里培养,每1~2 d传代一次。细胞分为Ad-Ctrl组、Ad-*Pp2cm*组、Ad-Ctrl+*S. aureus*组和Ad-*Pp2cm*+*S. aureus*组。分别在细胞中加入对照腺病毒(Ad-Ctrl)或针对*Pp2cm*基因的腺病毒(Ad-*Pp2cm*),使用或不使用金黄色葡萄球菌进行炎症诱导。

1.2 试剂

DMEM高糖培养基(HyClone,美国),胎牛血清(FBS, Biological Industries,美国),青链霉素(HyClone,美国),PBS缓冲液(PBS, biosharp,中国),Trizol(Vazyme,中国),HiScriptQ RT SuperMix试剂盒(Vazyme,中国),SYBR Green PCR mix(Bio-rad,美国),Cell Counting Kit-8细胞计数试剂(CCK-8, Beyotime,中国),RIPA裂解液(Beyotime,中国),蛋白酶抑制剂(Roche,瑞士),LB培养液(Sigma,日本),流式凋亡检测试剂盒(4A Biotech,中国)。

1.3 细胞转染

采用腺病毒(吉凯基因,中国)对Raw264.7小鼠巨噬细胞系*Pp2cm*基因进行敲低。处理时按2×10^{5}细胞/孔,接种于6孔板。培养24 h后按感染复数(multiplicity of infection, MOI)=150加入Ad-Ctrl或者Ad-*Pp2cm*培养48 h,更换培养基并根据分组进行处理,腺病毒载体序列见表1。

表 1 腺病毒载体序列

Table 1 Adenovirus vector sequence

Gene	Vector	Target sequence
Pp2cm	hU6-MCS-CMV-EGFP	CCTAGCATCAAGTACGGCAAA
Ctrl	hU6-MCS-CMV-EGFP	TTCTCCGAACGTGTCACGT

1.4 实时荧光定量聚合酶链式反应(real-time fluorescent quantitative polymerase chain reaction, RT-qPCR)检测

将处理后的细胞去除上清后用PBS清洗2次,去除上清后加入0.5 mL Trizol,吹打混匀收集于1.5 mL无酶EP管。室温裂解5 min后加入0.1 mL氯仿,剧烈涡旋振荡15 s至上下层彻底混合,室温放置3 min。在4 ℃、13 000×*g*离心15 min,吸取上层水相至另一个无酶EP管中,加入等

体积异丙醇, 轻轻振荡混匀, 室温放置10 min。4 ℃, 13000×*g* 离心10 min, 弃去上清, 用1 mL 75%DEPC酒精洗涤沉淀。再次离心, 弃上清, 室温干燥后用无酶水重悬, 使用酶标仪检测RNA浓度后, 用HiScriptQ RT SuperMix试剂盒制成DNA模板。使用SYBR green进行RT-qPCR。引物序列见表2。检测Ad-Ctrl+*S. aureus*组和Ad-*Pp2cm*+*S. aureus*组的*TNF-α*与*IL-1β*基因表达, Ad-Ctrl组、Ad-*Pp2cm*组的*Pp2cm*、*TLR*2、*TLR*4、*Tirap*与*Myd*88基因表达。以*GAPDH*为内参, 将基因水平标准化, 并报告为相对于对照组的倍数变化(以Ad-Ctrl组或Ad-Ctrl+*S. aureus*组表达量的均数为1)。

表 2　RT-qPCR引物序列

Table 2　RT-qPCR primer sequence

Gene	Primer sequence (5′-3′)	Product length/bp
GAPDH	F: AGGTCGGTGTGAACGGATTTG	123
	R: TGTAGACCATGTAGTTGAGGTCA	
Pp2cm	F: AAGTACGGCAAACCAATTCCC	132
	R: GACTGCGAAGTATAGCACCTC	
TNF-α	F: CCCTCACACTCAGATCATCTTCT	61
	R: GCTACGACGTGGGCTACAG	
IL-1β	F: GCAACTGTTCCTGAACTCAACT	89
	R: ATCTTTTGGGGTCCGTCAACT	
TLR2	F: GCAAACGCTGTTCTGCTCAG	231
	R: AGGCGTCTCCCTCTATTGTATT	
TLR4	F: ATGGCATGGCTTACACCACC	129
	R: GAGGCCAATTTTGTCTCCACA	
Tirap	F: CCTCCTCCACTCCGTCCAA	100
	R: CTTTCCTGGGAGATCGGCAT	
*Myd*88	F: TCATGTTCTCCATACCCTTGGT	175
	R: AAACTGCGAGTGGGGTCAG	

GAPDH: glyceraldehyde-3-phosphate dehydrogenase; *Pp2cm*: protein phosphatase 2cm; *TNF-α*: tumor necrosis factor-alpha; *IL-1β*: interleukin 1 beta; *TLR*: Toll-like receptor; *Tirap*: Toll-like receptor adaptor protein; *Myd*88: myeloid differentiation factor 88.

