成熟树突状细胞在肝脏缺血再灌注中的作用

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[摘 要] **目的**:探讨成熟树突状细胞(dendritic cells,DCs)在小鼠肝脏缺血再灌注中的作用。**方法**:收集骨髓源性树突状细胞(bone marrow dendritic cells,BMDCs),经过不同处理(正常肝细胞培养上清或缺氧再氧合原代肝细胞培养上清培养24 h)后,在细胞流式仪上检测细胞成熟相关指标(CD40、CD80、CD86、MHC Ⅱ);然后将20只健康雄性C57BL/6小鼠随机分成缺血再灌注组(IR)、未经刺激的骨髓源性树突状细胞处理组(IR+NEG-BMDCs)、正常肝细胞上清刺激的骨髓源性树突状细胞处理组(IR+CON-BMDCs)、经缺氧再氧合的原代肝细胞上清刺激成熟骨髓源性树突状细胞处理组(IR+H/R-BMDCs),各组5只。采用70%肝脏缺血再灌注模型(缺血1 h,再灌注6 h)。各组分别于术前1 h给予PBS、NEG-BMDCs、CON-BMDCs、H/R-BMDCs尾静脉注射。酶联免疫吸附实验(ELISA法)分别测定血清中丙氨酸氨基转移酶(ALT)、天门冬氨酸氨基转移酶(AST)、白介素-10(IL-10)、白介素-17(IL-17)水平。通过光镜观察组织HE染色改变。反转录酶-聚合酶链式反应(RT-PCR)检测肝组织转化生长因子β(TGF-β)、叉头框蛋白P3(FOXP3)、IL-10、IL-17水平。**结果**:H/R-BMDCs处理组肝酶水平明显低于其他3组($P<0.05$),形态学分析及Suzuki评分明显优于其他3组。H/R-BMDCs处理组血清及肝组织中IL-10、肝组织中TGF-β、FOXP3表达水平明显高于其他组,IL-17低于其他组($P<0.05$)。**结论**:H/R-BMDCs预处理可以通过调节调节性T细胞(Treg)和辅助性T细胞17(Th17)的平衡来减轻小鼠肝脏缺血再灌注损伤。

[关键词] 肝脏;缺血再灌注;树突状细胞;调节性T细胞;辅助性T细胞17

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Effects of mature dendritic cells on mouse liver ischemia/reperfusion injury

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[**Abstract**] **Objective**: To investigate effects of mature dendritic cells (DCs) on mouse liver ischemia/reperfusion (I/R) injury. **Methods**: After different treatment of bone marrow-derived dendritic cells collected, staining was performed according to the reagent protocol, and the relevant indexes were detected on the cell flow meter (CD40, CD80, CD86, and MHC Ⅱ). A total of 20 healthy male C57BL/6 mice were randomly divided into four main experimental groups (n=5 each), including the ischemia/reperfusion (I/R) group, the NEG-BMDCs pretreatment group, the CON-BMDCs pretreatment group, and the H/R-BMDCs pretreatment group. We chose a nonfatal model of 70% liver I/R (treated with 1 h ischemia, and then 6 h reperfusion). The mice of I/R group were injected with PBS, the NEG-BMDCs pretreatment group with NEG-BMDCs, the CON-BMDCs pretreatment group with CON-BMDCs, the H/R-BMDCs pretreatment group with H/R-BMDCs, at 1 h before operation. The levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), interleukin-10 (IL-10), interleukin-17 (IL-17) in serum were detected by enzyme-linked immunosorbent assay (ELISA). The mRNA expression of TGF-β, FOXP3, IL-10, IL-17 were examined by reverse transcription-polymerase chain reaction (RT-PCR). Histological haema (HE) stained sections were histopathologically examined using light microscopy. **Results**: The liver enzyme level were significantly decreased in the H/R-BMDCs pretreatment group, compared to those in the other groups ($P<0.05$). Morphometric analysis and Suzuki's scores showed that H/R BMDCs improved liver ischemia/reperfusion injury (IRI) to a greater extent than BMDCs from the negative and control groups. The expressions of IL-10 of liver tissue blood serum and liver tissue were upregulated and the expressions of TGF-β and FOXP3 of liver tissue were upregulated in the H/R-BMDCs pretreatment group, while the expression of IL-17 was downregulated in the H/R-BMDCs pretreatment group. **Conclusion**: Pretreatment with H/R-BMDCs protects mouse from I/R

