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· 基础研究 ·

抗CD19嵌合受体修饰的NK-92细胞的构建及其对CD19阳性非霍奇金淋巴瘤细胞的杀伤作用

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[摘要] **目的:** 构建表达CD19的二代CAR-NK-92细胞系, 探讨其对CD19阳性非霍奇金淋巴瘤细胞的特异性杀伤作用。 **方法:** 构建表达CD19-CAR基因的载体并包装慢病毒颗粒, 感染NK-92细胞, 流式细胞术检测感染率, 并进一步分选阳性细胞, Western blotting检测CD19-CAR在NK-92细胞中的表达; 以CD19阴性骨髓瘤细胞U-266、CD19阳性的非霍奇金淋巴瘤细胞ARH-77和HS-Sultan细胞为靶细胞, 以CD19CAR-NK-92为效应细胞, 流式细胞术检测细胞杀伤实验中存活肿瘤细胞的绝对数量并计算杀伤率。 **结果:** 成功构建慢病毒载体pLVX-CD19-CAR并包装病毒颗粒, 感染NK-92细胞后CD19CAR-NK-92细胞纯度高于90%; Western blotting分析显示CD19-CAR已经成功表达在NK-92中。CD19CAR-NK-92对ARH-77[(70.10±1.86)% vs (1.95±0.63)%, $P<0.01$]和HS-Sultan[(74.98±1.60)% vs (0.58±1.49)%, $P<0.01$]细胞的杀伤率显著高于空载体对照组ZsGreen-NK-92细胞, 对U266的杀伤率无显著差别($P>0.05$)。 **结论:** 成功构建了表达CD19CAR的NK-92细胞系, 其对CD19阳性的非霍奇金淋巴瘤细胞有特异性杀伤作用。

[关键词] 非霍奇金淋巴瘤; 嵌合受体; CD19; NK-92; 细胞毒性

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The construction of anti-CD19 chimeric receptor modified NK-92 cells and the killing effect of CD19 positive non-Hodgkin lymphoma cells

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[Abstract] **Objective:** A second generation CAR-NK-92 cell line expressing CD19 was constructed to investigate its specific killing effect on CD19 positive non-Hodgkin lymphoma cells. **Methods:** First, build CD19-CAR gene expression vector and packaged slow virus particles, then the infection rate was detected by flow cytometry after infected NK-92 cells and positive cells were further separated. Finally, detected the expression of CD19-CAR in NK-92 cells by Western blotting. U-266 with CD19 negative myeloma cells, ARH-77 and HS-Sultan with CD19 positive non-Hodgkin's lymphoma cells as target cells, and CD19CAR-NK-92 as effector cells, then the killing rate was calculated by the absolute number of tumor cells alive in the cell killing experiment. **Results:** Construct lentivirus vector pLVX-CD19-CAR and packaged virus particles successfully, the purity of CD19-CAR-NK-92 cells also was over 90% after infected with NK-92 cells; and Western blotting analysis showed that CD19-CAR had been successfully expressed in NK-92 cell. The killing effect of CD19CAR-NK-92 on ARH-77 [(70.10±1.86)% vs [1.95±0.63]%, $P<0.01$] and HS-Sultan [(74.98±1.60)% vs [0.58±1.49]%, $P<0.01$] cells was significantly higher than the empty vector control group of ZsGreen-NK-92, but there was no difference in killing U266 ($P>0.05$). **Conclusion:** The NK-92 cell lines expressing CD19CAR were successfully constructed, and also has specific killing effects on CD19 positive non-Hodgkin lymphoma cells.

