

doi: 10.13241/j.cnki.pmb.2018.08.008

LMP-1 单链抗体基因修饰人外周血单个核细胞的构建及鉴定*

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摘要 目的:构建携带抗-LMP-1型潜伏膜蛋白(anti-LMP-1)的单链抗体序列的过表达载体并包装慢病毒,构建及鉴定 LMP-1 单链抗体基因修饰的人外周血单个核细胞(PBMCs)。**方法:**根据 GenBank 中 LMP-1 单链抗体、CD28 及 CD3 ζ 序列设计引物并合成,从已有 pVito-IgG-1 载体钓取 IgG-1-Fc 段,并分别插入慢病毒表达载体,获得 pCDH-anti-LMP-1-IgG-Fc-CD28-CD3 ζ (CAR)及 pCDH-CD28-CD3 ζ (对照 VEC)并测序鉴定,测序成功后采用三质粒包装系统(核心质粒 pCDH、包装质粒 pSPAX2 和 pMD2.G)分别包装慢病毒 CAR 及 VEC;浓缩慢病毒并离心感染 PBMCs,经荧光显微镜观察绿色荧光蛋白(GFP)的表达,并利用流式细胞术检测 GFP+ 比率以鉴定目的蛋白的表达。**结果:**构建了过表达 LMP-1 单链抗体基因的质粒并成功包装慢病毒,浓缩慢病毒感染 PBMCs 后 GFP+ 细胞数可达 42.5%(VEC-PBMCs)及 51.0%(CAR-PBMCs)。**结论:**为进一步研究 anti-LMP-1 修饰的 PBMCs 对 LMP-1 阳性肿瘤细胞的靶向杀伤奠定了实验基础。

关键词:潜伏膜蛋白 1 型;慢病毒;嵌合抗原受体

中图分类号:R-33; R392.1; Q78; R733 文献标识码:A 文章编号:1673-6273(2018)08-1441-06

Construction and Identification of Anti-EBV LMP-1 Single Chain Variable Fragment Antibody Modified Peripheral Blood Mononuclear Cells*

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ABSTRACT Objective: To prepare the recombinant lentivirus over-expressing anti-latent membrane protein -1 (anti-LMP-1) single chain variable fragment (scFv) antibody gene, then construct and identify anti-LMP-1 gene modified human peripheral blood mononuclear cells (PBMCs). **Methods:** The primers of anti-LMP-1 scFv, CD28 and CD3 ζ were designed according to the GenBank. IgG1-Fc were obtained from existing pVito-IgG-1 vector. All these sequences mentioned above were amplified and inserted into the lentiviral expression plasmid pCDH to construct protein expression vectors (pCDH-anti-LMP-1-IgG-Fc-CD28-CD3 ζ , hereafter refer to as CAR; control vector pCDH-CD28-CD3 ζ , hereafter refer to as VEC). CAR and VEC lentiviruses were prepared through three-plasmid packaging system (shuttle plasmid pCDH, packaging plasmids pSPAX2 and pMD2.G). The PBMCs were isolated from healthy donors and infected with concentrated pCDH-VEC and pCDH-CAR lentivirus separately. The expression of green fluorescent protein (GFP), as a surrogate reporter of target protein, was detected by fluorescence microscope and flow cytometry. **Results:** Over-expression vectors, VEC and CAR, were successfully constructed. The concentrated recombinant lentiviruses containing VEC or CAR could infect PBMCs and GFP as a reporter protein could be observed through fluorescence microscope and flow cytometry. The percentage of GFP+ cells among VEC or CAR lentivirus transfected PBMCs were 42.5% and 51%. **Conclusion:** The anti-LMP-1 gene modified PBMCs were successfully established, which lays an experimental foundation for further research on its cytotoxic effect on LMP-1 positive tumor cells.

