Supplemental materials

Ethics Statement

Mouse studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animal studies were performed with the prior approval of the Animal Care and Use Committee of Johns Hopkins University (protocol MO08M19).

Rabbit studies were performed according to the quality service and ethical treatment policy of Covance Inc. All Covance programs are AAALAC International accredited, and meet or exceed USDA Research License requirements, as well as those of the "Guide for Care and Use of Laboratory Animals". All animal studies are performed with the prior approval of the Institutional Animal Care and Use Committee.

Conventional in-vitro neutralization method

293TT cells were seeded at 1.5 x 10^4 per well in a flat bottom 96-well cell culture plate with neutralization medium (DMEM without phenol red, 10% FBS, 1% non-essential amino acids, 1% GlutaMax, 10mM HEPES) and incubated at 37^{o}C culture 5% CO_2 incubator overnight. Antisera were serially diluted with the neutralization medium in a 96-well plate, and 50 µL of diluted serum was mixed with 50 µL of PsV working solution in a round bottom 96-well plate and incubated at 37^{o}C for 2 hr. The entire volume was transferred to corresponding well of the plate which was seeded with 293TT cells a day before. Plate was returned to the incubator for 67 hr, and the culture supernatant was analyzed for the presence of SEAP Chemilumimescence kit (Clontech laboratories, CA). The in-vitro neutralization titer was defined as the reciprocal of the highest dilution of serum that reduces the SEAP activity by half[1].

Cloning of recombinant Fla-L2 fusions

HPV L2 sequences derived from HPV6B, 16, 18, 31, 39, 51, 56, and 73 were codon optimized for *E. coli* expression by construction using synthetic DNA oligonucleotides (Blue Heron Biotechnology, Bothell WA). Flagellin B gene (Gene Bank accession# NP_461698) was amplified by PCR from genomic DNA purified from *Salmonella typhimurium LT2* (ATCC, Manassas, VA). The Fla only construct was generated by replacement of flagellin B domain D3 region (aa188-296) with a multicloning site linker GGSGGSPRGTGSEFGSGGS. Fla~1x11-200 was constructed by replacement of domain D3 with HPV16 L2 aa 11-200 via recombinant PCR. A synthetic concatamer containing the coding sequence for L2 aa11-88 from multiple HPV types was fused to the C-terminus of Fla-62, resulting in Fla~5x11-88 (5 HPV types in order as 6B-16-18-31-39) or Fla~8x11-88 (8 HPV types as 6B-16-18-31-39-51-56-73) as described in Jagu et al, [2]) excepting that triplet-glycine spacers were inserted at each of the type-to-type junctions. A histidine tag was added at the C-terminus of Fla~5x11-88 and Fla~8x11-88. All constructs were cloned into pET24a+ vector between NdeI and XhoI restriction sites and expressed in BLR(DE3) cells (Novagen, San Diego, CA.). The details of the bacterial expression and purification are provided in the supplemental materials section.

Generation of HPV pseudoviruses (PsV)

Pseudoviruses were generated as previously described[3]. Briefly, plasmid double expression vectors for codon-optimized L1 and L2 genes were transfected with either a luciferase or alkaline phosphatase (SEAP) reporter gene plasmid into 293TT cells[3]using lipofectamin 2000 transfection reagent (Life technologies, CA). Three days after transfection, cell pellets were collected and rinsed with DMEM and Dulbecco's PBS (DPBS; Life technologies, CA). The pellets were resuspended in a small volume of DPBS-Mg (DPBS supplemented with 9.5 mM $MgCl_2$) and then transferred into siliconized tubes (Bio Plas Inc, CA).

Cells were pelleted by low speed centrifugation, and the supernatant was discarded. For PsV maturation, an equal volume of lysis buffer (DPBS-Mg supplemented with 0.5% of Brij 58 and 0.2% benzonase) was added to the cell pellet, which was allowed to incubate at 37°C for 24 h. After maturation, lysates were adjusted to 850 mM NaCl and extracted with high salt buffer (DPBS with 0.8 M NaCl). Lysates were clarified by centrifugation at 10,000 x *g* for 10 min, loaded onto an OptiPrep (Sigma, MO) step gradient (27, 33 and 39%) and spun at 40,000 rpm in a Beckman (CA) SW40 rotor for 16 hr at 16°C. After centrifugation, 0.5 mL fractions were collected from the top of the gradient. A sample representing each fraction was diluted and tested on 293TT cultures[3] for reporter gene expression. Fractions with highest reporter gene expression SEAP Chemilumimescence kit (Clontech laboratories, CA) were pooled and used for the serum neutralization assay and the *in vivo* challenge experiments

