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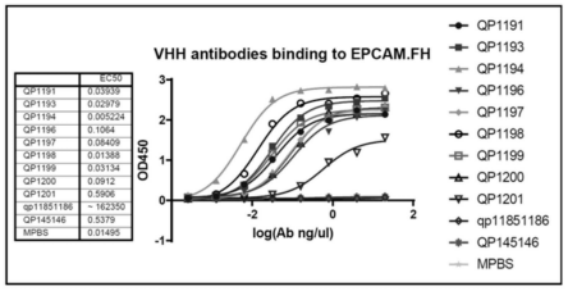
(54)发明名称

一种特异性靶向肿瘤EpCAM抗原的抗体及应用

(57)摘要

本发明属于基因工程和生物免疫治疗领域，提供了一种特异性靶向肿瘤EpCAM抗原的嵌合抗原受体，包括胞外抗原识别结构域、跨膜结构域和胞内信号结构域，所述胞外抗原识别结构域包括VHH抗体或其片段，所述VHH抗体或其片段具有特异性靶向肿瘤EpCAM抗原的互补决定区CDR，其能有效识别EpCAM表达阳性的肿瘤细胞，同时不会攻击正常的EpCAM低表达上皮细胞。



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1.一种特异性靶向肿瘤EpCAM抗原的嵌合抗原受体,包括胞外抗原识别结构域、跨膜结构域和胞内信号结构域,其特征在于,所述胞外抗原识别结构域包括VHH抗体,所述VHH抗体具有特异性靶向肿瘤EpCAM抗原的互补决定区CDR;所述VHH抗体为Seq ID NO.6所示的氨基酸序列。

2.如权利要求1所述的嵌合抗原受体,其特征在于,所述跨膜结构域包括CD3ζ、CD3ε、CD4、CD8α、CD28、CD5、CD16、CD9、CD22、CD33、CD27、CD37、CD45、CD64、CD80、CD86、CD127、CD137、CD134、CD152、CD154、PD-1或Dectin-1中的一个或多个。

3.如权利要求1所述的嵌合抗原受体,其特征在于,所述胞内信号结构域包括CD3ζ、CD27、CD28、CD30、CD137、CD134、CD154、Dectin-1、FcRγ或ICOS中的一个或多个。

4.如权利要求1所述的嵌合抗原受体,其特征在于,所述VHH抗体的核苷酸为Seq ID NO.24所示的序列。

5.一种CAR-T细胞,其特征在于,表达权利要求1-4任一项所述的嵌合抗原受体。

6.如权利要求5所述的CAR-T细胞,其特征在于,所述嵌合抗原受体的氨基酸序列如Seq ID NO.34所示;或者,所述嵌合抗原受体的核苷酸序列如Seq ID NO.43所示。

7.一种CAR-NK细胞,其特征在于,表达权利要求1-4任一项所述的嵌合抗原受体。

8.如权利要求7所述的CAR-NK细胞,其特征在于,所述嵌合抗原受体的氨基酸序列如Seq ID NO.34所示;或者,所述嵌合抗原受体的核苷酸序列如Seq ID NO.43所示。

9.一种抗肿瘤药物,其特征在于,含有权利要求5所述的CAR-T细胞和药学上允许添加的辅料和/或助剂;所述肿瘤为结肠癌和卵巢癌。

10.权利要求1-4任一项所述的嵌合抗原受体的合成方法,其特征在于,包括以下步骤:(1)构建Anti-EpCAM-VHH-CAR完整基因;(2)使用引物对Primer1和Primer2扩增所述Anti-EpCAM-VHH-CAR基因;(3)利用BamHI和EcoRI限制酶消化扩增出的基因序列并用病毒载体包装。

11.如权利要求10所述的合成方法,其特征在于,所述病毒载体包括慢病毒载体、腺病毒载体或反转录病毒载体。

12.如权利要求10所述的合成方法,其特征在于,所述引物Primer1的序列如Seq ID NO.46所示,引物Primer2的序列如Seq ID NO.47所示。

13.一种特异性靶向肿瘤EpCAM抗原的VHH抗体在制备双特异性抗体和抗体-药物偶联药物ADC中的应用,其特征在于,所述VHH抗体具有特异性靶向肿瘤EpCAM抗原的互补决定区CDR,所述VHH抗体为Seq ID NO.6所示的氨基酸序列。

14.一种特异性靶向肿瘤EpCAM抗原的VHH抗体在制备肿瘤诊断试剂盒的应用,其特征在于,所述VHH抗体具有特异性靶向肿瘤EpCAM抗原的互补决定区CDR,所述VHH抗体为Seq ID NO.6所示的氨基酸序列;所述肿瘤为结肠癌和卵巢癌。

一种特异性靶向肿瘤EpCAM抗原的抗体及应用

技术领域

[0001] .本发明属于基因工程和生物免疫治疗领域,具体涉及一种特异性靶向肿瘤EpCAM抗原的抗体及其应用。

背景技术

[0002] .在肿瘤免疫治疗过程中,诱导患者产生有效的抗肿瘤免疫是疾病治疗的关键。肿瘤细胞可以产生一系列的逃逸机制来避开免疫系统监视。包括下调主要组织相容性复合物(MHC)、下调自身抗原表达、下调免疫检查点分子配体,形成局部抑制微环境等,从而限制自身效应细胞对肿瘤细胞的识别和攻击[1]。

[0003] .目前已知的肿瘤抗原靶点主要分为两大类,一类是仅在肿瘤细胞中表达,而在正常细胞中不表达的抗原,具有肿瘤细胞特异性,被认为是肿瘤特异性抗原;另一类是在肿瘤细胞中高表达,在正常细胞中低表达或不表达的抗原,其表达具有肿瘤相关性,称为肿瘤相关抗原。EpCAM即为肿瘤相关抗原的一种,它在结直肠癌,卵巢癌等肿瘤细胞中高表达,在人的上皮来源细胞中也表达,虽然表达量相对较低,但是也缺乏肿瘤特异性。因此,选择EpCAM作为免疫治疗靶点需要考虑其免疫耐受。

[0004] .靶向EpCAM阳性肿瘤的治疗目前集中于抗体药物的开发和疗效评估。如Edrecolomab,Adecatumumab和Catumaxomab,以及抗体-药物偶联药(ADC)。EpCAM单抗药物Edrecolomab在III期结肠癌的辅助治疗中没有观察到其对患者总体生存率和无负荷生存期的改善[19-21]。Adecatumumab在转移性乳腺癌单药治疗临床研究中发现其具有剂量依赖性和靶抗原依赖性,但未观察到客观肿瘤消退[22]。Catumaxomab为靶向CD3和EpCAM的双特异性抗体药物,在上皮细胞癌继发的恶性腹水治疗中显示出明显临床获益,并且安全性可控[23,24],已于2009年获EMA批准上市用于恶性腹水的治疗,令人遗憾的是该药物由于销售惨淡于2017年退市。此外,抗EpCAM的抗体-药物偶联药如Oportuzumab monatox[25]和Tucotuzumab[26]在临床研究中显示出良好的耐受,但是对患者疾病改善和生存期的延长需要更多的临床数据来加以证实。总体来说,靶向EpCAM的抗体药物取得了一定的进展,但仍需要开发新的免疫治疗药物/方法来补充现有医疗技术的不足。

[0005] .嵌合抗原受体修饰的T细胞(CAR-T)疗法被认为是一种有效的肿瘤免疫治疗手段。该疗法是在T细胞中表达称为嵌合抗原受体(CAR)的一类重组受体,该受体能将T细胞重定向到所选靶点的肿瘤细胞。CAR分子由多个结构原件组成,分别为胞外的抗原识别结构域,铰链区、跨膜域,和胞内的信号转导结构域。抗原识别结构域包括但不限于免疫球蛋白来源的单链抗体(Single-chain variable fragment,scFv),该单链抗体由免疫球蛋白来源的重链和轻链的可变区通过G4S等结构连接组成;以及其它非抗体来源的分子,如自然杀伤细胞受体NKG2D能与靶细胞表面的配体NKG2DL结合而识别肿瘤抗原等[2]。铰链区则为CAR分子的胞外域和胞内域提供柔性连接作用,通常为免疫球蛋白重链的C_H2和C_H3结构域来源。跨膜区能够将CAR分子锚定在细胞膜上,其来源通常为CD4、CD8、或CD28、4-1BB等共刺激分子的跨膜区。胞内信号转导域由共刺激分子如CD28,4-1BB,ICOS等和胞内激活分子如CD3ζ

或FcRγ等组成,能够将胞外域的抗原配体识别信号传导并放大,使T细胞或NK细胞等被激活。

[0006] .CAR分子通常是由胞外的抗原识别结构域提供特异性抗原识别功能,将T细胞或NK细胞重定向抗原表达的肿瘤细胞位置。靶抗原的识别能激活CAR分子下游的信号转导,启动T细胞或NK细胞的效应功能。激活的T细胞或NK细胞通过分泌IFN-γ、TNF-α、IL-2、颗粒酶、穿孔素等效应分子,直接作用于靶细胞或募集其它免疫效应细胞参与免疫反应,最终导致靶细胞裂解死亡。因此,目前对CAR分子结构的改造主要思路都是增强CAR-T细胞的功能和靶细胞毒性。

[0007] .综上所述,本发明将提供一种新型纳米抗体应用于CAR分子结构的设计中,使其能够特异性识别EpCAM阳性表达的肿瘤细胞,以补充现有医疗技术的不足。

发明内容

[0008] .有鉴于此,本发明旨在提供一种特异性靶向肿瘤EpCAM抗原的抗体及应用,具体技术方案如下。

[0009] .一种特异性靶向肿瘤EpCAM抗原的嵌合抗原受体,包括胞外抗原识别结构域、跨膜结构域和胞内信号结构域,所述胞外抗原识别结构域包括VHH抗体或其片段,所述VHH抗体或其片段具有特异性靶向肿瘤EpCAM抗原的互补决定区CDR。

[0010] .VHH全称为重链单域抗体,为羊驼来源的一类抗体,与传统的IgG不同,VHH只包含重链,其体积小,分子量更小(~15kDa),因此又称为纳米抗体。与传统的单克隆抗体相比较,它具有独特优势,比如更容易进入抗原表位、免疫原性低、溶解性好、稳定性高,亲和力不逊于scFv等。

[0011] .VHH抗体与EpCAM靶点特异性识别的机制即抗体的重链可变区的CDR部位与肿瘤抗原识别结合的过程,其中,VHH的CDR结构决定其靶向EpCAM的特异性。