Key words: Latent membrane protein 1; Lentivirus; Chimeric antigen receptor

Chinese Library Classification(CLC): R-33; R392.1; Q78; R733 **Document code:** A

Article ID: 1673-6273(2018)08-1441-06

前言

近年来,嵌合抗原受体(Chimeric antigen receptor, CAR)修饰的 T 细胞(CAR-T)免疫疗法在血液系统肿瘤的临床治疗中显示出良好的疗效,使其成为生物治疗领域中的新热点^[1,2]。CAR 主要由胞外肿瘤相关抗原结合区,跨膜区及胞内共刺激

域所构成的融合蛋白。其中胞外区是 CAR-T 细胞发挥其靶向杀伤能力的核心,它通常为针对于靶细胞表面分子的单链抗体可变区片段(Single chain variable fragment, scFv),赋予 T 细胞 HLA 非依赖性的靶向杀伤活性。胞内区包括共刺激分子(如 CD28, 4-1BB 等)及信号区 CD3 ζ , 主要促进 T 细胞的活化、细胞因子的释放,提高 T 细胞的细胞毒性^[3]。

* 基金项目:国家自然科学基金项目(81601749)

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(收稿日期:2017-10-29 接受日期:2017-11-23)

将 HEK293T 细胞铺于 T75 培养瓶中, 用含 10% FBS 的

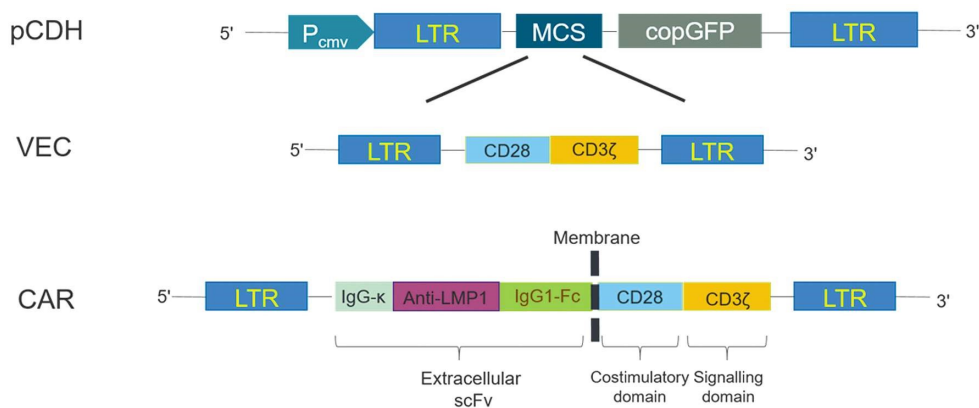


图1 嵌合抗原受体 CAR 及对照 VEC 结构插入慢病毒表达载体 pCDH 示意图

Fig.1 Schematic structure of chimeric antigen receptor(CAR) and control VEC inserted into lentiviral vector pCDH

DMEM 培养液于 37 °C、5% CO₂ 孵箱培养至 60%~80%融合度时,换 10 mL 无血清 DMEM 培养液,继续孵育。30 min 后,采用慢病毒表达质粒 (CAR 或 VEC)、包装质粒 pSPAX2 和 pMD2.G 组成的三质粒系统分别包装 CAR 及 VEC 慢病毒,转染试剂 MAX(μL)和转染质粒总量(μg)按照 3:1~5:1 的比例。慢病毒表达质粒、pSPAX2 和 pMD2.G 分别为 12、8、4 μg,于 250 μL 无血清 DMEM 培养基中混匀后室温静置 5 min,MAX 转染试剂 72 μL 于无血清 DMEM 培养基中混匀后室温静置 5 min,将质粒与转染试剂混匀,室温静置 20 min。逐滴加入至上述 T75 培养瓶中,37 °C、5%CO₂ 孵箱培养 6~8h 后换新鲜含 10% FBS 的 DMEM 于 37 °C、5% CO₂ 孵箱继续培养。48 h 和 72 h 后分别收集培养瓶中病毒上清液,离心去除细胞碎片。病毒液经 0.45 μm 滤膜(Millipore)过滤后与 5× PEG 8000(8.766 g NaCl,50 g PEG-8000,定容至 100 mL 后高压灭菌)按 4:1 混合,每 20 min 充分混匀一次,共 3~5 次;4 °C 过夜;4 °C,4000× g 离心 20 min。弃上清,病毒沉淀用 RPMI 1640 重悬,分装后储存于 -80 °C 冰箱中备用。

