Influence of Oxidation and Multimerization on the
Immunogenicity of a Thioredoxin-L2 Prophylactic
Papillomavirus Vaccine

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L2-Oxidation and Multimerization

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Abstract

Current commercial prophylactic human papillomavirus (HPV) vaccines are based on virus-like particles assembled from the major capsid protein L1 and show an excellent safety and efficacy profile. Still, a major limitation is their rather narrow range of protection against different HPV types. In contrast, the minor capsid protein L2 contains a so-called major cross-neutralizing epitope that can induce broad-range protective responses against multiple HPV types. This epitope is conserved among different papillomaviruses (PV) and contains two cysteine residues that are present in the L2 proteins of all known PV types. The main challenge in developing L2-directed vaccines is to overcome the intrinsically low immunogenicity of the L2 protein. Previously we have developed a recombinant, L2-based prototype vaccine by inserting peptide epitopes spanning the cross-neutralizing L2 sequence into a bacterial thioredoxin (Trx) scaffold. These antigens induced high-titer neutralizing antibodies in mice. Here we address the question as to whether Trx scaffold multimerization may further enhance immunogenicity of the TrxL2 vaccine. We also demonstrate that the oxidation state of the conserved cysteine residues is not essential for vaccine functionality, but contributes to immunogenicity.
Introduction

To date, at least thirteen different types of human papillomaviruses (HPVs) are defined as ‘high-risk’ (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59) or ‘probably high-risk’ (HPV 68) for they have been linked to cancer development (3). These HPV types are consistently detected in biopsies from invasive cervical cancers. Still, there is a great discrepancy between the number of cancer cases and the frequency of HPV infections, which are very common among adults. It is assumed that most infections are cleared by the immune system and, in fact, only a small fraction of benign, HPV-positive lesions progresses to cancer.

Worldwide, the eight most frequent high-risk HPV types associated with cervical cancer include HPV 16, HPV 18, HPV 45, HPV 31, HPV 33, HPV 35, HPV 52 and HPV 58 (2). Although other, as yet poorly understood factors contribute to cervical cancer development, HPV infection is considered a key determinant of neoplastic progression (24, 39). Two commercial vaccines, Gardasil® and Cervarix® have been licensed in 2006 and 2007, respectively (13, 32). They are ‘virus-like particle’ (VLP) vaccines based on the L1 major capsid protein. Up to date, more than 100 million doses have been administered and both vaccines show an impressive safety and efficacy profile (17, 27). It is expected that the two vaccines will reduce the rate of cervical cancer in vaccinated women by 70-80%.

Despite their clinical success, VLP vaccines have some important limitations, the major one being their rather narrow range of protection. The principle underlying VLP vaccines is the induction of neutralizing antibodies that block virus infection by binding to surface, L1 protein loops that are highly heterogeneous among different HPV types (6-8, 10).
For this reason, anti-L1 neutralizing antibodies are highly HPV-type specific. For example, anti-HPV 16 antibodies usually fail to neutralize any other HPV type, besides HPV 16, although a limited degree of HPV 31 and HPV 33 protection is observed.

In contrast to L1, the minor capsid protein L2 contains a number of conserved epitopes that are targets for virus neutralization (11, 18, 28). One of these epitopes, spanning the amino acids (aa) 17-38 region of HPV 16 L2, has gained special attention as antibodies recognizing this region show neutralizing activity against a broad range of different papillomavirus (PV) types (12, 28, 29). This major cross-neutralizing epitope, that we mapped to the aa region 20-38 of L2, contains two cysteine residues (positions 22 and 28) that are conserved among the L2 proteins of all known PVs. These cysteine residues are buried and disulfide-bonded in mature HPV virions, and it has been suggested that disulfide bond reduction, after viral entry, may be critical for endosomal escape and infectivity (5).

The main challenge in developing L2-directed vaccines is to overcome the intrinsically low immunogenicity of the L2 protein. Previously, we have developed a recombinant, L2-based prototype vaccine by inserting the cross-neutralizing L2 20-38 epitope into a bacterial thioredoxin (Trx) scaffold (28). Despite the encouraging results obtained with the prototype TrxL2 vaccine, a detailed knowledge of all the factors (especially, higher-order multimerization and aggregation state of the antigen) potentially influencing immunogenicity and virus neutralization capacity posed an important aspect for further vaccine development. In fact, in various subunit vaccine settings, including L1-based vaccines, where VLPs were superior to pentameric L1 capsomeres in terms of immunogenicity (30, 35), antigen multiplicity and assembly state have been shown to be important determinants of vaccine immunogenicity and efficacy. Thus, the multimerization...
state of the L1 antigen is likely to be a major factor influencing immunogenicity, as observed
with other antigens (1, 26).

