论著·军事医学

PD-1介导的CD4⁺T细胞耗竭加剧脓毒症小鼠的肠道菌群异位

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[摘要] **目的** 探究脓毒症中免疫耗竭的特征并分析其与肠道菌群异位的相关性。**方法** 采用随机数字表法将130只小鼠分为盲肠穿刺结扎(cecal ligation and puncture,CLP)组(n=100)和假手术(sham)组(n=30),通过CLP建立小鼠脓毒症模型;流式细胞术检测分析小鼠外周血CD4⁺T、CD8⁺T和PD-1⁺T细胞亚群比例变化;心、肝、肾等脏器匀浆涂板检测细菌定植量变化;HE染色分析免疫器官的病理改变;免疫组化染色检测免疫器官内CD4、CD8和PD-1阳性细胞表达及定位,使用Image J软件对阳性细胞数变化进行定量。**结果** HE染色提示各级免疫器官随病程进展出现不同程度病理损伤;与Sham组小鼠相比,CLP组小鼠各脏器及外周血细菌定植量均显著增高(P<0.05),以肝脏感染最为严重;CLP组小鼠在术后第1、3、5天时外周血CD4⁺T淋巴细胞比例较Sham组分别下降56%、70.57%和87.42%(P<0.001),CD8⁺T淋巴细胞比例只在第5天时下降48.33%(P<0.001),而表达PD-1的CD4⁺T细胞亚群比例分别升高为Sham组的673.08倍、423.08倍和600倍(P<0.001);免疫组化结果显示,CLP术后胸腺、脾脏和肠系膜淋巴结中CD4阳性细胞比例在早期最高时分别上升为Sham组的7.65倍、2.66倍和3.7倍(P<0.001),后期最低时分别较早期下降82.8%(P<0.001)、41.9%(P<0.01)和60.15%(P<0.001);CD8阳性细胞比例在早期升高后下降不显著;PD-1阳性细胞占比持续升高,最高时分别上升为Sham组的6.24倍、13.9倍和20.96倍(P<0.001),且其表达区域与CD4阳性细胞大致相同。**结论** 脓毒症时炎症反应可引起免疫器官严重损伤,CD4⁺T淋巴细胞持续耗竭,机体预防感染的能力下降,可能是造成肠道菌群异位加重,导致全身感染的重要原因。

[关键词] 脓毒症;细菌异位;T细胞耗竭;PD-1

[中图法分类号] R378.2;R392.32;R631.2 [文献标志码] A

PD-1-mediated CD4⁺ T cell exhaustion exacerbates gut microbiota translocation in mouse model of sepsis

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[**Abstract**] Objective To investigate the characteristics of immune exhaustion in sepsis and analyze its association with gut microbiota translocation. Methods A total of 130 mice were randomly divided into a

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cecal ligation and puncture (CLP) group (n=100) and a Sham group (n=30) Mouse model of sepsis was established with CLP procedure. Flow cytometry was used to analyze the proportions of peripheral blood $CD4^+$T and $CD8^+$T cells and programmed cell death protein 1 (PD-1) positive T cell subsets in mice. Bacterial colonization in organs such as the heart, liver and kidneys was quantified by plating homogenates of the organs. Pathological changes in immune organs were observed with HE staining. The expression and localization of $CD4^+$, $CD8^+$, and PD-1^+cells in immune organs were detected with immunohistochemical staining, and Image J software was employed for subsequent quantification of the number of the positive cells. **Results** HE staining demonstrated that immune organs exhibited varying degrees of pathological damages with disease progression. Compared with the Sham mice, the CLP mice exhibited significantly increased bacterial colonization in parenchymal organs and peripheral blood (P<0.05), notably in the liver, which showed the most severe infection. In the CLP group, the proportion of $CD4^+$ T lymphocytes in peripheral blood at days 1, 3, and 5 postoperatively was decreased by 56%, 70.57%, and 87.42%, respectively, when compared with the Sham group (P<0.001). The proportion of $CD8^+$ T lymphocytes was decreased by 48.33% relative to the Sham group only at day 5 (P<0.001). In contrast, the proportion of $CD4^+$ T cell subsets expressing PD-1 was increased to 673.08, 423.08, and 600 times that of the Sham group, respectively, at the same postoperative time points (P<0.001). Immunohistochemical results showed that, in the CLP group, the proportion of $CD4^+$ T cells in the thymus, spleen, and mesenteric lymph nodes was increased to 7.65, 2.66, and 3.7 times that of the Sham group, respectively, at the early-stage peak (P<0.001), and then these proportions were decreased by 82.8% (P<0.001), 41.9% (P<0.01), and 60.15% (P<0.001), respectively, at the late-stage trough when compared with the early-stage peak in the corresponding organs. The proportion of $CD8^+$ positive cells was increased in the early stage and then decreased insignificantly, while the proportion of PD-1^+ positive cells was increased continuously, and reached 6.24, 13.9, and 20.96 times that of the Sham group at the peak in the thymus, spleen, and mesenteric lymph nodes respectively (P<0.001), with their expression regions showing a rough overlap with those of $CD4^+$ cells. **Conclusion** During sepsis, the inflammatory response can cause severe damage to immune organs and persistent exhaustion of $CD4^+$T lymphocytes, leading to declined defenses against infection, which may be the main causes for exacerbated gut microbiota translocation and then systemic infection.

[**Key words**] sepsis; bacterial translocation; T cell exhaustion; programmed cell death protein 1

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肠道菌群异位是导致脓毒症发生和进展的关键因素[1]。目前的研究主要认为,肠道缺氧性损伤、上皮完整性破坏等原因引起的肠道黏膜机械屏障功能损伤是导致肠道菌群异位,侵犯远隔脏器并大量繁殖,从而诱发全身感染和炎症反应综合征的重要病理环节[2-4],其进一步发展可导致感染性休克、多器官功能障碍,严重威胁患者的生命安全[5]。

免疫器官是免疫系统的重要组成部分[6],包括胸腺、脾脏和淋巴结、组织驻留细胞等,其关键功能是在炎症感染中的损伤控制和对肿瘤的识别与监测[7],可划分为中枢免疫器官、二级免疫器官及外周(三级)免疫器官3个等级。正常情况下,除肠道的屏障功能外,肠系膜淋巴结也通过吞噬并杀灭易位细菌等方式参与对机体的保护[8],提示免疫系统在抑制细菌易位过程中发挥了重要作用。脓毒症中持续的免疫微环境会导致T细胞发生耗竭[9],在抗感染过程中发挥着核心作用的$CD4^+$T细胞的缺失会严重削弱免疫防御功能[10],降低机体清除病原体的能力,而该过程与肠道菌群异位定植可能存在相关性,但目前尚无相关报道。

PD-1是免疫细胞表面重要的程序性死亡受体,能够对免疫应答进行负性调节,可导致淋巴细胞凋亡并抑制其功能[11],即出现免疫耗竭。PD-1通路已成为T细胞功能抑制甚至耗竭的重要机制。既往研究指出脓毒症时循环和脾脏等T细胞的PD-1表达升高,与T细胞增殖能力降低、继发性院内感染和死亡率增加相关[12-13],但对其他免疫器官T细胞的PD-1表达情况、变化趋势及其与肠道细菌异位的关联研究较少。因此,为了研究脓毒症病程中免疫器官T细胞耗竭对肠道菌群异位定植的影响,本研究通过建立小鼠盲肠结扎穿刺脓毒症模型,分析造模后小鼠脏器的细菌定

植量变化,免疫器官病理改变特征,以及外周血和免疫器官中T细胞表达的PD-1随时间变化情况,旨在探究脓毒症免疫耗竭与肠道菌群异位的相关联系,为脓毒症防治提供理论依据。

1 材料与方法

1.1 实验动物

130只SPF级雄性C57BL/6J小鼠(6~8周龄,体质量18~22 g),购自重庆腾鑫比尔实验动物销售有限公司,小鼠饲养在温度20~26 ℃,湿度40%~70%的环境中,12 h光暗交替,自由摄食饲料和洁净水,实验前适应性饲养5 d。本研究的实验设计、实验过程及动物处理方法,均通过陆军军医大学实验动物福利与伦理委员会批准(AMUWEC20245210)。