1.5　蛋白印迹法检测PP2Cm蛋白

取Ad-Ctrl组巨噬细胞与Ad-*Pp2cm*组巨噬细胞, 将处理的细胞去除上清后用PBS清洗2次, 收集细胞于1.5 mL EP管, 视细胞量加入含蛋白酶抑制剂的裂解液, 超声裂解后得到蛋白样品溶液并测定浓度后定量。每个泳道加入50 μg蛋白质。采用Werstern blot法, 恒压电泳, 恒压转膜, 封闭后4 ℃孵育一抗过夜, 清洗后二抗(Cell Signaling, 美国)孵育1 h, 清洗后曝光。PP2Cm抗体(Proteintech, 1∶1000), GAPDH(Servicebio, 1∶1000)。

1.6　流式细胞术检测细胞凋亡

取Ad-Ctrl组巨噬细胞与Ad-*Pp2cm*组巨噬细胞, 用PBS洗涤, 离心后收集细胞, 根据试剂盒说明, 用1× annexin-binding buffer重悬, 测定细胞密度后, 稀释细胞, 加入Alexa Flour 488 annexin Ⅴ和PI工作液, 室温培养后加入1× annexin-binding buffer混合, 通过流式细胞术分析染色细胞。

1.7　细胞活力检测

取Ad-Ctrl组巨噬细胞与Ad-*Pp2cm*组巨噬细胞, 计数后接种于96孔板, 设置空白孔并按照试剂盒说明处理。将CCK-8溶液添加到每个孔中, 并将96孔板置于37 ℃恒温孵箱中孵育2 h, 用酶标仪(BioTek, 美国)检测450 nm波长的吸光度值。细胞活力正比于吸光度值。

1.8　统计学方法

所有数据均以$\bar{x}\pm s$表示。使用未配对的双尾*t*检验进行两组间比较, $P<0.05$为差异有统计学意义。

2　结果

2.1　Ad转染降低巨噬细胞*Pp2cm* mRNA和PP2Cm蛋白表达

Ad-*Pp2cm*组巨噬细胞*Pp2cm* mRNA和PP2Cm蛋白表达水平均低于Ad-Ctrl组巨噬细胞, 差异有统计学意义($P<0.05$)(图1)。

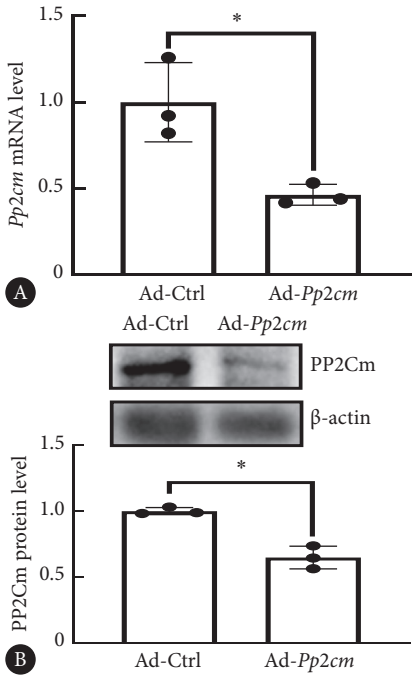

图 1　腺病毒载体对巨噬细胞PP2Cm表达水平的影响

Fig 1　Effect of adenoviral vectors on the expression level of PP2Cm in macrophages

A, Expression of *Pp2cm* mRNA (n=3 per group); B, expression of PP2Cm protein (n=3 per group). MOI=150; * $P<0.05$.

2.2　*Pp2cm*基因敲低增加巨噬细胞*TNF-α*与*IL-1β*表达

与Ad-Ctrl+*S. aureus*组巨噬细胞相比, Ad-*Pp2cm*+*S. aureus*组巨噬细胞中*TNF-α*与*IL-1β*基因表达水平升高, 差异有统计学意义($P<0.01$)(图2)。

