·基础研究·

BMMSCs输注对MRL/lpr狼疮鼠血液系统的影响

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[摘 要] **目的**:研究同种异体骨髓间充质干细胞(bone marrow mesenchymal stem cell,BMMSCs)输注对MRL/lpr狼疮鼠造血的影响,为BMMSCs输注治疗系统性红斑狼疮(systemic lupus erythematosus,SLE)的血液系统损伤提供依据。**方法**:分离培养C57BL/6小鼠的BMMSCs并对MRL/lpr狼疮鼠进行静脉输注,同时以PBS为对照。分为对照(C57BL/6)组、狼疮鼠(MRL/lpr)组、BMMSCs输注组1(MRL/lpr鼠12周注射BMMSCs)、PBS对照组1(MRL/lpr鼠12周注射PBS)、BMMSCs输注组2(MRL/lpr鼠12、16周两次注射BMMSCs)、PBS对照组2(MRL/lpr鼠12、16周两次注射PBS)。在20周末取小鼠的外周血进行血细胞计数,后4组取骨髓及脾脏进行流式细胞术和甲基纤维素半固体集落形成试验(CFU)检测分析输注组和对照组骨髓及脾脏各系细胞比例。**结果**:与C57BL/6小鼠相比,狼疮鼠外周血红细胞计数[$(9.3 \pm 0.8)\times10^{12}$个/L *vs.* $(10.5 \pm 0.8)\times10^{12}$个/L,$n$=5,$P<0.001$]、血红蛋白[(138.1 ± 11.6)g/L *vs.*(156.4 ± 11.0)g/L,n=5,$P<0.001$]和血小板计数[$(695.3 \pm 136.2)\times10^{9}$个/L *vs.* $(844.1 \pm 180.8)\times10^{9}$个/L,$n$=5,$P<0.05$]降低,与PBS对照组相比,BMMSCs输注组MRL/lpr狼疮鼠血小板数量改善[$(795.0 \pm 75.7)\times10^{9}$个/L *vs.* $(593.4 \pm 123.4)\times10^{9}$个/L,$n$=5,$P<0.05$],骨髓中干细胞($n$=5,$P<0.001$)和成熟T细胞比例减少($n$=5,$P<0.05$),髓系比例增加($n$=5,$P<0.01$),pre-B数量有所改善($n$=5,$P<0.05$),脾脏内干细胞比例上升($n$=5,$P<0.05$),成熟T细胞比例下降($n$=5,$P<0.05$)。**结论**:BMMSCs输注对狼疮鼠外周血、骨髓及脾脏不同系造血细胞有影响,其对狼疮鼠的血液系统损伤有治疗意义。

[关键词] 系统性红斑狼疮;骨髓间充质干细胞;成熟T细胞;髓系细胞;pre-B

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Effects of BMMSCs infusion on the blood system of MRL/lpr mice

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[**Abstract**] **Objective**: To investigate the effect of bone marrow mesenchymal stem cells (BMMSCs) infusion on hematopoiesis in MRL/lpr mice, and provide a basis for the infusion of BMMSCs to treat systemic lupus erythematosus (SLE) blood system damage. **Methods**: BMMSCs of C57BL/6 mice were isolated and cultured for intravenous infusion of MRL/lpr mice. The experimental group was divided into: ①C57BL/6 mice group; ②MRL/lpr mice group; ③once PBS control group: 12 weeks injection of PBS lupus mice group; ④once BMMSCs infusion group: 12 weeks injection of BMMSCs lupus mice group; ⑤twice PBS control group: 12 and 16 weeks were injected with PBS lupus mice group; ⑥twice BMMSCs infusion group: 12 and 16 weeks were injected BMMSCs lupus mice group. Blood cells were counted in peripheral blood of lupus mice at 20 weeks. Bone marrow and spleen were taken for flow cytometry and methylcellulose semi-solid colony formation assay (CFU). **Results**: Compared with C57BL/6 mice, the peripheral blood erythrocyte count, hemoglobin and platelet count of lupus mice were decreased. Compared with the PBS control group, the number of platelets in the BMMSCs infusion group was improved, the proportion of stem cells and mature T cells in the bone marrow was decreased, the proportion of myeloid cells was increased, the number of pre-B was improved. The proportion of stem cells in the spleen was increased, the proportion of T cells decreased. **Conclusion**: BMMSCs infusion has effects on peripheral blood, bone marrow and spleen hematopoietic cells of MRL/ lpr mice. It has therapeutic effect on the blood system injury of lupus mice.