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injury by modulating the balance between Treg and Th17 cells.
[**Key words**] liver; ischemia/reperfusion; dendritic cells; Treg; Th17
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肝脏缺血再灌注损伤多见于各种休克(尤其是失血性休克)及各种需要行肝门阻断的手术,与肝切除、肝移植术后的肝功能恢复密切相关。固有免疫和调节性免疫在肝脏缺血再灌注损伤中起着重要作用。最近有研究表明特定的T细胞群,如效应T细胞,通过分泌细胞因子和表达共刺激分子来促进或抑制固有免疫激活,从而促进组织修复,保持生理平衡[1]。还有研究表明树突状细胞和T细胞亚群,比如调节性T细胞(Treg)和辅助性T细胞17(Th17)在肝脏缺血再灌注中有重要作用[1-2]。本文旨在通过检测肝酶及相关细胞因子水平来探讨经缺氧再氧合处理的原代肝细胞上清刺激的骨髓源性树突状细胞(H/R-BMDCs)预处理对肝脏缺血再灌注的影响。

1 材料和方法

1.1 材料

25只健康雄性C57BL/6小鼠,体重(23 ± 3)g,由南京大学模式研究所提供。

反转录试剂盒、RT-PCR试剂盒(南京Vazyme公司);TRIzol及白介素-10(IL-10)、白介素-17(IL-17)、转化生长因子β(TGF-β)、叉头框蛋白P3(FOXP3)、actin引物(Invitrogen公司,英国);丙氨酸氨基转移酶(ALT)、天门冬氨酸氨基转移酶(AST)、IL-10、IL-17 ELISA检测试剂盒(BD公司,美国)。

1.2 方法

1.2.1 骨髓源性树突状细胞的制备、处理及成熟树突状细胞的鉴定

将5只C57BL/6小鼠颈椎脱臼法处死后,分离原代肝细胞,置于37 ℃ 5% CO_2培养箱中以DMEM/F12液培养2 d后,收取培养液上清,即为正常肝细胞培养上清(CON),另取部分肝细胞培养2 d后,先置于氮气培养箱3 h,再移至普通培养箱6 h,收取培养液上清,即为缺氧再氧合原代肝细胞培养上清(H/R)。

采用Lutz法获得骨髓源性树突状细胞(BM-DCs),分为3组:未经刺激的骨髓源性树突状细胞(NEG-BMDCs),用正常肝细胞上清刺激的骨髓源性树突状细胞(CON-BMDCs),经缺氧再氧合原代肝细胞上清刺激的骨髓源性树突状细胞(H/R-BMDCs)。分别处理24 h后,分别收集3组细胞,按照试剂说明书进行染色后,在细胞流式仪上进行检测树突状细胞成熟的相关指标(CD40、CD80、CD86、MHCⅡ)。

1.2.2 动物分组与处理

20只C57BL/6小鼠随机分成4组,每组5只。①缺血再灌注组(IR);②NEG-BMDCs处理组(IR+NEG-BMDCs);③CON-BMDCs处理组(IR+CON-BMDCs);④H/R-BMDCs处理组(IR+H/R-BMDCs)。各组分别于术前1 h尾静脉注射PBS、NEG-BMDCs、CON-BMDCs、H/R-BMDCs,再行缺血再灌注手术处理。具体步骤为:术前禁食12 h,不禁饮。水合氯醛腹腔注射麻醉。取腹部正中切口,暴露肝脏,用生理盐水棉签明确分离肝左、中叶后,使用无损伤钛夹夹闭肝左、中叶脉管主干,以实现70%肝脏组织缺血,此时可见肝脏缺血区域色泽明显改变。确认肝脏血供阻断成功后,予4-0针线缝合,同时以生理盐水纱布覆盖,保持体温。待预定1 h缺血时间后,松开钛夹,随后缝合关腹。待小鼠肝脏再灌注达到6 h时,处死各组小鼠,取血及肝脏组织。部分肝脏用4%甲醛固定,HE染色,其余肝脏生理盐水冲洗后置于液氮保存。

1.2.3 血清ALT、AST、IL-10、IL-17水平测定

血液标本离心15 min(3 000 r/min)后收集血清,使用ELISA法测定ALT、AST、IL-10、IL-17水平。具体方法参考ELISA试剂盒说明进行,每组实验重复3遍。

