H1N1流感病毒M1蛋白表位筛选及其在禽流感病毒交叉免疫中的作用机制

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摘要 流感病毒, 如2009甲型H1N1流感病毒(2009 pH1N1)以及发生跨种传播的H7N9禽流感病毒(H7N9)等的流行严重威胁了人类健康并造成了重大社会经济损失. 自2009年以来, 2009 pH1N1作为季节性流感病毒的流行使得健康人群存在一定的T细胞免疫本底水平. 本研究对2009 pH1N1和H7N9的主要T细胞免疫原M1蛋白存在的4个突变区段, 利用ELISPOT试验确定了2009 pH1N1在这4个区段来源的T细胞表位多肽H1-M42 (LMEWLKTR), H1-M102 (KLKREITFHGAK), H1-M202s (AMEVANQTR)和H1-M244 (MGVQMQRFK). 同时, 发现健康人群对H7N9来源的相应多肽预存有一定水平T细胞免疫, 但低于对2009 pH1N1本身多肽的免疫水平. 多肽与HLA分子的复性实验表明, H1-M102和H1-M244能够与HLA-A*1101稳定结合; 而H1-M42和H1-M202s虽然能够结合HLA-A*3303, 但H7N9相应的表位与这些分子的结合力相对较弱. 结果表明, 在健康人群中存在有限的针对H7N9的预存T细胞免疫, 而H7N9在新鉴定表位多肽中的氨基酸突变对于免疫逃逸具有一定的贡献. 对流感病毒交叉免疫的研究能够为了了解禽流感病毒逃逸T细胞免疫机制以及通用流感疫苗的研发提供重要参考.

关键词 2009 pH1N1, H7N9, 抗原变异, 交叉免疫, T细胞表位

2013年春季, 我国东部部分省份出现人感染H7N9亚型流感病毒H7N9病例. 随后, 其他多个地区也陆续出现该病毒感染病例^[1~3]. 在2013~2017年间H7N9引发了5次人感染禽流感疫情流行, 累计病例1000多例^[4]. 该病毒主要由禽到人实现跨种传播, 同时也存在有限的人际传播^[5], 对人类公共卫生安全造成了严重威胁. 流感病毒主要表达由8个RNA片段编码的14种蛋白, 包括表面蛋白和内部蛋白等. 在抗病毒免疫中, B细胞通过识别病毒表面蛋白血凝素(HA)和神经氨酸酶(NA)产生相应抗体来清除病毒. 由于HA和NA突变频率较高, 使得不同亚型流感病毒之间的抗体交叉反应较少^[6]. 而细

胞毒性T淋巴细胞(CTLs)主要靶向其内部的非糖基化蛋白, 如NP, M1和PB1^[7], 这些蛋白包含较多CTL表位, 并且与HA和NA相比显著保守. 对抗原表位具有高亲和力和交叉识别能力的CTLs对于抵抗流感病毒感染至关重要. 流感病毒之间常存在T细胞交叉免疫, 对季节性流感和H7N9之间的交叉免疫研究, 对于了解H7N9的逃逸机制以及开展科学防控非常重要.

2009年, 引起全球大流行的甲型H1N1亚型流感病毒2009 pH1N1是一种人-猪-禽流感重配病毒, 目前仍然是各国主要的季节性流感病毒之一^[6,8,9]. 既往研究表明, 2009年之前的季节性流感病毒感染激发的CD4⁺和

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CD8⁺T细胞具有对2009 pH1N1亚型的交叉免疫反应^[10,11]。我们最近的研究也发现,近年来健康人群建立的针对2009 pH1N1的T细胞免疫也可对H7N9产生交叉CD4⁺和CD8⁺T细胞免疫,但健康人群对H7N9的交叉T细胞免疫水平明显低于针对2009 pH1N1本身的T细胞免疫^[12,13]。在2009 pH1N1与多种禽流感的交叉T细胞免疫研究中发现,与NP相比,M1蛋白对于不同流感亚型的免疫原性差异贡献更大,而这种交叉T细胞免疫反应所产生的保护作用也在动物试验中得到了证实^[12]。