1.2 主要试剂和仪器

本研究使用的主要试剂有:无菌PBS缓冲液(中乔新舟生物公司),LB琼脂培养基(生工生物科技有限公司),4%多聚甲醛(重庆川东化工公司),小鼠外周血淋巴细胞分离液(天津灏洋生物有限公司),红细胞裂解液(Biosharp生物公司),藻红蛋白(PE)标记的小鼠PD-1(biolegend公司),异硫氰酸荧光素(FITC)标记的抗小鼠CD3(biolegend公司),多甲藻叶绿素蛋白-Cy5.5偶联物(PercpCy5.5)标记的抗小鼠CD4(biolegend公司),别藻蓝蛋白(APC)标记的抗小鼠CD8(biolegend公司),以及PE、FITC、PercpCy5.5、APC标记的小鼠IgG(biolegend公司)。

本研究使用的主要仪器有:10 cm细菌培养皿(卧宏生物科技有限公司),超净工作台(Thermo公司),低温水平离心机(Thermo公司),光学显微镜CX31(奥林巴斯有限公司),细菌培养箱(Thermo公司),全自动细胞计数仪(北京高分生物科技公司),Gallios流式细胞分析仪(BECKMAN公司)。

1.3 方法

1.3.1 动物模型的制备 CLP组:采用RITTIRSCH等[14]描述的标准化CLP操作流程,即小鼠腹腔注射戊巴比妥钠麻醉后固定四肢,腹部备皮并消毒后从腹中线切口进腹,向左寻找并轻柔拉出盲肠。用3-0丝线结扎盲肠下1/3段,接着使用22G针头贯穿结扎部位远端的盲肠2次,挤出少量的肠内容物后将盲肠还纳入腹,逐层缝合腹膜及皮肤,予小鼠背部皮下注射37 ℃预热的无菌生理盐水抗休克,待小鼠复苏后正常饲养。Sham组:小鼠称量后麻醉,固定四肢备皮,开腹暴露盲肠2~3 min后直接将盲肠回纳,余同CLP组。观察2组小鼠7 d内生存情况。

1.3.2 脏器细菌定植检测 分别于造模后24 h摘Sham组和CLP组小鼠眼球取血,75%乙醇浸泡消毒后转移至超净工作台,解剖取出心、肝、脾、肺和肾等主要实质脏器,称量后放入无菌研磨管中,随后加入3 mL PBS进行匀浆。取血和脏器匀浆液各20 μL置于96孔板进行连续的倍数梯度稀释,共稀释8个梯度即10^8倍。取每个梯度匀浆液各5 μL点涂于LB平板上,倒扣置于37 ℃细菌培养箱中培养12 h,再选取起始出现清晰菌落的梯度匀浆液50 μL均匀涂布至LB平板上进行培养,根据生长的菌落数计算脏器细菌定植量。

1.3.3 流式细胞术检测外周血T淋巴细胞比例和PD-1表达比例 分别于造模后1、3、5 d摘小鼠眼球取血于EDTA抗凝管中,使用小鼠外周血淋巴细胞分离液400×*g*,30 min梯度离心,取中间层云雾状细胞,PBS洗涤重悬并计数,使用抗小鼠PD-1(PE)、CD3(FITC)、CD4(PercpCy5.5)、CD8(APC)单克隆抗体对细胞进行染色。同型对照分别加入相应荧光标记的小鼠IgG抗体。Gallios流式细胞仪收集细胞进行检测,通过正散射(forward scatter,FSC)和侧向散射(side scatter,SSC)对淋巴细胞进行门控,使用$CD3^+$、$PD\text{-}1^+$圈画表达PD-1的T淋巴细胞。同时,在淋巴细胞门中使用SS-A和$CD3^+$圈画T淋巴细胞,再在此门中使用$CD4^+$、$PD\text{-}1^+$以及$CD8^+$、$PD\text{-}1^+$分别圈画表达PD-1的$CD4^+$和$CD8^+$T淋巴细胞。从每管样品中至少分析了5 000个$CD3^+$T细胞,阈值由同型对照定义,结果使用FlowJo 10软件进行分析。

1.3.4 脏器病理切片HE染色 取Sham组小鼠和CLP术后24 h小鼠的心、肝、肺、肾,以及Sham组小鼠和CLP术后1、3、5 d小鼠的脾、胸腺、肠系膜淋巴结,冲洗后使用4%多聚甲醛固定,再脱水12 h,石蜡包埋,使用切片仪制作成3~5 μm厚的白片,使用HE染色后封片,镜下观察各器官切片中组织结构及细胞组成变化情况。

1.3.5 小鼠免疫器官免疫组化检测 取Sham组小鼠以及CLP术后1、3、5 d小鼠的脾脏、胸腺、肠系膜淋巴结。4%多聚甲醛固定,石蜡包埋切片。放入二甲苯Ⅰ、Ⅱ、Ⅲ中各10 min脱蜡,再通过乙醇梯度各5 min水化。冲洗切片,浸入PH 6.0柠檬酸盐缓冲液并高压加热沸腾后自然冷却,3% H_2O_2浸泡15 min。冲洗切片,滴加5%BSA室温封闭20 min,吸干BSA后滴加稀释的一抗(CD4、CD8、PD-1)20 μL/张切片,4 ℃过夜。冲洗切片,滴加20 μL/张切片二抗室温孵育30 min。冲洗切片,DAB显色10 min,水洗,苏木素复染30 s,水洗,盐酸酒精分化3 s,水洗。乙醇梯度各5 min脱水,二甲苯10 min透明,中性树胶封片,加盖玻片,晾干,在显微镜下拍照观察。对实验信息不知

情的独立研究人员在组化染色区域随机选取3~5个视野进行观察,利用Image J软件计算CD4、CD8和PD-1阳性细胞占比。

1.3.6　统计学分析　　用GraphPad Prism 10.1.2软件进行统计分析,计量资料以$\bar{x}\pm s$表示,多组数据之间比较选择单因素方差分析(one-way ANOVE),两组数据之间比较采用Student's t检验,生存率计算使用Kaplan-Meier法,并采取Log-rank检验比较生存曲线之间的差异,$P<0.05$差异具有统计学意义。

2　结果

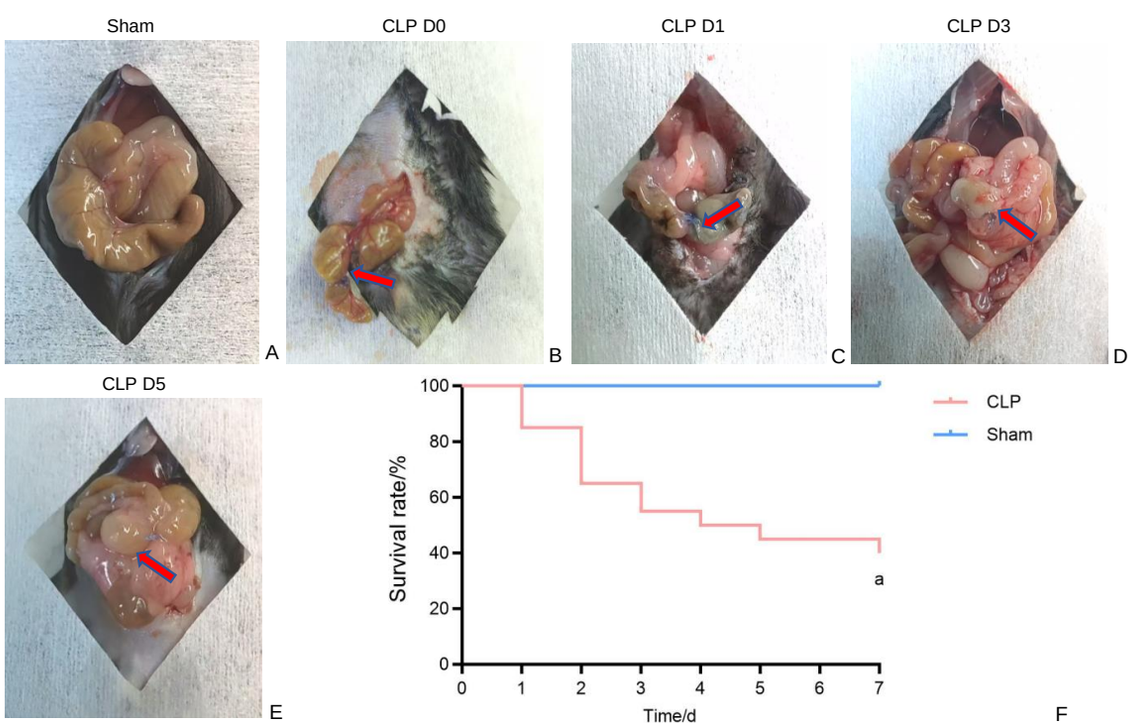
2.1　CLP小鼠术后表现及腹腔感染情况

小鼠在CLP手术苏醒8~10 h后陆续出现萎靡,寒颤、呼吸浅快、眼周脓性分泌物,体温降低,竖毛等症状和体征,行动迟缓,少部分拒绝进食,并在术后24 h内开始出现死亡。Sham组小鼠在苏醒后,无显著寒战、呼吸急促、竖毛等症状体征,能较快恢复正常。观察2组小鼠7 d存活率,结果显示CLP组小鼠7 d存活率为40%,而Sham组小鼠7 d存活率为100%,具有显著的差异($P<0.01$),提示CLP脓毒症小鼠模型造模成功,术后小鼠有较高死亡率,可能与肠道菌群异位感染相关。