1.2.4 组织学检测

取甲醛固定后的肝组织,经梯度酒精脱水,二甲苯透明,石蜡包埋切片后。脱蜡水化进行HE染色,酒精脱水后树脂封片,并在光镜下行组织学观察拍照。

1.2.5 肝组织TGF-β、FOXP3、IL-10、IL-17 mRNA表达的测定

取液氮冻存的肝组织,严格按照TRIzol试剂盒操作步骤提取肝组织总RNA,测定RNA浓度及纯度后进行反转录。总RNA按照反转录体系:5×qT SuperMix Ⅱ 2 μL, ddH_2O 7 μL, RNA 1 μL配成10 μL体系。反应条件为50 ℃ 15 min, 80 ℃ 2 min;反应所得的cDNA可直接用于RT-PCR。所用引物序列见

表1。RT-PCR反应体系为SYBR 5.0 μL,上下游引物各0.2 μL,cDNA 1.0 μL,ddH_2O 3.6 μL,反应条件为:95 ℃预变性5 min;95 ℃变性10 s,60 ℃退火/延伸30 s,40个循环;最后95 ℃ 15 s,60 ℃ 1 min,95 ℃ 15 s。PCR结束后,使用ABI Prism 7000 SDS系统进行分析,最终结果用$2^{-\Delta\Delta C_t}$来表示。每组实验重复3遍。

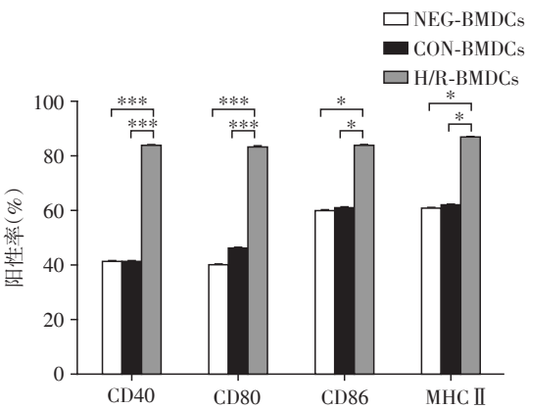
1.3 统计学方法

分别运用SPSS 15.0和Graphpad prism 6.0软件对数据进行分析和储存。计量资料用均数±标准差($\bar{x} \pm s$)表示。采用单因素方差分析比较组间均数差异,两两比较采用SNK-q法检验,$P \leqslant 0.05$为差异有统计学意义。

2 结 果

2.1 经缺氧再氧合处理的肝细胞上清促进树突状细胞成熟

NEG-BMDCs及CON-BMDCs处理组树突状细胞成熟标志物(CD40、CD80、CD86、MHC Ⅱ)的表达无明显差异,和这两组相比,H/R-BMDCs处理组中CD40、CD80、CD86、MHC Ⅱ的表达明显升高,差异有统计学意义(图1)。



两组比较,$^{*}P < 0.05$,$^{***}P < 0.001$。

图1 不同处理方法对骨髓源性树突状细胞成熟的影响

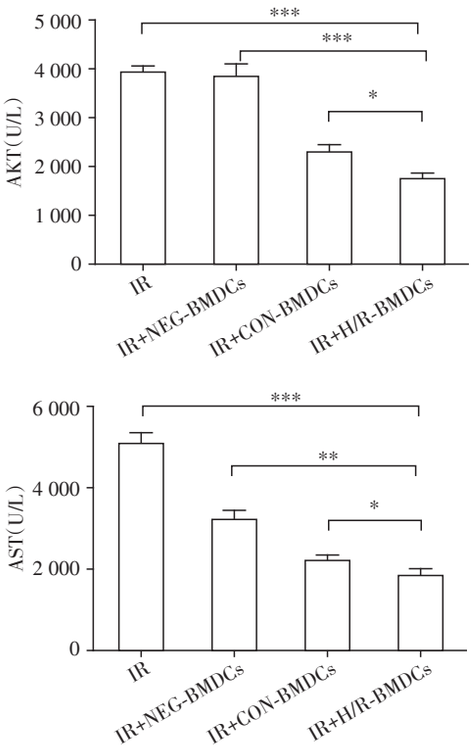
Figure 1 Effect of different treatments on the maturation of BMDCs

2.2 各组血清ALT、AST变化

IR组和IR+NEG-BMDCs组ALT无明显差异,IR+CON-BMDCs的ALT低于前两组,IR+H/R-BMDCs组的ALT明显低于其他3组,差异具有统计学意义($P < 0.05$)。IR+H/R-BMDCs组的AST也明显低于其他3组,差异具有统计学意义($P < 0.05$,图2)。

