

阻断乳腺癌细胞PD-L1减弱对共培养树突状细胞成熟的抑制作用

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摘要:目的 探讨表达PD-L1的乳腺癌细胞是否通过激活树突状细胞的PD-L1/PD-1信号通路抑制树突状细胞成熟。方法 人单核细胞用GM-CSF和IL-4诱导为不成熟树突状细胞,再用TNF-α诱导为成熟树突状细胞;表达PD-L1的乳腺癌细胞系MDA-MB-231与树突状细胞接触共培养;PD-L1阻断抗体处理共培养的的乳腺癌细胞和树突状细胞;重组人PD-L1蛋白处理TNF-α诱导的树突状细胞;流式细胞仪检测乳腺癌细胞膜PD-L1的表达和树突状细胞的成熟分化标志HLA-DR和CD83。结果 乳腺癌细胞MDA-MB-231细胞系中,细胞膜表面PD-L1阳性细胞高达(99.7±0.15)%;HLA-DR和CD83阳性细胞在成熟树突状细胞对照组分别为(88.8±6.96)%和(18.36±3.07)%,在MDA-MB-231共培养实验组树突状细胞群分别降至(42.76±10.52)%和(9.93±2.74)%,两组比较差异有统计学意义($P<0.01, P<0.05$);HLA-DR和CD83阳性细胞在PD-L1抗体同型对照组分别为(45.17±10.19)%和(10.15±2.54)%,在PD-L1抗体处理组分别升至(63.46±1.72)%和(16.46±2.58)%,两组相比差异均有统计学意义($P<0.05$);和成熟树突状细胞对照组相比,人重组PD-L1蛋白处理组HLA-DR和CD83阳性细胞率较低,组间统计学差异有统计学意义($P<0.05$)。结论 PD-L1抗体对三阴性乳腺癌患者的治疗的效果,可能部分基于抗体阻断乳腺癌细胞表面的PD-L1,从而减弱其对肿瘤微环境中的树突状细胞成熟的抑制作用。

关键词:乳腺癌,PD-L1,PD-1,树突状细胞成熟,肿瘤治疗,肿瘤微环境

Blocking programmed death-ligand 1 attenuates maturation inhibition of dendritic cells by co-cultured breast cancer cells

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Abstract: Objective To study if programmed death-ligand 1 (PL-L1) expression in breast cancer cell activates PD-L1/PD-1 pathway in dendritic cells to inhibit dendritic cell maturation. **Methods** Human monocytes were induced to differentiate into immature dendritic cells using GM-CSF and IL-4, and further to mature dendritic cells using TNF-α. PD-L1-expressing breast cancer cell line MDA-MB-231 was co-cultured in contact with the dendritic cells to observe the effects of the breast cancer cells on the maturation of the dendritic cells. A PD-L1 blocking antibody was applied to the co-culture, and the changes in the inhibitory effect of the MDA-MB-231 cells on dendritic cell maturation was observed. TNF-α-induced dendritic cells were treated with a recombinant human PD-L1 protein to study the effect of PD-L1/PD-1 pathway activation on the maturation of dendritic cells. The expression of PD-L1 in MDA-MB-231 cells and the dendritic cell maturation marker HLA-DR and CD83 were analyzed using flow cytometry. **Results** MDA-MB-231 cell line showed PD-L1 positivity on the cell membrane cells at a rate as high as (99.7±0.15)%. In mature dendritic cells, the positivity rates for HLA-DR and CD83 were (88.8±6.96)% and (18.36±3.07)%, respectively, but in the co-culture system, the positivity rates of the dendritic cells were significantly decreased to (42.76±10.52) ($P<0.01$) and (9.93±2.74) ($P<0.05$), respectively, indicating that MDA-MB-231 cells inhibited the maturation of dendritic cells. Following treatment with a PD-L1 antibody isotype control, the percentages of HLA-DR- and CD83-positive cells in the co-culture were (45.17±10.19)% and (10.15±2.54)%, which were significantly increased to (63.46±1.72)% and (16.46±2.58)% after treatment with PD-L1 antibody, respectively (both $P<0.05$). Compared with the mature dendritic cell controls, the cells treated with the recombinant human PD-L1 protein exhibited significantly lowered percentages of HLA-DR-positive [from (84.23±4.18)% to (2.56±2.39)%, $P<0.05$] and CD83-positive cells [(87.26±1.54)% to (60.67±1.63)%, $P<0.05$]. **Conclusion** The effect of PD-L1 antibody therapy on triple negative breast cancer can be partially mediated by blocking PD-L1 expression on breast cancer cell membrane, which attenuates the inhibition of dendritic cell maturation in the cancer microenvironment.

