Phb2/REA过表达慢病毒载体对体外Th17分化条件下小鼠单核细胞IL-17a表达的影响

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摘 要：目的 探讨Phb2/REA过表达慢病毒转染小鼠初始T细胞后，在Th17细胞分化条件下对该细胞IL-17a表达的影响。**方法** 提取小鼠脾脏淋巴细胞，分离纯化小鼠T淋巴细胞，将其分为空白组、空载体组、慢病毒组、Th17分化组和Th17+慢病毒组。空白组采用1640培养液培养，空载体组在37 ℃、5%CO_2条件下用空载体慢病毒感染T淋巴细胞，14 h后换回常规培养基；慢病毒组在37 ℃、5%CO_2条件下用含雌激素活性抑制因子(REA)过表达载体的慢病毒感染T淋巴细胞，14 h后换回常规培养基；Th17分化组在空白组培养条件下加用anti-CD3ε、anti-CD28、anti-IFN-γ、anti-IL-4抗体及IL-6、TGF-β1、IL-23等细胞因子，诱导原始T细胞向Th17细胞分化。Th17+慢病毒组在空载体组条件下加用Th17细胞分化。72 h后，收集5组细胞，采用Realtime-PCR检测REA、维甲酸依赖性孤核受体γt(ROR-γt)、IL-17a 的mRNA的表达，验证Th17细胞分化的效果，探讨Phb2/REA过表达慢病毒载体对Th17细胞的分化及IL-17a表达的影响。**结果** Th17+慢病毒组的REA、RORγt mRNA表达较慢病毒组高，且慢病毒组、Th17+慢病毒组REA、RORγt的mRNA明显高于Th17分化组，差异均有统计学意义(P<0.05或0.01)；空载体组与空白组比较差异无统计学意义(P>0.05)。Th17分化组的IL-17A mRNA表达明显上调(P<0.01)，且高于Th17+慢病毒组(P<0.01)；空白组、空载体组、慢病毒组的IL-17A mRNA表达差异无统计学意义(P>0.05)。**结论** Phb2/REA过表达慢病毒转染小鼠T淋巴细胞后，可以抑制Th17细胞的分化，同时抑制炎症因子IL-17的表达。

关键词：Phb2/REA；慢病毒；IL-17；细胞分化；Th17细胞

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Effect of Phb2/REA overexpression of lentiviral vector on the expression of IL-17a in mouse monocytes under in vitro Th17 differentiation

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Abstract: Objective To explore the effect of Phb2/REA overexpression of lentiviral vector on the expression of IL-17a in mouse monocytes under in vitro Th17 differentiation after transfection of mouse initial T cells. Methods Mouse spleen lymphocytes were extracted, and mouse T lymphocytes were isolated and purified. They were divided into the Blank Group, Empty Vector Group, Lentivirus Group, Th17 Differentiation Group and Th17+ Lentivirus Group. The Blank Group was cultured with 1640 culture solution, the Empty Vector Group had the T lymphocytes infected with the empty vector lentivirus under the conditions of 37 ℃ and 5% CO_2, which was changed back to routine culture medium after 14h; the Lentivirus Group had T lymphocytes infected with the lentivirus containing REA overexpression vector under the conditions of 37℃ and 5% CO_2, which was changed back to routine culture medium after 14 h; the Th17 Differentiation Group was induced with the anti-CD3ε, anti-CD28, antiIFN-γ and anti-IL-4 antibody and cytokines including IL-6, TGF-β1 and IL-23 based on the culture conditions for the Blank Group, inducing the differentiation of original T Cells to Th17 cells; the Th17+ Lentivirus Group was

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additionally differentiated with Th17 cells based on the conditions for the Empty Vector Group. After 72 h, the five groups of cells were collected, and the mRNA expression of REA, ROR-γt and IL-17a were detected by Realtime-PCR to verify the effect of Th17 cell differentiation and explore the effect of Phb2/REA overexpression lentiviral vector on the differentiation of Th17 cells and the expression of IL-17a. **Results** The mRNA expressions of REA and ROR-γt in the Th17+ Lentivirus Group were higher than those in the Lentivirus Group, and the mRNA expressions of REA and ROR-γt in the and Lentivirus Group the Th17+ Lentivirus Group were significantly higher than those of the Th17 Differentiation Group, and the differences were statistically significant (P<0.05 or 0.01); there was no statistical difference between the Empty Vector Group and the Blank Group (P>0.05). The mRNA expression of IL-17a in the Th17 Differentiation Group was significantly upregulated (P<0.01) and higher than that in the Th17+ Lentivirus Group; and there was no statistical difference in mRNA expression among the Blank Group, Empty Vector Group and Lentivirus Group (P>0.05). **Conclusion** Phb2/REA overexpression of lentivirus can inhibit the differentiation of Th17 cells and inhibit the expression of inflammatory factor IL-17 after transfection of mouth T lymphocyte.