Here we investigate whether the effectiveness of the TrxL2\textsubscript{20-38} prototype vaccine can be
enhanced by intermolecular multimerization of the Trx scaffold and whether the oxidation
state of the L2 antigen influences immunogenicity. We also show that a neutralizing
monoclonal antibody generated by using the L2\textsubscript{20-38} peptide grafted to Trx as antigen
preferentially binds to the oxidized L2 epitope.
Materials & Methods

Construction of TrxL2 multimers

The Trx-HPV 16 L2(20-38)₃ construct was generated as previously described (28). Briefly, the L2 (20-38)₃ DNA was inserted into a modified pET28 plasmid bearing the sequence for a dual 6xHis-tagged version of *E. coli* thioredoxin (pTrx) using the *CpOl* site of the Trx coding sequence as the cloning site. Phosphorylated oligonucleotides encoding the 20-38 L2 sequence of HPV16 were ligated to *CpOl* digested pTrx. Following bacterial transformation, constructs bearing a tripeptide insertion of 20-38 L2 were isolated. For multimerization, an internal *BglII* site was removed from the Trx coding sequence of pTrx, while a *SfiI* site flanking the coding sequence was introduced via PCR. In a second PCR, a flanking *BglII* site was introduced. The product of this second PCR was blunt-end cloned into the StrataClone PCR Cloning Vector pSC-B-amp/kan (Agilent, USA). The TrxL2 insert was excised from the pSC-B-amp/kan vector via an *Ndel/XhoI* double digestion and ligated into *Ndel/XhoI* pre-digested and dephosphorylated pTrx. This resulted in a monomeric TrxL2 plasmid capable of multimerization (pTrx 1x). For dimer generation: pTrx 1x was first cleaved with *SfiI*, followed by *SfiI*-mediated TrxL2 excision from pSC-B-amp/kan, and *BglII* digestion. Next, the linearized, *SfiI*-cleaved pTrx 1x plasmid was combined with the *SfiI/BglII*-digested TrxL2 insert to generate the dimeric construct (pTrx 2x). A similar procedure was used to generate the trimer (pTrx 3x) and the tetramer (pTrx 4x).

Expression and purification of TrxL2 proteins

TrxL2 protein expression was induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to *E. coli* Rosetta cells (Merck4Biosciences, Darmstadt, Germany) transformed with
either the monomeric or the multimeric constructs (pTrx 1x-4x), which were then allowed to
grow for 12 – 16 h at 23-30°C. Following resuspension of the bacterial pellet (300 mM NaCl,
25 mM Tris, 0.16% Tween-20, 0.5 mM PMSF and 0.1 mg/ml lysozyme, pH 8.0) and lysis with
EmulsiFlex (Avestin, Canada), His-tagged TrxL2 proteins were bound to Ni\(^{2+}\)-substituted
affinity columns (1 ml; Amersham, GE Healthcare, UK), purified as per manufacturer’s
instructions and dialyzed against phosphate buffered saline (PBS) containing 300 mM NaCl.
Protein concentration was determined with Coomassie-Blue G250 (Bio-Rad Protein Assay
Dye Reagent) using bovine serum albumin as standard, as well by UV absorbance at 280 nm
with the use of calculated extinction coefficients. The composition and purity of individual
polypeptide preparations was assessed by electrophoretic analysis on 12.5% and 15% SDS
polyacrylamide gels. The composition of the various TrxL2 protein preparations was further
confirmed by immunoblotting using anti-Trx (K12Trx) and anti-L2 (K4L2\textsubscript{20-38} and K18L2\textsubscript{20-38})
antibodies (28).