[0012] .进一步,所述VHH抗体或其片段可选地选自Seq ID NO.1-9中的任一氨基酸序列构成的多肽或表位。

[0013] .进一步,所述互补决定区CDR可选地选自Seq ID NO.10-18中氨基酸序列的一个或多个。

[0014] .现有研究已经证实,存在靶向多个靶点(一般是两个)的情况,这种情况下会在CAR中设计含有多个抗体的识别区,即有多个抗体的CDR区,这些CDR区可以是串联在一起的,也可以是整合在一个CAR结构中在细胞里表达后再分开识别抗原。

[0015] .进一步,所述跨膜结构域可选地包括CD3ζ、CD3ε、CD4、CD8α、CD28、CD5、CD16、CD9、CD22、CD33、CD27、CD37、CD45、CD64、CD80、CD86、CD127、CD137、CD134、CD152、CD154、PD-1或Dectin-1中的一个或多个。

[0016] .进一步,所述胞内信号结构域可选地包括CD3ζ、CD27、CD28、CD30、CD137、CD134、CD154、Dectin-1、FcRγ或ICOS中的一个或多个。

[0017] .进一步,所述VHH抗体或其片段的核苷酸可选地选自Seq ID NO.19-27中的任一序列。

[0018] .包含上述嵌合抗原受体的基因转移方式。

[0019] .进一步,所述基因转移方式可选地包含病毒载体、转座子系统、电穿孔法或

CRISPR/Cas9基因编辑工具。

[0020] .一种CAR-T细胞,表达上述的嵌合抗原受体。

[0021] .进一步,表达的所述嵌合抗原受体的氨基酸序列如Seq ID NO.28-36任一项所示。

[0022] .进一步,表达的所述嵌合抗原受体的核苷酸序列如Seq ID NO.37-45任一项所示。

[0023] .一种CAR-NK细胞,表达上述的嵌合抗原受体。

[0024] .进一步,表达的所述嵌合抗原受体的氨基酸序列如Seq ID NO.28-36任一项所示。

[0025] .进一步,表达的所述嵌合抗原受体的核苷酸序列如Seq ID NO.37-45任一项所示。

[0026] .一种抗肿瘤药物,含有本发明所述的CAR-T细胞和药学上允许添加的辅料和/或助剂。

[0027] .进一步,所述肿瘤包括上皮细胞来源的实体肿瘤,且EpCAM表达为阳性。

[0028] .进一步,所述肿瘤包括上皮来源的恶性肿瘤和循环肿瘤细胞,以及肿瘤干细胞。

[0029] .进一步,所述肿瘤包括肠癌、肺癌、卵巢癌、肝癌或胃癌。

[0030] .EpCAM全称上皮细胞粘附分子(Epithelial cell adhesion molecule),是由肿瘤相关钙信号转导1基因编码的一个40KD的单次跨膜糖蛋白,参与调节细胞黏附和迁移,调控增殖和分化以及介导信号转导[14]。它在1979年被确定为肿瘤相关抗原[15],表达于肠癌,肺癌,前列腺癌,卵巢癌等大多数上皮来源的恶性肿瘤细胞[16]。

[0031] .本发明所述的嵌合抗原受体的合成方法,包括以下步骤:(1)构建Anti-EpCAM-VHH-CAR完整基因;(2)使用引物对Primer1和Primer2扩增所述Anti-EpCAM-VHH-CAR基因;(3)利用BamHI和EcoRI限制酶消化扩增出的基因序列并用病毒载体包装。

[0032] .进一步,所述病毒载体包括慢病毒载体、腺病毒载体或反转录病毒载体。

[0033] .进一步,所述引物Primer1的序列如Seq ID NO.46所示,引物Primer2的序列如Seq ID NO.47所示。

[0034] .一种特异性靶向肿瘤EpCAM抗原的VHH抗体或其片段在制备双特异性抗体和抗体-药物偶联药物ADC中的应用,所述VHH抗体或其片段具有特异性靶向肿瘤EpCAM抗原的互补决定区CDR,所述互补决定区CDR可选地选自Seq ID NO.10-18中氨基酸序列的一个或多个。

[0035] .一种特异性靶向肿瘤EpCAM抗原的VHH抗体或其片段在制备肿瘤诊断试剂盒的应用,所述VHH抗体或其片段具有特异性靶向肿瘤EpCAM抗原的互补决定区CDR,所述互补决定区CDR可选地选自Seq ID NO.10-18中氨基酸序列的一个或多个。

[0036] .进一步,所述肿瘤为上皮细胞来源的实体肿瘤,且EpCAM表达为阳性。

[0037] .有益效果

[0038] .本发明首先提供了一种特异性靶向肿瘤EpCAM抗原的抗体。如本领域技术人员所知,EpCAM是一种肿瘤相关性抗原,其在肿瘤细胞中高表达,但在正常上皮来源细胞中也表达,虽然表达量相对较低,但是该靶点缺乏肿瘤特异性。本发明团队在选择靶向EpCAM的抗体时,着重考虑了抗体的亲和力问题。当抗体亲和力太高时,虽然能识别EpCAM高表达的肿

瘤细胞,但同时会导致CAR-T细胞识别并攻击EpCAM低表达的正常上皮来源细胞;而当抗体的亲和力太低时,则不能有效识别EpCAM表达的肿瘤细胞,导致治疗失败。所以本发明筛选的抗EpCAM的VHH抗体亲和力适中,能够保证有效识别到肿瘤细胞,同时保证其不会识别攻击正常的EpCAM低表达上皮细胞。

[0039] .本发明利用筛选出的亲和力适中的纳米级VHH抗体或其片段来构建第二代和第三代嵌合抗原受体分子核酸序列,所述嵌合抗原受体分子在T细胞、NK细胞等免疫效应细胞中表达能够赋予这些免疫细胞靶向识别并裂解表达EpCAM分子的肿瘤细胞的能力,达到治疗肿瘤疾病的目的。

[0040] .具体来说,本发明构建的Anti-EpCAM-VHH纳米抗体对EpCAM表达阳性细胞具有高亲和力,用Anti-EpCAM-VHH-CAR转导待治疗患者的T淋巴细胞,将转导后的T淋巴细胞回输给患者,能使成功转导的T细胞重定向到EpCAM阳性表达的靶细胞,产生免疫响应。该方法能增强T细胞对靶细胞的免疫应答。用Anti-EpCAM-VHH-CAR转导T细胞表达嵌合抗原受体的过程能够在体外完成,也能够在体内进行,最终转导成功的细胞都为Anti-EpCAM-VHH-CAR-T细胞。

[0041] .此外,本发明筛选出的该抗体或其片段也可用于双特异性结合抗体、抗体-药物偶联药物等靶向治疗产品的研究,同时也可应用于抗体类检测产品。

附图说明

[0042] .为了更清楚地说明本发明实施例或现有技术中的技术方案,下面将对实施例或现有技术描述中所需要使用的附图作一简单地介绍。显而易见地,下面描述中的附图是本发明的一些实施例,对于本领域普通技术人员来讲,在不付出创造性劳动性的前提下,还可以根据这些附图获得其它的附图。

[0043] .图1为Anti-EpCAM-VHH hFc蛋白分子量鉴定:经HEK293细胞表达的VHH-hFc融合蛋白经二硫苏糖醇(DTT)还原,用SDS-PAGE电泳鉴定相对分子量大小位于43-55kD之间;

[0044] .图2为不同VHH hFc结合EpCAM-His的ELISA检测结果;

[0045] .图3为Anti-EpCAM-VHH-CAR结构:Anti-EpCAM-VHH-CAR基因装载在本发明自构的慢病毒载体上,利用EF1-α启动子启动CAR基因表达,胞外域为IL-2引导序列和EpCAM-VHH,胞内域为CD8a铰链区和跨膜区、CD28和4-1BB以及CD3ζ胞内段氨基酸残基;

[0046] .图4为Anti-EpCAM-VHH-CAR在细胞膜上的表达模式:Anti-EpCAM-VHH-CAR的EpCAM-VHH和CD8a铰链区位于细胞膜外侧,用于识别靶抗原,CD8a跨膜区镶嵌在细胞膜上,用于在膜上固定CAR分子,信号转导域位于膜内,用于传导放大细胞激活信号;

[0047] .图5为Anti-EpCAM-VHH-CAR在T细胞中表达检测:利用流式细胞术,使用异硫氰酸酯(FITC)标记的兔抗羊驼抗体能够检测到CAR分子的表达情况;

[0048] .图6为Anti-EpCAM-VHH-CAR-T靶向EpCAM表达阳性细胞释放细胞因子;

[0049] .图7为Anti-EpCAM-VHH-CAR-T细胞毒性检测。

具体实施方式

[0050] .为使本发明实施例的目的、技术方案和优点更加清楚,下面将结合本发明实施例中的附图,对本发明实施例中的技术方案进行清楚、完整地描述。显然,所描述的实施例是

本发明一部分实施例,而不是全部的实施例。基于本发明中的实施例,本领域普通技术人员在没有作出创造性劳动前提下所获得的所有其他实施例,都属于本发明保护的范围。

[0051] .需要说明的是,在本文中,术语“包括”、“包含”或者其任何其他变体意在涵盖非排他性的包含,从而使得包括一系列要素的过程、方法、物品或者装置不仅包括那些要素,而且还包括没有明确列出的其他要素,或者是还包括为这种过程、方法、物品或者装置所固有的要素。在没有更多限制的情况下,由语句“包括一个……”限定的要素,并不排除在包括该要素的过程、方法、物品或者装置中还存在另外的相同要素。

[0052] .如在本说明书中使用的,术语“大约”,典型地表示为所述值的+/-5%,更典型的是所述值的+/-4%,更典型的是所述值的+/-3%,更典型的是所述值的+/-2%,甚至更典型的是所述值的+/-1%,甚至更典型的是所述值的+/-0.5%。

[0053] .在本说明书中,某些实施方式可能以一种处于某个范围的格式公开。应该理解,这种“处于某个范围”的描述仅仅是为了方便和简洁,且不应该被解释为对所公开范围的僵化限制。因此,范围的描述应该被认为是已经具体地公开了所有可能的子范围以及在此范围内的独立数字值。例如,范围1～6的描述应该被看作已经具体地公开了子范围如从1到3,从1到4,从1到5,从2到4,从2到6,从3到6等,以及此范围内的单独数字,例如1,2,3,4,5和6。无论该范围的广度如何,均适用以上规则。

