Robust In Vitro and In Vivo Neutralization against Multiple High-Risk HPV Types Induced by a Thermostable Thioredoxin-L2 Vaccine

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Abstract

Current prophylactic virus-like particle (VLP) human papillomavirus (HPV) vaccines are based on the L1 major capsid protein and provide robust but virus type-restricted protection. Moreover, VLP vaccines have a high production cost, require cold-chain storage, and are thus not readily implementable in developing countries, which endure 85% of the cervical cancer–related death burden worldwide. In contrast with L1, immunization with minor capsid protein L2 elicits broad cross-neutralization, and we previously showed that insertion of a peptide spanning amino acids 20–38 of L2 into bacterial thioredoxin (Trx) greatly enhances its immunogenicity. Building on this finding, we use, here, four different neutralization assays to demonstrate that low doses of a trivalent Trx-L2 vaccine, incorporating L2(20–38) epitopes from HPV16, HPV31 and HPV51, and formulated in a human-compatible adjuvant, induce broadly protective responses. Specifically, we show that this vaccine, which uses a far-divergent archaeobacterial thioredoxin as scaffold and is amenable to an easy one-step thermal purification, induces robust cross-neutralization against 12 of the 13 known oncogenic HPV types. Immune performance measured with two different in vitro neutralization assays was corroborated by the results of mouse cervico-vaginal challenge and passive transfer experiments indicating robust cross-protection also in vivo. Altogether, our results attest to the potential of Trx-L2 as a thermostable second-generation HPV vaccine particularly well suited for low-resource countries. Cancer Prev Res; 8(10); 932–41. ©2015 AACR.

Introduction

Infectious agents are responsible for approximately 2 million new cancer cases/year worldwide. Human papillomaviruses (HPV) are responsible for 30% of infection-induced cancers, most notably cervical cancer (1). In fact, after breast, colorectal, and stomach cancer, cervical cancer is the fourth most frequent cancer in women. Although cervical cancer is the most common HPV-induced malignancy in the developing world, HPV-related cancers, including those of the anus and oropharynx, are on the rise in developed countries, with a combined total incidence matching that of cervical cancer in the United States (2, 3).

Eight years ago two prophylactic HPV vaccines, Gardasil and Cervarix, became available. Both are subunit vaccines composed of 360 monomers of the major capsid protein L1 assembled into noninfectious virus-like particles (VLP). Gardasil, which is produced in yeast cells and adjuvanted with aluminum hydroxide plus monophosphoryl lipid A, is bivalent and only contains HPV16 and HPV18 VLPs. Because both vaccines include HPV16 and HPV18 VLPs, they provide >70% protection against cervical cancer. Standard vaccination includes three doses administered intramuscularly over 6 months, but a similar efficacy against HPV16 and HPV18 infections has been reported in women receiving three, two, or even one dose(s) of Cervarix (4). However, a likely disadvantage of fewer doses is reduction of the minimal cross-protection against heterologous (“non-vaccine”) HPV types. Indeed, fewer doses of Cervarix correlate with less HPV31 neutralization (5). In December 2014, a nonavalent vaccine targeting seven of the thirteen oncogenic HPVs, namely types 16, 18, 31, 33, 45, 52, and 58, was licensed by the FDA. Besides inefficient cross-protection, another drawback of current VLP vaccines is their costly production/distribution platform, which requires eukaryotic cell culture, laborious purification, and cold-chain storage.

In contrast with conformation-dependent, type-specific L1-immunogens, immunizations with linear, conserved regions of minor capsid protein L2 elicit broadly neutralizing responses. Cross-neutralizing epitopes have been mapped to various N-terminal regions of HPV16 L2 (e.g., amino acid residues 17–36, 20–38, 56–75, and 96–120; refs. 6–8). Importantly, immunogens from different laboratories spanning amino acid (aa) residues 17–38 of L2, a region exposed on the cell surface during initial stages of infection, elicit cross-neutralization irrespective of the vaccine scaffold or formulation used. For
example, concatenated as L1–88 L2 fusion proteins elicit broad neutralization in mice and rabbits (9–11). Similarly, cross-neutralization was obtained with HPV16 and HPV31 17–36 L2 peptides displayed on adenovirus scaffold capsids (12). Also, chimeric HPV16-L1-VLPs bearing the 17–36 L2 peptide grafted onto the surface-exposed DE loop of L1 elicit durable and broadly neutralizing responses in mice and rabbits (13, 14). Although production of chimeric HPV16 L1-L2 VLPs and AAV2-L2(17–36)-HPV16–31 particles requires eukaryotic cell culture, bacteriophage-derived VLPs displaying 17–31 L2 yield a similarly broad protection even in the absence of adjuvant, and can be inexpensively purified from E. coli (15–17).