**Chemical reduction, size-exclusion chromatography and DTNB assays of TrxL2 proteins**
Purified monomeric and multimeric TrxL2 proteins were adjusted to equal protein
concentrations ranging from 0.5 to 1.0 mg/ml. For chemical reduction, DL-Dithiothreitol
(DTT; BioUltra grade, Sigma-Aldrich, USA) was added to the TrxL2 proteins at either a final 10
mM concentration and incubated for 24 h incubation at 4°C (“mild treatment”), or at a 20
mM concentration and incubated at 37°C for 48 h (“harsh treatment”). Following DTT
treatment, protein samples were either analyzed immediately or snap-frozen in liquid
nitrogen.
Size-exclusion chromatograph (SEC) was performed with a Superdex 200 10/300 GL column
using a Äkta FPLC system, at a flow rate of 0.5 ml/min and a maximum pressure of 1.5 MPa,
L2-Oxidation and Multimerization

operated with Unicorn 5.0 (Amersham, GE Healthcare, UK). The running buffer was PBS for untreated proteins, or PBS supplemented with DTT for the reduced (DTT-treated) proteins. Elution was carried out with 2 column volumes (48 ml) of running buffer; 1 ml fractions were collected, stored at 4°C and analyzed for protein content and composition with the Coomassie dye-binding assay (see above) and SDS-PAGE.

The Ellman’s reagent (5,5'-dithiobis-(2-nitrobenzoic acid), DTNB; \( \varepsilon_{412\text{nm}} = 14,150 \text{ M}^{-1} \text{ cm}^{-1} \)) was used to quantify free cysteine, SH groups (20, 22). The assay was conducted in a final volume of 150 µl containing 0.1 mM DTNB and 2% SDS in 50 mM Na-phosphate buffer (pH 8.0); a calibration curve was constructed with increasing concentrations of N-acetyl-cysteine (Sigma-Aldrich, USA). The number of free cysteines/ protein molecule was determined by dividing the concentration of DTNB-reactive SH groups (OD_{412nm}/\varepsilon_{412nm}) by the concentration of protein utilized for the assay.

ELISA analysis of purified monomeric TrxL2

Individual wells of a flexible 96-well plate (BD Falcon, USA) were coated overnight at 4°C with 50 µl/well of purified monomeric or multimeric TrxL2 proteins (0.5 mg/ml) diluted 1:500 in PBS. After washing three times with PBS-0.3%Tween20, the plates were blocked for 1h at 37°C with 0.2% casein dissolved in PBS (50 µl/well). Next, monoclonal K18L220-38 (0.5 mg/ml) was serially diluted 1:2 on the plate (50 µl/well) starting from a 1:100 dilution. Incubation was allowed to proceed at 37°C for 1 hour before washing with PBS-0.3% Tween20. A secondary, goat-anti-mouse HRP-conjugated antibody (10 mg/ml, Dianova, Germany) was then added to the plate (50 µl/well) at a 1:3000 dilution and incubated for 1h at 37°C. After washing with PBS-0.3%Tween20 (100 µl/well), 1 mg/ml ABTS substrate (2,2'-
L2-Oxidation and Multimerization

Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; Sigma-Aldrich, USA), was added to the plate (100 µl/well) followed by the measurement of color development at 405 nm.

Immunization protocol

Six- to eight-weeks-old female BALB/c mice were purchased from Charles River (Sulzfeld, Germany) and kept in the animal house facility of the German Cancer Research Center under specific-pathogen-free conditions. Mice were immunized subcutaneously, three to four times at biweekly intervals, with 2–50 µg of the various TrxL2 antigens adjuvanted with 50% v/v Montanide ISA720 (Seppic, France). Doses used in initial immunization experiments (50 µg and 25 µg) were higher than those employed in later experiments (2 µg). While within this window, similar antibody responses are induced, comparisons were only made between groups receiving the same dose. Intermediate blood samples were taken after the second and/or the third immunization by puncture of the submandibular vein. Eight weeks after the last immunization, final blood samples were collected by cardiac puncture and neutralizing antibody titers were determined with an in vitro pseudovirion-based neutralization assay.

Pseudovirion-based neutralization assays

Pseudovirions were prepared as described previously (4, 19) with some modifications (34). Absence of cell culture contamination was confirmed by the Multiplex Cell Contamination Test (33). Neutralization assays were performed as described (34). Briefly, 50 µl of diluted polyclonal or monoclonal antibodies were combined with 50 µl of diluted pseudovirion stocks and incubated at room temperature for 30 minutes. Next, 50 µl of HeLaT cells (2.5 x
10^5 cells/ml) were added to the pseudovirion-antibody mixture and incubated for 48 hours at 37°C (5% CO₂). The amount of secreted Gaussia-luciferase was determined in 10 µl of cell culture medium using the coelenterazine substrate and Gaussia glow juice (PJK, Germany) according to the manufacturer’s instructions. A microplate luminometer (Victor³, Perkin Elmer) was used to measure culture medium-associated luminescence 15 minutes after substrate addition.