[0054] .名词解释

[0055] .本发明所述的“特异性靶向肿瘤EpCAM抗原”是指本发明提供的VHH抗体或其片段能靶向肿瘤细胞上表达丰度较高的EpCAM,而对其他EpCAM低表达的上皮细胞识别能力较弱。

[0056] .本发明所述的“表位”是指抗原上被抗体识别、结合并与抗体相互作用的部位。

[0057] .在本发明的其中一个实施例中,Anti-EpCAM-VHH-CAR分子的胞外抗原识别域Anti-EpCAM-VHH能够识别并结合人EpCAM分子。

[0058] .在本发明的其中一个实施例中,铰链区和跨膜区来源于CD8α,也可以是CD8α以外的其它分子,如CD4,CD28等。共刺激信号转导域来源包括但不限于CD28,4-1BB,也可以来源于ICOS,OX40等,且共刺激分子数量可以为1个或多个。

[0059] .在本发明的其中一个实施例中,CD3ζ信号转导域为CD3ζ分子胞内段61-164氨基酸残基。CD3ζ的61-164氨基酸残基含3个受体酪氨酸激活基序(ITAM),分别为氨基酸残基61-89,100-128,131-159,三个ITAM均能独立介导CD3ζ激活信号转导[28]。因而在CAR分子结构中使用CD3ζ胞内段、单独或任意组合使用CD3ζITAM构建的CAR分子均包含在本发明范围内。

[0060] .在本发明的其中一个实施例中,CD3ζ信号转导域也可以由其它信号转导结构替换,如FcRγ,数目可以为一个或多个。通过改变胞外抗原识别域以外的其它区域构建的嵌合抗原受体都在本发明范围内。

[0061] .在本发明的其中一个实施例中,通过在T淋巴细胞中表达Anti-EpCAM-VHH-CAR核酸序列,能够使在T细胞中产生Anti-EpCAM-VHH-CAR分子。此外,该核酸序列也能够在NK细胞中能够表达,因此构建的CAR-NK细胞,也在本发明范围内。

[0062] .在本发明的其中一个实施例中,Anti-EpCAM-VHH-CAR核酸分子是构建在慢病毒载体质粒上,利用EF1-α启动子启动CAR基因表达,除慢病毒载体外,使用其它的基因转移方式表达本发明的CAR,如逆转录病毒载体、转座子系统,电穿孔法、CRISPR/Cas9等基因编辑

工具,也包含在本发明范围内。

[0063] .在本发明的其中一个实施例中,VHH抗体或其片段通过大肠杆菌表达蛋白免疫对羊驼定期免疫得到。

[0064] .在本发明的其中一个实施例中,VHH抗体或其片段经过转录和扩增后为单域抗体基因片段。

[0065] .以下通过具体实施例进行说明:

[0066] .实施例1

[0067] .特异性靶向人肿瘤EpCAM抗原的VHH抗体制备

[0068] .(1)羊驼免疫和cDNA文库构建。使用人EpCAM抗原1.5mg与佐剂1:1混合后多点注射到羊驼颈部淋巴结附近进行免疫,每周免疫两次,一共进行7次免疫。从第4次免疫开始每次采集外周血进行免疫评估并分离外周血淋巴细胞。用Trizol液裂解淋巴细胞后提取淋巴细胞总RNA,利用反转录试剂盒将提取到的RNA反转录成cDNA,再利用cDNA文库构建试剂盒构建大肠杆菌cDNA文库。

[0069] .(2)噬菌体文库制备。将大肠杆菌cDNA文库菌接种到含四环素和氨苄抗生素的2X YT培养基中,37℃震荡培养直到OD_{600}=0.5。向培养体系中加入辅助噬菌体M13KO7至终浓度1E12 cfu/ml,37℃培养一小时后加入卡那霉素,后30℃培养过夜。之后将培养液5000g离心10分钟取上清,加入PEG/NaCl溶液混匀后4℃静置20分钟。再将溶液4000g离心20分钟弃上清,向沉淀中加入PBS悬浮沉淀,后16000g离心10min即得到噬菌体文库。

[0070] .(3)噬菌体筛选VHH。将文库噬菌体和提前包被EpCAM-camFc(人EpCAM与羊驼Fc重组蛋白)的免疫管用3%BSA液室温封闭2h.将封闭后的免疫管用PBS清洗多次,然后将封闭后的噬菌体加入免疫管中,室温旋转孵育1h。用PBS-T液清洗免疫管多次后,加入100mM TEA洗脱,室温孵育10分钟,再加入1M Tris-HCl,所得的洗脱液即为筛选后的噬菌体。该筛选过程需重复2次以去除非特异性噬菌体。

[0071] .(4)噬菌体ELISA鉴定VHH。将步骤(3)得到的噬菌体稀释后接种到OD_{600}=0.5的SS320菌液中,37℃培养30分钟后取培养液涂布2X YT平板。37℃培养过夜后,挑取单克隆接种于含2X YT的48孔板中,37℃培养3-4小时,再向孔板中的加入卡那霉素和辅助噬菌体,30℃过夜培养,离心培养物获得上清液。用EpCAM-camFc提前包被96孔板,并用3%BSA封闭孔板,加入上清液室温孵育1小时,经PBS清洗后,用anti-M13 HRP结合,再用TMB显色,测定OD_{450}值,根据OD_{450}值大小判断不同VHH和EpCAM的亲和力。对比样本孔和阴性对照的OD_{450}值,OD_{450}显著升高的孔对应的蛋白样品进行测序,即获得不同VHH氨基酸序列。通过序列对比去除具有相同CDR区的序列和冗余序列后,得到9条单一氨基酸序列(ID NO.1-9)。利用IMGT数据库(国际免疫遗传学数据库)分析序列确定每条序列的CDR区。在下面列出的序列中标黑加粗的片段,从左到右依次为CDR1、CDR2、CDR3。不同的CDR区序列的组合有不同的VHH-EpCAM亲和力。

[0072] .NO.1:

[0073] DVQLVESGGGSVQSGGSLRLSCAAS***GYTYRRYYMG***WFRQAPGEQRE***GVAVINNDGRTNY ADSVKG***RFRISRDNAENTLHLEMNSLKPEDTAMYYC***AATGNILPPMTAVPPLGRQWYPY***WGRGTLVTVSS

[0074] .NO.2:

[0075] HVQLVESGGGSVQSGGSLRLSCAAS***GYAVKNCMG***WFRQAPGKERE***GVAVINRNGITTYAD***

[0076] ***SVKG***RFTISQDKDKNTLDLQMNSLKPEDTAMYYC***AATPTLLTIPARFLCDVRNPSGFTD***WGQGTLVTVSS

[0077] .NO.3:

[0078] QVQLVESGGGSVQAGGSLRLSCVVS***AYSAYTYKTMCMG***WFRQAPGKERE***GVAAIYRGGLNTY***YADSVKGRFIISRDNAESTMYLQMNSLKPEDTAMYYC***AADWLRGDDCNIGANFDY***WGQGTQVTVSS

[0079] .NO.4:

[0080] QVQLVESGGGSVQAGGSLRLSCVAT***GFTISRKCMG***WFREAPGKKREVIA***TINTGSSSPYYADGVKG***RFTISQDNAKNTVYLQMNSLKPEDTAMYYC***AATKGVVVGTGYCGGPYVERPNSAY***WGQGTQVTVSS

[0081] .NO.5:

[0082] DVQLVESGGGSVQAGRSLRLSCELS***DYTWSTVCMG***WFRQAPGKEREGVAV***IYTRSGGTTYADSAKG***RFTISRDNAKDTLYLQMDSLKPEDTAMYYC***AAGPLYDGRCTYRSPAFHY***WGQGTQVTVSS

[0083] .NO.6:

[0084] DVQLVESGGGSAQAGGSLRLSCAAS***GPTSSLRTMG***WFRQASGKERERVA***VIWDGRTTDYDDSVQD***RFTISQDNAKSTVYLQMNTLKPEDTAMYYC***AASPRIVPFASTYFQH***WGQGTQVTVSS

[0085] .NO.7:

[0086] HVQLVESGGGSVQAGGSLKLSCAAS***GSIFSGSIFSRCGMR***WYRQAPGKERELVS***STSKDGFTSYTDSVKG***RFTISQDNANNTLYLQMSSLKTEDTAVYSC***AAICAVGGYSLSTYTY***WGQGTQVTVSS

[0087] .NO.8:

[0088] EVQLVESGGDSVQAGGSLRLSCAAS***GYSPGSYCMG***WFRQAPGKERERVA***IIESRGTVTYVDSVKG***RFTISKDNAKNTLYLQMNSLKPEDTAMYYC***AASRPWSGVRCLHDKYDY***WGQGTQVTVSS

[0089] .NO.9:

[0090] HVQLVESGGGSVQSGGSLRLSCAVS***GYAYSSLA***WFRQAPGKEREGVA***ALLTAIGGPTRTTYADSVKG***RLAISQDHAKNTLYLQMSSLKPEDTAMYYC***AAGRPAGTPRWLLLAPRDYNY***WGQGTQVTVSS

[0091] .实施例2

[0092] .Anti-EpCAM-VHH抗体鉴定。为了在分子水平和细胞水平鉴定Anti-EpCAM-VHH,将筛选到的Anti-EpCAM-VHH融合到人源信号肽和humanFc(hFc,约29kD)的N端,转换到哺乳动物瞬转表达载体pQDFc上,进行HEK293细胞瞬转表达。经过亲和纯化,得到VHH-hFc融合蛋白。经HEK293细胞表达的VHH-hFc融合蛋白经二硫苏糖醇(DTT)还原,用SDS-PAGE电泳鉴定相对分子量大小在43-55kD之间(图1)。

[0093] .实施例3

[0094] .VHH-hFc结合能力鉴定。VHH-hFc结合能力鉴定用ELISA方法,鉴定VHH-hFc与EpCAM抗原的结合能力。将EpCAM-His蛋白1ng/ul包被在96孔板中,4度置放过夜,后用5倍梯度稀释的VHH-hFc蛋白(起始溶度为20ng/ul)与孔板中的EpCAM-His蛋白结合1小时,再用anti-hFc-HRP二抗结合VHH-hFc,显色后检测450nm波长下的OD值,根据OD450判断VHH-hFc与EpCAM-His蛋白的结合能力。

[0095] .实验结论:筛选到的9条Anti-EpCAM-VHH序列与EpCAM有不同程度的亲和力,其中1194亲和力最强,1201亲和力最弱,VHH 1191与EpCAM的亲和力处于中等水平(图2)。