1.5 慢病毒感染 PBMCs

利用 Ficoll 法分离健康志愿者 PBMC,用含 10% FBS 的 RPMI 1640 培养基重悬细胞调整细胞浓度,以 1×10⁴/孔的密度接种于 24 孔板中,加入 OKT3 至终浓度为 2.5 μg/mL 刺激 PBMCs 中原代 T 细胞转化为增殖状态,加入 IL-2 至终浓度为 40 ng/mL 维持细胞活性。孵育 24 h 后离心,弃上清,实验组及对照组每孔各加入 200 μL 纯化 VEC-T 及 CAR-T 病毒液重悬细胞,每孔加入 0.1 μL 8 μg/mL Polybrene,1000× g 离心 60 min,重悬细胞后继续孵育 24 h,重复感染。之后每天半换液,继续培养至 7 d。

1.6 Anti-LMP-1 基因修饰 PBMCs 的建立及鉴定

离心收集慢病毒感染后实验组及对照组 PBMCs,弃上清,用 4%多聚甲醛重悬并固定于载玻片上,荧光显微镜下观察绿色荧光表达。流式细胞术检测 GFP+ 细胞比率。

2 结果

2.1 pCDH-CAR 质粒构建及测序

CAR 慢病毒表达载体酶切后产物经核酸电泳后,在 Gel Imaging 分析系统下可观察到 anti-LMP-1 目的片段长 804 bp;

CD28-3ζ 段长 549 bp; 整体 CAR 融合蛋白 anti-LMP-1-IgG-Fc-CD28-CD3ζ 共长 2061 bp(图 2A)。质粒经测序后显示无突变位点(图 2B)。

2.2 pCDH-CAR 慢病毒的包装及鉴定

利用 pCDH-CAR 浓缩慢病毒上清液感染 293T 细胞后,随着感染时间的延长,细胞荧光率逐渐增加,感染后第 48 h GFP+ 细胞比率可达 80%左右(图 3)。证明包装成功的慢病毒液能够成功感染靶细胞。

2.3 pCDH-VEC 慢病毒的包装及鉴定

利用 pCDH-VEC 浓缩慢病毒上清液感染 293T 细胞后,随着感染时间的延长,细胞荧光率逐渐增加,感染后第 48 h GFP+ 细胞比率可达 80%左右(图 3)。证明包装成功的慢病毒液能够成功感染靶细胞。

2.4 慢病毒感染 PBMCs 及鉴定

经重复平板离心法感染 PBMCs 后 5d,在荧光显微镜下观察 PBMCs GFP 表达水平,其阳性率大约为 40%-50%左右,且 GFP 示踪报告基因表达于细胞膜(图 4)。经流式细胞术检测,GFP+ PBMCs 比率分别为 42.5%和 51.0%(图 5)。证明慢病毒能成功感染 PBMCs 并表达报告基因。

3 讨论

自从 1964 年人类首次从 Burkitt 淋巴瘤中观察到 EBV,针对 EBV 的研究已经超过 50 余年。作为第一个被发现的致癌病毒,科学家逐渐证实其与多种淋巴源性上皮细胞性肿瘤的发生密切相关^[1]。据估计,每年约有 120,000 例新发肿瘤与 EBV 感染相关^[4]。