We have successfully used a non-virus display system based on bacterial thioredoxin as an immunogenicity-providing scaffold protein. Previously, we demonstrated that multipetide insertions of the 20–38 HPV16 L2 epitope into the active-site loop of E. coli thioredoxin creates an immunogen that elicits in vitro-detectable cross-neutralization in mice (18). These studies, however, were performed with high antigen doses (up to 100 μg) formulated in human-incompatible Freund’s adjuvant. Furthermore, cross-neutralization was not extended to notable high-risk HPV types, including HPV31. We recently showed that a trivalent mixture of monovalent E. coli thioredoxin-L2 antigens bearing L2 (20–38) epitopes from HPV16, HPV31, and HPV51 elicits a broader and more robust neutralization than individual monovalent antigens (19). We also showed that Trx-HPV16-L2 based on a far-divergent and hyperthermostable thioredoxin from Pyrococcus furiosus is as immunogenic as E. coli Trx-L2, but comes with the added benefits of (i) reduced cross-reactivity with human thioredoxin; (ii) a simplified purification; and (iii) a strikingly increased thermal stability (>12 h at 100°C, apparent Tm>95°C; ref. 20).

Taking into account the above scaffold improvement, the availability of different neutralization assays and the need for a L1-VLP comparison, we present here the results of a comprehensive immunization study evaluating the relative performance of monovalent P. furiosus thioredoxin-HPV16 L2 (20–38), (PTrx-16L2), a mixture of thioredoxin-L2 antigens derived from HPV16, 31, 51 (PTrx-L2 mix), and HPV16-L1-VLPs. The PTrx-L2 antigens were administered at moderate doses and formulated in the human-compatible adjuvant aluminium hydroxide and were tested in vitro using the L1- and the L2-pseudovirion-based neutralization assays (PNBA) and in vivo using the cervico-vaginal challenge and the passive transfer mouse model. Similarly protective responses for PTrx-16L2 and PTrx-L2 mix, both broader than those attained with HPV16-L1-VLPs, were revealed by the challenge assay. However, passive transfer experiments and especially the L1-PNBA highlighted the more prominent cross-protection achieved with the PTrx-L2 mix compared with PTrx-16L2 and HPV16-L1-VLPs.

**Materials and Methods**

**Expression and purification of the Trx-L2 proteins**

PTrx-L2 constructs were generated by subcloning chemically synthesized sequences (Eurofins MGW Operon) coding for HPV16-, HPV31- and HPV51-L2 (20–38) polypeptides into the CpoI site of an engineered version of Pyrococcus furiosus thioredoxin inserted into a 6xHis-tag-lacking pET-PTrx plasmid. The resulting constructs were used for recombinant expression and one-step heat purification of the three Trx-L2 proteins as described previously (20). The composition and purity of protein preparations were assessed by electrophoretic analysis on 11% to 15% SDS-polyacrylamide gels and MALDI-TOF analysis. Protein concentration was determined by A280 measurements using calculated extinction coefficients as well as with the use of a Qubit 2.0 Fluorometer (Life Technologies).

**Expression and purification of HPV16-L1-VLPs**

Trichoplusia ni (TN) High Five cells (Invitrogen) were cultivated in Ex-Cell 405 serum-free medium (SAFC Biosciences) at 27°C. Papillomavirus VLPs were produced as described previously (21). Purity and L1 content of individual fractions were assessed by SDS-PAGE and Coomassie-staining. Capsid quality was verified by electron microscopy.

**Mouse and guinea pig and immunization**

Six- to 8-week-old female BALB/c mice were obtained from Charles River Laboratories and kept at the German Cancer Research Center under specific pathogen-free conditions. Animals were immunized intramuscularly four times at monthly intervals with 15 μg PTrx-L2 or HPV16-L1-VLPs adjuvanted with 50 μg aluminium hydroxide (Brenntag) and 10 μg synthetic monophosphoryl lipid A (AvantiLipids).