[0096] .实施例4

[0097] .靶向EpCAM的嵌合抗原受体(Anti-EpCAM-VHH-CAR)构建及慢病毒载体构建。在Anti-EpCAM-VHH-CAR构建过程中,包含Anti-EpCAM-VHH的胞外段、CD8α跨膜区与胞内信号转导域(8α28BBζ)为合成的完整基因。使用Primer1:5‘-CGGGATCCATGTACCGGATGCAG-3’(SEQ ID NO.46)和Primer2:5‘-CGGAATTCTTAGCGAGGGGGC-3’(SEQ ID NO.47)扩增Anti-EpCAM-VHH-CAR基因。在引物序列ID NO.46和ID NO.47中分别添加了BamHI和EcoRI限制性内切酶位点,扩增得到的Anti-EpCAM-VHH-CAR核苷酸在5’段引入了BamHI位点,在3’端引入了EcoRI位点,利用BamHI和EcoRI限制酶消化Anti-EpCAM-VHH-CAR产物和自构载体PCLK质粒,能够在两种产物中获得相同的BamHI和EcoRI粘性末端,便于两种基因片段在相同的粘性末端利用T4连接酶连接成完整的DNA质粒环。

[0098] .实施例5

[0099] .人淋巴细胞培养及慢病毒转导。提取健康人的外周血单核细胞,培养在人淋巴细胞培养基X-vivo-15中,添加5%FBS和100IU/ml人IL-2。用CD3/CD28抗体包被的磁珠刺激细胞(磁珠:细胞=3:1)24小时,使T细胞被激活。再在包被了纤维连接蛋白(50ug/ml)的孔板中加入慢病毒颗粒和激活后的T细胞,感染复数为2-4,1000xg离心2小时促进慢病毒感染T细胞。成功被慢病毒感染的T细胞表达Anti-EpCAM-VHH-CAR基因,即为Anti-EpCAM-VHH-CAR-T细胞。

[0100] .实施例6

[0101] .人淋巴细胞表达嵌合抗原受体检测在逆转录感染48-72小时进行。由于Anti-EpCAM-VHH-CAR胞外域的Anti-EpCAM-VHH为羊驼来源,使用兔抗羊驼VHH抗体(Genscript,南京)能够进行检测,检测方法为流式细胞术。

[0102] .实施例7

[0103] .靶向特异性细胞因子释放检测。由于Anti-EpCAM-VHH-CAR-T对EpCAM阳性表达的细胞具有特异性细胞毒作用,本发明选用了三种肿瘤细胞作为靶细胞,分别是高表达EpCAM的人结肠癌肿瘤细胞HT29,低表达EpCAM的人卵巢癌细胞SKOV3和不表达EpCAM的人宫颈癌细胞Hela。在细胞因子检测前,将$1*10^5$个(E:T=10:1)或$5*10^4$(E:T=5:1)个Anti-EpCAM-VHH-CAR-T细胞分别与三种肿瘤细胞在96孔板中共培养,肿瘤细胞数为$1*10^4$个/孔,共培养24小时候后取共培养上清检测IFN-γ、TNF-α和IL-2,检测方法为酶联免疫吸附试验。

[0104] .实验结论:Anti-EpCAM-VHH-CAR-T细胞在共培养体系中识别并结合靶细胞表面的EpCAM,促使CAR-T细胞效应功能被激活。释放大量的细胞因子,如IFN-γ,TNF-α等,通过对共培养上清中的细胞因子分泌量进行检测,能够评估CAR-T细胞的靶向特异性激活程度。图6中的CAR-T细胞能够被EpCAM阳性的HT29和SKOV3细胞刺激释放细胞因子,但是不能被EpCAM阴性的Hela细胞所激活,且CAR-T细胞的因子释放水平随着靶细胞抗原表达的增强而升高,具有抗原依赖性。

[0105] .实施例8

[0106] .细胞毒性检测。使用实时细胞毒性检测法测定Anti-EpCAM-VHH-CAR-T对肿瘤细胞毒性。具体方案为取HT29和Hela细胞铺实施标记检测板,细胞数为$1*10^4$个/孔,至于TRCA检测仪上记录细胞贴壁情况,铺板24小时细胞贴壁稳定后按照设定的效应细胞:靶细胞(E:T)比例添加Anti-EpCAM-CAR-T细胞,后置于TRCA检测仪上记录细胞贴壁情况。靶细胞被

CAR-T细胞杀伤后将失去贴壁性。

[0107] .实验结论:通过实时检测靶细胞的贴壁性来评估CAR-T细胞的细胞毒性。图7显示在靶细胞HT29和Hela中加入T细胞后,ControlT细胞组HT29细胞数有所增加后缓慢减少,而CAR-T细胞组的HT29细胞迅速减少;在Hela细胞中加入Control T细胞和CAR-T细胞后,细胞均同等程度的减少,无显著变化。证实CAR-T细胞具有靶向EpCAM+细胞毒性。

[0108] .本发明涉及的序列如下表所示:

[0109]

编号	实验室命名	序列
ID NO.1	VHH 氨基酸 1191	DVQLVESGGGSVQSGGSLRLSCAASGYTYRRYYMGWFRQAPGEQREGVAVINNDGRTNYADSVKGRFRISRDNAENTLHLEMNSLKPEDTAMYYCAATGNILPPMTAVPPLGRQWYPYWGRGTLVTVSS
ID NO.2	VHH 氨基酸 1201	HVQLVESGGGSVQSGGSLRLSCAASGYAVKNCMGWFRQAPGKEREGVAVINRNGITTYADSVKGRFTISQDKDKNTLDLQMNSLKPEDTAMYYCAATPTLLTIPARFLCDVRNPSGFTDWGQGTLVTVSS
ID NO.3	VHH 氨基酸 1193	QVQLVESGGGSVQAGGSLRLSCVVSAYSAYTYKTMCMGWFRQAPGKEREGVAAIYRGGLNTYYADSVKGRFIISRDNAESTMYLQMNSLKPEDTAMYYCAADWLRGDDCNIGANFDYWGQGTQVTVSS
ID NO.4	VHH 氨基酸 1200	QVQLVESGGGSVQAGGSLRLSCVATGFTISRKCMGWFREAPGKKREVIATINTGSSSPYYADGVKGRFTISQDNAKNTVYLQMNSLKPEDTAMYYCAATKGVVVGTGYCGGPYVERPNSAYWGQGTQVTVSS
ID NO.5	VHH 氨基酸 1194	DVQLVESGGGSVQAGRSLRLSCELSDYTWSTVCMGWFRQAPGKEREGVAVIYTRSGGTTYADSAKGRFTISRDNAKDTLYLQMDSLKPEDTAMYYCAAGPLYDGRCTYRSPAFHYWGQGTQVTVSS
ID NO.6	VHH 氨基酸 1196	DVQLVESGGGSAQAGGSLRLSCAASGPTSSLRTMGWFRQASGKERERVAVIWDGRTTDYDDSVQDRFTISQDNAKSTVYLQMNTLKPEDTAMYYCAASPRIVPFASTYFQHWGQGTQVTVSS
ID NO.7	VHH 氨基酸 1197	HVQLVESGGGSVQAGGSLKLSCAASGSIFSGSIFSRCGMRWYRQAPGKERELVSSTSKDGFTSYTDSVKGRFTISQDNANNTLYLQMSSLKTEDTAVYSCAAICAVGGYSLSTYTYWGQGTQVTVSS
ID NO.8	VHH 氨基酸 1199	EVQLVESGGDSVQAGGSLRLSCAASGYSPGSYCMGWFRQAPGKERERVAIIESRGTVTYVDSVKGRFTISKDNAKNTLYLQMNSLKPEDTAMYYCAASRPWSGVRCLHDKYDYWGQGTQVTVSS
ID NO.9	VHH 氨基酸 1198	HVQLVESGGGSVQSGGSLRLSCAVSGYAYSSLAWFRQAPGKEREGVAALLTAIGGPTRTTYADSVKGRLAISQDHAKNTLYLQMSSLKPEDTAMYYCAAGRPAGTPRWLLLAPRDYNYWGQGTQVTVSS
ID NO.10	VHH 1191CDR	DVQLVESGGGSVQSGGSLRLSCAASGYTYRRYYMGWFRQAPGEQREGVAVINNDGRTNYADSVKGRFRISRDNAENTLHLEMNSLKPEDTAMYYCAATGNILPPMTAVPPLGRQWYPY

[0110]