EBV 主要通过唾液传播,病毒穿过黏膜上皮屏障,感染下层淋巴组织(如扁桃体 B 细胞)。EBV 的生命周期一般包括五个部分,在原发感染中,EBV 感染 naïve B 细胞后随即表达完整的病毒潜伏基因复合体,包括 10 个蛋白和两个小 RNAs,此为 III 型潜伏期,与淋巴瘤的发生密切相关。该期 EB 病毒抗核抗原(EBV nuclear antigens,EBNA)调控驱动 B 细胞的大量增殖,此后因为其高度的免疫原性迅速激活 EBV 特异性 T 细胞参与病毒的清除。为了躲避机体免疫系统的攻击,EBV 下调其免疫原性蛋白的表达,感染的 B 细胞继而进入淋巴滤泡,仅表达 EBNA1 及 LMP1&2,进入 II 型潜伏期,该期与淋巴瘤、霍奇

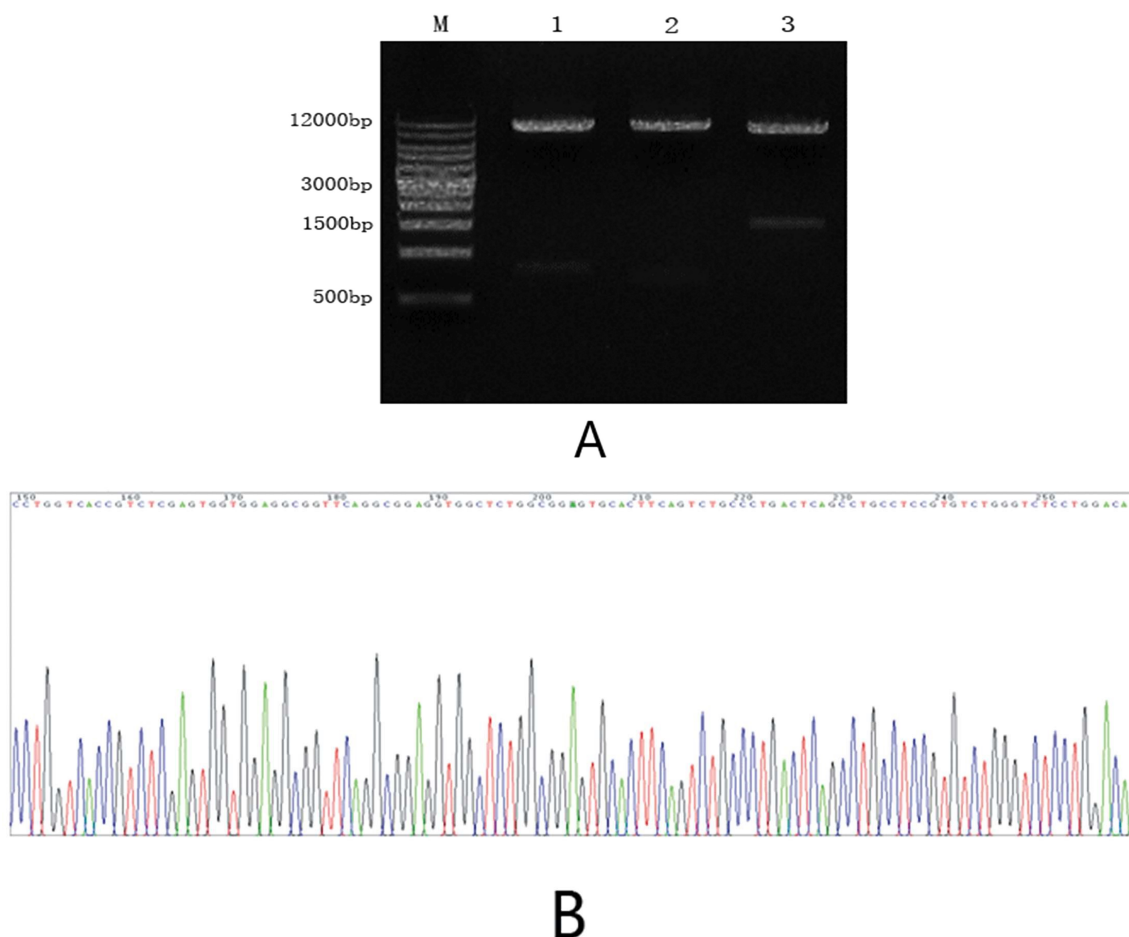


图 2 pCDH-CAR 质粒酶切鉴定及测序部分结果

Fig. 2 Restriction-digest identification and sequencing results of pCDH-CAR plasmid

