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Combined synthetic and recombinant techniques for the development of lipoprotein-based, self-adjuvanting vaccines targeting human papillomavirus type-16 associated tumors

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ABSTRACT

Human papillomaviruses (HPVs) are associated with various cancers, with HPV16 linked to more than half of cervical cancer cases. Vaccines to prevent HPV infection and cancer development have proven effective, but are not useful in individuals with prior HPV exposure. Treatment vaccines to eradicate or control HPV-associated lesions are therefore desirable for these patients. Herein we describe the development of a process to enable the production of semisynthetic vaccines based on the site-specific attachment of synthetic bacterial lipid analogs (e.g. Pam2Cys) to a non-oncogenic mutant HPV16 E7 protein to generate molecularly defined vaccines. Many cytotoxic lymphocyte (CTL) epitopes from E7 are delivered by this approach; potentially ensuring that large numbers of immunized individuals can generate CTLs to clear HPV infected cells. Delivery of this construct reduced the growth of HPV16-associated tumors in a TC1 mouse model, the effects of which were better than the potent CTL epitope HPV16 E7(44-57) administered with Montanide ISA51 adjuvant.

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Human papillomavirus (HPV) is the causative agent for cervical cancer, the third most common female cancer, and various other cancers (e.g. certain head and neck tumors).¹ This association has inspired research into vaccines to prevent HPV infection, culminating in the commercialization of L1-based virus-like particles (VLPs; e.g. Gardasil, Merck & Co and Cervarix, GSK). Despite their success, these vaccines are ineffective in individuals who have had prior HPV infection.¹ Vaccines to eradicate or control HPV-associated lesions are therefore of interest, with therapeutic vaccines that enhance T cell-mediated killing of HPV infected cells (expressing E6 and E7 HPV oncogenes) the subject of human trials (reviewed in references 1 and 2C).

Many groups¹⁻² have investigated therapeutic HPV peptide vaccines, with most focused on E7-derived peptides from HPV type 16 (HPV16), which causes over 50 % of cervical cancers.¹ Several cytotoxic lymphocyte (CTL) epitopes have been mapped from the E7 protein (see Fig. 1A; CTL epitopes marked with a line over the sequence),³ which have controlled or eradicated HPV16-associated tumors in mouse^{2a, 2b} and human trials.⁴ Using these epitopes, simple, chemically defined synthetic vaccines can

be produced.¹ The simplification of proteins down to defined peptide antigens however reduces the vaccine CTL epitope mix, limiting efficacy to individuals expressing human leukocyte antigen (HLA) molecules that bind these epitopes. The utility of such vaccines could therefore be improved by increasing the number of CTL epitopes. Methods to achieve this include mixing overlapping peptides covering the entire HPV16 E6 and E7 sequences (known as “synthetic long peptides”),⁵ or selected epitopes from these proteins.⁴ These approaches however greatly increase vaccination costs due to the need to synthesize and purify multiple peptides.

Recombinant expression of engineered proteins provides one means to overcome these difficulties. Full-length or truncated E6 and E7 proteins, and E6-E7 fusions,¹ that have been mutated to remove their oncogenic potential, have the capacity to be overexpressed at lower cost compared with synthetic approaches incorporating multiple peptide antigens, and include significantly more CTL epitopes. The low immunogenicity of these proteins however requires their delivery with adjuvants,^{1a} of which there are very few known to be capable of stimulating potent CTL responses in humans without being overly toxic.

