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CCL4 在病毒性心肌炎中的作用及其机制研究

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摘要 目的：观察巨噬细胞炎性蛋白 -1β(MIP-1β/CCL4)在病毒性心肌炎小鼠血清以及心肌组织中的变化，初步探讨 CCL4 在病毒性心肌炎中发挥的作用。**方法**：雄性 Balb/c 小鼠随机分成对照组 15 只和病毒性心肌炎组 25 只，病毒性心肌炎组腹腔注射病毒液，对照组腹腔注射 Eagle's 培养液。15 d 处死小鼠，通过 HE 染色观察小鼠心肌病理的变化；RT-PCR 观察 CCL4 以及促炎因子的 mRNA 水平，Western blotting 观察 CCL4 蛋白水平的变化。**结果**：与正常对照组比，病毒性心肌炎组小鼠生存质量降低，生存率也显著降低；病毒性心肌炎组小鼠心肌炎性浸润严重，心肌组织中的 CCL4、IL-1β、TNF-α mRNA 水平显著增高，CCL4 蛋白水平也明显增高，并且 CCL4 的浓度和蛋白表达水平与心肌病变积分呈正相关。**结论**：病毒性心肌炎 CCL4 的表达显著增加，并且与心肌病变程度相关。

关键词：CCL4；病毒性心肌炎；IL-1β；TNF-α

中图分类号：R-33; R542.21 **文献标识码**：A **文章编号**：1673-6273(2017)04-636-04

Research on the Function and Mechanism of CCL4 in Viral Myocarditis

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ABSTRACT Objective: To explore the expression of CCL4 in viral myocarditis and to investigate the mechanism. **Methods:** Male Balb/c mice were randomly divided into control group of 15 cases and viral myocarditis group of 25 cases, viral myocarditis group was injected intraperitoneally, and the control group was injected with Eagle's culture solution. 15d mice were sacrificed and the pathological changes of myocardium were observed by HE staining. CCL4 and mRNA levels of pro-inflammatory factors were observed through RT-PCR, and changes of CCL4 protein levels were observed by blotting Western. **Results:** Compared with the normal control group, the survival rate of mice with viral myocarditis was decreased, and the survival rate was decreased significantly.severe infiltration of myocarditis in mice with viral myocarditis, and the levels of CCL4, IL-1, TNF-α, mRNA and were significantly increased, and the level of CCL4 protein was significantly increased, and the concentration of CCL4 and protein expression were positively correlated with the score of myocardial lesion. **Conclusion:** The expression of CCL4 is significantly increased in viral myocarditis, and correlated with the degree of myocardial lesion.

Key words: CCL4;Viral myocarditis; Interleukin-1β;Tumor necrosis fator-α

Chinese Library Classification(CLC): R-33; R542.21 Document code: A

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前言

病毒性心肌炎（viral myocarditis）是由嗜心性病毒感染引起，以非特异性间质性炎症为主要病变的心肌炎[1,2]。病毒性心肌炎无特殊的发病年龄，各个年龄段均可能发病，近年来逐渐成为是儿童和青壮年不明原因猝死的重要原因之一，严重影响人类的健康[3,4]。导致心肌炎的病毒有 30 余种，其中以柯萨奇病毒最为常见[5]。目前，病毒性心肌炎的治疗主要是以休息为主，辅以抗病毒治疗、心肌保护治疗以及一些对症治疗[6,7]，但疗效并不十分明确。在心肌炎症发生的过程中，多种免疫细胞和细胞因子参与发挥了重要的作用，其中有一类重要的具有趋化作用的细胞因子的作用越来越受到人们的关注，它们在炎症反应中起核心作用[8]。巨噬细胞炎性蛋白 -1β (MIP-1β /CCL4)是由上皮细胞、淋巴细胞等分泌的对单核细胞、自然杀伤细胞、嗜酸性粒细胞和树突状细胞具有趋化作用的一种细胞因子。其生物学活性非常复杂，它可以刺激细胞释放多种促炎介质，在细菌感染疾病和炎症进程中发挥着重要的作用[9,10]。但是目前病毒性心肌炎不同病程中 CCL4 的表达变化和在该疾病中的作用研究甚少。

1 材料与方法

1.1 实验动物分组以及造模

雄性 Balb/c 小鼠 40 只购于南京大学实验动物中心，随机分成对照组和病毒性心肌炎组，分别为 15 只、25 只。病毒性心肌炎组采用腹腔注射法注射 0.1 mL 的病毒液，内含 1×10^2 TCID50CVB3，对照组腹腔注射 0.1 mL Eagle's 培养液。两组动物饲养在相同的条件下，每天记录小鼠存活数。