Keywords: breast cancer, programmed death-ligand 1; programmed from cell death protein 1; dendritic cell maturation; cancer therapy; cancer microenvironment

PD-L1(B7-H1,CD274)是PD-1的配体,在多种细胞中均有表达,包括肿瘤细胞、单核细胞、巨噬细胞、T细

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胞和树突状细胞。PD-L1与PD-1结合并激活PD-L1/PD-1信号通路,在不同的细胞中导致不同的结果。激活T细胞的PD-L1/PD-1通路可抑制T细胞增殖、减少T细胞INF-γ和IL-12的分泌、导致T细胞衰竭和凋亡,从而导致T细胞的免疫抑制。癌细胞表达PD-L1抑制T细胞功能是癌症患者肿瘤细胞逃避免疫监视的主要机制,因此,用肿瘤细胞表面表达PD-L1作为选择接受

PD-1或PD-L1抗体治疗的癌症患者的标志^[1-6]。虽然有研究表明其他免疫细胞,如树突状细胞^[7-10]表面也表达PD-1,但和T细胞相比,PD-L1/PD-1通路激活对其它免疫细胞影响的研究却很少。

树突状细胞是最强的抗原提呈细胞,它由骨髓祖细胞或单核细胞^[11]分化而来。在肿瘤微环境中,树突状细胞的成熟是受到抑制的^[12]。肿瘤组织中未成熟树突状细胞数量多提示预后较差。有研究认为树突状细胞的成熟抑制是由肿瘤细胞分泌的细胞因子所引起的^[11-17]。未成熟树突状细胞通过多种机制引起肿瘤微环境中T细胞耐受^[11]。除了对T细胞的影响,有研究认为,肿瘤相关树突状细胞可以通过分泌HB-EGF、CXCL5或双调蛋白促进肿瘤细胞的侵袭^[18-20]。免疫组化结果显示,在肺癌组织中树突状细胞的不成熟与癌细胞PD-L1的表达相关^[21]。Lim等^[22]研究结果表明树突状细胞表面表达的PD-1可以抑制CD8⁺T细胞的功能和其抗肿瘤免疫的作用。然而,PD-L1/PD-1信号通路在树突状细胞分化成熟中起何作用未见报道。

基于以上研究背景,本研究拟探讨表达在乳腺癌细胞细胞膜的PD-L1是否通过激活树突状细胞PD-L1/PD-1信号通路抑制树突状细胞成熟。

1 材料和方法

1.1 材料

1.1.1 细胞及其培养相关试剂 MDA-MB-231、MCF-7和T47-D细胞系(ATCC);DMEM、RPMI 1640、L-15、胎牛血清、青霉素、链霉素和L-谷氨酰胺(Gibco);人单个核细胞,从健康志愿者新鲜血液分离;细胞示踪剂CMFDA(Abcam)。

1.1.2 人单核细胞分离纯化与树突状细胞诱导分化有关试剂 淋巴细胞分离液(灏洋华科);抗CD14⁺的磁珠(美天旎);细胞因子IL-4、GM-CSF和TNF- α (R&D)。

1.1.3 抗体与重组人PD-L1蛋白 PD-L1抗体及同型对照抗体(Biolegend);PD-L1-APC抗体及同型对照抗体(e-Bioscience);CD83-APC、HLA-DR-PE以及同型对照抗体(BD);人重组PD-L1蛋白(Sino Biological)。

1.2 方法

1.2.1 乳腺癌细胞系与细胞培养 MCF-7和T47-D细胞培养于DMEM,MDA-MB-231细胞培养于L-15,含10%胎牛血清、100 U/mL青霉素、100 μ g/mL链霉素以及2 mmol/L谷氨酰胺,细胞培养于37 °C含5% CO₂的培养箱中。

1.2.2 分离人外周血单核细胞及体外诱导树突状细胞 健康志愿者新鲜血液分离,用肝素抗凝,用淋巴细胞分离液通过密度梯度离心法分离人单个核细胞(PBMC),PBMC再用抗CD14⁺的磁珠分选出单核细胞,单核细胞培养于RPMI 1640中,含10%胎牛血清及2 mmol/L谷