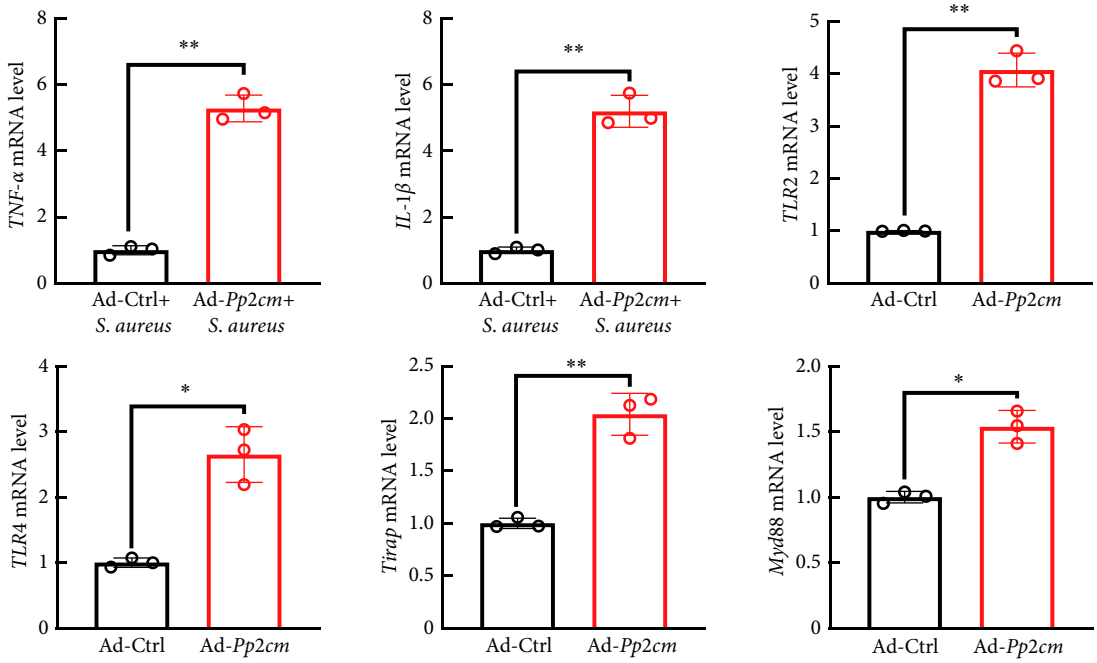

图 2 *Pp2cm*基因敲低对巨噬细胞炎症因子与TLR受体表达的影响

Fig 2 Effect of *Pp2cm* knockdown on the expression of inflammatory factors and TLR receptors in macrophages

n=3 per group; ** *P*<0.01, * *P*<0.05.

2.3 *Pp2cm*基因敲低增加巨噬细胞*TLR2*与*TLR4*的表达

与Ad-Ctrl组巨噬细胞相比，Ad-*Pp2cm*组巨噬细胞中*TLR2*与*TLR4*基因表达水平升高，差异有统计学意义（*P*<0.05）（图2）。

2.4 *Pp2cm*基因敲低增加巨噬细胞*Tirap*与*Myd88*表达

与Ad-Ctrl组相比，Ad-*Pp2cm*组巨噬细胞中*Tirap*和*Myd88*基因表达水平升高，差异有统计学意义（*P*<0.05）（图2）。

2.5 *Pp2cm*基因敲低不影响巨噬细胞凋亡和增殖

结果如图3。Ad-Ctrl组巨噬细胞与Ad-*Pp2cm*组巨噬细胞的细胞凋亡差异无统计学意义，巨噬细胞的细胞增殖差异无统计学意义。

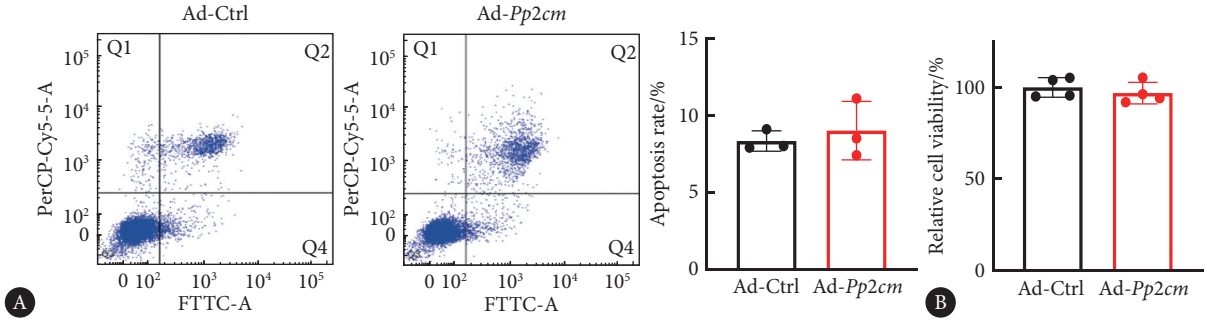

图 3 *Pp2cm*基因敲低对巨噬细胞增殖和凋亡的影响

Fig 3 Effect of *Pp2cm* knockdown on the proliferation and the apoptosis of macrophages

A, Apoptosis of RAW 264.7 macrophages was determined by flow cytometry (*n*=3 per group); B, cell proliferation of RAW 264.7 macrophages was determined by CCK-8 (*n*=4 per group).