**Statistical analysis**

The nonparametric Mann-Whitney test, performed with GraphPad Prism 5.00 (GraphPad Software, San Diego, CA), was used to determine the statistical significance of differences between neutralization titers. Differences between groups were considered significant at \( p < 0.05 \).
Results

Intermolecular multimerization of the TrxL2 antigen

Our standard TrxL2 antigen already contains three intramolecular, tandemly repeated copies of the L220-38 epitope, but we reasoned that additional intermolecular multimerization of TrxL2(20-38)3 might further increase anti-L2 immune responses. To test this hypothesis, we generated a set of multimerized TrxL2 proteins by covalently connecting individual TrxL2 building blocks to each other via a 15-amino acids linker (Figure 1A). Each building block comprises a Trx protein bearing three copies of the HPV 16 L2 20-38 epitope, with individual epitopes separated from each other by a GGP spacer sequence. The monomeric and the multimeric TrxL2 proteins were all expressed at high levels in \textit{E. coli} (Figure 1B) and were purified by metal-affinity chromatography. All multimer preparations contained degradation products migrating as monomers (2x-TrxL2, 3x-TrxL2, 4x-TrxL2), dimers (3x-TrxL2, 4x-TrxL2) and trimers (4x-TrxL2), probably resulting from spacer sequence cleavage. An attempt to remove these degradation products by size-exclusion chromatography (SEC; see ‘Material & Methods’ for details) was not successful, due to the relatively small differences in size (and elution times) between cleavage products and the corresponding intact proteins. While performing these experiments, however, we found that the dimer, and to a greater extent the trimer and the tetramer, were eluting much earlier than one would expect simply based on the predicted molecular weight of these proteins. In other words, multimerized TrxL2 proteins but not the monomer appeared to assemble into soluble, high molecular weight aggregates, even though a fraction of the loaded multimeric proteins eluted at the expected volume, indicating that at least a subset of the protein is present in a non-aggregated form.
We determined that these soluble aggregates are composed of cross-linked TrxL2 proteins with an average molecular mass $\geq 600$ kDa (Figure 2).

Are multimeric or aggregated TrxL2 antigens more immunogenic?

To address this question, different mice were immunized with monomeric or multimeric TrxL2. No significant difference was observed in the HPV 16 neutralization titers of the four immunization groups, although the immunogenicity of the dimeric TrxL2 antigen was apparently lower than that of the other groups (Figure 3). The latter group had a mean titer of 390 (range: 50-800) while the 1x-TrxL2, 3x-TrxL2 and 4x-TrxL2 groups yielded mean titers of 3,080 (range: 200-12,800), 2,000 (range: 400-3,200) and 8,650 (range: 50-25,600), respectively.

Next, we wished to determine whether higher-order aggregation, on top of intermolecular multimerization, might influence Trxl2 immunogenicity. To this end, we set up a further immunization experiment based upon the following tetrameric antigens: i) unfractionated 4x-TrxL2, i.e., the mixture of purified non-aggregated and aggregated proteins derived from bacterial lysates by metal-affinity chromatography; ii) SEC-fractionated 4x-TrxL2 aggregates; iii) the purified, non-aggregated 4x-TrxL2 antigen obtained upon SEC fractionation; and iv) fractionated 4x-TrxL2 aggregates plus the corresponding non-aggregated antigen mixed together prior to immunization. As shown in Figure 4, all forms of the 4x-TrxL2 antigen, whether aggregate, non-aggregate or the two forms together, were found to be equally immunogenic, as indicated by the almost identical mean titers of approximately 2,000. A somewhat lower titer (800) was observed for the group that received the reconstituted aggregate/non-aggregate mix, but the difference was not statistically significant. Thus, under
the presently examined conditions, the aggregation state of TrxL2 does not appear to influence immunogenicity.