ID NO.11	VHH 1201CDR	HVQLVESGGGSVQSGGSLRLSCAASGYAVKNCMGWFRQAPGKEREGVAVINRNGITTYADSVKGRFTISQDKDKNTLDLQMNSLKPEDTAMYYCAATPTLLTIPARFLCDVRNPSGFTD
ID NO.12	VHH 1193CDR	QVQLVESGGGSVQAGGSLRLSCVVSAYSAYTYKTMCMGWFRQAPGKEREGVAAIYRGGLNTYYADSVKGRFIISRDNAESTMYLQMNSLKPEDTAMYYCAADWLRGDDCNIGANFDY
ID NO.13	VHH 1200CDR	QVQLVESGGGSVQAGGSLRLSCVATGFTISRKCMGWFREAPGKKREVIATINTGSSSPYYADGVKGRFTISQDNAKNTVYLQMNSLKPEDTAMYYCAATKGVVVGTGYCGGPYVERPNSAY
ID NO.14	VHH 1194CDR	DVQLVESGGGSVQAGRSLRLSCELSDYTWSTVCMGWFRQAPGKEREGVAVIYTRSGGTTYADSAKGRFTISRDNAKDTLYLQMDSLKPEDTAMYYCAAGPLYDGRCTYRSPAFHY
ID NO.15	VHH 1196CDR	DVQLVESGGGSAQAGGSLRLSCAASGPTSSLRTMGWFRQASGKERERVAVIWDGRTTDYDDSVQDRFTISQDNAKSTVYLQMNTLKPEDTAMYYCAASPRIVPFASTYFQH
ID NO.16	VHH 1197CDR	HVQLVESGGGSVQAGGSLKLSCAASGSIFSGSIFSRCGMRWYRQAPGKERELVSSTSKDGFTSYTDSVKGRFTISQDNANNTLYLQMSSLKTEDTAVYSCAAICAVGGYSLSTYTY
ID NO.17	VHH 1199CDR	EVQLVESGGDSVQAGGSLRLSCAASGYSPGSYCMGWFRQAPGKERERVAIIESRGTVTYVDSVKGRFTISKDNAKNTLYLQMNSLKPEDTAMYYCAASRPWSGVRCLHDKYDY
ID NO.18	VHH 1198CDR	HVQLVESGGGSVQSGGSLRLSCAVSGYAYSSLAWFRQAPGKEREGVAALLTAIGGPTRTTYADSVKGRLAISQDHAKNTLYLQMSSLKPEDTAMYYCAAGRPAGTPRWLLLAPRDYNY
ID NO.19	VHH 核苷酸 1191	GACGTGCAGCTGGTGGAGAGCGGCGGCGGCAGCGTGCAGAGCGGCGGCAGCCTGAGGCTGAGCTGCGCCGCCAGCGGCTACACCTACAGGAGGTACTACATGGGCTGGTTCAGGCAGGCCCCCGGCGAGCAGAGGGAGGGCGTGGCCGTGATCAACAACGACGGCAGGACCAACTACGCCGACAGCGTGAAGGGCAGGTTCAGGATCAGCAGGGACAACGCCGAGAACACCCTGCACCTGGAGATGAACAGCCTGAAGCCCGAGGACACCGCCATGTACTACTGCGCCGCCACCGGCAACATCCTGCCCCCCATGACCGCCGTGCCCCCCCTGGGCAGGCAGTGGTACCCCTACTGGGGCAGGGGCACCCTGGTGACCGTGAGCAGC
ID NO.20	VHH 核苷酸 1201	CACGTGCAGCTGGTGGAGAGCGGCGGCGGCAGCGTGCAGAGCGGCGGCAGCCTGAGGCTGAGCTGCGCCGCCAGCGGCTACGCCGTGAAGAACTGCATGGGCTGGTTCAGGCAGGCCCCCGGCAAGGAGAGGGAGGGCGTGGCCGTGATCAACAGGAACGGCATCACCACCTACGCCGACAGCGTGAAGGGCAGGTTCACCATCAGCCAGGACAAGGACAAGAACACCCTGGACCTGCAGATGAACAGCCTGAAGCCCGAGGACACCGCCATGTACTACTGCGCCGCCACCCCCACCCTGCTGACCATCCCCGCCAGGTTCCTGTGCGACGTGAGGAACCCCAGCGGCTTCACCGACTGGGGCCAGGGCACCCTGGTGACCGTGAGCAGC

[0111]

ID NO.21	VHH 核苷酸 1193	CAGGTGCAGCTGGTGGAGAGCGGCGGCGGCAGCGTGC AGGCCGGCGGCAGCCTGAGGCTGAGCTGCGTGGTGAG CGCCTACAGCGCCTACACCTACAAGACCATGTGCATGG GCTGGTTCAGGCAGGCCCCCGGCAAGGAGAGGGAGG GCGTGGCCGCCATCTACAGGGGCGGCCTGAACACCTA CTACGCCGACAGCGTGAAGGGCAGGTTCATCATCAGC AGGGACAACGCCGAGAGCACCATGTACCTGCAGATGA ACAGCCTGAAGCCCGAGGACACCGCCATGTACTACTG CGCCGCCGACTGGCTGAGGGGCGACGACTGCAACATC GGCGCCAACTTCGACTACTGGGGCCAGGGCACCCAGG TGACCGTGAGCAGC
ID NO.22	VHH 核苷酸 1200	CAGGTGCAGCTGGTGGAGAGCGGCGGCGGCAGCGTGC AGGCCGGCGGCAGCCTGAGGCTGAGCTGCGTGGCCAC CGGCTTCACCATCAGCAGGAAGTGCATGGGCTGGTTCA GGGAGGCCCCCGGCAAGAAGAGGGAGGTGATCGCCA CCATCAACACCGGCAGCAGCAGCCCCTACTACGCCGA CGGCGTGAAGGGCAGGTTCACCATCAGCCAGGACAAC GCCAAGAACACCGTGTACCTGCAGATGAACAGCCTGA AGCCCGAGGACACCGCCATGTACTACTGCGCCGCCACC AAGGGCGTGGTGGTGGGCACCGGCTACTGCGGCGGCC CCTACGTGGAGAGGCCCAACAGCGCCTACTGGGGCCA GGGCACCCAGGTGACCGTGAGCAGC
ID NO.23	VHH 核苷酸 1194	GACGTGCAGCTGGTGGAGAGCGGCGGCGGCAGCGTGC AGGCCGGCAGGAGCCTGAGGCTGAGCTGCGAGCTGAG CGACTACACCTGGAGCACCGTGTGCATGGGCTGGTTCA GGCAGGCCCCCGGCAAGGAGAGGGAGGGCGTGGCCG TGATCTACACCAGGAGCGGCGGCACCACCTACGCCGA CAGCGCCAAGGGCAGGTTCACCATCAGCAGGGACAAC GCCAAGGACACCCTGTACCTGCAGATGGACAGCCTGA AGCCCGAGGACACCGCCATGTACTACTGCGCCGCCGG CCCCCTGTACGACGGCAGGTGCACCTACAGGAGCCCC GCCTTCCACTACTGGGGCCAGGGCACCCAGGTGACCG TGAGCAGC
ID NO.24	VHH 核苷酸 1196	GACGTGCAGCTGGTGGAAAGCGGAGGAGGAAGCGCC CAGGCCGGAGGAAGCCTGAGACTGAGCTGCGCCGCCA GCGGACCCACCTCTTCACTGAGAACAATGGGATGGTTC AGACAGGCCTCTGGCAAAGAAAGAGAGAGGGTGGCC GTCATTTGGGATGGCAGAACAACCGACTACGACGACTC CGTGCAGGACAGATTCACCATCAGCCAGGACAACGCC AAGAGCACAGTCTATCTGCAGATGAACACACTGAAGC CCGAAGATACCGCCATGTACTACTGCGCAGCCAGCCCC AGAATCGTGCCCTTCGCCAGCACCTACTTCCAGCACTG GGGACAGGGAACCCAGGTGACCGTCAGCTCC
ID NO.25	VHH 核苷酸 1197	CACGTGCAGCTGGTGGAGAGCGGCGGCGGCAGCGTGC AGGCCGGCGGCAGCCTGAAGCTGAGCTGCGCCGCCAG CGGCAGCATCTTCAGCGGCAGCATCTTCAGCAGGTGCG GCATGAGGTGGTACAGGCAGGCCCCCGGCAAGGAGAG GGAGCTGGTGAGCAGCACCAGCAAGGACGGCTTCACC AGCTACACCGACAGCGTGAAGGGCAGGTTCACCATCA GCCAGGACAACGCCAACAACACCCTGTACCTGCAGAT

[0112]

		GAGCAGCCTGAAGACCGAGGACACCGCCGTGTACAGC TGCGCCGCCATCTGCGCCGTGGGCGGCTACAGCCTGAG CACCTACACCTACTGGGGCCAGGGCACCCAGGTGACC GTGAGCAGC
ID NO.26	VHH 核苷酸 1199	GAGGTGCAGCTGGTGGAGAGCGGCGGCGACAGCGTG CAGGCCGGCGGCAGCCTGAGGCTGAGCTGCGCCGCCA GCGGCTACAGCCCCGGCAGCTACTGCATGGGCTGGTTC AGGCAGGCCCCCGGCAAGGAGAGGGAGAGGGTGGCC ATCATCGAGAGCAGGGGCACCGTGACCTACGTGGACA GCGTGAAGGGCAGGTTCACCATCAGCAAGGACAACGC CAAGAACACCCTGTACCTGCAGATGAACAGCCTGAAG CCCGAGGACACCGCCATGTACTACTGCGCCGCCAGCA GGCCCTGGAGCGGCGTGAGGTGCCTGCACGACAAGTA CGACTACTGGGGCCAGGGCACCCAGGTGACCGTGAGC AGC
ID NO.27	VHH 核苷酸 1198	CACGTGCAGCTGGTGGAGAGCGGCGGCGGCAGCGTGC AGAGCGGCGGCAGCCTGAGGCTGAGCTGCGCCGTGAG CGGCTACGCCTACAGCAGCCTGGCCTGGTTCAGGCAG GCCCCCGGCAAGGAGAGGGAGGGCGTGGCCGCCCTGC TGACCGCCATCGGCGGCCCCACCAGGACCACCTACGC CGACAGCGTGAAGGGCAGGCTGGCCATCAGCCAGGAC CACGCCAAGAACACCCTGTACCTGCAGATGAGCAGCC TGAAGCCCGAGGACACCGCCATGTACTACTGCGCCGCC GGCAGGCCCGCCGGCACCCCCAGGTGGCTGCTGCTGG CCCCCAGGGACTACAACTACTGGGGCCAGGGCACCCA GGTGACCGTGAGCAGC
ID NO.28	CAR 氨基酸 1191	MYRMQLLSCIALSLALVTNSADVQLVESGGGSVQSGGSL RLSCAASGYTYRRYYMGWFRQAPGEQREGVAVINNDGR TNYADSVKGRFRISRDNAENTLHLEMNSLKPEDTAMYY CAATGNILPPMTAVPPLGRQWYPYWGRGTLVTVSSTTTP APRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFAC DIYIWAPLAGTCGVLLLSLVITRSKRSRLLHSDYMNMTPR RPGPTRKHYQPYAPPRDFAAYRSKRGRKKLLYIFKQPFM RPVQTTQEEDGCSCRFPEEEEGGCELAPAYQQGQNQLYN ELNLGRREEYDVLDKRRGRDPEMGGKPQRRKNPQEGLY NELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTAT KDTYDALHMQALPPR
ID NO.29	CAR 氨基酸 1201	MYRMQLLSCIALSLALVTNSAHVQLVESGGGSVQSGGSL RLSCAASGYAVKNCMGWFRQAPGKEREGVAVINRNGITT YADSVKGRFTISQDKDKNTLDLQMNSLKPEDTAMYYCA ATPTLLTIPARFLCDVRNPSGFTDWGQGTLVTVSSTTTPAP RPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIY IWAPLAGTCGVLLLSLVITRSKRSRLLHSDYMNMTPRRP GPTRKHYQPYAPPRDFAAYRSKRGRKKLLYIFKQPFMRP VQTTQEEDGCSCRFPEEEEGGCELAPAYQQGQNQLYNEL NLGRREEYDVLDKRRGRDPEMGGKPQRRKNPQEGLYNE LQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKD TYDALHMQALPPR
ID NO.30	CAR 氨基酸 1192	MYRMQLLSCIALSLALVTNSAHVQLVESGGGSVQAGGSL RLSCAASGATRRTTCVSWFRQAPGKERERLATILTGTSYT