[Key words] non-Hodgkin's lymphoma; chimeric receptor; NK-92; cytotoxicity

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B细胞非霍奇金淋巴瘤是一组常见异质性疾病,临床上常用的治疗方法为利妥昔单抗合用化疗药物环磷酰胺、阿霉素、长春新碱以及强的松方案(R-CHOP)^[1-2]。患者对这种一线治疗方案的首次应答较高,但是有30%~60%会复发且对R-CHOP治疗产生抵抗,临床上尚无有效的药物来控制这一类病情,患者的生存期非常有限^[2-3]。因此,需要开发新的治疗方式来满足病情需求,提高患者生活质量。靶向CD19的嵌合抗体(chimeric antigen receptor, CAR)修饰的自体T细胞是一种新的治疗方法,目前FDA已经批准Novartis和Kite Pharma两家公司生产该CAR T细胞,用于急性淋巴细胞白血病(acute lymphocyte leukemia, ALL)和弥漫性大B细胞淋巴瘤(diffuse large B cell lymphoma, DLBCL)治疗^[4-5]。前期的治疗效果非常出色,在ALL中有效率与完全缓解率达到了90%^[6]。同时,DLBCL患者对这种治疗方法的反应率达79%,完全缓解率约为50%^[7]。但是,很多患者在经历化疗之后在体外扩增T细胞相对困难,无法生产出CD19CAR-T细胞;同时,CD19CAR-T细胞生产过程需要至少10 d^[4],有些患者的病情在生产期间恶化并死亡^[8]。这表明,生产一种能即时应用(off-the-shelf)的细胞治疗产品非常重要。NK-92是一种来源于NK的细胞系,是一个理想的细胞治疗载体^[9]。I期临床研究^[10-11]发现,NK-92本身可以非特异性杀伤一系列的肿瘤且不攻击正常细胞,患者静脉给予辐射后的NK-92并无出现严重的副反应,且肿瘤被有效控制,该试验证明了NK-92临床应用的安全性。CAR修饰的NK-92可以让NK-92获得CAR特异性靶向的性能,从而实现对肿瘤发展的控制。CD19是B细胞特异性表面抗原,在大多B细胞肿瘤中均呈现高表达^[12],是靶向该类肿瘤的理想靶点。本研究以CD19作为靶点,构建表达二代CAR的NK-92细胞系,并探讨其对CD19阳性非霍奇金淋巴瘤细胞的特异性杀伤作用。

1 材料与方法

1.1 细胞系和主要试剂

细胞系均购自ATCC,其中多发性骨髓瘤细胞U-266和HS-Sultan、人外周血淋巴瘤细胞ARH-77用RPMI-1640培养基培养,人肾上皮细胞HEK-293T用DMEM培养基培养;所有培养基含双抗和10%FBS。DMEM培养基、RPMI-1640培养基均购自Gibco公司,抗MYC抗体、APC标记的山羊抗鼠IgG抗体和PI均购自BD公司,CFSE-Far Red、Lipofectamine 3000均购自Thermo公司,EcoR I、BamH I限制性内切酶均购自NEB公司,CD19-CAR基因DNA由Invitrogen

公司合成,慢病毒包装质粒psPAX2和pMD2.G购自Addgene公司,病毒载体pLVX-IRES-ZsGreen购自TaKaRa公司。

1.2 DNA合成和载体构建

人工合成含EcoR I、BamH I酶切位点的CD19-CAR基因,酶切后连接入病毒载体pLVX,构建慢病毒载体pLVX-CD19-CAR并进行双酶切鉴定。

1.3 CD19CAR-ZsGreen病毒包装

将HEK-293T细胞以 1×10^5 /ml的密度养在T150培养瓶中,培养过夜。病毒包装前用新鲜预热的DMEM培养基换液,继续培养2 h。将一定比例的pLVX-CD19-CAR或pLVX-IRES-ZsGreen与慢病毒包装质粒、Lipofectamine 3000混合后转染细胞(0 h),包装CD19CAR-zsGreen和ZsGreen病毒颗粒。收集转染后24和48 h的细胞上清,滤膜过滤后。转至高速离心管中于 $20\ 000 \times g$ 离心2 h。浓缩后的CD19CAR-ZsGreen病毒用含1% BSA的PBS溶液重悬。分装后保存于 $-80\ ^\circ\text{C}$ 。

1.4 CD19CAR-ZsGreen感染NK-92细胞

将 1×10^6 个处于对数生长期的NK-92细胞与ZsGreen或CD19CAR-ZsGreen病毒混合,加入硫酸鱼精蛋白, $1\ 200 \times g$ 离心30 min,重悬细胞后培养12 h,次日重复感染步骤。感染后的细胞转移到T25培养瓶中继续培养。感染4 d后,流式细胞仪检测感染阳性率。选用未感染NK-92为对照组,感染后的NK-92细胞为实验组。于各组中取 5×10^5 个细胞,重悬在 $100\ \mu\text{l}$ 2% PBS缓冲液中,加入 $2\ \mu\text{g}$ MYC抗体于 $4\ ^\circ\text{C}$ 孵育30 min;孵育完成后用上述缓冲液清洗细胞,加入APC-抗鼠二抗, $4\ ^\circ\text{C}$ 孵育30 min。仅表达ZsGreen(绿色荧光)的细胞为空载体感染成功的NK-92,ZsGreen和APC双阳性细胞则为CD19CAR-NK-92。阳性细胞通过流式细胞仪进行再分选、纯化,将所得到的阳性细胞继续扩大培养,保种。