[**Key words**] systemic lupus erythematosus; bone marrow mesenchymal stem cells; mature T cells; myeloid cells; pre-B

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系统性红斑狼疮(systemic lupus erythematosus,SLE)是一种多脏器受累的弥漫性结缔组织疾病,其特点是存在自体抗体和多种形式的临床表现[1]。血液系统损伤包括白细胞、血红蛋白或血小板减少,在SLE中很常见。严重的血液异常可危及生命。骨髓间充质干细胞(bone marrow mesenchymal stem cells,BMMSCs)是具有多种分化潜力的非造血祖细胞,可以分化成多个间叶细胞组织,重建功能性的造血微环境,并且对造血干细胞(hematopoietic stem cells,HSCs)的功能及分化具有支持作用[2-4]。已有多个临床研究利用BMMSCs治疗SLE并获得较好的临床效果[5]。有研究显示,BMMSCs输注可有效纠正SLE患者的外周血三系的损伤[6],但BMMSCs对患者骨髓造血情况的影响尚未见报道。本研究首次检测了MRL/lpr狼疮鼠外周血的改变,进一步研究了同种异体BMMSCs输注对狼疮鼠骨髓及髓外造血的影响。

1　材料和方法

1.1　材料

MRL/lpr鼠(SPF级),雌性,6周龄,25只[上海灵畅生物科技有限公司,许可证号:SCXK(沪)2013-0018]。C57BL/6小鼠(SPF级),雌性,6周龄,5只[江苏大学实验动物中心,许可证号:SCXK(苏)2013-0011]。所有实验小鼠均于江苏大学动物实验中心SPF级饲养区饲养至20周龄。

胎牛血清(FBS)、低糖DMEM培养液(Sigma公司,美国);MethoCult™ M3630、MethoCult™ SF M3436、MethoCult™ GF M3534半固体培养基(Stemcell公司,美国);APC-CD3e、APC-Ter-119、PE-Sca-1、PE-B220、FITC-CD11b、PE-ISO、APC-ISO、FITC-ISO荧光抗体(BD公司,美国);流式细胞仪CANTO 10C(BD公司,美国);全自动血常规分析仪BC-5310(深圳迈瑞公司)。

1.2　方法

1.2.1　细胞培养

C57BL/6小鼠采用全骨髓贴壁筛选法分离培养BMMSCs,从小鼠的股骨和胫骨中分离骨髓,将全部骨髓细胞置于含有20%FBS的低糖DMEM培养液中,置于37 ℃、5%CO_2的培养箱中进行原代培养。培养48 h后首次全量换液,去除没有贴壁的细胞,之后每3 d半量换液1次。待贴壁细胞覆盖培养皿底部面积80%~90%时,用0.25%的胰蛋白酶消化传代。扩增至第3代BMMSCs用于BMMSCs输注实验。

1.2.2　实验动物分组

实验动物分为6组,每组5只小鼠,分别为:对照(C57BL/6)组、狼疮鼠(MRL/lpr)组、BMMSCs输注组1(MSC1组,MRL/lpr鼠于12周注射BMMSCs)、PBS对照组1(PBS1组,MRL/lpr鼠于12周注射PBS)、BMMSCs输注组2(MSC2组,MRL/lpr鼠于12、16周两次注射BMMSCs)、PBS对照组2(PBS2组,MRL/lpr鼠于12、16周2次注射PBS)。