[0113]

		NYADSVKGRFIISQDNAKNTVYLQMSSLKPEDTAMYYC AANLGGLCPPAEYAYWGQGTQVTVSSTTTPAPRPPTPAPT IASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAG TCGVLLLSLVITRSKRSRLLHSDYMNMTPRRPGPTRKHY QPYAPPRDFAAYRSKRGRKKLLYIFKQPFMRPVQTTQEE DGCSCRFPEEEEGGCELAPAYQQGQNQLYNELNLGRREE YDVLDKRRGRDPEMGGKPQRRKNPQEGLYNELQKDKM AEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALH MQALPPR
ID NO.31	CAR 氨基酸 1193	MYRMQLLSCIALSLALVTNSAQVQLVESGGGSVQAGGSL RLSCVVSAYSAYTYKTMCMGWFRQAPGKEREGVAAIYR GGLNTYYADSVKGRFIISRDNAESTMYLQMNSLKPEDTA MYYCAADWLRGDDCNIGANFDYWGQGTQVTVSSTTTP APRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFAC DIYIWAPLAGTCGVLLLSLVITRSKRSRLLHSDYMNMTPR RPGPTRKHYQPYAPPRDFAAYRSKRGRKKLLYIFKQPFM RPVQTTQEEDGCSCRFPEEEEGGCELAPAYQQGQNQLYN ELNLGRREEYDVLDKRRGRDPEMGGKPQRRKNPQEGLY NELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTAT KDTYDALHMQALPPR
ID NO.32	CAR 氨基酸 1200	MYRMQLLSCIALSLALVTNSAQVQLVESGGGSVQAGGSL RLSCVATGFTISRKCMGWFREAPGKKREVIATINTGSSSP YYADGVKGRFTISQDNAKNTVYLQMNSLKPEDTAMYYC AATKGVVVGTGYCGGPYVERPNSAYWGQGTQVTVSSTT TPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFA CDIYIWAPLAGTCGVLLLSLVITRSKRSRLLHSDYMNMTP RRPGPTRKHYQPYAPPRDFAAYRSKRGRKKLLYIFKQPF MRPVQTTQEEDGCSCRFPEEEEGGCELAPAYQQGQNQLY NELNLGRREEYDVLDKRRGRDPEMGGKPQRRKNPQEGL YNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTA TKDTYDALHMQALPPR
ID NO.33	CAR 氨基酸 1194	MYRMQLLSCIALSLALVTNSADVQLVESGGGSVQAGRSL RLSCELSDYTWSTVCMGWFRQAPGKEREGVAVIYTRSG GTTYADSAKGRFTISRDNAKDTLYLQMDSLKPEDTAMY YCAAGPLYDGRCTYRSPAFHYWGQGTQVTVSSTTTPAPR PPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYI WAPLAGTCGVLLLSLVITRSKRSRLLHSDYMNMTPRRPG PTRKHYQPYAPPRDFAAYRSKRGRKKLLYIFKQPFMRPV QTTQEEDGCSCRFPEEEEGGCELAPAYQQGQNQLYNELN LGRREEYDVLDKRRGRDPEMGGKPQRRKNPQEGLYNEL QKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDT YDALHMQALPPR
ID NO.34	CAR 氨基酸 1196	MYRMQLLSCIALSLALVTNSADVQLVESGGGSAQAGGSL RLSCAASGPTSSLRTMGWFRQASGKERERVAVIWDGRTT DYDDSVQDRFTISQDNAKSTVYLQMNTLKPEDTAMYYC AASPRIVPFASTYFQHWGQGTQVTVSSTTTPAPRPPTPAP TIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLA GTCGVLLLSLVITRSKRSRLLHSDYMNMTPRRPGPTRKH YQPYAPPRDFAAYRSKRGRKKLLYIFKQPFMRPVQTTQE EDGCSCRFPEEEEGGCELAPAYQQGQNQLYNELNLGRRE

[0114]

		EYDVLDKRRGRDPEMGGKPQRRKNPQEGLYNELQKDK MAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDAL HMQALPPR
ID NO.35	CAR 氨基酸 1197	MYRMQLLSCIALSLALVTNSAHVQLVESGGGSVQAGGSL KLSCAASGSIFSGSIFSRCGMRWYRQAPGKERELVSSTSK DGFTSYTDSVKGRFTISQDNANNTLYLQMSSLKTEDTAV YSCAAICAVGGYSLSTYTYWGQGTQVTVSSTTTPAPRPP TPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWA PLAGTCGVLLLSLVITRSKRSRLLHSDYMNMTPRRPGPTR KHYQPYAPPRDFAAYRSKRGRKKLLYIFKQPFMRPVQTT QEEDGCSCRFPEEEEGGCELAPAYQQGQNQLYNELNLGR REEYDVLDKRRGRDPEMGGKPQRRKNPQEGLYNELQKD KMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDA LHMQALPPR
ID NO.36	CAR 氨基酸 1198	MYRMQLLSCIALSLALVTNSAHVQLVESGGGSVQSGGSL RLSCAVSGYAYSSLAWFRQAPGKEREGVAALLTAIGGPTR TTYADSVKGRLAISQDHAKNTLYLQMSSLKPEDTAMYY CAAGRPAGTPRWLLLAPRDYNYWGQGTQVTVSSTTTPA PRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDI YIWAPLAGTCGVLLLSLVITRSKRSRLLHSDYMNMTPRR PGPTRKHYQPYAPPRDFAAYRSKRGRKKLLYIFKQPFMRP VQTTQEEDGCSCRFPEEEEGGCELAPAYQQGQNQLYNEL NLGRREEYDVLDKRRGRDPEMGGKPQRRKNPQEGLYNE LQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKD TYDALHMQALPPR
ID NO.37	CAR 核苷酸 1191	ATGTACCGGATGCAGCTGCTGAGCTGTATCGCCCTGAG CCTGGCCCTGGTGACCAACAGCGCCGACGTGCAGCTG GTGGAGAGCGGCGGCGGCAGCGTGCAGAGCGGCGGC AGCCTGAGGCTGAGCTGCGCCGCCAGCGGCTACACCT ACAGGAGGTACTACATGGGCTGGTTCAGGCAGGCCCC CGGCGAGCAGAGGGAGGGCGTGGCCGTGATCAACAAC GACGGCAGGACCAACTACGCCGACAGCGTGAAGGGCA GGTTCAGGATCAGCAGGGACAACGCCGAGAACACCCT GCACCTGGAGATGAACAGCCTGAAGCCCGAGGACACC GCCATGTACTACTGCGCCGCCACCGGCAACATCCTGCC CCCCATGACCGCCGTGCCCCCCCTGGGCAGGCAGTGGT ACCCCTACTGGGGCAGGGGCACCCTGGTGACCGTGAG CAGCACCACGACGCCAGCGCCGCGACCACCAACACCG GCGCCCACCATCGCGTCGCAGCCCCTGTCCCTGCGCCC AGAGGCGTGCCGGCCAGCGGCGGGGGGCGCAGTGCA CACGAGGGGGCTGGACTTCGCCTGTGATATCTACATCT GGGCGCCCCTGGCCGGGACTTGTGGGGTCCTTCTCCTG TCACTGGTTATCACCAGGAGTAAGAGGAGCAGGCTCCT GCACAGTGACTACATGAACATGACTCCCCGCCGCCCCG GGCCCACCCGCAAGCATTACCAGCCCTATGCCCCACCA CGCGACTTCGCAGCCTATCGCTCCAAACGGGGCAGAA AGAAACTCCTGTATATATTCAAACAACCATTTATGAGAC CAGTACAAACTACTCAAGAGGAAGATGGCTGTAGCTG CCGATTTCCAGAAGAAGAAGAAGGAGGATGTGAACTG GCCCCCGCGTACCAGCAGGGCCAGAACCAGCTCTATAA

[0115]

		CGAGCTCAATCTAGGACGAAGAGAGGAGTACGATGTT TTGGACAAGAGACGTGGCCGGGACCCTGAGATGGGGG GAAAGCCGCAGAGAAGGAAGAACCCTCAGGAAGGCC TGTACAATGAACTGCAGAAAGATAAGATGGCGGAGGC CTACAGTGAGATTGGGATGAAAGGCGAGCGCCGGAGG GGCAAGGGGCACGATGGCCTTTACCAGGGTCTCAGTA CAGCCACCAAGGACACCTACGACGCCCTTCACATGCA GGCCCTGCCCCCTCGCTAAGAATTCCG
ID NO.38	CAR 核苷酸 1201	ATGTACCGGATGCAGCTGCTGAGCTGTATCGCCCTGAG CCTGGCCCTGGTGACCAACAGCGCCCACGTGCAGCTG GTGGAGAGCGGCGGCGGCAGCGTGCAGAGCGGCGGC AGCCTGAGGCTGAGCTGCGCCGCCAGCGGCTACGCCG TGAAGAACTGCATGGGCTGGTTCAGGCAGGCCCCCGG CAAGGAGAGGGAGGGCGTGGCCGTGATCAACAGGAA CGGCATCACCACCTACGCCGACAGCGTGAAGGGCAGG TTCACCATCAGCCAGGACAAGGACAAGAACACCCTGG ACCTGCAGATGAACAGCCTGAAGCCCGAGGACACCGC CATGTACTACTGCGCCGCCACCCCCACCCTGCTGACCA TCCCCGCCAGGTTCCTGTGCGACGTGAGGAACCCCAG CGGCTTCACCGACTGGGGCCAGGGCACCCTGGTGACC GTGAGCAGCACCACGACGCCAGCGCCGCGACCACCAA CACCGGCGCCCACCATCGCGTCGCAGCCCCTGTCCCTG CGCCCAGAGGCGTGCCGGCCAGCGGCGGGGGGCGCA GTGCACACGAGGGGGCTGGACTTCGCCTGTGATATCTA CATCTGGGCGCCCCTGGCCGGGACTTGTGGGGTCCTTC TCCTGTCACTGGTTATCACCAGGAGTAAGAGGAGCAG GCTCCTGCACAGTGACTACATGAACATGACTCCCCGCC GCCCCGGGCCCACCCGCAAGCATTACCAGCCCTATGCC CCACCACGCGACTTCGCAGCCTATCGCTCCAAACGGGG CAGAAAGAAACTCCTGTATATATTCAAACAACCATTTAT GAGACCAGTACAAACTACTCAAGAGGAAGATGGCTGT AGCTGCCGATTTCCAGAAGAAGAAGAAGGAGGATGTG AACTGGCCCCCGCGTACCAGCAGGGCCAGAACCAGCT CTATAACGAGCTCAATCTAGGACGAAGAGAGGAGTAC GATGTTTTGGACAAGAGACGTGGCCGGGACCCTGAGA TGGGGGGAAAGCCGCAGAGAAGGAAGAACCCTCAGG AAGGCCTGTACAATGAACTGCAGAAAGATAAGATGGC GGAGGCCTACAGTGAGATTGGGATGAAAGGCGAGCGC CGGAGGGGCAAGGGGCACGATGGCCTTTACCAGGGTC TCAGTACAGCCACCAAGGACACCTACGACGCCCTTCA CATGCAGGCCCTGCCCCCTCGCTAAGAATTCCG
ID NO.39	CAR 核苷酸 1192	ATGTACCGGATGCAGCTGCTGAGCTGTATCGCCCTGAG CCTGGCCCTGGTGACCAACAGCGCCCACGTGCAGCTG GTGGAGAGCGGCGGCGGCAGCGTGCAGGCCGGCGGC AGCCTGAGGCTGAGCTGCGCCGCCAGCGGCGCCACCA GGAGGACCACCTGCGTGAGCTGGTTCAGGCAGGCCCC CGGCAAGGAGAGGGAGAGGCTGGCCACCATCCTGACC GGCACCAGCTACACCAACTACGCCGACAGCGTGAAGG GCAGGTTCATCATCAGCCAGGACAACGCCAAGAACAC CGTGTACCTGCAGATGAGCAGCCTGAAGCCCGAGGAC