(A:pCDH-CAR 载体酶切鉴定;B:测序部分结果;M:DNA maker;1:pCDH-CAR 经 *Xba*I 及 *Nhe*I 双酶切后,anti-LMP-1 目的片段长 804 bp;

2:pCDH-CAR 经 *Eco*RI 及 *Bam*HI 双酶切后,CD28-3 ζ 段长 549 bp;

3:pCDH-CAR 经 *Xba*I 及 *Bam*HI 双酶切后,目的基因 CAR 融合蛋白基因长 2061bp)

(A: Restriction-digest identification of pCDH-CAR vector; B: Part of the sequencing data; M: DNA marker; I: The length of anti-LMP-1 fragment is 804 bp when digested by restrictive enzymes *Xba*I and *Nhe*I. 2: The length of CD28-3 ζ is 549 bp, and the target fragment can be seen when pCDH-CAR vector was digested by *Eco*RI and *Bam*HI. 3: The length of recombinant fusion gene, CAR., is 2061 bp, and the target fragment can be seen when pCDH-CAR vector was digested by *Xba*I and *Bam*HI.)

金淋巴瘤和非霍奇金淋巴瘤的发生均密切相关。接下来 B 细胞离开淋巴结进入外周循环,并下调所有的病毒蛋白,进入 0 期潜伏期。EBV 感染的 B 细胞在外周循环中稳定分裂增殖,仅表达 ENBA1 病毒蛋白以确保病毒基因稳定存在,即为 I 型潜伏期^[12]。EBV 相关肿瘤的发生通常由潜伏 II 期及 I 期转化而来,这些肿瘤的发生与病毒在宿主细胞中的复制及病毒致癌蛋白的表达密不可分^[13]。

其中,LMP 作为 EBV 感染后 B 淋巴细胞转化的重要影响因素,是促进 EBV 致癌的重要因素^[9]。LMP-1 是 EB 病毒唯一能够转化体外及动物细胞为永生化细胞的致癌蛋白,在 EBV 相关肿瘤的多个阶段发挥重要的生物学功能,其参与细胞粘附性的改变,降解细胞外基质,促进肿瘤血管的生成等肿瘤形成过程^[14]。既往的大量研究显示,LMP-1 参与在鼻咽癌的发生、生长及转移中发挥重要作用,可通过多种信号通路诱导分泌细胞因子、趋化因子、促血管生成因子等,并通过刺激产生或分泌免疫调节性外泌体,抑制周边免疫细胞参与免疫应答^[15,16]。

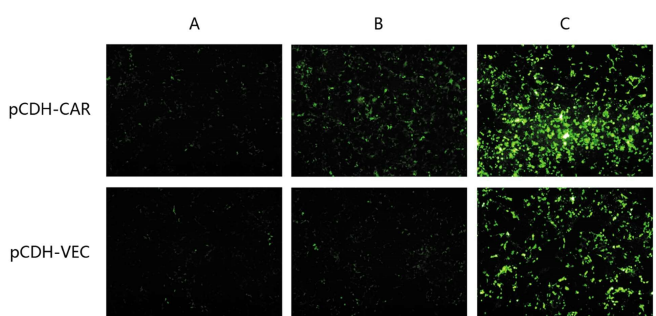


图 3 浓缩慢病毒感染 293T 细胞免疫荧光图(200 $\times$)

Fig.3 Observation of lentivirus infected 293T cells by fluorescence microscope(200 $\times$)

(A、B、C 分别为 CAR 及 VEC 浓缩慢病毒感染 293T 细胞后 12 h、24 h、48 h)

(The pictures grouped in A,B and C showed 293T cells were infected by purified CAR and VEC lentiviral solution after 12 h, 24 h and 48 h respectively.)