在2009 pH1N1与H7N9的M1蛋白中,我们和国际上其他研究组已经鉴定了一系列能够激发不同HLA超抗原型个体T细胞免疫反应的保守表位^[10,13-15]。为了进一步探究2009 pH1N1流感病毒与H7N9亚型流感病毒M1蛋白突变区段对于二者的交叉免疫水平的贡献,并且进一步利用筛选出的T细胞表位分析导致其免疫原性差异的分子机制,我们招募健康志愿者展开了此次研究。本研究将为了了解流感病毒的免疫逃逸分子机制以及流感通用疫苗研发提供科学参考。

1 材料与方法

(i) 研究对象. 本研究中的16位研究对象为北京市健康志愿者,在过去5年内未接种过流感疫苗,采集外周血并分离单核细胞(PBMCs)。研究对象通过SSO分型技术完成测定(北京曼泰里)。研究对象签署知情同意书,该研究方案由中国疾病预防控制中心病毒病预防控制所伦理委员批准通过。

(ii) 样本的采集与处理. 对每个研究对象用EDTA抗凝采血管采取静脉血20 mL用于PBMCs的分离,抗凝采血管采得的新鲜血液用等体积的PBS或1640培养基稀释,缓慢加于50 mL离心管中的等体积Ficoll中,采用水平转子室温离心,600×g离心30 min,升速为最低降速为0。吸出中间白膜层至15 mL离心管中,并向管中加入PBS洗两次(600×g /10 min)。

(iii) 2009 pH1N1与H7N9突变区段肽库的构建. 将A/California/7/2009(H1N1)和A/Anhui/1/2013(H7N9)的M1蛋白序列用重叠法分成15~18个氨基酸的肽段(<https://hcv.lanl.gov/content/sequence/PEPTGEN/pept-gen.html>),相邻肽段间重叠11个氨基酸,并避免C端出现Gly, Pro, Ser, Thr, Cys, Asp, Gln, Asn, Glu这几种氨基酸。可以观察到H7N9相较于2009 pH1N1存在大量保守区段,将该毒株中M1蛋白保守区的长肽剔除后组成特异性多肽库。H7-M5至H7-M9共5条多肽构成H7

(Pool1); H7-M15至 H7-M20共6条多肽构成H7 (Pool2); H7-M22至H7-M27共6条多肽构成H7 (Pool3); H7-M33至H7-M39共7条多肽构成 H7 (Pool4)(表S1)。

(iv) ELISPOT实验. 通过IFN-γ特异性的酶联免疫斑点试验(ELISPOT)(BD)检测抗原特异性T淋巴细胞反应。在使用前,将96孔ELISPOT平板用抗人IFN-γ的抗体稀释液在4℃包被过夜。来自健康志愿者的PBMCs (2.5×10^5 /孔用于新鲜分离的PBMC和 5×10^4 /孔用于体外培养的T细胞)与肽库(单个肽浓度为2 μg/mL)于孔中混合,在37℃和5% CO₂条件下孵育18 h。以植物血凝素(PHA)作为非特异性刺激的阳性对照,以没有刺激的情况下孵育的细胞作为阴性对照。孵育后,除去细胞,然后用生物素化IFN-γ抗体作为一抗,酶标亲和素作为二抗,辣根过氧化物酶底物(HRP)显色,通过用水彻底冲洗来终止显色。使用自动ELISPOT读取器和图像分析软件(Cellular Technology Limited)捕获斑点的数量并进行量化。该ELISPOT设定的阳性反应的阈值是20个酶联免疫斑点/10⁶个PBMC。

(v) HLA体外复性试验. 将人源重链HLA-A*0201和HLA-A*3301以及人源的轻链β₂m通过原核表达质粒表达。重链和轻链在大肠杆菌BL21(DE3)菌株中过表达产生包涵体,将纯化的蛋白质包涵体溶解于6 mol/L盐酸胍缓冲液中,浓度为30 mg/mL。然后,在复性缓冲液(100 mmol/L Tris-HCl [pH 8], 2 mmol/L EDTA, 400 mmol/L L-Arg, 0.5 mmol/L氧化型谷胱甘肽和5 mmol/L还原型谷胱甘肽)中以1:1:3(多肽:轻链:重链)的摩尔比进行重链,轻链和多肽的滴加和稀释。蛋白质复性24 h后,浓缩MHC复合物并换液到20 mmol/L Tris-HCl (pH 8)和50 mmol/L NaCl的缓冲液中,然后使用Superdex™ Increase 200 10/300 GL分离柱(GE Healthcare, China)进行纯化。