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ASSRVV-IHHHHH-GSG-LVPRGM-HGDDPTLHEYMLDLPGETTILYQYGLNDSESEEDIDGPAQGAEP
 1-12 (A*01) 11-20 (A*02) 21-30 (A*03) 31-40 (A*04) 41-50 (A*05) 51-60 (A*06) 61-70 (A*07) 71-80 (A*08) 81-90 (A*09) 91-100 (A*10) 101-110 (A*11) 111-120 (A*12) 121-130 (A*13) 131-140 (A*14) 141-150 (A*15) 151-160 (A*16) 161-170 (A*17) 171-180 (A*18) 181-190 (A*19) 191-200 (A*20) 201-210 (A*21) 211-220 (A*22) 221-230 (A*23) 231-240 (A*24) 241-250 (A*25) 251-260 (A*26) 261-270 (A*27) 271-280 (A*28) 281-290 (A*29) 291-300 (A*30) 301-310 (A*31) 311-320 (A*32) 321-330 (A*33) 331-340 (A*34) 341-350 (A*35) 351-360 (A*36) 361-370 (A*37) 371-380 (A*38) 381-390 (A*39) 391-400 (A*40) 401-410 (A*41) 411-420 (A*42) 421-430 (A*43) 431-440 (A*44) 441-450 (A*45) 451-460 (A*46) 461-470 (A*47) 471-480 (A*48) 481-490 (A*49) 491-500 (A*50) 501-510 (A*51) 511-520 (A*52) 521-530 (A*53) 531-540 (A*54) 541-550 (A*55) 551-560 (A*56) 561-570 (A*57) 571-580 (A*58) 581-590 (A*59) 591-600 (A*60) 601-610 (A*61) 611-620 (A*62) 621-630 (A*63) 631-640 (A*64) 641-650 (A*65) 651-660 (A*66) 661-670 (A*67) 671-680 (A*68) 681-690 (A*69) 691-700 (A*70) 701-710 (A*71) 711-720 (A*72) 721-730 (A*73) 731-740 (A*74) 741-750 (A*75) 751-760 (A*76) 761-770 (A*77) 771-780 (A*78) 781-790 (A*79) 791-800 (A*80) 801-810 (A*81) 811-820 (A*82) 821-830 (A*83) 831-840 (A*84) 841-850 (A*85) 851-860 (A*86) 861-870 (A*87) 871-880 (A*88) 881-890 (A*89) 891-900 (A*90) 901-910 (A*91) 911-920 (A*92) 921-930 (A*93) 931-940 (A*94) 941-950 (A*95) 951-960 (A*96) 961-970 (A*97) 971-980 (A*98) 981-990 (A*99) 991-1000 (A*100) 1001-1010 (A*101) 1011-1020 (A*102) 1021-1030 (A*103) 1031-1040 (A*104) 1041-1050 (A*105) 1051-1060 (A*106) 1061-1070 (A*107) 1071-1080 (A*108) 1081-1090 (A*109) 1091-1100 (A*110) 1101-1110 (A*111) 1111-1120 (A*112) 1121-1130 (A*113) 1131-1140 (A*114) 1141-1150 (A*115) 1151-1160 (A*116) 1161-1170 (A*117) 1171-1180 (A*118) 1181-1190 (A*119) 1191-1200 (A*120) 1201-1210 (A*121) 1211-1220 (A*122) 1221-1230 (A*123) 1231-1240 (A*124) 1241-1250 (A*125) 1251-1260 (A*126) 1261-1270 (A*127) 1271-1280 (A*128) 1281-1290 (A*129) 1291-1300 (A*130) 1301-1310 (A*131) 1311-1320 (A*132) 1321-1330 (A*133) 1331-1340 (A*134) 1341-1350 (A*135) 1351-1360 (A*136) 1361-1370 (A*137) 1371-1380 (A*138) 1381-1390 (A*139) 1391-1400 (A*140) 1401-1410 (A*141) 1411-1420 (A*142) 1421-1430 (A*143) 1431-1440 (A*144) 1441-1450 (A*145) 1451-1460 (A*146) 1461-1470 (A*147) 1471-1480 (A*148) 1481-1490 (A*149) 1491-1500 (A*150) 1501-1510 (A*151) 1511-1520 (A*152) 1521-1530 (A*153) 1531-1540 (A*154) 1541-1550 (A*155) 1551-1560 (A*156) 1561-1570 (A*157) 1571-1580 (A*158) 1581-1590 (A*159) 1591-1600 (A*160) 1601-1610 (A*161) 1611-1620 (A*162) 1621-1630 (A*163) 1631-1640 (A*164) 1641-1650 (A*165) 1651-1660 (A*166) 1661-1670 (A*167) 1671-1680 (A*168) 1681-1690 (A*169) 1691-1700 (A*170) 1701-1710 (A*171) 1711-1720 (A*172) 1721-1730 (A*173) 1731-1740 (A*174) 1741-1750 (A*175) 1751-1760 (A*176) 1761-1770 (A*177) 1771-1780 (A*178) 1781-1790 (A*179) 1791-1800 (A*180) 1801-1810 (A*181) 1811-1820 (A*182) 1821-1830 (A*183) 1831-1840 (A*184) 1841-1850 (A*185) 1851-1860 (A*186) 1861-1870 (A*187) 1871-1880 (A*188) 1881-1890 (A*189) 1891-1900 (A*190) 1901-1910 (A*191) 1911-1920 (A*192) 1921-1930 (A*193) 1931-1940 (A*194) 1941-1950 (A*195) 1951-1960 (A*196) 1961-1970 (A*197) 1971-1980 (A*198) 1981-1990 (A*199) 1991-2000 (A*200) 2001-2010 (A*201) 2011-2020 (A*202) 2021-2030 (A*203) 2031-2040 (A*204) 2041-2050 (A*205) 2051-2060 (A*206) 2061-2070 (A*207) 2071-2080 (A*208) 2081-2090 (A*209) 2091-2100 (A*210) 2101-2110 (A*211) 2111-2120 (A*212) 2121-2130 (A*213) 2131-2140 (A*214) 2141-2150 (A*215) 2151-2160 (A*216) 2161-2170 (A*217) 2171-2180 (A*218) 2181-2190 (A*219) 2191-2200 (A*220) 2201-2210 (A*221) 2211-2220 (A*222) 2221-2230 (A*223) 2231-2240 (A*224) 2241-2250 (A*225) 2251-2260 (A*226) 2261-2270 (A*227) 2271-2280 (A*228) 2281-2290 (A*229) 2291-2300 (A*230) 2301-2310 (A*231) 2311-2320 (A*232) 2321-2330 (A*233) 2331-2340 (A*234) 2341-2350 (A*235) 2351-2360 (A*236) 2361-2370 (A*237) 2371-2380 (A*238) 2381-2390 (A*239) 2391-2400 (A*240) 2401-2410 (A*241) 2411-2420 (A*242) 2421-2430 (A*243) 2431-2440 (A*244) 2441-2450 (A*245) 2451-2460 (A*246) 2461-2470 (A*247) 2471-2480 (A*248) 2481-2490 (A*249) 2491-2500 (A*250) 2501-2510 (A*251) 2511-2520 (A*252) 2521-2530 (A*253) 2531-2540 (A*254) 2541-2550 (A*255) 2551-2560 (A*256) 2561-2570 (A*2