通过解剖发现,CLP术后1 d,结扎穿孔的盲肠段呈现灰黑,明显坏死,余肠段可有少量充血。第3天时,结扎穿孔的盲肠开始出现纤维包裹,腹腔少量积液,脾脏部分充血。第5天时,盲肠结扎处形成脓肿,与周围组织粘连,腹壁可见少许出血点,部分小鼠脾脏可见明显肿大。以上结果提示CLP脓毒症的感染演变过程,从早期炎症性充血甚至坏死,到后期逐渐机化局限,然而脓肿的形成可导致感染慢性化发展。

2.2　小鼠脏器细菌定植量变化及病理损伤

为了了解脓毒症期间各脏器受细菌侵袭情况,将Sham组及CLP组小鼠于造模24 h后处死,收集外周血及心、肝、脾、肺、肾等实质脏器的研磨匀浆液,于LB平板上涂匀培养,统计菌落数并计算脏器细菌载量。结果显示,与Sham组小鼠相比,CLP术后24 h的小鼠心脏、肝脏、脾脏、肺脏、肾脏及外周血中的细菌定植量均呈现出显著增加的趋势,其中以肝脏中的细菌定



A: Mice in the sham group receiving no treatment after cecum exposure; B: Mice in the CLP group undergoing ligation and puncture of the distal 1/3 of the cecum; C: Necrosis of the ligated cecum at day 1 post-CLP; D: Ligated cecum showing encapsulation and adhesion at day 3 post-CLP; E: An abscess in the ligated segment of the cecum with adhesion to surrounding tissues at day 5 post-CLP; F: Survival curve of mice in different groups. CLP: cecal ligation and puncture. a: $P<0.01$, compared with the Sham group

图1　小鼠CLP术后感染情况及7 d存活率

Figure 1　Post-CLP infection status and 7-day survival rate in mice

植量最高,可达(3.27±1.07)×10^6 CFU/g,其余脏器中则多在10^5 CFU/g数量级,但血液中细菌定植量相对较低,约为(4.01±1.86)×10^4 CFU/mL。

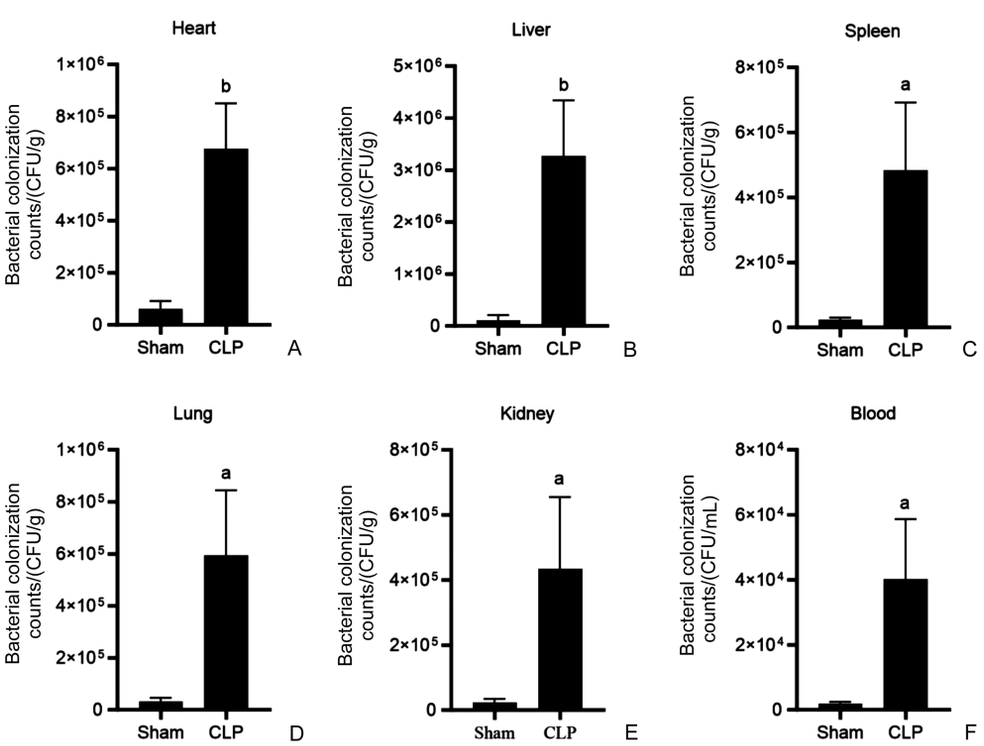
小鼠造模后24 h时分别取Sham组和CLP组心脏、肝脏、肺脏、肾脏制作组织切片并进行HE染色。结果显示:Sham组小鼠各脏器细胞形态正常,排列有序。与Sham组相比,在CLP术后24 h组中,小鼠的心脏中心肌纤维排列紊乱,部分断裂,局部心肌细胞变性,少量炎症细胞浸润;肝脏中细胞水肿,肝窦扩张充血,肝小叶结构破坏,炎性浸润;肺脏出现肺泡间隔显著增宽,肺泡腔内出血、渗出,间质充血、水肿;肾脏中肾小管上皮细胞变性、坏死、水肿,肾小球充血,间质炎症细胞浸润。以上结果表明,CLP术后随着肠道屏障破损,肠道菌群可能出现异位并定植于心、肝、肾等实质脏器,造成病理性损伤。

2.3 脓毒症小鼠免疫器官病理变化

为了探究脓毒症小鼠免疫器官是否存在病理损伤,本研究采集Sham组和CLP术后1、3、5 d小鼠的脾脏、胸腺及肠系膜淋巴结,切片并进行HE染色后观察其显微结构改变情况。结果显示Sham组小鼠脾脏、胸腺及肠系膜淋巴结细胞形态正常,排列有序,边界清晰。而在CLP组小鼠中第1天时,脾脏红髓区充血,白髓区少量中性粒细胞浸润,淋巴小结结构尚清晰;胸腺皮质轻度水肿,皮质与髓质分界尚清;肠系膜淋巴结被膜下窦扩张充血,中性粒细胞浸润,皮质区淋巴滤泡增生。第3天,脾脏红髓区出血及水肿加重,多量炎性细胞浸润,红白髓分界模糊,淋巴小结结构紊乱;胸腺皮质显著变薄,皮髓分界模糊,髓质结构紊乱;肠系膜淋巴结髓质区炎性细胞浸润,淋巴滤泡结构紊乱,生发中心萎缩。第5天,脾脏白髓区明显萎缩,红髓区纤维化倾向,以单核巨噬细胞浸润为主,组织疏松;胸腺整体积明显缩小,皮髓分界消失,胸腺小叶结构破坏;肠系膜淋巴结组织疏松,髓质区纤维化,单核巨噬细胞浸润为主,副皮质区萎缩,以浆细胞浸润为主。以上结果表明,随着脓毒症进程,各级免疫器官中炎症持续存在,并出现不同程度的损伤。