(vi) 分析软件. 本文中序列比对使用DNAMAN(7.0)软件分析,两株病毒的蛋白组序列从Influenza Virus Database中获取,差异性对比分析使用GraphPad Prism 7软件,文章作图利用了Origin 8.0, Pymol2.3.0, Adobe Illustrator CS6及Photoshop CS6软件。

2 结果

2.1 两种流感病毒2009 pH1N1与H7N9的M1蛋白保守性分析

基于序列比对结果发现,相较于2009 pH1N1,

H7N9存在4个发生氨基酸突变的区段(图1)。我们针对2009 pH1N1与H7N9的M1蛋白全长,各合成了38条15~18个氨基酸的长肽(表S1),相邻多肽重叠11个氨基酸。H7N9相比于2009 pH1N1, 63.16%(24/38)长肽中存在氨基酸突变位点,其中M1蛋白末端发生氨基酸突变率较高,有9个氨基酸位点发生了突变。

2.2 M1蛋白突变区段肽库的T细胞免疫原性及其交叉免疫

我们招募了6位健康志愿者Z1-Z6 (表1),以研究健康人群对于2009 pH1N1和H7N9 M1蛋白突变区段的T细胞应答。将健康志愿者的PBMCs中的2009 pH1N1特异性T细胞在体外扩增以评估T细胞免疫水平,并随后研究其针对H7N9的交叉免疫水平。结果发现,来自2009 pH1N1 M1肽库的9条长肽(H1-M8, H1-M9, H1-M17, H1-M18, H1-M23, H1-M24, H1-M33, H1-M35和H1-M39)能够激发受试者的T细胞免疫应答,而健康人群对来自H7N9对应的突变长肽交叉免疫应答较低(图2)。通过检索免疫表位数据库(IEDB)发现,这9条具有免疫原性的长肽中,含有多个先前研究鉴定的HLA I类或II类限制性T细胞表位(表2),而这些表位对于2009

pH1N1和H7N9之间的免疫原性差异可能具有一定的贡献。

2.3 2009 pH1N1 M1蛋白长肽可以引起健康人群的T细胞免疫反应

随后我们针对性纳入了10名HLA-A3超家族的健康志愿者,从受试者外周血中分离PBMCs进行培养和体外扩增,将PBMCs在9条2009 pH1N1来源长肽构成的肽库以及20 U/mL rhIL-2存在下刺激培养7 d,通过ELISPOT测定IFN- γ 的分泌。结果表明,10位健康人中多数都对先前鉴定出的9条突变长肽产生较强的T细胞免疫反应(图3)。在9条突变长肽中,H1-M8, H1-M9, H1-18和H1-M39引起的T细胞免疫反应,与Mock组(用培养基替代多肽刺激物)相比具有显著性差异。

2.4 健康人中存在H7N9来源短肽的T细胞交叉免疫反应

为进一步探究2009 pH1N1与H7N9的交叉T细胞免疫,我们结合网站评分(<http://www.cbs.dtu.dk/services/NetMHCpan/>),根据HLA-A3和HLA-A2等HLA超家族结合多肽的特点,预测并合成了9条长肽中对应的潜在

图1 H7N9与2009 pH1N1 M1蛋白差异性比对模式图。浅蓝色方框代表保守序列,深蓝色方框代表非保守序列,黑点标出了具体的氨基酸突变位点,虚线方框圈出了非保守长肽。深绿色方框代表2009 pH1N1来源的预测短肽,浅绿色方框代表H7N9来源的预测短肽

Figure 1 Comparison of H7N9 and 2009 pH1N1 M1 protein. The light blue boxes represent non-conserved sequences, dark blue boxes represent non-conserved sequences, black dots indicate specific amino acid mutation sites, and dotted boxes include non-conserved peptides. The dark green box represents the predicted short peptide from 2009 pH1N1 and the light green box represents the predicted short peptide from H7N9