Outbred Hartley (Crl:HA) guinea pigs (350–400 g female animals) were obtained from Charles River Laboratories and immunized intramuscularly four times at triseweekly intervals with 50 μg PTrx-L2 or HPV16-L1-VLPs formulated in 300 μg aluminium hydroxide and 20 μg synthetic monophosphoryl lipid A.

**L1-pseudovirion-based neutralization assay**

HPV pseudovirions (PSV) were produced as described previously (22) with minor modifications (23). L1-PBNAs were performed as detailed recently (23) with a pseudovirion input of approximately 0.5 ng L1 per 1 × 10⁶ HeLaT cells, generated in house by stable large T-antigen transfection and routinely authenticated with the Multiplex human Cell line Authentication Test (MCA; ref. 24).

**L1-pseudovirion-based neutralization assay**

The L1-PBNA was performed as described (25) with a pseudovirion input of approximately 2.5 ng L1 per 8 × 10⁵ pgsa745 cells. All L2-PBNA cell lines, including pgsa745, CHO.Afurin and MCF10A, were kindly provided by John Schiller’s laboratory (NIH, Bethesda, MD) and were routinely authenticated with the MCA (24).

**Challenge and passive transfer in the cervico-vaginal mouse model of HPV pseudovirion infection**

The mouse cervico-vaginal model was used as described (26) with minor modifications. Both challenge and passive transfer assays were performed over the course of 8 days. On day 1, BALB/c male cage bedding was transferred to the cages of female mice to induce hormonal synchronization (Whitten effect). On day 3, 100 μl of 30 mg/ml Medroxyprogesteroneacetat (Pharmacia) was injected s.c. In the case of passive transfer, 50 μl of serum (diluted 1:2 with PBS) was delivered i.p. to each mouse on day 5. For challenge assays, no treatment was performed on day 5. On day 6, mice were treated with 50 μl of 4% Nonoxynol-9 (N9; Spectrum) plus 4% carbosymmetriccellulose. Four hours after N9 treatment,
HPV pseudovirions (~250 ng L1/mouse for HPV16, 31, 33 and 51; ~75 ng L1/mouse for HPV18) encapsidating a firefly luciferase plasmid were instilled intravaginally. Luminescence-based imaging using a Xenogen IVIS imager (Xenogen Corporation; PerkinElmer) was performed on day 8. Images were acquired before and after intravaginal instillation of 20 µL luciferin substrate (15 mg/mL; Promega). Three minutes after substrate addition, luminescence was recorded (30 seconds–1 minute exposure time, medium binning). A region-of-interest (ROI) analysis was performed using the Living Image 2.50.1 software (Xenogen; PerkinElmer).

Statistical analysis
Statistical analysis was performed with GraphPad Prism 5.00 (GraphPad Software) using the non-parametric Mann–Whitney test; differences were considered significant at $P \leq 0.05$.

Results
To move forward with thioredoxin-L2 as a second-generation HPV vaccine, we initially focused on antigenic dose and adjuvant formulation using monovalent Trx-16L2 as a test antigen. We found that Trx-16L2 doses comprised between 50 ng and 15 µg elicited potent type-specific and cross-neutralizing responses (Fig. 1). We tested several human-compatible adjuvants and selected aluminum hydroxide-MPLA because it produced neutralization titers most comparable with those achieved with Freund’s adjuvant (data not shown).

The lead thioredoxin-L2 vaccine investigated in this work is an equimolar mixture of three monomeric, thermally purified and fully oxidized Pyrococcus furiosus (Pf) Trx-L2 proteins ("mix"), each bearing a 3-fold repeated L2(20–38) peptide from HPV16, 31 or 51. The PfTrx-L2 mix was compared with monovalent PfTrx-16L2 and HPV16-L1-VLPs, and neutralizing responses were analyzed in vitro on groups of 35 Balb/c mice per immunogen using the standard L1-PBNA and the L2-PBNA (25). Twenty-five of the 35 mice in each group were randomly selected and destined to in vivo vaginal challenge with pseudovirions from five different oncogenic HPV types. Final sera were collected from the remaining 10 animals in each group, including 10 negative control animals immunized with the empty PfTrx scaffold, pooled, L1-PBNA titrated, and passively transferred into naïve mice.