Expression of target antigens

Frozen glycerol stocks were stabbed and seeded into 125 mL baffled flasks containing 50 mL of Animal free Luria Broth (AFLB, Teknova, CA) supplemented with 30 µg/ml Kanamycin (Sigma-Aldrich, MO). Seed cultures were grown overnight shaken at 200 rpm in an Innova (New Brunswick, NJ)) incubator at 30°C. Production cultures consisting of 1L AFLB/Kanamycin (30µg/ml) in 4L Baffled flasks were inoculated from the overnight seed culture to a starting cell density OD at 600nm of 0.1. The production cultivation conditions were set to 250 rpm at 37°C with hourly sampling. Upon achieving a cell density of OD_{600nm} = 0.8, a pre-induction sample was taken and the culture was induced with 1 mM Isopropyl β-D-1thiogalactopyranoside (IPTG, Sigma-Aldrich, MO). The temperature was reduced to 28°C at the time of induction; agitation remained set at 250 rpm. Samples were taken at 1 hour intervals for 4 to 6 hours post-induction, at which time the culture was harvested by centrifugation in pre-weighed 450 mL bottles at 8000 rpm for 10 minutes at 4°C in RC4 (Sorval, Thermoscientific, MA) centrifuge and

Ultralight 3000 rotor at 7000 rpm. Supernatant was discarded and cell pellets were stored at -80°C for purification. Biomass yields (g/L wet cell weights were calculated based gross weight (bottle + wet cell pellet) minus bottle tare weight. The post induction samples were analyzed for target protein expression via SDS/PAGE gel electrophoresis and Western Blot with anti-penta-hisitidine antibody (Qiagen, Germany) or monoclonal antibody directed against the RG-1 domain of HPV16 L2 to verify target antigen.

Quasivirion production for rabbit challenge studies

The production of quasivirion (QV) production has been performed as described before[4;5]. Briefly, 293TT producer cells were cotransfected with circular CRPV genomes and plasmids expressing codon-modified HPV L1 and L2 of the same papillomavirus type, resulting in HPV L1/L2 particles encapsidating the CRPV genome. On the second day after transfection, the cells were lysed with Brij-58, incubated to allow particle maturation, and treated with Benzonase (Sigma, MO) and Plasmid Safe (Epicenter, WI) to destroy unprotected DNA. Cell lysates were centrifuged on an OptiPrep density gradient (27,33-39.0%) at 40, 000 rpm in SW40 rotor (Backman, CA). Fractions (300 µL) were collected dropwise from the bottom of the column and analyzed for the presence of capsid proteins by immunoblotting and/or nondenaturing enzyme-linked immunosorbent assay. Fractions with appropriate densities and positive for capsid proteins were assayed for their infectivity in RK13 cells by quantitative reverse transcription-PCR (QRT-PCR) measuring viral E1^E4 transcripts[6]. Those fractions capable of generating viral transcripts in inoculated cells were retested for their ability to be neutralized by type-specific monoclonal antibodies. Fractions (and particles) termed “infectious” both induced the production of E1^E4 transcripts in the in vitro infection assay and lost this ability in the presence of a specific MAb[6-8].

Cutaneous challenge with QV (rabbits)

To evaluate the infectivity of quasivirions in vivo, several sites of scarified rabbit skin were exposed to 5 µL aliquots of stock preparations as previously described[9;10]. Animals were monitored for 10 weeks after challenge. Papilloma volumes were charted as height x width x depth. Additionally back of each rabbit was individually photographed.

Prior to a transfer of pre-challenge serum into naïve donor animals, all donor animals were bled and small samples stored as pre-bleed samples and the remainder used as diluents for the pre-challenge sera derived during the active immunization phase. For that purpose, a minimum of ten naïve New Zealand White rabbits per pre-challenge serum group were bled by ear vein to obtain 40 mL of blood yielding 15-20 mL of naïve serum per rabbit. Pre-challenge serum from each of the active immunization groups was taken at 1 month after the last antigen dose and pooled. Each pool of serum was mixed with naïve serum to generate a five-fold dilution series composed of 30 mL each of the following: naïve serum (negative control), 1/2, 1/10, 1/50, 1/250 and 1/1250. The 30 mL of diluted serums were stored at -80°C for up to 2 weeks prior to the start of the study. Three days prior to challenge, rabbits were anesthetized with a mixture of Ketamine HCl (40 mg/kg) and xylazine (5 mg/kg) and their backs shaved. Up to twenty four evenly spaced 1 cm x 2 cm areas were scarified by scalpel along the back of each rabbit. On the day of challenge, pre-challenge pooled dilutions were administered to pairs of rabbits at 1 ml per minute by ear vein for a total of a 15 ml volume. Final dilution in-vivo of the pre-challenge pools were estimated as 0 (naïve serum only), 1/20, 1/100, 1/500, 1/2500 and 1/12,500. Six hours after intra-venous serum administration, rabbits were challenged with HPV quazivirus types 6, 16, 18, 31, 45, 58 and CRPV as described previously(50). Briefly, animals were anesthetized with a mixture of Ketamine HCl (40 mg/kg) and xylazine (5 mg/kg), a small pre-challenge serum sample was taken and the 1 cm x 2 cm patches were abraded by scalpel. Each pair of rabbits was challenged with an administration of 5 µL of QV stocks per pair/triplicate of scarified sites on the back. Each 5 µL volume was scraped into the site with a

26 g needle and syringe. Weekly caliper measurements of papilloma “wart” volume and digital photos of individual animals were recorded during post-challenge weeks 3 to 10.