3 讨论

本研究证明了*Pp2cm*基因沉默通过增加TLR受体成分*TLR2*、*TLR4*、*Tirap*和*Myd88*表达，引起巨噬细胞在有金黄色葡萄球菌存在的情况下*TNF-α*和*IL-1β*表达水平的升高，这种炎症表型加重不伴有巨噬细胞增殖和凋亡的改变。研究不仅揭示了糖尿病患者金黄色葡萄球菌感染预后差的原因，而且为糖尿病患者金黄色葡萄球菌感染的治疗提供了潜在的靶点。

BCAA代谢障碍与T2DM风险增加密切相关，促进

PP2Cm表达可改善糖代谢，*Pp2cm*基因突变在引起BCAA代谢紊乱的同时可引发糖尿病[16-17]。既往的研究通过敲低或过表达*Pp2cm*来干预BCAA代谢[7, 18-19]。本研究通过腺病毒感染的方式对巨噬细胞*Pp2cm*表达进行干预并利用RT-qPCR技术和Western blot技术验证*Pp2cm*基因和PP2Cm蛋白表达水平，发现Ad-*Pp2cm*组这两者表达水平相对于Ad-Ctrl组而言均有下降。

既往的体外研究表明，BCAA代谢对免疫细胞具有潜在的免疫调节作用，但对补充BCAA是促炎还是抑炎的结论并不统一[20-22]。有文献报道BCAA代谢障碍加重了糖尿病小鼠巨噬细胞炎症，也有文献报道抑制BCAA代谢可减少巨噬细胞促炎表型[8, 23]。本研究证明了*Pp2cm*基因敲低使巨噬细胞在接受金黄色葡萄球菌刺激后的*TNF-α*和*IL-1β*炎症因子基因表达水平升高，这些结论可以部分解释糖尿病患者金黄色葡萄球菌感染预后较差的原因。

在金黄色葡萄球菌侵入机体时，巨噬细胞主要以TLR2受体响应刺激极化为M1型巨噬细胞发挥作用，TLR4同样参与其中。本研究验证了*Pp2cm*基因增加*TLR2*与*TLR4*基因表达水平，提示*Pp2cm*基因敲低通过影响巨噬细胞识别细菌受体的方式促进巨噬细胞炎症反应。

巨噬细胞识别金黄色葡萄球菌抗原成分后，TLR2、TLR4、MyD88和TIRAP与下游信号分子相互作用，激活巨噬细胞极化并上调炎症相关通路，如NF-κB和PI3K信号通路[24-25]。本研究发现*Pp2cm*基因敲低增加巨噬细胞*Tirap*和*Myd88*基因表达，这与炎症水平改变相一致，提示*Pp2cm*基因敲低可通过上调TLR2和TLR4受体，介导巨噬细胞炎症水平的升高。

为了探索*Pp2cm*基因是否影响巨噬细胞细胞活性进而影响巨噬细胞炎症功能，本研究通过CCK-8法和流式细胞术检测了*Pp2cm*基因对于巨噬细胞增殖和凋亡的影响。结果发现*Pp2cm*表达水平改变并不会影响巨噬细胞细胞增殖和凋亡。因此T2DM的*Pp2cm*水平降低在增加巨噬细胞炎症反应的同时并不改变静息态巨噬细胞的增殖和凋亡。

本研究仅使用小鼠巨噬细胞，未纳入动物模型和临床样本进行验证；*Pp2cm*基因敲低对于巨噬细胞TLR通路的影响还需在动物水平进一步验证；PP2Cm影响TLR通路的具体作用机制还需要更进一步解析。

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作者贡献声明　罗凯腾、李涛和姜春玲负责论文构思和审读与编辑写作，罗凯腾、李涛和姜春玲负责数据审编，罗凯腾、谢茂迪和杨为负责正式分析，李涛和姜春玲负责经费获取、研究项目管理、提供资源和监督指导，罗凯腾、谢茂迪和杨为负责调查研究、研究方法和可视化，罗凯腾、谢茂迪、杨为、李涛和姜春玲负责验证，罗凯腾负责初稿写作。所有作者已经同意将文章提交给本刊，且对将要发表的版本进行最终定稿，并同意对工作的所有方面负责。

利益冲突　所有作者均声明不存在利益冲突

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