氨酰胺,在37 °C含5% CO₂的培养箱中。单核细胞用IL-4与GM-CSF诱导为未成熟树突状细胞,再加入TNF- α 诱导为成熟的树突状细胞^[23]。单核细胞以1×10⁵/孔的密度种于12孔板,于第1天加入细胞因子IL-4(40 ng/mL)与GM-CSF(40 ng/mL);于第3天更换新鲜培养基以及细胞因子;在第4天得到未成熟树突状细胞,再加入TNF- α (40 ng/mL)继续培养2 d,于第6天得到成熟的树突状细胞。

1.2.3 细胞示踪剂CMFDA预染MDA-MB-231细胞并与未成熟树突状细胞共培养 当MDA-MB-231细胞生长密度达到80%后,吸出培养基,加入含0.5 μ mol细胞示踪剂CMFDA^[24-25]的预热培养基,预染乳腺癌细胞45 min,而后弃去染液,更换为新鲜培养基,MDA-MB-231细胞继续培养30 min。预染后的MDA-MB-231细胞用胰酶消化为单个细胞,按1:1的比例和未成熟树突状细胞共培养48 h,培养基中含有IL-4、GM-CSF和TNF- α ,用胰酶消化共培养的细胞,并用流式细胞术检测树突状细胞群表面标记。

1.2.4 PD-L1抗体与共培养MDA-MB-231和未成熟树突状细胞的孵育以及人重组PD-L1蛋白处理单独培养的树突状细胞 在共培养的预染MDA-MB-231细胞与未成熟树突状细胞中加入PD-L1抗体(10 μ g/mL)、同型对照抗体(10 μ g/mL)和TNF- α ,继续培养48 h。单独培养的单核细胞由IL-4及GM-CSF诱导4 d成为未成熟树突状细胞,再加入TNF- α 和人重组PD-L1蛋白(终浓度在2 μ g/mL)^[26],继续培养48 h。

1.2.5 流式细胞术检测细胞膜表面蛋白 用流式细胞术检测肿瘤细胞膜表面表达的PD-L1蛋白以及树突状细胞表面的CD83和HLA-DR。流式细胞仪检测的细胞用流式细胞仪缓冲液洗2遍,随后在4 °C与荧光抗体(PD-L1-APC、CD83-APC或HLA-DR-PE抗体)或同型对照抗体孵育30 min;1000 r/min 4 °C离心细胞5 min弃去多余抗体并加入200 μ L流式细胞仪缓冲液,用BD CytoFLEX流式细胞仪进行检测,检测结果用FlowJo进行分析。

1.2.6 统计分析 所有实验均重复3次,数据表达为均数±标准差。应用SPSS21.0软件进行统计分析,两组数据之间的差异比较用独立样本t检验,多组数据之间的差异比较应用ANOVA统计方法。 $P<0.05$ 视为差异有统计学意义。

2 结果

2.1 3个乳腺癌细胞系细胞表面PD-L1蛋白表达的百分率

在3种细胞系中,MDA-MB-231细胞表达PD-L1的阳性细胞高达99.7±0.15%,相比之下MCF-7与T47D分别为(2.85±1.77)%和(1.44±0.51%)(图1)。

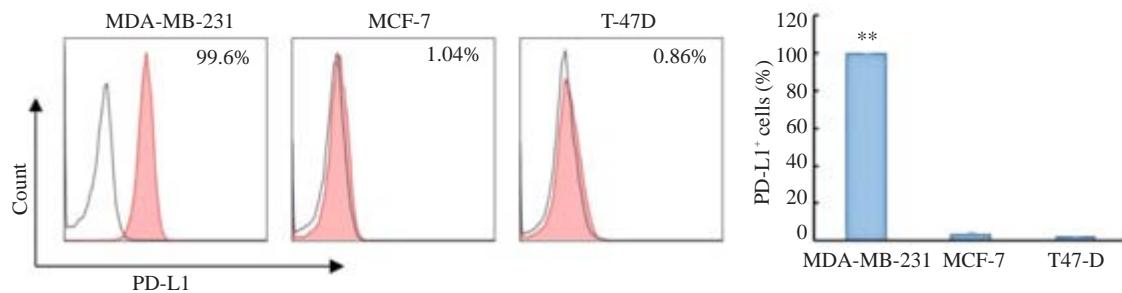


图1 3个乳腺癌细胞系细胞膜表面PD-L1蛋白表达的百分率

Fig.1 Percentages of PD-L1 expression on cell membrane of the 3 breast cancer cell lines (**P<0.01 vs MCF-7, T47-D).