			ACCGCCATGTACTACTGCGCCGCCAACCTGGGCGGCCT GTGCCCCCCCGCCGAGTACGCCTACTGGGGCCAGGGC ACCCAGGTGACCGTGAGCAGCACCACGACGCCAGCGC CGCGACCACCAACACCGGCGCCCACCATCGCGTCGCA GCCCCTGTCCCTGCGCCCAGAGGCGTGCCGGCCAGCG GCGGGGGGCGCAGTGCACACGAGGGGGCTGGACTTCG CCTGTGATATCTACATCTGGGCGCCCCTGGCCGGGACT TGTGGGGTCCTTCTCCTGTCACTGGTTATCACCAGGAG TAAGAGGAGCAGGCTCCTGCACAGTGACTACATGAAC ATGACTCCCCGCCGCCCCGGGCCCACCCGCAAGCATTA CCAGCCCTATGCCCCACCACGCGACTTCGCAGCCTATC GCTCCAAACGGGGCAGAAAGAAACTCCTGTATATATTC AAACAACCATTTATGAGACCAGTACAAACTACTCAAGA GGAAGATGGCTGTAGCTGCCGATTTCCAGAAGAAGAA GAAGGAGGATGTGAACTGGCCCCCGCGTACCAGCAGG GCCAGAACCAGCTCTATAACGAGCTCAATCTAGGACGA AGAGAGGAGTACGATGTTTTGGACAAGAGACGTGGCC GGGACCCTGAGATGGGGGGAAAGCCGCAGAGAAGGA AGAACCCTCAGGAAGGCCTGTACAATGAACTGCAGAA AGATAAGATGGCGGAGGCCTACAGTGAGATTGGGATG AAAGGCGAGCGCCGGAGGGGCAAGGGGCACGATGGC CTTTACCAGGGTCTCAGTACAGCCACCAAGGACACCTA CGACGCCCTTCACATGCAGGCCCTGCCCCCTCGCTAAG AATTCCG
[0116]	ID NO.40	CAR 核苷酸 1193	ATGTACCGGATGCAGCTGCTGAGCTGTATCGCCCTGAG CCTGGCCCTGGTGACCAACAGCGCCCAGGTGCAGCTG GTGGAGAGCGGCGGCGGCAGCGTGCAGGCCGGCGGC AGCCTGAGGCTGAGCTGCGTGGTGAGCGCCTACAGCG CCTACACCTACAAGACCATGTGCATGGGCTGGTTCAGG CAGGCCCCCGGCAAGGAGAGGGAGGGCGTGGCCGCC ATCTACAGGGGCGGCCTGAACACCTACTACGCCGACAG CGTGAAGGGCAGGTTCATCATCAGCAGGGACAACGCC GAGAGCACCATGTACCTGCAGATGAACAGCCTGAAGC CCGAGGACACCGCCATGTACTACTGCGCCGCCGACTGG CTGAGGGGCGACGACTGCAACATCGGCGCCAACTTCG ACTACTGGGGCCAGGGCACCCAGGTGACCGTGAGCAG CACCACGACGCCAGCGCCGCGACCACCAACACCGGCG CCCACCATCGCGTCGCAGCCCCTGTCCCTGCGCCCAGA GGCGTGCCGGCCAGCGGCGGGGGGCGCAGTGCACAC GAGGGGGCTGGACTTCGCCTGTGATATCTACATCTGGG CGCCCCTGGCCGGGACTTGTGGGGTCCTTCTCCTGTCA CTGGTTATCACCAGGAGTAAGAGGAGCAGGCTCCTGC ACAGTGACTACATGAACATGACTCCCCGCCGCCCCGGG CCCACCCGCAAGCATTACCAGCCCTATGCCCCACCACG CGACTTCGCAGCCTATCGCTCCAAACGGGGCAGAAAG AAACTCCTGTATATATTCAAACAACCATTTATGAGACCA GTACAAACTACTCAAGAGGAAGATGGCTGTAGCTGCC GATTTCCAGAAGAAGAAGAAGGAGGATGTGAACTGGC CCCCGCGTACCAGCAGGGCCAGAACCAGCTCTATAACG AGCTCAATCTAGGACGAAGAGAGGAGTACGATGTTTTG

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[0121] .本发明涉及到的参考文献

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[0150] .上面结合附图对本发明的实施例进行了描述,但是本发明并不局限于上述的具体实施方式,上述的具体实施方式仅仅是示意性的,而不是限制性的,本领域的普通技术人员在本发明的启示下,在不脱离本发明宗旨和权利要求所保护的范围情况下,还可做出很多形式,这些均属于本发明的保护之内。

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[0193] Val Lys Gly Arg Leu Ala Ile Ser Gln Asp His Ala Lys Asn Thr Leu
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[0219] Ala Thr Gly Asn Ile Leu Pro Pro Met Thr Ala Val Pro Pro Leu Gly

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[0235] 50 55 60

[0236] Arg Phe Thr Ile Ser Gln Asp Lys Asp Lys Asn Thr Leu Asp Leu Gln

[0237] 65 70 75 80

[0238] Met Asn Ser Leu Lys Pro Glu Asp Thr Ala Met Tyr Tyr Cys Ala Ala

[0239] 85 90 95

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[0254] 35 40 45

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[0257] Asp Ser Val Lys Gly Arg Phe Ile Ile Ser Arg Asp Asn Ala Glu Ser

[0258] 65 70 75 80

[0259] Thr Met Tyr Leu Gln Met Asn Ser Leu Lys Pro Glu Asp Thr Ala Met

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[0262] 100 105 110

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[0264] 115

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[0272] Ser Leu Arg Leu Ser Cys Val Ala Thr Gly Phe Thr Ile Ser Arg Lys

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[0276] Ala Thr Ile Asn Thr Gly Ser Ser Ser Pro Tyr Tyr Ala Asp Gly Val

[0277] 50 55 60

[0278] Lys Gly Arg Phe Thr Ile Ser Gln Asp Asn Ala Lys Asn Thr Val Tyr

[0279] 65 70 75 80

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[0281] 85 90 95

[0282] Ala Ala Thr Lys Gly Val Val Val Gly Thr Gly Tyr Cys Gly Gly Pro

[0283] 100 105 110

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[0302] 85 90 95
[0303] Ala Ala Gly Pro Leu Tyr Asp Gly Arg Cys Thr Tyr Arg Ser Pro Ala
[0304] 100 105 110
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[0317] 35 40 45
[0318] Ala Val Ile Trp Asp Gly Arg Thr Thr Asp Tyr Asp Asp Ser Val Gln
[0319] 50 55 60
[0320] Asp Arg Phe Thr Ile Ser Gln Asp Asn Ala Lys Ser Thr Val Tyr Leu
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[0336] 35 40 45

[0337] Glu Arg Glu Leu Val Ser Ser Thr Ser Lys Asp Gly Phe Thr Ser Tyr

[0338] 50 55 60

[0339] Thr Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Gln Asp Asn Ala Asn

[0340] 65 70 75 80

[0341] Asn Thr Leu Tyr Leu Gln Met Ser Ser Leu Lys Thr Glu Asp Thr Ala

[0342] 85 90 95

[0343] Val Tyr Ser Cys Ala Ala Ile Cys Ala Val Gly Gly Tyr Ser Leu Ser

[0344] 100 105 110

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[0346] 115

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[0353] 1 5 10 15

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[0355] 20 25 30

[0356] Cys Met Gly Trp Phe Arg Gln Ala Pro Gly Lys Glu Arg Glu Arg Val

[0357] 35 40 45

[0358] Ala Ile Ile Glu Ser Arg Gly Thr Val Thr Tyr Val Asp Ser Val Lys

[0359] 50 55 60

[0360] Gly Arg Phe Thr Ile Ser Lys Asp Asn Ala Lys Asn Thr Leu Tyr Leu

[0361] 65 70 75 80

[0362] Gln Met Asn Ser Leu Lys Pro Glu Asp Thr Ala Met Tyr Tyr Cys Ala

[0363] 85 90 95

[0364] Ala Ser Arg Pro Trp Ser Gly Val Arg Cys Leu His Asp Lys Tyr Asp

[0365] 100 105 110

[0366] Tyr

[0367] <210> 18

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[0375] 20 25 30

[0376] Ala Trp Phe Arg Gln Ala Pro Gly Lys Glu Arg Glu Gly Val Ala Ala

[0377] 35 40 45

[0378] Leu Leu Thr Ala Ile Gly Gly Pro Thr Arg Thr Thr Tyr Ala Asp Ser
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[0382] Tyr Leu Gln Met Ser Ser Leu Lys Pro Glu Asp Thr Ala Met Tyr Tyr
[0383] 85 90 95
[0384] Cys Ala Ala Gly Arg Pro Ala Gly Thr Pro Arg Trp Leu Leu Leu Ala
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[0434] ggcgtggtgg tgggcaccgg ctactgcggc ggcccctacg tggagaggcc caacagcgcc 360