Superior in vitro cross-neutralization capacity of the PfTrx-L2 mix formulation
Intermediate sera were collected from mice vaccinated with PfTrx-L2 mix, PfTrx-16L2, or HPV16-L1-VLPs 2 weeks after the final immunization and analyzed on HPV16, 18, 31, 33, and 51 pseudovirions in the L1- and L2-PBNAs (Fig. 2). Consistent with our previous results (19), the PfTrx-L2 mix elicited comparable HPV16 type-specific responses (Fig. 2A) in addition to significantly broader cross-reactivity (Fig. 2B–E). Its robustness and cross-neutralization capacity were especially apparent in the L2-PBNA, where most sera neutralized HPV31 and HPV51 pseudovirions well above the 50% threshold (Fig. 2B and C). Also in line with previous findings, monovalent PfTrx-16L2 did not elicit cross-neutralizing responses against HPV31 and HPV51, but effectively neutralized HPV18 and HPV33 (Fig. 2D and E). Although HPV16-L1-VLPs induced exceptionally strong HPV16 neutralization responses, they largely failed at cross-neutralization. In particular, anti–HPV16-L1-VLP immune sera showed neither HPV18 nor HPV51 cross-neutralization capacity and limited activity against HPV31 and HPV33. These results are consistent with the observation that Cervarix, which also contains aluminum hydroxide-MPLA as adjuvant, is able to induce cross-protection against HPV31 and HPV33 (27).

To determine in vitro cross-neutralization titers, pools of the 35 sera were titrated against 13 HPV pseudovirion types presently recognized as “high-risk” (HPV16, 18, 45, 31, 33, 52, 58, 35, 59, 56, 31, 39, and 68; ref. 28). Three different pools from PfTrx-16L2 and PfTrx-L2 mix immune sera were generated on the basis of the magnitude of their HPV16 neutralization titers. Pool #1 included sera with HPV16 titer greater than 2,000, whereas pool #2 contained sera with titers greater than 1,000 but lower than 2,000, and pool #3 comprised sera with titers below 1,000. For HPV16-L1-VLP immune sera, two pools were created: pool #1 comprised of sera with anti-HPV16 titers greater than 3,000,000 and pool #2 with titers of 3,000,000 or lower. The PfTrx-L2 mix sera, especially the high-titer pool #1, performed best in terms of neutralization breadth and potency, with neutralization activity

Figure 1. Dose-effect analysis of the monovalent Pyrococcus furiosus (Pf) Trx-16L2 antigen. Mice were immunized intramuscularly four times at monthly intervals with the PfTrx-16L2 antigen formulated in aluminum hydroxide-MPLA. Intermediate sera were collected 2 weeks after the third immunization and L1-PBNA titrated against HPV16 (left) and HPV18 (right) pseudovirions; dots represent neutralization titers against the indicated HPV types measured in individual mouse sera. Antigen doses between 1 and 15 µg elicited higher type-specific and cross-neutralizing titers than 50 to 200 ng doses. Mean titers are indicated by horizontal bars; statistical significance of the differences between the immune responses elicited by different antigen doses was determined with the Mann–Whitney test; ns, non-significant.
Figure 2.
Cross-neutralization breadth: Comparison between monovalent PfTrx-16L2, PfTrx-L2 mix and HPV16-L1-VLPs. Sera collected 2 weeks after the fourth immunization were tested at a 1:200 dilution against HPV16 (A), HPV31 (B), HPV51 (C), HPV18 (D), and HPV33 (E) pseudovirions using the standard L1- and the modified L2-PBNAs (left and right columns, respectively). Each dot represents one mouse serum; data are the mean plus SD of neutralization percentages measured within individual assay groups.
against all 13 pseudovirions except HPV56 (Table 1). The PfTrx-16 L2 sera, specifically the high- and medium-titer pools (#1 and #2), neutralized 10 of the 13 pseudovirions, with no activity against HPV31, 51, 59, and 56. As expected, HPV16-L1-VLP sera showed high type-specific neutralization against HPV16, but minimal cross-neutralization against two (pool #1) or four (pool #2) of the other tested pseudovirions. Importantly, and in stark contrast with the outstanding titers measured against HPV16, the cross-neutralization capacity of anti-VLP immune sera was particularly low on HPV31, 33, 58, 35, and HPV56 pseudoviruses. Notably, anti–HPV16-L1-VLP pool #1 and pool #2 displayed significantly different cross-neutralization patterns. Although pool #1 cross-neutralized HPV38 and HPV56, pool #2 cross-neutralized HPV31, 33, 35, and 56.