Key words: Phb2/REA; lentivirus; IL-17; cell differentiation; Th17 cells

国内外大量研究表明IL-17可参与系统性红斑狼疮(SLE)、银屑病、类风湿性关节炎、脑脊髓炎等多种自身免疫性疾病的发病环节[1-4]，也可以通过诱导炎症介质及趋化因子的产生而导致机体组织器官的损伤。我们的前期研究发现，雌二醇(E2)与ER结合后可以募集雌激素活性抑制因子(REA)，作用于维甲酸依赖性孤核受体γt(ROR-γt)启动子区域的雌激素反应元件(ERE)，抑制ROR-γt的表达，从而抑制Th17分化[5]。但E2有抑制炎症和促进炎症的双重作用，不但可以通过上调REA、抑制RORγt表达以遏制Th17细胞的分化，还可促进Th2细胞及浆细胞分化分泌自身免疫性抗体，参与SLE发病，因此不能直接采用E2治疗SLE。抑制素2(PHB2)是蛋白抑制素家族中的一员[6]，可选择性地抑制ER的转录活性，有效抑制Th17细胞分化及IL-17表达。由于REA-慢病毒过表达技术能够稳定表达REA，因此我们通过该项技术上调REA的表达、抑制Th17细胞的分化，进而减少IL-17的分泌，以期验证该方法治疗SLE的可行性。

1 材料和方法

1.1 Phb2过表达慢病毒载体

慢病毒载体的构建、合成、测试均由上海吉凯基因化学技术有限公司提供，本实验最终所制备的GV358-Phb2过表达慢病毒载体元件顺序：Ubi-MCS-3FLAG-SV40-EGFP-IRES-puromycin。阴性对照病毒CON248(对应目的病毒)元件顺序：Ubi-MCS-3FLAG-SV40-EGFP。

1.2 小鼠脾脏T淋巴细胞的分离和纯化

颈椎脱臼法处死C57/BL6J小鼠后置于75%酒精溶液中浸泡2 min，超净工作台(SW-CJ-2FD苏净安泰)中取出小鼠脾脏，置于盛有PBS(20012-043)的培养皿(60 mm)中；剔除脾脏以外多余的脂肪组织，并予PBS漂洗2次；剪碎脾脏，用磨砂玻片轻轻研磨至组织发白，再将研磨液转移至细胞筛(70 μm)中过滤，获得细胞悬浊液，混合均匀后用吸管将细胞悬液转移至盛有小鼠淋巴细胞分离液的离心管中(注意不要将分离液粘在管壁上)，使细胞悬浊液悬于淋巴细胞分离液上，再将悬液以500 r/min离心30 min，吸取从上数第二层悬液至新的离心管中，并予PBS重悬，吹打混匀，以320 r/min离心5 min，弃上清，加2 mL红细胞裂解液(PBS : 红细胞裂解液=4 : 1)，用吸管伸至离心管底部吹打混匀5 min后再加8 mL PBS终止裂解反应，再次吹打混匀，以320 r/min 离心5 min，弃上清。再次加入PBS液清洗1遍，弃上清，予PBS重悬。采用免疫磁珠法分离纯化T淋巴细胞[6]，最后加入1 mL(FBS+RPMI-1640)培养基重悬，细胞计数，鉴定细胞活力。

1.3 慢病毒转染淋巴细胞

实验设立5个组：空白组、空载体组、慢病毒组、Th17分化组和Th17+慢病毒组。每皿加入2 mL培养基并接种1×10^7个淋巴细胞，置37 ℃、5%CO_2恒温培养箱中培养30 min。按照预实验所得的细胞最适MOI(multiplicity of infection) 值为10，计算出所需要的病毒体积[病毒体积=(MOI×细胞数)/病毒滴度]；空载体组加入不含目的基因的病毒液；慢病毒组加入病毒液，置37 ℃、5% CO_2恒温培养箱中培养14 h后，将其分别转移至5支15 mL离心管中，以300 r/min 离心 5 min后弃上清，予4 mL常规培养液重悬，如表1分别加入刺激因子。提前2 h以anti-CD1=3ε(2 mg/L，Biolegend，Cat.100314) 分别覆盖6个直径6 cm的培养皿中，37 ℃ 孵化2 h，再予PBS漂洗