表1 志愿者血样信息

Table 1 Blood sample donor information

ID	性别 ^{a)}	年龄(岁)	HLA-A分型	HLA-B分型
Z1	M	33	A*1101, A*3101	— ^{b)}
Z2	F	30	A*0201, A*0201	B*0801, B*4001
Z3	M	24	A*2402, A*2402	B*1801, B*4001
Z4	M	28	A*1101, A*0201	B*1302, B*1501
Z5	M	39	A*0206, A*0206	—
Z6	F	25	A*2402, A*3303	—
C1	F	29	A*0210, A*3001	B*1302, B*4006
C2	M	28	A*0201, A*1101	B*1501, B*4601
C3	F	24	A*1101, A*2402	B*1501
C4	M	25	A*1101, A*3101	B*4001, B*5101
C5	F	26	A*1101, A*2402	B*1501, B*1511
C6	M	25	A*1101, A*2402	B*1302
C7	F	25	A*0201, A*3001	B*4403, B*5401
C8	F	23	A*0207, A*6801	B*3801, B*4601
C9	M	37	A*1101, A*1101	B*1501, B*4001
C10	M	29	A*1101, A*3303	B*5101, B*5801

a) M, 男性; F, 女性. b) —, 志愿者的具体HLA分型未检测

CD8⁺ T细胞表位多肽^[16], 共计16条(表3), 其相应位置也在图中标示(图1). 检测结果显示, 4条2009 pH1N1来源的HLA-A3超家族限制性短肽可在健康个体中引起较强的免疫反应, 而HLA-A2超家族限制性短肽均为阴性, 未能激发免疫反应(图4). 同时我们发现, 健康人群中针对2009 pH1N1这4条新鉴定表位的T细胞存在对A(H7N9)突变肽的交叉免疫反应. 结果显示, 短肽H1-M42 (LMEWLKTR), H1-M102 (KLKREITFHGAK), H1-M202s (AMEVANQTR)以及H1-M244 (MGVQMQRFK)能够激发较强的T细胞免疫应答; 与2009 pH1N1来源多肽相比, A(H7N9)相应位点来源的突变肽H7-M42 (LMEILKTR), H7-M102 (KLKREMTFHGAK), H7-M202s (AMEVASQAR)和H7-M244 (MGVQLQRFK)诱导较低的T细胞免疫应答, 表明H1-M42, H1-M102, H1-M202s和H1-M244作为2009 pH1N1的M1蛋白来源表位, 对于2009 pH1N1与H7N9的T细胞免疫原性差异具有一定贡献.

2.5 M1蛋白来源表位多肽与HLA I的亲和力分析

多肽-HLA分子的结构学研究表明, T细胞表位突变对其诱导的T细胞活化产生影响的机制主要有两种:

一是表位多肽在HLA分子上的锚定残基突变影响二者结合力; 二是表位多肽残基的突变影响T细胞受体的识别. 为了进一步阐明H7-M42, H7-M102, H7-M202s和H7-M244逃逸机体预存免疫的机制, 我们利用体外复性试验检测这些多肽及其相应的2009 pH1N1来源多肽与HLA I的结合力. H1-M102和H1-M244T细胞免疫应答人群分型主要为HLA-A*1101, H1-M42和H1-M202s反应人群主要分型为HLA-A*3303, 因此我们分别利用HLA-A*1101和HLA-A*3303验证这4条多肽与HLA I的结合力. 结果表明, 4条短肽均可帮助相应的HLA I分子实现体外复性, 这进一步验证了2009 pH1N1来源的这4条短肽是HLA-A3超家族限制性表位(图5(a)~(d)). 序列比对显示, H7-M102相较于2009 pH1N1同一区段突变了第6位氨基酸, H7-M244突变了第5位氨基酸, H7-M202s突变了第6和第8位氨基酸, 表明这些多肽的突变主要对T细胞受体的直接识别产生影响, 从而造成2009 pH1N1和H7N9的T细胞免疫原性差异. 同时, 从模拟的结构图中可以看出, H1-M244中第五位的甲硫氨酸不作为多肽的锚定残基, 故该位点的改变不影响与HLA-A*1101的结合(图5(e)), H1-M244中第五位的甲硫氨酸相较于突变后的亮氨酸, 更