2.3 各组肝脏组织学改变

形态学分析显示,H/R-BMDCs处理组相较于其他3组,更能减轻肝脏缺血再灌注损伤。Suzuki's评分H/R-BMDCs处理组明显低于其他3组,差异具有统计学意义(图3),与图2中肝酶变化相符合。



两组比较,$^{*}P < 0.05$,$^{**}P < 0.01$,$^{***}P < 0.001$。

图2 成熟树突状细胞对缺血再灌注小鼠血清ALT、AST的影响

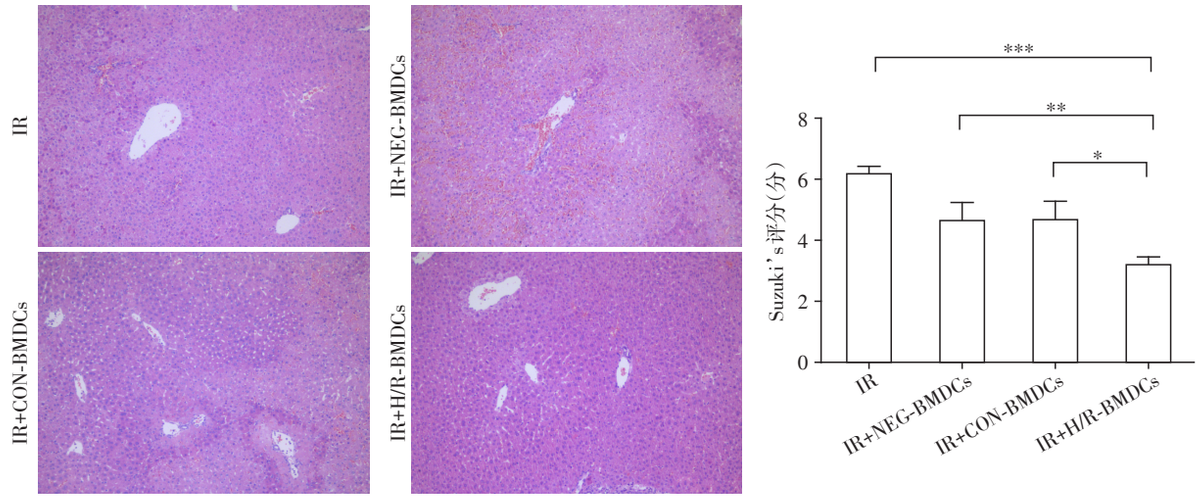
Figure2 Effect of mature BMDCS on ALT and AST in serum of mice with liver ischemia/reperfusion injury

2.4 各组血清IL-10、IL-17和肝组织TGF-β、FOXP3、IL-10、IL-17表达量的变化

在肝组织中,IR+H/R-BMDCs组抗炎因子TGF-β、FOXP3、IL-10的mRNA表达明显高于其他组,炎症因子IL-17则明显低于其他组。ELISA实验显示,H/R-BMDCs处理组血清中IL-10明显高于其他组,而IL-17则相反,差异具有统计学意义(均$P < 0.05$,图4)。