2次备用。将加入上述刺激因子的重悬液分别转移到备用的培养皿中，培养72 h后收集细胞，详细过程可参考文献[8]，于荧光显微镜下观察并测定转染效率，最后提取RNA行Realtime-PCR检测。

1.4　RT-qPCR检测

收集上述5组细胞，使用RNeasy Mini kit (QIAGEN，Cat.74106) 提取细胞RNA，用iScript cDNA合成试剂盒(BIO-RAD，Cat.170-8891)进行逆转录，加入iQ SYBR Green Supermix DNA聚合酶混合物(BIO-RAD，Cat.170-8882)后，使用Master Cycler Gradient PCR仪(扩增条件为95 ℃ 30 s预变性→95 ℃ 5 s，60 ℃ 34 s，共40个循环→荧光融解得到融解曲线)检测REA、IL-17a、RORγt、GAPDH的mRNA表达情况。REA、IL-17a、RORγt、GAPDH引物序列见表2。

表1　5组细胞的处理方法

组别	10%FCS+1640培养基+anti-CD28	阴性病毒	Phb2/REA过表达慢病毒	anti-IFN-γ、anti-IL-4、IL-6、TGF-β1、IL-23
空白组	+	−	−	−
空载体组	+	+	−	−
慢病毒组	+	−	+	−
Th17分化组	+	−	−	+
Th17+慢病毒组	+	−	+	+

各刺激因子质量浓度及体积如下：anti-CD28(5 mg/L) 25 μL，anti-IL-4 (10 mg/L) 50 μL，anti- IFN-γ(10 mg/L)50 μL，IL-6 (50 μg/L) 25 μL，TGF-β1 (1 μg/L) 50 μL和IL-23 (5 μg/L)25 μL

表2　REA、IL-17a、RORγt、GAPDH引物序列

基因	序列(5'→3')	碱基数
REA	F: AAGGACTTAGCTGGACGCCT	20
	R: CTGCACGCCACCAATACGAT	20
IL-17A	F: GCCAAGGACTTCCTCCAGAATGTG	20
	R: TGGAACGGTTGAGGTAGTCTGAGG	24
RORγt	F: AGACTCCGTTCTGGCCTCAC	20
	R: TGTGTCAACAGACGCAGCAA	20
GAPDH	F: TGCACCACCAACTGCTTAG	19
	R: GGATGCAGGGATGATGTTC	19

F：前引物；R：后引物

1.5　统计学处理

采用SPSS17.0统计软件分析数据。计量资料以$\bar{x}\pm s$表示，采用单因素方差分析和Dunnett's T3检验，P<0.05表示差异有统计学意义。

2　结果

2.1　Phb2/REA慢病毒转染小鼠淋巴细胞

荧光表达约80%，细胞生长良好，REA过表达慢病毒转染成功，见图1。

2.2　RT-qPCR检测

Th17+慢病毒组的REA、RORγt mRNA表达较慢病毒组高，且慢病毒组、Th17+慢病毒组REA、RORγt的mRNA明显高于Th17分化组，差异均有统计学意义(P<0.05或0.01)；空载体组与空白组比较差异无统计学意义(P>0.05)，见图2、3。Th17分化组的IL-17A mRNA表达明显上调(P<0.01)，且高于Th17+慢病毒组(P<0.01)；空白组、空载体组、慢病毒组的IL-17A mRNA表达差异无统计学意义(P>0.05)，见图4。