图 2 确定H7N9中具有抗原性降低的突变单肽. 使用2009 pH1N1 M1蛋白长肽库对PBMCs进行体外扩增, 通过ELISPOT检测来自2009 pH1N1和H7N9的长肽识别情况. 2009 pH1N1 M1蛋白来源的突变长肽(共24条)与H7N9来源的长肽肽库(共4个)作为刺激物, 检测健康人群的T细胞免疫反应((a)–(d)). 将H7N9来源的H7-M5至H7-M9混为H7-Pool1; H7-M15至H7-M20混为H7-Pool2; H7-M22至H7-M27混为H7-Pool3; H7-M33至H7-M39混为H7-Pool4. PBMCs来自健康志愿者Z1, Z2, Z3, Z4, Z5和Z6

Figure 2 Determination of the individual peptide variants in H7N9 with decreased antigenicity. Recognition of M1 peptides derived from 2009 pH1N1 and H7N9 influenza virus were performed by ELISPOT using the *in vitro* expanded PBMC specific for 2009 pH1N1 M1. The individual peptides from the 2009 pH1N1 M1 peptides were used as stimulus. The peptides from H7N9 were mixed as peptide pool to stimulate the cells due to the limitation of the cell numbers. H7-M5 to H7-M9 derived from H7N9 were mixed into H7-Pool1; H7-M15 to H7-M20 derived from H7N9 were mixed into H7-Pool2; H7-M22 to H7-M27 of H7N9 into H7-Pool3; H7-M33 to H7-M39 of H7N9 into H7-Pool4. The PBMCs are from donors Z1, Z2, Z3, Z4, Z5, and Z6

靠近TCR作用区, 可能更有利于下游TCR的识别, 因此此位点氨基酸突变后相较于突变前激起的T细胞免疫反应程度较低(图5(f)).

3 结论与讨论

自2009年2009 pH1N1流感疫情暴发以来, 流感病人临床样本中不断检测到该病毒, 2009 pH1N1已成为主要的流感病毒流行株之一^[8,17]. 研究表明, 健康人群中存在相当高比例的2009 pH1N1特异性抗体, 这意味着人群中的2009 pH1N1暴露率很高^[18], 使得多数健康人体内存在针对2009 pH1N1的T细胞免疫. 而对于新发H7N9病毒, 健康人群中没有预先存在的特异性抗体^[13]. 与B细胞表位不同, T细胞主要识别来源于流感病毒相对保守的内部蛋白表位, 流感病毒特异的CD4⁺和CD8⁺T细胞交叉免疫普遍存在于健康群体中, 为继发同种异

型病毒感染提供保护^[7,11,19–21]. 研究表明, 2009 pH1N1感染之所以产生相对温和临床症状, 部分是因为季节性流感病毒感染或疫苗接种产生了交叉免疫T细胞从而产生保护效力^[11,22]. 季节性H1和H3甲型流感病毒感染产生的记忆性T细胞对禽流感H5N1病毒具有交叉免疫应答^[7,20], 并且对H7N9也能产生CD4⁺和CD8⁺T细胞交叉免疫^[12,23,24]. 新型禽流感病毒H7N9具有哺乳动物适应性的氨基酸替换, 与其从禽类到人类的宿主转换相关^[1,25], 同时该病毒蛋白序列上相应的T细胞表位存在突变, 影响了人类免疫系统识别和病毒清除^[19]. 在本文中, 我们揭示了在健康人群中对于2009 pH1N1 M1蛋白的突变区段, 存在一定水平的针对H7N9的交叉反应性T细胞应答, 主要由未突变关键锚定残基的T细胞表位激发, 有助于了解流感病毒的免疫逃逸并对通用疫苗开发提供有用参考.

表 2 2009pH1N1与H7N9来源M1蛋白多肽分析

Table 2 M1-derived peptides with low T-cell cross-reactivities due to the mutations in H7N9