1.5 Western blotting 鉴定 CD19CAR-NK-92 细胞中 CD19-CAR 的表达

取 1×10^6 个ZsGreen-NK-92和CD19CAR-NK-92细胞,分别加入100、300 μl 的蛋白裂解液,冰上静置30 min,期间5~6 min震荡1次, $4\ ^\circ\text{C}$, $120\ 000 \times g$ 离心10 min,吸取上清,根据上清体积加入5 \times 非变性非还原蛋白上样缓冲液25、75 μl ,两个样品分别吸出5、10 μl 并加入10% PAGE分离胶上样孔中,电泳结束后将蛋白转移至NC膜上,转膜结束后5%脱脂牛奶封闭液封闭2 h,抗CD3 ζ 作为一抗,孵育3 h,加入山羊抗鼠IgG二抗(1:1 000)孵育12 h,室温中显像检测CD19-CAR在NK-92里的表达水平。

1.6 流式细胞术检测 CD19CAR-NK-92 细胞对淋巴瘤细胞的杀伤作用

选择U-266(NK-92敏感的CD19阴性骨髓瘤细胞系,阴性对照组)、ARH-77和HS-Sultan细胞(均为CD19阳性的非霍奇金淋巴瘤细胞系,实验组)为靶细胞,以 $1 \times 10^5/\text{ml}$ 的密度铺于96孔板中,每孔 $100 \mu\text{l}$ 体系,加入CFSE-Far Red于 37°C 孵育15 min,用完全培养基洗涤后备用。标记好的细胞分别与ZsGreen-NK-92或者CD19CAR-NK-92以3:1比例混合,培养5 h后,细胞用PI标记,用流式细胞仪纪录存活肿瘤细胞的绝对数量,计算出CD19CAR-NK-92对淋巴瘤细胞系的杀伤率。杀伤率($\%$)= $[(0 \text{ h时存活靶细胞数} - 5 \text{ h时存活靶细胞数} - \text{自然死亡细胞数})/0 \text{ h时存活靶细胞数}] \times 100\%$ 。

1.7 统计学处理

应用Graphpad prism软件对数据进行处理分析,两样本均数比较采用Student-*t*检验。以 $P < 0.05$ 或 $P < 0.01$ 表示差异具有统计学意义。

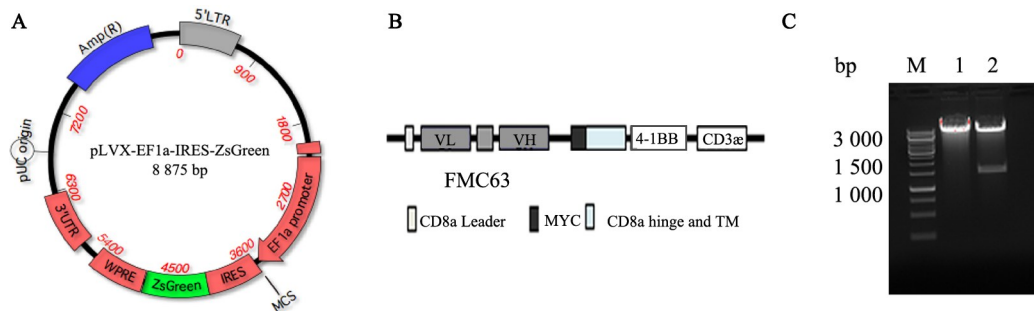
2 结果

2.1 成功构建慢病毒载体

图1A和B分别展示慢病毒载体pLVX-IRES-ZsGreen以及CD19CAR序列。pLVX-CD19-CAR载体构建完成后,*EcoR* I、*Bam*H I双酶切鉴定结果(图1C)显示,在1 500 bp处有一条新条带,而空载体没有,表明CAR基因已经正确连接到慢病毒载体中。

2.2 成功构建CD19CAR-NK-92细胞

流式细胞术检测结果(图2A、B)显示,空载体病毒感染的NK-92细胞仅表达绿色荧光且细胞表面无MYC标签表达;pLVX-CD19-CAR病毒感染的NK-92则同时表达绿色荧光和MYC标签。对两组细胞中的阳性细胞进一步用流式细胞仪进行分选,分选后的细胞阳性率均达到90%以上。对得到的阳性细胞进行Western blotting分析CD3V表达结果(图2C)显示,CD19CAR-NK-92细胞中表达相对分子质量50 000的蛋白,与CD19CAR大小相符,而此条带并未在ZsGreen-NK-92细胞中被检测到。