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[0439] <213> 人工序列

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[0447] caggtgaccg tgagcagc 378

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[0468] ttcaccagct acaccgacag cgtgaagggc aggttcacca tcagccagga caacgccaac 240

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[0471] acccaggtga ccgtgagcag c 381

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[0473] <211> 372

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[0508] 50 55 60

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[0582] 195 200 205

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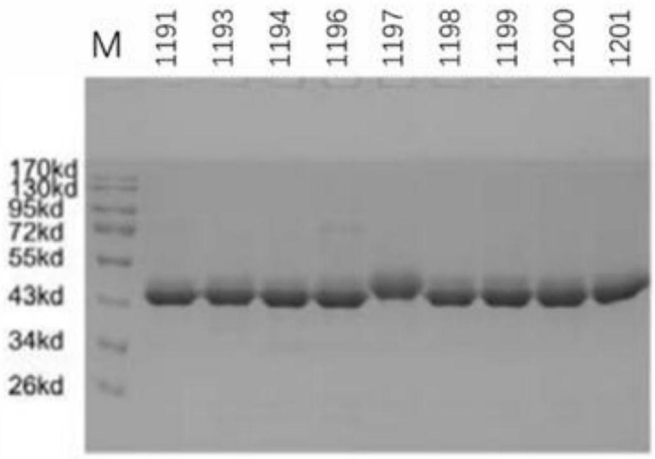

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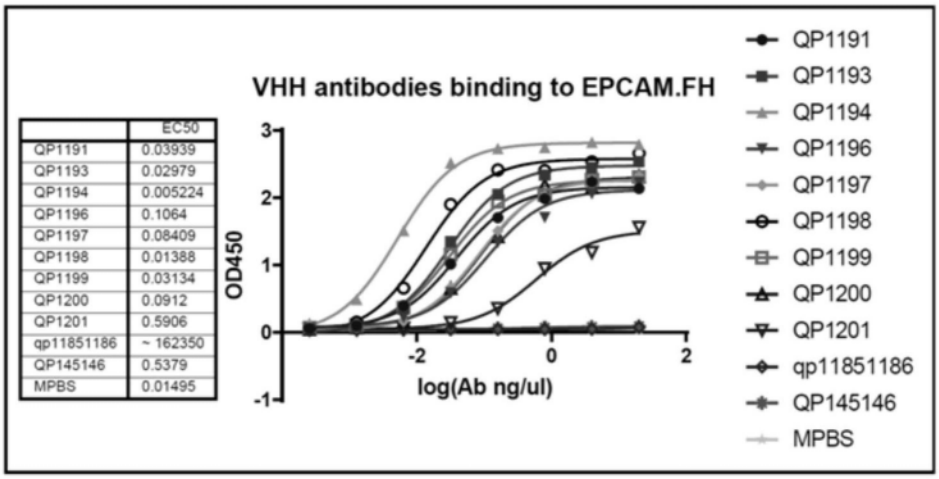

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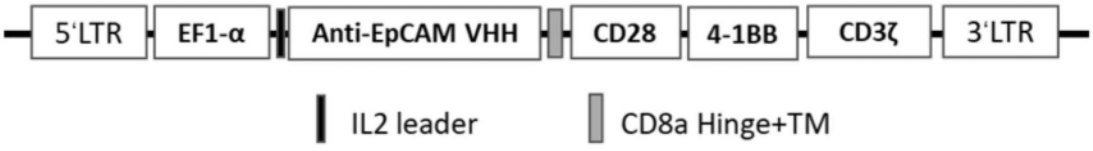

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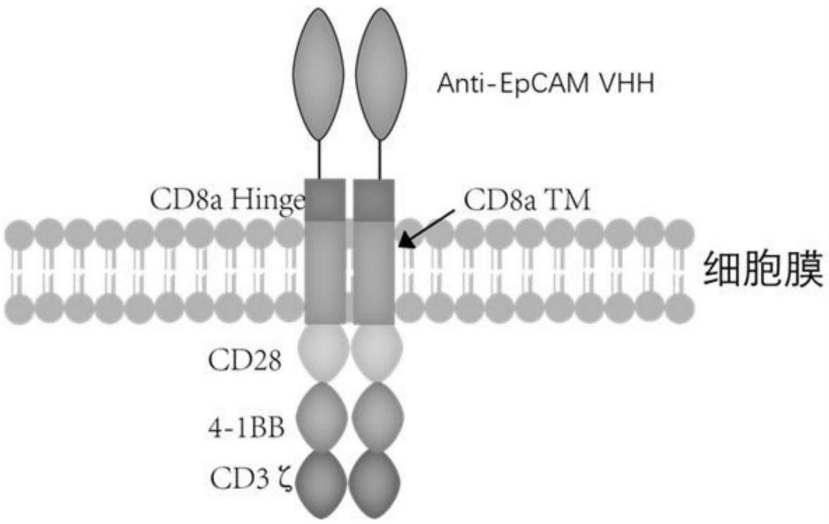
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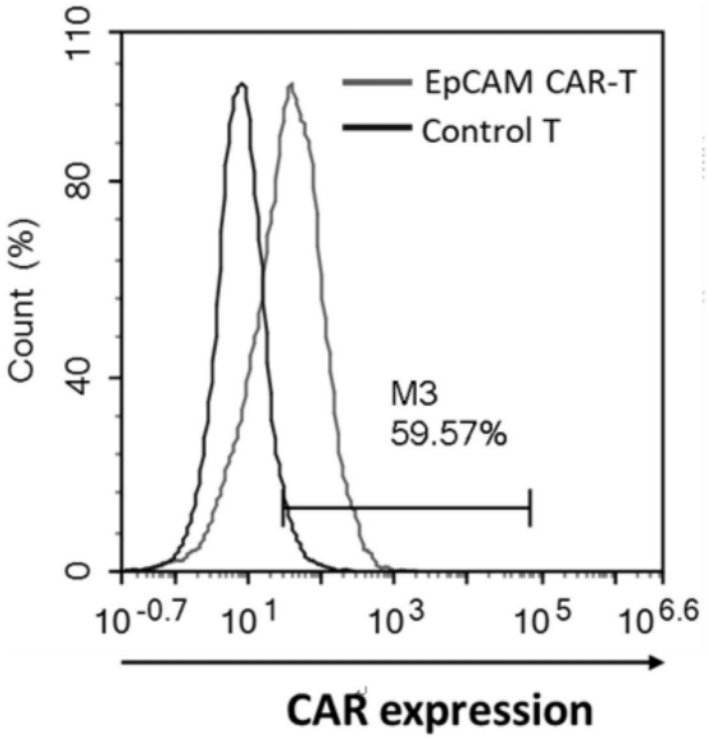
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CAR expression

图5

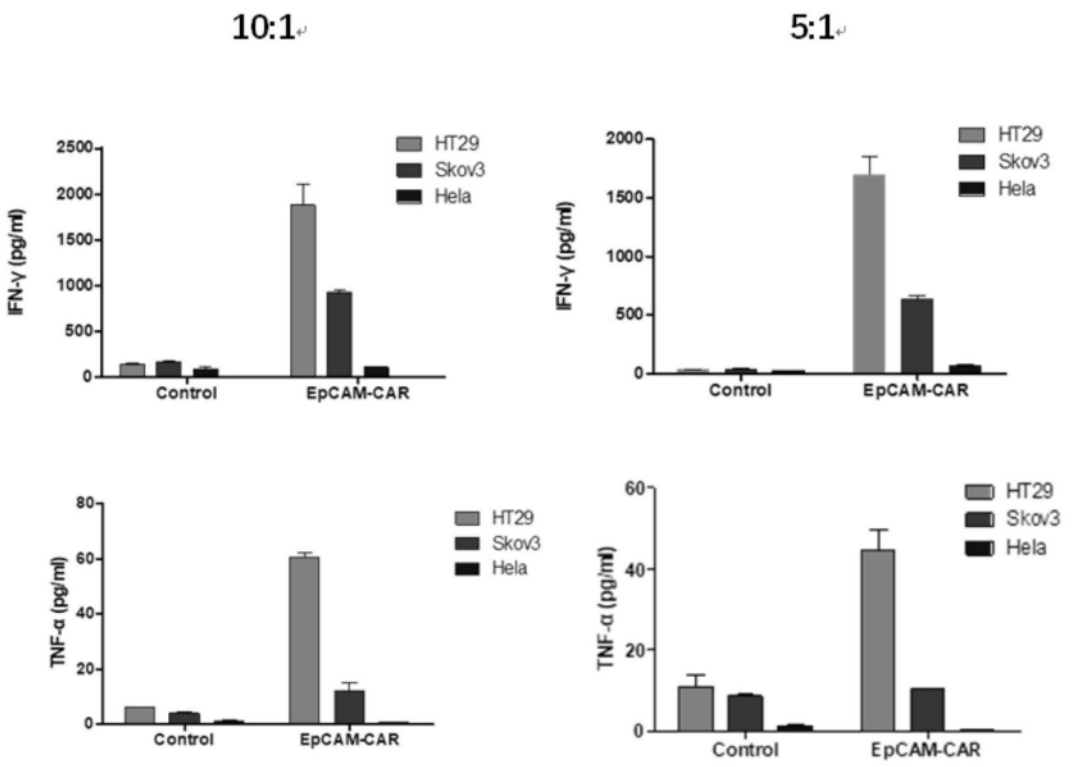
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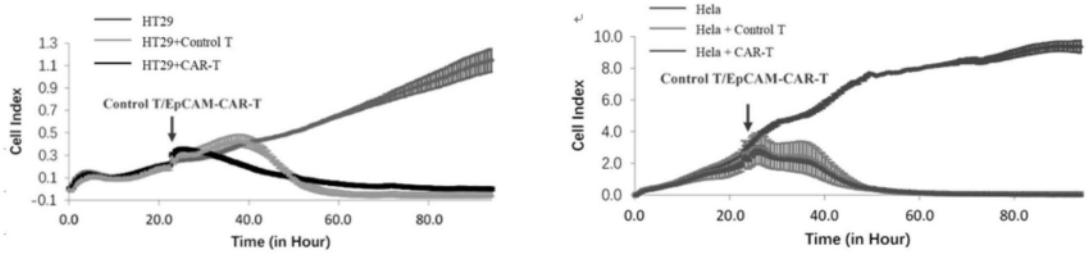
图6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Control T/EpCAM-CAR-T
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Time (in Hour)
Hela
Hela + Control T
Hela + CAR-T

图7