Sup. Fig. 1 Purification and biological (TLR5 signaling) properties of Fla-L2 vaccines

Sup. Fig. 1 A, B, C - purified protein samples of Fla~1X11-200, Fla~5X11-88, Fla~8X11-88 respectively. Lanes 1 and 2 are SDS PAGE; lanes 3 and 4 – Western Blots. Western blot of Fla~1X11-200 (Fig. 2-A) membrane has been performed with mAb RG1 specific to AA17-36 of HPV16 L2, while Western blot with Fla~5X11-88 (Fig. 2-B) and Fla~8X11-88 (Fig. 2-C) membranes have been processed with anti-His mAbs.

Sup. Fig. 1-D. Induction of TLR-5 by Fla-L2 vaccines.

TLR5 stimulation has been tested by assessing NF-κB activation in HEK293 cells expressing a human TLR5 and containing secreted alkaline phosphatase (SEAP) reporter gene under the control of an NF-κB promoter. TLR5 signaling was quantified by measurement of SEAP activity in the supernatants of stimulated cells. Each protein was applied to HEK293-hTLR5 cells at the four concentrations indicated and compared to control sample Fla~ (delta D3 flagellin backbone). Expression of NF-κB induced SEAP has been detected at OD at 650nm after 16-20 hr of incubation.

For that purpose HEK293 cells stably transfected with pUNO-hTLR5 (Invivogen, CA) to express human TLR5 (hTLR-5) gene are used to bind the target agonist (flagellin). Upon binding to its receptor flagellin initiates a signaling cascade that eventually leads to a translocation of transcription factor NF-κB which induces transcription of the reporter gene pNiFty-SEAP (Invivogen, CA) coding for secreting soluble embryonic alkaline phosphotase (SEAP) controlled by NF-κB–dependent promoter. SEAP catalysed hydrolysis of p-Nitrohenyl

phosphate producing a colorometric change in culture media or to added reagents which is detectable in the range of OD=405-650 nm.

Detection procedure briefly contains the following steps: (1) 96-well plate (200µL total volume) containing (25,000-50,000 cells/well) of HEK293pNiFty-SEAP and 20µL of sample or the positive control ligands were added to the plate; (2) each Fla~, Fla~fusion or control was assayed in the concentration range of 100 ng/ml, 10 ng/ml, 1 ng/ml and 0.1 ng/ml. Each sample was titrated two fold in triplicate and added to the wells. (3) after a 16-20 hr incubation the supernatant was removed in a 96 well format and the plate is centrifuged to pellet cell debris; (4) 10 µl of the clarified culture supernatant was transferred to a clean 96 well tray and heated at 65^{o}C for 5-10 minutes to inhibit endogenous alkaline phosphatase activity; (5) 50 µl of a 1X Dilution Buffer (Proprietary, Invivogen, Inc) 100 µl of 1X Assay Buffer*, 20 µl of 100mM L-Homoarginine and 20 µl of water was added to each well; (6) the plate was incubated at 37^{o}C for 10 minutes and 20 µl of Staining Solution (Proprietary, Invivogen, Inc) was added; (7) plate was incubated for an additional 10 minutes at 37^{o}C in the dark and then the OD was red at 405nm to 650nm.

Preparation of vaccine antigens:

To purify HPV L2 flagellin fusion constructs we developed an original purification method based on the selectivity of HiTrap Q HP resin (GE Healthcare Life Sciences, Pittsburgh, PA) in binding/elution mode to capture protein from the soluble fraction. The method was used for all constructs with slight modifications for Fla~5x11-88 and Fla~8x11-88 to overcome nonspecific interaction of the target protein with the chromatography matrix.