2.4 脓毒症小鼠外周血CD4$^+$T、CD8$^+$T和PD-1$^+$T细胞亚群比例变化

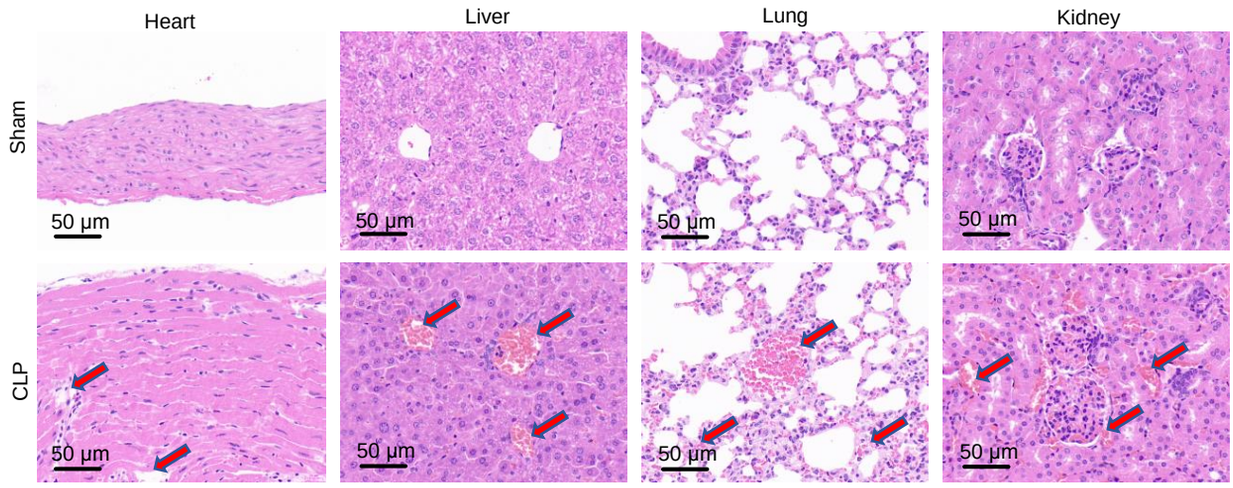
分别采集Sham组和CLP术后1、3、5 d小鼠外周血,提取淋巴细胞并染色后流式细胞仪检测CD4$^+$T和CD8$^+$T细胞比例变化情况。结果显示,与Sham组相比,CLP术后第1、3、5天时CD4$^+$T淋巴细胞比例分别



A~F: Histograms of bacterial colonization in the heart (A), liver (B), spleen (C), lungs (D), kidneys (E), and peripheral blood (F) from mice in the Sham group and the CLP group at 24 h after modeling. CLP: cecal ligation and puncture. a: $P<0.05$, b: $P<0.01$, compared with the Sham group

图2 小鼠外周血及不同脏器细菌定植数(n=3,$\bar{x}\pm s$)

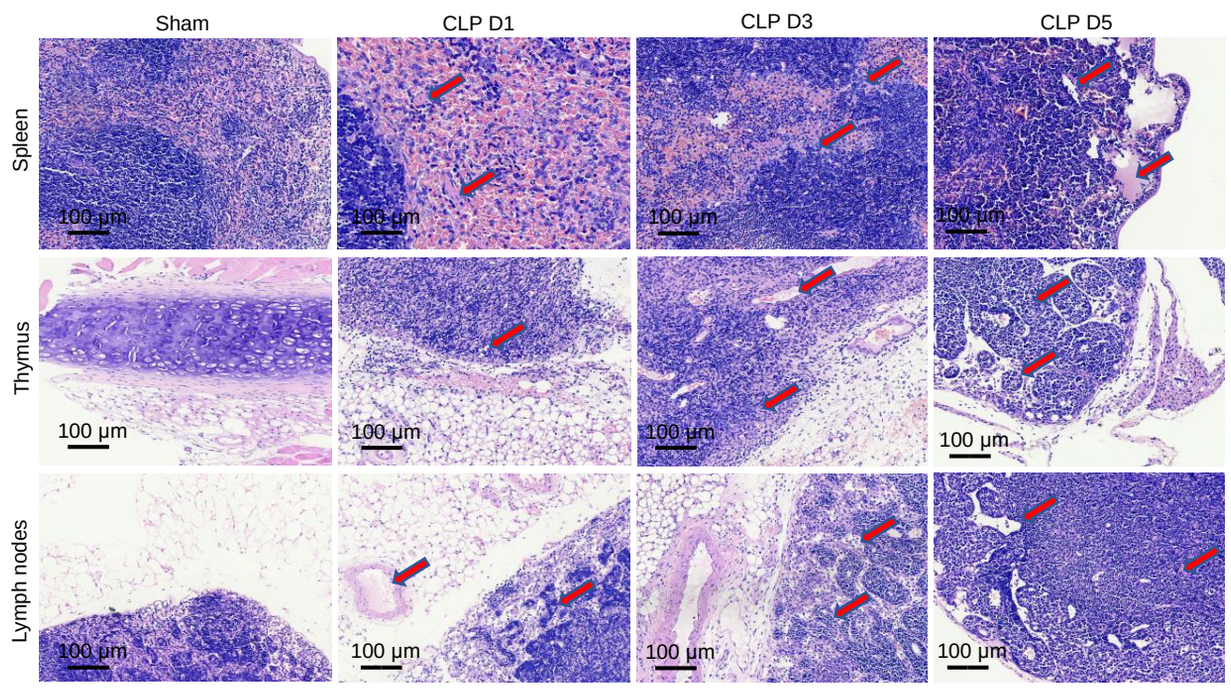
Figure 2 Bacterial colonization counts in peripheral blood and various organs of mice



Pathological changes of the heart, liver, lungs, and kidneys in mice from the Sham group and the CLP group at 1, 3, and 5 d after modeling; Red arrows indicate pathological changes. CLP: cecal ligation and puncture

图3 脓毒症小鼠主要实质脏器病理变化

Figure 3 Pathological changes of major parenchymal organs in septic mice (HE×200)



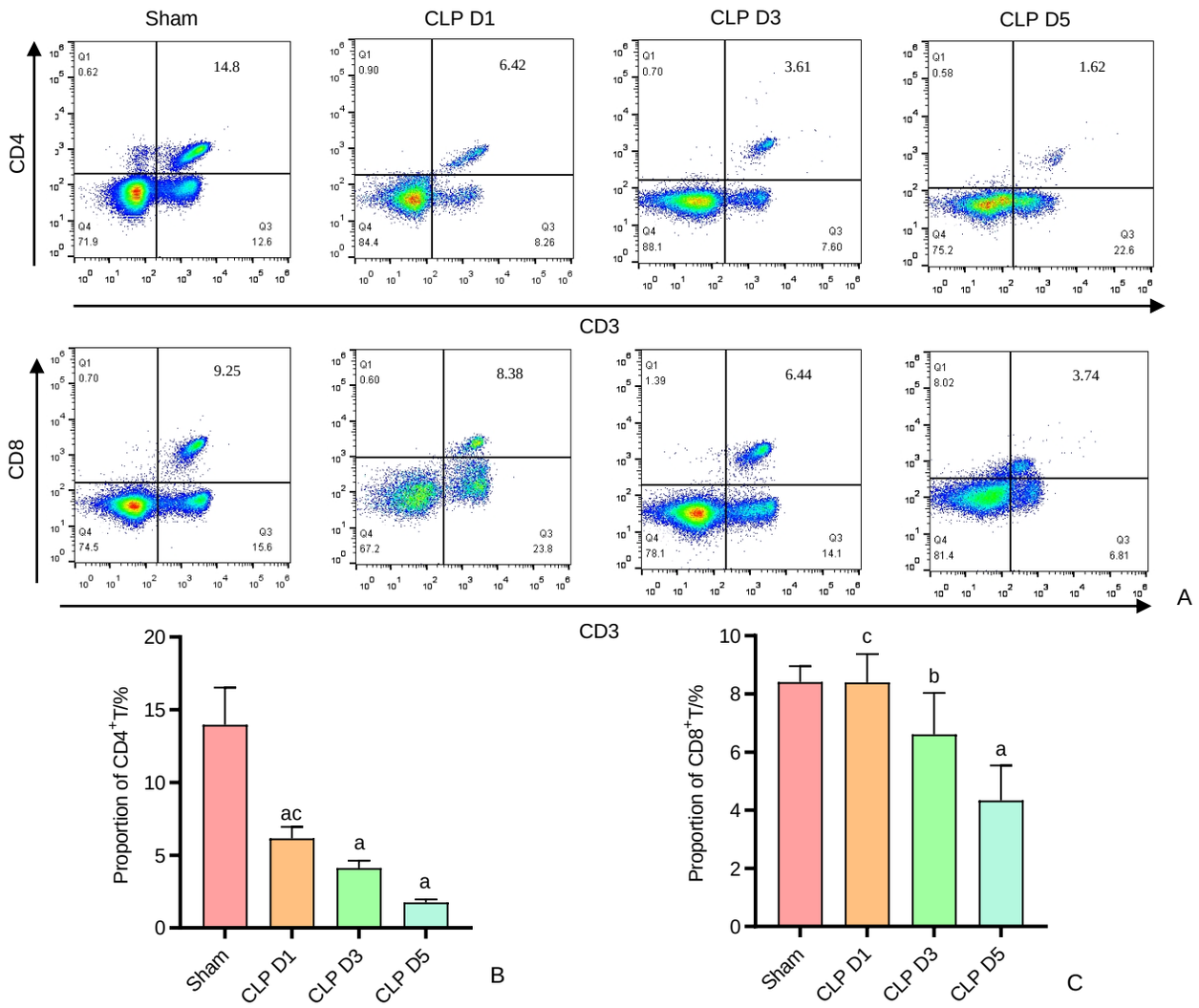
Pathological changes of the spleen, thymus, and mesenteric lymph nodes in mice from the Sham group and the CLP group at 1, 3, and 5 d after modeling; Red arrows indicate pathological changes. CLP: cecal ligation and puncture