MSC1组、MSC2组均在对应周龄末经尾静脉注射BMMSCs,细胞浓度为1×10^6个/mL PBS,体积为0.1 mL/10 g体重,PBS1组、PBS2组注射相同体积的PBS,C57BL/6组、MRL/lpr组未予处理,所有动物统一在20周龄第7天进行静脉取血。

1.2.3　外周血细胞计数

各组小鼠经眼球内眦的眼眶静脉丛采集外周血500~1 000 μL,EDTA抗凝,采用全自动血细胞分析仪进行外周血细胞计数。

1.2.4　流式细胞仪检测

从注射BMMSCs或PBS后的小鼠股骨和胫骨中分离骨髓,并取脾脏研磨过筛,使用含5%FBS的PBS缓冲液重悬、离心,裂解红细胞后进行有核细胞计数,调整终浓度为1×10^7个/mL,各取100 μL分别与相应的荧光直标单抗4 ℃避光孵育20 min,于流式细胞仪检测,每份标本检测1×10^6个细胞。所用荧光抗体包括:B淋系抗体B220;髓系抗体CD11b;T淋系抗体CD3E;幼红系抗体TER-119,造血干细胞抗体Sca-1。

1.2.5　集落形成实验

将注射BMMSCs或PBS后小鼠骨髓细胞按有核细胞数1×10^5个/mL、5×10^4个/mL、2×10^5个/mL分别接种于MethoCult™ M3630、MethoCult™ SF M3436、MethoCult™ GF M3534半固体培养基中,检测前B细胞集落形成单位(CFU-pre-B)、红细胞爆式集落形成单位(BFU-E)和粒细胞-巨噬细胞集落形成单位(CFU-GM)。脾脏细胞按有核细胞数3×10^5个/mL接种于MethoCult™ SF M3436、MethoCult™ GF M3534半固体培养基中,使用35 mm培养皿进行集落单位形成实验,在37 ℃、5%CO_2饱和湿度条件下培养7 d后计数CFU-pre-B,培养10~12 d后计数CFU-GM、BFU-E,每个样本均种植2个培养皿,计数后取平均值。具体操作按MethoCult™说明书进行。

1.3　统计学方法

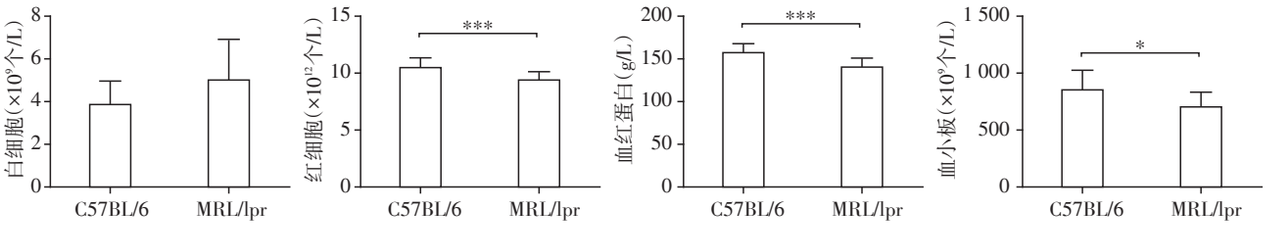
采用SPSS20软件进行分析,数据采用均数±标准差($\bar{x}\pm s$)表示,两组样本之间比较采用t检验,多

组之间的均数比较采用单因素方差分析，进一步组间比较采用配对样本 t 检验。$P \leqslant 0.05$ 则认为差异有统计学意义。

2　结　果

2.1　MRL/lpr狼疮鼠和C57BL/6小鼠外周血三系情况

20周龄MRL/lpr狼疮鼠较C57BL/6小鼠外周血红细胞计数[(9.3 ± 0.8)×10¹²个/L *vs.*(10.5 ± 0.8)×10¹²个/L，n=5，$P < 0.001$]、血红蛋白[(138.1 ± 11.6) g/L *vs.*(156.4 ± 11.0)g/L，n=5，$P < 0.001$]和血小板计数[(695.3 ± 136.2)×10⁹个/L *vs.*(844.1 ± 180.8)×10⁹个/L，n=5，$P < 0.05$]降低，且差异有统计学意义。与C57BL/6小鼠相比，狼疮鼠的白细胞计数无减少(图1)。