2.2 MDA-MB-231 细胞对共培养树突状细胞成熟的影响及PD-L1抗体对这一影响的作用

人单核细胞在 IL-4 (40 ng/mL) 和 GM-CSF (40 ng/mL) 诱导 4d 后可分化为不成熟的树突状细胞, 其特征为低表达 CD83 与 HLA-DR; 加入 TNF- α (40 ng/mL) 继续诱导 2d, 树突状细胞分化成熟, 其特征为两种表面标记的阳性细胞百分比均有上升, HLA-DR 由 (7.18±1.73)% 上升至 (76.8±4.13)% , CD83 由 (41.77±3.61)% 上升至 (99.37±0.40)% (图 2A)。

流式细胞仪分析表明, 共培养的两种细胞可被区分为两个细胞群(数据未展示), 一群为预染 CMFDA 的 MDA-MB-231 细胞, 另一群为单核细胞诱导而来的树突状细胞。CD83 和 HLA-DR 的阳性细胞百分率只在树突状细胞群中进行分析。和成熟树突状细胞对照组相比, 共培养细胞中树突状细胞群 HLA-DR 和 CD83 阳性细胞的百分率下降, HLA-DR 由 (88.8±6.96)% 降至 (42.76±10.52)% , CD83 由 (18.36±3.07)% 降至 (9.93±2.74)% (图 2B)。

和同型对照组相比, PD-L1 抗体处理组树突状细胞亚群 HLA-DR 和 CD83 阳性细胞的百分比下降, HLA-DR 阳性细胞百分比由 (45.17±10.19)% 升至 (63.46±1.72)% , CD83 的阳性细胞百分比由 (10.15±2.54)% 升至 (16.46±2.58)% (图 2B), 统计结果显示 PD-L1 抗体组与同型对照组相比 HLA-DR 和 CD83 的阳性细胞百分比均有统计学意义 ($P<0.05$)。

2.3 人重组PD-L1蛋白对树突状细胞成熟分化的影响

流式细胞术检测结果(图3)显示, 和成熟树突状细胞对照组比, 人重组 PD-L1 蛋白处理可以降低树突状细胞 HLA-DR 和 CD83 阳性细胞的百分率, HLA-DR 由 (84.23±4.18)% 降至 (62.56±2.39)% , CD83 由 (87.26±1.54)% 降至 (60.67±1.63)% ($P<0.01$)。

3 讨论

虽然肿瘤微环境中树突状细胞成熟受到抑制的机制已有报道, 但对这一机制的认识并无定论。本研究表明, PD-L1 抗体可以减弱表达 PD-L1 的乳腺癌细胞

MDA-MB-231 对共培养树突状细胞成熟分化的抑制作用, 而人重组 PD-L1 蛋白可抑制单核细胞来源的未成熟树突状细胞向成熟分化, 这些结果提示, PD-1 信号通路的激活可抑制 TNF- α 诱导未成熟树突状细胞向成熟方向分化的过程。

PD-L1 在多种肿瘤细胞表面均有表达, 其中包括乳腺癌细胞^[2, 27-28], PD-L1 抗体也对多种实体瘤的治疗有效果^[3, 29]。临床实验结果表明表达 PD-L1 的三阴性乳腺癌对 PD-L1 抗体的治疗有效应^[2, 30-31], 疾病控制率达到 25%。有研究报道乳腺癌细胞系 MDA-MB-231 细胞高表达 PD-L1 蛋白^[32-33], 本实验的数据与之前的报道一致, 即 MDA-MB-231 细胞膜表面高表达 PD-L1 蛋白, 而乳腺癌细胞系 MCF-7 与 T47D 基本不表达。有报道指出, 肿瘤微环境中 T 细胞分泌的 INF- γ 上调乳腺癌细胞表达的 PD-L1^[34-35]。单独培养的 MDA-MB-231 细胞表面表达 PD-L1, 说明肿瘤细胞膜表面组成性表达 PD-L1 可不依赖于肿瘤微环境中 T 细胞分泌的 INF- γ , 反而, 肿瘤细胞通过组成性表达的 PD-L1 抑制 T 细胞、巨噬细胞和树突状细胞。