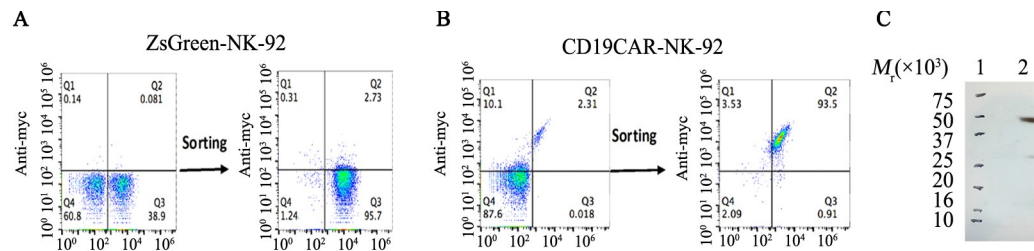


A: Map of self-inactivating third generation lentivirus vector with EF1 promoter. Chimeric antigen receptor (CAR) gene is inserted to MCS site; B: A schematic representation of CAR is shown, single chain variable region of antibody is derived from FMC63(GenBank: ADM64594.1), followed by myc tag, CD8 hinge and transmembrane (TM) domain. Cytoplasmic domain of 4-1BB and CD3 are used as signal transduction moiety; C: *EcoR* I and *Bam*H I double digestion demonstrate CAR gene was inserted into lentivirus vector.

1: Empty vector; 2: pLVX-CD19-CAR; M: Marker

图1 慢病毒载体pLVX-CD19-CAR构建成功

Fig. 1 Lentivirus vector pLVX-CD19-CAR was constructed successfully



A, B: NK-92 cells are transduced either with vector virus (A) or CD19CAR virus(B), ZsGreen and CAR positive cells were then isolated by flow cytometry sorting; C: CD19CAR expression in NK-92 was further determined by Western blotting against human CD3V; M: Marker; 1: ZsGreen-NK-92 cells; 2: CD19CAR-NK-92 cells

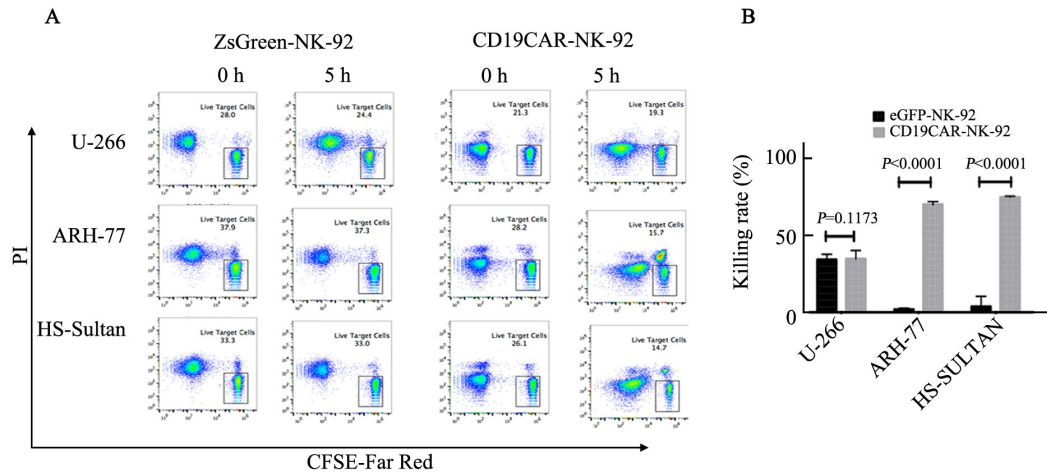
图2 成功构建CD19CAR-NK-92细胞

Fig. 2 CD19CAR-NK-92 cells were constructed successfully

2.3 CD19CAR-NK-92 对 CD19 阳性非霍奇金淋巴瘤细胞有特异性杀伤作用

流式细胞术检测后计算细胞杀伤率,结果(图3)显示,CD19CAR-NK-92 细胞对 ARH-77 [(70.10±1.86)% vs (1.95±0.63)% , P<0.01] 和 HS-Sultan

