论著·“乳腺癌精准靶向与转化”重点号

槐耳多糖通过调控JAK1/STAT3/CCL2轴减少瘤内髓源性抑制细胞募集以抑制三阴性乳腺癌进展

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[**摘要**] **目的** 髓源性抑制细胞(myeloid-derived suppressor cells,MDSCs)常在三阴性乳腺癌(triple negative breast carcinoma,TNBC)免疫微环境中过度募集形成免疫抑制屏障,致其对现有的免疫治疗响应率低。本研究旨在验证槐耳多糖(polysaccharide of trametes robiniophila murr,PS-T)对三阴性乳腺癌免疫微环境的影响及其通过抑制JAK1/STAT3/CCL2轴抑制MDSCs募集的分子机制。**方法** 用36只6~8周、体质量18~20 g雌性Balb/c小鼠构建三阴性乳腺癌移植瘤模型,将小鼠随机分成(n=6):PS-T(以含3 mg PS-T2灌胃)组,对照(以等渗氯化钠溶液灌胃)组。自接种第5、7、9、11、13、15、17、19、21天测量肿瘤体积,并在第21天剖取小鼠肿瘤组织并拍照记录;将肿瘤组织切片及制成单细胞悬液,行免疫组化染色和流式细胞术检测组织中MDSCs及T细胞的浸润情况。GO及KEGG富集分析RNA测序数据,探索PS-T影响三阴性乳腺癌中MDSCs的相关信号通路。RT-qPCR、Western blot及免疫组化染色检测*JAK1*、*STAT3*及*CCL2*的表达情况,Transwell共培养反向验证PS-T抑制通过抑制CCL2的分泌从而抑制MDSCs的募集。构建JAK1敲低细胞系,Western blot及ELISA检测敲低JAK1后槐耳多糖对磷酸化STAT3及CCL2的影响。**结果** ①与对照组相比,PS-T显著抑制三阴性乳腺癌生长(P=0.003)并重塑免疫微环境。流式细胞术显示PS-T组的瘤内$CD8^+$ T细胞比例增加(P=0.002),免疫组化证实PS-T上调瘤内CD8阳性细胞的数量(P=0.003)。②单细胞RNA测序显示PS-T治疗后瘤体内的T细胞浸润从3.6%增加至9.33%,而MDSCs细胞数量则从15.64%降至0.17%,免疫组化显示CD11b阳性细胞数量减少(P=0.008)。流式细胞术显示PS-T显著减少$CD45^+CD11b^+Ly6G^+Ly6C^{Low}$的比例($P$=0.012)。③GO及KEGG富集发现,2组小鼠肿瘤组织的差异基因显著富集于细胞因子相关通路及JAK/STAT信号通路,RT-qPCR及Western blot实验发现PS-T处理后4T-1细胞内CCL2的mRNA及蛋白表达水平下降(P<0.001),免疫组化结果显示PS-T治疗后肿瘤组织中CCL2的免疫组化评分下降(P=0.001)。流式分选得到的MDSCs与4T-1细胞共培养,显示加入MK-0821拮抗MDSCs中的CCR2受体后,PS-T处理组下室募集的MDSCs数量增加(P<0.01)。④免疫组化及Western blot实验均提示PS-T处理后肿瘤组织及4T-1细胞内p-STAT3的表达水平下降,但STAT3的总蛋白水平无变化。⑤Western blot实验发现PS-T处理后4T-1细胞内STAT3的磷酸化酶JAK1的蛋白水平下降(P=0.003),而敲低4T-1中的JAK1后,Western blot及ELISA结果显示PS-T处理组4T-1细胞表达p-STAT3及CCL2的水平增加。**结论** 本研究证实PS-T通过抑制TNBC肿瘤细胞中的JAK1/STAT3/CCL2信号通路,进而抑制瘤内MDSCs的募集,重塑免疫抑制微环境,发挥抗肿瘤作用。

[**关键词**] 三阴性乳腺癌;槐耳多糖;髓源性抑制细胞;STAT3转录因子

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Polysaccharide of *Trametes robiniophila* Murr inhibits the recruitment of myeloid-derived suppressor cells by modulating the JAK1/STAT3/CCL2 axis to suppress triple-negative breast cancer progression

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[**Abstract**] **Objective** Myeloid-derived suppressor cells (MDSCs) are frequently over-recruited in the immune microenvironment of triple-negative breast cancer (TNBC), forming an immunosuppressive barrier that contributes to the poor response rates to existing immunotherapies. This study aims to investigate the effects of polysaccharide of *Trametes robiniophila* Murr (PS-T) on the immune microenvironment in TNBC and clarifying the underlying molecular mechanism by which PS-T inhibits MDSC recruitment through suppression of the JAK1/STAT3/CCL2 axis. **Methods** TNBC xenograft model was established in 36 female BALB/c mice (6 to 8 weeks, weighing 18 to 20 g). Mice were randomly divided into 2 groups (n=6) for intragastric administration: PS-T group (100 μL solution containing 3 mg of PS-T) and control group (100 μL isotonic sodium chloride solution). Tumor volume was measured on days 5, 7, 9, 11, 13, 15, 17, 19, and 21 post-inoculation. On day 21, tumor tissues were harvested and photographed, and then processed into single-cell suspensions and tissue sections for flow cytometry and immunohistochemical staining to evaluate the infiltration of MDSCs and T cells. RNA sequencing data were analyzed using GO and KEGG enrichment analyses to identify signaling pathways involved in the PS-T-mediated MDSC regulation in TNBC. RT-qPCR, Western Blot, and immunohistochemical staining were used to detect JAK1, STAT3, and CCL2 expression. Transwell co-culture assay was performed to verify that PS-T inhibits MDSCs recruitment by suppressing CCL2 secretion. JAK1-knockdown 4T-1 cell line was constructed, and Western blot and ELISA were used to assess the effects of JAK1 knockdown on PS-T-mediated inhibition of phosphorylated STAT3 and CCL2. **Results** ① Compared with the control group, PS-T significantly inhibited the growth of TNBC (P=0.003) and remodeled the immune microenvironment. Flow cytometry showed that the proportion of intratumoral $CD8^+$ T cells was increased in the PS-T group (P=0.002), which was confirmed by immunohistochemistry demonstrating elevated numbers of intratumoral $CD8^+$ T cells (P=0.003). ② Single-cell RNA sequencing of the immune cell composition in tumor tissues from TNBC-bearing mice revealed that PS-T treatment increased $CD8^+$ T cell infiltration from 3.6% to 9.33%, while markedly reducing the proportion of MDSCs from 15.64% to 0.17%. Immunohistochemical staining further showed a significant decrease in the number of $CD11b^+$ cells in the PS-T group (P=0.008). Flow cytometric analysis of MDSC subsets indicated that PS-T significantly reduced the proportion of PMN-MDSCs (gated as $CD45^+CD11b^+Ly6G^+Ly6C^{low}$ cells) (P=0.012). ③ GO and KEGG enrichment analyses of differentially expressed genes between the control and PS-T groups revealed significant enrichment in cytokine-related pathways and the JAK/STAT signaling pathway. RT-qPCR and Western blot assays demonstrated that PS-T significantly downregulated the mRNA and protein levels of CCL2 in 4T-1 cells (P<0.001), which was further confirmed by immunohistochemistry showing reduced CCL2 expression in vivo (P<0.001). Co-culture of flow cytometry-sorted MDSCs with 4T-1 cells in Transwell chambers in the presence of MK-0821, an antagonist of the CCR2 receptor, attenuated the PS-T-mediated inhibition of MDSC recruitment to the lower chamber (P<0.01). ④ Both immunohistochemistry and Western blot analyses demonstrated that PS-T inhibited the phosphorylation of STAT3 (p-STAT3) without altering the total STAT3 protein level. ⑤ Western blot analysis showed that PS-T reduced the protein level of JAK1, the

