(19)国家知识产权局

(12)发明专利

(10)授权公告号 CN 109069615 B
(45)授权公告日 2025.03.04

(21)申请号 201780028507.X
(22)申请日 2017.04.13
(65)同一申请的已公布的文献号
申请公布号 CN 109069615 A
(43)申请公布日 2018.12.21
(30)优先权数据
62/322,167 2016.04.13 US
(85)PCT国际申请进入国家阶段日
2018.11.08
(86)PCT国际申请的申请数据
PCT/IB2017/052160 2017.04.13
(87)PCT国际申请的公布数据
WO2017/179017 EN 2017.10.19

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(51)Int.Cl.
*A61K 39/12*(2006.01)
*A61P 31/14*(2006.01)
(56)对比文件
WO 2013188315 A1,2013.12.19
CN 105451763 A,2016.03.30
审查员 白静

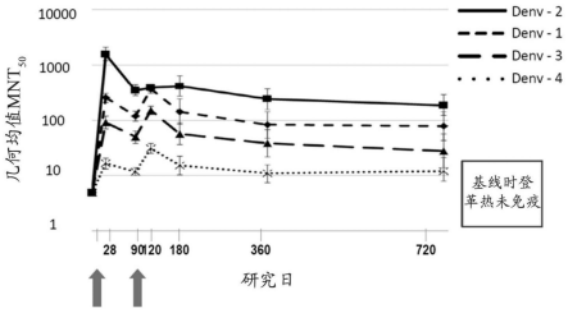
权利要求书1页 说明书43页
序列表267页 附图14页

(54)发明名称

# 在儿童和年轻人中针对登革热病毒接种疫苗的组合物和方法

(57)摘要

本文的实施方案涉及用于在年龄约1岁至约20岁的儿童或年轻人中诱导针对所有四种登革热病毒血清型的免疫应答的组合物、方法和用途。一些实施方案涉及可以组合物,包括但不限于登革热病毒嵌合体,其单独或与其他构建体组合可以用于针对所有四种登革热病毒血清型的疫苗组合物。在某些实施方案中,组合物可以包含各种浓度或比率的超过一种登革热病毒血清型的构建体,如登革热-1(DEN-1)病毒、登革热-2(DEN-2)病毒、登革热-3(DEN-3)病毒和/或登革热-4(DEN-4)病毒以在儿童和年轻人中改善免于感染的保护。在某些实施方案中,配制剂的病毒限于登革热病毒血清型。其他实施方案涉及使用单一、双重或其他方案施用针对登革热病毒的免疫原性组合物的方法,所述免疫原性组合物可以包含嵌合登革热构建体和活的减毒登革热病毒。

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1.一种四价疫苗配制剂在制备用于针对登革热病毒感染的个体的治疗方法的药物组合物中的用途,所述四价疫苗配制剂由所有四种登革热病毒血清型组成,包括活的减毒登革热-2病毒、登革热-2/登革热-1嵌合体、登革热-2/登革热-3嵌合体和登革热-2/登革热-4嵌合体,所述方法包括:对所述个体皮下施用所述药物组合物,其中施用所述药物组合物由在第0天施用第一剂所述药物组合物和在第一次施用的3个月或90天内施用第二剂相同的针对登革热病毒的药物组合物组成,其中所述药物组合物在所述个体中诱导针对登革热病毒的免疫应答;

其中所述个体为2至17岁龄;

并且其中编码所述活的减毒登革热-2病毒的氨基酸序列如SEQ ID NO:12所示,编码所述登革热-2/登革热-1嵌合体的氨基酸序列如SEQ ID NO:4所示,编码所述登革热-2/登革热-3嵌合体的氨基酸序列如SEQ ID NO:20所示,并且编码所述登革热-2/登革热-4嵌合体的氨基酸序列如SEQ ID NO:28所示。

2.一种四价疫苗配制剂在制备用于针对登革热病毒感染的个体的治疗方法的药物组合物中的用途,所述四价疫苗配制剂由所有四种登革热病毒血清型组成,包括活的减毒登革热-2病毒、登革热-2/登革热-1嵌合体、登革热-2/登革热-3嵌合体和登革热-2/登革热-4嵌合体,所述方法包括:对所述个体皮下施用所述药物组合物,其中施用所述药物组合物由在第0天施用第一剂所述药物组合物和在第一次施用的3个月或90天内施用第二剂相同的针对登革热病毒的药物组合物组成,其中所述药物组合物在所述个体中诱导针对登革热病毒的免疫应答;

其中所述个体为1至11岁龄;

并且其中编码所述活的减毒登革热-2病毒的氨基酸序列如SEQ ID NO:12所示,编码所述登革热-2/登革热-1嵌合体的氨基酸序列如SEQ ID NO:4所示,编码所述登革热-2/登革热-3嵌合体的氨基酸序列如SEQ ID NO:20所示,并且编码所述登革热-2/登革热-4嵌合体的氨基酸序列如SEQ ID NO:28所示。

3.根据权利要求1或2所述的用途,其中所述登革热-2/登革热-1嵌合体与所述活的减毒登革热-2病毒与所述登革热-2/登革热-3嵌合体与所述登革热-2/登革热-4嵌合体的浓度比率按噬菌斑形成单位计算是4:4:5:5。

4.根据权利要求1或2所述的用途,其中所述登革热-2/登革热-1嵌合体与所述活的减毒登革热-2病毒与所述登革热-2/登革热-3嵌合体与所述登革热-2/登革热-4嵌合体的浓度比率按噬菌斑形成单位计算是5:4:5:5。

5.根据权利要求1或2所述的用途,所述药物组合物还包含稳定缓冲液。

6.根据权利要求5所述的用途,其中所述稳定缓冲液包含海藻糖和清蛋白。

7.根据权利要求6所述的用途,其中所述稳定缓冲液进一步包含泊洛沙姆407。

8.根据权利要求1或2所述的用途,其中所述个体在施用所述药物组合物之前对登革热病毒呈血清阴性或未免疫。

9.根据权利要求1或2所述的用途,其中所述个体在施用所述药物组合物之前对所述登革热病毒呈血清阳性。

# 在儿童和年轻人中针对登革热病毒接种疫苗的组合物和方法

[0001] 相关申请

[0002] 本PCT申请根据35USC§119(e)要求于2016年4月13日提交的美国临时申请序列号62/322,167的权益,其通过引用完整并入本文用于所有目的。

## 发明领域

[0003] 本文公开的实施方案涉及用于在约1岁龄至约20岁龄的儿童或年轻人(young adult)中诱导针对所有四种登革热病毒血清型的免疫应答的组合物、方法和用途。一些实施方案涉及可以包括但不限于嵌合和非嵌合黄病毒病毒构建体的组合物,所述黄病毒病毒构建体单独或组合可以用于疫苗组合物中以诱导针对所有四种登革热病毒血清型的免疫应答。在某些实施方案中,组合物可以包含各种浓度或比率的超过一种登革热病毒血清型的构建体,如登革热-1(DEN-1)病毒、登革热-2(DEN-2)病毒、登革热-3(DEN-3)病毒和/或登革热-4(DEN-4)病毒,以改善儿童和年轻人中针对登革热感染的保护。其他实施方案涉及使用单一、双重或其他施用方案施用可以包含嵌合登革热构建体和活的减毒登革热病毒的疫苗组合物的方法。

[0004] 发明背景

[0005] 登革热是由登革热病毒感染引起的蚊传播性疾病。登革热病毒感染可导致虚弱和疼痛的症状,包括突然的高烧、头痛、关节和肌肉疼痛、恶心、呕吐和皮疹。迄今为止,已鉴定出四种登革热病毒血清型:登革热-1(DEN-1)、登革热-2(DEN-2)或登革热-3(DEN-3)组合登革热-4(DEN-4)。将来可能会发现其他亚型(例如,DEN-5)。登革热病毒血清型1-4也可引起登革热出血热(DHF)和登革热休克综合征(DSS)。在最严重的情况下,DHF和DSS可以危及生命。登革热病毒每年造成5000至1亿例虚弱性登革热病例、500,000万例DHF/DSS病例、超过20,000例死亡,其中较大部分是儿童。迄今为止,没有有效的疫苗来预防登革热和没有针对该疾病的药物治疗。蚊控制努力在预防地方性区域的登革热暴发中或在防止疾病的进一步地理传播中一直是低效的。估计35亿人受到登革热病毒感染的威胁。此外,登革热病毒是诸如亚洲、中南美洲和加勒比地区等地方性地区旅行者发烧的主要原因。此外,DHF/DSS是一些亚洲和拉丁美洲国家儿童中严重疾病和死亡的主要原因。

[0006] 所有四种登革热病毒血清型在世界的热带地区是地方性的,并且构成全世界热带地区对人最重要的蚊传播性病毒威胁。登革热病毒主要通过埃及伊蚊(Aedes aegypti)传播给人。感染一种登革热病毒血清型导致针对所述血清型的再感染的终生保护,但不能防止其他三种登革热病毒血清型之一的继发感染。事实上,先前感染一种登革热病毒血清型导致被不同血清型的继发感染后严重疾病(DHF/DSS)的风险增加。开发有效的疫苗(特别是在儿童和年轻人中)是预防和控制此全球疾病的重要方法。

[0007] 发明概述

[0008] 本文公开的实施方案涉及嵌合登革热病毒构建体的组合物、方法和用途。在一些实施方案中,组合物可以包含单独的或者与活的减毒登革热病毒组合的嵌合登革热病毒构建体,其具有减毒的登革热病毒骨架,所述登革热病毒骨架具有来自至少一种其他登革热

病毒血清型的结构基因。其他实施方案涉及至少一种活的减毒病毒与一种或多种嵌合登革热病毒的组合,所述嵌合登革热病毒含有针对至少一种另外的登革热病毒血清型的结构元件。其他实施方案可以包括嵌合登革热组合物,其具有经修饰的DEN-2骨架(例如,PDK-53作为P1(传代-1)中的起始骨架和使用传代变异性和选择修饰的PDK-53,如对P2、P3...P8..P10等指出)和DEN-1、DEN-2、DEN-3或DEN-4的一种或多种结构组分。在一些实施方案中,可以产生免疫原性组合物,其中当引入受试者时,所述组合物在受试者中产生对一种或多种登革热病毒的免疫应答。因此,可以产生和传代本文涵盖的构建体,并且每次传代提供涵盖用于药学可接受疫苗组合物的减毒登革热病毒。

[0009] 本文公开的实施方案涉及用于在年龄约1岁龄至约45岁龄的儿童或年轻人中诱导对所有四种登革热病毒血清型的免疫应答的组合物、方法和用途。目前的配制剂针对年幼儿童无效,并且对用于所有年龄组的针对登革热感染使用的登革热疫苗存在未满足的需求。根据这些实施方案,本文公开的组合物和方法针对年龄为约1至20岁龄、约1至15岁龄、约1至11岁龄或约2至约9岁龄或约1至约5岁龄的儿童使用。

[0010] 在某些实施方案中,用于疫苗的登革热病毒血清型的嵌合登革热病毒构建体可以包括传代8(P8)活的、减毒的病毒或嵌合病毒,其具有由SEQ ID NO:1、3、5和7鉴定的核酸序列或由SEQ ID NO:2、4、6和8指示的多肽序列。本文涵盖的是,本文所述的任何活的减毒病毒的任何传代可以用于免疫原性组合物中以诱导针对所表示的登革热病毒血清型(例如血清型1-4)的免疫应答。根据这些实施方案,可以将包含P-8分离的活的减毒病毒的免疫原性组合物施用于受试者,以根据选择的构建体诱导针对一种或多种登革热病毒血清型的免疫原性应答。此外,活的减毒病毒可以与这些嵌合病毒中的一种或多种组合。对于在每个后续细胞传代中分离/产生的每种活的减毒病毒(例如,非洲绿猴Vero细胞产生,下文中:Vero细胞或其他合适的细胞系)考虑这点。本文涵盖的是,能够产生登革热病毒的任何细胞系(例如,GMP批准)用于以制造规模传代任何病毒构建体或在适当时本文考虑用于随后用于针对登革热病毒的疫苗或免疫原性组合物。

[0011] 在其他实施方案中,本文涵盖的组合物可以与针对其他黄病毒(Flaviviruses)的其他免疫原性组合物,例如寨卡病毒(Zika virus)、西尼罗河病毒(West Nile virus)、日本脑炎(Japanese encephalitis),圣路易斯脑炎病毒(St.Louis encephalitis virus)、黄热病病毒(yellow fever virus)或任何其他黄病毒嵌合构建体和/或活的减毒病毒组合。在某些实施方案中,单一组合物可以针对多种黄病毒使用。在一些实施方案中,即使疫苗组合物限于登革热病毒构建体和活的减毒登革热病毒,本文公开的组合物也能够诱导针对寨卡病毒的免疫应答。

[0012] 在某些实施方案中,本发明的免疫原性组合物可以包括单独或与活的减毒登革热病毒组合物组合的针对DEN-1、DEN-2、DEN-3和/或DEN-4中的一种或多种的嵌合登革热病毒。

[0013] 在其他实施方案中,构建体可以包括在病毒的结构或非结构区域中具有适应性突变的构建体,所述适应性突变在引入受试者时增加生长或产生而不影响病毒的减毒或安全性。在某些实施方案中,任何涵盖的嵌合登革热病毒构建体可以包括用作骨架的具有特定突变的活的减毒DEN-2病毒,其中活的减毒DEN-2PDK病毒可以进一步包含其他登革热病毒血清型的prM(前膜(premembrane))和E(包膜)结构蛋白中一种或多种的结构蛋白。此外,

DEN-2骨架可以包括额外突变或突变的回复,以便在施用时增强受试者中对预定组合物的免疫应答(例如,嵌合登革热病毒2/1、2/3或2/4)。

[0014] 在一些实施方案中,结构蛋白基因可以包括在DEN-2骨架上具有一个或两个回复以改善免疫原性的DEN-1、DEN-2、DEN-3或DEN-4的prM和E基因。例如,在某些实施方案中,登革热构建体可以包含称为TDV-1-A、TDV-2-F、TDV-3-F和TDV-4-F的那些构建体(参见实施例部分),其中DEN-2骨架具有来自活的减毒DEN-2病毒的对野生型DEN-2的一个或多个回复(例如,在非编码区(NCR)或非结构区(NS1等)或在P1或其他先前传代的病毒中未发现的其他突变),所述活的减毒DEN-2病毒以前证明对于诱导免疫应答是安全且有效的。本申请的活的减毒DEN-2病毒是最初使用的活的减毒DEN-2病毒的改善形式。本文公开的一些实施方案的嵌合构建体可以包括经修饰的减毒DEN-2PDK-53骨架,其具有第二登革热病毒血清型的一种或多种结构蛋白,其中结构蛋白可以包含额外的突变以增加对嵌合构建体的免疫原性应答。在一些实施方案中,通过减毒的DEN-2PDK-53获得的某些突变可以恢复为对照或另一种氨基酸以产生不同于P1构建体的嵌合构建体,其可以导致增加的免疫原性、增加的生长、增加的噬菌斑大小而不影响疫苗安全性和减毒并且可以影响活的减毒病毒的生长和/或复制。

[0015] 在其他实施方案中,活的减毒DEN-2基因组可以用于产生登革热病毒血清型1(DEN-1)、登革热病毒血清型3(DEN-3)和登革热病毒血清型4(DEN-4)的嵌合构建体,其中DEN-2病毒基因组的一种或多种结构蛋白基因可以分别被DEN-1、DEN-3或DEN-4的一种或多种结构蛋白基因替换。在一些实施方案中,结构蛋白可以包含第三登革热病毒的C、prM或E蛋白。在某些实施方案中,结构蛋白基因可以包含DEN-1、DEN-3或DEN-4的prM和E基因。根据这些实施方案,这些嵌合病毒可以表达DEN-1、DEN-3或DEN-4的表面抗原,同时保留亲本减毒DEN-2的减毒表型。此外,本文公开的用于儿童和年轻人疫苗的嵌合构建体可以包括使用减毒的DEN-2病毒作为骨架表达DEN-1、DEN-3和DEN-4表面抗原的DEN-4、DEN-2、DEN-1、和DEN-3的嵌合构建体。

[0016] 在某些实施方案中,本发明的组合物可以包括组合物,其可以包含本文公开的单一嵌合登革热病毒构建体和药学可接受载体或赋形剂。在其他实施方案中,本文公开的组合物可以包含两种或更多种,或三种或更多种嵌合登革热病毒构建体和药学可接受载体或赋形剂。根据这些实施方案,本文涵盖的一种或多种登革热病毒嵌合构建体可以与一种或多种活的减毒登革热病毒组合。在某些实施方案中,活的减毒病毒可以是活的减毒DEN-2病毒,其中在NCR、NS1区域或其他区域中对野生型氨基酸的回复对于本文公开的配制剂中使用的改善的活减毒登革热病毒构建体增加免疫应答,增加病毒生长或其他改善。

[0017] 在某些实施方案中,活的减毒登革热病毒可以包括DENV-2的核苷酸5'NCR-57-T、NS1-53-Asp、和NS3-250-Val的突变或取代,其可以由四种TDV构建体病毒的共同PDK-53病毒特异性遗传背景共享。已经针对cGMP制造的TDV种子,仔细监测三种减毒基因座的遗传序列以及先前建立的这些疫苗候选物的体外和体内减毒表型。本文公开了用于产生用于制备登革热病毒疫苗组合物的主病毒种子(master virus seed,MVS)以及登革热病毒的其他相关传代的策略。这些MVS可以用于制备临床材料并最终用于制备商业疫苗供应。

[0018] 在某些实施方案中,免疫原性组合物可以包含能够在儿童和年轻人中诱导对至少三种或所有四种登革热病毒血清型的免疫应答的三价或四价配制剂。例如,免疫原性组合

物可以包括四种登革热病毒血清型中每种的前主要病毒种子(Pre-Master Virus Seed)、主病毒种子(MVS)、工作病毒种子(Working Virus Seed,WVS)和散装病毒种子(Bulk Virus Seed,BVS)构建体。在其他实施方案中,免疫原性组合物可以包含一种或多种多核苷酸,其具有编码经修饰的活的减毒登革热-2病毒血清型的核酸序列,所述核酸序列由SEQ ID NO:9(前主要)、11(MVS)、13(WVS)或15(BVS)表示;登革热-1/登革热-2嵌合多核苷酸,其具有编码来自经修饰的活的减毒登革热-2病毒血清型的非结构蛋白和来自登革热-1病毒血清型的结构蛋白的核酸序列,所述核酸序列由SEQ ID NO:1(前主要)、3(MVS)、5(WVS)或7(BVS)表示;登革热-3/登革热-2嵌合多核苷酸,其具有编码来自经修饰的活的减毒登革热-2病毒血清型的非结构蛋白和来自登革热-3病毒血清型的结构蛋白的核酸序列,所述核酸序列由SEQ ID NO:17(前主要)、19(MVS)、21(WVS)或23(BVS)表示;登革热-4/登革热-2嵌合多核苷酸,其具有编码来自经修饰的活的减毒登革热-2病毒血清型的非结构蛋白和来自登革热-4病毒血清型的结构蛋白的核酸序列,所述核酸序列由SEQ ID NO:25(前主要)、27(MVS)、29(WVS)或31(BVS)表示。一些实施方案公开了能够在儿童或年轻人中引发针对一种、两种、三种或所有四种登革热病毒血清型的免疫应答的免疫原性组合物。

[0019] 在某些实施方案中,免疫原性组合物可以包括配制剂,其中组合物中所述编码经修饰的活的减毒登革热-2病毒血清型的多核苷酸的浓度比率为比一种或多种登革热病毒多核苷酸的对数(log)噬菌体形成单位(PFU)低至少0.5-1个对数PFU。在其他实施方案中,免疫原性组合物可以包括配制剂,其中组合物中编码经修饰的活的减毒登革热-2病毒血清型的多核苷酸的浓度比率为比一种或多种多核苷酸的对数噬菌体形成单位(PFU)低至少1.5或大于2或3个对数PFU。

[0020] 在某些实施方案中,单独或与其他病毒疫苗组合的针对登革热的免疫原性组合物可以是多肽和多核苷酸的混合物。根据这些实施方案,免疫原性组合物可以包含本文所述的多核苷酸或具有或没有由多核苷酸编码的各种多肽的其他黄病毒多核苷酸。例如,多肽可以包括由SEQ ID NO:10(前主要)、12(MVS)、14(WVS)或16(BVS)表示的对应于经修饰的活的减毒登革热-2病毒血清型的多肽;对应于由SEQ ID NO:2(前主要)、4(MVS)、6(WVS)或8(BVS)表示的登革热-1/登革热-2嵌合多核苷酸的多肽;对应于由SEQ ID NO:18(前主要)、20(MVS)、22(WVS)或24(BVS)表示的登革热-3/登革热-2嵌合多核苷酸的多肽;和对应于由SEQ ID NO:26(前主要)、28(MVS)、30(WVS)或32(BVS)表示的登革热-4/登革热-2嵌合多核苷酸的多肽。

[0021] 在某些实施方案中,本文公开的免疫原性组合物可以包括各种浓度比率的多肽和/或多核苷酸,包括登革热-1/登革热-2嵌合体与经修饰的活的减毒登革热-2病毒血清型与登革热-3/登革热-2嵌合体与登革热-4/登革热-2嵌合体的比率4:4:5:5噬菌斑形成单位(PFU)。在其他实施方案中,登革热-1/登革热-2嵌合体与经修饰的活的减毒登革热-2病毒血清型与所述登革热-3/登革热-2嵌合体与所述登革热-4/登革热-2嵌合体的比率可以是约5:4:5:5对数PFU。本文涵盖的其他比率可以包括组合物,其中与登革热-3或登革热-4嵌合体中的至少一种相比活的减毒登革热-2和/或登革-1嵌合病毒减少。在其他实施方案中,四价配制剂比率可以是约5:3:5:5、3:3:5:5、或任何比率,所述比率在某些实例中在配制剂中具有较低的活的减毒登革热-2或减少的呈现或活的减毒登革热-2和登革热-1/登革热-2嵌合体两者。

[0022] 在其他实施方案中,本文公开的组合物可以是药物组合物,其包含本文公开的一种或多种免疫原性组合物和药学可接受赋形剂。在某些实施方案中,药物组合物可以包含免疫原性组合物,所述免疫原性组合物由活的减毒登革热病毒和/或登革热嵌合体构成,所述登革热嵌合体具有针对一种或多种登革热血清型的多核苷酸和多肽。

[0023] 其他实施方案可以包括试剂盒,其具有至少一种本公开的免疫原性组合物和至少一个在儿童和年轻人中针对登革热病毒感染使用的容器。

[0024] 某些实施方案包括用于针对至少一种至所有四种登革热病毒血清型使儿童和年轻人免疫的方法。方法可以包括对受试者施用一剂或多剂的本文公开的药物组合物。根据这些实施方案,具有这些表征的比率可以包括活的减毒病毒和/或嵌合体,其在受试者中诱导针对所有四种登革热病毒血清型的免疫应答。

[0025] 在某些实施方案中,方法可以包括对儿童或年轻人施用免疫原性组合物,其除了多核苷酸外还包括由本文公开的多核苷酸编码的一种或多种多肽。方法可以包括多肽,其包括以下一种或多种:由SEQ ID NO:10(前主要)、12(MVS)、14(WVS)或16(BVS)表示的多肽;由S SEQ ID NO:2(前主要)、4(MVS)、6(WVS)或8(BVS)表示的多肽;由SEQ ID NO:18(前主要)、20(MVS)、22(WVS)或24(BVS)表示的多肽;和由SEQ ID NO:26(前主要)、28(MVS)、30(WVS)或32(BVS)表示的多肽。在某些实施方案中,方法可以包括对年龄范围为约1岁至约20岁的受试者施用具有本文中公开的免疫原性组合物中表示的所有四种登革热病毒血清型的免疫原性组合物,并在受试者中引发对所有四种登革热病毒血清型的免疫应答(四价配制剂)。在其他实施方案中,方法可以包括对年龄范围为约1岁至约45岁的受试者使用本文公开的免疫原性组合物。

[0026] 在某些实施方案中,方法可以包括通过任何可接受的手段对受试者施用一剂或多剂本公开的四价配制剂,所述手段包括例如皮下、静脉内、皮内、透皮、口服、通过吸入、阴道内、局部、鼻内或直肠。根据这些实施方案,本文公开的配制剂可以以单剂或以两剂或更多剂量施用于受试者。在一些实施方案中,本文公开的免疫原性配制剂可以在彼此的约90天内、在彼此的约60天内、在彼此的约30天内,以及在彼此的小于约30天内施用。在一些实施方案中,本文公开的免疫原性配制剂可以在第0天施用于儿童或年轻人,并且距第一次施用的约3个月再施用。

[0027] 在某些实施方案中,本文公开的四价配制剂可以在已经接受一剂或多剂四价组合物的儿童和年轻人的至少60%中引发针对所有四种登革热病毒血清型的免疫应答。

[0028] 附图简述

[0029] 以下附图构成本说明书的一部分,并且包括在内以进一步表明某些实施方案。可以通过单独或与呈现的具体实施方案的详细描述结合参考这些附图中的一个或多个更好地理解一些实施方案。

[0030] 图1表示示例性图,其反映与先前产生的构建体和野生型登革热病毒相比本发明的示例性嵌合构建体DEN-2/DEN-4。

[0031] 图2表示示例性直方图,其比较使用活的减毒DEN-2骨架(具有额外突变)和第二登革热病毒血清型作为替换登革热-2结构组分的结构组分(例如TDV-1MVS)的各种应答。此图显示了TDV MVS的噬菌斑大小。包括野生型登革热病毒和先前发表的研究级疫苗候选病毒用于对照和比较。图显示了与对照登革热病毒嵌合构建体相比改善的登革热病毒构建体的

产生。

[0032] 图3表示示例性直方图,其表示TDV MVS(主病毒种子,本文表示为DENVax)的温度敏感性。包括野生型登革热病毒和先前发表的研究级疫苗候选病毒用于与MVS级进行比较。

[0033] 图4表示示例性直方图,其表示与对照相比C6/36细胞中TDV(也称为DENVax)MVS的病毒生长。包括野生型登革热病毒和研究级疫苗候选病毒以用于与TDV MVS(本文表示为DENVax)进行比较。

[0034] 图5A-5C表示新生小鼠中神经毒力的示例性图。几个实验的汇总结果,汇总用$10^4$pfu病毒ic攻击的CDC-ICR(n=72)和Taconic-ICR(n=32)新生小鼠中的wt DENV-2 16681病毒的神经毒力。用$10^4$pfu(B)或$10^3$pfu(C)剂量在Taconic-ICR小鼠中测试的TDV MVS(主病毒种子,本文表示为DENVax)的神经毒力。指示一个实验(n=16)或两个合并实验(n=31或32)中每组测试的动物数目。

[0035] 图6表示示例性直方图,其显示了TDV MVS、WVS和BVS的噬菌斑大小。在pi第9天测量的琼脂糖覆盖下的Vero或LLC-$MK_2$细胞中病毒噬菌斑的平均噬菌斑直径±SD(误差条)。包括野生型DENV和先前发表的研究级疫苗候选病毒用于对照和比较。

[0036] 图7表示示例性直方图,其显示了在两个温育温度下C6/36细胞中TDV(本文表示为DENVax)MSV、WVS和BVS的生长,以验证它们在大规模制备后此体外减毒标志物的保留。包括某些野生型登革热病毒和先前发表的研究级疫苗候选病毒用于比较。

[0037] 图8表示绘制C6/36细胞中TDV MVS(主病毒种子,本文表示为DENVax)、WVS和BVS的限制性生长的示例性直方图。在第7天pi在C6/36细胞中复制的病毒的平均滴度±SD(误差条)。包括某些野生型登革热病毒和先前发表的研究级疫苗候选病毒用于比较。

[0038] 图9A-9B表示新生儿ICR小鼠中TDVMVS(主病毒种子,本文表示为DENVax)的神经毒力数据的示例性图。(A)以$10^4$PFU剂量的病毒的IC接种。(B)以$10^3$PFU剂量的病毒的IC接种。

[0039] 图10表示示例性图,其将新的活减毒登革热-2病毒与先前产生的活减毒登革热-2病毒进行比较。

[0040] 图11A是本公开的活的减毒登革热病毒和登革热-登革热嵌合构建体(表示为TDV)的代表性图,包括代表性蛋白质域和突变位点。

[0041] 图11B是根据本公开的一个实施方案的一种四价配制剂的代表性配制剂。

[0042] 图12A是根据本公开的一个实施方案,作为临床试验的一部分进行的受试者中的给药方案的代表性图。

[0043] 图12B是根据本公开的一个实施方案,作为临床试验的一部分进行的受试者中的给药方案的代表性图。

[0044] 图13A是描绘根据本公开的一个实施方案的微中和测试(MNT)的结果的代表性线图,其中在对血清阳性受试者施用四价配制剂后中和性抗体应答。

[0045] 图13B是描绘根据本公开的一个实施方案的微中和测试(MNT)的结果的代表性线图,其中在对登革热未免疫的受试者施用四价配制剂后中和性抗体应答。

[0046] 图14是代表性条形图,其描绘了根据本公开的一个实施方案,在四价配制剂施用后的各个时间点对所有四种登革热血清型呈血清阳性的临床试验受试者的百分比。

[0047] 图15是代表性条形图,其描绘了根据本公开的一个实施方案,在四价配制剂施用后的各个时间点对所有四种登革热血清型呈血清阳性的临床试验受试者的百分比。

[0048] 图16是代表性条形图,其描绘根据本公开的一个实施方案,在四价配制剂施用后的各个时间点接受所有四种登革热血清型的药物组合物之前未免疫的血清阳性临床试验受试者的百分比。

[0049] 图17是代表性条形图,其描绘根据本公开的一个实施方案,接受所有四种登革热血清型的药物组合物之后对多种血清型的血清阳性临床试验受试者(儿童和成人)的百分比。

[0050] 定义

[0051] 如本文所用,“一个”或“一种”可以表示一个/种或超过一个/种项。

[0052] 如本文说明书中所用,“受试者”可以包括但不限于哺乳动物,如人(例如儿童至成人)或哺乳动物(驯养或野生),例如狗、猫、其他家庭宠物(例如,仓鼠、豚鼠、小鼠、大鼠)、雪貂、兔、猪、马、牛、草原土拨鼠、野生啮齿动物或动物园动物。

[0053] 如本文所用,术语“病毒嵌合体”、“嵌合病毒”、“黄病毒嵌合体”和“嵌合黄病毒”可以指包含登革热-2病毒的核苷酸序列的一部分和另外的核苷酸序列的构建体,所述另外的核苷酸序列不来自登革热-2病毒或来自不同的黄病毒。“登革热嵌合体”包含至少两种不同的登革热病毒血清型,但不包括不同的黄病毒。因此,其他登革热病毒或黄病毒的实例可以包括但不限于来自登革热-1病毒、登革热-3病毒、登革热-4病毒、寨卡病毒、西尼罗河病毒、日本脑炎病毒、圣路易斯脑炎病毒、蜱传脑炎病毒、黄热病病毒及其任何组合的序列。

[0054] 如本文所用,“核酸嵌合体”可以指本公开的构建体,其包含具有登革热病毒(例如登革热-2病毒)核苷酸序列的一部分和与此登革热病毒不是相同起源的另外的核苷酸序列的核酸。根据这些实施方案,本文公开的任何嵌合登革热或黄病毒嵌合体可以认为是核酸嵌合体的实例。