两组比较，$^{*}P < 0.05$，$^{***}P < 0.001$，n=5。

图1　MRL/lpr狼疮鼠与C57BL/6小鼠外周血中白细胞、红细胞、血红蛋白及血小板的比较

Figure 1　Comparison of WBC，RBC，hemoglobin and platelet in peripheral blood between MRL/lpr mice and C57BL/6 mice

2.2　BMMSCs输注对MRL/lpr狼疮鼠外周血细胞计数的影响

MRL/lpr小鼠12周龄、16周龄进行BMMSCs输注，并以相同周龄注射PBS作为对照。与PBS1组相比，接受1次BMMSCs输注的MSC1组外周血白细胞、红细胞及血红蛋白均无明显变化；MSC2组与PBS2组相比，血小板升高[(795.0 ± 75.7)×10⁹个/L *vs.*(593.4 ± 123.4)×10⁹个/L，n=5，$P < 0.05$]，差异具有统计学意义(图2)。

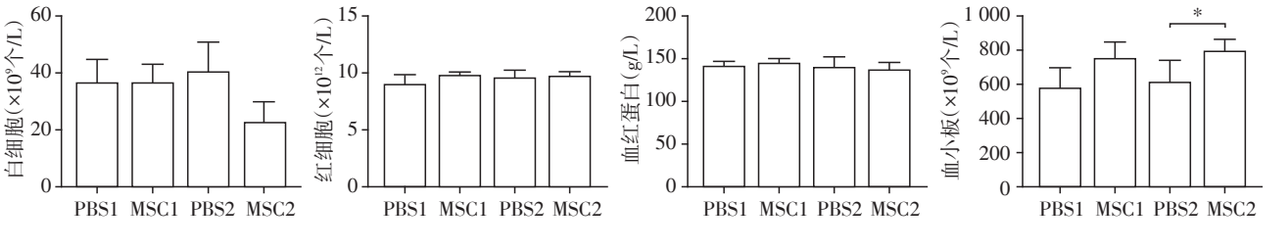
2.3　BMMSCs输注对MRL/lpr狼疮鼠骨髓造血的影响

骨髓流式细胞仪检测结果显示，MSC1组与PBS1组、MSC2组与PBS2组比较，Sca-1⁺的干细胞比例均减少[(14.8 ± 2.1)% *vs.*(22.2 ± 2.7)%，(12.6 ± 0.4)% *vs.*(21.5 ± 2.3)%，n=5，$P < 0.001$]；CD3e⁺的成熟T细胞比例减少[(9.1 ± 1.4)% *vs.*(11.8 ± 1.8)%，(10.4 ± 2.4)% *vs.*(11.8 ± 2.4)%，n=5，$P < 0.05$]；CD11b⁺的髓系细胞比例增加[(39.8 ± 4.9)% *vs.*(30.6 ± 5.3)%，n=5，$P < 0.01$；(39.6 ± 4.3)% *vs.*(32.8 ± 1.9)%，n=5，$P < 0.05$]；骨髓内B淋系、幼红系比例均无统计学差异。

骨髓集落形成实验结果显示：MSC2组与PBS2组及MSC1组相比，CFU-pre-B数目增高[(3.0 ± 1.3)个*vs.*(1.3 ± 0.3)个，(3.0 ± 1.3)个*vs.*(1.5 ± 0.4)个，n=5，$P < 0.05$]；BMMSCs输注后MRL/lpr狼疮鼠BFU-E、CFU-GM以及总集落数与对照无差异(图3)。