1.2 标本收集和病理学检查

小鼠断颈处死，眼球取血，-20℃冰箱保存。分离小鼠心脏，

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一半用于病理切片检查;另一半 -70℃保存,用于提取总 RNA 和蛋白。组织切片用 HE 染色,显微镜下观察心肌病理改变并计算心肌组织病理评分,每张病理切片下观察 5 个视野,计算炎性细胞浸润及坏死区域与整个视野的面积之比,以病变面积计分,无病变为 0 分,病变面积 <25%为 1 分,25%～50%为 2 分,51%～75%为 3 分,>75%为 4 分。

1.3 Western blotting 测蛋白含量

提取蛋白,取 100 μL蛋白裂解液储存液加 900 μL细胞裂解液,吹打混匀,冰上静止 30-40 min,取上清分装、测蛋白浓度。以 β-actin 为内参照,胶片条带扫描后分析产物积分光密度值,CCL4 蛋白表达量 =CCL4 积分光密度值 /β-actin 的积分光密度值。

1.4 Realtime PCR 检测 CCL4、IL-1β、TNF-α mRNA 的表达

采用 Trizol 法提取总 RNA,按逆转录试剂盒说明转录成 cDNA,用 PCR 试剂盒扩增 DNA。CCL4 引物序列:上游 5-ATG-AAG-CTC-TGC-GTG-TCT-GCC-TTC-3′ ,anti-sense 5-TCA-GTT-CAA-CTC-CAA-GTC-ATT-CAC-3′ ;GAPDH 引物序列:上游:5'- CGGAATTCGCCACCATGCAGGTCCCT - 3,下游:5'- GCTCTAGACTAGTT CACTGTCACACT- 3'。PCR 的反应条件为 94℃预变性 5 min,然后 94℃变性 30 s,50℃退火 45 s,72℃延伸 1 min,共 33 个循环,72℃延伸 7 min。

1.5 统计学处理

采用 SPSS13.0 进行统计学分析,两独立组间比较用 student's t 检验,生存分析为 Kaplan-Meier method 乘积限法,两组生存率比较为 Logrank 检验,相关分析用 Pearson 分析,显著性水准 α=0.05。

2 结果

2.1 两组小鼠生存情况比较

对照组小鼠活动正常,无死亡。病毒性心肌炎组小鼠在第 4～8d 各死一只,第 11 d 死亡 2 只,第 13d 死亡 1 只,生存率为 68%(17/25)。现按 Kaplan-Meier method 乘积限法进行生存分析,并进行生存率比较的假设检验,见表 1、表 2。

表 1 两组生存率

Table 1 The survival rate of the two groups

Viral myocarditis group			Control group		
Mean survival time	Median survival time	1 month survival rate	Mean survival time	Median survival time	1 month survival rate
13.4	15.4	-	15.0	15.5	-
Survival days	Survival rate over the day	Confidence interval of survival rate	Survival days	Survival rate over the day	Confidence interval of survival rate
tj	s(t)	α=0.95	tj	s(t)	α=0.95
1	0.960	0.748～0.994	15	1.000	-
4	0.920	0.716～0.979	-	-	-
8	0.880	0.673～0.960	-	-	-
11	0.800	0.584～0.911	-	-	-
15	0.800	0.584～0.911	-	-	-

Note: by Kaplan-Meier product-limit method.

表 2 生存率比较的假设检验

Table 2 Hypothesis test for comparison of survival rates

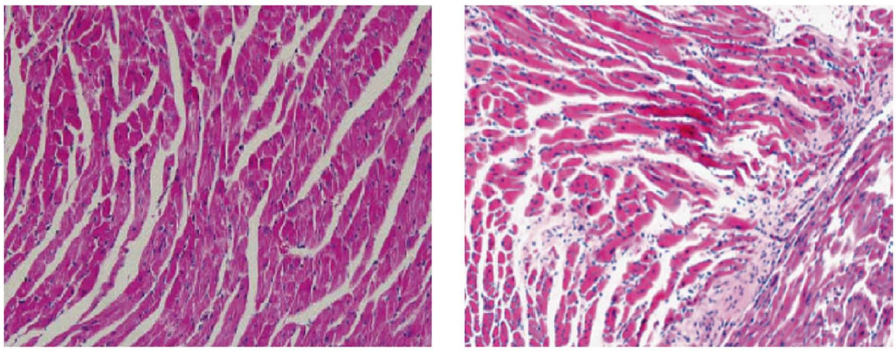
Survival days	Early stage cases		Actual death number		Theoretical death number		Variance V
	Viral myocarditis group	Control group	Viral myocarditis group	Control group	Viral myocarditis group	Control group	
1	25	15	1	0	0.6	0.4	0.23
4	24	15	1	0	0.6	0.4	0.24
8	23	15	1	0	0.6	0.4	0.24
11	22	15	2	0	1.2	0.8	0.47
15	20	15	0	0	0.0	0.0	0.00
Totals	-	-	5	0	3.0	2.0	1.18