图4 脓毒症小鼠免疫器官病理变化

Figure 4 Pathological changes of immune organs in septic mice (HE×200)

下降56%(P<0.001)、70.57%(P<0.001)和87.42%(P<0.001),第5天下降程度较第1天更显著(P<0.001)。而$CD8^+$T淋巴细胞比例在第1天时较Sham组无明显变化,第3天时下降21.3%,差异不显著,在第5天时下降48.33%(P<0.001)。以上结果表明脓毒症能够引起外周血中T细胞数量下降,尤以$CD4^+$T淋巴细胞数量下降最早且最为显著。

为了探索脓毒症期间外周血T淋巴细胞中PD-1的表达比例随时间(即感染进程)的变化关系,我们检测了Sham组和CLP术后1、3、5 d小鼠外周血中表达PD-1的T淋巴细胞比例变化。结果显示,与Sham组小鼠相比,CLP组小鼠术后1 d表达PD-1的T淋巴细



A: Flow cytometry results of the proportions of $CD4^+$T and $CD8^+$T cells, respectively, in the peripheral blood sample collected from mice in the Sham group and the CLP group at 1, 3, and 5 d after modeling; B and C: Histograms showing the proportions of $CD4^+$T (B) and $CD8^+$T (C) cells in peripheral blood samples from each group of mice. CLP: cecal ligation and puncture. a: $P<0.001$, compared with the Sham group; b: $P<0.001$, compared with the CLP D1 group; c: $P<0.01$, compared with the CLP D3 group

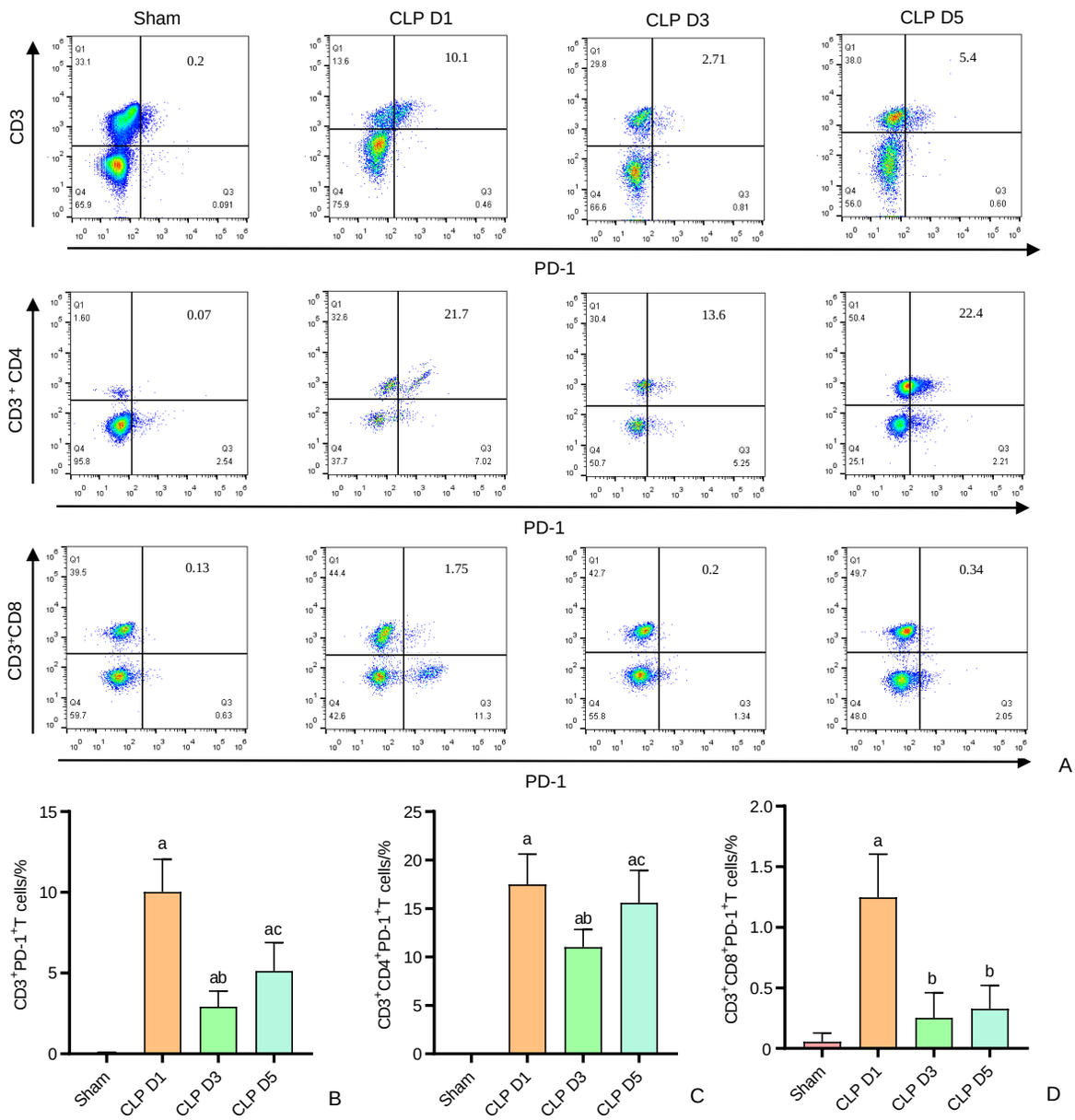
图5 脓毒症小鼠外周血T淋巴比例随时间的变化($n=5,\bar{x}\pm s$)

Figure 5 Temporal changes in the proportion of T lymphocytes in peripheral blood of septic mice

胞($CD3^+PD\text{-}1^+$T细胞)即大量增加,上升至Sham组的217.4倍($P<0.001$),在术后第3天表达比例较第1天下降70.70%($P<0.001$),第5天时又较第3天上升75.42%($P<0.01$),但后两者均显著高于Sham组,分别为其63.7倍和111.74倍($P<0.001$)。表达PD-1的$CD4^+$T淋巴细胞亚群($CD3^+CD4^+PD\text{-}1^+$T细胞)比例升降趋势同整体T淋巴细胞,在CLP术后1、3、5 d分别上升为Sham组的673.08倍、423.08倍和600倍($P<0.001$)。而表达PD-1的$CD8^+$T淋巴细胞亚群($CD3^+CD8^+PD\text{-}1^+$T细胞)比例在术后第1天时上升为Sham组的22.32倍($P<0.001$),但在第3天和5天时分别为Sham组的4.52倍和5.84倍,差异不显著($P>0.05$)。这一结果表明脓毒症病程中外周血T淋巴细胞可能出现PD-1途径的持续性耗竭,并且以$CD4^+$T淋巴细胞亚群的耗竭为主。