2.4　BMMSCs输注对MRL/lpr狼疮鼠脾脏细胞的影响

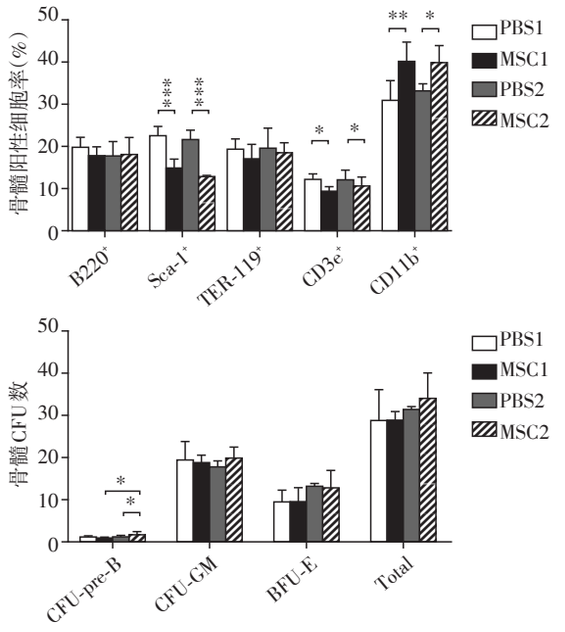
脾脏流式细胞仪检测结果显示：MSC2组与PBS2组及MSC1组相比，狼疮鼠脾脏内Sca-1⁺的干细胞比例增高[(40.2 ± 6.3)% *vs.*(30.5 ± 4.4)%，(40.2 ± 6.3)% *vs.*(28.9 ± 5.8)%，n=5，$P < 0.05$]。MSC2组与PBS2组及MSC1组相比，CD3e⁺的成熟T细胞比例减少[(57.1 ± 1.9)% *vs.*(67.6 ± 5.2)%，(57.1 ± 1.9)% *vs.*(67.2 ± 5.3)%，n=5，$P < 0.05$]，差异具有统计学意义。MSC1组与PBS1组、MSC2组与PBS2组比较，BFU-E、CFU-GM以及总集落数均没有统计学差异(图4)。



两组比较，$^{*}P < 0.05$，n=5。

图2　狼疮鼠输注BMMSCs后与PBS对照组外周血差异

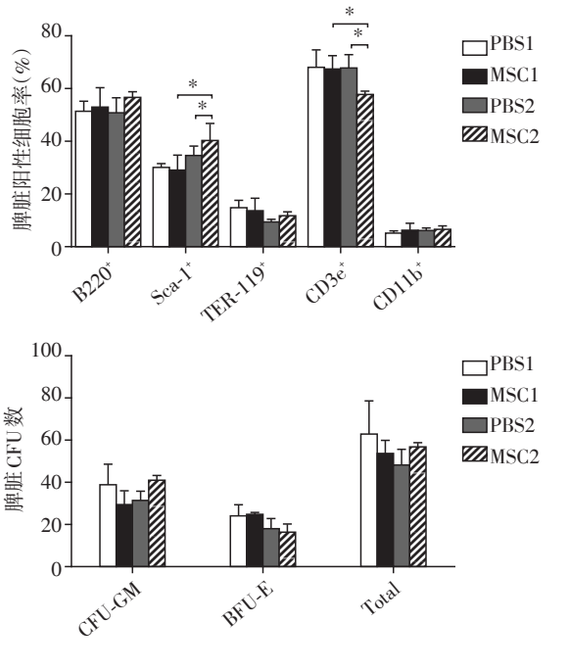
Figure 2　The difference of peripheral blood between the BMMSCs and PBS control group in MRL/lpr mice after infusion



两组比较，$^{*}P<0.05$，$^{**}P<0.01$，$^{***}P<0.001$，n=5。

图3　BMMSCs输注后与PBS对照组狼疮鼠骨髓流式细胞检测与骨髓细胞集落形成单位的比较

Figure 3　Comparison of bone marrow flow cytometry and bone marrow cell colony forming units in MRL/lpr mice after BMMSCs infusion