PfTrx-L2 mix and PfTrx-16L2–immunized mice are similarly protected from vaginal in vivo challenge with heterologous HPV types

Twenty-five mice each from the PfTrx-L2 and L1-VLP groups were challenged with HPV16, 31, 51, 33, or 18 pseudovirions (5 mice/HPV type). The rationale for choosing these particular high-risk types rests on the fact that HPV16 and HPV18 are the two most prominent HPV types associated with cervical cancer, whereas HPV31, HPV33, and HPV51 are the most divergent (from HPV16) prominent HPV types associated with cervical cancer, whereas risk types rests on the fact that HPV16 and HPV18 are the two most

Table 1. L1-PBNA titers (IC50 values) of serum pools #1, #2, and #3 derived from PfTrx-16L2, PfTrx-L2 mix, or HPV16-L1-VLPs vaccinated mice

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<th>#1</th>
<th>#2</th>
<th>#3*</th>
<th>#1</th>
<th>#2</th>
<th>#3*</th>
<th>#1</th>
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NOTE: Sera from 35 mice immunized with the indicated antigens were collected 2 weeks after the fourth immunization and titrated against the 13 high-risk pseudoviruses. For PfTrx-16L2 and PfTrx-L2 mix, pool #1 included sera with HPV16 neutralization titers higher than 2,000; pool #2 comprised sera with titers greater than 1,000; and pool #3 contained sera with neutralization titers below 1,000. For HPV16-L1-VLPs, pool #1 comprised sera with anti–HPV16 neutralization titers higher than 3,000,000, whereas pool #2 contained sera with titers below 3,000,000.

Passive transfer of PfTrx-L2 mix immune sera provides more robust cross-protection than PfTrx-16L2 or L1-VLP immune sera

As revealed by the above data, the challenge assay is extremely sensitive and capable to uncover neutralization activities undetectable in vitro. Multiple immune mechanisms, including innate and cellular immunity in addition to humoral immunity, likely contribute to protection in this experimental set-up. Instead, passive transfer of immune-sera is humorally defined and probably only relies on vaccine-induced antibodies. After the final immunization, sera from 10 mice in each group (PfTrx-16L2, PfTrx-L2 mix, HPV16-L1-VLP, and empty PfTrx-negative control) were pooled and L1-PBNA titrated against HPV16, HPV31, HPV51, HPV53, and HPV33 pseudoviruses. Pools were passively transferred i.p. into five groups of animals, which were subsequently infected with the above pseudovirions (Fig. 4).

As observed in the challenge and anticipated by the high L1-PBNA titers, passively transferred sera (diluted ~1:40; serum: blood volume ratio) from PfTrx-16L2, PfTrx-L2 mix, and HPV16-L1-VLP-immunized mice provided protection against HPV16 infection (Fig. 4A). However, at variance with the challenge results, where PfTrx-16L2 and PfTrx-L2 mix immunized animals appeared to be protected and all yielded significant L1-PBNA titers, with no or very little in vivo detectable firefly luciferase activity. In contrast, L1-VLP-immunized mice were infected and to a greater extent than negative control animals. PfTrx-16L2 and PfTrx-L2 mix-immunized animals were similarly protected from challenge with HPV33, with higher L1-PBNA titers for the latter antigen formulation (Fig. 3E). By comparison, no in vitro titers nor protection against HPV33 challenge were observed in L1-VLP-immunized mice (with the exception of one animal).
Figure 3.
Broader in vivo challenge protective responses elicited by monovalent and mix PfTrx-L2 formulations compared with HPV16-L1-VLPs. After four immunizations with the indicated immunogens, sera from 5 animals were collected and analyzed in the L1-PBNA (individual titers are reported below each immunogen). Immunized mice were subsequently challenged with HPV16 (A), HPV31 (B), HPV51 (C), HPV18 (D), and HPV33 (E) pseudovirions. A similarly broad protection against the five tested HPV types was detected with sera from PfTrx-L2–vaccinated mice, despite low (or absent) L1-PBNA titers in some animals. A type-restricted (HPV16 and HPV31 only) protection is apparent, instead, in HPV16-L1-VLP–immunized animals. Average radiance values indicating the extent of infection are reported for each mouse; nonimmunized animals served as negative controls. Representative images showing the magnitude of vaginal infection by HPV51 PSVs are shown in F. The colors (scale shown on the left) indicate the intensity of luciferase expression; an ROI analysis for average radiance (p/s/cm²/sr) was performed with the Living Image 2.50.1 software.
HPV16-L1-VLP sera without a detectable HPV31 L1-PBNA titer failed to confer protection against HPV31 (Fig. 4B). In fact, only the PfTrx-L2 mix serum pool with an L1-PBNA titer of 300 provided HPV31 protection upon passive transfer. Instead, both PfTrx-16L2 and PfTrx-L2 mix sera afforded protection against HPV51, HPV18, and HPV33 (Fig. 4C–E). Importantly, however, protection conferred by the PfTrx-L2 mix sera was significantly more robust. Under the same conditions, passively transferred HPV16-L1-VLP sera lacking L1-PBNA measurable titers against these three HPV types did not provide any protection against HPV51, HPV18, or HPV33 (Fig. 4C–E).