kinase responsible for STAT3 phosphorylation(P=0.003). Knockdown of JAK1 in 4T-1 cells abrogated the inhibitory effects of PS-T on p-STAT3 and CCL2 expression, as verified by both Western blot and ELISA assays. **Conclusion** PS-T suppresses intratumoral MDSC recruitment by inhibiting the JAK1/STAT3/CCL2 signaling pathway in TNBC tumor cells, thereby remodeling the immunosuppressive microenvironment and exerting anti-tumor effects. These findings provide novel theoretical support for the application of PS-T in combination immunotherapy for TNBC.

[**Key words**] triple-negative breast cancer; polysaccharide of *Trametes robiniophila*; myeloid-derived suppressor cells; STAT3 transcription factor

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依据国家癌症中心2022年国内肿瘤监测数据:乳腺癌已经成为我国女性发病率最高、严重威胁女性健康的恶性肿瘤[1]。三阴性乳腺癌(triple-negative breast cancer,TNBC)是一种特殊类型的乳腺癌,这部分患者由于缺少雌激素受体、孕激素受体及人表皮生长因子受体2(human epidermal growth factor receptor 2,HER2)的表达,因此无法从现有的内分泌治疗及靶向HER2治疗中获益。尽管化疗是目前治疗TNBC重要手段,但并非所有患者均能从化疗中获益,部分患者在化疗结束后短期内即发生复发与转移,整体预后不佳。TNBC已被证明与其他亚型相比,具有更多的肿瘤浸润性淋巴细胞[2-3],以及与T细胞和B细胞浸润程度呈正相关的总生存率[4],使得免疫治疗成为治疗TNBC最有前景的选择[5]。尽管免疫检查点阻断治疗能够改善TNBC患者总生存率,且以帕博利珠单抗为代表的免疫抑制剂已获批应用于TNBC的临床治疗[5],但免疫治疗还存在着响应率不高导致治疗失败的缺陷[6]。因此,寻找新的治疗手段以增敏免疫治疗是当前TNBC临床治疗急需解决的焦点问题。传统中医药及其提取物在预防肿瘤发生、抑制瘤灶形成、防止复发转移等方面有着独特的优势。槐耳作为一种传统的药用真菌,在中国已有悠久的临床治疗史[7-8]。其成品制剂槐耳颗粒已获得国家药品监督管理局批准上市,目前广泛应用于多种恶性肿瘤的辅助治疗[9]。该药物的主要活性成分为槐耳多糖(polysaccharides of trametes robiniophila murr, PS-T)。随着研究的不断进展,PS-T在乳腺癌治疗中展现出重要的临床价值与潜在应用前景。

骨髓来源的抑制性细胞(myeloid-derived suppressor cells,MDSCs)是一群来自粒细胞或单核细胞的髓系细胞,在多种肿瘤中具有免疫抑制作用[10]。在乳腺癌中,外周血循环MDSCs数量与更高的临床分期、转移进展及微环境免疫抑制状态密切相关[11]。MDSCs可以通过多种机制介导肿瘤免疫抑制,包括产生ROS和通过表达精氨酸酶(arginase, Arg)和吲哚胺2,3-双加氧酶(indoleamine 2,3-dioxygenase)耗尽T细胞增殖所需的关键氨基酸[12-14];MDSCs还能产生一系列免疫抑制性和促进癌症的细胞因子,包括IL-10和TGF-β[15]。除了它们的免疫抑制功能外,MDSCs还可能与乳腺癌细胞、周围间质相互调控,积极塑造肿瘤微环境,导致血管生成增加、肿瘤侵袭和转移复发,进而限制TNBC的免疫治疗效果[6, 16-17]。在小鼠中,MDSCs为共表达CD11b与Gr1的髓系细胞,并可根据Ly6G和Ly6C的表达水平进一步划分为两个亚群:$CD11b^{+}Ly6G^{+}Ly6C^{Low}$定义为粒细胞样MDSCs(polymorphonuclear myeloid-derived suppressor cells, PMN-MDSCs),$CD11b^{+}Ly6G^{-}Ly6C^{high}$定义为单细胞样MDSCs(monocytic myeloid-derived suppressor cells, M-MDSCs)。在多数实体瘤中,PMN-MDSCs约占MDSCs总数的80%,通过产生ROS及Arg1等介质直接抑制T细胞的活化与增殖,现有研究已经证明PMN-MDSC通过抑制适应性免疫、调控肿瘤血管生成等途径在肿瘤的发生发展中发挥了重要的作用[18-19]。M-MDSCs则在缺氧及IL-6、M-CSF等细胞因子等信号的驱动下分化为肿瘤相关巨噬细胞,从而进一步加剧免疫抑制[20]。本研究拟通过体内外研究证实PS-T通过抑制TNBC的JAK1/STAT3/CCL2信号轴,减少MDSCs的募集及浸润数量,逆转免疫抑制微环境,为PS-T在TNBC的免疫辅助治疗探索新的研究视角。

1 材料与方法

1.1 材料

1.1.1 细胞及动物来源 本研究所用的小鼠三阴性乳腺癌4T-1细胞系购于上海富衡生物公司。36只7~8周正常健康雌性Balb/c小鼠,平均体质量约为19 g,购自重庆恩斯维尔生物公司。小鼠饲养于SPF级动物

房,环境温度22~25 ℃、湿度50%~60%,实行12 h光暗交替,饲养用品均灭菌处理,小鼠自由摄食饮水。实验操作轻柔规范,全程规避动物疼痛损伤。实验期间每日早晚监测小鼠状态,设定标准人道终点,实验全程无不良事件及动物异常情况发生。本实验经第三军医大学动物福利伦理审查委员会审查通过(AMUWEC20224228),严格遵循动物福利3R原则。