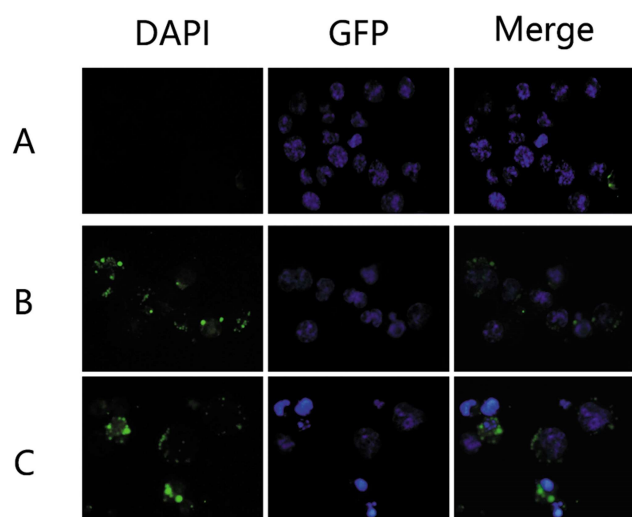


图4 荧光显微镜观察浓缩慢病毒感染 PBMCs(200×)

Fig.4 Observation of lentivirus infected PBMCs by fluorescence microscope(200×)

(A: 未经慢病毒感染 PBMCs; B: pCDH-VEC 慢病毒感染 PBMCs; C: pCDH-CAR 慢病毒感染 PBMCs.)

(A: PBMCs were not infected by any lentivirus.

B: PBMCs were infected by pCDH-VEC lentivirus;

C: PBMCs were infected by pCDH-CAR lentivirus.)

EBV 感染机体后形成长期潜伏感染,在潜伏感染阶段,病毒限制性表达病毒蛋白以逃避免疫系统的监视。因而,增强免疫细胞对于 EBV 相关抗原的识别能力成为了治疗 EBV 相关疾病的重要途径。已有研究表明,EBV 相关鼻咽癌患者输入 EBV 特异性细胞毒性 T 淋巴细胞可以有效控制疾病进展^[17],在体外利用 LMP-2 多表位肽段诱导 EBV 特异性 T 细胞的产生并大量扩增,继而回输至鼻咽癌患者体内可有效控制鼻咽癌的进展^[18]。体外诱导 LMP-1 特异性杀伤性 T 细胞(CTLs)可控制 EBV 潜伏、增殖、维持及转化过程,且 LMP-1 特异性 CTLs 经

体外扩增再回输至患者体内,能达到良好的临床疗效^[19,21]。因此,靶向 LMP-1 这一 EBV 关键致瘤分子的生物治疗具有较好的安全性及有效性。

近年来,CAR-T 细胞过继免疫疗法在血液系统肿瘤中取得显著的临床疗效而使其成为过继性免疫治疗最有前景的热点研究之一^[2]。该方法可将特异性识别肿瘤细胞表面靶分子的嵌合抗体在体外通过电转染或慢病毒感染的方式转入 T 细胞,并使其在 T 细胞表面表达。经修饰后的 T 细胞在体外进行大量扩增再回输至患者体内,从而赋予 T 细胞特异性的识别肿瘤抗原的能力及 MHC 的非限制性^[3]。