For purification of the Fla~ only and Fla~1x11-200 immunogens, cells were harvested by centrifugation and cell paste was stored at -80º C. Cell paste was thawed and

diluted to 10% solids in 50 mM Tris-HCl, 150 mM NaCl, pH 8.0 with Complete EDTA-free protease inhibitors cocktail tablets (Roche Applied Science, Indianapolis, IN). To disrupt cells, the cell suspension was passed twice through a microfluidizer (Microfluidics, Newton, MA, model 110Y) at 18,000 psi, and cooled to 4-8 ºC. The resultant lysate was clarified by centrifugation on Beckman Avanti J-25 centrifuge (Beckman Coulter, Inc., Indianapolis IN) with JA-25.50 rotor at 29,000 X g for 40-60 min at 4º to 8ºC. The target protein was precipitated with 30% ammonium sulfate by adding a 3 M stock solution dropwise into clarified cell lysate on a stir plate and continually stirring for 90–120 min at 4ºC. Precipitated protein was collected by centrifugation at 29,000 X g for 40-60 min at 4º to 8ºC; the pellet was resuspended in the same volume of 50 mM Tris-HCl, pH 8.0 with Complete EDTA-free protein inhibitors. The resuspended material was clarified by centrifugation at 29,000 X g for 40 min at 4º to 8ºC and the soluble fraction was applied to two 5 ml HiTrap Q HP pre-packed columns attached together for a total column volume of 10 ml pre-equilibrated with 50 mM Tris-HCl, pH 8.0. At bigger scales we utilized HiTrap FF media in XK 50/30 housings (both from GE Healthcare Life Sciences, Pittsburgh, PA) with column volumes of 150 -180 ml. Bound protein was eluted with a linear salt gradient from 0 to 1.0 M NaCl in 50 mM Tris-HCl, pH 8.0. For final polishing, peak elution fractions were pooled, spiked with 3 M Ammonium Sulfate to 1 M, filtered through a 0.45 µm Acrodisc syringe filters with hydrophilic polyethersulfone Supor membrane (Pall, Inc, Port Washington, NY) and loaded onto a HiTrap Phenyl HP 5 ml pre-packed column (GE Healthcare Life Sciences, Pittsburgh, PA) pre-equilibrated with 50 mM Sodium Phosphate, 1 M Ammonium Sulfate, pH 8.0. Bound protein was eluted with a linear gradient from 1.0 M to 0

Ammonium Sulfate in 50 mM Sodium Phosphate, pH 8.0. Peak fractions were pooled, dialyzed against Phosphate Buffered Saline (PBS buffer) solution, 5 mM EDTA, pH 8.0, followed by sterile filtration through a 0.2 µm Acrodisc syringe filters with Supor membrane (Pall, Inc, Port Washington, NY) and storage at -80 ºC.

For purification of Fla~5x11-88 and Fla~8x11-88 immunogens, the purification procedure described above was modified as described below: (1) post-Ammonium sulfate precipitation, pellets containing target protein were resuspended in 50 mM Tris-HCl, 8 M urea, pH 8.6 buffer and clarified from insoluble material by centrifugation at 29,000 X g for 40 min at 4 to 8 ºC. The soluble fraction was applied to a 5 ml HiTrap Q HP pre-packed column or to 180 ml of HiTrap FF resin packed in an XK 50/30 housings pre-equilibrated with the same buffer containing 8 M urea at pH 8.6. Bound protein was eluted with the same salt gradient as before, under denaturing conditions; (2) Similarly, 8 M urea was added to the HiTrap Phenyl HP resin binding (50 mM Tris-HCl, 1 M Ammonium Sulfate) and elution (50 mM Tris-HCl) buffers at pH 8.6; (3) Peak elution fractions containing target protein were refolded by extensive dialysis against 50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, pH 8.0, followed by sterile filtration and storage at -80ºC.

Protein purity and integrity were visualized by NuPAGE Novex 4-12% Bis-Tris 1.0 mm Gels in MES SDS Running buffer at 200 V constant voltage and stained with SimplyBlue SafeStain (all from Life Technologies, Carlsbad, CA). Protein concentrations were determined using the Pierce BCA Protein Assasy kit (Thermo Fisher Scientific Inc., Waltham, MA) according to their standard protocol with sample incubation at 37 ºC

for 30 minutes, BSA standards and readi at 562 nm absorbance on a SpectraMax M5 spectrophotometer (Molecular Devices, LLC, Sunnyvale, CA).

Sup. Fig. 2 Diagram of preclinical study design

Mouse and rabbit experimental designs are shown on the upper and lower parts of diagram respectively. Either 25 or 125 µg of vaccines were administered three times to mice or rabbits respectively. Mice were immunized biweekly, while rabbits in three week intervals. Both species were bled before challenges which happened after either 2 or 24 weeks (mice; vaginal challenge) or 3 weeks (rabbits; cutaneous challenge) with types specified on the diagram. A passive immunization study was also performed in rabbits to elucidate the role of antibodies in protection. For this purpose pooled rabbit serum samples harvested a day 77 were serially diluted and transferred into naive animals. 6 hours later animals were challenged with HPV6, 16, 18, 31, 45 and 58 quasivirions and CRPV.