名称	位置	序列信息	阳性志愿者	CD8 ⁺ 表位 ^{a)}	HLA 限制性 ^{b)}	CD4 ⁺ 表位	HLA 限制性
H1-M8 ^{e)}	39~55	LEALMEWLKTRPILSPL	2/6	ALMEWLKTRP ¹	A2	ALMEWLKTRPILSPLTKGIL ¹	DRB1*07:01
H7-M8		----- I ^{d)} -----					
H1-M9 ^{e)}	45~62	WLKTRPILSPLTKGILGF	5/6	KTRPILSPLTK ¹	A11	TRPILSPLTKGILGFVF ¹	DRB1
H7-M9		- I -----		PILSPLTKG ¹	A2		
H1-M17	95~112	RAVKLYKKLKREITTFHGA	2/6	LYKKLKREITF ¹	A24	KKLKREITTFHGAK ¹	DRB1*04:01
H7-M17		K ----- M -----		LYKKLKREMTF ⁷	A24		
H1-M18	102~119	KLKREITTFHGAKEVSLSY	3/6	ITTFHGAKEIA ¹	A2	REITTFHGAKEISL ¹	DRB1*09:01
H7-M18		----- M ----- A -----		TFHGAKEVSL ¹	A24		
H1-M23	134~149	RMGTVTTEAAFGVLVCA	3/6	/	/	/	/
H7-M23		----- A - G - I -----					
H1-M24	139~155	TTEAAFGVLVCAATCEQIA	3/6	GLVCATCEQIA ¹	A2	/	/
H7-M24		- A - G - I -----					
H1-M33	196~213	SEQAAEAMEVANQTRQMV	2/6	SEQAAEAMEV ¹	B44	/	/
H7-M33		----- S - A -----		AAEAMEVA ¹	B7		
H1-M35	209~227	TRQMVHAMRTIGTHPSSSA	1/6	/	/	/	/
H7-M35		A ----- Q ----- V ----- N - T					
H1-M39 ^{d)}	238~252	QAYQKRMGVQMQRFK	1/6	AYQKRMGVQM ¹	A11	YQKRMGVQMQRFK ¹	HLA-DQ1
H7-M39		----- N ----- I -----					

a) 统计总结先前已报道的表位, 1代表来源于H1N1, 7代表来源于H7N9; b) 对应该确定多肽表位的HLA型; (c) II类T细胞多肽表位在多肽H1-M8和H1-M9发生重叠; d) 与2009 pH1N1流感病毒相比, H7N9特异性肽序列中的突变位点以粗体和下划线标出

图 3 M1蛋白突变长肽的免疫原性比较. 该数据使用平均值±SEM (平均值的标准误差), 使用ANOVA比较统计对照组和突变长肽(H1-M8, H1-M9, H1-M17, H1-M18, H1-M23, H1-M24, H1-M33, H1-M35和H1-M39)之间的差异. *, $P<0.05$; **, $P<0.01$

Figure 3 T-cell responses to the mutant overlapping peptides derived from 2009 pH1N1 M1. Data are shown as means $\pm$ SEM (standard errors of the means), the differences among mock and overlapping peptides (H1-M8, H1-M9, H1-M17, H1-M18, H1-M23, H1-M24, H1-M33, H1-M35 and H1-M39) were compared using ANOVA. *, $P<0.05$; **, $P<0.01$

研究证明其他流感病毒毒株感染产生的记忆性T淋巴细胞识别H7N9时依赖于不同种族的不同HLA I类等位基因^[26]. 通过对异源亚型流感病毒保守的优势免疫表位如M1蛋白表位的识别应答可以产生一定的

免疫保护^[13,27]. 然而, 很少有研究关注M1蛋白突变区段表位的取代如何影响异源亚型T细胞免疫应答和流感病毒免疫逃逸. 正如先前的结构研究所示, T细胞表位内的氨基酸突变有助于源自不同流感病毒株的表位之间的抗原产生差异^[12,28,29]. 在本研究中, 我们鉴定出了2009 pH1N1来源的H1-M42, H1-M102, H1-M202s和H1-M244 4条T细胞表位, 4条多肽均来源于M1蛋白突变区.

本研究发现, 健康人群中预存一定水平的针对H7N9和2009 pH1N1的非保守T细胞表位的免疫应答. 同时观察到H7N9的氨基酸残基突变导致T细胞免疫反应降低, 其在异源亚型流感免疫逃逸中起到重要作用. 我们研究发现, 在H7N9M1蛋白突变区段中, 其T细胞表位H1-M42仅第四位氨基酸Trp突变为Ile, 导致了T细胞免疫反应的显著下降. 而其他表位, 即使多个氨基酸位点发生突变如H7-M202, 仍可引起较强的交叉免疫反应. 本研究验证了H7N9和2009 pH1N1特异性表位之间的T细胞抗原变异主要是由MHC/表位结合力的差异和/或TCR识别差异造成, 为理解流感病毒不同亚型之间T细胞免疫原性差异的分子机制提供了参考. 同时, 考虑到少数氨基酸的突变会完全改变肽的免疫原性,