两组比较，$^{*}P<0.05$，n=5。

图4　BMMSCs输注后与PBS对照组狼疮鼠脾脏流式细胞检测与脾脏细胞集落形成单位的比较

Figure 4　Comparison of spleen flow cytometry and spleen cell colony forming units in MRL/lpr mice after BMMSCs infusion

3　讨　论

SLE是一种慢性自身免疫性和炎症性疾病，涉及多个器官和系统，其中常累及造血系统。约有10%的SLE患者在病程中发生严重的血液系统损伤，SLE患者血液系统损伤的临床治疗主要是以糖皮质激素及免疫抑制剂为主，仍有部分患者死于严重的出血及感染。BMMSCs被认为是非造血的多潜能祖细胞，具有自我更新能力，能够分化成多细胞谱系，包括成骨细胞、软骨细胞、肌细胞和脂肪细胞等多种细胞类型[7]。现在认为，BMMSCs也为造血干细胞提供了支持性的微环境，在维持造血干细胞生长、分化和正常功能方面起重要作用[8]。目前SLE亦被认为是一种干细胞疾病[9]。有研究发现，SLE患者BMMSCs存在多种异常[10]，临床使用MSCs治疗包括SLE在内的多种自身免疫性疾病已被认可[6,11]。

本实验主要研究输注同种异体BMMSCs对MRL/lpr鼠的外周血三系、骨髓及髓外造血的影响。实验所用的MRL/lpr狼疮鼠是广泛使用的狼疮鼠模型。该狼疮鼠在8周出现肾脏损害[12]，16周出现白细胞、血小板及红系的改变[13]。Sun等[10]研究结果也表明SLE患者不仅存在HSCs的异常，BMMSCs细胞结构功能也存在缺陷，其分泌细胞因子的能力下降，导致造血系统的异常，SLE患者多存在三系单独或同时减少。实验发现MRL/lpr鼠外周血红细胞、血红蛋白和血小板均比正常对照组低，但未检测到外周血白细胞的异常。BMMSCs输注治疗后血小板数量有所改善，这与临床输注后结果相符[5]。Liu等[14]研究发现，BMMSCs能够通过细胞间相互接触，提供造血所需要的多种造血因子包括：SDF-1α、血小板生成素（TPO）、IL-11、干细胞生长因子（SCF）等[15-19]，这些可能都是BMMSCs输注治疗血小板改善的原因之一。

狼疮患者骨髓存在多种异常，包括血细胞生成、成熟障碍，骨髓纤维化[20]，骨髓增生异常综合征等[21]。本研究中外源性BMMSCs输注治疗后骨髓中Sca-1^{+}的干细胞比例减少，而脾脏中比例增加，Sca-1^{+}的干细胞比例变化，考虑原因可能为：①BMMSCs产生多种细胞因子，包括粒细胞集落刺激因子（G-CSF）、粒细胞-巨噬细胞集落刺激因子（GM-CSF）、巨噬细胞集落刺激因子（M-CSF）等[22-27]，促使BMMSCs输注后的狼疮鼠骨髓中干细胞被动员到脾脏中；②外源性BMMSCs通过静脉灌注后在肝脏和脾脏中累积[28]，BMMSCs通过多种黏附分子选择性黏附

HSCs,使HSCs进入外周循环并归巢于脾脏[29],具体机制有待进一步研究。

实验发现BMMSCs输注后狼疮鼠骨髓及脾脏中$CD3e^+$T细胞比例均减少,且二次输注后脾脏内成熟T细胞较一次输注下降明显,考虑原因为:①已有文献报道MSCs剂量依赖性抑制T细胞的增殖[30],并抑制Akt/GSK3β介导的狼疮T细胞的G1/S期转变[31];②既往研究证实,SLE患者经MSCs输注后,血清细胞因子向Th1极化[32];NZB/NZW F1狼疮鼠经MSCs输注后,血液和脾脏的滤泡辅助性T细胞(Tfh细胞)比例及其前体细胞均明显降低。Tfh细胞是T细胞亚群,在生发中心形成和B细胞分化中起重要作用[33]。有文献报道SLE患者BMMSCs输注治疗后外周血T淋巴细胞减少[34],本研究发现经BMMSCs输注后狼疮鼠骨髓中成熟T细胞低于对照组,考虑骨髓的成熟减少亦可能为外周血减少的原因之一。