Influence of the oxidation and aggregation state of the antigen on immune performance

The above results (see the immune performance of the 4x-TrxL2 antigen in Figure 3) hinted at a slight improvement of immunogenicity produced by TrxL2 multimerization. At the same time, however, multimerization is accompanied by the appearance of soluble, high molecular weight aggregates, likely resulting from extensive, disulfide bond-mediated intermolecular cross-linking of the multimeric TrxL2 antigens. Each L2\textsubscript{20-38} epitope contains two cysteine residues, for a total of 6 cysteines/tripeptide insert, plus two Trx scaffold-associated cysteine residues flanking each L2 tripeptide insert. This corresponds to 8, 16, 24, and 32 cysteine residues for the 1x-TrxL2, 2x-TrxL2, 3x-TrxL2 and 4x-TrxL2 antigens, respectively.

We performed free SH-group titrations with DTNB to determine the degree of reduction of the various purified proteins. Untreated proteins extracted and purified from \textit{E. coli} were found to be almost completely (>95%) oxidized. Only a prolonged (“harsh”) treatment with DTT led to a significant disulfide reduction (53-100% after incubation with 20 mM DTT for 48 h at 37°C), thus indicating that the oxidized state is very stable (Figure 5).

We then analyzed the impact of disulfide-bond reduction on the aggregation state of the proteins by SEC. As shown by the representative results of the 3x-TrxL2 and 4x-TrxL2 proteins presented in Figure 6, most of the untreated protein was in the form of high molecular weight aggregates but a “mild” DTT treatment (24 h at 4°C) led to a strong...
decrease of such aggregated species. This indicates that at least a fraction of the disulfide bonds (S-S) is in fact intermolecular and that these bonds can be readily reduced and disrupted by a relatively mild DTT treatment. Only the “harsh” DTT treatment (48 h at 37”) led to a complete reduction of 4x-TrxL2 multimers. Although the SEC profiles were identical to those produced by the mild DTT treatment, intramolecular S-S bonds are also disrupted under these stronger conditions.

Next, we tested the immunogenic capacity of 4x-TrxL2 proteins with different cysteine oxidation states, using the fully oxidized monomeric protein as a reference antigen. As shown in Figure 7, the HPV 16 type-specific titers in animals immunized with untreated, oxidized 1x-TrxL2 and 4x-TrxL2 were similar (mean titers of approximately 10,000 and 5,000, respectively) even though there was a trend for higher titers in the 1x-TrxL2 group. Partial reduction of the 4x-TrxL2 protein led to a significantly reduced induction of anti-L2 neutralizing antibodies (mean titer of ~2,000) compared to the untreated tetrameric TrxL2 antigen, and this difference became even more pronounced in the case of sera from mice treated with fully reduced 4x-TrxL2 antigen preparations (mean titer ~900). A similar trend was observed when the same sera (raised against the HPV 16 L2 epitope) were tested for their capacity to cross-neutralize heterologous HPV 58 and HPV 33 pseudovirions.

Most of the untreated, oxidized 4x-TrxL2 protein is in the form of high molecular weight aggregates and this aggregation state is disrupted by partial reduction. Thus, the observed decrease in immunogenicity could reflect the breakage of intermolecular disulfide bonds. In contrast, the monomeric TrxL2 protein only contains minor amounts of aggregates. To determine whether the observed decreased immunogenicity of reduced 4x-TrxL2 is a result of intermolecular disulfide bond breakage we, therefore used oxidized and reduced
monomeric TrxL2 for immunization applying the same immunization schedule as done for
the previous 1x-TrxL2 versus 4x-TrxL2 immunization. As shown in Figure 8, in contrast to
what we observed with the 4x-TrxL2 antigen, reduced and oxidized 1x-TrxL2 were found to
be equally immunogenic with regard to HPV 16 type-specific neutralization (mean titers of
approximately 5,000 and 4,000, respectively). The same sera were also tested against other
HPV types. As there are sequence differences in the 20-38 epitope between different HPV
types, we reasoned that cross-protective responses are better indicators for the robustness
of the induced immune responses. We selected HPV 58 and HPV 33 for this analysis, as we
usually observe robust and impaired neutralization of these types using anti-HPV 16 L2
antisera, respectively. In fact, contrary to the finding for HPV 16 pseudovirions, the sera of
mice that had received the reduced monomer neutralized HPV 58 pseudovirions with a
lower efficiency compared to sera from mice immunized with the oxidized antigen. This
difference in immunogenicity of reduced versus oxidized monomer became even more
visible for neutralization of HPV 33.