表 3 本次研究中所用短肽信息

Table 3 Characteristics of the predicted peptides used in this study

名称	位置	来源	序列	HLA 分型	得分 ^{a)}	复性 ^{b)}
H1-M102	102-113	H1-M18	KLKREITFHGAK	A*1101	0.0265590	+
H7-M102	102-113	H7-M18	-----M-----	A*1101	0.0230080	+
H1-M244	244-252	H1-M39	MGVQMQRFK	A*1101	0.0584090	+
H7-M244	244-252	H7-M39	----L----	A*1101	0.0705220	+
H1-M42	42-49	H1-M8	LMEWLKTR	A*3301	0.0273900	+
H7-M42	42-49	H7-M8	---I----	A*3301	0.0389110	+
H1-M202s	202-210	H1-M33	AMEVANQTR	A*3301	0.0488980	+
H7-M202s	202-210	H7-M33	-----S-A-	A*3301	0.0610020	+
H1-M45s	45-52	H1-M9	WLKTRPIL	A2	0.0017820	-
H7-M45s	45-52	H7-M9	I-----	A2	0.0029750	-
H1-M107	107-117	H1-M18	ITFHGAKEVSL	A2	0.0100740	-
H7-M107	107-117	H7-M18	M-----A	A2	0.0027310	-
H1-M139	139-147	H1-M23	TTEAAFGLV	A2	0.0039870	-
H7-M139	139-147	H7-M23	-A-G-L---	A2	0.0020070	-
H1-M202	202-212	H1-M33	AMEVANQTRQM	A2	0.0015840	-
H7-M202	202-212	H7-M33	-----S-A---	A2	0.0008670	-

a) 结合力通过<http://www.cbs.dtu.dk/services/NetMHCpan/>预测. b) 可以通过体外复性证明结合的用“+”表示, 其他用“-”表示

图 4 通过ELISPOT法鉴定健康人群PBMC的特异性短肽。斑点数目是用来衡量多肽刺激的PBMCs的分泌IFN- γ 的量。蓝色柱代表是2009 pH1N1来源的短肽，而绿色柱代表H7N9来源的短肽。柱状图代表来自多个独立捐赠者的SFC的平均数量，并且它们都去除了阴性对照组数值。该数据表示为平均值 $\pm$ SEM，用 T 检验比较两组之间的差异。*， $P < 0.05$

Figure 4 Identification of candidate peptides with the PBMCs of healthy donors by ELISPOT assay. The spots are a measure of IFN- γ secretion from PBMCs stimulated with various candidate peptides. The blue columns are peptides derived from 2009 pH1N1, while the green columns are peptides derived from H7N9. Bars represent the mean number of SFCs from independent donors, and all of them cut off the value of mock. Values are expressed as means $\pm$ SEM. T test was used for the statistical analyses. *, $P < 0.05$

图 5 通过体外复性验证T细胞表位与MHC分子的结合。通过体外复性证明多肽H1-M42/H7-M42和M202s/H7-M202s可以在HLA-A*3303重链和轻链帮助下实现体外复性((a), (b))。H1-M102/H7-M102和H1-M244/H7-M244可以与HLA-A*1101结合((c), (d))。复性后，正确折叠的MHC I类分子在SuperdexTM Increase 200 10/300 GL柱上吸光度峰值出现在16 mL左右，预测的分子量为45 kD。该分子量标准标记的近似位置为75.0, 44.0和13.7 kD。波峰代表聚集的重链，正确复性的MHC I复合物(45 kD)和多余的 β_2 m(d)。基于已有HLA-A*1101 (PDB:1q94) 呈递H1-M244多肽(e)和呈递H1-M244, H7-M244(f)的模拟结构