在本实验模型中, CD14⁺ 人单核细胞可以被 IL-4 和 GM-CSF 诱导为不成熟的树突状细胞, 再加入 TNF- α 则可诱导分化为成熟的树突状细胞; 成熟的树突状细胞 HLA-DR 和 CD83 的阳性细胞比例较高。在 MDA-MB-231 细胞与未成熟树突状细胞的接触共培养实验中, 发现即使加入了 TNF- α , MDA-MB-231 细胞仍可以抑制树突状细胞的成熟, 而加入 PD-L1 抗体后可以减弱这一抑制作用, 部分恢复树突状细胞向成熟分化, 可能的机制是, PD-L1 抗体改变了树突状细胞和/或肿瘤细胞分泌的细胞因子, 但另一种可能性是 PD-L1 抗体阻断了 MDA-MB-231 细胞的 PD-L1, 使其不能通过 PD-1 信号通路影响与其共培养的未成熟树突状细胞, 从而减弱了 MDA-MB-231 细胞对树突状细胞分化成熟的抑制作用。事实上, Mu 等^[21]的研究表明, 肺癌组织切片中, 未成熟树突状细胞与 PD-L1 的表达相关, 虽然他们没有提供更多的数据证明 PD-L1 可以向树突状细胞传递信号从而抑制树突状细胞的成熟分化。因有多篇论文报道