[0055] 如本文所用,“活的减毒病毒”可以指针对用于疫苗或其他免疫原性组合物的性状突变或选择的野生型病毒,其中一些性状可以包括降低的毒力、安全性、功效或改善的生长等。

[0056] 如本文所用,“登革热病毒配制剂”或“免疫原性组合物”或“疫苗配制剂”或其药物配制剂可以包括本文公开的多核苷酸和/或多肽的各种组合,其可以施用于受试者和可以在所述受试者中引发对一种或多种登革热病毒血清型的免疫应答。

[0057] 描述

[0058] 在以下部分中,描述了各种示例性组合物和方法以详述各种实施方案。对于本领域技术人员显而易见的是,实践各种实施方案不需要使用本文概述的所有或甚至一些具体细节,而是可以通过常规实验来修改浓度、时间和其他具体细节。在某些情况下,公知的方法或组件尚未包括在说明书中。

[0059] 根据本公开的实施方案,可以使用本领域技术范围内的常规分子生物学、蛋白质化学、微生物学和重组DNA技术。在文献中完整解释这些技术。参见,例如Sambrook,Fritsch&Maniatis,Molecular Cloning:A Laboratory Manual,第二版1989,Cold Spring Harbor Laboratory Press,Cold Spring Harbor,N.Y.;Animal Cell Culture,R.I.Freshney编,1986)。

[0060] 本文公开的实施方案涉及用于在受试者,如约1岁龄至约20岁龄的儿童或年轻人中诱导一种或多达所有四种登革热病毒血清型的免疫应答的组合物、方法和用途。其他实

施方案涉及在受试者中单独或同时诱导针对一种或多种登革热病毒血清型的免疫应答的组合物、方法和用途。根据这些实施方案,可以产生减毒登革热病毒和核酸嵌合体并将其用于本文公开的疫苗组合物中。一些实施方案涉及经修饰的或突变的登革热构建物或嵌合体。其他实施方案涉及引入突变以修饰登革热病毒的结构蛋白的氨基酸序列,其中突变增加对病毒的免疫原性。

[0061] 已经通过在细胞培养物中传代野生型病毒开发所有四种血清型的活的减毒登革热病毒。这些是用于针对黄病毒,特别是登革热病毒感染和/或疾病的免疫的一些最有希望的活的减毒疫苗候选物。这些疫苗候选物已经通过它们的登革热血清型、将它们传代的细胞系和将它们传代的次数的组合来命名。因此,将在PDK细胞中传代13次的登革热血清型1野生型病毒命名为DEN-1PDK-13病毒。其他候选疫苗是DEN-2PDK-53、DEN-3PGMK-30/FRhL-3(例如,在原代绿猴肾细胞中传代30次,然后在胎儿猕猴肺细胞传代3次和DEN-4PDK-48中)。这四种候选疫苗病毒分别通过野生型亲本DEN-1 16007、DEN-2 16681、DEN-3 16562和DEN-4 1036病毒的组织培养传代而得到。

[0062] DEN-2PDK-53病毒疫苗候选物(下文为PDK-53)具有几种与减毒相关的可测量的生物学标志物,包括温度敏感性、小噬菌斑大小、蚊C6136细胞培养物中复制减少、完整蚊中复制减少、哺乳小鼠的神经毒力丧失和猴中病毒血症的发生率降低。候选PDK-53疫苗的临床试验证明了其在人中的安全性和免疫原性。此外,PDK-53疫苗在人疫苗接种者中诱导登革热病毒特异性T细胞记忆应答。本文的一些实施方案描述了用于本文公开的嵌合构建体中的改善的DEN-2PDK-53,所述嵌合构建体用于在儿童和年轻人中诱导针对登革热病毒的免疫应答。

[0063] 具有登革热-2病毒骨架和至少一种另一种登革热病毒血清型结构蛋白的免疫原性黄病毒嵌合体可以用于制备登革热病毒嵌合体,并描述了产生登革热病毒嵌合体的方法。在药学可接受载体中以免疫原性组合物单独或组合提供免疫原性登革热病毒嵌合体,以最小化、抑制单独或组合的一种或多种血清型,诸如登革热病毒血清型DEN-1、DEN-2、DEN-3和DEN-4的感染或针对所述感染免疫个体。当组合时,免疫原性登革热病毒嵌合体可以用作多价疫苗(例如,双价、三价和四价),以同时赋予保护而免受超过一种黄病毒物种或毒株的感染。在某些实施方案中,登革热病毒嵌合体可以在免疫原性组合物中组合,所述免疫原性组合物用作针对已知登革热病毒血清型的二价、三价或四价疫苗,其可以通过包含编码来自不同黄病毒的一种或多种蛋白质的核酸来赋予对其他致病性黄病毒的免疫力。

[0064] 在一些实施方案中,本文提供的无毒力、免疫原性登革热病毒嵌合体含有减毒的登革热-2病毒(例如,PDK-53)或其等同物的非结构蛋白基因和受试者中要诱导的免疫原性针对的黄病毒的结构蛋白基因或其免疫原性部分中的一种或多种。例如,一些实施方案涉及嵌合体,其具有减毒的登革热-2病毒PDK-53基因组作为病毒骨架,并且将编码PDK-53基因组的壳体、前膜/膜或包膜的一种或多种结构蛋白基因或其组合替换为来自DEN-1、DEN-3或DEN-4或要提供保护所免于的其它黄病毒,诸如不同黄病毒或不同登革热病毒血清型的一种或多种相应的结构蛋白基因。根据这些实施方案,本文公开的核酸嵌合体可以具有减毒的登革热-2病毒的功能特性并且是无毒力的,但是在其他黄病毒外表达DEN-1、DEN-3或DEN-4的结构基因产物的抗原性表位且是免疫原性的(例如,在受试者中诱导对基因产物的免疫应答)。然后,将这些构建体传代1次或更多次超出53代,以产生活的减毒病毒组合物,

其用于产生针对一种或多种登革热病毒血清型(例如P1-P10)的免疫原性组合物。

[0065] 在另一个实施方案中,核酸嵌合体可以是核酸嵌合体,其具有但不限于编码来自减毒登革热-2病毒的非结构蛋白的第一核苷酸序列和编码来自登革热-4病毒的结构蛋白的第二核苷酸序列,单独或与另一种黄病毒组合。在其他实施方案中,减毒的登革热-2病毒可以是疫苗株PDK-53,其具有一种或多种向野生型氨基酸的回复氨基酸(参见实施例),其针对改善的物理性质或增加的免疫原性选择。这些特定的回复赋予在儿童和年轻人中针对登革热病毒感染的方案中作为活的减毒登革热-2或作为本文所述的嵌合构建体使用的期望特性。一些实施方案包括第二登革热病毒的C、prM或E蛋白中的一种或多种的结构蛋白。

[0066] 在本文公开的其他实施方案中,核苷酸和氨基酸序列可以包括例如在PDK-53登革热-2基因组中的取代、缺失或插入以减少对其他靶向的登革热病毒血清型的干扰。这些修饰可以单独或与本文公开的示例性修饰组合在结构和非结构蛋白中进行,并且可以通过传代减毒病毒并获得用于诱导针对一种或多种登革热病毒血清型的免疫应答的改善组合物来产生。

[0067] 本文公开的某些实施方案提供了使用重组技术制备嵌合黄病毒的方法,例如,通过将一种血清型的替代序列插入另一种血清型骨架中进行。本文的其他实施方案涉及传代经确认的(例如,安全且有效的)活的减毒嵌合病毒以实现额外的改善并且选择期望的性状。在某些实施方案中,本文使用的活的减毒登革热-2可以包括表3中所示的一种或多种突变。在其他实施方案中,本申请的登革热-登革热嵌合体可以包括如表3中所示的一种或多种突变。在其他实施方案中,登革热-登革热嵌合体可以包括每种嵌合体的所有突变,如表3中针对Den-2/Den-1、Den-2/Den-3或Den-2/Den-4所示。涵盖包含由表3的构建体表示的活的减毒病毒的药物组合物。例如,涵盖本文使用如表3中所示的登革热-登革热嵌合体和活的减毒登革热-2病毒的用途的单价、二价、三价、或四价组合物。

[0068] 在某些实施方案中,本文涵盖的活的减毒DEN-2变体可以配制成药物组合物,其中药物组合物可以单独施用或与登革热-登革热嵌合体或登革热-黄病毒嵌合体组合施用。在某些实施方案中,二价、三价、或四价组合物可以在单次应用中或在多次应用中施用于受试者。

[0069] 黄病毒嵌合体

[0070] 登革热病毒1-4型(DEN-1至DEN-4)是蚊传播性黄病毒病原体。黄病毒基因组包含5'-非编码区(5'-NC)、壳体蛋白(C)编码区、前膜/膜蛋白(prM)编码区、包膜蛋白(E)编码区、编码非结构蛋白(NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5)的区域和3'非编码区(3'NC)。黄病毒结构蛋白包括C、prM和E。非结构蛋白包括NS1-NS5。结构蛋白和非结构蛋白翻译为单一多蛋白,并由细胞和病毒蛋白酶加工。

[0071] 黄病毒嵌合体可以是通过将来自登革热病毒或黄病毒科的病毒物种的一种类型或血清型的非结构蛋白基因与来自登革热病毒或其他黄病毒的不同类型或血清型的蛋白基因(例如,结构蛋白基因)融合来形成的构建体。在其他实施方案中,黄病毒嵌合体构建体可以通过融合来自登革热病毒或黄病毒科的病毒物种的一种类型或血清型的非结构蛋白基因与指导选自其他登革热病毒血清型或其他黄病毒的多肽或蛋白质合成的其他核苷酸序列来形成。在其他实施方案中,涵盖一种黄病毒至另一种的序列替换。

[0072] 在其他实施方案中,登革热嵌合体可以含有活的减毒登革热病毒或其等同物的非

结构蛋白基因和赋予的免疫原性所针对的登革热病毒或其他黄病毒的一种或多种结构蛋白基因或其抗原性部分。

[0073] 用于构建登革热嵌合体的其他合适的登革热病毒可以是野生型、毒性DEN-1 16007、DEN-2 16681、DEN-3 16562和DEN-4 1036以及减毒的疫苗株DEN-1PDK-13、DEN-2PDK-53、DEN-3PMK-30/FRhL-3和DEN-4PDK-48。涵盖DEN-1、DEN-2、DEN-3和DEN-4野生型/减毒病毒对之间的遗传差异以及由病毒基因组编码的氨基酸序列的变化。

[0074] 在一些实施方案中,本文使用的登革热-2病毒可以包括DEN-2PDK-53-V变体,其中基因组核苷酸位置5270从A蛋白突变为T,并且多蛋白的氨基酸位置1725或NS3蛋白的氨基酸位置250含有登革热-2全长序列的缬氨酸残基。一种没有此核苷酸突变DEN-2PDK-53-E的DEN-2PDK-53变体在此位置上与PDK-53-V不同。DEN-2PDK-53-E具有核苷酸位置5270处的A和多肽位置1725处的谷氨酸,NS3蛋白氨基酸位置250。在一些实施方案中,经修饰的PDK 53登革热-2可以包含向天然序列的这些位置的一个或多个回复以获得本文涵盖的疫苗组合物中使用的更期望的性状。

[0075] 在某些实施方案中,登革热-登革热嵌合体可以包含DEN-2病毒特异性感染性克隆,其具有经修饰的骨架和从其他登革热病毒或其他黄病毒中插入的结构基因(prM-E或C-prM-E)。在一些实施方案中,可以从登革热-2 16681毒株(P)、PDK-53-E(E)或PDK-53-V(V)产生登革热-2骨架变体;最后一个字母指示来自亲本(P)毒株或其疫苗衍生物(V)的C-prM-E结构基因或来自亲本(P)或其疫苗衍生物(V1)的prM-E结构基因。例如;DEN-2/1-VP表示嵌合体,其包含含有NS3-250处的缬氨酸的减毒DEN-2PDK-53V骨架和来自野生型DEN-1 16007的C-prM-E基因;DEN-2/1-VV表示DEN-2PDK-53V骨架与登革热-1,DEN-1PDK-13疫苗株;DEN-2/1-VP1表示DEN-2PDK-53V骨架和来自野生型DEN-1 16007的prM-E基因;DEN-2/3-VP1表示DEN-2PDK-53V骨架和来自野生型DEN-3 16562的prM-E基因;DEN-2/4VP1表示DEN-2PDK-53V骨架和来自野生型DEN-4 1036的prM-E基因。本文公开的其他嵌合体用类似的命名法表示。

[0076] 在一些实施方案中,本文公开的登革热-登革热嵌合体可以含有减毒登革热-2病毒PDK-53基因组作为骨架,其中编码PDK-53基因组的C、prM和E蛋白或其组合的结构蛋白基因可以用来自登革热-1、登革热-3或登革热-4病毒和任选地提供保护所针对的另一种黄病毒,诸如不同黄病毒(例如寨卡病毒或黄热病或其组合)或不同登革热病毒株的相应结构蛋白基因替换。活的减毒登革热-2PDK-53病毒株在核苷酸位置5270处具有混合基因型。相当大部分(约29%)的病毒群体编码野生型DEN-2 16681中存在的未突变的NS3-250-Glu而非NS3-250-Val突变。由于这两种遗传变体都是无毒力的,此突变在无毒力的嵌合体中可能不是必需的。

[0077] 在某些实施方案中,NS1-53处的单突变、NS1-53处和5'NC-57处的双重突变、NS1-53处和NS3-250处的双突变以及NS1-53、5'NC-57和NS3-250处的三重突变导致DEN-2病毒的减毒。因此,在这些基因座处含有此类非保守氨基酸取代或核苷酸取代的任何登革热-2病毒的基因组可以用作衍生本文公开的经修饰的PDK-53病毒的基础序列。若期望的话,5'非编码区中茎/环结构的茎中的另一个突变可以提供额外的无毒力表型稳定性。对此区域的突变破坏对病毒复制重要的潜在二级结构。

[0078] 本文公开的突变可以通过本领域已知的任何方法实现,包括但不限于在感兴趣的细胞系(例如,Vero细胞)中传代后具有额外特征的选择的克隆。本领域技术人员应当理解,

如本文所述和本领域公知的毒力筛选测定法可以用于区分毒力和无毒力的骨架结构。

[0079] 黄病毒嵌合体的构建

[0080] 本文公开的黄病毒嵌合体可以使用重组工程将相应的PDK-53基因除去并且将它替换为登革热-1、登革热-3或登革热-4病毒基因或本领域已知的其他基因,通过将期望的免疫所针对的一种黄病毒的结构蛋白基因中的一种或多种剪接到PDK-53登革热-2病毒基因组骨架,或本领域中已知的其它方法来产生。

[0081] 或者,使用序列表中提供的序列,可以使用已知的核酸合成技术合成编码黄病毒蛋白的核酸分子,并将其插入合适的载体中。本文公开的活的减毒病毒可以使用本领域技术人员已知的重组工程技术产生。

[0082] 含有编码其他黄病毒或登革热病毒血清型结构蛋白的核苷酸序列的合适嵌合病毒或核酸嵌合体可以通过对它们筛选指示无毒力的前述减毒表型标志物并通过对它们筛选免疫原性来评估其作为疫苗的可用性。可以使用本领域技术人员已知的常规筛选方法,使用与黄病毒抗体或免疫反应性血清的体外或体内反应性来评估抗原性和免疫原性。

[0083] 登革热病毒疫苗

[0084] 在某些实施方案中,嵌合病毒和核酸嵌合体可以提供可用作免疫原或疫苗的活的减毒病毒。一些实施方案包括对登革热-4病毒表现出高免疫原性而不产生危险的致病或致死效应的嵌合体。

[0085] 为了减少仅针对一种登革热病毒血清型接种疫苗的儿童和年轻人中DHF/DSS的发生,需要四价疫苗来为所有四种病毒血清型提供同时免疫。可以通过在合适的药物载体中组合本申请的活的减毒登革热-2病毒与本文所述的登革热-2/1、登革热-2/3和登革热-2/4嵌合体或其他登革热病毒构建体产生四价疫苗以作为多价疫苗施用。

[0086] 在某些实施方案中,本公开的嵌合病毒或核酸嵌合体可以包括减毒DEN-2病毒骨架上的野生型或活的减毒病毒的结构基因。例如,登革热-2/登革热-4嵌合体可以表达野生型DEN-41036病毒的结构蛋白基因。

[0087] 在某些方法中,可以使用本领域已知的技术培养本文所述的嵌合体中使用的病毒。然后,可以进行病毒噬菌斑滴定并计数噬菌斑以评估生长培养物的存活力和表型特征。野生型病毒可以通过培养的细胞系传代以获得减毒的候选起始材料。

[0088] 在某些实施方案中,嵌合克隆可以由本领域技术人员可获得的各种登革热血清型克隆构建。还可以实现病毒特异性cDNA片段的克隆。含有结构蛋白或非结构蛋白基因的cDNA片段可以从登革热病毒RNA用各种引物通过逆转录酶-聚合酶链式反应(RT-PCR)扩增。可以将扩增的片段克隆到其他中间克隆的切割位点中。然后可以对中间的嵌合登革热病毒克隆进行测序以验证插入的登革热病毒特异性cDNA的准确性。

[0089] 通过将登革热血清型病毒的结构蛋白和/或非结构蛋白基因区插入载体中构建的全基因组长度嵌合质粒可以使用本领域技术人员公知的重组技术获得。

[0090] 核苷酸和氨基酸分析

[0091] DEN-2PDK-53疫苗病毒中的NS1-53突变对于此病毒的减毒表型是有意义的,因为DEN-2 16681病毒的NS1-53-Gly在至今测序的几乎所有黄病毒中都是保守的,包括蜱传播性病毒。DEN-4疫苗病毒也可以在位置253处在NS1蛋白中含有氨基酸突变。此基因座(其是DEN-4PDK-48疫苗病毒中的Gln至His突变)在所有四种登革热病毒野生血清型中均为Gln。

此Gln残基对于黄病毒属内的登革热病毒是独特的。NS1蛋白是从黄病毒感染的细胞分泌的糖蛋白。它存在于感染细胞的表面上,并且NS1特异性抗体存在于病毒感染个体的血清中。已经报道了用NS1蛋白免疫或用NS1特异性抗体被动免疫的动物的保护。NS1蛋白似乎参与早期病毒RNA复制。

[0092] 在某些实施方案中,在DEN-1、-2、-3和-4减毒株的NS2A、NS2B、NS4A、和NS4B蛋白中发生的突变本质上是保守的。DEN-2和DEN-4疫苗病毒分别的NS4A-75和NS4A-95突变在登革热病毒间,但一般不在黄病毒间的氨基酸保守位点处发生。

[0093] 可以将编码DEN-4、DEN-3或DEN-1病毒的核酸序列(例如,结构元件)插入载体(例如质粒)中,并在活生物体中重组表达(例如,进入登革热-2骨架)以产生重组登革热病毒肽和/或多肽和/或病毒。

[0094] 核酸检测方法

[0095] 本公开提供了对本文所述的每种疫苗病毒诊断性的快速遗传测试。本公开的此实施方案增强从形成病毒血症的接种人的血清中分离的病毒的分析,以及增强用候选疫苗病毒免疫的非人灵长类中的病毒血症的表征。

[0096] 这些序列可以包括诊断性TaqMan探针,其用来报告通过使用逆转录酶/聚合酶链式反应(RT/PCR)以及设计用于扩增cDNA扩增子的正向和反向扩增引物从病毒基因组RNA模板扩增的cDNA扩增子的检测,如下所述。在某些情况下,扩增产物之一已经设计为在扩增产物的3'端末端含有疫苗病毒特异性突变,其有效地使测试对疫苗毒株更具特异性,因为靶位点处引物延伸以及因此扩增仅当病毒RNA模板含有所述特定突变时才发生。

[0097] 可以使用基于PCR的自动核酸序列检测系统,或用于核酸检测的其他已知技术。TaqMan测定法是一种高度特异性和灵敏的分析,其允许从样品核酸模板中自动实时显现和定量PCR产生的扩增子。TaqMan可以测定特定序列的存在或缺乏。在该测定法中,设计正向和反向引物设计为分别在靶突变位点上游和下游退火。特异性探测器探针构成该测定法的第三引物组分,所述特异性探测器探针设计为比任一种扩增引物具有高约10℃的熔解温度并且含有疫苗病毒特异性核苷酸突变或其互补物(取决于所检测的RT/PCR扩增子链)。

[0098] 设计用于特异性检测疫苗病毒基因组之一中的突变基因座的探针将在探针的中间含有疫苗特异性核苷酸。若病毒RNA模板是疫苗病毒特异性的,则此探针将在TaqMan测定法中产生可检测的荧光。然而,来自野生型DEN病毒的基因组RNA模板由于单核苷酸错配(在亲本病毒DEN病毒的情况下)或可能超过一个错配(如可以在其他野生型DEN病毒中发生)而具有降低的探针杂交效率并且不会导致相当大的荧光。DNA聚合酶相比于去切割错配探针更有可能从RT/PCR扩增子模板中置换错配的探针以释放报告物染料(TaqMan等位区分测定法,Applied Biosystems)。

[0099] 用于诊断性遗传测试的一种策略利用分子信标。分子信标策略还利用引物进行扩增子的RT/PCR扩增,并通过在探针末端含有报告物和淬灭染料的探针检测扩增子内的特定序列。在该测定法中,探针形成茎-环结构。分子信标测定法采用淬灭剂和报告染料,其不同于TaqMan测定中使用的那些。药物组合物

[0100] 本文的实施方案提供了以适于体内药物施用的生物相容形式向受试者施用组合物。"适于体内给药的生物相容形式"是指待施用的活性剂(例如,实施方案的药剂)的形式,其中活性剂的治疗效果超过任何毒性作用。治疗有效量的治疗组合物的施用定义为在实现

期望结果必需的剂量和时间段上有效的量。例如,化合物的治疗活性量可以随诸如个体的疾病状态、年龄、性别和体重以及抗体在个体中引发期望响应的能力等因素而变化。可以调整剂量方案以提供最佳治疗响应。

[0101] 在一个实施方案中,化合物(例如,实施方案的活的减毒病毒)可以以方便的方式施用,例如,皮下、静脉内、通过口服施用、吸入、皮内、透皮应用、阴道内应用、局部施用、鼻内或直肠施用。根据施用途径,活性化合物可以包含在保护性缓冲液(例如清蛋白和海藻糖、泊洛沙姆407/海藻糖/清蛋白(FTA))中。在一个实施方案中,组合物可以口服施用。在另一个实施方案中,组合物可以静脉内施用。在一个实施方案中,组合物可以鼻内施用,例如吸入。在另一个实施方案中,组合物可以使用无针系统(例如Pharmajet®)或其他皮内施用系统皮内施用。

[0102] 在某些实施方案中,组合物可以在合适的载体或稀释剂中施用于儿童或年轻人。在某些实施方案中,活的减毒登革热病毒疫苗可以以稳定配制剂(例如清蛋白和海藻糖;FTA,或用于稳定活的减毒病毒的其他配制剂)施用于儿童或年轻人。如本文所用,“药学可接受载体”还可以包括稀释剂,例如盐水和水性缓冲溶液。可能有必要将活的减毒病毒配制剂与防止其失活的材料组合或将化合物与防止其失活的材料共施用。在某些实施方案中,活的减毒登革热病毒的一种或多种配制剂可以以初始剂量,接着在加强剂中对儿童或年轻人皮下或皮内施用。也可以在甘油、液体聚乙二醇及其混合物中以及在油中制备分散剂。在通常的储存和使用条件下,这些制剂可以含有防腐剂,以防止微生物的生长或其他稳定化配方(例如,用于稳定化活的减毒黄病毒或其嵌合体的FTA或其他稳定配制剂)。

[0103] 适于可注射使用的药物组合物可以通过本领域已知的手段施用。例如,可以使用无菌水溶液(在水溶性的情况下)或分散体和用于即时制备无菌可注射溶液或分散体的无菌粉末。在一些实施方案中,组合物可以是无菌的并且可以是流体以易于可注射性。药学可接受载体可以是溶剂或分散介质,其含有例如水、乙醇、多元醇(例如,甘油、丙二醇和液体聚乙二醇等)及其合适的混合物。例如,可以通过使用诸如卵磷脂的涂层材料,通过在分散体的情况下维持需要的粒度以及通过使用表面活性剂来维持适当的流动性。可以通过添加各种抗细菌剂或抗真菌剂或其他试剂来实现微生物的预防。

[0104] 在一些实施方案中,在配制时,溶液可以以与剂量配制剂相容的方式并以治疗有效的量施用。根据这些实施方案,配制剂可以容易地以多种剂型施用,例如上述可注射溶液的类型。在某些实例中,活的减毒登革热病毒和/或嵌合体可以以具有特定比率,例如5:4:5:5、4:4:5:5、或其他比率(例如,给定的登革热病毒血清型的PFU)的配制剂递送,其中三价或四价配制剂中的登革热-3和/或登革热-4构建体就pfu而言比配制剂中表示的登革热-1和/或登革热-2大至少0.5个对数。在某些实施方案中,登革热-3和登革热-4活的减毒者或嵌合体的pfu浓度比四价配制剂中登革热-1和/或登革热-2的浓度至少大1个对数。在一些实施方案中,DEN2/4嵌合体可以比其他登革热病毒血清型,例如活的减毒登革热-1和/或活的减毒登革热-2以更高的浓度存在。

[0105] 在某些实施方案中,单剂或多剂登革热配制剂也可以施用于约1岁至约20岁龄的儿童或年轻人。在一些实施方案中,可以用单剂配制剂治疗儿童或年轻人。在其他实施方案中,可以用至少两剂活的减毒登革热病毒配制剂治疗儿童或年轻人。在某些实施方案中,儿童或年轻人可以在第0天施用登革热-登革热配制剂的组合物,并在第一剂的约3个月内施

用加强剂量。在某些实施方案中,儿童和年轻人是从未暴露于登革热病毒的未免疫受试者(血清阴性)。在其他实施方案中,儿童和年轻人可以先前暴露于登革热病毒和/或登革热病毒感染(血清阳性)。根据这些实施方案,血清阴性儿童和/或血清阴性的年轻人可以在第0天进行治疗,然后在第一剂的6个月内、5个月内、4个月内、3个月内或更小接受加强剂,以便对登革热病毒产生增强的免疫应答。在某些实施方案中,儿童可以是约2岁至约17岁龄的儿童。在其他实施方案中,儿童是2岁至17岁龄。

[0106] 在另一个实施方案中,鼻用溶液或喷雾剂、气溶胶或吸入剂可以用于将活的减毒登革热病毒配制剂递送至受试者。某些配制剂可以包含赋形剂,例如,药物级的甘露醇、乳糖、淀粉、硬脂酸镁、糖精钠、纤维素、碳酸镁等。

[0107] 可以用载体制备药物组合物,所述载体保护活性成分免于从体内快速消除,例如定时释放配制剂或涂层材料。此类载体可以包括受控释放配制剂,例如但不限于微包囊的递送系统和可生物降解的生物相容性聚合物,例如乙烯乙酸乙烯酯、聚酐、聚乙醇酸、聚原酸酯(polyorthoesters)、聚乳酸和已知的其它物质。

[0108] 在某些实施方案中,活的减毒登革热-登革热嵌合体、活的减毒登革热病毒或黄病毒嵌合体的剂量范围可以是最初施用的约$10^2$至约$10^6$PFU,并且任选地,然后是根据需要在30天内或之后长达12个月的至少第二次施用。在某些实施方案中,受试者可以接受本文公开的单价、二价、三价或四价组合物的双重施用,其中组合物是单一组合物混合物或具有不同登革热病毒血清型的预先确定的组合物。

[0109] 明显的是,对于任何特定的儿童或年轻人或任何特定的大龄儿童(aged child),可以根据个体需要调整特定的剂量方案。在一些实施方案中,调整剂量方案以适应青少年受试者,诸如约20岁龄或更小的儿童、或约18岁龄或更小或约15岁龄或更小或约12岁龄或更小或约9岁龄或更小或约6岁龄或更小,或约3岁龄或更小或约1.5岁龄或更小的儿童。在某些实施方案中,儿童可以是2岁至17岁龄。在其他实施例中,儿童可以是4至16岁龄。

[0110] 在某些实施方案中,本文公开的免疫原性组合物可以在一剂或多剂中施用于儿童或年轻人。在一些实施方案中,本文公开的免疫原性组合物可以以单剂或两剂或更多在预先确定的时间段内,包括但不限于约6个月内、约120天内、约90天内、约80天内、约70天内、约60天内、约50天内、约40天内、约30天内、约20天内、约10天内、约5天内或更小,或者在同一天在几小时或几分钟内施用于受试者或者在相同或不同解剖位置中同时施用。在一些实施方案中,本文公开的免疫原性组合物可以在彼此的约90天内、在彼此的约60天内、在彼此的约30天内、以及在彼此的小于约30天内施用。在一些实施方案中,本文公开的组合物可以皮下或皮内施用于受试者。在两个或更多个解剖部位中的施用可以包括施用的任何组合,包括在两个或更多个解剖部位中通过相同模式或者通过包括两个分开的解剖部位的两种不同模式。根据这些实施方案,两个或更多个解剖部位可以包括身体的不同肢体或不同区域。在某些实施方案中,可以在第0天在相同或多个解剖学位置中将两剂疫苗组合物连续导入受试者,例如以提供保护而免于所有登革热血清型(例如,交叉保护)。在某些实施方案中,本文公开的免疫原性组合物可以包括但不限于对受试者施用的诱导对所有四种血清型的免疫原性反应(例如,四价配制剂)的单剂,其能够诱导针对所有登革热病毒血清型的免疫应答。在某些实施方案中,受试者可以接受单剂组合物,其中单剂能够诱导对所有四种登革热病毒血清型的充分免疫应答。在其他实施方案中,免疫原性组合物可以包括活的减毒

登革热病毒血清型与针对其他黄病毒(例如,寨卡病毒、日本脑炎、西尼罗河病毒、圣路易斯脑炎病毒、黄热病或其他病毒)的其他免疫原性试剂的组合。在某些实施方案中,本文公开的针对登革热病毒的疫苗可以用于减少其他相关的病毒感染,例如寨卡病毒感染。

[0111] 在一些实施方案中,可以使用本文中公开的药物组合物来增加儿童或年轻人中靶配制剂的免疫应答,其通过在接受此类针对登革热病毒的药物组合物的儿童或年轻人中组合针对登革热病毒的免疫原性组合物与加强$CD8^+$T细胞应答或针对一种或多种登革热病毒血清型(例如登革热病毒的一种或多种结构或非结构组分)的其他免疫应答的试剂进行。

[0112] 通过局部应用的含有治疗有效量的丝氨酸蛋白酶抑制剂的乳膏、凝胶、冲洗液等完成局部施用。通过应用能够使丝氨酸蛋白酶抑制剂穿透皮肤并进入血流的乳膏、冲洗液、凝胶等完成经皮施用。另外,渗透泵可以用于施用。必要的剂量将随着所治疗的具体状况、施用方法和分子从身体清除的速率而变化。

[0113] 其他实施方案涉及减少活的减毒病毒失活的方法,包括但不限于组合一种或多种活的减毒病毒与能够减少活的减毒病毒(例如黄病毒)失活的组合物。这些组合物可以包括但不限于一种或多种蛋白质试剂;一种或多种糖或多元醇试剂;和任选地一种或多种EO-PO嵌段共聚物,其中所述组合物可以减少失活或稳定化活的减毒病毒。