[(74.98±1.60)% vs (0.58±1.49)% , P<0.01]细胞的杀伤率显著高于 ZsGreen-NK-92 细胞,对 U266 细胞的杀伤率无显著差异 [(34.82±5.45)% vs (34.28±3.48)% , P>0.05]。



A: CD19CAR-NK92 cells exhibit specific cytotoxicity toward CD19 positive lymphoma cell lines. U266, ARH-77, HS-Sultan were labeled with CFSE-Far red before co-culture. ZsGreen-NK92 or CD19CAR-NK92 cells were incubated with CFSE labeled lymphoma cell lines at an effector: Target ratio of 3 for 5 h. PI staining is used to discriminate dead cell from live cells; B: Absolute number of target cells were counted by flow cytometry for cytotoxicity calculation

图3 CD19CAR-NK-92 细胞特异性杀伤 CD19 阳性肿瘤细胞

Fig. 3 The specific cytotoxicity of CD19CAR-NK-92 cells against tumor cells expressed CD19

3 讨论

自体 NK 细胞难以开展临床试验研究的原因主要是血液中的 NK 细胞很难被现有的基因递送系统,如慢病毒、电转等技术进行修饰,而且原代的 NK 细胞在体外增殖速度慢,很难在短时间内生产足够量的细胞用于患者治疗^[13]。因此,开发一种临床应用安全的 NK 细胞系用于细胞治疗有着重要的价值。

NK-92 细胞是一种可以不断扩增、增殖的细胞系,对一系列小鼠体内移植肿瘤有着广泛的杀伤作用^[14-16]。此外,NK-92 的 I 期临床试验^[11]表明,给予患者反复静脉注射 NK-92 细胞不会出现显著的不良反应,证实了 NK-92 细胞对于肿瘤治疗的有效性和安全性。尽管亲代 NK-92 细胞对很多肿瘤细胞有着细胞毒性作用,但是其杀伤作用有限,不能够完全清除肿瘤细胞。因此,本课题组希望通过用 CD19CAR 修饰 NK-92 细胞以增加其对 CD19 阳性肿瘤细胞的特异性杀伤作用,为此构建了靶向 CD19 的 CAR-NK-92 细胞,探讨其对 CD19 阳性淋巴瘤细胞的特异性杀伤作用。CD19CAR 中的 CD19 抗原识别序列别于杂交瘤 FMC63 克隆,这个序列现在应用在 CD19CAR-T 的临床治疗。该识别序列在本实验室

中的应用保证了靶向的准确性、有效性及进一步临床研究的相关性。

空白载体病毒感染 NK-92 细胞后,ZsGreen 的阳性率达到了 38.9%;CD19CAR 的表达仅有 2.3%。但是 NK-92 细胞是一种可以无限增殖的细胞系,所以通过流式细胞分选技术对 CAR 阳性的 NK-92 细胞进行浓缩,然后扩大培养。分选后的细胞中 CD19CAR 阳性率达到 90% 以上,在培养瓶中持续培养 3 个月均未发现 CD19CAR 丢失(未出示该数据)。说明 CD19CAR 基因已经整合到 NK-92 的基因组中,并可以被持续表达。同时检测到了 CD19CAR-NK-92 细胞中的 CD3 表达,而亲代 NK-92 细胞是一种 CD3 阴性的细胞系,进一步表明 CD19CAR 在 NK-92 中的成功表达。

接下来探讨了所得到的 CD19CAR-NK-92 细胞系对 CD19 阳性肿瘤细胞的特异性杀伤作用。U266 是一种对 NK-92 细胞敏感的 CD19 阴性骨髓瘤细胞系。CD19CAR 表达未改变 NK-92 细胞对该种细胞系的敏感性,表明慢病毒修饰并未改变 NK-92 细胞的内在特性。而在两组 CD19 阳性的淋巴瘤细胞系中,与亲代 NK-92 细胞相比,CD19CAR-NK-92 细胞表现出了显著的 CD19 驱动的特异性杀伤作用。

综上,本研究成功构建了表达CD19CAR的NK-92细胞系,其对CD19阳性的淋巴瘤细胞有着特异性的杀伤作用,后续期望进一步探究CD19CAR-NK-92对小鼠异种移植瘤的抗肿瘤作用;同时还可以进一步研究该细胞系对临床上原代肿瘤的杀伤作用,来逐步明确CD19CAR-NK-92对B细胞肿瘤治疗的有效性,为进一步的临床试验提供实验依据。

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