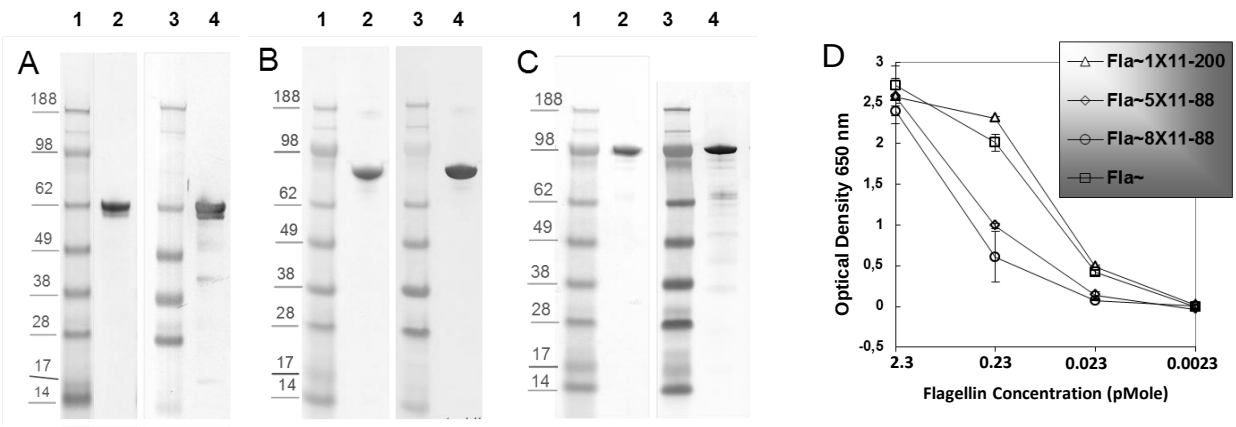
Sup. Fig. 3 Example of End Point Protection Titer determination (EPPT)

This figure illustrates the EPPT concept exemplified by the efficacy of anti-Fla~5x11-88 sera passively administered into naïve rabbits and challenged with quasivirus (QV) 45 **Panel A -** Efficacy graph: individual anti-Fla~5X11-88 sera dilutions (X axes) versus wart volumes (Y axes; mm^3; Geometric means are shown) at week 8 after the challenge

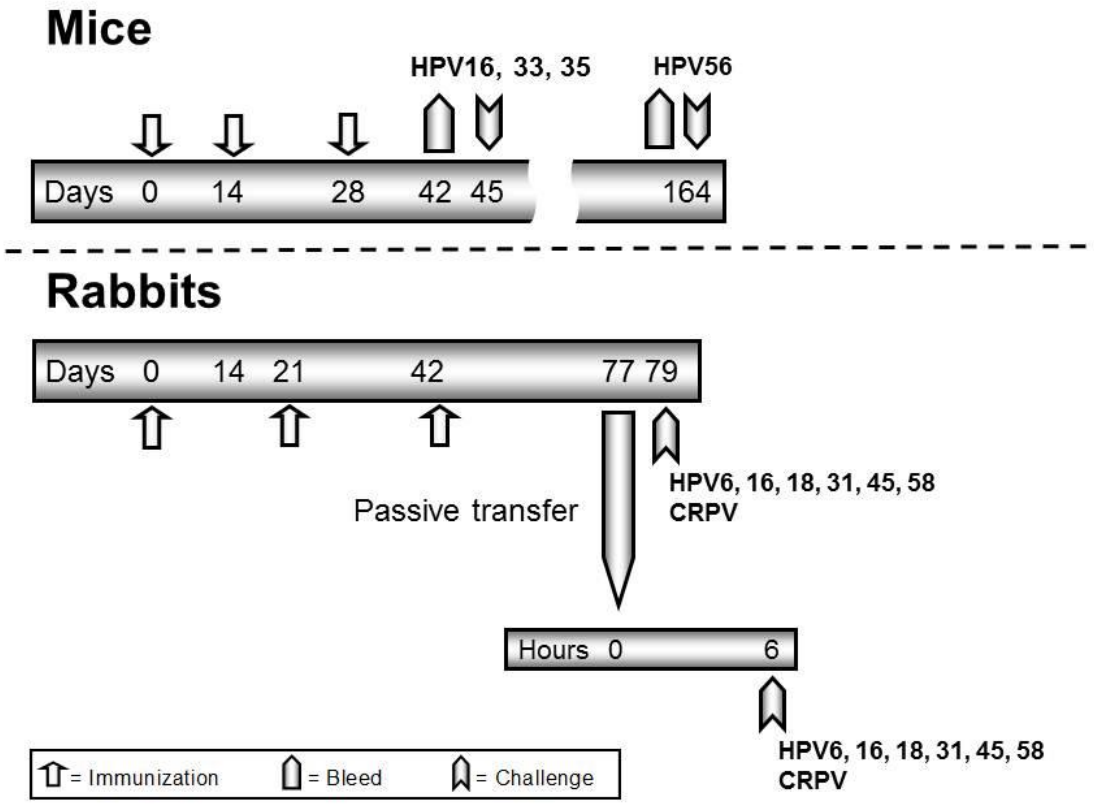
with QV45. EPPT is determined by the highest sera dilution achieving complete protection is indicated by a block arrow (dilution 100; see Material and Methods). **Panel B –** Representative images of rabbits passively transferred with anti-Fla~5X11-88 sera at dilutions of 100, 500 or 2,500 and challenged with QV45 and maintained 8 weeks post challenge. Black arrows show tattoo dots corresponding to QV45 challenge locations. Note that two challenge spots were located on either side of each of QV-specific tattoo dots. 6 viruses have been used for challenge of each rabbit (tattoo dots from the top to the bottom): QV6, 16, 18, 31, 45, 58 and CRPV. The location of QV45 challenge is also highlighted by dashed frame. As on panel A, block arrow signify a rabbit corresponding to EPPT=100.