[0114] 在某些实施方案中,本文涵盖的组合物可以部分或完全脱水或水合。在其他实施方案中,涵盖用于本文的药物或非药物组合物的稳定蛋白质试剂可包括但不限于乳清蛋白、人血清清蛋白、重组人血清清蛋白(rHSA)、牛血清清蛋白(BSA)、其他血清清蛋白或清蛋白基因家族成员。糖或多元醇试剂可以包括但不限于单糖、二糖、糖醇、海藻糖、蔗糖、麦芽糖、异麦芽糖、纤维二糖、龙胆二糖、laminaribose、木二糖、甘露二糖、乳糖、果糖、山梨糖醇、甘露醇、乳糖醇、木糖醇、赤藓糖醇、棉子糖、淀粉酶、环糊精、壳聚糖或纤维素。在某些实施方案中,表面活性剂可以包括但不限于非离子表面活性剂,例如烷基聚(环氧乙烷)、聚(环氧乙烷)和聚(环氧丙烷)的共聚物(EO-PO嵌段共聚物)、聚(乙烯基吡咯烷酮)、烷基多聚葡萄糖苷(例如蔗糖单硬脂酸酯,月桂基二葡糖苷或单月桂酸山梨糖醇酯(sorbitan monolaureate)、辛基葡糖苷和癸基麦芽糖苷)、脂肪醇(鲸蜡醇或油醇(olelyl alcohol))或椰油酰胺(cocamides)(椰油酰胺MEA、椰油酰胺DEA和椰油酰胺TEA)。

[0115] 在其他实施方案中,表面活性剂可以包括但不限于泊洛沙姆407(例如Pluronic F127®)、替换泊洛沙姆407或除泊洛沙姆407之外的泊洛沙姆335、338或238、或与F127®具有类似特性的其他EO-PO嵌段共聚物。

[0116] 在一些实施方案中,疫苗组合物可以包括但不限于一种或多种蛋白质试剂,其为血清清蛋白;一种或多种海藻糖糖类;和一种或多种表面活性剂聚合物试剂,例如EO-PO嵌段共聚物、泊洛沙姆407或更具体地,Pluronic F127®。

[0117] 在其他实施方案中,用于稳定化活病毒的配制剂可以包含一种或多种活黄病毒、一种或多种碳水化合物试剂和一种或多种氨基酸或其盐、酯或酰胺衍生物。在其他实施方案中,本文使用的配制剂稳定化活的减毒黄病毒用于商业用途。在一些实施方案中,组合物还包含缓冲剂。根据这些实施方案,缓冲液可以包括但不限于磷酸盐缓冲盐水(PBS)。根据这些实施方案,缓冲剂可以包括氯化钠(NaCl)、磷酸一钠和/或磷酸二钠($Na_2HPO_4$)、氯化钾(KCl)和磷酸钾($KH_2PO_4$)中的至少一种。在一些实施方案中,组合物的缓冲剂可以包含浓度25mM至200mM的氯化钠。在其他实施方案中,本文公开的组合物可以包含尿素和/或MSG其他

合适的试剂。

[0118] 在一些实施方案中,活的减毒黄病毒如登革热病毒可以在配制剂中稳定化,所述配制剂包含但不限于浓度0.1%至0.2%(w/v)的重组HSA;和/或浓度约4.0%至约6.0%(w/v)的蔗糖;和/或浓度约2%至4%(w/v)的甘露醇浓度;和/或浓度约8.0mM至约22.0mM的丙氨酸;和/或浓度约1.0mM至约5.0mM的甲硫氨酸;和/或浓度约8.0mM至12.0mM的MSG;和/或浓度约0.1%至约0.3%(w/v)的尿素。在某些实施方案中,组合物可以包含重组HSA、海藻糖、甘露醇、丙氨酸、甲硫氨酸、MSG和尿素。在其他实施方案中,稳定化组合物可以包含浓度约0.1%至约0.2%(w/v)的HSA;浓度约4%至约6%(w/v)的海藻糖;浓度约2%至约4%(w/v)的甘露醇;其中丙氨酸浓度为8mM至22mM;其中甲硫氨酸浓度为1mM至5mM;其中MSG浓度为8mM至12mM;其中尿素浓度为0.1%至0.3%(w/v)。用于稳定化活的减毒病毒的某些配制剂可以包含但不限于重组HSA、蔗糖、丙氨酸和尿素。根据这些实施方案,HSA浓度可为约0.1%至约0.2%(w/v);蔗糖浓度可以为约4%至约6%(w/v);丙氨酸浓度可以为约8.0mM至约22mM;并且尿素浓度可以为约0.1%至约0.3%(w/v)。其他稳定化配制剂可以包括重组HSA、蔗糖、甲硫氨酸和尿素。重组HSA浓度可以为约0.1%至0.2%(w/v);蔗糖浓度可以为约4.0%至约6.0%(w/v);甲硫氨酸浓度可以为约1.0mM至约5.0mM;尿素浓度可以为约0.1%至约0.3%(w/v)。在其他实施方案中,稳定化配制剂可以包含重组HSA、蔗糖、精氨酸和尿素,其中重组HSA浓度可以为0.1%至0.2%(w/v);蔗糖浓度可以为4%至6%(w/v);精氨酸浓度可以为10mM至50mM;尿素浓度可以为0.1%至0.3%(w/v)。其他稳定化配制剂可以包括重组HSA、海藻糖、精氨酸和尿素,其中重组HSA浓度为约0.1%至0.2%(w/v);海藻糖浓度为约4%至6%(w/v);精氨酸浓度为约10mM至50mM;尿素浓度为约0.1%至0.3%(w/v)。在其他实施方案中,稳定化组合物可以包括重组HSA、海藻糖、MSG和尿素。根据这些实施方案,重组HSA浓度可以为约0.1%至约0.2%(w/v);海藻糖浓度可以为约4.0%至约6.0%(w/v);其中MSG浓度为8mM至12mM;并且其中尿素浓度为0.1%至0.3%(w/v)。

[0119] 本文的一些实施方案涉及用于运输或其他原因的部分或完全脱水的活的减毒病毒组合物。根据这些实施方案,组合物可以是20%或更多;30%或更多;40%或更多;50%或更多;60%或更多;70%或更多;80%或更多;或90%或更多脱水的。根据这些实施方案,在将药学可接受组合物施用儿童或年轻人之前,病毒疫苗组合物可以脱水并在任何已知的稳定化组合物中再水合。

[0120] 在某些实施方案中,受试者可以是哺乳动物,诸如人或兽医和/或家养动物或家畜或野生动物。在某些实施方案中,本公开的免疫原性组合物可以有效免疫青少年受试者,例如20岁龄或更小的人儿童。尽管与儿童使用的当前登革热病毒疫苗/免疫原性组合物(例如登革热/黄热病嵌合体)相关的现有技术报道了9岁龄或更小的儿童的低效力和/或免疫原性,但本文公开的免疫原性组合物可以在约1岁至约17岁或约1岁至约9岁龄或更大的儿童中产生有效免疫应答。与现有技术相比,本文公开的免疫原性组合物表现出优异的功效和免疫原性。

[0121] 在一个示例性方法中,可以将具有呈现的所有四种登革热病毒血清型的本文公开的免疫原性组合物(例如,四价配制剂)施用于受试者,其中免疫原性组合物可以在受试者中引发针对所有四种登革热病毒血清型的免疫应答。在某些实施方案中,本公开的免疫原性组合物可以包括各种登革热病毒血清型构建体的多核苷酸和/或多肽的组合或本文公开

的活的减毒登革热病毒。在其他实施方案中,免疫原性组合物可以包括具有由SEQ ID NO:9(前主要)、11(MVS)、13(WVS)或15(BVS)表示的编码经修饰的活的减毒登革热-2病毒血清型的核酸序列的多核苷酸;登革热-1/登革热-2嵌合多核苷酸,其具有编码来自经修饰的活的减毒登革热-2病毒血清型的非结构蛋白和来自登革热-1病毒血清型的结构蛋白的核酸序列,其由SEQ ID NO:1(前主要)、3(MVS)、5(WVS)或7(BVS)表示;登革热-3/登革热-2嵌合多核苷酸,其具有编码来自经修饰的活的减毒登革热-2病毒血清型的非结构蛋白和来自登革热-3病毒血清型的结构蛋白的核酸序列,其由SEQ ID NO:17(前主要)、19(MVS)、21(WVS)或23(BVS)表示;和登革热-4/登革热-2嵌合多核苷酸,其具有编码来自经修饰的活的减毒登革热-2病毒血清型的非结构蛋白和来自登革热-4病毒血清型的结构蛋白的核酸序列,其由SEQ ID NO:25(前主要)、27(MVS)、29(WVS)或31(BVS)表示。这些多核苷酸可以与或不与它们编码的各种多肽一起包含在内。在某些实施方案中,本文公开的免疫原性组合物可以包含一种或多种多肽,其对应于由SEQ ID NO:10(前主要)、12(MVS)、14(WVS)或16(BVS)表示的经修饰的活的减毒登革热-2病毒血清型;一种或多种多肽,其对应于由SEQ ID NO:2(前主要)、4(MVS)、6(WVS)或8(BVS)表示的登革热-1/登革热-2嵌合多核苷酸;一种或多种多肽,其对应于SEQ ID NO:18(前主要)、20(MVS)、22(WVS)或24(BVS)表示的登革热-3/登革热-2嵌合多核苷酸;和/或一种或多种多肽,其对应于由SEQ ID NO:26(前主要)、28(MVS)、30(WVS)或32(BVS)表示的登革热-4/登革热-2嵌合多核苷酸。

[0122] 本文公开的各种多核苷酸和多肽(例如TDV-1、TDV-2、TDV-3和TDV-4)的浓度可以大致相同,或者某些登革热病毒血清型可以在免疫原性组合物中超过其他血清型呈现,取决于一种或多种血清型的需要或爆发。根据这些实施方案,各种浓度或比率可以表示为彼此相比的对数PFU浓度,如图11A-11B所示。例如,基于TDV-1与TDV-2与TDV-3与TDV-4的比率,可以将浓度应用于本文公开的免疫原性组合物的各种四价配制剂。浓度可以包括但不限于任何登革热病毒血清型的对数PFU浓度3:3:3:3、4:4:4:4、5:5:5:5、5:1:5:5、3:2:3:3、4:2:4:4、5:3:5:5、4:3:4:4、5:4:5:5、4:4:5:5或任何浓度比率,取决于例如配制剂中表示的血清型的数目、预先确定的响应和期望的效果,如本领域技术人员基于本公开将容易认识到的。

[0123] 在某些实施方案中,活的减毒登革热-2病毒的浓度可以比免疫原性组合物中的一种或多种其他登革热病毒血清型低0.5 1、1.5 2、2.5 3或4个对数PFU,以诱导对所有四种登革热病毒血清型的更平衡的免疫应答并减少病毒干扰。尽管与现有登革热病毒疫苗/免疫原性组合物有关的现有技术报道登革热-2病毒的较低的效力和/或免疫原性,但本文公开的免疫原性组合物提供配制剂,其可以在大于60%受试者和某些情况下约85%中产生对所有四种登革热病毒血清型,包括登革热-2的有效的免疫应答(参见例如图15-16),即使活的减毒登革热-2病毒可以比其他登革热病毒血清型低至少1个对数PFU。对于20岁龄或更小的受试者,与现有技术相比,本公开的免疫原性组合物表现出优异的功效和免疫原性,并且在任何年龄提供针对所有四种登革热病毒血清型的更平衡的免疫应答。在某些实施方案中,可以评估血清转化率,以便在施用一剂或多剂活的减毒疫苗后分析某种配制剂随时间的功效。

[0124] 在某些实施方案中,针对登革热病毒的免疫原性组合物可以包括下列一种或多种:浓度约1.0x $10^3$至约5x $10^5$PFU的登革热-1/登革热-2嵌合体;浓度约1.0x $10^3$至约5x

$10^5$PFU的活的减毒登革热-2;浓度约5.0x $10^3$至约5x$10^5$PFU的登革热-3/登革热-2嵌合体;和/或浓度约1.0x $10^4$至约5x $10^6$PFU的登革热-4/登革热-2嵌合体。在一些实施方案中,四价配制剂可以在三价或四价配制剂中包含比登革热-1/登革热-2嵌合体和活的减毒登革热-2大至少0.5至1个对数的浓度的登革热-3/登革热-2嵌合体,而登革热-4/登革热-2可以在三价或四价配制剂中包含比登革热-1/登革热-2嵌合体和活的减毒登革热-2大至少0.5至1个对数的浓度。在某些实施方案中,登革热-3/登革热-2和登革热-4/登革热-2嵌合体的浓度可以是相同的或登革热-4/登革热-2嵌合体的浓度在本文涵盖的二价、三价或四价配制剂中比登革热-3/登革热-4大。在一些实施方案中,通过本文公开的组合物和方法治疗的受试者可以一年2次、每年、18个月或类似方案治疗,这取决于例如受试者的位置和旅行计划。在某些实施方案中,针对登革热病毒的免疫原性组合物可以包含下列一种或多种:浓度约2.0x $10^4$PFU的登革热-1/登革热-2嵌合体;浓度约5.0x $10^3$PFU的活的减毒登革热-2;浓度约1x $10^5$PFU的登革热-3/登革热-2嵌合体;和/或浓度约3.0x $10^5$PFU的登革热-4/登革热-2嵌合体。

[0125] 在某些实施方案中,可以在一段时间内对接受本文公开的组合物的儿童或年轻人评估与登革热感染或其他黄病毒感染有关的任何症状或体征的形成。例如,可以评估儿童或年轻人的登革热发作或与登革热感染有关的其他症状或体征。

[0126] 治疗方法

[0127] 在本公开的一个实施方案中,方法提供了使用本文涵盖的活的减毒者和/或嵌合病毒构建体的单、双、三或四价配制剂诱导对登革热病毒血清型的免疫应答。

[0128] 通过以下非限制性实施例进一步示例本发明的实施方案,所述实施例不应以任何方式解释为对其范围施加限制。相反,应当清楚地理解,可以采用各种其他实施方案、其修改和等同物,在阅读本文的描述之后在不脱离本发明的精神或所附权利要求书的范围的情况下,本领域技术人员可以想到它们。

## 实施例

[0129] 包括以下实施例以说明本文提供的某些实施方案。本领域技术人员应当理解,以下实施例中公开的技术表示被发现在本文公开的实践中良好起作用的技术,因此可以认为是构成其实践的优选模式。然而,根据本公开,本领域技术人员应当理解,在不脱离本文的精神和范围的情况下,可以在所公开的特定实施方案中进行许多改变并仍然获得相同或相似的结果。

[0130] 实施例1

[0131] 在一些示例性方法中,公开了用于产生本文称为“主病毒种子(MVS)”的组合物。这些组合物可以源自一种或多种活的减毒登革热病毒,例如DEN-1、DEN-2、DEN-3、和DEN-4。在某些方法中,组合物可以源自一种或多种活的减毒登革热病毒,其包括但不限于本文称为TDV-1、TDV-2、TDV-3、和TDV-4(例如,先前称为DENVax)公开的特定构建体。在其他示例性方法中,提供了用于产生和表征这些组合物的策略。在其他实施方案中,提供了四价登革热病毒配制剂和这些配制剂的遗传和表型表征。这些构建体可以用于产生用于治疗1岁至20岁龄的儿童或年轻人的免疫原性组合物。

[0132] 生产和分析前主要TDV病毒

[0133] 进行某些程序以产生前主要登革热病毒种子,诸如登革热病毒(例如TDV)的连续扩增和纯化。首先,通过将从全长重组TDV cDNA转录的病毒RNA转染到生产验证的细胞(例如Vero细胞)中再衍生TDV病毒,产生P1(第1代)病毒种子。然后,扩增来自登革热-1至登革热-4中每种的四种P1病毒并进行噬菌斑纯化以获得候选的前主要疫苗P7种子(参见表1)。进行某些测试以分析登革热病毒的传代。例如,全长基因组测序证明所有四种P2(第2代)种子病毒在遗传上与其同源祖先、研究衍生的、研究级候选疫苗病毒相同。初始噬菌斑表型也保留在P2病毒中。对于来自P2种子的登革热病毒(例如,TDV1-4)的每种血清型分离6个噬菌斑纯化的病毒(P3A-F),并且将每个分离的噬菌斑再直接进行噬菌斑纯化两次。将每种病毒的第三次噬菌斑纯化(P5)在Vero细胞中扩增两次(P6A-F和P7A-F),以产生潜在的前主要P7TDV种子(表1)。

[0134] 表1 WCB-Vero细胞中TDV病毒的cGMP再衍生的实例

[0135]

| 传代 | 种子生产/纯化 | 表征 |
|---|---|---|
| P1 | 用转录的病毒RNA转染WCB-Vero | 噬菌斑滴定 |
| P2 | 扩增P1病毒 | 全基因组序列 |
| P3 | 从P2噬菌斑测定法挑出6个噬菌斑(A-F)/血清型 | 噬菌斑纯化 |
| P4 | 从P3噬菌斑测定法挑出噬菌斑A-F | 噬菌斑纯化 |
| P5 | 从P4噬菌斑测定法挑出噬菌斑A-F | 噬菌斑纯化 |
| P6 | 扩增P5 A-F噬菌斑 | 噬菌斑滴定 |
| P7 | 前主要种子: 扩增P6 A-F | 全基因组序列,TaqMAMA,噬菌斑表型 |
| P8* | MVS: 扩增选定的P7病毒种子 | 全遗传和表型表征 |
| P9 | WVS: 扩增P8主要种子病毒 | 全基因组序列,TaqMAMA |
| P10 | BVS: 扩增P9工作种子病毒 | 全基因组序列,TaqMAMA |
| * 一种最佳的P7种子(A、B、C、D、E或F)基于遗传和噬菌斑分析选择以制备P8 MVS | | |

[0136] 进一步进行一些测试以表征P7TDV种子,诸如分析P7种子的基因组序列和噬菌斑表型,以及与P2种子比较(表2)。P7病毒的噬菌斑表型通常与P2种子的噬菌体表型相似。在一些示例性实验中,监测病毒滴度。除5种病毒外,大多数P7种子的病毒滴度达到超过6.0个对数pfu/ml。在Vero细胞中连续传代10次或更多次后对超过60种候选疫苗病毒种子的基因组测序鉴定DENV-2PDK-53遗传载体的三个主要减毒决定簇的NS1-53和NS3-250处无回复事件,这表明这2个基因座在候选疫苗病毒种子中非常稳定。开发了灵敏的错配扩增测定法(TaqMAMA)以通过实时RT-PCR精确测量5'NCR-57基因座处的回复率。在一些研究中,通过TaqMAMA测量所有24个P7种子的5'NCR-57回复率。根据测定法中每种病毒的输入病毒RNA浓度,TaqMAMA的灵敏度限度介于0.01%和0.07%回复之间,这比通过共有基因组序列分析可检测的10-30%回复灵敏性限度灵敏得多。得到的数据表明24种P7病毒中的15种具有最小或检测不到的回复(<0.07%),1种病毒(TDV-3-D)具有几乎100%回复,8种病毒(例如TDV-1、1TDV-2、2TDV-3、和4种TDV-4)具有0.08%至12.85%的部分回复(表2)。如通过TaqMAMA测

量,对具有低水平的5'NCR57回复的24种P7病毒中的16种进行全长基因组测序。所有测序的病毒维持其它两种TDV减毒决定簇(NS1-53,NS3-250),并且所有病毒都获得了初始工程化重组cDNA克隆中不存在的其他突变(表2)。在一种示例性靶疫苗组合物中,选择TDV-1-A、TDV-2-F、TDV-3-F、和TDV-4-F作为每种血清型的靶标前主要种子,因为它们的基因型和噬菌斑表型最接近类似于最初设计的疫苗重组体的那些。TDV-1-A、TDV-2-F、和TDV-4-F具有两个非同义突变,并且TDV-3-F具有1个。证据表明在这4种前主要种子中观察到的这些额外突变不引起病毒的安全性忧虑或免疫原性改变。进一步扩增这些前主要种子以产生MVS(主种子,称为P8,表1)。

[0137] 本文提供的示例性方法使用来自克隆的cDNA质粒的纯化的体外转录的病毒RNA作为纯来源,以转染疫苗验证的Vero细胞,从而产生疫苗病毒。将连续噬菌斑纯化和全基因组序列分析掺入制造程序中以确保具有最佳纯度和遗传稳定性的制造的疫苗种子。制备6种克隆病毒作为每种TDV血清型的潜在前主要种子。通过基因组分析,包括TaqMAMA和完整基因组测序,以及病毒噬菌斑表型的表征,选择前主要种子以促进每种血清型(血清型1-4)的主要病毒种子生产。所选择的前主要种子在5'NCR-57基因座处具有不可检测的回复(<0.01%或<0.07%),在其基因组中具有1或2个氨基酸取代,并保留先前观察到的小噬菌斑表型。

[0138] 表2.前主要(P7)种子的表征

[0139]

| 病毒 | 克隆[a] | TaqMAMA[b] | $Log_{10}$ pfu/ml | 噬菌斑[c] | 基因组中鉴定的突变[d] |
|---|---|---|---|---|---|
| TDV-1 | **A** | ** | 6.85 | P2 | NS2A-116 I-L, NS2B-92 E-D, 1个沉默 |
| | B | * | 6.93 | P2 | nd[e] |
| | C | * | 6.93 | D | nd |
| | D | ** | 7.02 | D | C-67 K-A; 1个沉默 |
| | E | 0.57% | 7.28 | P2 | nd |
| | **F** | ** | 7.18 | P2 | E473 T-M; 1个沉默 |
| TDV-2 | A | 0.03% | 6.33 | P2 | NS1-341 K-N |
| | B | * | 6.33 | P2 | E-305 K-T, 2个沉默 |
| | C | * | 5.84 | L | NS4A-18 T-A, 4个沉默 |
| | D | 0.08% | 6.20 | P2 | NS2B-99 I-L, 1个3'NCR |
| | E | 0.03% | 6.31 | P2 | prM-52 K-E, NS5-412 I-V, 2个沉默 |
| | **F** | ** | 6.15 | P2 | prM-52 K-E, NS5-412 I-V |

[0140]

| | | | | | |
|---|---|---|---|---|---|
| TDV-3 | A | * | 6.00 | P2 | NS5-200 K-N, 1个沉默, 1个3'NCR |
| | B | 0.05% | 6.27 | P2 | NS2A-33 I-T, NS2A-59 M-T |
| | C | 0.30% | 6.25 | P2 | nd |
| | D | 100.00% | 6.27 | P2 | nd |
| | E | 0.31% | 6.00 | P2 | nd |
| | **F** | ** | 6.30 | P2 | E-223 T-S, 1个沉默 |
| TDV-4 | A | 0.47% | 5.60 | P2 | E323 K-R/K, NS2B-21 L-F/L, NS2B-39 T-S, 1个沉默 |
| | B | * | 5.65 | D | NS2A-126 A-V ; NS4A-5 N-D; NS5-383 K-R, 1个沉默 |
| | C | 4.50% | 5.90 | P2 | nd |
| | D | 12.85% | 5.97 | D | nd |
| | E | 0.52% | 6.85 | S | prM-85 E-D, NS2B-45 T-A, NS5-320 M-T, NS5-551 E-G, 2个沉默 |
| | **F** | 0.02% | 6.93 | S | NS2A-66 D-G, NS4A-21 A-V, 4个沉默 |

[a]选择用于进一步开发MVS的克隆病毒(通过连续噬菌斑纯化)以粗体表示。
[b] *: 回复率< 0.07% (检测限)。 **:回复率< 0.01% (检测限)
[c] 噬菌斑表型: P2: 类似于P2病毒; L= 大于P2病毒, D= 相似大小, 但是在噬菌斑的清晰度上表现略微不同; S =小于P2。
[d] 与工程化TDV cDNA克隆不同的取代。氨基酸突变以病毒蛋白的氨基位置和变化(wt-突变)列出。列出每种种子的结构和非结构基因中的沉默突变的总数。还记录非编码区(NCR)处的突变。
[e] nd = 未完成。这些克隆比其它克隆具有更高的5'NCR-57回复率(通过TaqMAMA), 因此从进一步序列分析排除。

[0141] 实施例2

[0142] 在另一个示例性方法中,描述了主病毒种子、工作病毒种子和散装病毒种子的组合物以及它们的遗传和表型特征。提供这些组合物用于制造临床材料并最终用于商业疫苗供应。将连续噬菌斑纯化和全基因组序列分析掺入制造过程中以确保具有最佳安全性和遗传稳定性的疫苗种子的组合物,用于制造临床试验材料。

[0143] MVS、WVS和BVS的生产和制造质量控制

[0144] 在一些研究中,通过在经验证的Vero细胞中扩增前主要P7种子来产生4种TDV的MVS。在其他研究中,使用MVS在细胞工厂中制备大量的WVS。此外,从WVS扩增TDV的BVS原液并用于人临床试验。在一些示例性方法中进行产物释放的质量控制,包括对MVS、WVS和BVS所有测试身份、感染滴度、无菌性、支原体和体外和体内外来媒介(adventitious agent)。所有种子通过使用血清型特异性RT-PCR测定法的病毒身份测试,其显示对应于其血清型的阳性扩增和对异源血清型呈阴性(数据未显示)。在MVS、WVS或BVS原液中未检测到可检测的支原体或外来媒介。

[0145] 在一个示例性方法中,具有呈现的所有四种登革热病毒血清型的本文公开的免疫原性组合物(例如,四价)可以施用于儿童或年轻人,其中免疫原性组合物可以在儿童或年

轻人中引发针对所有四种登革热病毒血清型的免疫应答。

[0146] MVS、WVS和BVS的遗传分析

[0147] 在某些示例性方法中,在从选择的前MVS(P7)产生MVS后,产生上文选择的毒株,并再次对相应的病毒RNA进行测序。全长基因组测序显示TDV-1的MVS与其前主要种子相同,而WVS和随后的BVS在E-483和NS4B-108处获得2个额外的取代(参见表2和3)。E-483处的Ala取代表示MVS中基因型的一部分,但是成为BVS中的主要基因型。TDV-2和TDV-3与它们各自的前主要种子相同(表2和3)。TDV-2MVS与其前主要种子相同,并且WVS和BVS在NS4A-36和NS4B-111处具有2个额外突变。这两种突变在WVS中都是部分的,并且是BVS中的主要基因型。TDV-3的MVS再次与前主要种子相同,但WVS和BVS在NS4A-23处含有额外的氨基酸取代。在MVS的产生期间,TDV-4MVS在基因座NS2A-99处获得额外的氨基酸突变(从Lys至Lys/Arg混合基因型)(表3)。其WVS和BVS保留了NS2A-99Lys/Arg混合基因型,并且BVS具有额外的NS4B-238Ser/Phe混合基因型。共有序列结果还证实MVS、WVS以及BV在5'NCR-57、NS1-53和NS3-250基因座处保留三种减毒的遗传决定簇。通过TaqMAMA对最小稳定性的减毒基因座的分析表明,MVS中5'NCR-57回复率在<0.7%至0.13%之间,在WVS中≤0.07%,在BVS之间为<0.07至0.21%。5'NCR-57位点处的3%回复被认为是疫苗批次接受性的最大允许率(表3)。

[0148] 表3 TDV种子中的核苷酸和氨基酸取代

[0149]

| TDV | 核苷酸 | 氨基酸 | 前主要 | MVS[a] | WVS[a] | BVS[a] |
|---|---|---|---|---|---|---|
| TDV-1 | 2384 G-C | E-483 | - | - | Gly/ | Ala |

[0150]

| | | | | | | |
|---|---|---|---|---|---|---|
| | | Gly-Ala | | | **Ala** | |
| | 3823 A-C | NS2A-116 Ile-Leu | Leu | Leu | Leu | Leu |
| | 4407 A-T | NS2B-92 Glu-Asp | Asp | Asp | Asp | Asp |
| | 7148 C-T | NS4B-108 Thr-Ile | - | - | **Ile** | **Ile** |
| | 7311 A-G | 沉默 | G | G | G | G |
| | TaqMAMA 5'NCR-57 回复%[b] | | -- | - | - | - |
| TDV-2 | 592 A-G | prM-52 Lys-Glu | Glu | Glu | Glu | Glu |
| | 6481 G-C | NS4A-36 Ala-Pro | - | - | **Ala/Pro** | **Pro** |
| | 7156 C-T | NS4B-111 Leu-Phe | - | - | **Leu/Phe** | **Phe** |
| | 8803 A-G | NS5-412 Ile-Val | Val | Val | Val | Val |
| | TaqMAMA 5'NCR-57 回复 %[b] | | -- | - | 0.07 % | 0.21% |
| TDV-3 | 1603 A-T | E-223 Thr-Ser | Ser | Ser | Ser | Ser |
| | 6436 G-A | NS4A-23 Asp-Asn | - | - | **Asn** | **Asn** |
| | 7620 A-G | 沉默 | G | G | G | G |
| | TaqMAMA 5'NCR-57 回复 %[b] | | -- | - | - | - |
| TDV-4 | 225 A-T | 沉默 | T | T | T | T |
| | 3674 A-G | NS2A-66 Asp-Gly | Gly | Gly | Gly | Gly |
| | 3773 | NS2A-99 | - | **Lys/** | **Lys/** | **Lys/Arg** |

[0151]

| | | | | | | |
|---|---|---|---|---|---|---|
| | A-A/G | Lys-Lys/Arg | | **Arg** | **Arg** | |
| | 5391 C-T | 沉默 | T | T | T | T |
| | 6437 C-T | NS4A-21 Ala-Val | Val | Val | Val | Val |
| | 7026 T-C | 沉默 | T/C | T/C | T/C | T/C |
| | 7538 C-C/T | NS4B-238 Ser-Ser/Phe | - | - | **Ser/Phe** | **Ser/Phe** |
| | 9750 A-C | 沉默 | C | C | C | C |
| | TaqMAMA 5'NCR-57 回复 %[b] | | - | 0.13 % | - | - |
| [a] 粗体：在MVS原液开始的变化。 | | | | | | |
| [b] "--" 指示回复率< 0.01% (检测限), "-" 指示回复率<0.07% (检测限) | | | | | | |

[0152] 全基因组序列分析揭示在TDV-4MVS中形成的另外的氨基酸突变,而其他三个TDV MVS批次保留其前主种子的共有基因组序列。从P1种子到前主要(P7)种子,在给定种子中仅发生1或2个非同义突变。从P1到MVS(P8)种子,在任何给定的TDV种子中鉴定出2至7个核苷酸取代,并且这些取代中仅2至3个导致氨基酸变化。MVS中的沉默突变无一在可影响病毒复制的5'或3'NCR内。仅TDV-2的prM-52Lys-Glu的变化和TDV-4的NS2A-66Asp-Gly的取代不是保守的变化。TDV-4的NS2A-66突变位于DENV-2PDK-53的非结构骨架部分中。尽管在DENV-2的各种毒株中NS2A-66基因座通常是Asp,但它对于DENV-4通常是Gly。TDV-4中的Asp至Gly变化可能与Vero细胞中TDV-4的适应性(fitness)相关。TDV-2prM-52突变位于prM的C末端部分,其从成熟病毒颗粒中切除。在一些示例性方法中,进行表型表征以证实MVS种子中无一突变显著改变疫苗的减毒表型。