1.1.2 试剂与仪器 本研究所用的槐耳清膏由江苏启东盖天力药业公司提供,并按课题组前期使用的方法提纯分离制成槐耳多糖。细胞培养基、胎牛血清及转染试剂Lipofectamine3000均购自美国赛默飞公司。流式细胞术实验抗体CD45抗体、CD3抗体、CD8抗体、CD11b抗体、Gr1抗体、Ly6C抗体及Ly6G抗体购自美国BioLegend公司,CD4抗体购自中国四正柏生物公司。CCL2抗体、GM-CSF抗体、CXCL12抗体、STAT3抗体及JAK1抗体购自中国三鹰科技有限公司,CCL5抗体及JAK2抗体购自美国Cell Signaling Technology公司,p-STAT3及GAPDH抗体购自中国亲科生物研究中心有限公司。小鼠趋化因子CCL2 ELISA定量试剂盒购自中国亚科因生物公司。可穿透性细胞培养小室购自美国康宁公司。RNA提取试剂盒、反转录体系及SYBR Green qPCR Master Mix购自中国艾博泰克公司。BCA蛋白定量试剂盒、多聚甲醛及质粒抽提试剂盒均购自中国碧云天公司。

1.2 方法

1.2.1 动物模型建立与分组 4T-1细胞分为对照组(PBS)、PS-T组(5 μg/mL PS-T处理24 h)。将处于对数生长期的4T-1细胞消化后用细胞培养基将单细胞悬液浓度调整为1×10^7个/mL。随后,将100 μL单细胞悬液注射于小鼠右侧第二对乳腺脂肪垫内以构建移植瘤模型。动物实验将小鼠随机分成2组(n=6),自接种后第2天进行药物干预,对照组用100 μL等渗氯化钠溶液进行隔天灌胃处理;PS-T组用100 μL含3 mg PS-T溶液进行隔天灌胃处理。自接种第5天开始每隔一天测量肿瘤体积。接种第21天后采用颈椎脱臼法处死小鼠,完整剖离其肿瘤组织拍照记录。

1.2.2 免疫组化染色检测MDSCs及T细胞浸润 将剖取的小鼠肿瘤组织制成蜡块,并切成4 μm的切片,放入烘箱中,60 ℃烘片1 h。将切片进行脱蜡处理,放入二甲苯中浸泡10 min,通过梯度乙醇(无水乙醇5 min、90%乙醇5 min、80%乙醇5 min、70%乙醇5 min)进行水化。蒸馏水洗涤后,用苏木素染色液孵育30 s。蒸馏水冲洗后,滴入盐酸乙醇分化液分化30 s。蒸馏水冲洗后,加入伊红染色液染色20 s至1 min。染色完成后,再将石蜡切片经梯度乙醇(70%乙醇3 min、80%乙醇3 min、90%乙醇3 min、无水乙醇3 min)脱水,最后用二甲苯浸泡5 min。用中性树脂封片并在显微镜下观察并记录图像。组化评分标准如下:①阳性细胞百分比:0~25%记为1分,26%~50%记为2分,51%~75%记为3分,>75%记为4分;②阳性细胞的显色强度:无着色记为0分,浅黄色记为1分,棕黄色记为2分,棕褐色记为3分。两次计分的乘积为最终得分。

1.2.3 流式细胞术检测MDSCs与T细胞浸润 将小鼠肿瘤组织用眼科剪将组织剪成小碎块,放入组织消化液中室温消化1 h并用细胞筛网过滤,4 ℃ 1 300 r/min离心5 min。弃上清,用PBS缓冲液重悬洗涤细胞,加入红细胞裂解液在冰上裂解5 min。弃上清,加入适量流式缓冲液洗涤重悬细胞,向离心得到的细胞沉淀加入配好的抗体稀释液,重悬混匀后在冰上避光孵育30 min。4 ℃ 1 300 r/min离心5 min,加入流式缓冲液稀释重悬细胞沉淀。上机分析,并按照以下步骤进行划分亚群:①将$CD45^+CD11b^+Gr1^+$细胞亚群定义为MDSCs细胞;②将$CD45^+CD11b^+Ly6G^-Ly6C^{high}$细胞亚群定义为M-MDSC细胞;将$CD45^+CD11b^+Ly6G^+Ly6C^{Low}$定义为PMN-MDSC细胞;③将$CD45^+CD3^+CD4^+CD8^-$细胞亚群定义为$CD4^+$T细胞;④将$CD45^+CD3^+CD4^-CD8^+$细胞亚群定义为$CD8^+$T细胞。

1.2.4 Transwell细胞共培养实验检测MDSCs募集 将细胞接种到24孔板中培养6 h,取100 μL流式分选得到的MDSCs细胞悬液加入经过贴壁处理的Transwell小室内与4T-1细胞共培养,PS-T处理组在下室培养基中加入10 μg/mL PS-T。24 h后取出小室并用PBS缓冲液洗涤死细胞,加入4%多聚甲醛在室温下固定细胞20 min,用PBS缓冲液洗涤后加入结晶紫染液在室温下避光染色30 min。用PBS缓冲液洗涤并在镜下观察计数。

1.2.5 Western blot检测蛋白表达 用PBS洗涤细胞后在冰上用预冷好的细胞刮刀刮取贴壁细胞并在冰上裂解20 min。12 000 r/min离心20 min后测定上清蛋白的样品浓度,加入1/4体积的5×LoadingBuffer缓冲液,充分混匀,放入95~100 ℃金属浴10 min。制备SDS-PAGE凝胶,加入Marker和蛋白样品,90 V恒压电泳30 min,120 V恒压电泳1 h。转膜后在冰上300 mA恒流电转2 h。取出PVDF膜在室温下加入10%封闭液摇床封闭30 min。用TBST缓冲液晃洗PVDF膜后在4 ℃条件下摇床孵育一抗。吸取一抗缓冲液用TBST缓冲液洗涤PVDF膜并室温孵育二抗1 h。用

TBST洗涤并配置显影液,上机显影并用Image J软件测量条带灰度值。

1.2.6 ELISA实验 收集4T-1的细胞上清样品离心去掉沉淀,将上清及不同浓度的标准品加入孔板中室温孵育2 h。配置洗涤液并洗板5次,倒置将水分晾干后每孔加入100 μL辣根过氧化物酶标记Streptavidin,贴上封板膜,在室温下避光孵育20 min。洗板后倒置将水分晾干。加入100 μL显色剂TMB溶液室温避光孵育30 min。每孔加入50 μL终止液,上机测量。

1.2.7 反转录实时荧光定量PCR(RT-qPCR) 利用RNA快速提取试剂盒提取细胞RNA并检测RNA浓度,按每10 μL混合液中含有1 μg RNA计算配置混合液所需要的RNA及无酶水的体积。按照RNA反转录试剂盒的配置说明在冰上配置反应混合液,上机完成反转录得到cDNA。按照PCR试剂盒的说明在冰上配置反应液,添加引物,离心减少挂壁。在实时荧光定量PCR仪上完成扩增反应并分析数据。所用引物序列见下表1。