Sup. Fig. 4-6 Protective efficacy of Fla~1X11-200 (A-panels), Fla~5X11-88 (B-panels), Cervarix®(C-panels), Gardasil™ (D-panels); Placebo (E-panels) against challenges with quazivirus types 6, 16, 18, 31, 45, 58 and CRPV as detected at 1 (Supp.Fig. 4), 6 (Supp.Fig. 5) and 12 (Supp. Fig. 6) months postimmunizations respectively.

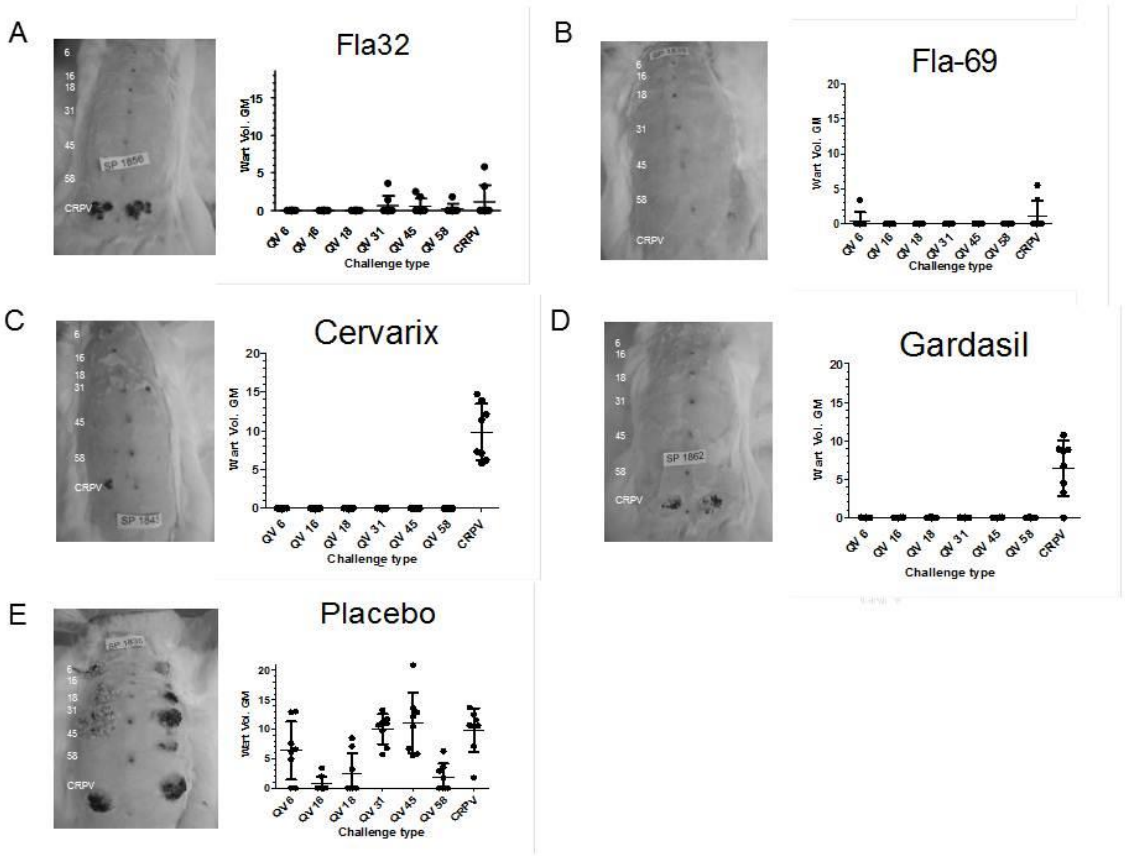
Geometric means of wart volumes are shown on Y axes on the graph; Antigens are listed on X axis. Representative photographs of rabbit backs within each panel are provided for illustration purposes