PfTrx-L2 immunogens elicit higher magnitude responses in guinea pigs than in mice

Despite the favorable results obtained in Balb/c mice, inbred animals only partially mirror the human response and parallel studies on additional animal models are desirable, and required, for further vaccine development. To this end, we immunized outbred guinea pigs with a subset of PfTrx-L2 immunogens, including PfTrx-16L2, PfTrx-31L2, PfTrx-51L2, the PfTrx-L2 mix, and HPV16-L1-VLPs, adjuvanted with aluminium hydroxide-MPLA, using the same protocol applied to Balb/c mice. Final sera were titrated in the L1-PBNA against HPV16, HPV31, HPV51, HPV18, and HPV33. Each group was then challenged with HPV16 pseudovirions (Fig. 4A), HPV31 pseudovirions (Fig. 4B), HPV51 pseudovirions (Fig. 4C), HPV18 pseudovirions (Fig. 4D), or HPV33 pseudovirions (Fig. 4E). The broader protection afforded by mix-elicited antibodies compared with monovalent PfTrx-L2 is apparent in B, C, and E; a significantly lower cross-protection against all viral types except HPV16 was observed with anti-HPV16-L1-VLP sera. Data are average radiance values, indicating the extent of infection in individual mice. L1-PBNA titers of individual serum pools are shown on a gray background above each group; the lack of a value, as in the case of PfTrx-16L2 in B, indicates the absence of a measurable neutralization titer.
HPV18, HPV45, HPV33, HPV59, and HPV56 pseudovirions (Table 2).

When comparing PfTrx-L2–induced immune responses in mice and guinea pigs, it is apparent that HPV16 and HPV51 neutralization is higher in the latter species. In contrast, L1-VLPs induced equally high HPV16 titers in both species. Interestingly, and in contrast with what we observed in mice (Table 1), PfTrx-16L2 elicited similar HPV51 neutralization titers as PfTrx-51L2 in guinea pigs. Also at variance with the situation in mice, PfTrx-16L2 elicited weak HPV18 neutralization responses in guinea pigs. However, PfTrx-L2 mix and PfTrx-51L2 both induced strong HPV18 neutralization responses in guinea pigs. In this species, PfTrx-L2 mix yielded rather low HPV31 neutralization, whereas the opposite was observed for HPV16-L1-VLPs. Thus, despite the rather small sample size, cross-neutralization in guinea pigs seems to be somewhat different from that in mice. However, the best immune performance was still achieved with the PfTrx-L2 mix.

Discussion

We recently demonstrated the superior cross-neutralization responses elicited by a trivalent mixture of E. coli thioredoxin-L2 immunogens bearing L2 sequences from HPV16, 31, and 51 compared with the corresponding monovalent antigens (19). Because the latter study only measured in vitro neutralization, we wished to further investigate the immune performance of the Trx-L2 mix via a comprehensive set of in vivo (vaginal challenge and passive transfer) and in vitro (L1- and L2-PBNA) assays, using monovalent Trx-L2 and HPV16-L1-VLPs as reference immunogens. In doing so, we also gained information on the relative sensitivity and predictive value of the four assays. Another major variation introduced in the present study was the use of heat-purified PfTrx-L2 immunogens built on the non–cross-reactive and highly thermostable thioredoxin from archaeabacterium Pyrococcus furiosus (Pf; ref. 20).