The six cysteines of the three-fold repeated L2 epitope are flanked by two cysteine residues
provided by the Trx scaffold. In the oxidized form of the monomeric TrxL2 protein purified
from E. coli it can be assumed that also these Trx-associated cysteines form disulfide bonds,
either with each other or with L2-epitope cysteines. The lower immunogenicity of the
reduced TrxL2 antigen might, therefore, be a consequence of an altered, more relaxed
structure of the Trx scaffold. To address this question, we generated monomeric TrxL2
proteins in which the two Trx cysteines were replaced by serine residues (1xDM-TrxL2).
Immunization experiments indicated a significantly reduced immunogenicity of 1xDM-TrxL2
compared to the unmodified 1x-TrxL2 antigen, as reflected by type-specific titers on HPV 16
(~1,000 and ~4,000, respectively) as well as by the further reduced cross-protective titers measured on heterologous HPV pseudovirions (Figure 8). In keeping with previous observations that the conformational quality (21) as well the immunogenicity (S.O and A.B., unpublished results) of multi-epitope Trx fusion constructs tend to deteriorate above a certain insert size threshold, this finding further indicates that intra-scaffold S-S bond formation is critical for epitope presentation as well as for the structural integrity and immunogenicity of Trx-based peptide antigens. Interestingly and most unexpectedly, reducing 1xDM-TrxL2 restored its immunogenicity to a large degree, at least when considering HPV 16 neutralization (mean titer ~3,500). However, when looking at neutralization of HPV 58 and HPV 33 pseudovirions, four out of the ten mice immunized with reduced 1xDM-TrxL2 failed to develop measurable responses, confirming the above described observation that oxidized L2 epitopes are better immunogens in respect to cross-protective responses.

**Preferential recognition of the oxidized L2\textsubscript{20-38} epitope by a neutralizing anti-L2 monoclonal antibody**

Using the monomeric TrxL2\textsubscript{(20-38)} antigen, we previously generated a monoclonal anti-L2 antibody (named K18L2\textsubscript{20-38}) that recognizes the L2\textsubscript{20-38} epitope and thereby neutralizes a number of HPV types with high efficiency (29). To determine the preference of this antibody for either the oxidized or reduced from of the antigen, we performed ELISA analyses using different forms of the monomeric TrxL2 protein as capture antigens. As shown in Figure 9A, the K18L2\textsubscript{20-38} mAb binds to the reduced and to the oxidized form of Trx-HPV 16 L2 equally well. However, when we used TrxL2 antigens in which the natural HPV 16 L2 sequence was
replaced by a tripeptide of a variant epitope (“HPV 16 L2 mimotope”) presenting a cysteine-
containing, but otherwise suboptimal target sequence, K18L220-38 reacted more strongly with
the oxidized than the reduced form of the antigen (Figure 9B). In line with the results
obtained with homologous and heterologous neutralization assays, this indicates that the
intramolecular disulfide bond is not absolutely required for epitope recognition, yet
contributes to the binding of HPV neutralizing antibodies.
Discussion

Commercial prophylactic HPV vaccines are based on virus-like particles, composed of 360 units of the L1 major capsid protein. Although it has been shown that L1 capsomeres also mount neutralizing antibody responses, albeit lower ones than those elicited by VLPs, it is clear that neutralizing L1 epitopes are highly conformation-dependent. All epitopes identified so far are formed by at least two L1 loops displayed on the surface of L1 capsomeres or VLPs. In contrast to L1, all neutralizing epitopes associated with the minor capsid protein L2 seem to be ‘linear’ epitopes, i.e. the neutralizing antibodies they elicit react with, and bind to the denatured L2 protein as well as to short synthetic L2 peptides. The possible existence of conformational neutralizing epitopes has not been demonstrated for the L2 protein so far, and this may explain the lack of a native and highly efficient L2 immunogen. While linear antigens are usually less immunogenic than the conformational ones, they often come with the benefit of a greater ease of production. Of the several linear L2 epitopes that have been described as neutralizing (12, 18, 28), only a few are sufficiently conserved to provide a broad range of protection. In particular, there is general consensus as to the presence of such a cross-neutralizing epitope within aa 17-38 of the L2 protein (12, 28). This region shows a remarkable conservation among papillomaviruses and contains two fully conserved cysteine residues. By forming disulfide bonds, these residues could promote the formation of a self-sustained epitope structure even in the absence of other L2 regions, and outside of a capsid assembly context. Various strategies are being actively pursued by different groups to confer immunogenicity to L2-derived (poly)peptides of different lengths. With the exception of a monomeric L2 (aa 17-36) lipopeptide formulation (14), most of these strategies rely on displaying neutralizing L2 epitope-bearing regions, most notably the 17-36
L2-Oxidation and Multimerization