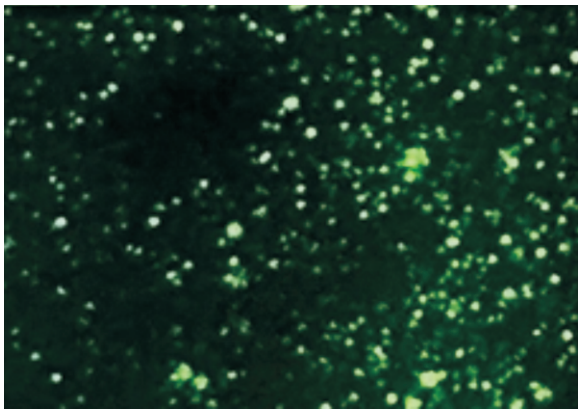

图1　病毒转染后72 h荧光图

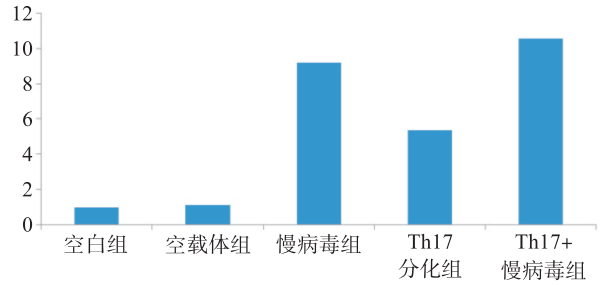

图2 细胞培养72 h后REA的mRNA 表达情况

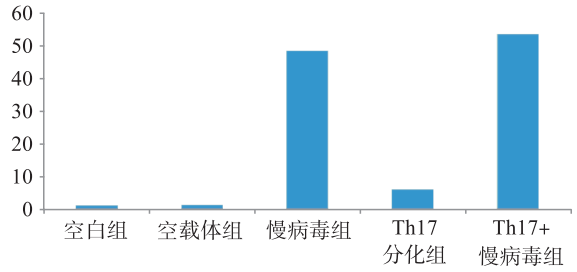

图3 细胞培养72 h后RORγt的mRNA表达情况

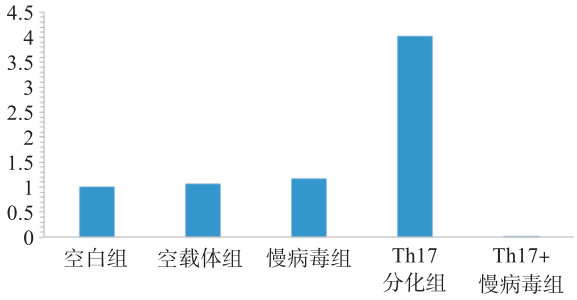

图4 细胞培养72 h后 IL-17a 的mRNA表达情况

3 讨论

Th17细胞是2005年发现的T细胞亚群，以选择性分泌细胞因子IL-17为特征[8]，其特异性转录调控因子主要是RORγt[10-11]。Th17细胞的分化过程不同于Th1和Th2。当机体遭受病原体时，其先合成IL-6和TGFβ，继而诱导T细胞表达转录因子RORγt，调控Th17细胞的分化。而IL-23既可以诱导IL-17的产生，与受体结合后也可以启动JAK-STAT信号通路，促进Th17细胞成熟[1, 12]。

E2参与细胞免疫和体液免疫的调节及抗原的呈递，在抑制炎症的同时也能促进炎症的发生。E2可以通过上调REA募集HDAC1、HDAC2的方式抑制RORγt的表达[13]，遏制Th17细胞分化。其过程可能是通过抑制IL-17的分泌，亦可能是通过促进Th2细胞的分化参与SLE的发病，因此不能直接采用E2治疗SLE。

我们预期采用Phb2(REA)慢病毒过表达技术促进REA表达，从而下调RORγt抑制Th17细胞的分化，探讨治疗SLE的可行性。结果发现，Th17+慢病毒组REA和RORγt的mRNA表达较慢病毒组高，且慢病毒组和Th17+慢病毒组REA和RORγt的mRNA明显高于TH17分化组，空载体组与空白组比较差异无统计学意义(P>0.05)。TH17分化组IL-17A的mRNA表达明显上调(P<0.01)，且TH17分化组IL-17的mRNA表达明显高于Th17+慢病毒组(P<0.01)。实验结果显示REA上调的同时RORγt也上调，但Phb2(REA)慢病毒过表达载体在Th17分化条件下可明显抑制IL-17A表达，表明Th17细胞的分化过程受到了抑制。虽然这一机制与E2通过RORγt抑制Th17的分化不同，但该方法却能有效抑制IL-17A的表达，提示Phb2(REA)慢病毒过表达载体有望成为治疗Th17细胞免疫紊乱相关性疾病如SLE、银屑病等的新方案。我们推测Phb2(REA)慢病毒过表达载体未能抑制RORγt的原因可能与细胞对REA高表达的反应性，即反馈性上调RORγt的表达有关。由于实验只是初步探讨了Phb2/REA过表达慢病毒载体对Th17细胞分化的影响，至于慢病毒是通过何种途径参与抑制Th17细胞的分化过程，其具体影响机制有待我们进一步研究。

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