表1 RT-qPCR实验使用引物序列

Table 1 Primer sequences for RT-qPCR

Gene	Primer sequence (5′→3′)
CCL2	Forward: CAGGTCCCTGTCATGCTTCT
	Reverse: GTGGGGCGTTAACTGCATCT
CCL5	Forward: TGCTGCTTTGCCTACCTCTC
	Reverse: CGAGTGACAAACACGACTGC
CXCL12	Forward: TGACGGTAAACCAGTCAGCC
	Reverse: CGTGCAACAATCTGAAGGGC
GM-CSF	Forward: TTGGAAGCATGTAGAGGCCA
	Reverse: TTCATTCAACGTGACAGGCA
STAT3	Forward: GCCAAATGCTTGGGCATCAA
	Reverse: AGGTTCCAATTGGCGGCTTA
GAPDH	Forward: GGTTGTCTCCTGCGACTTCA
	Reverse: TGGTCCAGGGTTTCTTACTCC

1.2.8 质粒转染 利用Lipofectamine 3000转染试剂盒进行质粒转染,siJAK1敲低质粒购于丰晖生物公司。在转染前一天铺板,待细胞汇合度达到80%加入转染试剂及稀释质粒,放入培养箱转染6 h。更换完全培养基培养48 h后,提取蛋白。

1.2.9 单细胞RNA测序 将小鼠种瘤组织用眼科剪将组织剪成小碎块,消化并制备成单细胞悬液。使用自动细胞计数器将单细胞悬液的浓度调整为1×10^3~2×10^3个/μL。生成条形码互补DNA片段,使用10× Genomics平台构建测序文库。得到的文库在Illumina平台上进行高通量测序,配对端配置为150 bp。使用Cell Ranger(10× Genomics)生成原始基因表达矩阵并注释,分析小鼠肿瘤组织中的各免疫细胞亚群数量。利用R语言工具包进行KEGG及GO分析。

1.3 统计学分析

所有生物实验均独立完成至少3次。统计数据均使用SPSS 26.0和GraphPad Prism 10.0软件进行分析和作图。数据以$\bar{x}\pm s$表示,采用双尾独立样本t检验进行组间比较,$P<0.05$为差异有统计学意义。

2 结果

2.1 PS-T抑制三阴性乳腺癌生长并重塑免疫微环境

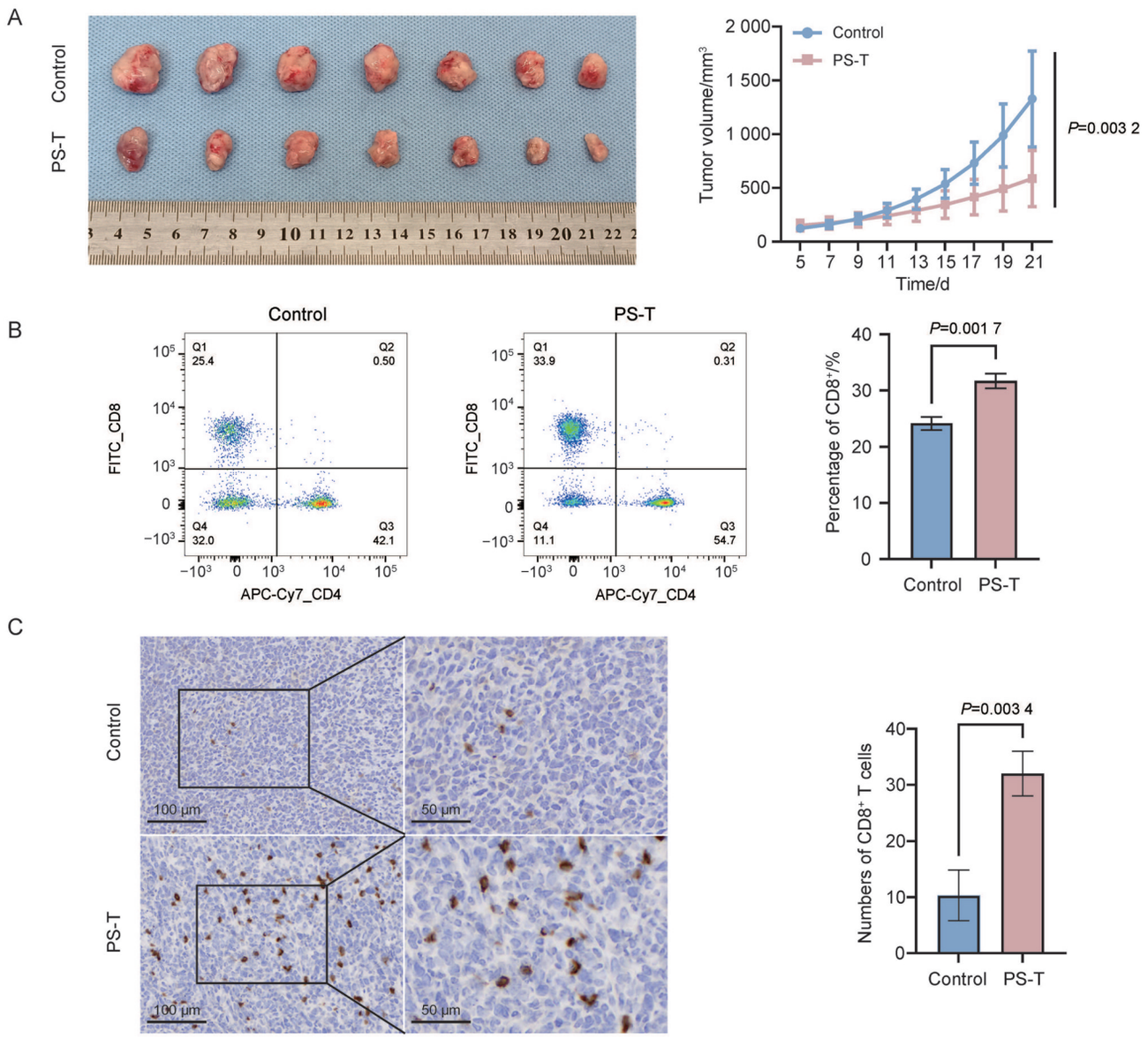
与对照组相比,PS-T治疗显著抑制了三阴性乳腺肿瘤的生长($P=0.003\,2$,图1A),表现出较强的抗肿瘤作用。流式细胞术结果显示,PS-T治疗组的小鼠瘤体内浸润的$CD8^+$ T细胞的数量明显增多($P=0.001\,7$,图1B);免疫组化染色结果同样显示$CD8^+$ T细胞浸润增加($P=0.003\,4$,图1C)。

2.2 PS-T抑制三阴性乳腺癌中PMN-MDSCs的浸润与募集

经对数标准化处理的单细胞表达矩阵的聚类结果显示(图2A~C),PS-T治疗组瘤体内的T细胞浸润从3.6%增加至9.33%,而MDSCs细胞数量则显著减少,从15.64%降至0.17%。通过免疫组化对小鼠肿瘤组织中的CD11b进行染色,结果显示,PS-T治疗组中CD11b阳性染色细胞数量显著减少($P=0.007\,9$,图2D、E)。Transwell实验结果显示,PS-T处理后4T-1细胞向下室募集MDSCs的效果减弱($P<0.000\,1$,图2F、G)。流式细胞术结果显示,PS-T治疗后,小鼠肿瘤组织内的MDSCs细胞亚群在免疫细胞中的比例显著减少($P=0.002\,2$,图2H、I),通过Ly6G及Ly6C的表达情况对小鼠肿瘤组织内的CD11b细胞进一步分群,结果显示,PS-T显著减少了PMN-MDSCs的比例,而M-MDSCs未表现出差异($P=0.012\,8$,图2J、K)。