A
B
C
D
1 2 3 4
188
98
62
49
38
28
17
14
Fla~1X11-200
Fla~5X11-88
Fla~8X11-88
Fla~
Optical Density 650 nm
3
2,5
2
1,5
1
0,5
0
-0,5
2.3
0.23
0.023
0.0023
Flagellin Concentration (pMole)

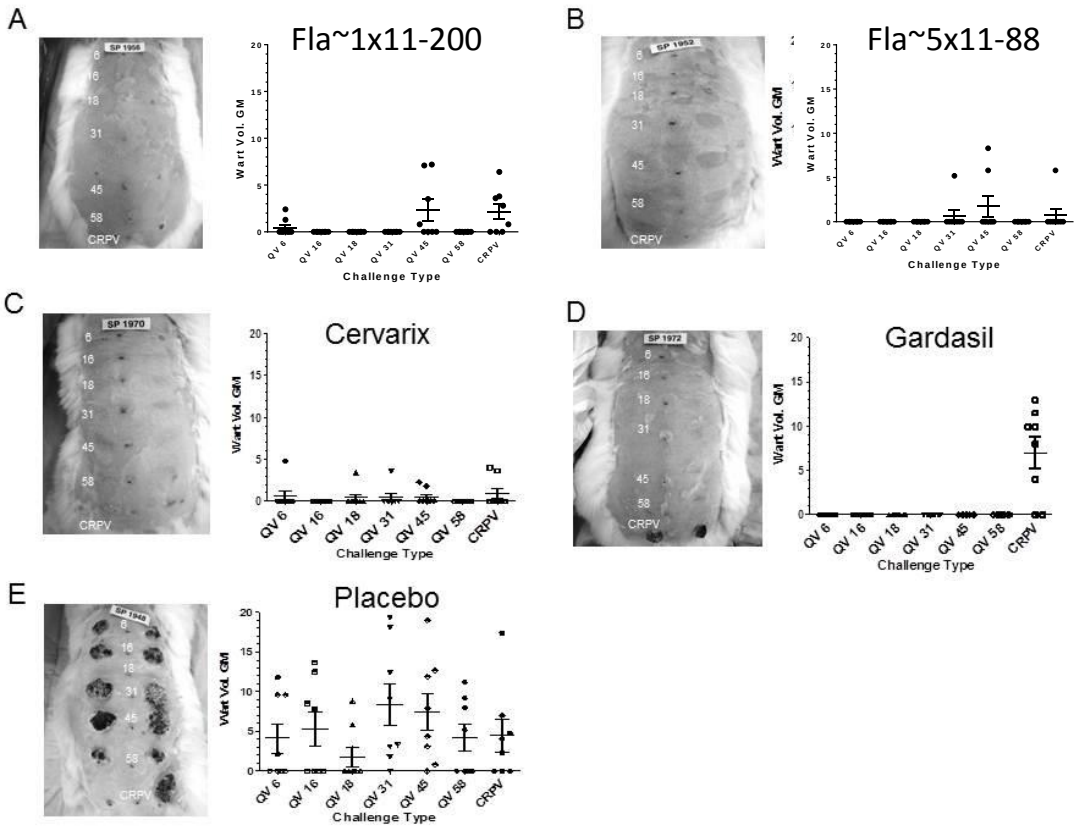
Sup. Fig 1


Mice
HPV16, 33, 35
HPV56
Days 0 14 28 42 45 164
Rabbits
Days 0 14 21 42 77 79
HPV6, 16, 18, 31, 45, 58
CRPV
Passive transfer
Hours 0 6
HPV6, 16, 18, 31, 45, 58
CRPV
= Immunization
= Bleed
= Challenge