2.5 免疫器官CD4、CD8及PD-1阳性细胞表达检测

结果显示,在Sham组小鼠胸腺中CD4、CD8阳性细胞较为均匀地分布在皮质和髓质区。CLP术后第1天时CD4、CD8阳性细胞比例分别为Sham组的7.65倍和4.93倍,均明显增多($P<0.001$),并相对集中于髓质区域。从第3天开始至第5天胸腺中CD4阳性细胞比例相较于第1天分别减少54.1%、82.8%,均显著下



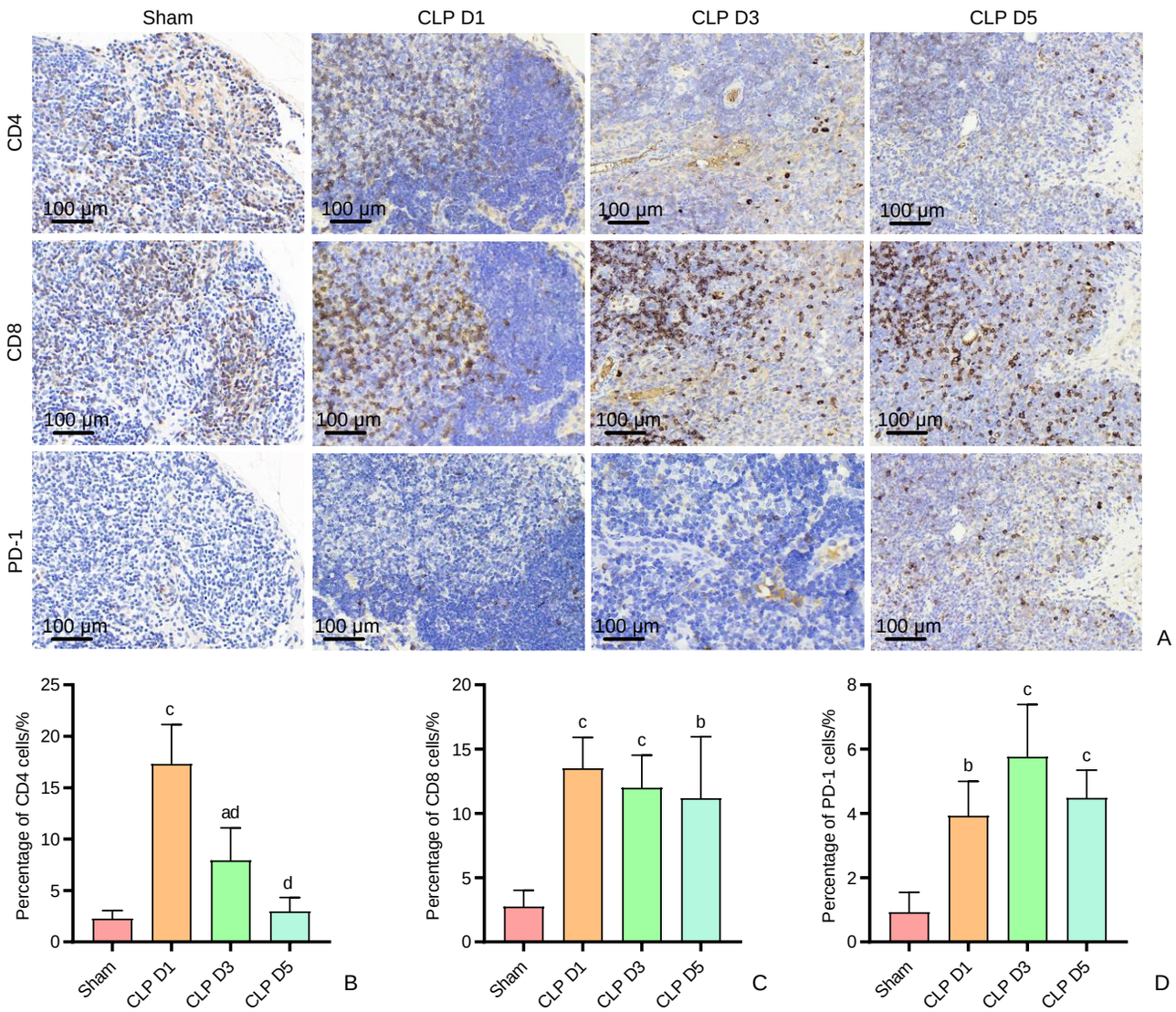
A: Flow cytometry results of the proportion of PD-1 expression in $CD3^+$T, $CD3^+CD4^+$T, and $CD3^+CD8^+$T cells, respectively, in the peripheral blood samples collected from mice in the Sham group and the CLP group at 1, 3, and 5 d after modeling; B~D: Histograms showing the proportion of PD-1 expression in $CD3^+$T (B), $CD3^+CD4^+$T (C), and $CD3^+CD8^+$T (D) cells in each time group, respectively. CLP: cecal ligation and puncture. a: P<0.001, compared with the Sham group; b: P<0.001, compared with the CLP D1 group; c: P<0.01, compared with the CLP D3 group

图6 脓毒症小鼠外周血T淋巴细胞PD-1表达比例变化(n=10,$\bar{x}\pm s$)

Figure 6 Changes in the proportion of PD-1 expression on T lymphocytes in peripheral blood of septic mice

降(P<0.001),而CD8阳性细胞分别下降11.11%和17.04%,变化不显著(P>0.05)。Sham组小鼠胸腺内PD-1阳性表达的细胞比例很少,CLP术后第1、3、5天时分别上升为Sham组的4.25倍(P<0.01)、6.24倍(P<0.001)和4.85倍(P<0.001),呈持续性高比例表达,且表达区域主要与CD4阳性细胞分布相同。

相较于Sham组,CLP术后小鼠脾脏内CD4、CD8阳性细胞比例随时间延长而持续增加,到第3天时最显著(P<0.001),分别上升为Sham组的2.66倍和7.35倍,细胞位置也由红髓变化至白髓和边缘区,并主要集中于脾动脉周围淋巴鞘(periarterial lymphatic sheath,PALS),此处含有大量T淋巴细胞。但到第5天时,CD4阳性细胞比例较第3天下降41.9%(P<0.01),与Sham组水平相当,而CD8阳性细胞变化不显著。CLP



A: Immunohistochemical staining results of CD4, CD8, and PD-1 in the thymus of mice from the Sham group and the CLP group at 1, 3, and 5 d after modeling; B~D: Histograms showing changes in the percentages of $CD4^+$ (B), $CD8^+$ (C), and $PD\text{-}1^+$ (D) cells in the thymus. CLP: cecal ligation and puncture. a: $P<0.05$, b: $P<0.01$, c: $P<0.001$, compared with the Sham group; d: $P<0.001$, compared with the CLP D1 group