[0153] TDV病毒在制造过程中表现出高遗传稳定性。位于5'NCR、NS1-53和NS3-250中的三个限定的DENV-2PDK-53减毒基因座在TDV从前主要毒株连续传代至散装疫苗制剂后在共有基因组序列中保持稳定。5'NCR-57基因座的高度灵敏的TaqMAMA在登革热病毒血清型的MVS,WVS(P9/工作)和BVS(疫苗的散装病毒种子)中表现出最小或不可检测的回复。TDV BVS制剂(P10等同物)的5'NCR-57回复率显著低于Vero细胞中10次连续传代后研究级疫苗候选物中进化的5'NCR-57回复率(4-74％回复)。大规模制造本文提供的TDV种子的策略产生遗传稳定的疫苗种子,其在候选疫苗病毒中保留减毒的标志物。

[0154] 所有TDV病毒构建体可以储存在稳定化的缓冲液,例如FTA、泊洛沙姆407 F127® (0.01％至约3.0％w/v)和约5％至约50％(w/v)海藻糖,2和约0.01％至约3.0％清蛋白(例如rHSA)中

[0155] 安全性和体内免疫原性

[0156] 在此实施例中,证明了示例性组合物在皮下注射后是安全的并且基本上是免疫惰性的。如下选择四种不同的示例性组合物用于在小鼠中测试(数据未显示)。

[0157] 配制剂1:15%海藻糖、2%F-127、1%rHSA

[0158] 配制剂2:15%海藻糖、2%F-127、1%rHSA、1mM $CaCl_2$/0.5mM $MgSO_4$

[0159] 配制剂3:15%海藻糖、2%F-127、1%rHSA、0.5%壳聚糖

[0160] 配制剂4:22.5%海藻糖、3%F-127、1.5%rHSA

[0161] 配制剂5:PBS

[0162] TDV MVS的噬菌斑表型

[0163] 在一个示例性方法中,将TDV MVS的噬菌斑表型与野生型登革热病毒及其在Vero细胞中的同源研究级嵌合病毒进行比较(图2)。TDV-1、-2和-3的所有MVS产生的噬菌斑在Vero细胞中明显小于其野生型同源物,并且与其同源研究级病毒非常相似(在0.4mm差异内)。TDV-4MVS也显著小于野生型DENV-4,但比初始实验室衍生的D2/4-V嵌合体略大(0.9mm差异)。

[0164] 图2表示示例性直方图,其显示了与对照野生型病毒和研究级疫苗候选病毒形成对比的TDV MVS的噬菌斑大小。在第9天pi测量的琼脂糖覆盖下Vero细胞中病毒噬菌斑的平均噬菌斑直径(mm)±SD(误差条)。包括以黑条表示的野生型DEN病毒和以白条表示的以前发表的研究级疫苗候选病毒,用于与由灰色条表示的TDV主疫苗种子的对照和比较。

[0165] TDV MVS的温度灵敏度

[0166] 在另一个示例性方法中,在Vero细胞中对TDV MVS测试温度敏感性,并与其同源野生型和初始研究级嵌合疫苗病毒进行比较。野生型(WT)DENV-3 16562对温度不敏感。wt登革热病毒血清型1和登革热病毒血清型-4在39℃是中等温度敏感的(滴度在39℃比在37℃低约1.0个$log_{10}$pfu/ml,图3)。WT登革热病毒血清型-2 16681是所测试的WT登革热病毒中温度最敏感的,并且在39℃导致100倍的滴度下降。TDV-1、-2和-3与其初始同源研究级嵌合疫苗病毒一样是温度敏感的(图2)。对于这些TDV毒株,在39℃的滴度下降2.0至3.0个$log_{10}$pfu/ml。TDV-4也是温度敏感的,表现出滴度降低5倍。然而,初始研究级D2/4-V表明滴度降低约10倍。最终稳定化的TDV-4MVS含有泊洛沙姆407、F127®(以及其他稳定化这些配制剂的试剂(FTA或二价配制剂、清蛋白和海藻糖)),先前已证明其增强登革热病毒的热稳定性。TDV-4MVS中F127®的存在可能促成Vero培养测定法中病毒的不太明显的温度敏感性。在不同的实验中,进一步评估在缺乏F127®的情况下MVS衍生的TDV-4毒株的温度敏感性。为了从毒株中除去泊洛沙姆407,F127®,从TDV-4散装病毒制剂中分离病毒RNA,并将其转染到Vero细胞中。在缺乏泊洛沙姆407,F127®的情况下,该TDV-4病毒在感染后第3天似乎与D2/4V研究毒株(滴度降低1.5个$log_{10}$pfu/ml)一样是温度敏感的(图3)。

[0167] 图3显示的示例性直方图显示了TDV MVS温度敏感性。包括野生型登革热病毒和先前发表的研究级疫苗候选病毒用于比较。TDV-4MVS含有额外的poloxamer 407,F127®,其可以掩盖该测定法中病毒的温度敏感性结果。还包括在缺乏F127®的情况下分析替代物TDV-4的不同实验。在37℃或39℃在Vero细胞中复制的病毒的平均滴度±SD(误差条)。

[0168] TDV MVS在蚊C6/36细胞中的复制

[0169] 在一些示例性方法中,凭借研究级嵌合疫苗病毒保留骨架DENV-2PDK53病毒在这些蚊细胞中的减毒表型的知识,将TDV MVS在C6/36细胞中培养以验证它们对体外减毒表型的保留。与wt登革热病毒相比,TDV-1、TDV-2和TDV-4MVS在感染后(pi)第6天显示C6/36细胞

的显著生长降低(至少3个$log_{10}$pfu/ml降低)(图4)。与wt DENV-3 16562相比,TDV-3MSV也表现降低的生长,但降低不明显(1-2个$log_{10}$pfu/ml降低)。然而,TDV-3种子批次的C6/36效价与初始研究级嵌合D2/3-V疫苗病毒的C6/36效价相似(在1个$log_{10}$pfu/ml差异内)。

[0170] 图4显示的示例性直方图,其绘制与wt登革热病毒(黑色条)和研究级疫苗病毒(白色条)相比C6/36细胞中TDV MVS(灰色条)的受限生长。第6天pi在C6/36细胞中复制的病毒的平均滴度±SD(误差条)。

[0171] 整个蚊中的病毒感染、散播(dissemination)和传播率(transmission rate)

[0172] 在一些示例性方法中,将TDV的感染和散播速率与其亲本wt登革热病毒进行比较。在某些示例性实验中,在埃及伊蚊中进行口腔感染实验。对感染性血粉进行返滴定以测量病毒滴度,并且仅包括亲本登革热病毒和每种血清型的TDV之间在血粉中具有相似病毒滴度(小于1个$log_{10}$pfu/ml差异)的实验,用于表4中的比较。TDV-1、TDV-2和研究级D2PDK-53-VV45R未通过口服喂养感染蚊,这与其亲本病毒DENV-1 16007(44%感染)和DENV-216681(43.3%感染)显著不同($p<0.0001$)。由于没有蚊被TDV-1和-2感染,因此对这两种疫苗病毒几乎没有传播忧虑。虽然TDV-4确实通过口服喂养感染一些蚊(55只中的2只),但感染率显著低于其亲本野生型病毒DENV-4 1036(50只中的8只)($p<0.05$)。TDV-3在血粉病毒滴度为5.2±0.02$log_{10}$pfu/ml的两次实验中没有感染任何蚊(表4),并且在血粉病毒滴度为6.0$log_{10}$pfu/ml的不同实验中,仅30只蚊中的1只被感染(数据未显示)。然而,野生型登革热病毒-3 6562在5.2$log_{10}$pfu/ml也具有非常低的感染率(8%),并且在具有更高的血粉病毒滴度6.2$log_{10}$pfu/ml的不同实验中该比率没有增加(3%,30只蚊中1只阳性,数据未显示)。虽然野生型(WT)登革热病毒-3和登革热病毒-4的感染率显著低于野生型登革热病毒-1和登革热病毒-2,但感染蚊的平均病毒滴度相似(3.1至3.9$log_{10}$pfu/蚊)。相比之下,来自两只受感染蚊的TDV-4滴度都是最小的(0.7$log_{10}$pfu/蚊),其比来自感染野生型登革热病毒血清型-4 1036的蚊的滴度(3.9±1.5pfu/蚊)低1000倍。

[0173] 对于那些被感染的蚊,可以通过确定腿中是否存在病毒来评估中肠外的传播。四种亲本DENV导致范围为36.3%和62.5%之间的传播率,并且其来自腿的平均病毒滴度(以$log_{10}$pfu计)在0.9±0.3和2.2±0.7之间(不包括阴性样品)。两只TDV-4感染的蚊都未导致病毒传播到腿(表4)。虽然在腿中可检测到传播病毒,但是四种wt登革热病毒无一在口腔感染的蚊的唾液中检出,这表明口服喂养条件对于测量这些DENV的传播率可能不是足够灵敏的。因此,在其他示例性方法中,随后进行通过直接IT接种的高严格的人工蚊感染(表4)。除TDV-4外,所有病毒(wt和TDV)均实现IT接种的埃及伊蚊的100%感染。TDV-4接种物的病毒滴度略低于其他三种病毒接种物,但它仍成功感染70%的接种蚊。尽管通过IT接种实现高的身体感染率,但与wt登革热病毒(43-87%,表4)相比,所有四种TDV病毒表现出显著更低($p<0.005$)或检测不到的传播率(0-10%)。TDV病毒表明在口服喂养后很少至无感染和传播,并且高严格的IT结果证实了这些TDV病毒在埃及伊蚊中的最小传播能力。

[0174] 表4:整个蚊中的病毒感染、散播和传播率

[0175]

| 病毒 | 口服喂养 | | | | | IT接种 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 血粉[a] | 感染[b] | 身体滴度[c] | | 散播[c] | 接种物 | 感染[b] | 身体滴度[c] | 唾液[f] | |
| | 均值±SD | %(P/N) | 均值±SD | P[d] | %(P/N)[f] | pfu/剂 | %(P/N) | 均值±SD | %(P/N) | P[d] |
| DENV-116007 | 6.6 | 44.0%(11/25) | 3.6±1.5 | | 36.3%(4/11) | 53.9 | 100%(30/30) | 4.7±0.48 | 43%(13/30) | |
| TDV-1 | 6.9 | 0%(0/30) | NA | <0.0001 | NA | 67.8 | 100%(30/30) | 3.4±0.39 | 10%(3/30) | <0.005 |
| DENV-216681 | 6.6 | 43.3%(13/30) | 3.1±1.5 | | 38.5%(5/13) | 67.8 | 100%(30/30) | 5.2±0.34 | 87%(26/30) | |
| D2PDK53-VV45R | 6.4 | 0%(0/30) | NA | <0.0001 | NA | 56.4 | 100%(30/30) | 4.0±0.20 | 0%(0/30) | <0.0001 |
| TDV-2 | 6.4 | 0%(0/30) | NA | <0.0001 | NA | 52.7 | 100%(30/30) | 3.5±0.27 | 7%(2/30) | <0.0001 |
| DENV-316562 | 5.2 | 8%(2/25) | 3.8±0.2 | | 50%(1/23) | 34.0 | 100%(30/30) | 4.2±0.50 | 67%(20/30) | |
| TDV-3 | 5.2±0.02 | 0%(0/50) | NA | 0.108 | NA | 37.3 | 100%(30/30) | 3.3±0.36 | 3%(1/30) | <0.0001 |
| DENV-41036 | 5.8±0.5 | 16%(8/50) | 3.9±1.5 | | 62.5%(5/8) | 69.4 | 100%(30/30) | 5.2±0.45 | 70%(21/30) | |
| TDV-4 | 5.4±0.4 | 3.6%(2/55) | 0.7±0.0 | 0.033 | 0%(0/2) | 11.8 | 70%(21/30) | 1.1±0.46 | 0%(0/21) | <0.0001 |

[a] 病毒滴度或均值±标准差，若通过返滴定来自血粉中的超过1个实验($log_{10}$ pfu/ml)
[b] 蚊体中检测的病毒率。*P/N*= 阳性/总蚊
[c] 蚊体中均值病毒滴度±标准差($log_{10}$ pfu/蚊)，仅包括阳性样品以进行计算
[d] 通过Fisher确切概率法(Fisher Exact probability)，wt DENV和TDV之间的差异的统计学分析
[e] 在阳性感染蚊的腿中检测的病毒率
[f] 在阳性感染蚊唾液中检测的病毒率。用于测量传播效率

[0176] 传病媒介能力(vector competence)是活减毒黄病毒疫苗病毒的重要安全性组分。以前,研究级DENV-2PDK-53-VV45R病毒和wt回复突变体衍生物在埃及伊蚊中进行了测试,并且发现NS1-53-Asp减毒突变是蚊复制受损的主要决定因素。DENV-2PDK-53疫苗的其它两个主要减毒基因座,核苷酸5'NCR-57-T和NS3-250-Val,也对蚊的复制表现出一定的抑制效果,因此为蚊传病媒介能力提供了额外的冗余的限制。本文描述的一些示例性方法用于测试所有四种TDV毒株的蚊口腔和IT感染和复制。TDV-1、-2和-3没有通过口腔感染感染任何埃及伊蚊(表4)。TDV-4仅以比wt DENV-4感染的蚊的复制平均滴度低的蚊体内复制平均滴度感染3.6%口暴露的蚊,水平显著低于wt DENV-4的水平。令人惊讶的是,在感染的蚊的腿中检测到TDV-4,表明TDV-4在口感染后不能从蚊中肠散播。TDV-1、-2和-4的感染率均显著低于其野生型对应物,但是TDV-3和WT DENV-3 16562之间的差异不显著,这是由于这

两种病毒的感染率非常低。与在来自相同泰国湄索(Mae Sot)省收集的埃及伊蚊中评估的其他野生型DENV毒株相比,用于工程化TDV的亲本野生型登革热病毒株通过口腔感染似乎具有较低的感染率和散播率。用于工程化基于黄热病(YF)17D疫苗的ChimeriVax-DEN疫苗的wt DENV-1PUO359、DENV-2PUO218、DENV-3PaH881/88、和DENV-4 1288具有范围为47-77％的感染率。相反,YF 17D疫苗不能感染埃及伊蚊。尽管ChimeriVax毒株含有来自这些高感染性wt DENV的prM-E,但ChimeriVax保留它们的YF 17D复制骨架的蚊减毒表型。本文提供的结果还指示DENV-2PDK-53骨架的蚊减毒在TDV毒株中得到维持。此外,在本文所述组合物中包含的构建体中使用具有较低蚊感染性的wt登革热病毒株提供了额外的安全特征。

[0177] 在一个示例性方法中,口腔感染结果表明TDV具有最小的蚊感染性和散播能力。此外,进行更灵敏且严格的IT感染实验以进一步分析TDV由埃及伊蚊传播的潜力。IT结果证明了所有四种TDV病毒与其wt对应物相比具有检测不到或最小的蚊传播潜力。TDV传播仅可以在以下情况下在理论上发生:(1)传病媒介以具有足够病毒血症滴度的已接种疫苗者为食以感染蚊中肠,(2)病毒能够在中肠上皮中复制并能够随后从中肠散播出去,并且(3)散播的病毒可以在唾液腺中复制,并在唾液中咳出足够的病毒进行传播。尚未充分建立感染蚊需要的人病毒血症的阈值,但是在自然wt DENV感染后人病毒血症可以是$10^6$-$10^8$蚊感染剂量$_{50}$($MID_{50}$)/ml。该$MID_{50}$基于用稀释的人血浆直接IT接种蚊。对非人灵长类动物中TDV的分析指示TDV免疫后的病毒血症滴度非常低(小于2.4$log_{10}$pfu/ml)并持续2-7天。鉴于TDV的低病毒血症水平和低蚊感染、散播和传播能力,这些疫苗病毒不太可能可被蚊自然传播或导致病毒血症。

[0178] 因此,提出任何血清型的任何传代(P1-P10)可以在组合物中使用以产生针对一种、两种、三种或所有四种登革热病毒血清型的安全且有效的疫苗。哺乳小鼠中的神经毒力

[0179] 初始研究级疫苗病毒在DVBD/CDC内部维持的新生ICR小鼠中的神经毒力方面高度减毒。所有这些小鼠幸免于用$10^4$pfu每种疫苗病毒的ic(脑内)攻击。另一方面,wt登革热病毒血清型-2 16681病毒在各种实验中导致这些CDC-ICR小鼠的死亡率62.5％-100％。在一些实验中,从Taconic Labs(Taconic-ICR)获得的商业ICR小鼠用于研究新生小鼠中的神经毒力。观察到新生Taconic-ICR小鼠对登革热病毒血清型-2感染的易感性显著高于先前的CDC-ICR小鼠。图5A总结了用$10^4$pfu病毒ic攻击的Taconic-ICR新生小鼠和CDC-ICR集落中的wt登革热病毒血清型-2 16681的神经毒力。Taconic-ICR小鼠(32只小鼠中100％死亡率,平均存活时间8.3±0.5天)比以前的CDC-ICR小鼠(72只小鼠中91％死亡率,平均存活时间14.6±2.3天)对ic登革热病毒血清型-216681更易感。

[0180] 在其他示例性方法中,为了评估TDVMVS的神经毒力,最初在一个(n＝16)或两个(n＝31-32)实验中用约$10^4$pfu wt登革热病毒血清型-2 16681、D2PDK-53VV45R、D2/3-V、或TDV 1-4病毒的剂量ic(脑内)攻击Taconic-ICR小鼠。在该剂量,D2/3-V研究级病毒以及TDV-1和TDV-3MVS表现出完全减毒的神经毒力表型(无疾病或死亡)。如预期,发现wt登革热病毒血清型-2是“致命的”,平均小鼠存活时间(AST)为8.3±0.8天。在这些登革热病毒血清型-2敏感性Taconic-ICR小鼠中,D2PDK-53-VV45R研究级病毒导致81.3％的死亡率。TDV-2MVS和TDV-4MVS在Taconic-ICR中均为致命,显示AST值分别为9.8±1.7、10.2±1.4、和11.3±0.4天。

[0181] 在一些示例性方法中,在低10倍的剂量($10^3$pfu,图5C),将wt登革热病毒血清型-2

16681病毒的神经毒力与D2PDK-53VV45R、TDV-2MVS和TDV-4MVS以及D2/4-V研究级病毒的神经毒力进行比较。在此较低的攻击剂量下,wt登革热病毒血清型-2保留匀一致命的神经毒性表型,AST为9.0±1.4天。其他4种病毒表现出中间神经毒力表型,并且神经毒力程度是血清型特异性的。D2PDK-53-VV45R病毒及其TDV-2MVS关联物显示显著的减弱(分别为在13.1±3.8天的AST的情况下32.3%存活和在10.5±3.4天的AST的情况下31.2%存活)。TDV-4MVS和研究级D2/4-V病毒两者在神经毒力方面都是高度减毒的(分别为在18.8±5.8天的AST的情况下81.3%存活和100%存活)。结果表明,TDV-1和-3的MVS表现出神经毒力的完全减弱,而TDV-2和-4MVS批次保留与其同源研究级病毒候选疫苗非常相似的减毒表型。

[0182] 图5A-5C表示显示用各种组合物测试的新生小鼠中的神经毒力的示例性图,所述组合物包含wt登革热病毒血清型-2和不同的减毒登革热病毒。许多实验的汇总结果总结了用$10^4$pfu病毒(A)ic攻击的CDC-ICR(n=72)和Taconic-ICR(n=32)新生小鼠中wt登革热病毒血清型-2 16681病毒的神经毒力。用$10^4$pfu(B)或$10^3$pfu(C)的剂量在Taconic-ICR小鼠中测试的TDVMVS的神经毒力。指示在一个实验(n=16)或两个合并实验(n=31或32)中每组测试的动物数目。

[0183] WVS和BVS噬菌斑表型

[0184] 进行某些研究以在Vero细胞中比较WVS和BVS与MVS、wt登革热病毒及其同源实验室衍生的研究级嵌合体的噬菌斑表型(图6)。从对于每种疫苗病毒的10个噬菌斑计算平均噬菌斑大小,但是从减少的wt DENV-1、-3和-4数目计算平均噬菌斑大小。TDV-1、-2和-3的所有MVS病毒产生的噬菌斑在Vero细胞中显著小于它们的wt同源物,并且与其同源研究级病毒非常相似(在0.4mm差异内)。TDV-4MVS也显著小于wt DENV-4,但比初始实验室衍生的D2/4-V嵌合体略(0.9mm)大。除TDV-2外,TDV-1、-3、-4的所有WVS和BVS保留的噬菌斑尺寸显著小于从其wt同源物产生的噬菌斑尺寸。TDV-2WVS和BVS产生的噬菌斑在Vero细胞中类似于wt DENV-2病毒的噬菌斑,但是当在LLC-$MK_2$细胞中测试时,所有TDV-2制造的种子产生的噬菌斑略小于wt DENV-2(1.4±0.4)的噬菌斑并且类似于实验室衍生的D2PDK-53-VV45R(1.0±0.3)(图6)。

[0185] 针对MVS原液的组成评估病毒减毒的表型标志物的评估,包括小噬菌斑表型、温度敏感性、蚊细胞中降低的复制、蚊的感染/散播/传播减少和新生ICR小鼠中的神经毒力降低。结果指示所有TDV保留与初始研究级疫苗病毒相似的预期减毒表型。鉴于负责减毒的突变在所有MVS、WVS和BV中都是保守的,可以预期减毒表型保留在为人临床测试制造的材料中。

[0186] 图6表示显示TDVMVS、WVS和BVS的噬菌斑大小的示例性直方图。在第9天pi测量的琼脂糖覆盖下的Vero或LLC-$MK_2$细胞中病毒噬菌斑的平均噬菌斑直径±SD(误差条)。包括野生型DENV和先前发表的研究级疫苗候选病毒用于对照和比较。

[0187] 病毒在蚊C6/36细胞中复制

[0188] 先前的研究证明,基于PDK-53的研究级嵌合疫苗病毒保留C6/36细胞中骨架DENV-2PDK53病毒的减毒表型。在一些示例性方法中,在C6/36细胞中培养TDV MSV、WVS和BVS,以验证在大规模制造后此体外减毒标志物的其保留。与野生型登革热病毒相比,除TDV-3外,所制造的种子在第6天pi显示C6/36细胞中明显的生长降低(至少3$log_{10}$PFU/ml降低)(图7)。与wt DENV-3 16562相比,TDV-3种子也表现降低的生长,但降低不明显(1-2$log_{10}$PFU/ml降

低)。然而,TDV-3种子批次的滴度与初始研究级嵌合D2/3-V疫苗病毒相似(在1$log_{10}$PFU/ml差异内)。

[0189] 图8表示绘制C6/36细胞中TDV MVS、WVS和BVS的限制性生长的示例性直方图。在第7天pi在C6/36细胞中复制的病毒的平均滴度±SD(误差条)。包括wt登革热病毒和先前发表的研究级疫苗候选病毒用于比较。

[0190] 哺乳小鼠中的神经毒力

[0191] 进行另外的实验以分析新生ICR小鼠中的神经毒力。在颅内剂量$10^4$PFU,wt DENV-2 16681和D2PDK-53-VV45R的存活在ICR小鼠中分别为0%和18.8%(图9A),但是在CDC ICR小鼠中,对于wt DENV-2 16681为约20%且对于D2PDK-53-VV45R为100%。在这项研究中,TDV-1和TDV-3MVS在$10^4$PFU的剂量对于小鼠是减毒的(100%存活),但是TDV-2和TDV-4的MVS在超过$10^4$PFU的剂量导致100%死亡率(图5A)。然而,当以$10^3$PFU的病毒剂量进行测试时,相对于野生型登革热病毒血清型-2 16681(0%存活率),TDV-2(31.3%存活率)和TDV-4(81.3%存活率)显示神经毒力降低,并且它们的存活率分别与研究级疫苗候选物D2PKD-53-VV45R(32.3%)和D2/4-V(100%)的存活率相似(图9B)。虽然在本研究中未包括野生型DENV-1、-3或-4用于比较,但先前的工作证明野生型DENV-1 16007通过ic途径在CDC-ICR小鼠中是减毒的,而野生型DENV-3 16562和DENV-4 1036两者对于CDC-ICR小鼠是高毒力的(0%存活率)。这3种野生型DENV可能在更易感的Taconic ICR小鼠中表现出相似或更高的毒力。因此,包含这些野生型登革热病毒以与其同源TDV MVS进行比较被认为是无信息的。该研究指示相对于野生型DENV-2 16681,所有4种TDV MVS和初始实验室衍生的候选疫苗病毒表现出相当的小鼠减毒表型。

[0192] 图9A-9B表示新生ICR小鼠中TDVMVS的神经毒力数据的示例性图。(A)以$10^4$PFU剂量的病毒IC接种。(B)以$10^3$PFU剂量的病毒IC接种。

[0193] 对TDV的所有种子批次测试身份、无菌性和不期望的作用剂的免除。全基因组序列分析揭示在TDV-4MVS中进化出一个额外的氨基酸突变,而其他3个TDV MVS保留其前主要种子的共有基因组序列。在WVS批次中,TDV-3获得额外的氨基酸突变,而其他3种血清型相对于其前主要种子积累2个额外的氨基酸取代。所有4个BVS批次的基因组序列与其WVS批次相同。总体而言,从P2种子至前主要(P7)种子,在给定的种子中仅发生1或2个非沉默突变。在前主要和BCS(P10)种子之间,仅观察到1到2个核苷酸取代,除了导致残基E-483处的保守甘氨酸和丙氨酸的单核苷酸变化外,所有这些取代都发生在NS2A、4A或4B中。从P2至BVS(P10)种子,在任何给定的TDV种子中鉴定出总共3至8个核苷酸取代,并且这些取代中仅2至4个导致氨基酸改变。BVS中的无一沉默突变在可能影响病毒复制的5'-或3'-NCR区域内。这些结果表明TDV病毒在制造过程中在遗传上是高度稳定的。位于5'NCR、NS1-53和NS3-250中的三个限定的DENV-2PDK-53减毒基因座在连续传代TDV以产生BVS原液时在共有基因组序列中保持不变。5'-NCR-57基因座的高度灵敏的TaqMAMA在TDV的MVS、WVS和BVS中显示最小或检测不到的回复。在TDV-2BVS中鉴定出0.21%的最高回复率。P10等同BVS的回复率(<0.07%至0.21%)显著低于Vero细胞中连续传代后其他疫苗候选物中进化的回复率(到P10,4-74%回复)。这表明就维持候选疫苗病毒中的遗传稳定性和减毒标志物的保留而言,此种用于大规模制造TDV种子的策略是成功的。

[0194] 由于本文公开的MVS原液将用于WVS和BVS批次的未来制造,因此对所有MVS或其等

同替代原液进行完全的病毒减毒表型评估组,包括小噬菌斑表型、温度敏感性、蚊细胞中的复制减少、整个蚊中降低的感染/散播/传播、和新生ICR小鼠中降低的神经毒力。对于WVS和BVS原液,还进行了噬菌斑大小,蚊细胞中的感染性以确认它们的减毒。结果指示4种TDV血清型的所有MVS原液保留预期的减毒表型,如小噬菌斑、C6/36细胞中降低的复制、降低的小鼠神经毒力,与初始实验室衍生的疫苗病毒相似(图6、8和9)。除TDV-4外,TDV的所有其他3种MVS原液在39℃为TS,如图3和图7所示。

[0195] 对于WVS和BVS原液,分析和确认了两种减毒表型/小噬菌斑和C6/36细胞中的限制性复制。由于MVS和BVS之间存在非常小的遗传变化,因此预计它们将保留作为MVS的减毒表型。除了本报告中描述的实验之外,还测试了制造的TDV在Ag129小鼠和非人灵长类动物中的安全性和免疫原性。

[0196] 本文提供了示例性方法以证明在cGMP下制造TDV MVS、WVS和BVS原液。BVS原液用于配制目前在人临床试验评估中的四价TDV。在一些示例性方法中提供了用于优化制造的MVS的遗传稳定性和安全性的独特制造策略。由于先前已经充分表征了TDV的主要减毒基因座并且高度灵敏且可量化的SNP测定法,开发TaqMAMA以整合基因组序列和TaqMAMA,从而鉴定用于制备MVS的最佳前主要种子。对MVS的遗传和表型特征全面分析,以确认这些病毒对疫苗的安全性保持期望的减毒。这可以是唯一的活的减毒病毒疫苗,其可以有效地分析制造过程中从前主要原液一直至BVS原液的所有主要减毒遗传基因座。本文提供的结果例示策略性设计的减毒活疫苗在疫苗安全性方面的优势。

[0197] 实施例3

[0198] 在另一个示例性方法中,除了编码登革热-2血清型的非结构蛋白域和来自登革热-1(TDV-1)、登革热-3(TDV-3)或登革热-4(TDV-4)血清型中的任一种的结构蛋白的一种或多种构建体之外,构建体还可包括基于活的减毒登革热-2血清型(TDV-2)的经修饰的活的减毒登革热病毒。另外,本文公开的免疫原性组合物中的单独构建体可以以基于彼此相比的对数PFU的PFU的比率或浓度配制。例如,可以配制免疫原性组合物,使得TDV-1以约$2x10^4$PFU的浓度存在,TDV-2以约$5x10^4$PFU的浓度存在,TDV-3以约$1x10^5$PFU的浓度存在,并且TDV-4以$3x10^5$PFU的浓度存在,如图11B所示。免疫原性配制剂中不同登革热血清型构建体的相对浓度也可以表示为基于PFU的对数数的比率,例如约4:4:5:5,对应于TDV-1:TDV-2:TDV-3:TDV-4的比率,如图11B所示。在一些实施方案中,比率可以是约5:4:5:5以及其他预定比率,其中一种或多种活的减毒登革热病毒血清型与其他登革热病毒血清型相比更高或更低。在一个实例中,与TDV-1、TDV-3和TDV-4相比,TDV-2的相对浓度可以降低,并且当向受试者施用所有四种登革热血清型时,这可以产生具有更好的平衡响应的免疫原性组合物。例如,TDV-1、TDV-2、TDV-3和TDV-4可以诱导针对所有四种登革热病毒血清型的中和性抗体(活的减毒登革热-2和登革热病毒-1、-3和-4的嵌合体),并且使用登革热-2作为骨架可以诱导多功能和交叉反应的CB8+T细胞对登革非结构蛋白的应答。

[0199] 在另一个示例性方法中,为了测试本文公开的登革免疫原性组合物在儿童(历史上难以有效针对登革热病毒免疫的群体,并且先前的疫苗在该群体中未能起作用,尤其是9岁及以下的儿童)中的功效和耐受性,进行了两部分随机、双盲、安慰剂对照的临床试验。第1部分以年龄下降的方式进行,其中对年龄21-45岁的受试者(n=38)、年龄12-20岁的受试者(n=36)、年龄6-11岁的受试者(n=38)和年龄1.5-5岁的受试者(n=36)施用一剂含有所

有四种构建体,TDV-1,TDV-2,TDV-3和TDV-4的登革热疫苗组合物(图12A)。第II部分在年龄1.5-11岁的健康儿童受试者(n=211)中进行,作为第I部分的扩展(图12B)。在第0天施用疫苗剂量,并且在第0天和贯穿整个试验的各个时间点从受试者中取出组织样品(例如血液样品)以测试病毒血症、免疫原性和总体安全性(图12A-12B)。对于第I部分,随机化剂量的疫苗和安慰剂以约2:1的疫苗与安慰剂比率施用。对于第II部分,随机化剂量的疫苗和安慰剂以约3:1的疫苗与安慰剂比率施用。在免疫原性组合物中,TDV-1以约$2x10^4$PFU的浓度存在,TDV-2以约$5x10^4$PFU的浓度存在,TDV-3以约$1x10^5$PFU的浓度存在,并且TDV-4以约$3x10^5$PFU的浓度存在。