2.3 PS-T通过下调肿瘤细胞CCL2的表达抑制MDSC的募集

肿瘤中MDSCs的募集和活化受多种趋化因子调控,GO富集分析结果发现对照组及PS-T处理组肿瘤组织间的差异基因显著富集于GO:0001819(positive regulation of cytokine production,细胞因子产生的正向调控)、GO:0005126(cytokine receptor binding,细胞因子受体结合)、GO:0002526(acute inflammatory response,



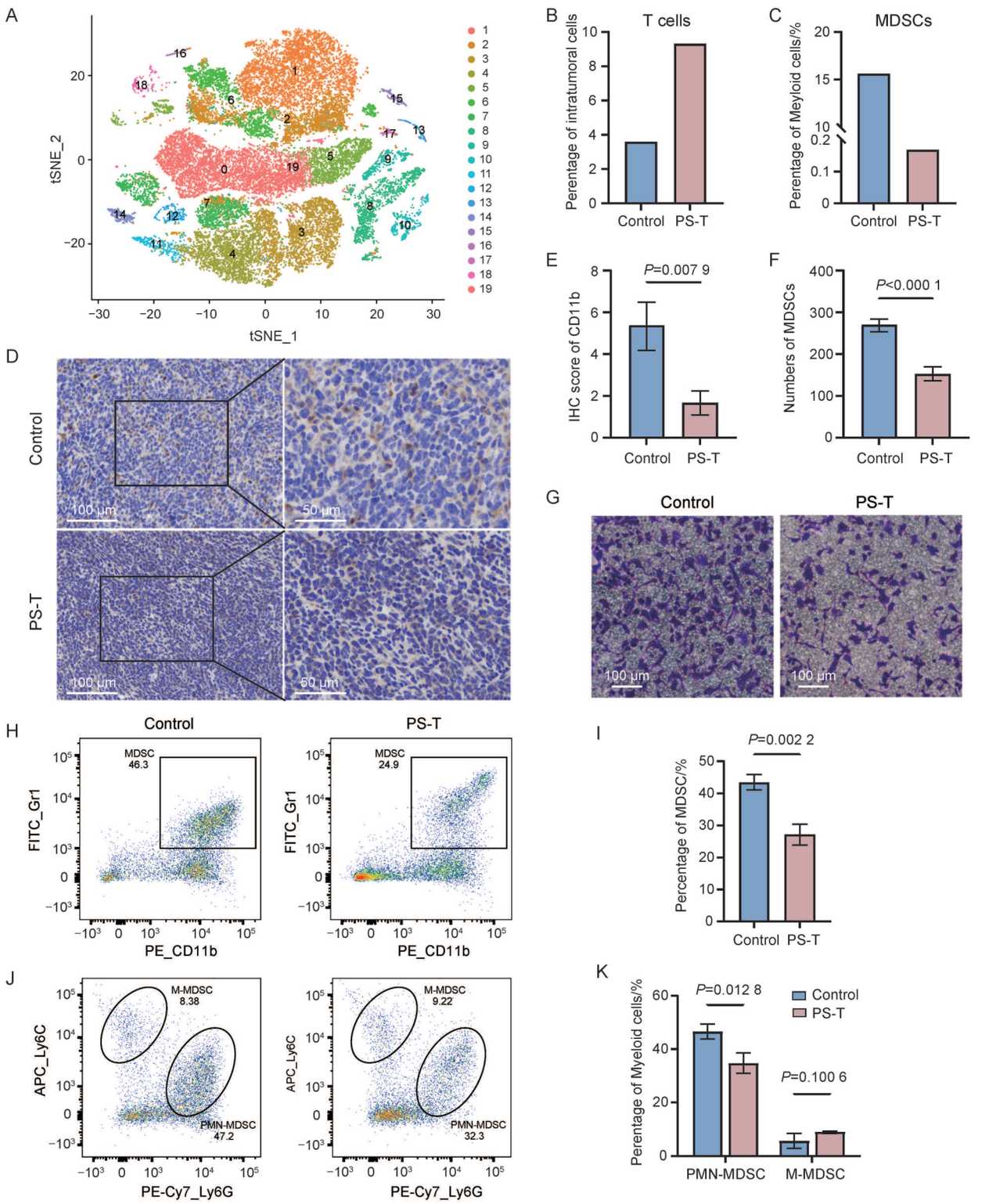
A: Representative gross images of resected tumors (on day 21 after tumor inoculation) and tumor volume growth curves (days 5 to 21 post-inoculation); B: Flow cytometric diagram for the proportion of T cells in tumor; C: Immunohistochemical staining of the infiltration of $CD8^+$ T cells. TNBC: triple-negative breast cancer

图1 PS-T抑制三阴性乳腺癌的发展并重塑免疫微环境 (n=6, $\bar{x}\pm s$)

Figure 1 PS-T inhibits TNBC progression and remodels the tumor immune microenvironment (n=6, $\bar{x}\pm s$)

急性炎症反应)、GO:0005125(cytokine activity,细胞因子活性)、GO:0016324(apical plasma membrane,细胞质膜)、GO:0008009(chemokine activity,趋化因子活性)等生物学过程(图3A)。KEGG通路富集分析进一步表明,上述差异基因显著富集于Cytokine-cytokine receptor interaction信号通路(图3B),因此PS-T可能是通过影响三阴性乳腺癌细胞的细胞因子相关通路抑制MDSCs的募集。