L1- and L2-PBNA data corroborated our previous finding that the trivalent PfTrx-L2 mix elicits stronger neutralization against HPV31 and HPV51 compared with PfTrx-16L2 and HPV16-L1-VLPs. Moreover, PfTrx-L2 mix induced higher-level neutralization of the non–vaccine-type HPV33 and neutralization of HPV18 similar to that of PfTrx-16L2. L1-PBNA titrations of pooled sera against the 13 oncogenic HPV types revealed the superior cross-neutralization capacity of sera from mice immunized with PfTrx-L2 mix, which neutralized 12 of the 13 tested oncogenic HPV types (Table 1). By comparison, sera from mice immunized with PfTrx-16L2 or HPV16-L1-VLPs neutralized 10 and five oncogenic HPV types, respectively. Interestingly, in vivo challenge experiments revealed protective effects in animals immunized with PfTrx-L2 mix, monovalent PfTrx-16L2 and HPV16-L1-VLPs, even in the absence of measurable in vitro titers. This was especially evident in animals immunized with PfTrx-16L2 and subsequently challenged with HPV31, HPV51, or HPV33 pseudovirions. In fact, if one only considered challenge data, PfTrx-16L2 would appear comparable with the PfTrx-L2 mix with regard to cross-protection breadth. Passive transfer data, instead, were more aligned with those obtained from in vitro neutralization assays. Only sera with a measurable in vitro neutralization titer conferred protection in vivo upon transfer to naïve mice. Also, higher-titer sera generally afforded greater in vivo protection than lower-titer sera. Thus, on the basis of in vitro neutralization and in vivo passive transfer data, the PfTrx-L2 mix appears to induce the broadest cross-neutralization, whereas an immunogenicity similar to that of PfTrx-16L2 is supported by challenge data.

If we rank the four assays according to their sensitivities, the in vivo challenge and the L1-PBNA emerge as the most and the least sensitive, respectively, whereas the L2-PBNA and the passive transfer (with a serum transfer volume of 50 μL) are of intermediate sensitivity. How long-lived can be a neutralization that cannot be measured in vitro? What immune mechanisms promote the apparently extended protection observed in the challenge assay? Which assay represents the most reliable correlate of multitype HPV protection in humans?

Neutralization measured in vitro reflects prevention of virus internalization by anti-capsid antibodies. However, additional mechanisms likely enhance antibody-mediated neutralization, including complement binding, FcR engagement, and recruitment of phagocytic immune cells. These mechanisms are not recapitulated in vitro, but probably contribute to neutralization efficiency measured in the challenge assay. Moreover, innate components such as complement, IgM, macrophages, and neutrophils, might play a significant role in in vivo neutralization. Although these in vivo-restricted mechanisms undoubtedly contribute to the overall response, an ideal L2 immunogen should elicit in vitro and in vivo neutralization. Although in vitro assays may underestimate in vivo neutralization capacity, they provide a reliable, albeit indirect indication on immunity robustness. Hence, it is reassuring that the PfTrx-L2 mix elicits L1-PBNA detectable titers against 12 of the 13 oncogenic HPV types. The latter results as well as those produced by the other assays were obtained at a fixed time (2 months) after the last vaccination. Thus, longevity of immunization-induced anti-HPV protection still needs to be addressed in future studies.

Interestingly, PfTrx-L2 immunogens elicited higher HPV16 and HPV51 neutralization titers in guinea pigs than in mice, whereas L1-VLPs induced similarly high HPV16-neutralizing responses in both species. Furthermore, and in contrast with the situation in

Table 2.

| L1-PBNA titers (IC50 values) of immune sera from PfTrx-16L2, PfTrx-51L2, PfTrx-L2 mix, or HPV16-L1-VLPs immunized guinea pigs |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | #1              | #2              | #1              | #2              | #1              | #2              |
| HPV16           | 4115            | 120,696         | 14,982          | 4,793           | 190,492         | 8,037           | 34,450          | 8.5 × 10⁴       | 7.1 × 10⁵       |
| HPV31           | —               | 184             | 2,300           | —               | —               | —               | —               | —               | —               |
| HPV51           | 1,247           | 3,537           | 465             | 267             | 4,826           | 294             | 2,650           | —               | —               |
| HPV68           | 115             | 250             | 604             | 1,474           | 1,324           | 901             | 1,537           | —               | —               |
| HPV45           | 428             | 443             | 943             | 926             | 721             | 257             | 597             | —               | —               |
| HPV33           | —               | —               | —               | 981             | —               | —               | —               | —               | —               |
| HPV59           | —               | 118             | 1,090           | —               | —               | —               | —               | —               | —               |
| HPV56           | —               | —               | —               | —               | —               | —               | —               | —               | —               |