To improve E7m protein expression, the E7m gene was codon optimized for B strain *E. coli* using DNAworks v3.2.2 (<http://helixweb.nih.gov/dnaworks/>) with a codon frequency table from the codon usage database (<http://www.kazusa.or.jp/codon/>). This gene was fused to: i) a promiscuous T helper epitope (PADRE1024.03)¹⁰ to help increase the potency of CTL responses against HPV16 E7;¹¹ and ii) the *Mxe* GyrA (N198A) intein-chitin-binding domain (CBD) fusion¹² from pTXB1 (NEB) (1; Fig. 1A; Table S1) to enable the incorporation of a C-terminal thioester modification. This gene overexpressed in *E. coli* to yield a large quantity of **1** as an insoluble inclusion body pellet (Fig. 1B, lane 4), which could be enriched by simple and inexpensive phosphate buffered saline (PBS) washing steps (Fig. S2A). The active intein was subsequently regenerated by solubilizing the inclusion bodies under denaturing conditions (8 M urea, 25 mM HEPES pH 7.5, 1 mM EDTA, 0.5 M NaCl, 1 mM TCEP), followed by dialysis against 50-volumes of a non-denaturing buffer (25 mM HEPES pH 7.5, 1 mM EDTA, 0.5 M NaCl, 1 mM TCEP) at 4 °C overnight for urea removal and intein refolding. The intein was then cleaved by adding ethanethiol to 3 % v/v, and incubating overnight at RT, followed by reversed-phase high-performance liquid chromatography (RP-HPLC) to yield the stable ethyl α -thioester **2** (Fig. 1A,C; Fig. S2B; > 10 mg/L culture) without any need for an expensive immobilized metal affinity chromatography (IMAC) enrichment step.

Figure 1. Inactivated HPV16 E7 (E7m) proteins **1-3**. (A) E7m sequence; CTL epitopes (line over) and HLA restriction; inactivating (bold) and cysteine mutations (italics); and conversion of intein fusion protein **1** to thioester proteins **2** and **3**. (B) SDS-PAGE of **1** protein expression (whole cell uninduced (UN) and induced (IN) bacteria; soluble cytoplasmic (SOL) and inclusion body pellet (PEL)). (C) RP-HPLC monitoring of *Mxe* GyrA intein-CBD cleavage from **1** with EtSH to yield **2**. (D) Analytical RP-HPLC and (E) electrospray ionization-mass spectrometry (ESI-MS) data (deconvoluted spectra inset) for pure E7m thioester protein **3**.