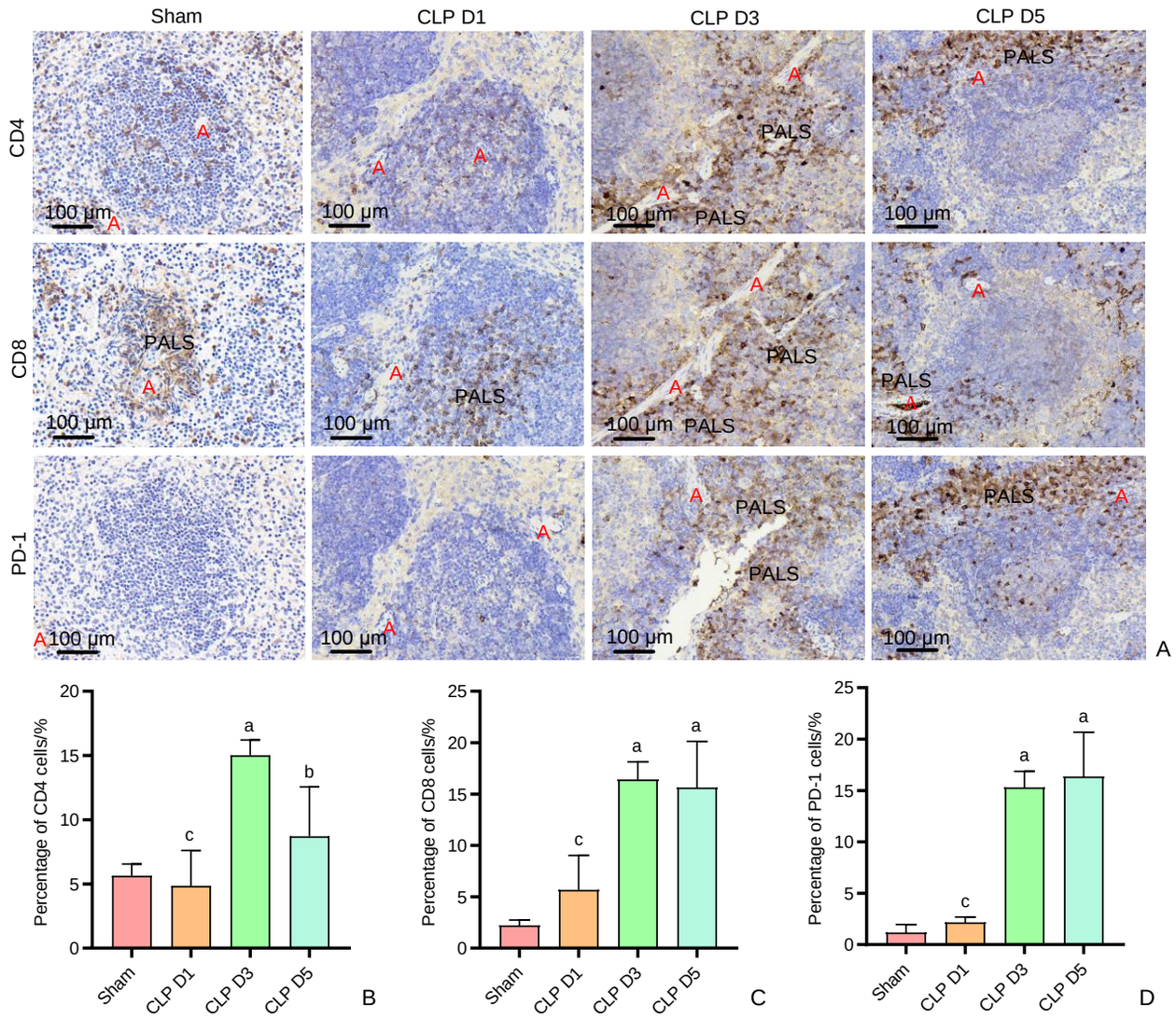
图7 脓毒症小鼠胸腺CD4、CD8及PD-1阳性细胞变化($n=5, \bar{x}\pm s$)

Figure 7 Changes in $CD4^+$, $CD8^+$, and $PD\text{-}1^+$ cells in the thymus of septic mice

术后第1、3、5天时,PD-1阳性表达细胞比例分别上升为Sham组的1.83倍($P>0.05$)、12.97倍($P<0.001$)和13.9倍($P<0.001$),第3天和第5天时增加较为显著,其表达区域也主要与CD4阳性细胞分布相同。

Sham组小鼠肠系膜淋巴结内CD4、CD8阳性细胞呈相对均匀分布,PD-1阳性细胞较少且分散。CLP术后第1天时肠系膜淋巴结内CD4、CD8阳性细胞比例分别上升为Sham组的3.7倍($P<0.001$)和2.52倍($P<0.01$),增加明显,且分布范围扩大。但第3、5天时,CD4阳性细胞比例较第1天分别下降47.52%($P<0.001$)、60.15%($P<0.001$),而CD8阳性细胞变化不显著。CLP术后第1、3、5天时PD-1阳性细胞比例分别上升为Sham组的1.43倍、13.86倍($P<0.001$)和20.96倍($P<0.001$),从第3天开始增加较为显著,其表达区域与CD4阳性细胞分布大致相同。

以上结果表明,脓毒症中各级免疫器官$CD4^+$T和$CD8^+$T淋巴细胞在炎症前期出现应激性增多,但随后$CD4^+$T比例显著下降,而$PD\text{-}1^+$细胞比例明显上升并与$CD4^+$细胞表达范围相重合,提示$CD4^+$T细胞出现了PD-1途径的持续性耗竭。



A: Immunohistochemical staining results of CD4, CD8, and PD-1 in the spleen of mice from the Sham group and the CLP group at 1, 3, and 5 d after modeling. Red A indicates the splenic artery, and Black PALS represents the periarteriolar lymphoid sheath. Scale bar: 100 μm; B~D: Histograms showing changes in the percentages of $CD4^+$ (B), $CD8^+$ (C), and $PD\text{-}1^+$ (D) cells in the spleen. CLP: cecal ligation and puncture. a: $P<0.001$, compared with the Sham group; b: $P<0.01$, c: $P<0.001$, compared with the CLP D3 group

图8 脓毒症小鼠脾脏CD4、CD8及PD-1阳性细胞变化($n=5, \bar{x}\pm s$)

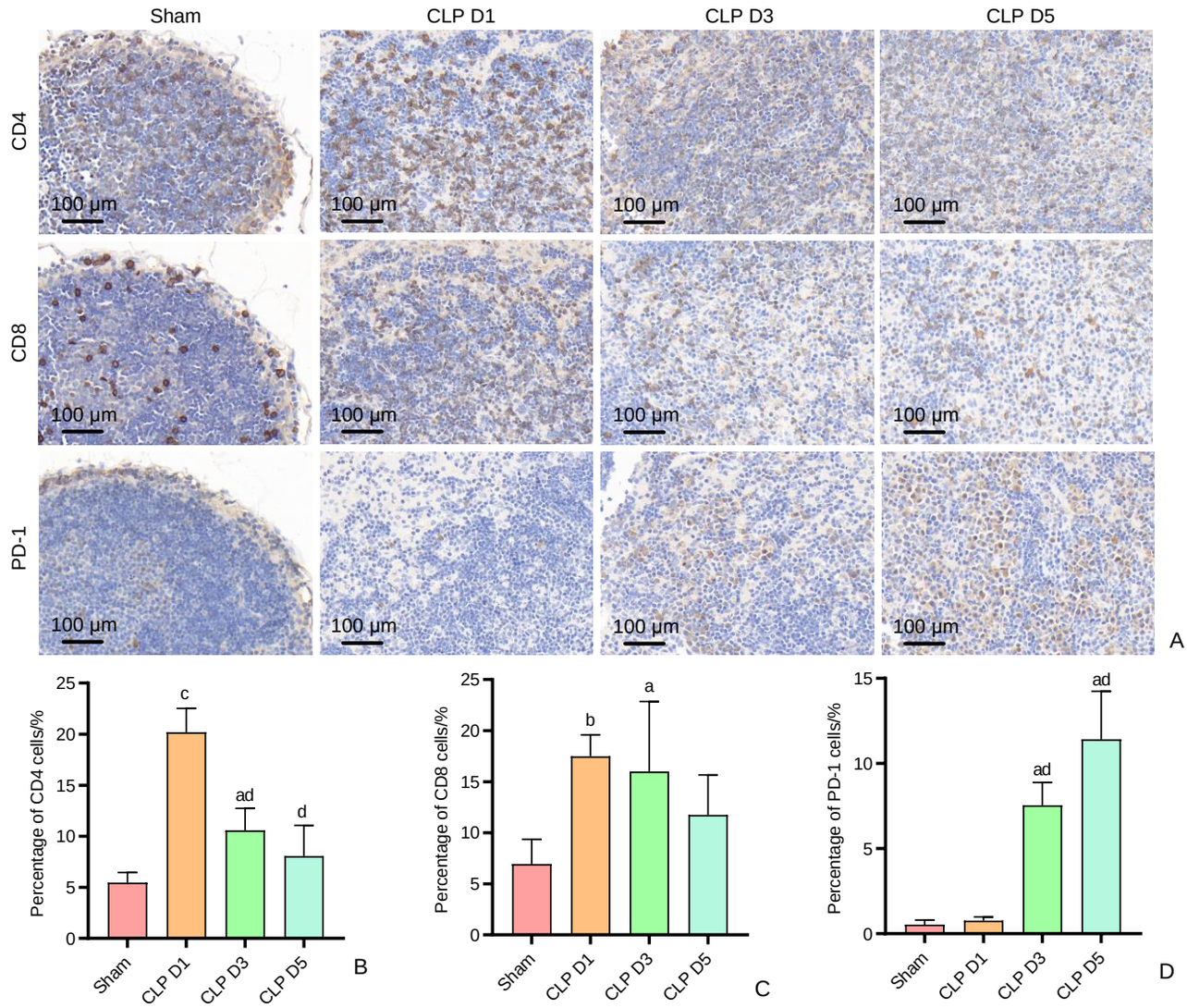
Figure 8 Changes in $CD4^+$, $CD8^+$, and $PD\text{-}1^+$ cells in the spleen of septic mice