[0200] 在另一种方法中,为了研究本文公开的针对登革热的免疫原性组合物在儿童中的耐受性,研究不良反应(AE)的发生率(数据未显示)。在TDV和安慰剂施用之间确定注射部位的疼痛、瘙痒和红斑以及水肿的发生率(自我报告)。在第一次施用TDV(n=249)或安慰剂(n=111)后14天内,以及在第二次施用TDV(n=249)或安慰剂(n=111)后14天内调查各种AE。总体而言,在研究受试者中没有报道严重的疫苗相关AE,并且血液化学或血液学没有显著变化,指示本公开的登革热疫苗组合物在儿童中良好耐受。(数据未显示)

[0201] 在另一个示例性方法中,为了测试本公开的免疫原性组合物在儿童中的功效,在注射TDV疫苗组合物后由受试者产生的中和性抗体的存在在注射后720天的各个时间点使用微中和测试测量。如图13A-13B所示,TDV疫苗施用引发持续至第720天的中和性抗体应答,而不管受试者的初始免疫状态(图13A中所示的基线时呈血清阳性;图13B中所示的基线时登革热未免疫)。Denv-1抗体水平由标记有实心黑色菱形的短虚线表示;Denv-2抗体水平由标记有实心黑色方块的实线暗线表示;Denv-3抗体水平由标记有实心黑色三角形的长虚线表示;并且Denv-4抗体水平用标记有X的虚线表示。剂量由向上指向的对应于第0天和第90天的实线箭头表示。

[0202] 在另一个实施例中,为了测试登革免疫原性组合物是否可以在儿童中引发四价应答而不管初始免疫状态,在注射TDV疫苗组合物后在各个时间点直至注射后720天测量研究受试者的血清阳性。如图14所示,在针对所有登革热血清型测试的所有注射后时间点,大于约60%的研究受试者呈血清阳性。在针对三种或更多种登革热血清型测试的所有注射后时间点,大于约80%的受试者呈血清阳性。并且在针对两种或更多种登革热血清型测试的所有注射后时间点,几乎100%的受试者呈血清阳性。第0、28、90和120天的数据获自临床试验的第I部分和第II部分(n=249),而第180、360和720天的数据获自临床试验的第I部分(n=90)。确定血清阳性是$MNT_{50}$滴度>10。

[0203] 在另一个示例性方法中,进行测试以确定本文公开的针对登革热的免疫原性组合物是否能够在基线时呈血清阴性(登革热未免疫)的儿童中引发针对所有四种登革热病毒血清型的免疫应答。如图15中所示,在针对所有登革热血清型测试的所有注射后时间点,大于约40%的研究受试者呈血清阳性。在针对三种或更多种登革热血清型测试的所有注射后时间点,大于约60%的受试者呈血清阳性。并且在针对两种或更多种登革热血清型测试的所有注射后时间点,几乎100%的受试者呈血清阳性。第0、28、90和120天的数据获自临床试验的第I部分和第II部分(n=133),而第180、360和720天的数据获自临床试验的第I部分(n=40)。确定血清阳性是$MNT_{50}$滴度>10。应当注意,本文公开的四价组合物能够诱导针对所有四种登革热病毒血清型的免疫应答,其能够在施用后持续到720天。

[0204] 实施例4

[0205] 在一种方法中,将参与者登记;1794名在第0和第3个月时接受皮下注射两个TDV剂量(n＝200;第1组);第0个月接受一剂(n＝398;第2组);第0个月接受一剂并且在第12个月接受加强剂(n＝998;第3组);或安慰剂(n＝198;第4组)。TDV引发针对所有DENV的中和性抗体,其在第1个月达到峰值并且在第6个月时仍然高于基线。第1-4组中的第6个月GMT(95％CI)分别为:对于DENV-1为89(321、746)、434(306、615)、532(384、738)、62(32、120);对于DENV-2为1565(1145、2140)、1638(1286、2088)、1288(1031、1610)、86(44、169);对于DENV-3为160(104、248)、151(106、214)、173(124、240)、40(23、71);和对于DENV-4为117(79、175)、110(80、149)、93(69、125)、24(15、38)。在最初血清阴性参与者中,两个TDV剂量比一剂引起更高的平均GMT和向DENV-3和-4的血清转化率,并且相对于一个TDV剂量后的67・6％,第6个月时的四价血清转化率为85・0％。

[0206] 研究设计和参与者

[0207] 这些研究是在巴拿马、菲律宾和多米尼加共和国的三家医院/诊所进行的多中心、随机化、双盲、安慰剂对照研究。登记年龄2-17岁的健康参与者并评估他们的合格性,然后以1:2:5:1比率随机化以在第0个月和第3个月接受两剂的TDV(第1组);第0个月接受一剂(第2组);第0个月接受一剂和第12个月接受加强剂(第3组);或安慰剂(第4组)。选择1:2:5:1的随机化比率以提供有关施用或不使用加强剂的单剂方案的数据,以支持该给药方案在3期开发中的潜在用途。先前对TDV的研究已经建立了2剂日程表(第0个月-第3个月)的安全性和免疫原性,并且该组(第1组)用于与较大的1个一剂组(第2组和第3组)进行比较。

[0208] 程序

[0209] TDV的血清型组成在冻干配制剂中分别为2.5x $10^4$、6・3x $10^3$、3・2x $10^4$和4・0x $10^5$噬菌体形成单位(PFU)的TDV-1、TDV-2、TDV-3和TDV-4。安慰剂是磷酸盐缓冲盐水;在施用时将TDV在注射用水中重建,并且皮下注射0・5mL的TDV或安慰剂(例如三角肌区域)。先前将样品在稳定化配制剂中冻干。

[0210] 在研究治疗施用之前的第0和第3个月,以及第1和第6个月从免疫原性子集的参与者收集用于测量中和性抗体的血液样品,并集中分析。

[0211] 一个端点是免疫原性,其通过使用微中和测试[$MNT_{50}$]在第1、3和6个月时针对四种DENV血清型中每种的中和性抗体的几何平均滴度(GMT)显示。次要免疫原性端点是第1、3和6个月时四种DENV血清型中每种的血清阳性率(其中通过$MNT_{50}$的血清阳性定义为倒数中和滴度(reciprocal neutralizing titer)≥10)。对按方案组(per protocol set,PPS)总结免疫原性端点,所述按方案组包括来自免疫原性子集的所有参与者,他们没有主要方案违背,并且对于他们,可获得有效的给药前和给药后血样。

[0212] 统计分析

[0213] 此试验被设计为主要描述性的,并且不基于测试正式无效假设(formal null hypotheses)。因此,不基于正式的统计功效计算(formal statistical power calculation)确定样本大小。认为计划的样本大小1800名参与者(免疫原性子集中600名)提供合理数目的参与者,用于评估单剂施用后免疫应答的持续性和加强剂量的效果,并提供足够的安全数据库,以支持大约20000名参与者中的3期效力试验。

[0214] 在基线和第1、3和6个月时单独对于四种TDV血清型中每种,以95％置信区间(CI)

计算登革热中和性抗体的GMT和血清阳性率。具有至少二价、三价和四价血清阳性的参与者的百分比在每次研究访视时按组总结。这些数据也通过基线登革热血清状态呈现;血清阳性定义为对于≥1种DENV血清型的倒数$MNT_{50}$≥10。描述性总结安全性数据,请求的AE按年龄(<6和≥6岁)表示。

[0215] 在第6个月,1743名参与者接受了计划的方案。研究参与者的平均年龄为7·3年(数据未显示)。PPS(免疫原性子集)的人口统计学数据通常反映了安全性组的人口统计学数据,只是PPS中比安全性组中更小的比例为年龄2-5岁。PPS中基线时任何DENV血清阳性的参与者比例在研究组之间相似(总体为54·7%;范围51·2-57·4%)。在接种疫苗的参与者中,TDV引发针对所有4种登革热血清型的中和性抗体。针对DENV-2观察到最高水平的登革热中和性抗体(图2),接着是DENV-1,并且针对DENV-3和DENV-4观察到更低水平。每种血清型的GMT在第1个月时达到峰值,并且在第6个月(第180天)从基线保持升高。第1-4组中的第6个月GMT(95%CI)分别为:DENV-1:489(321、746)、434(306、615)、532(384、738)、62(32、120);DENV-2:1565(1145、2140)、1638(1286、2088)、1288(1031、1610)、86(44、169);DENV-3:160(104、248)、151(106、214)、173(124、240)、40(23、71);和DENV-4:117(79、175)、110(80、149)、93(69、125)、24(15、38)。在基线时血清阴性的参与者中,两剂日程表在第6个月(第1组)引起的DEN-3和DEN-4GMT高于对第2组和第3组参与者给予的单剂量,尽管CI重叠(数据未显示)。在基线时血清阳性的参与者中,所有TDV组中的GMT应答是相似的(数据未显示)。

[0216] 到第1个月,所有研究组中接种TDV疫苗的参与者中对个别DENV的血清阳性率增加至87·3-100%,并且在第6个月时对于每种DENV仍然较高(85·0-100%;图3,左图)。在基线时血清阴性的参与者中,两剂日程表导致比一剂日程表在第6个月时更高的对DENV-3(97·5%)和DENV-4(87·5%)的血清阳性率(对于DENV-3为85·7%和85·3%以及对于DENV-4为81·4%和69·3%-数据未显示)。然而,95%CI重叠。

[0217] 在基线时,44·7%的参与者总体上具有对DENV的四价血清阳性。到第1个月,每个研究组中超过80%的接种TDV疫苗的参与者具有四价血清阳性,并且96%或更多具有至少三价血清阳性(数据未显示)。在第6个月时维持多价血清阳性率,在两个TDV剂量后6个月的比率稍高(数据未显示)。在基线血清阴性参与者中,6个月四价血清阳性率在接受两剂的参与者(第1组为85%)中比在接受一剂的参与者(第2组和第3组为70%和65·3%)中高,尽管95%CI重叠。

[0218] 总体而言,1402名参与者接受一个TDV剂量,并且194名接受两剂。32名参与者(即安全性组的1·8%)报告的40例SAE无一与研究疫苗或程序有关。三个SAE导致研究退出(对食物着色剂的过敏反应、免疫性血小板减少性紫癜和急性肾小球肾炎)。与研究疫苗或程序无关的一例死亡发生在6个月的分析截留后(由于肺炎、肺结核和脓毒性休克)。两例妊娠导致研究中断,但参与者随后正常分娩并且她们的婴儿是健康的。

[0219] 在TDV和安慰剂之间未看到未请求的AE率的主要差异,或是在第一剂相对于第二剂后,或是与基线时的血清阳性相关,并且大多数与研究疫苗接种无关(表3)。总体而言,免疫原性子集中562名参与者中的15名(2·7%)报告与疫苗相关的未请求的AE。未请求的AE在186名参与者中的161名(86·6%)中是轻度的。在基线血清阳性参与者中,相对于接受安慰剂的47名中的1名(2·1%)和252名中的2名(0·8%),250名中的5名(2·0%)和45名中

无一(0％)分别在第一次和第二次TDV注射后报告与疫苗接种相关的未请求的AE。在最初血清阴性参与者中,相对于接受安慰剂的43名中的无一(0％)和203名中的2名(1·0％),202名中的4名(2·0％)和42名中的1名(2·4％)分别在第一次和第二次TDV注射后报告与疫苗相关的未请求的AE。

[0220] 疫苗接种后的血清阳性可以是疫苗性能的重要量度,因为它提供了对疫苗接种的可测量响应的证据。在缺乏保护相关物的情况下,当然不可能说保护需要多大的响应。然而,产生体液和细胞免疫并且对大多数个体(即使那些没有事先暴露于登革热的个体)中表明对所有登革热血清型的可测量血清转化的疫苗表明它适合在大规模疫苗效力试验中进行评估,并且可以是儿童和青少年中针对登革热病毒的有效疫苗。为该试验选择的给药日程表应当在最大比例的最初血清阴性受试者中产生多价响应。

[0221] 在该示例性大研究中,2期分组从两个登革热地方流行地区(亚洲和拉丁美洲)取得并且接近将用TDV接种疫苗的真实世界群体。在评估间隔3个月给予的1个对2个TDV剂量的此第一项研究中,证明诱导的体液免疫原性在初始剂量后6个月仍保持稳健,初始血清阴性疫苗接种者中仅略微降低,但是第二剂的施用确实有助于减轻这些减少(例如DENV-4)以及增加免疫学响应疫苗接种(例如DENV-3)的受试者比例。在1596名接种TDV疫苗的参与者中,6个月内没有出现新的安全性忧虑。无论接种疫苗时登革热血清状态,证实TDV在儿童和年轻人中从2岁龄开始是安全且耐受良好的。

[0222] 尽管黄病毒嵌合体的其他疫苗在几个地区获得许可,但这些未被批准用于9岁以下的儿童。这些黄病毒嵌合体在登革热未免疫的接受体中诱导针对DENV-1和-2的35-50％血清型特异性保护和较低的功效。因此,仍然需要在所有年龄的接受体,特别是那些小于9岁的接受体(不论先前的登革热暴露和感染血清型)中针对所有四种DENV血清型的安全且有效的疫苗。TDV的有希望的2期结果支持设计为支持TDV在广泛年龄范围内使用的研究中两剂日程表的3期评估的启动,而不考虑接种疫苗时的登革热血清阳性(特别是在幼年儿童中)。

[0223] 材料和方法

[0224] 病毒和细胞

[0225] DENV-1 16007、DENV-2 16681、DENV-3 16562、和DENV-4 1034充当野生型(wt)DENV对照,并且它们是四种重组TDV疫苗候选物的亲本基因型病毒。先前制备并表征了称为D2/1-V、D2PDK-53-VV45R、D2/3-V、和D2/4-V的TDV祖先研究级病毒。用于制备用于疫苗生产的主要和工作细胞库的Vero(非洲绿猴肾)细胞源自美国典型培养物保藏中心(ATCC)CCL81细胞系,其已被世界卫生组织(WHO)表征以用于疫苗生产(WCB-Vero细胞)。

[0226] 从cDNA克隆衍生活重组TDV病毒

[0227] 为了在cGMP制造条件下再衍生候选疫苗病毒,含有全基因组长度病毒cDNA的先前工程化的DENV感染性cDNA克隆pD2-PDK-53-VV45R、pD2/1-V、pD2/4-V、和体外连接的pD2/3-V用于通过体外转录制备新鲜病毒RNA转录物,如先前描述。简言之,用蛋白酶K处理XbaI线性化的DENV基因组cDNA,用苯酚/氯仿提取并在乙醇中沉淀以除去任何残留的蛋白质,然后在转录前悬浮在无RNA酶的Tris-EDTA缓冲液中。使用AmpliScribe T7High Yield Transcription试剂盒(Epicenter Technologies)按照制造商推荐的方案进行体外转录。在2小时转录反应期间掺入RNA A-帽类似物m7G(5')ppp(5')A(New England BioLabs)以将

5'-末端A-帽添加至RNA转录物。然后用DNA酶I处理样品以消化模板cDNA,接着进行低pH苯酚/氯仿提取和乙醇沉淀以除去残留的DNA和蛋白质。将悬浮在无RNA酶水中的纯化的RNA转录物以20μl等分试样分配并储存在-80℃直至准备转染细胞。通过琼脂糖凝胶电泳分析RNA转录物的完整性和浓度。估计每个20-μl等分试样含有足够的基因组长度的病毒RNA,以允许通过电穿孔转染0.4-1x $10^7$个生产认证的Vero细胞。

[0228] 在ShanthaBiotechnics的cGMP设施中将每种RNA转录物转染到WCB-Vero细胞中。解冻TDV RNA转录物,与400μl Vero细胞悬浮液(1x $10^7$个细胞/ml)混合,并转移至预冷的无菌电穿孔皿(4-mm间隙),通过Gene PulserXcell总系统(BioRad Laboratories)进行电穿孔。每个样品在250V/∞Ohms/500μF脉冲一次,在室温下温育10-15分钟,转移到含有30ml细胞生长培养基(含有10%FBS的MEM)的75-$cm^2$烧瓶中,并在36℃±1℃、5%$CO_2$温育6至11天。收获培养基,通过离心澄清,稳定化,并低于-60℃以小等分试样储存。通过Vero细胞中的噬菌斑滴定测定法测定由转染产生的候选疫苗原液(称为P1,代表传代水平1)的病毒滴度,并用于进一步增殖TDV种子。TDV病毒种子的制造

[0229] P1病毒种子用于通过设计为确保制造批次的最佳遗传稳定性和安全性的策略来增殖TDV前主要、主要、工作和散装病毒种子批次。该策略包括三个连续的噬菌斑纯化,以及各种传代水平的病毒的遗传分析,以选择用于连续种子生产的最佳克隆病毒群体(表1)。简言之,通过以MOI为0.001感染Vero细胞扩增从转染细胞收获的P1种子一次以产生P2种子。通过噬菌斑形态学和完整的病毒基因组测序评估P2种子原液的等分试样。将遗传确认的P2原液接种在具有覆盖培养基的Vero细胞单层上,如下面的噬菌斑滴定部分所述,以产生良好分离的噬菌斑。在用中性红显现后,分离来自4种疫苗病毒血清型中每一种的6个单独噬菌斑(噬菌斑克隆A至F)并混合到0.5ml培养基中(第P3代)。将六个噬菌斑悬浮液中的每一个进行另外两轮噬菌斑纯化,分别在P4和P5传代产生两次和三次噬菌斑纯化的病毒种子。通过两个连续的Vero传代扩增P5病毒以产生P7种子原液。

[0230] 使用如先前公开的基于点测序和/或基于Taqman的错配扩增突变测定(TaqMAMA)对三个主要TDV减毒基因座进行遗传分析,并进行噬菌斑表型分析以筛选所有24种P7种子。然后,通过完整的基因组测序进一步表征拥有适当初始特征的种子。作为这些分析的结果,基于DENV-2PDK-53减毒突变的存在、最小基因组序列改变和预期的噬菌斑表型,选择每种TDV血清型的6个(克隆AF)P7种子之一为前主要种子。通过在多个175$cm^2$的Vero细胞培养烧瓶中以0.001的MOI一次性传代病毒,将每个选择的前主要种子扩增成主病毒种子(MVS或P8)。除TDV-4MVS外,在感染后(pi)8-10天收获主病毒种子。感染后(pi)6-10天收获MVS原液,通过离心澄清,通过加入蔗糖/磷酸盐/谷氨酸盐溶液(最终浓度分别为7.5%蔗糖、3.4mM磷酸二氢钾、7.2mM磷酸氢二钾、5.4mM谷氨酸钠)和0.95至1.90%FBS(终浓度)稳定化。以不同方式制备TDV-4MVS以优化其产量。简言之,在已经证明增强DENV病毒的热稳定性的0.1%F-127™,泊洛沙姆407(其它EO-PO嵌段共聚物已经经评估并且本文可以替换,参见公告专利)存在下,以MOI 0.001用TDV-4前主要种子感染多个细胞烧瓶。在感染后第6-10天收获感染性培养基,并用17%FBS(终浓度)稳定化,合并并冷冻。低于-60℃将所有四种TDV MVS原液作为1ml等分试样储存。

[0231] 通过在MVS的Vero细胞培养物中以MOI为0.001一次传代来制备TDV工作病毒种子(WVS)。程序类似于MVS的生产,只是它们在多层细胞工厂(6360$cm^2$)中培养。将WVS原液通过

10μM和0.45μM过滤器过滤,使用用于MVS的相同稳定剂稳定化,等分取样到30ml PETG瓶或2.0ml冷冻瓶中,并储存在-60℃以下。

[0232] 在某些方法中,通过用90mL稀释的WVS感染汇合的Vero细胞的多个细胞工厂(每个6360cm$^2$)产生散装病毒种子(BVS)以获得0.001的MOI。用于稀释WVS接种物的培养基含有0.1%F-127™,不含血清。吸附1.5小时后,用PBS洗涤细胞3次,并向每个细胞工厂加入800ml无血清DMEM培养基,并将工厂在36(±1)℃在5(±0.5)%$CO_2$中温育。温育4天后,收集培养基的小等分试样用于无菌性测试。在感染后第5天和第10天之间收获病毒,并通过0.45μm孔径过滤器立即澄清澄清,并通过加入500ml 3x FTA缓冲液(PBS,pH 7.4中终浓度15%海藻糖,1.0%Pluronic®F-127™泊洛沙姆407、0.1%人血清USP)使1L每个澄清的病毒合并物稳定化。将稳定化的病毒分配到1-L PETG瓶中并在-60℃以下冷冻储存,用于随后的合并和质量控制测试。具有高于$10^5$PFU/ml的病毒滴度和可接受的残留DNA水平的所有稳定化的病毒收获物在32℃的水浴中快速解冻,然后无菌合并并混合。将每个合并的单价BVS分配到标记的PETG容器中并在-60℃以下储存直至进一步使用。

[0233] 制造产品质量控制

[0234] 测试MVS、WVS和BVS种子的身份、无菌性和可检测的外来媒介。通过使用TDV血清型特异性引物的RT-PCR确认每种疫苗原液的身份。扩增的cDNA片段含有E/NS1嵌合连接位点,以便鉴定四种TDV血清型中的每一种。在所有4种血清型特异性RT-PCR反应中测试每种种子以确认病毒身份和免于异源TDV血清型的交叉污染。根据USP 71(美国药典,第71节)进行无菌性测试。进行支原体测试。

[0235] 以下用于病毒污染的体外和体内测试均使用在种子制造期间收集的未澄清的未稳定化的TDV收获物进行。首先用DENV兔多克隆抗血清(Inviragen)在36±1℃中和收获的感染性培养基1小时以灭活DENV。对于体外测试,将中和的种子接种到25cm$^2$烧瓶中的三个指示细胞系MRC5、VERO和MA104中。艾柯病毒(CPE对照)或腮腺炎病毒(血细胞吸附对照)分别用作阳性CPE或血细胞吸附对照。每天监测所有细胞的CPE总共14天。在14天结束时,除去培养物上清液并用10mL豚鼠红细胞(RBC)溶液(在磷酸盐缓冲盐水中的3mL0.5%豚鼠RBC,用细胞生长培养基制成10mL)替换。然后将烧瓶在5±3℃温育30分钟,然后在室温下温育30分钟。用PBS洗涤单层,并且在10X放大率下观察用于血细胞吸附的任何星形的RBC块的存在。

[0236] 在哺乳小鼠、断奶后小鼠和豚鼠中进行外来媒介的体内试验。通过腹膜内(ip)注射,用0.1ml或0.01ml(每个剂量组中10只小鼠)的DENV-抗血清中和的种子样品接种哺乳小鼠。类似地,对10只断奶后小鼠各自腹膜内接种0.5ml或0.03ml样品。每只豚鼠(5只/组)各自腹膜内接种5.0mL。在接种后每天观察哺乳小鼠的发病率和死亡率总共14天。在接种后,观察断奶后小鼠总共28天,并且观察豚鼠总共42天。若≥80%的接种动物在整个观察期内保持健康,则测试物品符合接受标准。

[0237] 还在含胚鸡蛋中进行污染物的体内测试并进行。对于每个样品,将10只含胚鸡蛋(9天龄)各自用0.5mL DENV抗血清中和的样品接种到尿囊液中并在35℃温育3天。收集来自这10只蛋的尿囊液,合并,并传代到10只新鲜含胚蛋(10-11天龄;0.5mL/蛋)的尿囊液中,并在35℃再温育3天。类似地,对于每个样品,通过注射到卵黄囊中对10只含胚蛋(6-7天龄)各接种0.5mL/蛋(TDV-2单价BVS)或0.25mL/蛋(TDV-1、TDV-3和TDV-4BVS)并在35℃温育9天。

收集并合并来自这10只蛋的卵黄囊,并且将10%悬浮液传代到10只新鲜含胚蛋(6-7天龄;0.5mL/蛋)的卵黄囊中,并在35℃再温育9天。在温育3天后观察接种到尿囊液(初始和传代接种两者)中的蛋的存活率。使用鸡、豚鼠和人O型红细胞在4℃和25℃测试尿囊液的这两种合并物的血凝集活性。在温育9天后观察接种到卵黄囊(初始和传代接种两者)中的蛋的存活率。

[0238] 病毒噬菌斑测定和免疫聚焦测定

[0239] 使用Vero细胞通过噬菌斑测定或免疫聚焦测定来测量病毒滴度。如前所述,在汇合的Vero细胞的六孔板中的双琼脂糖覆盖物中进行噬菌斑测定,并且它们还用于评估TDV种子的噬菌斑表型。为了准确比较,在同一实验中测量并比较所有病毒的噬菌斑大小。在感染后第9天用中性红显现后,测量每种病毒多达10个孔分离的噬菌斑的平均噬菌斑大小计算。测量wt DENV-1、-3和-4的较小的噬菌斑,其较大的噬菌斑大小通常不允许测量10个良好分离的噬菌斑。

[0240] 因为四价TDV含有所有四种DENV血清型,所以开发DENV血清型特异性免疫聚焦测定以定量四价配制剂中的每种TDV组分。将每个单独的TDV MVS的免疫焦点测定与噬菌斑测定进行比较,以确保两种测定之间的病毒滴定结果可比较。免疫聚焦测定在用连续稀释的病毒感染的汇合的Vero细胞的6孔板中进行。用含有0.7%高粘度羧甲基纤维素(Sigma)的平衡盐培养基(BSS/YE-LAH培养基)覆盖细胞,并在37℃,5%$CO_2$下温育7天。除去覆盖物后,将细胞片用PBS洗涤3次,用冷的80%丙酮在-20℃固定30分钟,用PBS洗涤一次,并用PBS中含有2.5%(w/v)脱脂奶粉、0.5%Triton X-100、0.05%Tween-20的封闭缓冲液于37℃封闭30分钟。将封闭的细胞与稀释的DENV血清型特异性MAbs、1F1(DENV-1)、3H5(DENV-2)、8A-1(DENV-3)、或1H10(DENV-4)在封闭缓冲液中于37℃温育1小时或4℃过夜,用洗涤缓冲液(在PBS中0.05%Tween-20)洗涤3次,并于37℃与碱性磷酸酶或辣根过氧化物酶(HRP)偶联的亲和纯山羊抗小鼠IgG(Jackson Immuno Research Laboratories)一起温育45-60分钟。将板洗涤3次,然后加入适当的底物,用于碱性磷酸酶的1步NBT/BCIP加抑制剂(Pierce)或用于HRP的Vector-VIP试剂盒(Vector Labs)用于显色。当焦点完全显影时,通过用水冲洗来停止显色。直接显现染色的免疫球蛋白并在灯箱上计数。

[0241] 基因序列

[0242] 对MVS和WVS的全长基因组进行测序(参见下文)。简言之,通过使用QIAamp病毒RNA试剂盒(Qiagen)从TDV种子中提取病毒RNA,并使用Titan One Tube RT-PCR试剂盒(Roche Applied Science,Inc.)扩增覆盖整个基因组的重叠cDNA片段。对扩增的cDNA片段进行凝胶纯化,然后使用BigDye Terminator v3.1循环测序试剂盒(Applied Biosystems)用正向和反向引物两者测序。使用BigDyeXTerminator纯化试剂盒(Applied Biosystems)清洁序列反应,并在DVBD/CDC的3130xl遗传分析仪(Applied Biosystems)上运行。LasergeneSeqMan软件(DNAStar,Inc)用于基因组分析和比较。

[0243] 基于Taqman的错配扩增突变试验(TaqMAMA)

[0244] TaqMAMA是一种灵敏的定量单核苷酸多态性测定法,其开发用于允许更精细评估5'NC-57减毒位点的回复水平,并进一步优化用于该研究。通过用两组对wt或疫苗5'NC-57区特异性的引物/Taqman探针的TaqMAMA分析来自MVS和WVS的提取的病毒RNA。用于检测DENV-2wt和疫苗序列的正向引物分别是D2-41-GC和D2-40-TT。每个正向引物的3'末端核苷

酸与每种病毒的特异性5'NCR-57核苷酸匹配,而每个引物中与3'-末端核苷酸相邻的核苷酸与DENV-2病毒基因组序列不同以增强错配效应。用于wt和疫苗组两者的反向引物CD-207和Taqman探针CD-169F是相同的。先前描述了引物和探针的序列以及循环条件。使用iR5或CFX-95系统(BioRad),使用BioRadiScript RT-PCR(用于探针)试剂盒,在含有5μl病毒RNA模板、0.4μM每种引物和0.2μM探针的25μl反应中进行实时RT-PCR。对每个样品进行每种wt和疫苗特异性测定的一式三份反应。相对于为每种病毒基因型制备的标准曲线确定基因组拷贝数,其中RNA标准品是源自含有每种基因型特异性cDNA的nt 1-2670的质粒的转录物。此外,通过用异源基因型引物/探针组测试每种RNA标准品来确认测定的特异性,以确保在每个实验中最小的交叉反应性。结果报告为显示回复的病毒基因组的百分比。以前,由于限制此测定的输入RNA水平的较高的交叉反应背景,初始检测灵敏度约为0.1%回复(鉴别能力)。从那时起,使用改善的实时PCR设备和反应试剂盒进一步优化了测定,并且在RNA模板输入的高得多的水平(7-8$log_{10}$拷贝)下交叉反应背景相当大地降低。此种优化导致检测灵敏度显著改善,降至0.01-0.07%回复。

[0245] 蚊C6/36细胞中的病毒复制和哺乳动物Vero细胞中的温度敏感性

[0246] 在C6/36蚊细胞(白纹伊蚊(Aedesalbopictus)中评估四种TDVMVS原液和wt DENV-1、-2、-3、和-4病毒的复制表型。将6孔板中培养的C6/36细胞以MOI为0.001用每种病毒一式两份感染,并与4ml/孔含有2%FBS的DMEM培养基在5%$CO_2$培养箱中于28℃温育。在感染后第6天,针对每种病毒收集培养物上清液的小等分试样,与等体积的含有40%FBS的培养基混合,并储存在-80℃直至准备用于病毒噬菌斑滴定。

[0247] 通过比较6孔板中Vero细胞的感染后第5天在39℃的病毒生长与37℃的生长进行温度敏感性。于37℃以MOI为0.001用每种病毒将细胞一式四份感染。吸附病毒后,将感染的培养物与4ml/孔含有2%FBS的DMEM培养基在2个不同的5%$CO_2$培养箱中温育,一组(一式两份板)在37℃,而另一组在39℃。在感染后第5天收集培养物上清液的等分试样(50-μl),与等体积的含有40%FBS的DMEM混合,并储存在-80℃直至准备用于病毒噬菌斑滴定。培养箱温度用NIST可溯源的工厂校准温度计(-1至51℃;ERTCO)校准。

[0248] 蚊感染、散播和传播

[0249] 用于研究的埃及伊蚊来自于2002年从泰国湄索(16'N,33'E)附近的村庄建立的集落。从幼虫出来后,将成年蚊以16:8(光照:黑暗)光周期保持在28℃,随意提供10%蔗糖溶液。五至七日龄雌性蚊用于感染性血粉喂养或胸腔内(IT)接种。在收获(没有任何冻融循环)后立即使用新鲜培养的TDV和wt DENV的等分试样以制备如下所示的病毒血粉用于口腔感染。对剩余的病毒上清液补充FBS至终浓度20%,并将等分试样储存在-80℃,用于将来的病毒噬菌斑滴定和IT接种实验。用于这些实验的新鲜制备的TDV种子从Vero细胞中的前主要种子扩增,并且认为是TDV MVS等同物。