In order to incorporate Pam2Cys into the E7m lipoprotein vaccine **5**, a Pam2Cys adjuvant peptide **4** (Fig. 2A; Fig. S1) was assembled by microwave-assisted 9-fluorenylmethyloxycarbonyl (Fmoc)-solid-phase peptide synthesis (SPPS) in 24 % overall yield (see supplementary data).^{7b} This peptide incorporated: a PEG linker, to increase its aqueous solubility; a dendritic cell (DC) targeting peptide (DCpep: FYPSYHSTPQRP);¹³ and an N-terminal cysteine to enable its conjugation to the E7m thioester protein **3** using the native chemical ligation (NCL) reaction.¹⁴ The Pam2Cys adjuvant was attached during SPPS through the side-chain of a C-terminal lysine residue by coupling a *S*-(2,3-dihydroxypropyl)-*N*-[(9*H*-fluoren-9-ylmethoxy)carbonyl]-L-cysteine (Fmoc-Dhc-OH) building block, followed by palmitoylation of the free thioglycerol hydroxyl groups, and an Fmoc-deprotection step using 2.5 % v/v DBU in DMF.¹⁵

To ensure site-specific conjugation of Pam2Cys-peptide **4** and the E7m protein **3**, a NCL¹⁶ reaction was used. This reaction requires two unprotected polypeptide species, one containing a C-terminal thioester (the E7m protein **3**), and the other an N-terminal cysteine (Pam2Cys-peptide **4**). Simple mixing of these compounds in aqueous buffers at neutral pH leads to their conjugation through a peptide bond. The aforementioned conversion of **1** to the ethyl α -thioester **2** yielded a highly stable thioester species. This had the advantage of being stable at -20 °C for at least 1 year without hydrolysis of the thioester, but was not reactive enough to generate the E7m-Pam2Cys lipoprotein **5**. In order to link **4** and **2**, the ethyl α -thioester **2** was converted to the more reactive ethanesulfonyl α -thioester **3** (Fig. 1A,D,E; Fig. S2C; 85 % yield) immediately prior to the reaction in order to improve the reaction rate. This was achieved by incubating **2** (2 mg/mL) in 6 M guanidine-HCl, 0.1 M NaPi pH 7.5, 0.1 M NaCl, 0.1 M 2-mercaptoethanesulfonate sodium (MESNa) for 2 h at 37 °C, followed by RP-HPLC purification. This conversion also resulted in a leftwards shift in the RP-HPLC retention time (Fig. S2B,C), thereby providing an additional purification step, which helped to ensure that **3** lacked any underlying impurities.

To ensure the solubility of each reaction component, the NCL reaction was performed under denaturing conditions (6 M guanidine; pH 7.5) in the presence of 20 % (v/v) acetonitrile. This was essential since the E7m ethanesulfonyl α -thioester protein **3** and the lipopeptide adjuvant **4** proved difficult to dissolve in PBS at sufficient concentration (> 1 mg/mL) to provide fast NCL reactions. To further accelerate the reaction: 4-mercaptophenylacetic acid¹⁷ (MPAA; to 30 mM) was added, which generates a highly reactive thioester population *in situ*; an excess (~ 4-5 eq) of the lipid adjuvant **4** was employed; and the reaction was heated (37 °C), leading to reactions that were essentially complete by 3 h (Fig. 2C). The product was purified by RP-HPLC to give the E7m lipoprotein **5** in high yield (89 %) and purity (Fig. 2D,E; Fig. S3).