目标抗原的选择对于 CAR 的特异性、有效性及安全性而言都是最关键的决定性因素。仅表达于肿瘤细胞且对肿瘤细胞存活具有重要意义的抗原是理想的目标抗原,然而大多数的肿瘤相关抗原(Tumor-associated antigen,TAA)并不具备肿瘤细胞的限制性,这是导致靶向/非肿瘤毒性产生的根源^[22]。一项靶向胶质母细胞瘤细胞表面表皮生长因子受体变异体 III(Epidermal growth factor receptor variant III,EGFRvIII)的 CAR-T 细胞免疫疗法中,9 例患者在输入 CAR-T 细胞后均表现出了良好的耐受性及极低的毒性反应,显示出了 EGFRvIII 作为肿瘤特异性抗原的优越性^[23]。然而,EGFRvIII 仅仅表达于极为有限的肿瘤细胞表面,如胶质母细胞瘤,从而极大的限制了应用范围^[24]。因此,寻找特异性靶点以降低 CAR-T 细胞对于正常细胞、组织的毒性,病毒特异性抗原成为近年来针对病毒感染相关肿瘤靶向治疗的热点。其中靶向 HBV 包膜蛋白^[25,26]、HCV 包膜 E2 糖蛋白^[27]、HIV gp120^[28,29]及利用针对 HIV 多种病毒株的广谱中和抗体修饰 T 细胞^[30]等的过继性细胞免疫疗法显示了病毒蛋白作为靶分子的良好潜能。

基于既往研究,本研究以 LMP-1 这一 EBV 重要致瘤病毒蛋白为靶点,设计并构建过表达抗-LMP-1 单链抗体基因的慢病毒表达载体,通过纯化慢病毒,提高病毒滴度且改善感染条件等方式成功将抗-LMP-1 的单链抗体基因转导至人原代

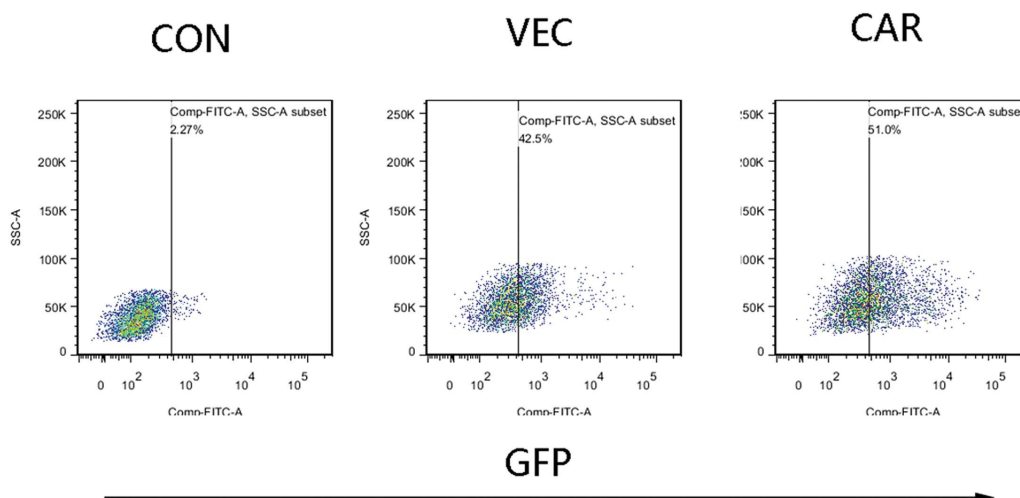


图5 慢病毒感染 PBMCs 后经流式检测 GFP+ 细胞比率

Fig.5 Detection of GFP+ cell ratio in lentivirus infected PBMCs by flow cytometry

(CON: 未经慢病毒感染的 PBMCs; VEC: 经对照组 pCDH-VEC 慢病毒感染的 PBMCs; CAR: 经 pCDH-CAR 慢病毒感染的 PBMCs)

(CON: PBMCs were not infected by any lentivirus; VEC: PBMCs infected by pCDH-VEC lentivirus; CAR: PBMCs were infected by pCDH-CAR lentivirus)

PBMCs 内, 赋予其靶向杀伤 LMP-1 阳性肿瘤细胞的能力, 从而克服肿瘤细胞局部抑制性的免疫微环境, 打破宿主免疫耐受状态, 拓宽 CAR-T 细胞免疫疗法在 EBV 相关肿瘤生物治疗中的应用。

而针对过表达抗 -LMP-1 抗体的 PBMCs 能否增强免疫细胞靶向 LMP-1 阳性肿瘤细胞杀伤能力的功能作用正在进一步验证之中。

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