NOTE: Guinea pigs (2 animals/group) were immunized intramuscularly four times at triweekly intervals with 50 μg antigen formulated in Alum-MPLA. Final sera were collected 8 weeks after the fourth immunization and titrated in the L1-PBNA.

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mice (19). PfTrx-L2 extended neutralization to HPV51 in guinea pigs as effectively as PfTrx-L1.2. However, fairly low anti-HPV31 neutralization titters were induced in guinea pigs by PfTrx-L2 mix, whereas the opposite was observed with HPV16-L1-VLPs. In summary, slightly different immune responses, in terms of magnitude and breadth, were observed with PfTrx-L2 and HPV16-L1-VLPs in guinea pigs compared with mice. In both animals, however, PfTrx-L2 mix was the most effective immunogen.

Why does PfTrx-L2 elicit higher neutralization in guinea pigs than in mice? Conceivably, immunoglobulin germ-line configurations may be more adapted to recognize, and be activated by, PfTrx-L2. Further, the TLR-4 agonist monophosphoryl lipid A may be more effective in guinea pigs than in mice (29). Also, although both Balb/c mice and guinea pigs express TLR-4 receptors on dendritic cells, signaling might be more sustained in the latter species. Although PfTrx-L2–associated T-helper epitopes have not been mapped yet, preliminary data indicate that such epitopes can have a strong, strain-dependent impact on immunogenicity and the immunogenicity plunge observed in certain mouse strains can be compensated by incorporation of pan-reactive T-helper epitopes. Ultimately, it is encouraging to see that even unmodified PfTrx-L2 immunogens elicit cross-neutralizing responses in more than one preclinical model organism.

Despite recent progress in the optimization of L2-based immunogens (11, 13, 30), the current benchmark of HPV protection is set by the licensed VLP vaccines. HPV16 neutralization endpoints measured with the HPV16-L1-VLPs we used as a surrogate L1-vaccine reference were nearly identical to those achieved with PfTrx-L2, both the monovalent and the mix formulation. However, similar to the results obtained in other L1 versus L2 vaccine comparisons (10, 14), neutralization titters were two-three orders of magnitude higher for L1-VLPs than for PfTrx-L2. This gap, whose clinical significance is unknown, was largely compensated by the superior cross-neutralization achieved with the PfTrx-L2 mix. Cross-protection remains the top goal of current VLP-HPV vaccine development. This is well documented by the recent FDA licensing of a nonavalent variant of Gardasil (V503), which includes five additional high-risk VLP types (HPV31, 33, 45, 52, and 58) besides HPV16, 18, 6, and 11. Despite the extended coverage of V503, its breadth of effective cross-protection remains to be determined. In addition to the six oncogenic types not present in V503, this includes a number of (i) alpha-types presently considered as low-risk and (ii) non-genital types which may be (co)causal agents of nonmelanoma skin cancer, especially in immunocompromised patients. Given the type specificity of L1, it is unlikely that the nonavalent vaccine will confer any appreciable cross-protection against these HPV types, while we know, for example, that the PfTrx-L2 vaccine induces significant L1-PBNA titters against the dermatotropic alpha type HPV51 implicated in squamous cell carcinomas (M. Müller; unpublished data). The increased complexity of the nonavalent vaccine may increase production costs and further reduce physicochemical (especially thermal) stability. This, in turn, makes the vaccine even less accessible to the populations most in need of HPV protection, that is, low-resource countries, which bear 85% of the global cervical cancer burden, often more strongly associated with types other than HPV16 and HPV18 (31). Along this view, the PfTrx-L2 vaccine embodies relevant features of a next-generation HPV vaccine, namely strong HPV16 and HPV18 protection coupled to extended cross-protection, thermal stability, and cost-effectiveness.

Disclosure of Potential Conflicts of Interest
M. Müller has ownership interest in a patent. No potential conflicts of interest were disclosed by the other authors.

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