To test the capacity of **5** to reduce the growth of HPV16-associated tumors, the TC-1 HPV16 tumor model¹⁸ was used. This involved subcutaneous administration of TC-1 cells (1 × 10⁵/mouse; C57BL/6 lung epithelial cells transformed with HPV16 E6 and E7, and the *c-Ha-ras* oncogenes)¹⁸ into the right flank of C57BL/6 mice (8/group). The mice were then rested to enable the formation of a solid tumor, and on day 3 received a single subcutaneous injection at the tail base with 25 μ g of **5** in 100 μ L phosphate buffered saline (PBS). No subsequent boosts were administered as part of this experiment. As a negative control, mice were immunized with PBS vehicle alone (100 μ L) to enable the rate of tumor growth to be assessed against non-treated mice. An additional group was immunized with a synthetic lipopeptide vaccine **6** (Fig. 3A; E7₄₄₋₅₇/E6₄₃₋₅₇-

Pam2Cys; 100 μ g in 100 μ L PBS), which incorporates two HPV16 CTL antigens: E7₄₄₋₅₇^{2b} (QAEPDRAHYNIIVTF; IEDB Epitope ID: 111662), and E6₄₃₋₅₇⁹ (QLLRREYDFAFRDL; IEDB Epitope ID: 111677) fused to Pam2Cys as an adjuvant. For each group, tumor growth was measured every two days using calipers, with mice sacrificed when the measured tumor volume was greater than 1 cm³ or where tumor bleeding was observed.

Figure 3. Structures of (A) Pam2Cys fused lipopeptide vaccine **6**; (B) the E7₄₄₋₅₇ positive control peptide **7**; and (C) star-polymer **8**, which incorporate the HPV16 E7₄₉₋₅₇ CTL epitope, and for **6** the HPV16 E6₄₉₋₅₇ CTL epitope in addition.

For the initial experiment, tumor growth was measured until the first mouse from any group was sacrificed (day 24). From this experiment, it was observed that mice treated with E7m-Pam2Cys **5** or E7₄₄₋₅₇/E6₄₃₋₅₇-Pam2Cys **6** demonstrated reduced rates of TC-1 tumor growth compared to non-treated (PBS) mice ($P < 0.001$; Fig. 4A; individual mouse TC-1 tumor growth data supplied as Fig. S4). Of these groups, **5** demonstrated the smallest average tumor volume on day 24, with **6** proving less effective (Fig. 4A). This promising preliminary data suggested that incorporating the E7m protein, rather than one or two defined CTL epitopes, could improve the efficacy of treatment vaccines targeting HPV16-associated tumors. This finding was further strengthened by the 12-fold lower dose (by mol) of **5** that was administered in this experiment compared to **6**. The likely explanation for this greater efficacy is due to lipoprotein **5** delivering more CTL and T helper epitopes compared to lipopeptide **6**, as both constructs include Pam2Cys as an adjuvant as well as the potent E7₄₉₋₅₇ CTL epitope.

Based on the promising preliminary data (Fig. 4A), a second experiment (Fig. 4B,C) was performed to compare lipoprotein **5** with a synthetic HPV16 E7₄₄₋₅₇^{2b} peptide **7** (Fig. 3B; 30 μ g/mouse) emulsified in 1:1 Montanide ISA51/PBS (100 μ L/mouse; 7/ISA51) as a positive control, and PBS vehicle alone (100 μ L/mouse) as a negative control. The Montanide ISA51 adjuvant used in this experiment has been successfully applied as an adjuvant in human trials of synthetic HPV16 E6 and E7 derived peptides, and has been demonstrated to induce CTL-mediated immune responses.⁵ A star-polymer **8** (Fig. 3C; S4-8Qmin; 100 μ g/mouse, equivalent to 30 μ g of **7**/dose; incorporates four copies of **7**), which was previously demonstrated to control TC-1 tumor growth (when administered 3 days after challenge with 1×10^5 TC-1 cells/mouse) was used as an additional control. Each of the components of this polymer (the E7₄₄₋₅₇ peptide **7** and the S4 polymer) have been demonstrated to have no effect on tumor growth unless they are conjugated together (e.g. **8**; S4-8Qmin), or administered with a suitable adjuvant (e.g. Montanide ISA51) in the case of E7₄₄₋₅₇ (**7**).^{2b}

For this second experiment, more stringent tumor model conditions were employed compared to the preliminary study. These included doubling the number of implanted TC-1 cells to 2×10^5 /mouse, and administering treatments on day seven instead of three, which resulted in larger, more difficult to shrink or eradicate tumors by the time treatments were administered. These changes were performed in order to increase the observed differences in treatment efficacy between groups. In addition to these changes, the number of mice per group was increased to 10; two different doses of **5** were assessed (25 and 5 μ g; Fig. S5); and the tumor growth was measured over a 60-day period. As per the preliminary experiment, mice were sacrificed when the measured tumor volume was greater than 1 cm³ or where tumor bleeding was observed.