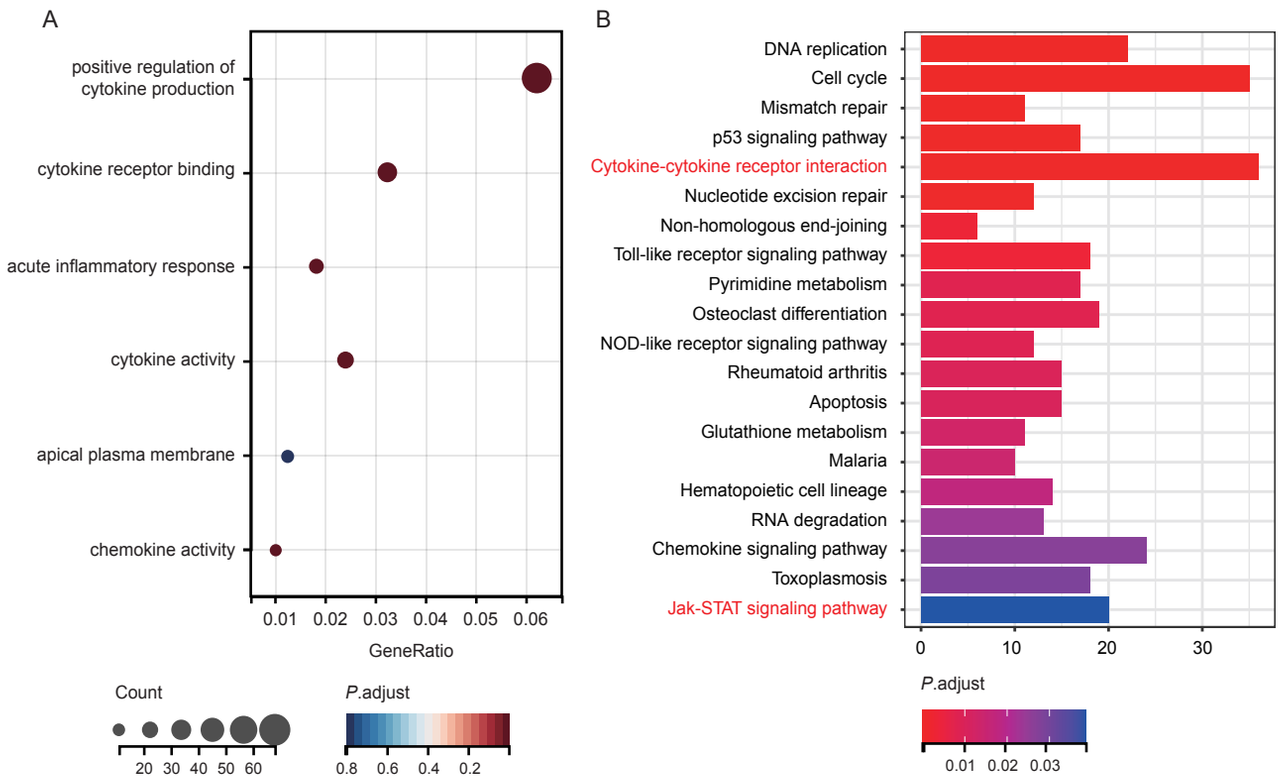
我们检测了几种MDSCs常见趋化因子的细胞内mRNA水平(图4A)及蛋白表达水平(图4B),结果显示PS-T显著下调了4T-1细胞内的CCL2水平。体内的免疫组化结果同样证实了这一点(图4C)。其中PS-T下调了CXCL12的mRNA水平,但其蛋白水平未表现出显著差异,这一结果提示PS-T虽然改变了瘤内CXCL12转录水平,但其分泌可能受到其他瘤内细胞的补偿调节。Transwell迁移实验结果显示,用CCR2拮抗剂MK-0812处理后,PS-T抑制MDSCs的效果减弱(图4D)。因此,PS-T通过抑制三阴性乳腺癌细胞分泌CCL2来抑制MDSCs的募集。



A: t-SNE map of 118 123 cells color-coded for the cell types; B: Frequency of T cells of intratumoral cells; C: Frequency of MDSCs of myeloid cells; D and E: Immunohistochemical staining of CD11b in tumor; F and G: The number of MDSCs recruited to the lower chamber; H and I: Flow cytometry of the proportion of MDSCs among the infiltrating immune cells; J and K: Flow cytometry showing the proportion of PMN-MDSCs and M-MDSCs. MDSCs: myeloid-derived suppressor cells

图2 PS-T抑制三阴性乳腺癌中PMN-MDSCs的浸润与募集 (n=6, $\bar{x}\pm s$)

Figure 2 PS-T inhibits the infiltration and recruitment of PMN-MDSCs in TNBCs (n=6, $\bar{x}\pm s$)



A: GO enrichment analysis showing differentially expressed genes (DEGs); B: Top 20 most significantly enriched KEGG pathways based on the DEGs

图3 PS-T影响三阴性乳腺癌组织的细胞因子相关的信号通路

Figure 3 PS-T affects enrichment analysis of cytokine-related signaling pathways in TNBC

2.4 PS-T抑制三阴性乳腺癌中的STAT3磷酸化

KEGG富集分析显示(图3B),PS-T治疗组的差异基因在JAK/STAT信号通路显著富集(P=0.038)。体外实验结果显示,PS-T处理后4T-1细胞内STAT3表达水平未表现出明显差异,但p-STAT3的表达水平则明显下降(图5A、B)。利用免疫组化检测三阴性乳腺癌瘤体内的p-STAT3表达水平,结果显示PS-T治疗组的免疫组化评分显著低于对照组(图5C),因此我们推测PS-T通过抑制STAT3的磷酸化来抑制三阴性乳腺癌细胞表达分泌CCL2。

2.5 PS-T通过抑制JAK1抑制STAT3磷酸化及CCL2

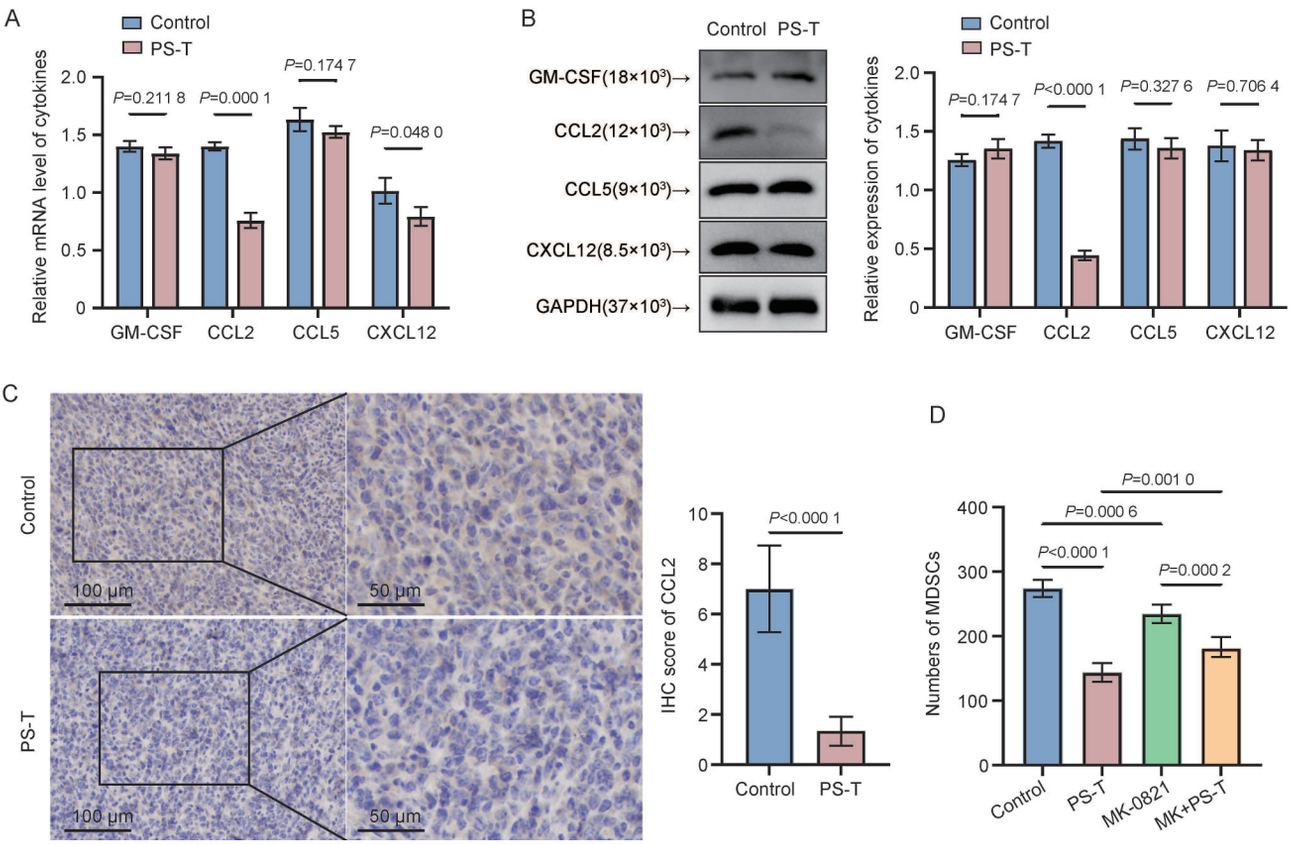
Western blot检测细胞内STAT3的2种磷酸化酶,结果显示,PS-T可以显著下调JAK1的蛋白水平(P=0.002 5,图6A)。成功构建JAK1敲低细胞株(图6B),并检测敲低JAK1后胞内p-STAT3及CCL2的蛋白水平,结果显示,JAK1敲低后,PS-T抑制STAT3磷酸化及CCL2表达水平能力消失(P=0.005 1,图6C)。使用ELISA试剂盒检测细胞上清中的CCL2水平,结果同样提示,敲低JAK1后,PS-T抑制CCL2表达分泌的效果下降(P=0.000 4,图6D)。