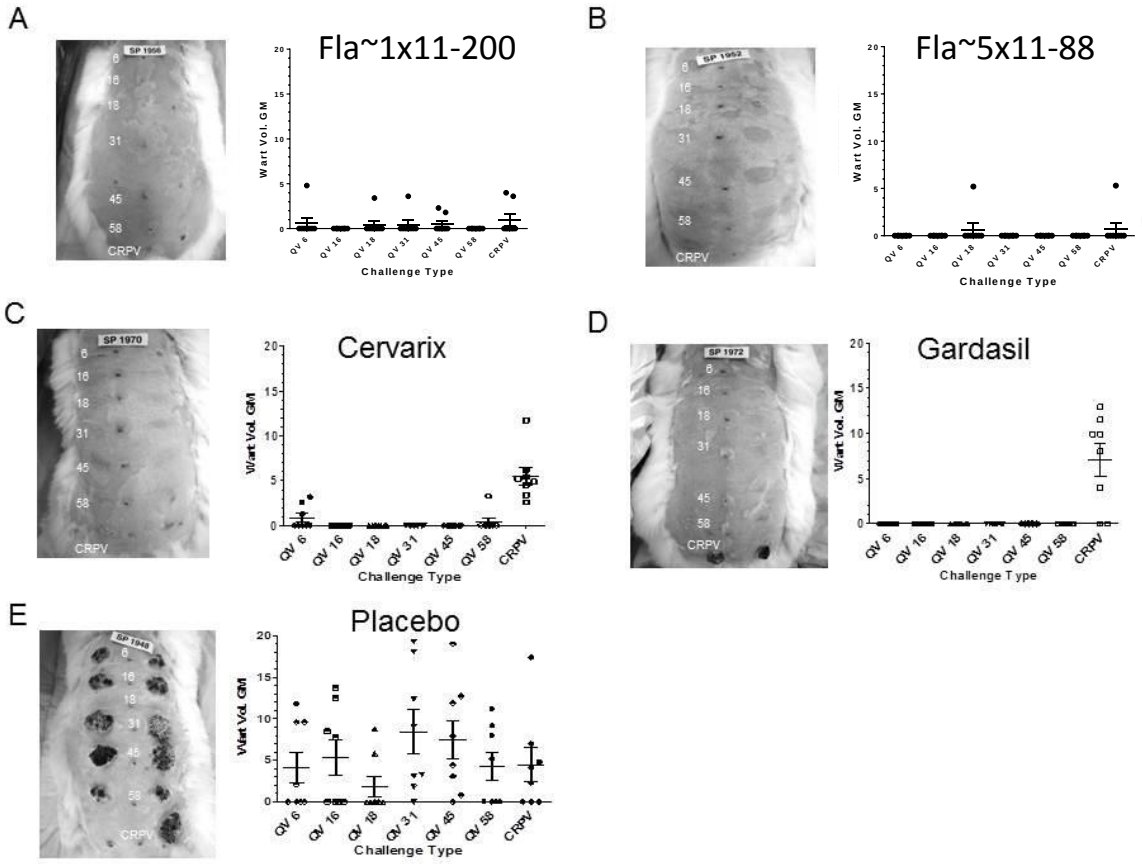
Sup. Fig. 2


A
Fla32
Wart Vol. GM
Challenge type
QV 6
QV 16
QV 18
QV 31
QV 45
QV 58
CRPV
B
Fla-69
C
Cervarix
D
Gardasil
E
Placebo

Sup. Fig. 3

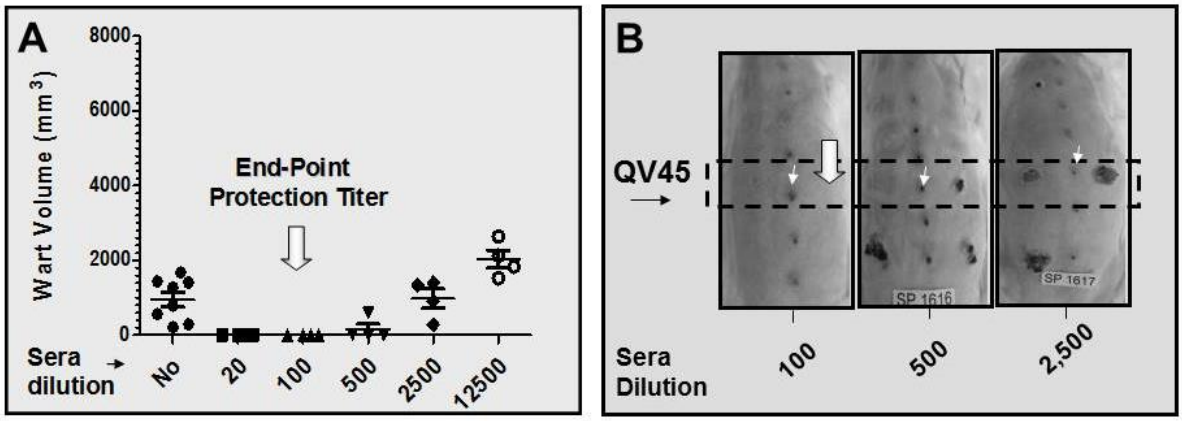

A
Fla~1x11-200
B
Fla~5x11-88
C
Cervarix
D
Gardasil
E
Placebo
Wart Vol. GM
Challenge Type
QV 6
QV 16
QV 18
QV 31
QV 45
QV 58
CRPV

Sup. Fig. 4


A
Fla~1x11-200
B
Fla~5x11-88
C
Cervarix
D
Gardasil
E
Placebo
Wart Vol. GM
Challenge Type
CRPV

Sup. Fig. 5

Passive transfer of anti-Fla~5x11-88 sera/challenge with QV45



Sup. Fig 6

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Ref Type: Serial (Book,Monograph)

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