A comparison of the average tumor burden (Fig. 4B; Fig. S5B; individual mouse TC-1 tumor growth data supplied as Fig. S6) and length of survival (Fig. 4C; Fig. S5A) in each group demonstrated that tumor growth was most rapid in the non-treatment (PBS) group. All ten mice in the PBS group were sacrificed by day 41 (Fig. 4C). Mice in each treatment group exhibited prolonged survival and reduced rates of TC-1 tumor growth compared to non-treated mice ($P < 0.05$), with the 25 μ g dose of lipoprotein **5** proving superior to the 5 μ g dose based on the rate of tumor growth (Fig. S4). No statistical significance ($P = 0.44$) was observed when comparing survival curves for polymer **8** and the lipoprotein **5** (25 μ g) group. At day 60, three mice survived in the polymer **8** group, and one in each of the lipoprotein **5** groups (Fig. 4C; Fig. S4A). In comparison, all mice were sacrificed by day 50 in the 7/ISA51 group (Fig. 4C).

As polymer **8** demonstrated significant protection against TC-1 tumor challenge in previous experiments (60 % survival on day 60), where less challenging conditions were employed (immunization on day 3; 1×10^5 TC-1 cells/mouse),^{2b} and the survival and tumor growth curves did not show significant differences between mice administered lipoprotein **5** (25 μ g) and polymer **8** in the current experiments (Fig. 4B,C), this data supports the potential for **5** to stimulate immune responses useful for controlling HPV16-associated tumors. Further, the 25 μ g dose of lipoprotein vaccine **5** contains 10-fold lower E7₄₄₋₅₇ antigen (by mol) compared to the administered doses of 7/ISA51 and the S4-8Q polymer **8** (which each delivered 30 μ g of antigen **7**). Based on the observed dose dependence, this suggests that further increases in efficacy may be afforded through increasing the dose of **5**, or administering boosts. The incorporation of a

non-oncogenic mutant of the HPV16 E6 protein,¹ or combined therapy with appropriately timed and dosed chemotherapy (e.g. cisplatin)¹⁹ represent additional means to increase the therapeutic effect of **5**.

Figure 4. Prolonged survival of TC-1 tumor bearing mice in response to therapeutic vaccination. (A) Tumor volume in C57BL/6 mice (8/group) challenged with 1×10^5 TC-1 cells/mouse (day 0), and immunized on day 3 with E7m-Pam2Cys **5**, or E7₄₄₋₅₇/E6₄₃₋₅₇-Pam2Cys **6**; (B) Tumor volume and (C) Kaplan-Meier survival curve (60 days) in C57BL/6 mice (10/group) challenged with 2×10^5 TC-1 cells/mouse (day 0), and immunized on day 7 with E7m-Pam2Cys **5**, 7/ISA51, or polymer **8**. Tumor volume is plotted until the first mouse from any group reached the endpoint. Down arrows in (A) and (B) indicate the day of tumor injection. * $P < 0.05$, *** $P < 0.001$.

Overall, this study has provided a method for the simple, high yielding, and site-specific incorporation of a synthetic Pam2Cys adjuvant onto the C-terminus of engineered recombinant HPV16 E7 proteins to generate chemically defined candidate lipoprotein vaccines for the treatment of HPV16-associated tumors. The

construct that was generated exhibited self-adjuvanting activity, and could stimulate immune responses that provided similar control of HPV16-associated tumor growth to a previously described successful therapeutic HPV16 polymer vaccine, which incorporates four copies of the HPV16 E7₄₄₋₅₇ antigen.^{2b} This polymer cannot be fully defined, and may therefore prove more difficult to commercialize when compared with the chemically defined lipoprotein vaccines produced herein. Further, the incorporation of a non-oncogenic, full-length HPV16 E7 protein enables the delivery of a variety of CTL epitopes, which are capable of binding different HLA molecules, offering the promise of therapeutic activity in a greater proportion of the population than a peptide-based approach.

Acknowledgments

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Supplementary Data

Supplementary data (detailed expression protocols for **1**; methods for generating thioester proteins **2** and **3**; synthesis of adjuvant peptide **4** and lipoprotein vaccine **5**; characterization data for **1-5**; and immunization protocols) associated with this article can be found in the online version, at <insert reference here>.

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