3 讨论

脓毒症是由感染引起宿主免疫反应失调导致的临床危重症[15]。据估计,我国ICU收治的23万脓毒症患者每年的医疗费用约为46亿美元,存在巨大的医疗和社会负担[16]。本研究复制了小鼠CLP脓毒症模型并观察到小鼠脏器中的细菌定植量在造模前后发生了变化,在此基础上进一步探索了免疫器官的病理学改变和免疫耗竭特征,发现机体免疫耗竭与肠道菌群异位之间存在一定关联。

3.1 脓毒症时肠道菌群在各脏器异位定植

肠道上皮、免疫系统和肠道微生物群构成了肠道屏障功能三道主要的防线,即机械屏障,免疫屏障和生物屏障,它们互相关联共同作用,以保护自身免受病原体损害[17]。肠道屏障功能障碍既是腹腔脓毒症病程发展的危险因素,也是预后不良的标志。既往研究表明,感染因素所造成的肠黏膜上皮细胞凋亡和坏死会导致黏膜机械屏障功能损伤、肠道菌群异位,肠



A: Immunohistochemical staining results of CD4, CD8, and PD-1 in mesenteric lymph nodes of mice from the Sham group and the CLP group at 1, 3, and 5 d after modeling; B~D: Histograms showing changes in the percentages of $CD4^+$, $CD8^+$, and $PD\text{-}1^+$ cells in mesenteric lymph nodes. CLP: cecal ligation and puncture. a: $P<0.05$, b: $P<0.01$, c: $P<0.001$, compared with the Sham group; d: $P<0.001$, compared with the CLP D1 group

图9 脓毒症小鼠肠系膜淋巴结CD4、CD8及PD-1阳性细胞变化(n=5,$\bar{x}\pm s$)

Figure 9 Changes in $CD4^+$, $CD8^+$, and $PD\text{-}1^+$ positive cells in mesenteric lymph nodes of septic mice

道内的致病菌经过受损的肠黏膜进入血液循环并播散至其他脏器,最终引起全身炎症反应以及靶器官功能损伤[18]。通过对Sham组和CLP组24 h的小鼠心、肝、肺等主要实质脏器进行匀浆培养,我们发现CLP组小鼠各脏器培养菌落数均显著高于Sham组,其中以肝脏细菌定植量最高。胃肠道和肝脏之间通过门静脉复杂相接,并且肝脏能够在一定程度上清除细菌及其产生的炎症介质[19-20],它是防止肠道菌群异位的另一道防线。因此,在肠道机械屏障损伤之后,细菌首先随血流涌入肝脏,这可能是导致小鼠CLP术后肝脏细菌定植量最高并伴有严重损伤的原因之一,与既往脓毒症中肝脏容易受到损害的报道相一致[21]。另外,CLP组小鼠外周血和其他脏器匀浆培养菌落数均较Sham组有显著增高,并且其余脏器也出现不同程度病理损伤,与前述脓毒症导致多种靶器官损伤的报道相同。

3.2 免疫器官发生以$CD4^+$T淋巴细胞为主的免疫耗竭

然而,正常情况下肠道菌群也存在异位现象,据报道,人群中其异位基线率为5%~10%,但健康个体中

不会出现任何病变,可能因为这种低水平的抗原暴露有助于机体产生有效免疫[22]。但当免疫系统受损时,会打破这种平衡并导致脓毒症的发生。PD-1可引起淋巴细胞凋亡,导致淋巴细胞数量下降[22],同时PD-1也是一种免疫抑制分子,其表达水平与T细胞的功能状态密切相关,如PD-1表达升高可引起T细胞的失能[23]。有实验表明,随着脓毒症的加重,表达PD-1的T细胞比例会升高[24],我们也在研究中发现CLP术后小鼠外周血表达PD-1的$CD4^+$T细胞比例持续性增高,$CD8^+$T细胞中PD-1表达比例则只在第1天升高,与此相反的是$CD4^+$T淋巴细胞自身的相对数量则从早期就持续下降。我们进一步研究发现,随着脓毒症进程,小鼠胸腺、脾脏、淋巴结等各级免疫器官中炎症持续存在,并出现不同程度病理损伤。免疫组化表明,$CD4^+$T和$CD8^+$T淋巴细胞在炎症早期免疫器官中呈现应激性增多,但$CD4^+$T淋巴细胞随后又显著减少,并且CD4阳性细胞表达分布与逐渐增多的PD-1阳性细胞表达区域大致相同。免疫器官中$CD4^+$T淋巴细胞减少的原因可能是一方面它们被紧急调往外周循环以弥补缺损,另一方面则发生了PD-1途径的凋亡。虽然第5天时免疫器官中$CD4^+$T淋巴细胞比例下降后仍与对照组水平相当,但其表面PD-1的表达会使其功能受到抑制,无法起到正常抗感染的作用。这一系列结果表明,脓毒症时外周血和各级免疫器官均出现$CD4^+$T淋巴细胞比例下降,伴表达PD-1的$CD4^+$T淋巴细胞比例显著升高,提示其发生了免疫耗竭。

3.3 免疫耗竭可导致肠道菌群异位加重

胸腺是负责幼稚T细胞发育、选择和输出的主要器官[25]。$CD4^+$T细胞等胸腺细胞亚群耗竭所引发的急性胸腺退化会对免疫系统产生严重影响,削弱了它们在脓毒症期间抵抗反复和继发感染的能力[26]。脾脏是体内最大的免疫器官,也是机体细胞和体液免疫的核心,据报道切除脾脏会导致免疫系统紊乱,更容易继发感染[27-28],因而脾脏中的T细胞耗竭可能进一步加重脓毒症免疫抑制[12]。正常情况下,易位细菌可被肠系膜淋巴结吞噬,但炎症引起淋巴结损伤和T淋巴细胞免疫耗竭可导致细菌易位失控及其分泌的毒性因子逃逸,有研究就表明从创伤导致出血性休克的动物身上采集的肠系膜淋巴液在注射到大鼠体内时足以诱导急性肺损伤[29]。总之,脓毒症中炎性环境等因素可诱导免疫器官损伤,出现$CD4^+$T细胞PD-1的持续表达并使其发生耗竭,这削弱了机体抵抗肠道菌群易位感染的能力,可能形成“炎症-免疫耗竭-菌群异位”的恶性循环,使细菌最终在各远隔脏器中成功定植进而加重病理损伤,甚至导致多器官功能障碍综合征的发生。而针对PD-1进行阻断治疗可能改善T细胞耗竭状态,进而提高存活率[12]。

本研究存在一定的局限性:①尽管小鼠CLP模型模拟了人类严重脓毒症的性质和演变而被认为是研究脓毒症的金标准[30],但CLP动物模型通过盲肠结扎穿孔直接导致肠道内容物泄漏,其细菌异位的触发机制与临床脓毒症可能存在差异,后续研究拟采用静脉注射LPS的方法复制脓毒症模型,消除模型复制方法可能带来的不确定性;②未纳入肠道菌群组成分析,不能很好区分异位细菌的来源,在后续研究我们拟增加菌群测序以明确异位细菌组成;③缺乏针对细菌异位或免疫耗竭的干预实验,不易验证两者的直接关联,未来将通过抗生素干预或针对PD-1的免疫调控进行更深入研究。

综上所述,本研究应用CLP小鼠脓毒症模型,发现脓毒症中各级免疫器官均出现病理损伤并发生与PD-1通路相关的$CD4^+$T淋巴细胞耗竭。同时表明脓毒症中除了肠道机械屏障破坏外,免疫系统受损也是导致肠道菌群异位定植并造成远隔脏器严重损伤的重要原因。该研究为更好地阐述脓毒症肠道菌群异位机制及采取相应防治措施奠定了理论基础。

利益冲突 本文所有作者声明不存在利益冲突

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