[0250] 通过在口腔感染当天将新鲜病毒以1:1的比率与去纤维蛋白的鸡血液(Colorado Serum Company)混合来制备感染性血粉。将蚊进行糖饥饿过夜,然后使用Hemotek膜进料系统(Discovery Workshops)提供病毒:血液混合物1小时。将50μl血粉等分试样保留在-80℃用于病毒剂量的返滴定。在冷麻醉下对完全充血的雌性进行分选,并将其置于纸箱中,随意提供10%蔗糖溶液。将纸箱置于28℃,光周期为16:8h(光照:黑暗)。14天后,通过暴露于三乙胺(Flynap®,Carolina Biological Supply Company)麻醉来自每个病毒组的25-30只

蚊,并且取出一条后腿并置于含有10％FBS和5％青霉素/链霉素(分别为100U/ml和100μg/ml)的0.5ml DMEM中。通过将麻醉的蚊的吻突插入含有2.5％FBS和25％蔗糖溶液的毛细管中来收集唾液。使蚊流涎至少15分钟,然后将毛细管和身体置于含有DMEM的单独管中。将蚊体、腿和唾液储存在-80℃直至它们被研磨并测定感染性病毒。对于IT接种,将蚊冷麻醉并在0.34μl接种物中接种约50pfu的病毒。将经接种的蚊在与上述相同的条件下保持7天。然后将蚊麻醉,并如上所述收集它们的唾液和身体。将样品储存在-80℃直至进一步处理。

[0251] 为了处理用于病毒滴定的样品,使用混合磨,使用铜涂布的BB(Crossman Corporation,NY)将身体和腿样品以24个循环/秒均化4分钟,然后通过以3,000xg离心3分钟澄清。将唾液样品以3,000×g离心3分钟以从毛细管中排出流体。通过噬菌斑测定法测试身体和腿部匀浆和唾液样品的十倍稀释液中感染性病毒的存在。来自身体、腿和唾液的结果分别用于确定感染、散布和传播率。

[0252] 小鼠神经毒力

[0253] 从Taconic Labs获得定时妊娠的雌性ICR小鼠,并且每天监测几次以确定一窝幼仔的近似出生时间。在给定的实验中,出生后约12-24小时,通过使用30号针的颅内(ic)接种使用20μl稀释剂中的$10^3$至$10^4$pfu的病毒攻击每个病毒的两窝8只幼仔(n=16)。在攻击后每天监测动物至少3次,持续至少32天。在疾病的第一体征(毛皮粗糙、驼背、体重减轻、异常运动、瘫痪或嗜睡)时,通过用异氟醚气体的致死麻醉,然后进行颈脱位法对动物实施安乐死。安乐死的感染后天数表示动物的“疾病/发病率前时间”或“存活时间”。按照DVBD/CDC IACUC批准的动物方案进行动物实验。

[0254] 主种子病毒的衍生

[0255] TDV-1主病毒种子(MVS)

[0256] 本文提供了嵌合病毒基因组的核苷酸序列和翻译的蛋白质的推导的氨基酸序列。例如,TDV-1多核苷酸序列包含由SEQ ID NO:1、3、5和7表示的那些,并且TDV-1多肽序列包括由SEQ ID NO:2、4、6和8表示的那些。prM-E基因的大部分(nt 457至-2379,下划线)是野生型(wt)DEN-1 16007病毒特异性的;剩余的基因组是DEN-2PDK-53病毒特异性的。所有工程化取代不同于wt病毒(D1 16007或D2 16681),以及在MVS中检测到的额外突变(自工程化cDNA克隆的变化)是标记的。

[0257] 基因组和蛋白质中包含的取代:

[0258] D1(prM-E)和D2骨架之间的接合位点:

[0259] a.MluI(nt 451-456):工程化沉默突变,nt-453A至G

[0260] b.NgoMIV(nt 2380-2385):工程化突变,nt-2381/2382TG至CC(导致E-482Val至Ala变化)

[0261] D2PDK-53病毒骨架(从wt D2 16681改变):均为粗体

[0262] a.5’-非编码区(NCR)-57(nt-57C至T):主要减毒基因座(为红色)

[0263] b.NS1-53Gly至Asp(nt-2579G至A):主要减毒基因座(为红色)

[0264] c.NS2A-181Leu至Phe(nt-4018C至T)

[0265] d.NS3-250Glu至Val(nt-5270A至T):主要减毒基因座(为红色)

[0266] e.nt-5547(NS3基因)T至C沉默突变

[0267] f.NS4A-75Gly至Ala(nt-6599G至C)

[0268] *PDK-53的nt-8571C至T沉默突变在疫苗病毒中未工程化改造

[0269] DEN-1prM-E(从wt D1 16007改变)

[0270] a.工程化nt-1575T至C沉默突变以除去天然XbaI位点

[0271] 在疫苗种子中发现的额外取代(与初始克隆不同的0.03％nt)

[0272] TDV-2主病毒种子(MVS)

[0273] a.NS2A-116Ile至Leu(nt-3823A至C,为粗体)

[0274] b.NS2B-92Glu至Asp(nt-4407A至T,为粗体)

[0275] c.nt-7311A至G沉默突变(为粗体)

[0276] 本文提供了嵌合病毒基因组的核苷酸序列和翻译的蛋白质的推导的氨基酸序列。例如,TDV-2多核苷酸序列包括由SEQ ID NO:9、11、13和15表示的那些,并且TDV-2多肽序列包括由SEQ ID NO:10、12、14和16表示的那些。工程化病毒是基于D2PDK-53病毒。所有与野生型DEN-2 16681病毒(也是PDK-53的亲本病毒)不同的工程化取代,以及在MVS中检测到的额外突变(自工程化cDNA克隆的变化)是标记的。

[0277] 基因组和蛋白质中包含的取代:

[0278] D2PDK-53病毒骨架(从wt D2 16681改变):均为粗体

[0279] a.5'-非编码区(NCR)-57(nt-57C至T):主要减毒基因座(为红色)

[0280] b.prM-29Asp至Val(nt-524A至T)

[0281] c.nt-2055C至T(E基因)沉默突变

[0282] d.NS1-53Gly至Asp(nt-2579G至A):主要减毒基因座(为红色)

[0283] e.NS2A-181Leu至Phe(nt-4018C至T)

[0284] f.NS3-250Glu至Val(nt-5270A至T):主要减毒基因座(为红色)

[0285] g.nt-5547(NS3基因)T至C沉默突变

[0286] h.NS4A-75Gly至Ala(nt-6599G至C)

[0287] *PDK-53的nt-8571C至T沉默突变在疫苗病毒中未工程化改造

[0288] 工程化克隆标志物(沉默突变):

[0289] a.nt-900T至C沉默突变:感染性克隆标志物

[0290] 在疫苗种子中发现的额外取代(与初始克隆不同的0.02％nt)

[0291] a.prM-52Lys至Glu(nt-592A至G),为粗体

[0292] b.NS5-412Ile至Val(nt-8803A至G),为粗体

[0293] TDV-3主病毒种子(MVS)

[0294] 本文提供了嵌合病毒基因组的核苷酸序列和翻译的蛋白质的推导的氨基酸序列。例如,TDV-3多核苷酸序列包括由SEQ ID NO:17、19、21和23表示的那些,并且TDV-3多肽序列包括由SEQ ID NO:18、20、22和24表示的那些。prM-E基因的大部分(nt-457至-2373,下划线)是野生型(wt)DEN-3 16562病毒特异性的;剩余的核苷酸序列是DEN-2PDK-53病毒特异性的。DEN-3病毒的E蛋白比DEN-2的E蛋白少两个氨基酸。因此,从NgoMIV开始的nt位置比初始DEN-2PDK-53nt位置小6nt。所有工程化取代与wt病毒(DEN-316562或DEN-2 16681)不同,并且额外的突变(自工程化cDNA克隆的变化)是标记的。

[0295] 基因组和蛋白质中包含的取代:

[0296] 接合点:

[0297] a.MluI(nt 451-456):工程化沉默突变,nt-453A至G

[0298] b.NgoMIV(nt 2374-2379):工程化突变,nt-2375/2376TG至CC(导致E-480Val至Ala变化)

[0299] D2PDK-53病毒骨架(从wt D2 16681改变):为粗体

[0300] a.5'-非编码区(NCR)-57(nt-57C至T):主要减毒基因座(为红色)

[0301] b.NS1-53Gly至Asp(nt-2573G至A):主要减毒基因座(为红色)

[0302] c.NS2A-181Leu至Phe(nt-4012C至T)

[0303] d.NS3-250Glu至Val(nt-5264A至T):主要减毒基因座(为红色)

[0304] e.nt-5541(NS3基因)T至C沉默突变

[0305] f.NS4A-75Gly至Ala(nt-6593G至C)

[0306] *PDK-53的nt-8565C至T沉默突变在疫苗病毒中未工程化改造

[0307] DEN-3prM-E中的工程化突变(自wt D3 16562的变化)

[0308] a.工程化nt-552C至T沉默突变:克隆标志物

[0309] b.工程化E-345His至Leu(nt-1970A至T),用于在培养物中有效复制

[0310] 疫苗种子中发现的额外取代(与初始克隆不同的0.02%nt)

[0311] a.E-223Thr至Ser突变(nt-1603A至T,为粗体)

[0312] b.nt-7620A至G沉默突变(为粗体)

[0313] TDV-4主病毒种子(MVS)

[0314] 本文提供了嵌合病毒基因组的核苷酸序列和翻译的蛋白质的推导的氨基酸序列。例如,TDV-4多核苷酸序列包括由SEQ ID NO:25、27、29和31表示的那些,并且TDV-4多肽序列包括由SEQ ID NO:26、28、30和32表示的那些。prM-E基因的大部分(nt-457至-2379,下划线)是野生型(wt)DEN-4 1036病毒特异性的;剩余的核苷酸序列是DEN-2PDK-53病毒特异性的。所有工程化取代与wt病毒(DEN-3 16562或DEN-2 16681)不同,并且额外的突变(自工程化cDNA克隆的变化)是标记的。

[0315] 基因组和蛋白质中包含的取代:

[0316] 接合点:

[0317] a.MluI(nt 451-456):工程化沉默突变,nt-453A至G

[0318] b.NgoMIV(nt 2380-2385):工程化突变,nt-2381/2382TG至CC(导致E-482Val至Ala变化)

[0319] D2PDK-53病毒骨架(自wt D2 16681的变化)

[0320] a.5'-非编码区(NCR)-57(nt-57C至T):主要减毒基因座(为红色)

[0321] b.NS1-53Gly至Asp(nt-2579G至A):主要减毒基因座(为红色)

[0322] c.NS2A-181Leu至Phe(nt-4018C至T,为粗体)

[0323] d.NS3-250Glu至Val(nt-5270A至T):主要减毒基因座(为红色)

[0324] e.nt-5547(NS3gene)T至C沉默突变(为粗体)

[0325] f.NS4A-75Gly至Ala(nt-6599G至C,为粗体)

[0326] *PDK-53的nt-8571C至T沉默突变在疫苗病毒中未工程化改造

[0327] cDNA克隆中的工程化取代

[0328] a.工程化C-100Arg至Ser(nt-396A至C):可以改善培养物中的病毒复制

[0329] b.工程化nt-1401A至G沉默突变

[0330] c.工程化E-364Ala至Val(nt-2027C至T):可以改善培养物中的病毒复制

[0331] d.工程化E-447Met至Leu(nt-2275A至C):可以改善培养物中的病毒复制

[0332] 疫苗种子中发现的额外取代(与初始克隆不同的0.06%nt)

[0333] a.nt-225(C gene)A至T沉默突变(为粗体)

[0334] b.NS2A-66Asp至Gly(nt-3674A至G)突变(为粗体)

[0335] c.NS2A-99Lys至Lys/Arg混合物(nt-3773A至A/G混合物,为粗体)

[0336] d.nt-5391C至T(NS3gene)沉默突变(为粗体)

[0337] e.NS4A-21Ala至Val(nt-6437C至T,为粗体)

[0338] f.nt-7026T至C/T混合物沉默突变(为粗体)

[0339] g.nt-9750A至C沉默突变(为粗体)

[0340] **********

[0341] 根据本公开,无需过度实验即可制备和实施本文公开和要求保护的所有组合物和方法。尽管已经根据优选实施方案描述了组合物和方法,但是对于本领域技术人员显而易见的是,可以在不脱离本发明的概念、精神和范围的情况下将变化应用于本文所述方法的组合物和方法以及方法的步骤或步骤顺序中。更具体地,显而易见的是,化学和生理学相关的某些试剂可以代替本文所述的试剂,同时可以获得相同或相似的结果。对于本领域技术人员显而易见的所有这些类似的替代和修改被认为是在由所附权利要求书限定的本发明的精神、范围和概念内。

[0001] 序列表

[0002] <110>武田疫苗股份有限公司

[0003] Wallace,Derek

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[10811] 580 585 590
[10812] Ala Glu Thr Gln His Gly Thr Val Leu Val Gln Val Lys Tyr Glu Gly
[10813] 595 600 605
[10814] Thr Asp Ala Pro Cys Lys Ile Pro Phe Ser Thr Gln Asp Glu Lys Gly
[10815] 610 615 620
[10816] Ala Thr Gln Asn Gly Arg Leu Ile Thr Ala Asn Pro Ile Val Thr Asp
[10817] 625 630 635 640
[10818] Lys Glu Lys Pro Val Asn Ile Glu Ala Glu Pro Pro Phe Gly Glu Ser
[10819] 645 650 655
[10820] Tyr Ile Val Val Gly Ala Gly Glu Lys Ala Leu Lys Leu Ser Trp Phe
[10821] 660 665 670
[10822] Lys Lys Gly Ser Ser Ile Gly Lys Met Phe Glu Ala Thr Ala Arg Gly
[10823] 675 680 685
[10824] Ala Arg Arg Met Ala Ile Leu Gly Asp Thr Ala Trp Asp Phe Gly Ser
[10825] 690 695 700
[10826] Ile Gly Gly Val Phe Thr Ser Met Gly Lys Leu Val His Gln Val Phe
[10827] 705 710 715 720
[10828] Gly Thr Ala Tyr Gly Val Leu Phe Ser Gly Val Ser Trp Thr Met Lys
[10829] 725 730 735
[10830] Ile Gly Ile Gly Ile Leu Leu Thr Trp Leu Gly Leu Asn Ser Arg Asn
[10831] 740 745 750
[10832] Thr Ser Leu Ser Met Met Cys Ile Ala Ala Ala Ile Val Thr Leu Tyr
[10833] 755 760 765
[10834] Leu Gly Val Met Val Gln Ala Asp Ser Gly Cys Val Val Ser Trp Lys
[10835] 770 775 780

[10836] Asn Lys Glu Leu Lys Cys Gly Ser Gly Ile Phe Ile Thr Asp Asn Val
[10837] 785 790 795 800
[10838] His Thr Trp Thr Glu Gln Tyr Lys Phe Gln Pro Glu Ser Pro Ser Lys
[10839] 805 810 815
[10840] Leu Ala Ser Ala Ile Gln Lys Ala His Glu Glu Asp Ile Cys Gly Ile
[10841] 820 825 830
[10842] Arg Ser Val Thr Arg Leu Glu Asn Leu Met Trp Lys Gln Ile Thr Pro
[10843] 835 840 845
[10844] Glu Leu Asn His Ile Leu Ser Glu Asn Glu Val Lys Leu Thr Ile Met
[10845] 850 855 860
[10846] Thr Gly Asp Ile Lys Gly Ile Met Gln Ala Gly Lys Arg Ser Leu Arg
[10847] 865 870 875 880
[10848] Pro Gln Pro Thr Glu Leu Lys Tyr Ser Trp Lys Thr Trp Gly Lys Ala
[10849] 885 890 895
[10850] Lys Met Leu Ser Thr Glu Ser His Asn Gln Thr Phe Leu Ile Asp Gly
[10851] 900 905 910
[10852] Pro Glu Thr Ala Glu Cys Pro Asn Thr Asn Arg Ala Trp Asn Ser Leu
[10853] 915 920 925
[10854] Glu Val Glu Asp Tyr Gly Phe Gly Val Phe Thr Thr Asn Ile Trp Leu
[10855] 930 935 940
[10856] Lys Leu Lys Glu Lys Gln Asp Val Phe Cys Asp Ser Lys Leu Met Ser
[10857] 945 950 955 960
[10858] Ala Ala Ile Lys Asp Asn Arg Ala Val His Ala Asp Met Gly Tyr Trp
[10859] 965 970 975
[10860] Ile Glu Ser Ala Leu Asn Asp Thr Trp Lys Ile Glu Lys Ala Ser Phe
[10861] 980 985 990
[10862] Ile Glu Val Lys Asn Cys His Trp Pro Lys Ser His Thr Leu Trp Ser
[10863] 995 1000 1005
[10864] Asn Gly Val Leu Glu Ser Glu Met Ile Ile Pro Lys Asn Leu Ala
[10865] 1010 1015 1020
[10866] Gly Pro Val Ser Gln His Asn Tyr Arg Pro Gly Tyr His Thr Gln
[10867] 1025 1030 1035
[10868] Ile Thr Gly Pro Trp His Leu Gly Lys Leu Glu Met Asp Phe Asp
[10869] 1040 1045 1050
[10870] Phe Cys Asp Gly Thr Thr Val Val Val Thr Glu Asp Cys Gly Asn
[10871] 1055 1060 1065
[10872] Arg Gly Pro Ser Leu Arg Thr Thr Thr Ala Ser Gly Lys Leu Ile
[10873] 1070 1075 1080
[10874] Thr Glu Trp Cys Cys Arg Ser Cys Thr Leu Pro Pro Leu Arg Tyr
[10875] 1085 1090 1095
[10876] Arg Gly Glu Asp Gly Cys Trp Tyr Gly Met Glu Ile Arg Pro Leu
[10877] 1100 1105 1110

[10878] Lys Glu Lys Glu Glu Asn Leu Val Asn Ser Leu Val Thr Ala Gly
[10879] 1115 1120 1125
[10880] His Gly Gln Val Asp Asn Phe Ser Leu Gly Val Leu Gly Met Ala
[10881] 1130 1135 1140
[10882] Leu Phe Leu Glu Glu Met Leu Arg Thr Arg Val Gly Thr Lys His
[10883] 1145 1150 1155
[10884] Ala Ile Leu Leu Val Ala Val Ser Phe Val Thr Leu Ile Thr Gly
[10885] 1160 1165 1170
[10886] Asn Met Ser Phe Arg Asp Leu Gly Arg Val Met Val Met Val Gly
[10887] 1175 1180 1185
[10888] Ala Thr Met Thr Asp Asp Ile Gly Met Gly Val Thr Tyr Leu Ala
[10889] 1190 1195 1200
[10890] Leu Leu Ala Ala Phe Lys Val Arg Pro Thr Phe Ala Ala Gly Leu
[10891] 1205 1210 1215
[10892] Leu Leu Arg Lys Leu Thr Ser Lys Glu Leu Met Met Thr Thr Ile
[10893] 1220 1225 1230
[10894] Gly Ile Val Leu Leu Ser Gln Ser Thr Leu Pro Glu Thr Ile Leu
[10895] 1235 1240 1245
[10896] Glu Leu Thr Asp Ala Leu Ala Leu Gly Met Met Val Leu Lys Met
[10897] 1250 1255 1260
[10898] Val Arg Asn Met Glu Lys Tyr Gln Leu Ala Val Thr Ile Met Ala
[10899] 1265 1270 1275
[10900] Ile Leu Cys Val Pro Asn Ala Val Ile Leu Gln Asn Ala Trp Lys
[10901] 1280 1285 1290
[10902] Val Ser Cys Thr Ile Leu Ala Val Val Ser Val Ser Pro Leu Phe
[10903] 1295 1300 1305
[10904] Leu Thr Ser Ser Gln Gln Lys Thr Asp Trp Ile Pro Leu Ala Leu
[10905] 1310 1315 1320
[10906] Thr Ile Lys Gly Leu Asn Pro Thr Ala Ile Phe Leu Thr Thr Leu
[10907] 1325 1330 1335
[10908] Ser Arg Thr Ser Lys Lys Arg Ser Trp Pro Leu Asn Glu Ala Ile
[10909] 1340 1345 1350
[10910] Met Ala Val Gly Met Val Ser Ile Leu Ala Ser Ser Leu Leu Lys
[10911] 1355 1360 1365
[10912] Asn Asp Ile Pro Met Thr Gly Pro Leu Val Ala Gly Gly Leu Leu
[10913] 1370 1375 1380
[10914] Thr Val Cys Tyr Val Leu Thr Gly Arg Ser Ala Asp Leu Glu Leu
[10915] 1385 1390 1395
[10916] Glu Arg Ala Ala Asp Val Lys Trp Glu Asp Gln Ala Glu Ile Ser
[10917] 1400 1405 1410
[10918] Gly Ser Ser Pro Ile Leu Ser Ile Thr Ile Ser Glu Asp Gly Ser
[10919] 1415 1420 1425

[10920] Met Ser Ile Lys Asn Glu Glu Glu Asp Gln Thr Leu Thr Ile Leu
[10921] 1430 1435 1440
[10922] Ile Arg Thr Gly Leu Leu Val Ile Ser Gly Leu Phe Pro Val Ser
[10923] 1445 1450 1455
[10924] Ile Pro Ile Thr Ala Ala Ala Trp Tyr Leu Trp Glu Val Lys Lys
[10925] 1460 1465 1470
[10926] Gln Arg Ala Gly Val Leu Trp Asp Val Pro Ser Pro Pro Pro Met
[10927] 1475 1480 1485
[10928] Gly Lys Ala Glu Leu Glu Asp Gly Ala Tyr Arg Ile Lys Gln Lys
[10929] 1490 1495 1500
[10930] Gly Ile Leu Gly Tyr Ser Gln Ile Gly Ala Gly Val Tyr Lys Glu
[10931] 1505 1510 1515
[10932] Gly Thr Phe His Thr Met Trp His Val Thr Arg Gly Ala Val Leu
[10933] 1520 1525 1530
[10934] Met His Lys Gly Lys Arg Ile Glu Pro Ser Trp Ala Asp Val Lys
[10935] 1535 1540 1545
[10936] Lys Asp Leu Ile Ser Tyr Gly Gly Gly Trp Lys Leu Glu Gly Glu
[10937] 1550 1555 1560
[10938] Trp Lys Glu Gly Glu Glu Val Gln Val Leu Ala Leu Glu Pro Gly
[10939] 1565 1570 1575
[10940] Lys Asn Pro Arg Ala Val Gln Thr Lys Pro Gly Leu Phe Lys Thr
[10941] 1580 1585 1590
[10942] Asn Ala Gly Thr Ile Gly Ala Val Ser Leu Asp Phe Ser Pro Gly
[10943] 1595 1600 1605
[10944] Thr Ser Gly Ser Pro Ile Ile Asp Lys Lys Gly Lys Val Val Gly
[10945] 1610 1615 1620
[10946] Leu Tyr Gly Asn Gly Val Val Thr Arg Ser Gly Ala Tyr Val Ser
[10947] 1625 1630 1635
[10948] Ala Ile Ala Gln Thr Glu Lys Ser Ile Glu Asp Asn Pro Glu Ile
[10949] 1640 1645 1650
[10950] Glu Asp Asp Ile Phe Arg Lys Arg Arg Leu Thr Ile Met Asp Leu
[10951] 1655 1660 1665
[10952] His Pro Gly Ala Gly Lys Thr Lys Arg Tyr Leu Pro Ala Ile Val
[10953] 1670 1675 1680
[10954] Arg Glu Ala Ile Lys Arg Gly Leu Arg Thr Leu Ile Leu Ala Pro
[10955] 1685 1690 1695
[10956] Thr Arg Val Val Ala Ala Glu Met Glu Glu Ala Leu Arg Gly Leu
[10957] 1700 1705 1710
[10958] Pro Ile Arg Tyr Gln Thr Pro Ala Ile Arg Ala Val His Thr Gly
[10959] 1715 1720 1725
[10960] Arg Glu Ile Val Asp Leu Met Cys His Ala Thr Phe Thr Met Arg
[10961] 1730 1735 1740

[10962] Leu Leu Ser Pro Val Arg Val Pro Asn Tyr Asn Leu Ile Ile Met
[10963] 1745 1750 1755
[10964] Asp Glu Ala His Phe Thr Asp Pro Ala Ser Ile Ala Ala Arg Gly
[10965] 1760 1765 1770
[10966] Tyr Ile Ser Thr Arg Val Glu Met Gly Glu Ala Ala Gly Ile Phe
[10967] 1775 1780 1785
[10968] Met Thr Ala Thr Pro Pro Gly Ser Arg Asp Pro Phe Pro Gln Ser
[10969] 1790 1795 1800
[10970] Asn Ala Pro Ile Ile Asp Glu Glu Arg Glu Ile Pro Glu Arg Ser
[10971] 1805 1810 1815
[10972] Trp Asn Ser Gly His Glu Trp Val Thr Asp Phe Lys Gly Lys Thr
[10973] 1820 1825 1830
[10974] Val Trp Phe Val Pro Ser Ile Lys Ala Gly Asn Asp Ile Ala Ala
[10975] 1835 1840 1845
[10976] Cys Leu Arg Lys Asn Gly Lys Lys Val Ile Gln Leu Ser Arg Lys
[10977] 1850 1855 1860
[10978] Thr Phe Asp Ser Glu Tyr Val Lys Thr Arg Thr Asn Asp Trp Asp
[10979] 1865 1870 1875
[10980] Phe Val Val Thr Thr Asp Ile Ser Glu Met Gly Ala Asn Phe Lys
[10981] 1880 1885 1890
[10982] Ala Glu Arg Val Ile Asp Pro Arg Arg Cys Met Lys Pro Val Ile
[10983] 1895 1900 1905
[10984] Leu Thr Asp Gly Glu Glu Arg Val Ile Leu Ala Gly Pro Met Pro
[10985] 1910 1915 1920
[10986] Val Thr His Ser Ser Ala Ala Gln Arg Arg Gly Arg Ile Gly Arg
[10987] 1925 1930 1935
[10988] Asn Pro Lys Asn Glu Asn Asp Gln Tyr Ile Tyr Met Gly Glu Pro
[10989] 1940 1945 1950
[10990] Leu Glu Asn Asp Glu Asp Cys Ala His Trp Lys Glu Ala Lys Met
[10991] 1955 1960 1965
[10992] Leu Leu Asp Asn Ile Asn Thr Pro Glu Gly Ile Ile Pro Ser Met
[10993] 1970 1975 1980
[10994] Phe Glu Pro Glu Arg Glu Lys Val Asp Ala Ile Asp Gly Glu Tyr
[10995] 1985 1990 1995
[10996] Arg Leu Arg Gly Glu Ala Arg Lys Thr Phe Val Asp Leu Met Arg
[10997] 2000 2005 2010
[10998] Arg Gly Asp Leu Pro Val Trp Leu Ala Tyr Arg Val Ala Ala Glu
[10999] 2015 2020 2025
[11000] Gly Ile Asn Tyr Ala Asp Arg Arg Trp Cys Phe Asp Gly Val Lys
[11001] 2030 2035 2040
[11002] Asn Asn Gln Ile Leu Glu Glu Asn Val Glu Val Glu Ile Trp Thr
[11003] 2045 2050 2055

[11004] Lys Glu Gly Glu Arg Lys Lys Leu Lys Pro Arg Trp Leu Asp Ala
[11005] 2060 2065 2070
[11006] Arg Ile Tyr Ser Asp Pro Leu Ala Leu Lys Glu Phe Lys Glu Phe
[11007] 2075 2080 2085
[11008] Ala Ala Gly Arg Lys Ser Leu Thr Leu Asn Leu Ile Thr Glu Met
[11009] 2090 2095 2100
[11010] Gly Arg Leu Pro Thr Phe Met Thr Gln Lys Ala Arg Asp Ala Leu
[11011] 2105 2110 2115
[11012] Asp Asn Leu Ala Val Leu His Thr Ala Glu Ala Gly Gly Arg Ala
[11013] 2120 2125 2130
[11014] Tyr Asn His Ala Leu Ser Glu Leu Pro Glu Thr Leu Glu Thr Leu
[11015] 2135 2140 2145
[11016] Leu Leu Leu Thr Leu Leu Ala Thr Val Thr Gly Gly Ile Phe Leu
[11017] 2150 2155 2160
[11018] Phe Leu Met Ser Ala Arg Gly Ile Gly Lys Met Thr Leu Gly Met
[11019] 2165 2170 2175
[11020] Cys Cys Ile Ile Thr Ala Ser Ile Leu Leu Trp Tyr Ala Gln Ile
[11021] 2180 2185 2190
[11022] Gln Pro His Trp Ile Ala Ala Ser Ile Ile Leu Glu Phe Phe Leu
[11023] 2195 2200 2205
[11024] Ile Val Leu Leu Ile Pro Glu Pro Glu Lys Gln Arg Thr Pro Gln
[11025] 2210 2215 2220
[11026] Asp Asn Gln Leu Thr Tyr Val Val Ile Ala Ile Leu Thr Val Val
[11027] 2225 2230 2235
[11028] Ala Ala Thr Met Ala Asn Glu Met Gly Phe Leu Glu Lys Thr Lys
[11029] 2240 2245 2250
[11030] Lys Asp Leu Gly Leu Gly Ser Ile Ala Thr Gln Gln Pro Glu Ser
[11031] 2255 2260 2265
[11032] Asn Ile Leu Asp Ile Asp Leu Arg Pro Ala Ser Ala Trp Thr Leu
[11033] 2270 2275 2280
[11034] Tyr Ala Val Ala Thr Thr Phe Val Thr Pro Met Leu Arg His Ser
[11035] 2285 2290 2295
[11036] Ile Glu Asn Ser Ser Val Asn Val Ser Leu Thr Ala Ile Ala Asn
[11037] 2300 2305 2310
[11038] Gln Ala Thr Val Leu Met Gly Leu Gly Lys Gly Trp Pro Leu Ser
[11039] 2315 2320 2325
[11040] Lys Met Asp Ile Gly Val Pro Leu Leu Ala Ile Gly Cys Tyr Ser
[11041] 2330 2335 2340
[11042] Gln Val Asn Pro Ile Thr Leu Ile Ala Ala Leu Phe Leu Leu Val
[11043] 2345 2350 2355
[11044] Ala His Tyr Ala Ile Ile Gly Pro Gly Leu Gln Ala Lys Ala Thr
[11045] 2360 2365 2370