3 讨 论

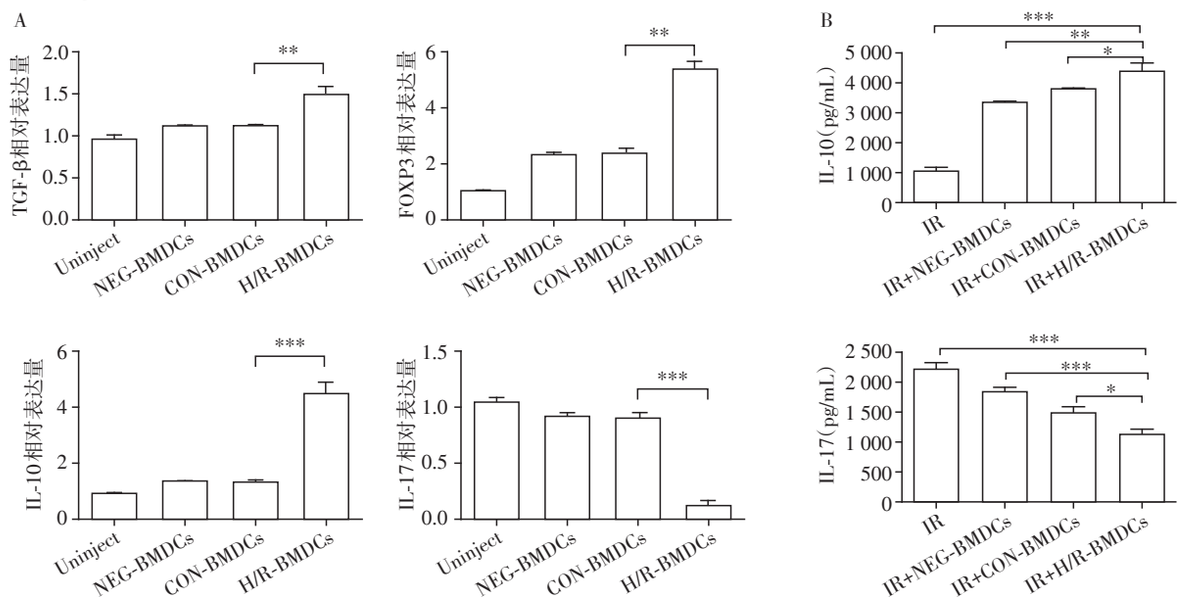
在肝脏疾病手术治疗中,特别是合并肝硬化的患者,为了减少术中出血量,降低手术风险等,需要行肝门阻断,而缺血再灌注损伤则是这个过程中无法避免的病理生理过程,减轻肝脏缺血再灌注的损伤,对患者术后恢复和预后有着重要意义。大量文献表明枯否细胞、中性粒细胞、黏附分子以及L-选择素等都在肝脏缺血再灌注损伤中起着重要作用。这些细胞及分子通过一系列过程,引发炎症,



两组比较，$^{*}P<0.05$，$^{**}P<0.01$，$^{***}P<0.001$。

图3 成熟树突状细胞对缺血再灌注小鼠肝脏组织病理学改变的影响(HE，×200)

Figure 3 Effect of mature BMDCS on histopathological changes in liver tissues of mice with liver ischemia/reperfusion injury(HE，×200)



A：PCR检测组织中TGF-β、FOXP3、IL-10、IL-17表达率；B：ELISA检测血清中IL-10、IL-17表达水平。两组比较，$^{*}P<0.05$，$^{**}P<0.01$，$^{***}P<0.001$。

图4 成熟树突状细胞对缺血再灌注小鼠血清中IL-10、IL-17及组织中TGF-β、FOXP3、IL-10、IL-17水平的影响

Figure 4 Effect of mature BMDCS on IL-10 and IL-17 in serum and TGF-β，FOXP3，IL-10 and IL-17 in liver tissues of mice with liver ischemia/reperfusion injury

损伤肝细胞，最终导致缺血再灌注损伤[3-9]。先前有文献表明树突状细胞在肝脏缺血再灌注损伤中有着重要作用[2,10-11]。

本研究显示了成熟树突状细胞、Treg和Th17平衡及肝脏缺血再灌注之间的关系。成熟树突状细胞通过Treg在肝脏组织中的比例及减少Th17的数量，缓解炎症，减轻肝脏缺血再灌注损伤。有研究表明$CD4^{+}$T细胞在肝脏缺血再灌注中有着重要作用[12]，活化的$CD4^{+}$T细胞表达不同的表型实现不同的免疫功能。不同的刺激会导致$CD4^{+}$T分化成Th1、Th2、Th17及Treg等。而Treg能够抑制巨噬细胞的活化，从而缓解肝脏缺血再灌注损伤[12-15]。通过观察血清转氨酶(ALT、AST)和肝组织形态学改变，我们发现H/R-BMDCs缓解肝脏缺血再灌注损伤明显优于NEG/CON-BMDCs。此外，根据PCR结果，IR+H/R-BMDCs组中的抗炎因子TGF-β、FOXP3、IL-10表达量明显高于其他3组，TGF-β、IL-10可以诱导Treg的产生，FOXP3是Treg的标志分子，而IL-17的表达量则

明显低于其他组,IL-17是Th17表达的重要细胞因子,结果显示成熟树突状细胞通过调节Treg和Th17之间的平衡来减轻肝脏缺血再灌注损伤。

据此,我们认为成熟的树突状细胞通过调节Treg/Th17的平衡,抑制炎症因子的表达,从而缓解肝脏缺血再灌注损伤。但是成熟的树突状细胞通过什么途径调节Treg/Th17的平衡,这一点我们将在后面的研究中进一步探索。

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