3 讨论

TNBC因其高度免疫抑制的"冷肿瘤"微环境而对免疫治疗反应不佳,其中MDSCs的过度浸润是导致T细胞功能耗竭的关键因素,且与患者的不良预后密切相关[21]。本研究系统阐明,传统中药槐耳提取物——PS-T,能够通过靶向TNBC细胞内的JAK1/STAT3信号通路,下调关键趋化因子CCL2的分泌,从而显著抑制MDSCs在肿瘤组织的募集,最终重塑免疫微环境、抑制肿瘤生长,为TNBC的治疗提供了新的中药辅助治疗视角。

3.1 PS-T抑制PMN-MDSCs并重塑TNBC免疫微环境

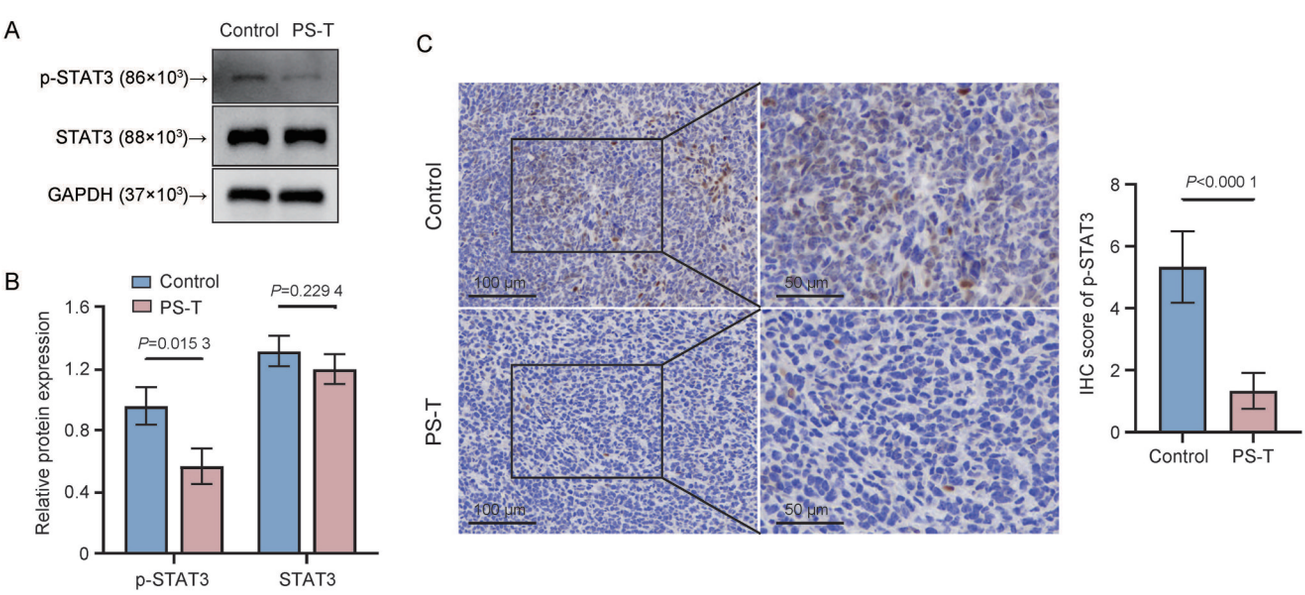
MDSCs是一群源于骨髓的异质性细胞,在肿瘤相关炎症因子和趋化因子的作用下异常分化扩增并向肿瘤微环境中募集,通过表达Arg-1、iNOS、ROS等分子抑制T细胞活化、促进Treg细胞增殖,在乳腺癌的发生发展中表现出显著的免疫抑制活性[22]。MDSCs在小鼠中表现为CD11b与Gr1共表达,并基于Ly6G和Ly6C的表达水平定义出两个亚群:PMN-MDSCs($CD11b^+Ly6G^+Ly6C^{low}$)和M-MDSCs($CD11b^+Ly6G^-Ly6C^{high}$),



A: Relative mRNA levels of chemokines in 4T-1 cells (n=3, $\bar{x}\pm s$); B: Western blot analysis of these chemokines (n=3, $\bar{x}\pm s$); C: Immunohistochemical staining showing CCL2 expression in tumor tissues between the 2 groups (n=6, $\bar{x}\pm s$); D: MDSCs co-cultured with 4T-1 cells and number of MDSCs recruited to the lower chamber (n=6, $\bar{x}\pm s$)