[11046] Arg Glu Ala Gln Lys Arg Ala Ala Ala Gly Ile Met Lys Asn Pro
[11047] 2375 2380 2385
[11048] Thr Val Asp Gly Ile Thr Val Ile Asp Leu Asp Pro Ile Pro Tyr
[11049] 2390 2395 2400
[11050] Asp Pro Lys Phe Glu Lys Gln Leu Gly Gln Val Met Leu Leu Val
[11051] 2405 2410 2415
[11052] Leu Cys Val Thr Gln Val Leu Met Met Arg Thr Thr Trp Ala Leu
[11053] 2420 2425 2430
[11054] Cys Glu Ala Leu Thr Leu Ala Thr Gly Pro Ile Ser Thr Leu Trp
[11055] 2435 2440 2445
[11056] Glu Gly Asn Pro Gly Arg Phe Trp Asn Thr Thr Ile Ala Val Ser
[11057] 2450 2455 2460
[11058] Met Ala Asn Ile Phe Arg Gly Ser Tyr Leu Ala Gly Ala Gly Leu
[11059] 2465 2470 2475
[11060] Leu Phe Ser Ile Met Lys Asn Thr Thr Asn Thr Arg Arg Gly Thr
[11061] 2480 2485 2490
[11062] Gly Asn Ile Gly Glu Thr Leu Gly Glu Lys Trp Lys Ser Arg Leu
[11063] 2495 2500 2505
[11064] Asn Ala Leu Gly Lys Ser Glu Phe Gln Ile Tyr Lys Lys Ser Gly
[11065] 2510 2515 2520
[11066] Ile Gln Glu Val Asp Arg Thr Leu Ala Lys Glu Gly Ile Lys Arg
[11067] 2525 2530 2535
[11068] Gly Glu Thr Asp His His Ala Val Ser Arg Gly Ser Ala Lys Leu
[11069] 2540 2545 2550
[11070] Arg Trp Phe Val Glu Arg Asn Met Val Thr Pro Glu Gly Lys Val
[11071] 2555 2560 2565
[11072] Val Asp Leu Gly Cys Gly Arg Gly Gly Trp Ser Tyr Tyr Cys Gly
[11073] 2570 2575 2580
[11074] Gly Leu Lys Asn Val Arg Glu Val Lys Gly Leu Thr Lys Gly Gly
[11075] 2585 2590 2595
[11076] Pro Gly His Glu Glu Pro Ile Pro Met Ser Thr Tyr Gly Trp Asn
[11077] 2600 2605 2610
[11078] Leu Val Arg Leu Gln Ser Gly Val Asp Val Phe Phe Ile Pro Pro
[11079] 2615 2620 2625
[11080] Glu Lys Cys Asp Thr Leu Leu Cys Asp Ile Gly Glu Ser Ser Pro
[11081] 2630 2635 2640
[11082] Asn Pro Thr Val Glu Ala Gly Arg Thr Leu Arg Val Leu Asn Leu
[11083] 2645 2650 2655
[11084] Val Glu Asn Trp Leu Asn Asn Asn Thr Gln Phe Cys Ile Lys Val
[11085] 2660 2665 2670
[11086] Leu Asn Pro Tyr Met Pro Ser Val Ile Glu Lys Met Glu Ala Leu
[11087] 2675 2680 2685

[11088] Gln Arg Lys Tyr Gly Gly Ala Leu Val Arg Asn Pro Leu Ser Arg
[11089] 2690 2695 2700
[11090] Asn Ser Thr His Glu Met Tyr Trp Val Ser Asn Ala Ser Gly Asn
[11091] 2705 2710 2715
[11092] Ile Val Ser Ser Val Asn Met Ile Ser Arg Met Leu Ile Asn Arg
[11093] 2720 2725 2730
[11094] Phe Thr Met Arg Tyr Lys Lys Ala Thr Tyr Glu Pro Asp Val Asp
[11095] 2735 2740 2745
[11096] Leu Gly Ser Gly Thr Arg Asn Ile Gly Ile Glu Ser Glu Ile Pro
[11097] 2750 2755 2760
[11098] Asn Leu Asp Ile Ile Gly Lys Arg Ile Glu Lys Ile Lys Gln Glu
[11099] 2765 2770 2775
[11100] His Glu Thr Ser Trp His Tyr Asp Gln Asp His Pro Tyr Lys Thr
[11101] 2780 2785 2790
[11102] Trp Ala Tyr His Gly Ser Tyr Glu Thr Lys Gln Thr Gly Ser Ala
[11103] 2795 2800 2805
[11104] Ser Ser Met Val Asn Gly Val Val Arg Leu Leu Thr Lys Pro Trp
[11105] 2810 2815 2820
[11106] Asp Val Val Pro Met Val Thr Gln Met Ala Met Thr Asp Thr Thr
[11107] 2825 2830 2835
[11108] Pro Phe Gly Gln Gln Arg Val Phe Lys Glu Lys Val Asp Thr Arg
[11109] 2840 2845 2850
[11110] Thr Gln Glu Pro Lys Glu Gly Thr Lys Lys Leu Met Lys Ile Thr
[11111] 2855 2860 2865
[11112] Ala Glu Trp Leu Trp Lys Glu Leu Gly Lys Lys Lys Thr Pro Arg
[11113] 2870 2875 2880
[11114] Met Cys Thr Arg Glu Glu Phe Thr Arg Lys Val Arg Ser Asn Ala
[11115] 2885 2890 2895
[11116] Ala Leu Gly Ala Ile Phe Thr Asp Glu Asn Lys Trp Lys Ser Ala
[11117] 2900 2905 2910
[11118] Arg Glu Ala Val Glu Asp Ser Arg Phe Trp Glu Leu Val Asp Lys
[11119] 2915 2920 2925
[11120] Glu Arg Asn Leu His Leu Glu Gly Lys Cys Glu Thr Cys Val Tyr
[11121] 2930 2935 2940
[11122] Asn Met Met Gly Lys Arg Glu Lys Lys Leu Gly Glu Phe Gly Lys
[11123] 2945 2950 2955
[11124] Ala Lys Gly Ser Arg Ala Ile Trp Tyr Met Trp Leu Gly Ala Arg
[11125] 2960 2965 2970
[11126] Phe Leu Glu Phe Glu Ala Leu Gly Phe Leu Asn Glu Asp His Trp
[11127] 2975 2980 2985
[11128] Phe Ser Arg Glu Asn Ser Leu Ser Gly Val Glu Gly Glu Gly Leu
[11129] 2990 2995 3000

[11130] His Lys Leu Gly Tyr Ile Leu Arg Asp Val Ser Lys Lys Glu Gly
[11131] 3005 3010 3015
[11132] Gly Ala Met Tyr Ala Asp Asp Thr Ala Gly Trp Asp Thr Arg Ile
[11133] 3020 3025 3030
[11134] Thr Leu Glu Asp Leu Lys Asn Glu Glu Met Val Thr Asn His Met
[11135] 3035 3040 3045
[11136] Glu Gly Glu His Lys Lys Leu Ala Glu Ala Ile Phe Lys Leu Thr
[11137] 3050 3055 3060
[11138] Tyr Gln Asn Lys Val Val Arg Val Gln Arg Pro Thr Pro Arg Gly
[11139] 3065 3070 3075
[11140] Thr Val Met Asp Ile Ile Ser Arg Arg Asp Gln Arg Gly Ser Gly
[11141] 3080 3085 3090
[11142] Gln Val Gly Thr Tyr Gly Leu Asn Thr Phe Thr Asn Met Glu Ala
[11143] 3095 3100 3105
[11144] Gln Leu Ile Arg Gln Met Glu Gly Glu Gly Val Phe Lys Ser Ile
[11145] 3110 3115 3120
[11146] Gln His Leu Thr Ile Thr Glu Glu Ile Ala Val Gln Asn Trp Leu
[11147] 3125 3130 3135
[11148] Ala Arg Val Gly Arg Glu Arg Leu Ser Arg Met Ala Ile Ser Gly
[11149] 3140 3145 3150
[11150] Asp Asp Cys Val Val Lys Pro Leu Asp Asp Arg Phe Ala Ser Ala
[11151] 3155 3160 3165
[11152] Leu Thr Ala Leu Asn Asp Met Gly Lys Ile Arg Lys Asp Ile Gln
[11153] 3170 3175 3180
[11154] Gln Trp Glu Pro Ser Arg Gly Trp Asn Asp Trp Thr Gln Val Pro
[11155] 3185 3190 3195
[11156] Phe Cys Ser His His Phe His Glu Leu Ile Met Lys Asp Gly Arg
[11157] 3200 3205 3210
[11158] Val Leu Val Val Pro Cys Arg Asn Gln Asp Glu Leu Ile Gly Arg
[11159] 3215 3220 3225
[11160] Ala Arg Ile Ser Gln Gly Ala Gly Trp Ser Leu Arg Glu Thr Ala
[11161] 3230 3235 3240
[11162] Cys Leu Gly Lys Ser Tyr Ala Gln Met Trp Ser Leu Met Tyr Phe
[11163] 3245 3250 3255
[11164] His Arg Arg Asp Leu Arg Leu Ala Ala Asn Ala Ile Cys Ser Ala
[11165] 3260 3265 3270
[11166] Val Pro Ser His Trp Val Pro Thr Ser Arg Thr Thr Trp Ser Ile
[11167] 3275 3280 3285
[11168] His Ala Lys His Glu Trp Met Thr Thr Glu Asp Met Leu Thr Val
[11169] 3290 3295 3300
[11170] Trp Asn Arg Val Trp Ile Gln Glu Asn Pro Trp Met Glu Asp Lys
[11171] 3305 3310 3315

[11172] Thr Pro Val Glu Ser Trp Glu Glu Ile Pro Tyr Leu Gly Lys Arg
[11173] 3320 3325 3330
[11174] Glu Asp Gln Trp Cys Gly Ser Leu Ile Gly Leu Thr Ser Arg Ala
[11175] 3335 3340 3345
[11176] Thr Trp Ala Lys Asn Ile Gln Ala Ala Ile Asn Gln Val Arg Ser
[11177] 3350 3355 3360
[11178] Leu Ile Gly Asn Glu Glu Tyr Thr Asp Tyr Met Pro Ser Met Lys
[11179] 3365 3370 3375
[11180] Arg Phe Arg Arg Glu Glu Glu Glu Ala Gly Val Leu Trp Lys Ala
[11181] 3380 3385 3390
[11182] Lys Leu Thr Asn Lys Ala Arg Ser Gln Val Gly Leu Ser His Ser
[11183] 3395 3400 3405
[11184] Thr Glu Lys Thr Met Leu Pro Val Ser Pro Val Gln Gly Arg Lys
[11185] 3410 3415 3420
[11186] Lys Ser Gly His His Lys Cys His Ser Leu Ser Lys Leu Cys Ser
[11187] 3425 3430 3435
[11188] Leu Leu His Leu Arg Arg Cys Lys Lys Ser Gly Arg Pro Gln Thr
[11189] 3440 3445 3450
[11190] Met Glu Ala Val Arg Met Ala Trp Thr Ser Gly
[11191] 3455 3460

D2/D4嵌合体间的遗传变异(与wt D2 16681和D4-1036比较)

| 基因组 | D2 | | | 接合prM | D4 | | | | 接合 | D2 | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 基因 | NCR | C | | | E | | | | | NS1 | NS2A | | | NS3 | | | NS4A | | | NS4B | NS5 | | |
| 突变类型* | PDK-53 | 种子 | Eng | MluI | 种子 | 标志物 | Eng | Eng | NgoMIV | PDK-53 | 种子 | 种子 | PDK-53 | PDK-53 | 种子 | PDK-53 | 种子 | PDK-53 | | 种子 | PDK53 | 种子 |
| 基因组NT位置 | C57T | A225T | A396C | A453G | C647G/C | A1401G | C2027T | A2275 | TG2380/1CC | G2579A | A3674G | A3773A/G | C4018T | A5270T | C5391T | T5547C | C6437T | G6599C | | T7026C/T | C8571T** | A9750C |
| 蛋白-AA位置 | NCR | C-沉默 | C-R100S | prM-沉默 | prM-T79R/T | E-沉默 | E-A364V | E-M447L | E-V482A | NS1-G53D | NS2A-D66G | NS2A-K99K/R | NS2A-L181F | NS3-E250V | NS3-沉默 | NS3-沉默 | NS4A-A21V | NS4A-G75A | | NS4B-沉默 | NS5-沉默 | NS5-沉默 |
| D2-16681 | C | A | A (R) | A | | | | | TG (V) | G (G0 | A (D) | A (K) | C (L) | A (E) | C | T | C (A) | G (G) | | T | C | A |
| D2-PDK-53 | T | - | - | | | | | | | A (D) | - | - | T (F) | T (V) | - | c | - | C (A) | | - | t | - |
| D4-1036 | | | | | G (T) | A | C (A) | A (M) | | | | | | | | | | | | | | |
| 克隆 D2/4-V1 (pD2/4-VP1) | T | - | C (S) | g | - | g | T (V) | C (L) | CC (A) | A (D) | - | - | T (F) | T (V) | - | c | - | C (A) | | - | - | - |
| DENVax-4 (MVS) | T | t | C (S) | g | - | g | T (V) | C (L) | CC (A) | A (D) | G (G) | A/G (K/R) | T (F) | T (V) | t | c | T (V) | C (A) | | c/t | - | c |

"-": 与wt D2 16681或D4-1036相同; 小的nt字母: 可读框中的沉默突变

*: PDK-53:D2-PDK-53 特定的基因型(相对于16681), 红色: 主要减毒PDK-53基因座; 种子: 仅在规定的病毒种子中而不在初始克隆中找到的突变; Eng: D2/4克隆的工程化突变; MluI和NgoMV: D2/4接合工程化RE位点;

**: 在大多数D2/4嵌合克隆中不包括C8571T (PDK-53沉默突变)

图1

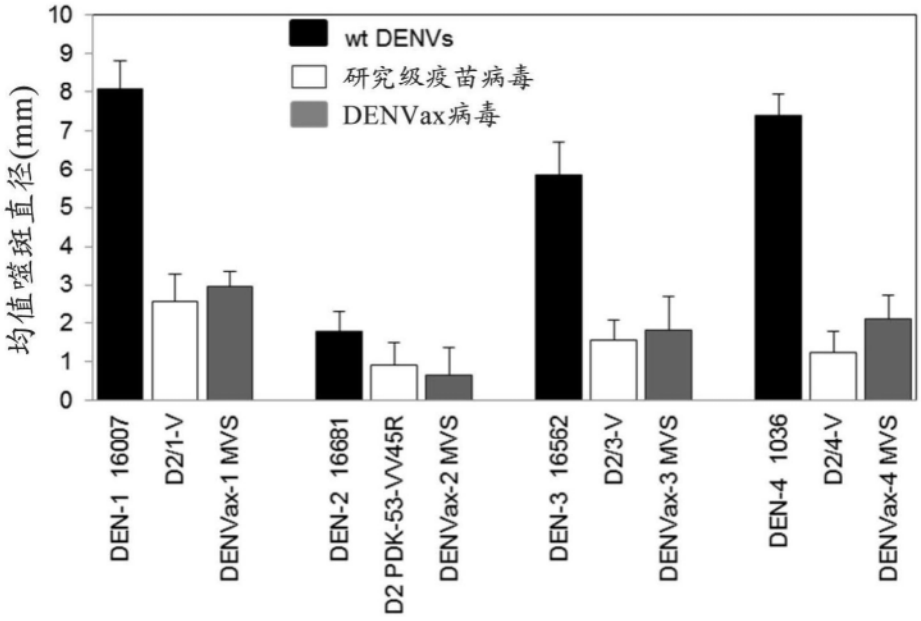

均值噬斑直径(mm)
wt DENVs
研究级疫苗病毒
DENVax病毒
DEN-1 16007
D2/1-V
DENVax-1 MVS
DEN-2 16681
D2 PDK-53-VV45R
DENVax-2 MVS
DEN-3 16562
D2/3-V
DENVax-3 MVS
DEN-4 1036
D2/4-V
DENVax-4 MVS

图2

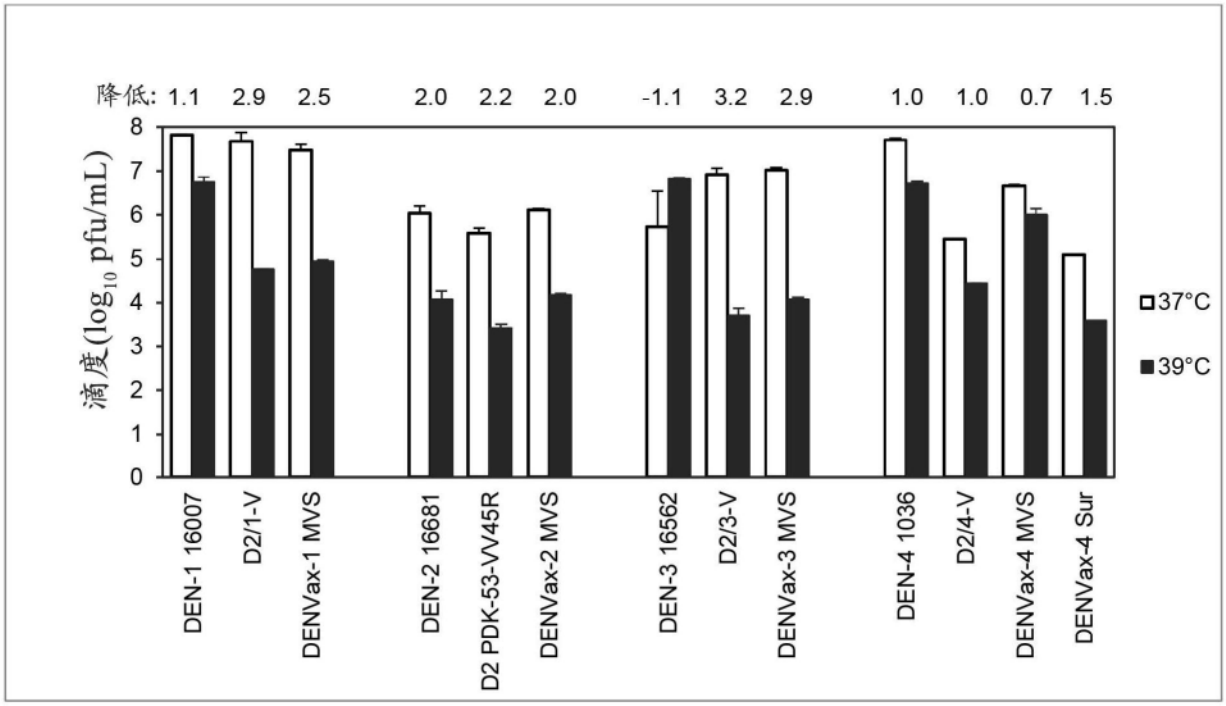

降低: 1.1 2.9 2.5 2.0 2.2 2.0 -1.1 3.2 2.9 1.0 1.0 0.7 1.5
滴度(log10 pfu/mL)
37°C
39°C
DEN-1 16007
D2/1-V
DENVax-1 MVS
DEN-2 16681
D2 PDK-53-VV45R
DENVax-2 MVS
DEN-3 16562
D2/3-V
DENVax-3 MVS
DEN-4 1036
D2/4-V
DENVax-4 MVS
DENVax-4 Sur

图3

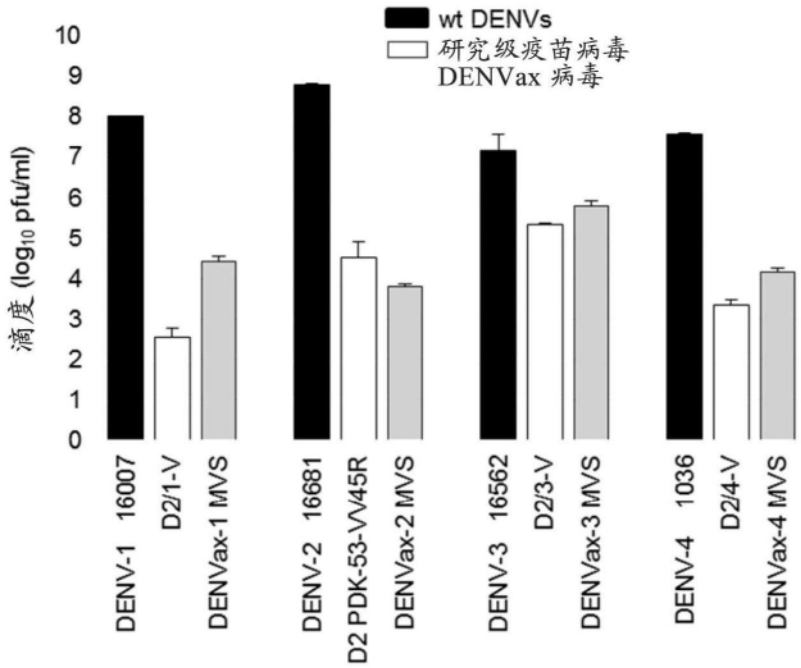

wt DENVs
研究级疫苗病毒
DENVax 病毒
滴度 (log10 pfu/ml)
10
9
8
7
6
5
4
3
2
1
0
DENV-1 16007
D2/1-V
DENVax-1 MVS
DENV-2 16681
D2 PDK-53-VV45R
DENVax-2 MVS
DENV-3 16562
D2/3-V
DENVax-3 MVS
DENV-4 1036
D2/4-V
DENVax-4 MVS

图4

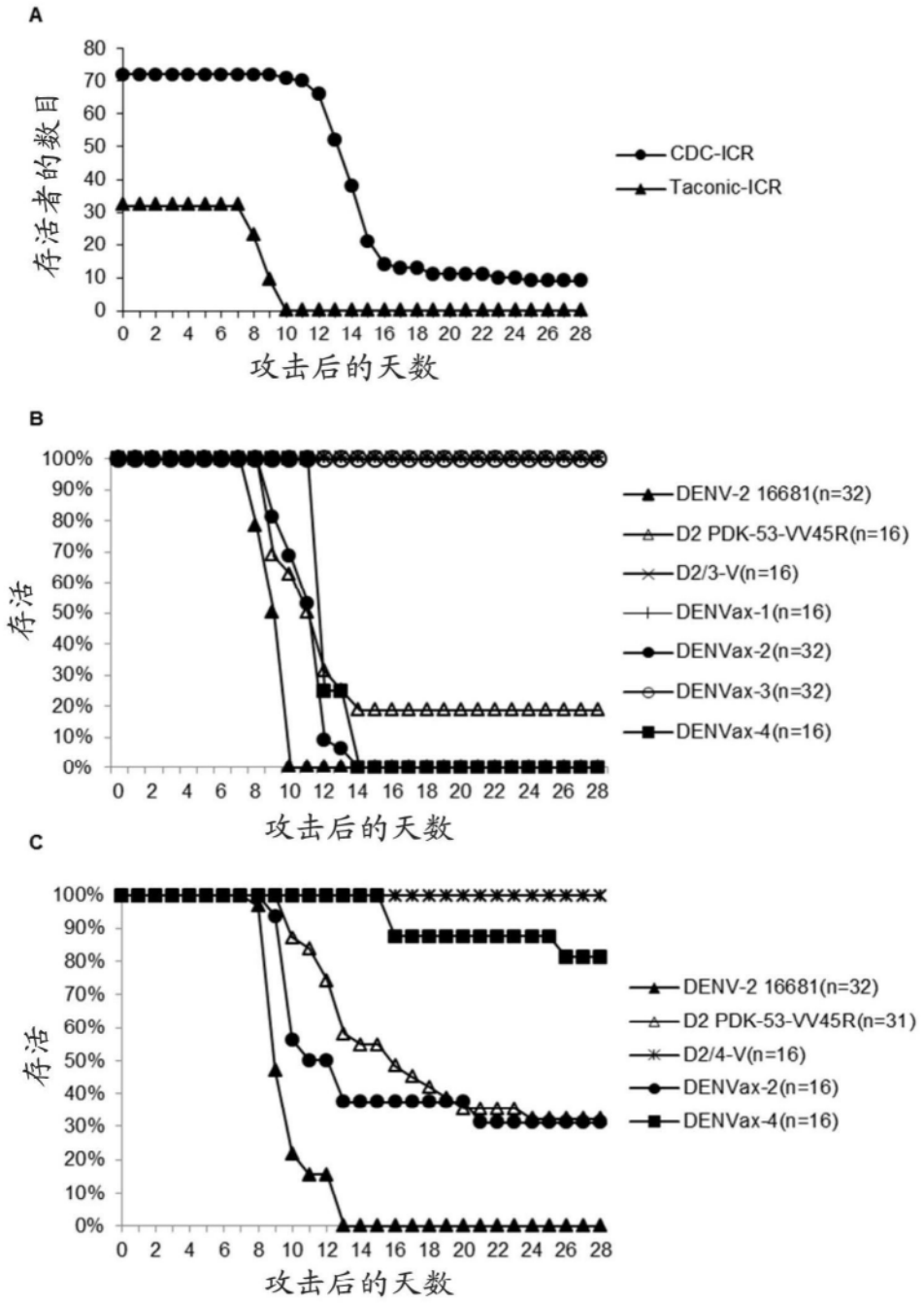

A
存活者的数目
攻击后的天数
CDC-ICR
Taconic-ICR
B
存活
攻击后的天数
DENV-2 16681(n=32)
D2 PDK-53-VV45R(n=16)
D2/3-V(n=16)
DENVax-1(n=16)
DENVax-2(n=32)
DENVax-3(n=32)
DENVax-4(n=16)
C
存活
攻击后的天数
DENV-2 16681(n=32)
D2 PDK-53-VV45R(n=31)
D2/4-V(n=16)
DENVax-2(n=16)
DENVax-4(n=16)

图5A-5C

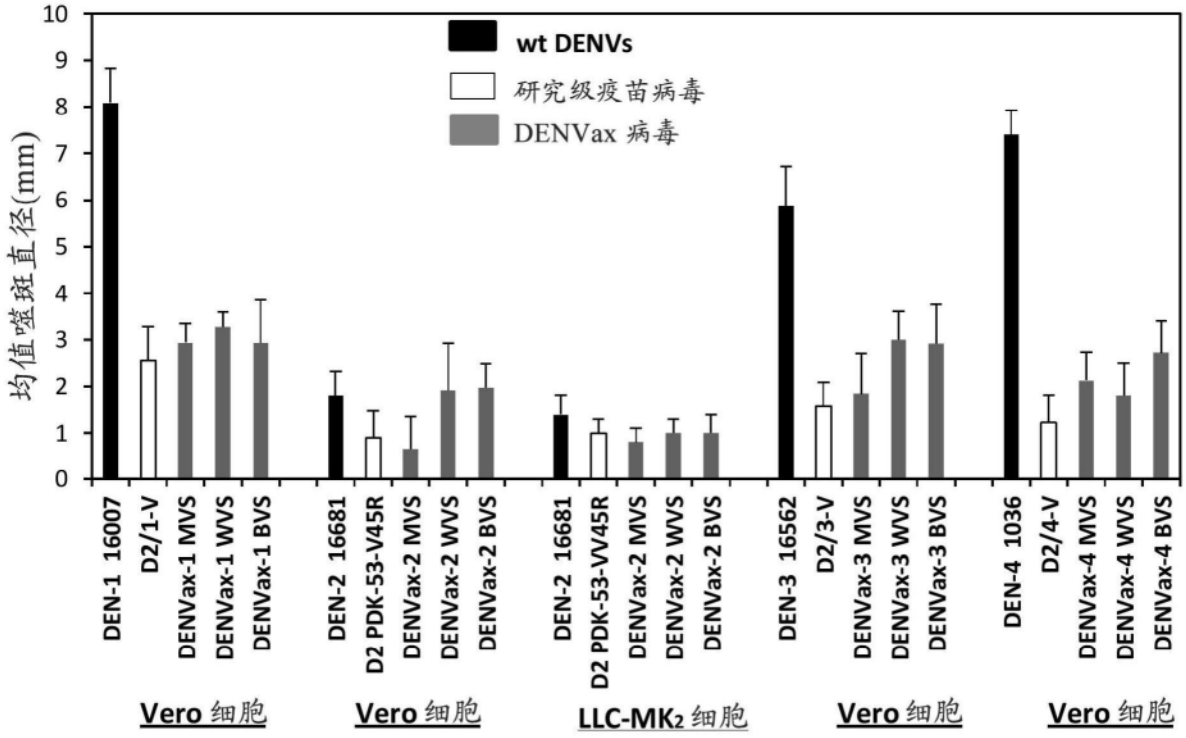

均值噬斑直径(mm)
wt DENVs
研究级疫苗病毒
DENVax 病毒
DEN-1 16007
D2/1-V
DENVax-1 MVS
DENVax-1 WVS
DENVax-1 BVS
DEN-2 16681
D2 PDK-53-V45R
DENVax-2 MVS
DENVax-2 WVS
DENVax-2 BVS
DEN-2 16681
D2 PDK-53-VV45R
DENVax-2 MVS
DENVax-2 WVS
DENVax-2 BVS
DEN-3 16562
D2/3-V
DENVax-3 MVS
DENVax-3 WVS
DENVax-3 BVS
DEN-4 1036
D2/4-V
DENVax-4 MVS
DENVax-4 WVS
DENVax-4 BVS
Vero 细胞
Vero 细胞
LLC-MK2 细胞
Vero 细胞
Vero 细胞

图6

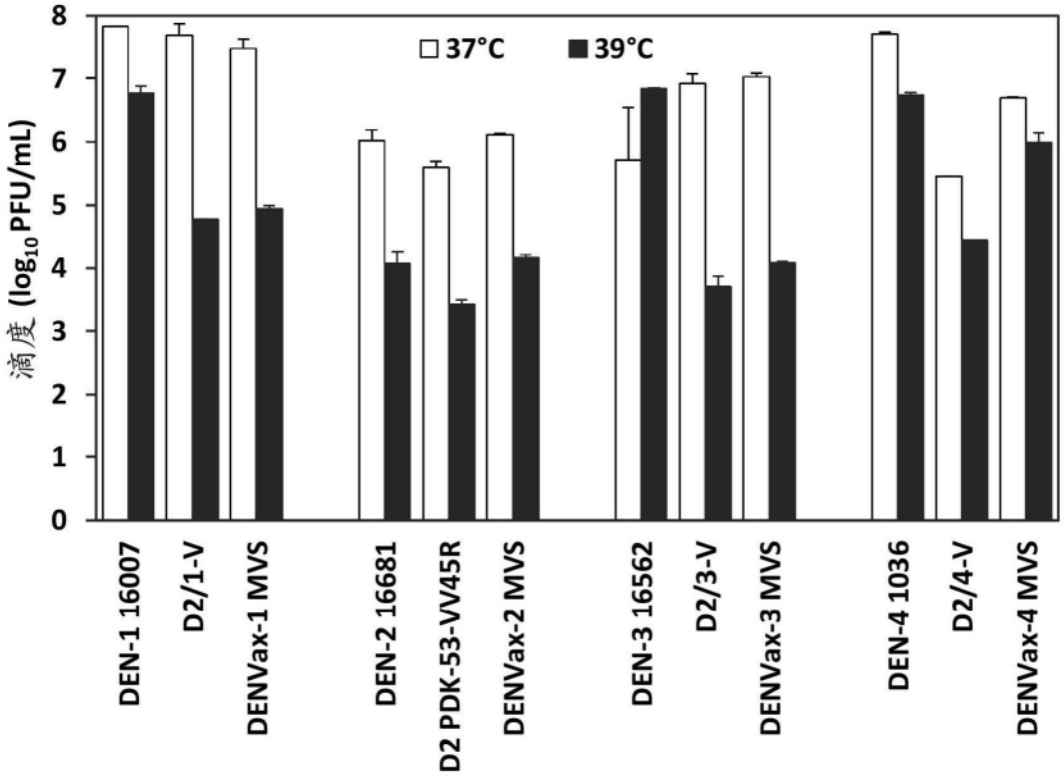

滴度 (log10 PFU/mL)
37°C
39°C
DEN-1 16007
D2/1-V
DENVax-1 MVS
DEN-2 16681
D2 PDK-53-VV45R
DENVax-2 MVS
DEN-3 16562
D2/3-V
DENVax-3 MVS
DEN-4 1036
D2/4-V
DENVax-4 MVS