Note: by Logrank test.

2.2 心肌病理及心脏重量 / 体重与心肌病变积分

正常对照组心肌细胞 HE 染色在镜下胞浆着色均匀,未发现任何病理改变。而在病毒性心肌炎组发现,心肌内有大量的炎性细胞浸润,包括淋巴细胞和单核细胞,见图 1。心肌炎组小

鼠的心脏重量 / 体重和心肌病变积分明显高于对照组，差异有统计学意义（P<0.05），见表 3。



Control group　　Viral myocarditis group

图 1 两组小鼠心肌细胞 HE 染色

Fig. 1 HE staining of cardiac muscle cells in two groups

表 3 两组小鼠心脏重量 / 体重与心肌病变积分

Table 3 The ratio of heart weight/body weight and the score of myocardial lesion in two groups

Groups	n	Heart weight/body weight	Score of myocardial lesion	
			Infiltration	Necrosis
Control group	15	3.23± 0.72	0	0
Viral myocarditis group	8	5.8± 0.69	2.53± 0.49	2.83± 0.47
t		8.27	-	-
p		0.000	0.000	0.000

2.3 心肌组织中 CCL4、IL-1β、TNF-α mRNA 的表达

实时定量 PCR 检测发现，心肌炎组小鼠的 CCL4、IL-1β、TNF-α 均显著高于对照组，差异有统计学意义（P<0.001），见图 2。

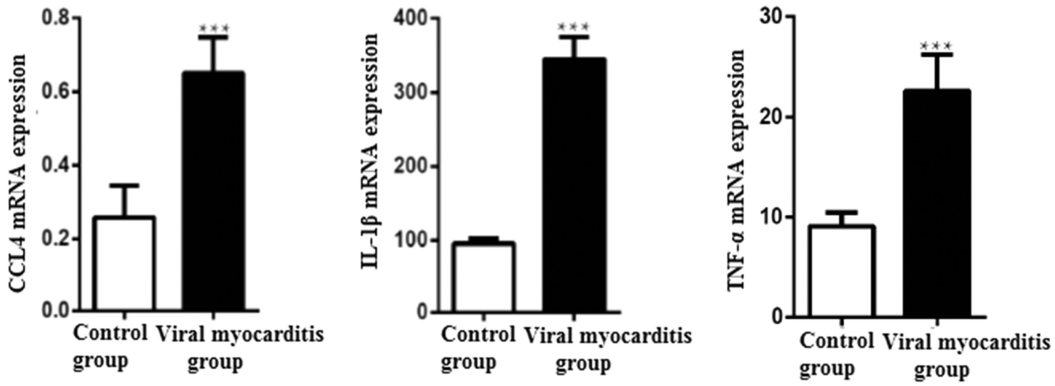


图 2 心肌组织中 CCL4、IL-1β、TNF-α mRNA 的表达（*P<0.001）**

Fig. 2 Expressions of CCL4, IL-1, TNF- and mRNA in cardiac muscle（***P<0.001）

2.4 血清中 CCL4 的含量

经检测血清中 CCL4 的水平，发现正常对照组血清中存在低水平的 CCL4，心肌炎组较正常对照组显著升高（t=58.55，P<0.001），见图 3，具有统计学意义。

2.5 心肌组织中 CCL4 蛋白的表达

进一步在蛋白水平上检测 CCL4 的表达，Western blotting 显示，见图 4。心肌炎组小鼠的 CCL4 蛋白含量显著高于对照组，差异具有统计学意义（t=7.03，P<0.001）。