本实验亦发现,BMMSCs输注后狼疮鼠骨髓中$CD11b^+$的髓系细胞比例增加,考虑原因如下:①Niu等[35]研究发现狼疮小鼠活动期HSCs向髓系分化增加,认为狼疮的炎性环境有促进作用,结合本实验输注BMMSCs后狼疮鼠骨髓中髓系增加,提示BMMSCs可增强HSCs短期扩增的能力,分泌多种造血因子,在骨髓内促进HSCs向髓系的分化;②已有研究发现,BMMSCs经静脉输注可显著增加$CD206^+$巨噬细胞(M2)在狼疮小鼠腹膜及肾组织中的表达,且巨噬细胞的吞噬活性有明显提高。进一步研究表明BMMSCs分泌的IL-6在其中起介导作用[36-37],狼疮鼠髓外巨噬细胞数量增加,可能与骨髓内$CD11b^+$的髓系细胞比例增加并进入到外周循环有关,但在实验中未发现脾脏中$CD11b^+$的髓系细胞比例增加。

本课题组前期研究发现狼疮鼠骨髓中B淋系及pre-B减少,考虑HSCs向B淋系祖细胞分化减少(结果待发表),Niu等[38]研究发现狼疮鼠的炎症环境可抑制淋巴细胞生成,实验发现BMMSCs输注后狼疮鼠CFU-pre-B增高,结合其他学者发现,原因可能与BMMSCs分泌的SDF-1[39]相关,SDF-1在pre-B细胞增殖分化发挥着重要作用,也被称为pre-B细胞生长刺激因子(pre-B-cell growth stimulating factor,PBSF),对祖B和pre-B细胞有很强的趋化作用,但对成熟的B淋巴细胞没有趋化作用[40],导致骨髓中pre-B细胞增高,而骨髓及脾脏中成熟B淋巴细胞在移植前后没有统计学差异。本课题组将对狼疮鼠骨髓SDF-1进行进一步研究。

实验发现二次输注BMMSCs的小鼠骨髓内$Sca\text{-}1^+$的干细胞比例低于一次输注,但没有统计学意义,脾脏内$Sca\text{-}1^+$的干细胞比例高于一次输注,BMMSCs二次输注组骨髓中pre-B细胞数量也高于一次输注组,二次BMMSCs输注组脾脏中成熟T细胞较一次输注组下降。临床研究显示,狼疮患者一次和两次输注BMMSCs在12个月的随访中临床反应率和安全性相似,考虑单次输注在临床的治疗足够有效。但是BMMSCs在体内的作用并不永久,16.7%的患者在随访9~12个月复发。提示在临床应用中,对难治性狼疮患者,可以重复使用BMMSCs输注治疗[32]。本研究提示,两次BMMSCs输注对血液系统的影响或许优于一次输注。

综上所述,本研究发现MRL/lpr狼疮小鼠存在血液系统损伤,主要表现为红细胞、血红蛋白及血小板的下降。对狼疮鼠进行同种异体BMMSCs输注,可改善狼疮鼠造血系统的损伤,增加外周血血小板的数量,减少骨髓中$Sca\text{-}1^+$的干细胞比例,亦减少骨髓及脾脏中$CD3e^+$T细胞比例,增加骨髓中$CD11b^+$的髓系细胞比例,改善了狼疮鼠pre-B细胞的数量,但对成熟的B淋巴细胞没有影响。本研究仅探讨了移植前后狼疮鼠的血液系统的改变,BMMSCs影响狼疮鼠的造血机制仍需进一步研究。

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