图7

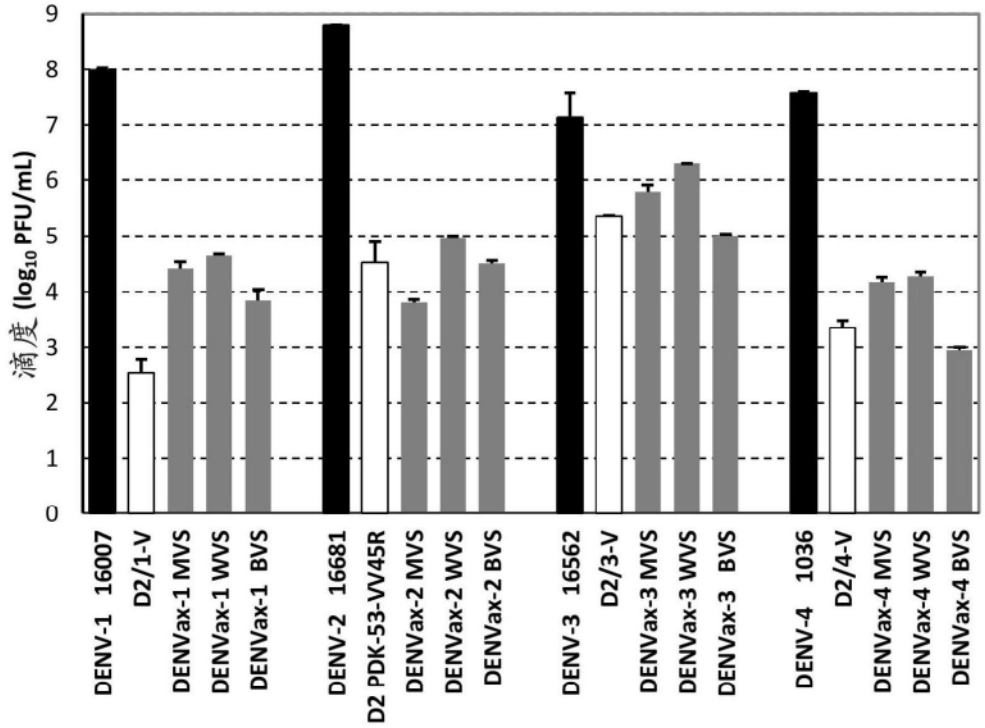

滴度 (log10 PFU/mL)
0
1
2
3
4
5
6
7
8
9
DENV-1 16007
D2/1-V
DENVax-1 MVS
DENVax-1 WVS
DENVax-1 BVS
DENV-2 16681
D2 PDK-53-VV45R
DENVax-2 MVS
DENVax-2 WVS
DENVax-2 BVS
DENV-3 16562
D2/3-V
DENVax-3 MVS
DENVax-3 WVS
DENVax-3 BVS
DENV-4 1036
D2/4-V
DENVax-4 MVS
DENVax-4 WVS
DENVax-4 BVS

图8

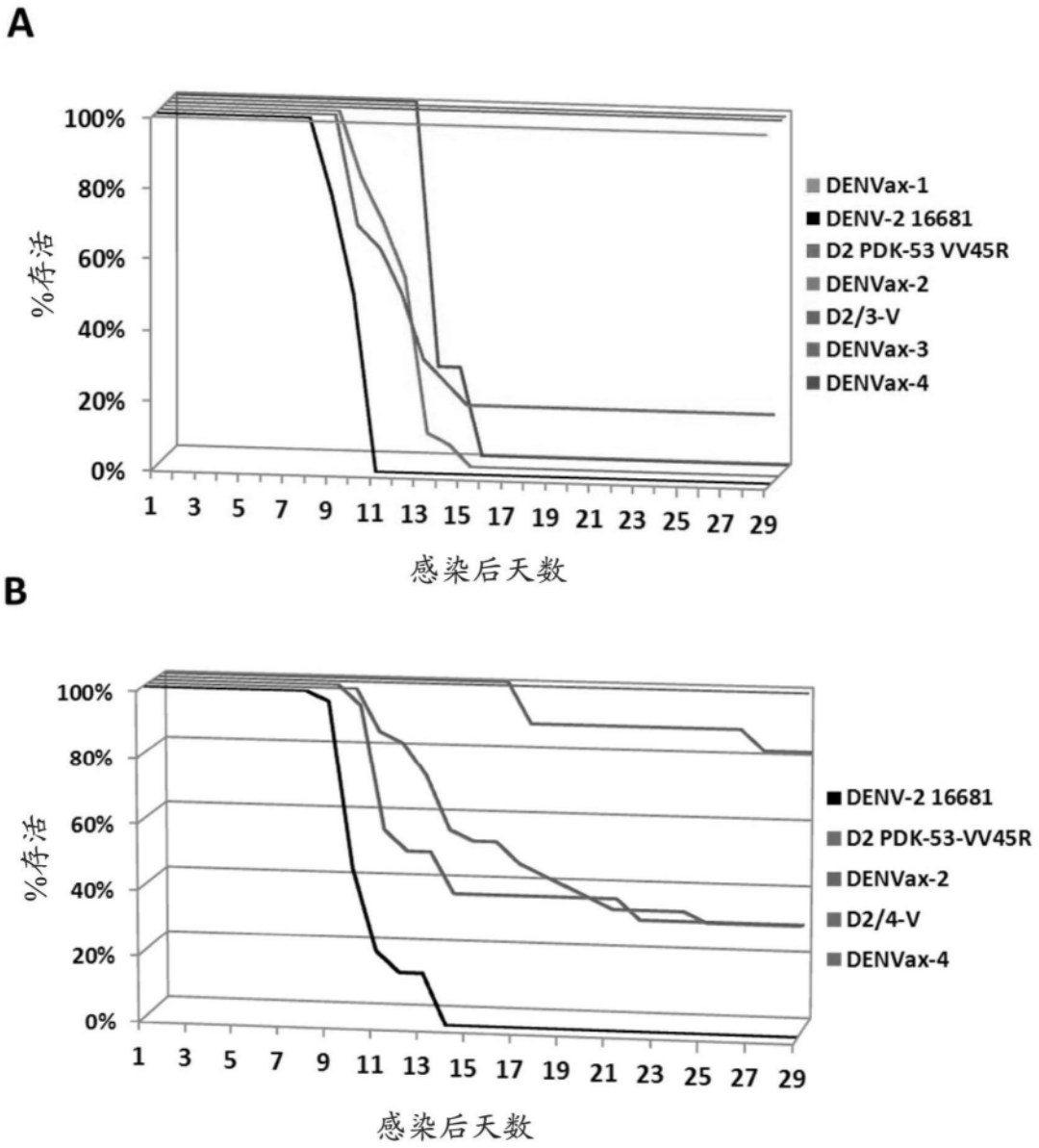

A
100%
80%
60%
40%
20%
0%
%存活
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29
感染后天数
DENVax-1
DENV-2 16681
D2 PDK-53 VV45R
DENVax-2
D2/3-V
DENVax-3
DENVax-4
B
100%
80%
60%
40%
20%
0%
%存活
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29
感染后天数
DENV-2 16681
D2 PDK-53-VV45R
DENVax-2
D2/4-V
DENVax-4

图9A和9B

| 血清型 | 毒株 | 病毒起源 | C57T<br>5'NCR | A524T<br>prM-D29V | T900C[a]<br>M(沉默) | C2055T<br>E(沉默) | G2579A<br>NS3-G53D | C4018T<br>NS2A-L181 | A52703<br>NS3-E350V | T5547C<br>NS3(沉默) | G6599C<br>NS4A-G75A | C8571T<br>NS5(沉默) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| DENV-2 | 16681 | 从人分离 | C | A | T | C | G | C | A | T | G | C |
| | PDK-53 | 16681的PDK细胞传代 | T | T | .. | t | A | T | T/A混合物 | c | C | t |
| | PDK53-V(VV45R) | 重组PDK-53-V | T | T | c | t | A | T | T | c | C | C |
| | PDK53-E(VE48R) | 重组PDK-53-E | T | T | c | t | A | T | A | c | C | C |

加下划线的突变：PDK-53的3个最重要的减毒基因座

红色字体：PDK-53特异性序列(从16681改变)

粗体字体：PDK-53和克隆衍生的V或E病毒之间的不同nt序列

[a]工程化沉默克隆标志物以区分初始PDK-53和重组(克隆衍生的)病毒

图10

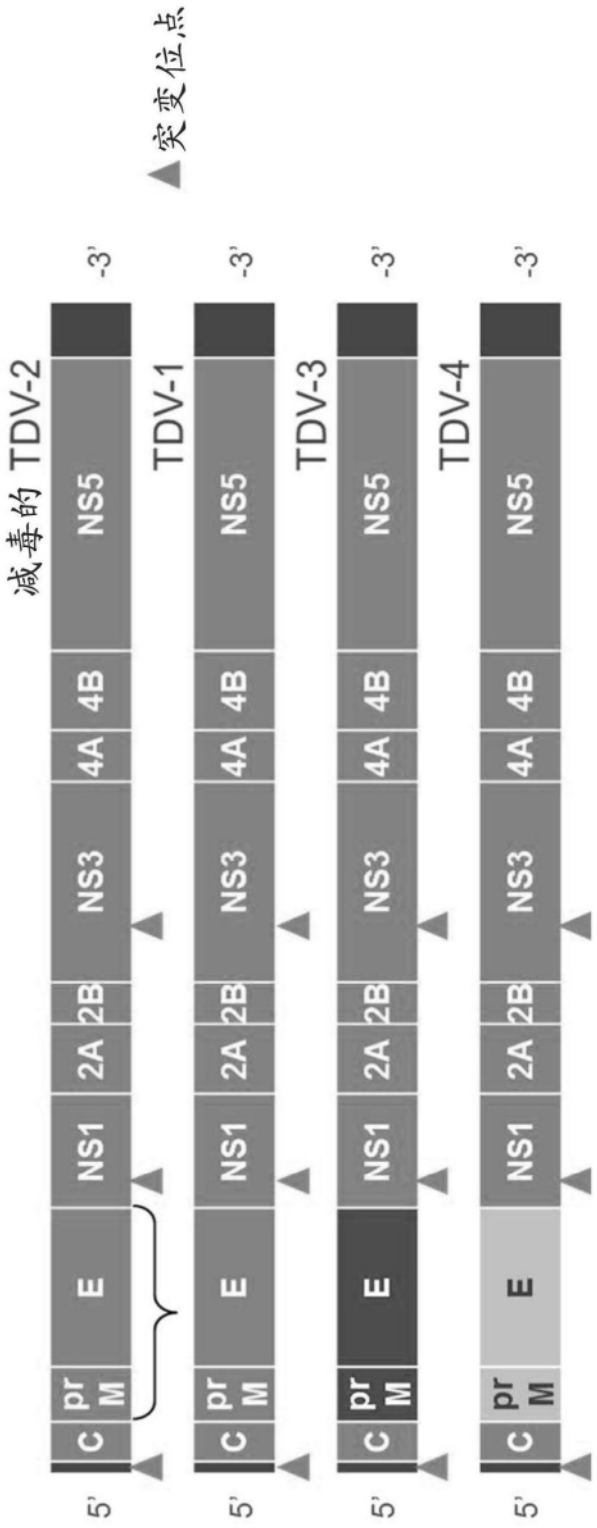



图11A

| 每剂 | TDV-1 | TDV-2 | TDV-3 | TDV-4 | 总计 |
|---|---|---|---|---|---|
| TDV配制剂 (噬斑形成单位) | 2 x $10^4$ PFU | 5 x $10^4$ PFU | 1 x $10^5$ PFU | 3 x $10^5$ PFU | 4.7 x $10^5$ PFU |

图11B

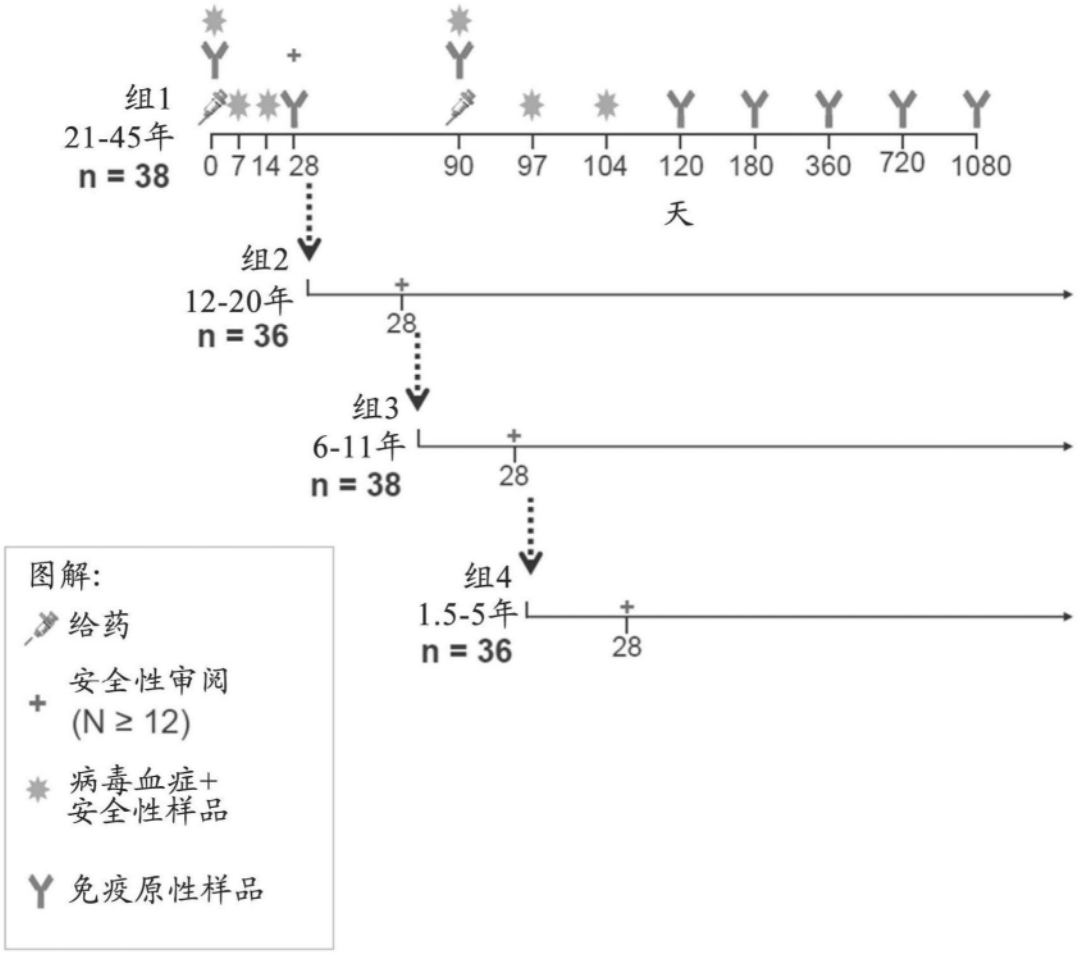



图12A

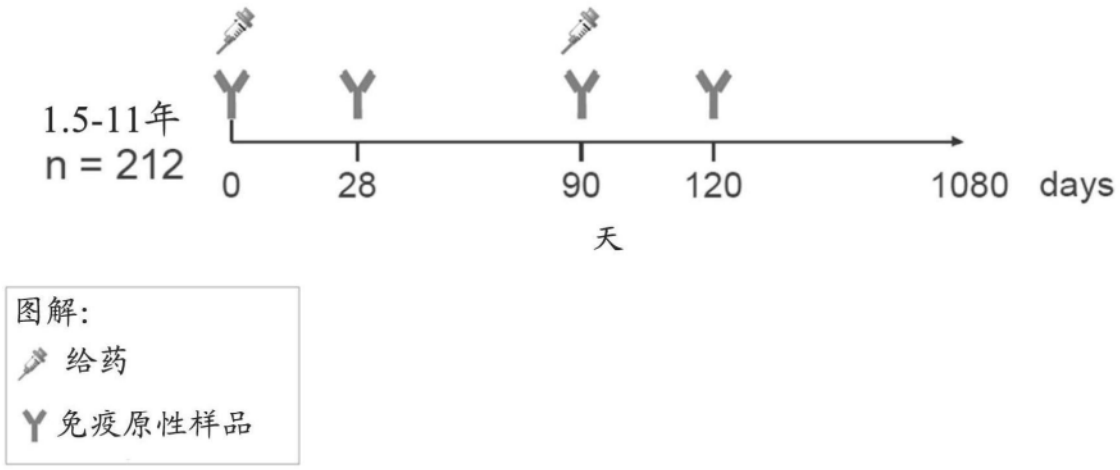

1.5-11年
n = 212
0
28
90
120
1080 days
天
图解:
给药
免疫原性样品

图12B

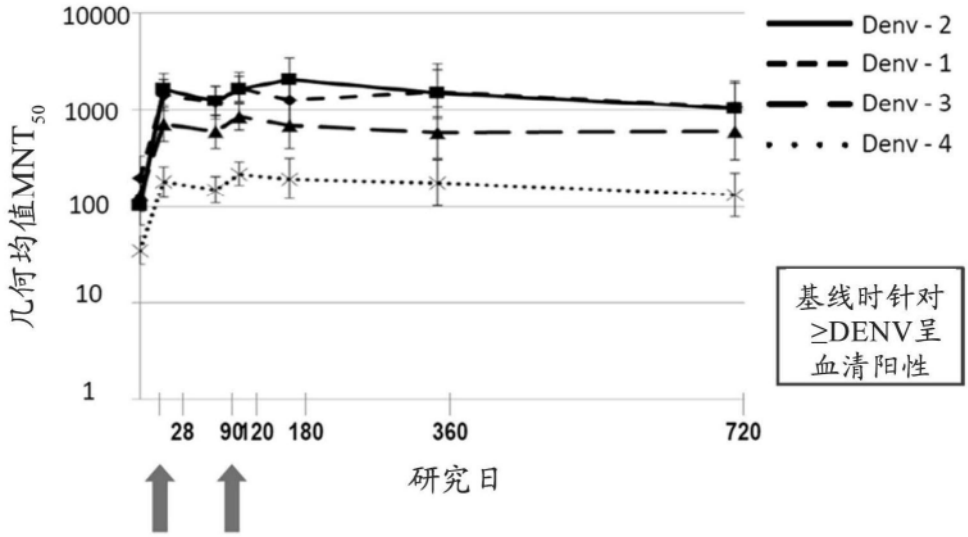

10000
1000
100
10
1
几何均值MNT50
28
90
120
180
360
720
研究日
Denv - 2
Denv - 1
Denv - 3
Denv - 4
基线时针对
≥DENV呈
血清阳性

图13A

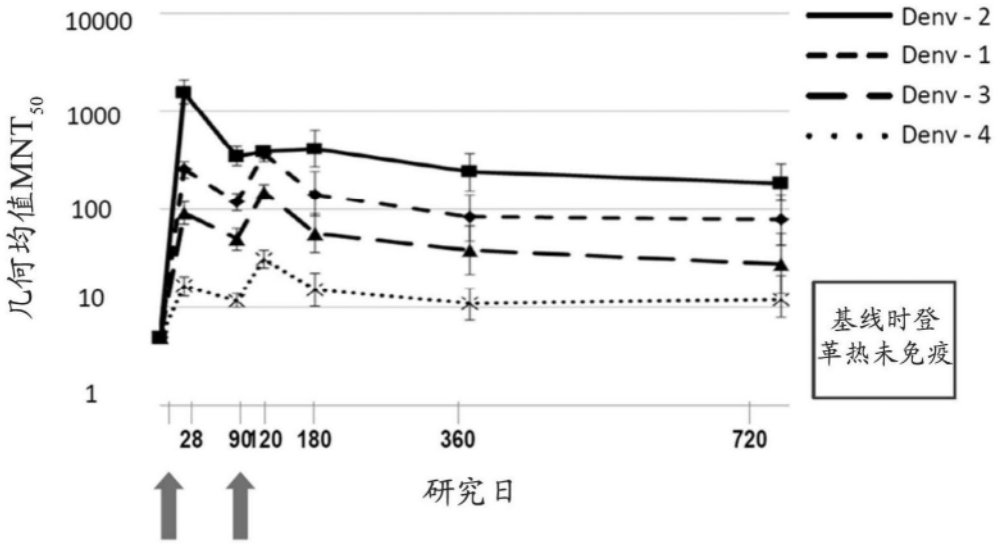

10000
1000
100
10
1
几何均值MNT50
Denv - 2
Denv - 1
Denv - 3
Denv - 4
基线时登革热未免疫
28
90
120
180
360
720
研究日

图13B

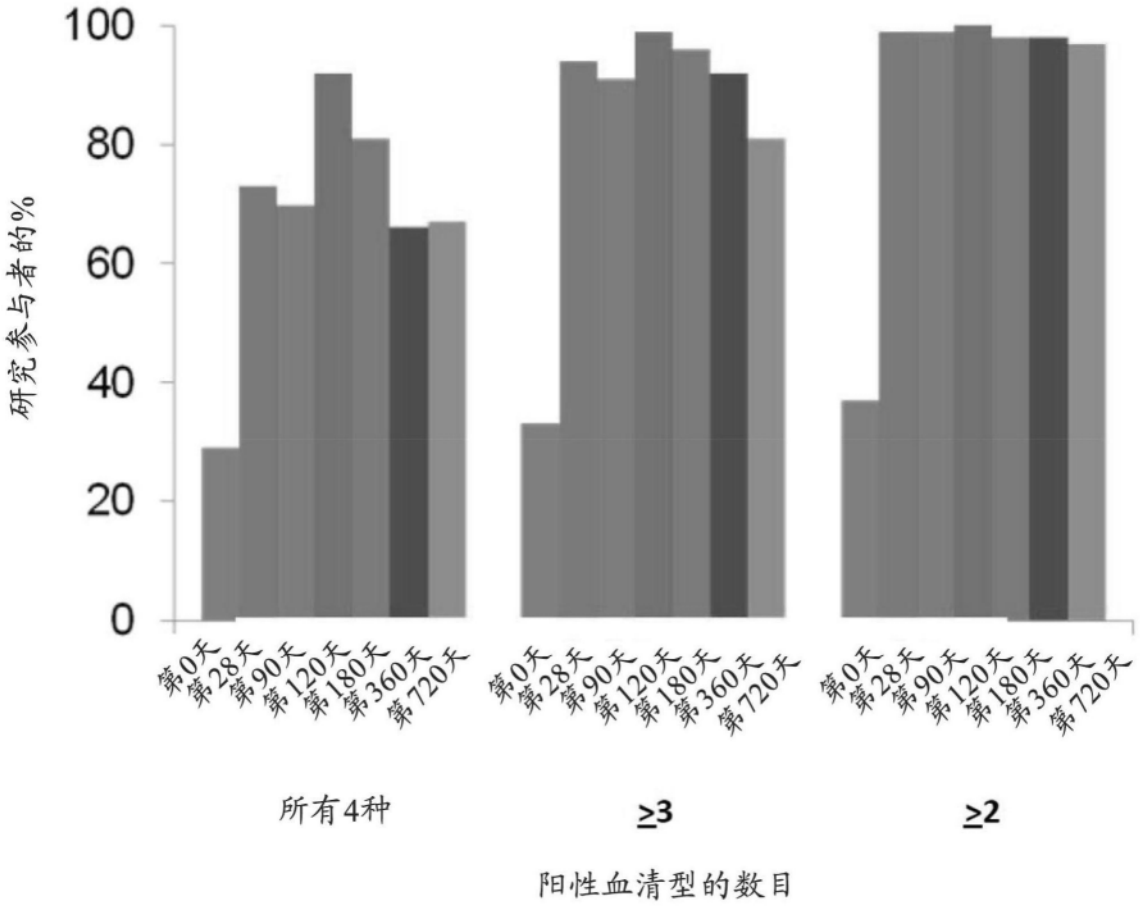

100
80
60
40
20
0
研究参与者的%
第0天
第28天
第90天
第120天
第180天
第360天
第720天
所有4种
≥3
≥2
阳性血清型的数目

图14

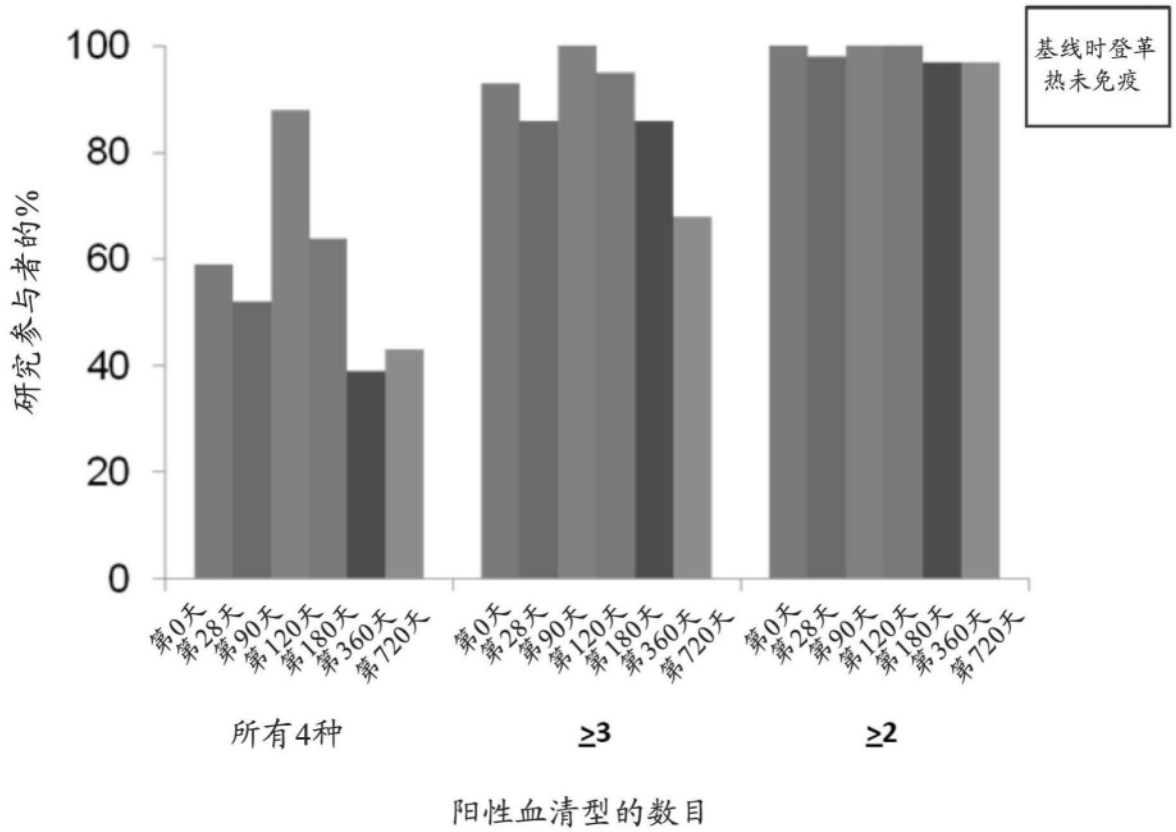
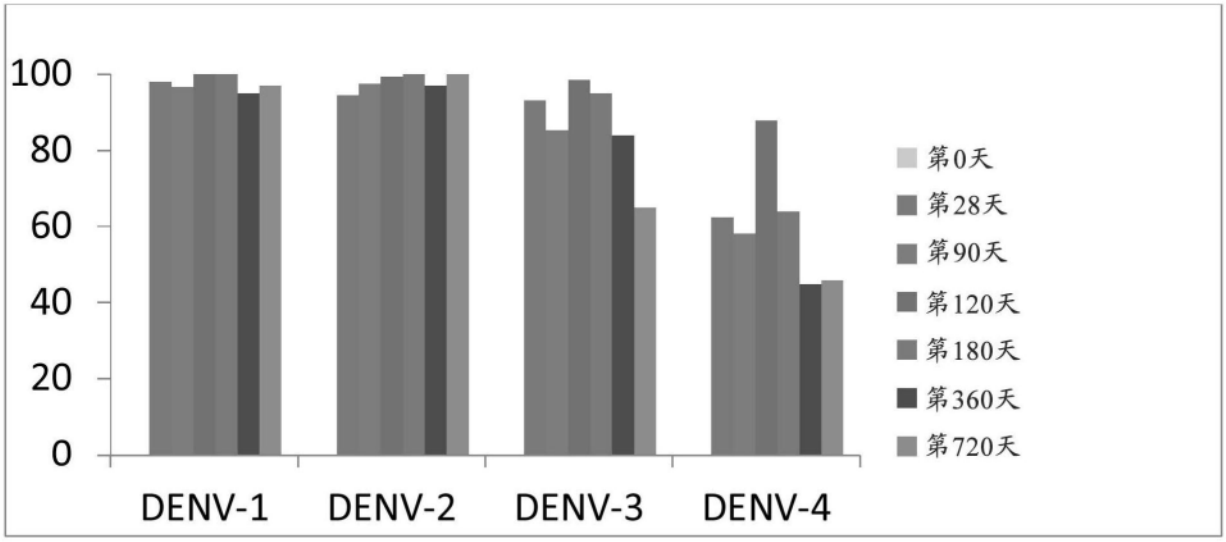
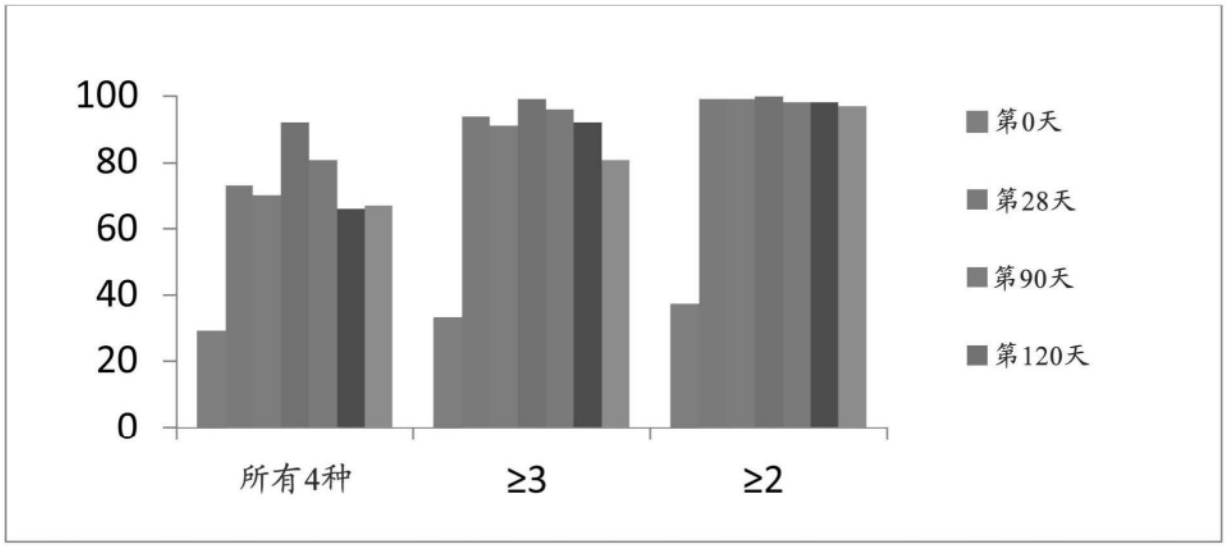



图15




*基线时和接受TDV (四价)后登革热未免疫的儿童/年轻人

图16




*基线时和接受TDV (四价)后登革热未免疫的儿童/年轻人

图17