Figure 5 The identification of T cell epitopes binding to HLA-A3. Binding of peptides H1-M42/H7-M42 and H1-M202s/H7-M202s to HLA-A*3303 was elucidated by *in vitro* refolding ((a), (b)). Peptides H1-M102/H7-M102 and H1-M244/H7-M244 could refold with the HLA-A*1101 chain and β_2 m ((c), (d)). After properly refolding, the high-absorbance peaks of the correctly refolded MHC I with the expected molecular mass of 45 kD eluted at the estimated volume of 16 mL on a SuperdexTM Increase 200 10/300 GL column. The profile is marked with the approximate positions of the molecular mass standards of 75.0, 44.0, and 13.7 kD. Peaks represent the aggregated heavy chain, the correctly refolded Ptal-N*01:01 complex (45 kD), and the extra β_2 m, respectively(d). Structure-based model of HLA-A*1101 (PDB Code:1q94) binding peptide H1-M244 (e) and H1-M244 compared with H7-M244 (f)

流感病毒主要T细胞免疫原如M1, NP和PB1的抗原变异性研究在通用疫苗的研发中至关重要，因此本研究

也为目前流感病毒通用疫苗的研发提供了一定的科学参考。

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补充材料

表S1 在2009 pH1N1和H7N9中M1蛋白的非保守肽库构建

本文以上补充材料见网络版csb.scichina.com. 补充材料为作者提供的原始数据, 作者对其学术质量和内容负责.

Summary for “H1N1流感病毒M1蛋白表位筛选及其在禽流感病毒交叉免疫中的作用机制”

Identification of H1N1 influenza virus-derived T-cell epitopes and the contribution in the cross-reactivities to avian influenza virus

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Influenza viruses, such as 2009 pandemic A(H1N1) influenza virus (2009 pH1N1) and avian influenza A (H7N9) virus (H7N9), which spread across the species, pose a great threat to human health and cause socioeconomic losses. Since 2009, circulation of 2009 pH1N1 has become a seasonal influenza virus. The sustaining epidemics have resulted in certain T-cell immune level among healthy populations.

T-cell epitopes are mainly derived from conserved internal proteins of influenza virus compared with B-cell epitopes, influenza virus-specific cross-reactive CD4⁺ and CD8⁺ T-cells broadly exist among the population, providing protection from subsequent infections by heterotypic viruses. Cytotoxic T lymphocytes (CTLs) specific for influenza A viruses mostly target internal, nonglycosylated proteins including M1, which are enriched with immunodominant CTL epitopes and markedly conserved among diverse strains compared to HA and NA. In comparison to M1 protein of 2009 pH1N1, the H7N9 M1 has 4 segments with clustering substitutions. In this study, we defined the cross-reactivity immunity between 2009 pH1N1 and H7N9 and identified four T-cell epitopes: H1-M42 (LMEWLKTR), H1-M102 (KLKREITFHGAK), H1-M202s (AMEVANQTR) and H1-M244 (MGVQMQRFK) in the mutant segments in 2009 pH1N1. T-cell responses were investigated using freshly isolated PBMCs from individuals ($n = 16$) through IFN- γ ELISPOT with these peptides derived from influenza virus M1 protein that are not conserved between 2009 pH1N1 and H7N9. We investigated the baseline of pre-existing immunity targeting M1 mutant segments of avian H7N9 influenza virus compared to 2009 pH1N1 among a healthy population. There was a certain level of T-cell immunity to H7N9 in the healthy population, but still weaker than that to 2009 pH1N1. Peptides derived from H7N9 which had mutations induced significant lower T-cell responses. The limited pre-existing T-cell immunity against H7N9 in healthy populations was partially contributed by H7N9 amino acid mutations in novel identified epitopes. Furthermore, we found that H1-M102 and H1-M244 could bind with HLA-A*1101 stably after renaturation *in vitro*, while the binding of H1-M42 and H1-M202s with HLA-A*3303 were relatively weak. Combined refolding and functional studies based on T-cell epitopes derived from influenza virus illustrated that minor mutation of an epitope can lead to a profound effect on the antigenicity of the peptide, which may also influence both HLA binding and TCR docking. Our study on T-cell immunity against influenza viruses provides an important reference for understanding the preexisting immune responses to avian influenza virus, and benefits the development of universal influenza vaccine. At the same time, considering that a few amino acid mutations will change the immunogenicity of the peptide completely, the study of antigenic variability of influenza virus major T cell immunogens such as M1, NP and PB1 are crucial in the development of universal vaccines.

2009 pH1N1, H7N9, antigenic variation, cross-reactivity, T-cell epitopes

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