2.6 CCL4 浓度和 CCL4 蛋白水平与心肌病变积分的相关性

Pearson 分析发现，心肌炎组小鼠血清中 CCL4 的浓度和心肌组织中 CCL4 的蛋白表达水平与心肌病变积分呈显著正相关（r=0.96，0.36；P<0.05）。

3 讨论

目前研究表明，病毒性心肌炎最具特征性的病理特征是心肌细胞的坏死以及心肌中炎性细胞的浸润[11,12]，其主要的发病

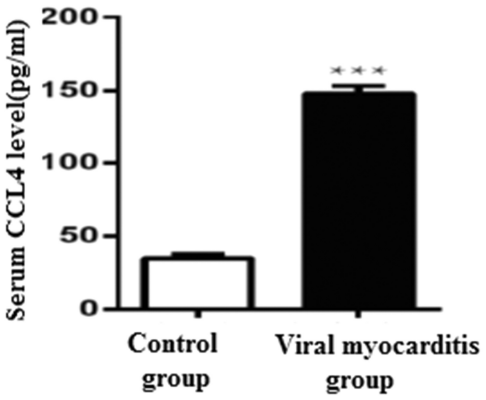


图 3 血清中 CCL4 的含量

Fig. 3 The content of CCL4 in serum

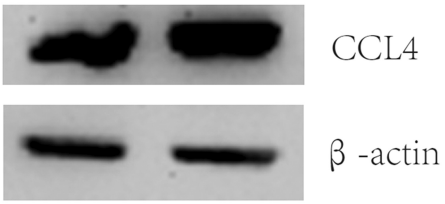


图 4 心肌组织中 CCL4 蛋白的表达

Fig. 4 Expression of CCL4 protein in cardiac tissue

机制是病毒的直接作用和机体的免疫反应[13,14]。趋化因子是一类具有趋化作用的分泌型小分子蛋白，它们参与白细胞的迁移、激活和趋化，在炎症反应中起着重要的作用[15,16]。CCL4 是一种近年来发现在感染免疫疾病中发挥着重要趋化作用的趋化因子[17,18]，它不仅可以趋化多种炎症细胞如单核巨噬细胞、淋巴细胞等到达炎症部位，还可以刺激细胞产生多种促炎因子从而发挥免疫效应[19,20]。

由于病毒性心肌炎严重影响动物模型的生存质量，在本研究中，我们首先观察了两组小鼠的生存情况，发现我们发现经过病毒性心肌炎的造模，小鼠生存率受到严重影响，在 15 d 时，小鼠死亡数量近一半。为了进一步的观察柯萨奇病毒对心脏的影响，我们运用 HE 染色在镜下观察心肌细胞的变化，并且计算两组小鼠心脏与体重的比，进一步探讨病毒性心肌炎的心肌损害发现，正常对照组心肌细胞 HE 染色在镜下胞浆着色均匀，未发现任何病理改变。而在病毒性心肌炎组发现，心肌内有大量的炎性细胞浸润，包括淋巴细胞和单核细胞。心肌炎组小鼠的心肌炎性细胞浸润积分和坏死积分显著高于正常对照组，并且心肌炎组小鼠的心脏重量与体重比明显高于对照组，说明病毒性心肌炎造模成功。以上结果发现病毒性心肌炎组小鼠心肌内大量炎性细胞浸润，基于此继续检测心肌组织中促炎因子 IL-1β、TNF-α 的表达，并观察了趋化因子 CCL4 的水平，发现病毒性心肌炎组 IL-1β、TNF-α 的水平明显高于对照组，我们进一步观察了心肌组织中 CCL4 的表达，以及血清中 CCL4 的表达水平，并分别从 mRNA 水平和蛋白水平分析 CCL4 的表达水平，发现无论是 mRNA 还是蛋白水平，病毒性心肌炎组的 CCL4 水平均显著高于对照组，提示 CCL4 在病毒性心肌炎的发病机制中起重要作用，进一步 Pearson 分析发现，心肌炎组小鼠血清中 CCL4 的浓度与心肌病变积分呈显著正相关，心肌炎组心肌组织中 CCL4 的蛋白表达水平与心肌病变积分呈显著正相关，进一步提示 CCL4 与病毒性心肌炎密切相关。

综上所述，CCL4 在病毒性心肌炎中发挥了重要的作用，其机制可能与促进炎症细胞趋向到炎症部位和刺激细胞产生大量促炎因子有关，控制 CCL4 的表达可能可以成为治疗病毒性心肌炎的一个潜在靶点，为病毒性心肌炎的防治提供新的途径。

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