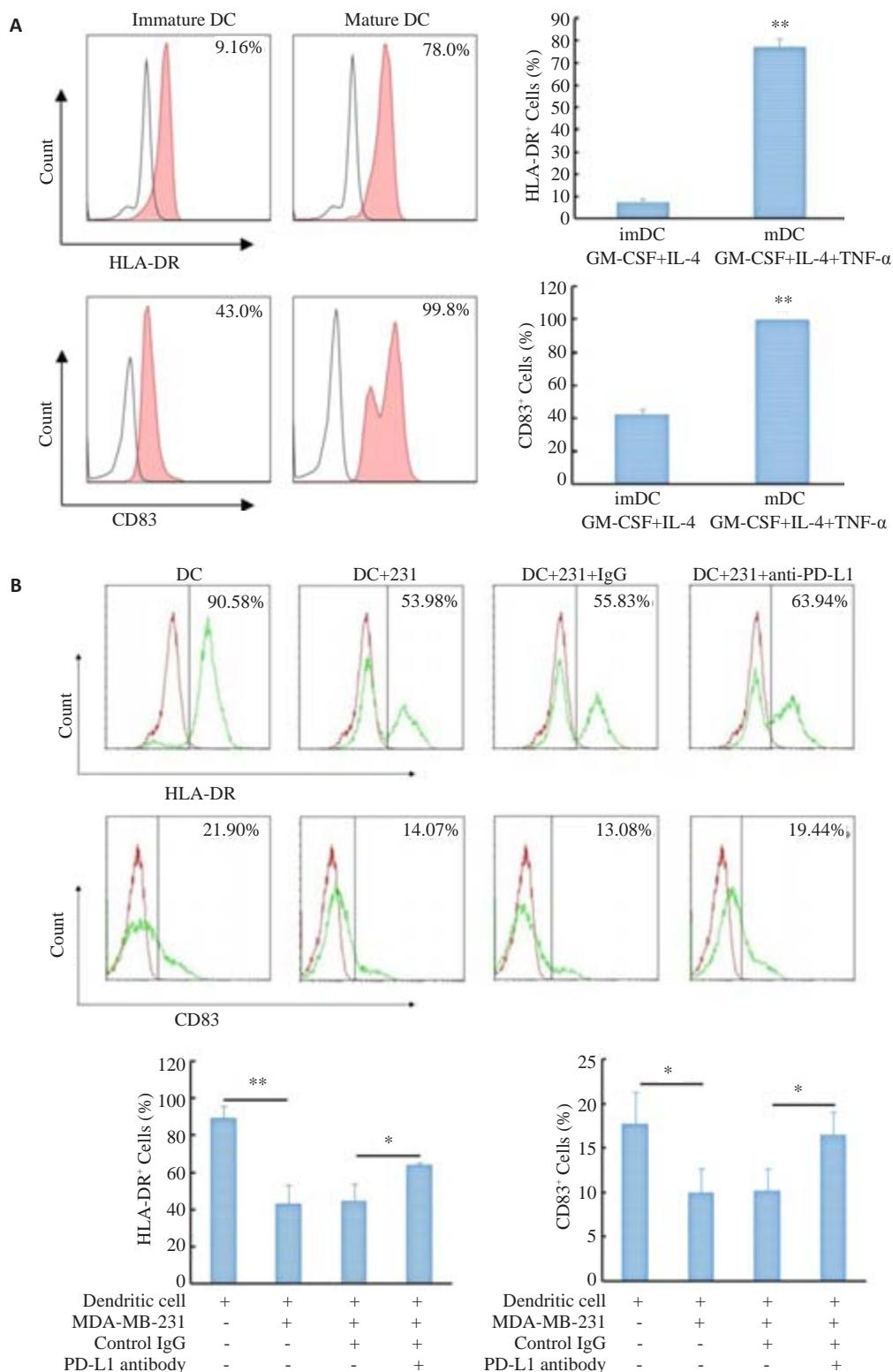


图2 MDA-MB-231细胞对共培养树突状细胞成熟分化的影响及PD-L1抗体对这一影响的作用
Fig.2 Effect of MDA-MB-231 cells on maturation of co-cultured dendritic cells and the impact of PD-L1 antibody on this effect. **A:** Percentages of HLA-DR- and CD83-positive cells in immature and mature dendritic cells (**P<0.01 vs imDC); **B:** Effects of MDA-MB-231 cells on percentages of HLA-DR- and CD83-positive cells in co-cultured dendritic sub-population cells and the impact of PD-L1 antibody on these effects (**P<0.01, *P<0.05).

树突状细胞膜表面表达PD-1^[7-9, 36], 所以有理由认为, PD-1信号途径的激活抑制TNF- α 诱导的未成熟的树突状细胞向成熟分化。

为了更进一步证明本研究的假设, 即PD-L1激活树突状细胞的PD-1信号通路从而抑制树突状细胞成熟, 我们将未成熟树突状细胞与人重组PD-L1共孵育,

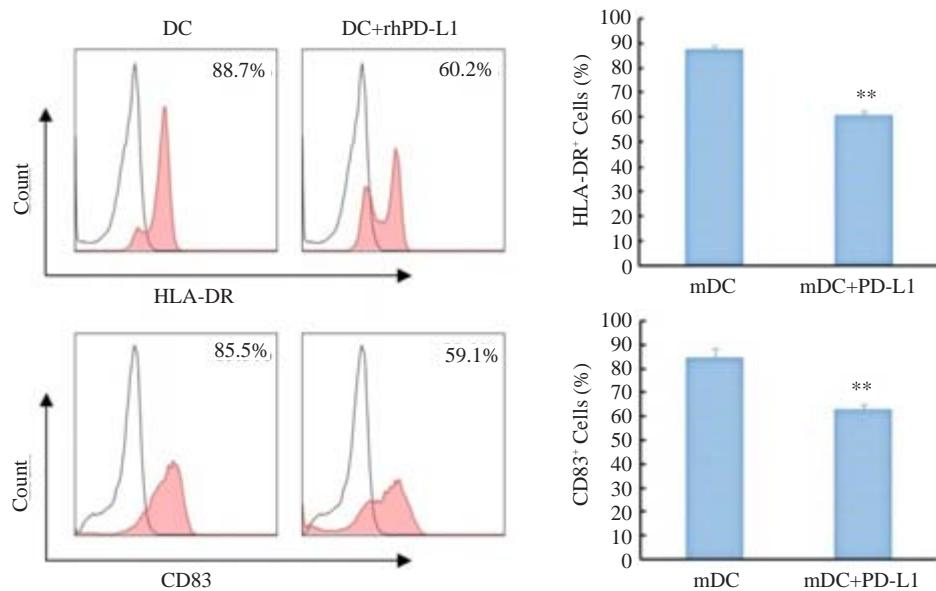


图3 人重组PD-L1蛋白对熟树突状细胞成熟分化的影响

Fig.3 Effect of recombinant human PD-L1 protein on maturation differentiation of dendritic cells (**P<0.01 vs mDC).

观察人重组PD-L1对未成熟树突状细胞的影响。我们的数据支持本研究的假设,即人重组PD-L1可以抑制TNF- α 诱导单核细胞来源的未成熟树突状细胞向成熟分化的过程。

基于本研究的数据,提出乳腺癌细胞表面表达的PD-L1可通过D-L1/PD-1信号通路抑制肿瘤微环境中的树突状细胞成熟,因此导致肿瘤微环境中的树突状细胞不能提呈抗原或使不成熟树突状细胞行使其他功能。PD-L1抗体对三阴性乳腺癌患者疗效的部分原因是抗体可以阻断乳腺癌细胞表达的PD-L1,从而削弱其对肿瘤微环境中树突状细胞成熟的抑制作用。

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