图4 PS-T抑制三阴性乳腺癌细胞分泌CCL2

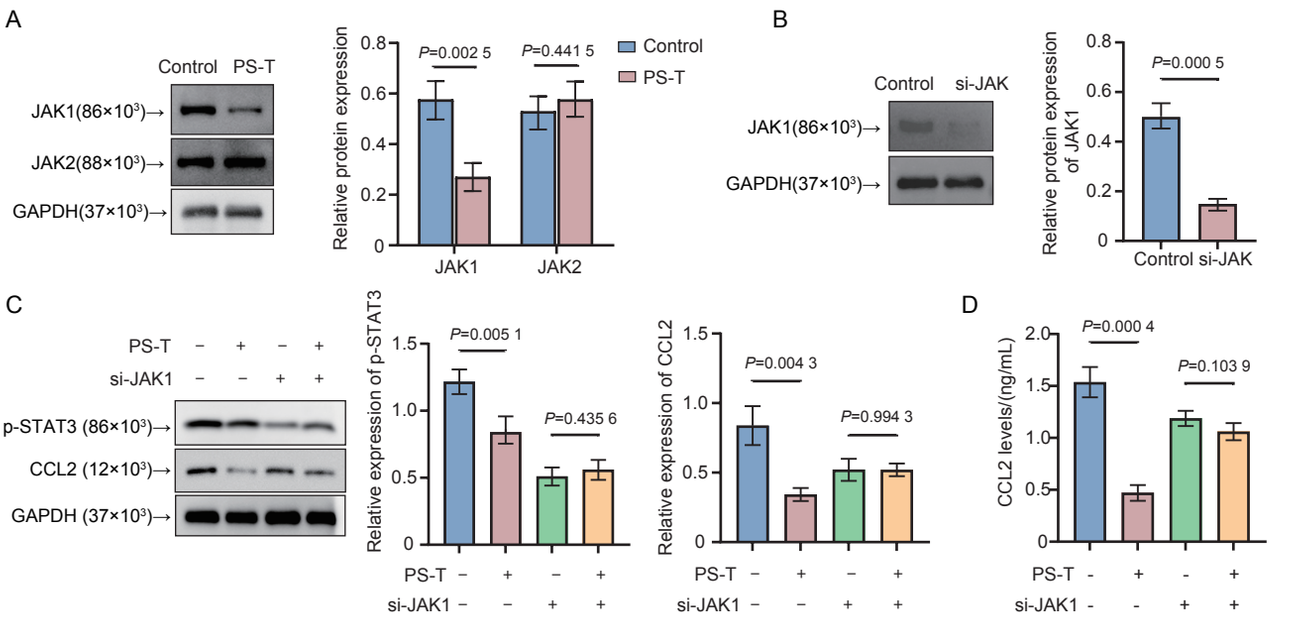
Figure 4 PS-T inhibits MDSC recruitment by suppressing CCL2 secretion from TNBC cells



A and B: Western blot analysis showing STAT3 and p-STAT3 protein levels in 4T-1 cells (n=3, $\bar{x}\pm s$); C: Immunohistochemical scoring of p-STAT3 expression in TNBC (n=6, $\bar{x}\pm s$)

图5 PS-T抑制三阴性乳腺癌中的STAT3磷酸化

Figure 5 PS-T inhibits STAT3 phosphorylation in TNBC



A: Western blot of JAK1 and JAK2 protein levels; B: Western blot of JAK1 after its knockdown; C: Western blot analysis of p-STAT3 and CCL2 after JAK1 knockdown; D: ELISA of CCL2 levels in cell culture supernatant

图6 PS-T通过抑制JAK1抑制STAT3磷酸化及CCL2 (n=3, $\bar{x}\pm s$)

Figure 6 PS-T suppresses STAT3 phosphorylation and CCL2 by inhibiting JAK1 (n=3, $\bar{x}\pm s$)

许多研究已经证明PMN-MDSC通过抑制适应性免疫、调控肿瘤血管生成等途径在肿瘤的发生发展中发挥了重要的作用[23-24]。目前对乳腺癌中MDSCs的研究尚不完善,尤其缺乏药物干预对PMN-MDSCs与M-MDSCs差异化调控的实验证据。本研究证实了PS-T可以抑制TNBC中MDSCs的募集,其中以PMN-MDSCs的减少为主。本研究还发现,PS-T作用后瘤内浸润的$CD8^+$ T细胞数量减少,正向调控了TNBC的免疫微环境。虽未能证实MDSCs的募集受限是否为TNBC组织内T细胞浸润增加的唯一原因,但依旧为PS-T作为一种有效的免疫辅助治疗药物在乳腺癌肿瘤免疫治疗中的应用提供了新的科学视角。

3.2 PS-T抑制TNBC表达分泌CCL2进而抑制MDSCs

CCL2作为CCR2受体的配体,是募集MDSCs的关键分子。现有的研究表明,靶向CXCL1/CXCL2的药物被证明可增强抗PD-L1疗法在TNBC中的疗效[25-26]。靶向细胞因子联合免疫治疗或可成为未来TNBC治疗的新思路,目前,Carlumab等靶向CCL2或其受体CCR2的单克隆抗体及小分子抑制剂已在多种实体瘤中进入临床探索阶段,其与免疫检查点抑制剂的联合应用展现出协同增效的潜力[27-29]。在本研究中,我们通过体内外实验证实了PS-T抑制TNBC表达和分泌CCL2,而CCR2拮抗剂证实了阻断CCL2-CCR2轴能削弱PS-T对MDSCs募集的抑制效果,为PS-T靶向CCL2与PD-1/PD-L1抑制剂的联合治疗方案提供了新的研究方向。

3.3 PS-T通过抑制STAT3磷酸化抑制TNBCCCL2的表达

为了揭示PS-T调控CCL2的上游分子机制,本研究对相关的信号通路进行了探查,明确了JAK1/STAT3/CCL2轴是PS-T抑制TNBC MDSCs募集的核心信号通路。JAK/STAT通路,尤其是STAT3,在乳腺癌中持续激活[30]。现有研究表明,抑制乳腺癌中的STAT3磷酸化后,*BCL-2*、*MCL-1*、*VEGF*、*MMP-2*和*MMP-9*等促癌基因的表达受到抑制[31],对肿瘤细胞启动促增殖、抗凋亡程序至关重要[32-33]。异常活化的STAT3还能通过影响*TWIST*基因促进乳腺癌的上皮-间充质转化[34];调控*Hif1a*及*VEGF*促进肿瘤血管生成[35]。本研究的实验数据显示,PS-T能特异性下调磷酸化STAT3水平,而非细胞中总STAT3的蛋白水平。与Li等[36]的研究结果一致,即磷酸化的STAT3是驱动肿瘤恶性表现的关键。本研究通过体内外实验证明,PS-T通过抑制JAK1来抑制STAT3磷酸化,进而导致了CCL2转录与分泌的减少。

此项研究也存在一定局限性:PS-T是一种多糖混合物,组分复杂,受分离纯化技术条件限制,本研究暂

未筛选出调控MDSCs的活性单体成分;其次,本研究仅初步证实PS-T可调控JAK1信号通路,尚未阐明PS-T直接或间接作用于JAK1的分子模式。综上所述,本研究揭示了PS-T抗TNBC的一种全新免疫调控机制:通过抑制肿瘤细胞JAK1/STAT3/CCL2信号轴,减少MDSCs募集,进而逆转免疫抑制微环境。这为传统中药PS-T作为免疫调节剂的联合治疗策略提供了新的依据。

利益冲突 本文所有作者声明不存在利益冲突

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