cross-neutralizing epitope, as part of repetitive, high molecular weight assemblies. Cross-
protective responses have been elicited by virus-like display of the 17-36 L2 epitope on VLPs
of bacteriophage PP7 (36) as well as on HPV16 L1 VLPs (31) and on adeno-associated virus
(AAV) particles (23). L2 (aa 13-47) has also been successfully incorporated into L1
capsomeres although the elicited cross-protection was quite narrow (37). Heat-killed
*Lactobacillus casei* cells displaying a larger portion of L2 (aa 1-224) were successful in
inducing cross-protective antibodies even after oral administration (38). Another potent
approach does not employ virus-like, capsomere or bacterial display assemblies but instead
relies on concatenated multitype L2 fusion proteins encompassing the aa 11-88 region from
various HPV types (16). Unlike the high molecular weight approaches mentioned above, our
first-generation TrxL2 antigens utilized the relatively small bacterial thioredoxin protein (109
aa) as a scaffold to display repetitive 20-38 L2 sequences. Also, while most approaches
include L2 sequence information spanning relatively large regions of the L2 N-terminus (aa 1-
120), TrxL2 contains repetitions of the shorter 20-38 L2 epitope sequence. This has the
advantage of eliciting targeted antibody responses focused on the conserved, cross-
protective L2 region. We recently found that immunization of mice with a mix of TrxL2
antigens bearing 20-38 L2 peptides from HPV 16, HPV 31 and HPV 51 elicits *in vitro-
detectable antibodies neutralizing 13 of 14 tested high-risk HPVs (Seitz et al., in preparation).
Further options to increase the cross-protection capacity of the TrxL2 prototype vaccine may
rely on the incorporation of additional L2 peptides, such as the aa 56-75 peptide, which has
recently been shown to represent another cross-protective epitope (25).

We reasoned that disulfide bond formation within the L2<sub>20-38</sub> epitope could be essential for
c vaccine efficacy and wished to determine its impact on immunogenicity. Our analysis was
complicated by the fact that the thioredoxin scaffold itself structurally constrains the
L2-Oxidation and Multimerization

inserted (multi)peptide epitope via disulfide bond formation. In fact, heterologous peptides are interposed between two cysteine residues provided by the active (‘display’) site of thioredoxin and this guarantees proper surface exposure of the inserted peptide epitopes (9). These cysteine residues are disulfide-bonded in native thioredoxin and, as revealed by the results of DTNB titrations carried out on untreated monomeric TrxL2 (>95% oxidized), formation of this disulfide bond is apparently compatible with the insertion of the L2_20-38 tripeptide and presumably increases immunogenicity.

Multimerization of TrxL2 led to aggregation of a substantial fraction of the multimeric proteins. This aggregation effect was not observed for monomeric TrxL2. Aggregate production was shown to be mediated by intermolecular disulfide (S-S) bond formation, and while these were easily disrupted by DTT treatment, intramolecular S-S bonds were much more resilient to reduction. This finding points to a marked stability and structural rigidity of the oxidized intramolecular multipeptide epitope, a feature of the TrxL2 antigen that may have important implications in terms of immunogenicity. In general, oxidized or reduced TrxL2 proteins proved to be extremely stable. Although this in vitro stability does not necessarily reflect the in vivo situation (i.e., administration of oxidized or reduced, adjuvanted antigens to mice), TrxL2 antigens maintained their oxidation state even after multiple freeze-thaw cycles and prolonged handling at the bench. Immunization experiments showed that neither the aggregates nor the non-aggregates of the multimers were more immunogenic than the non-aggregated monomeric TrxL. The Trx multimerization strategy we describe, even though it did not appreciably increase immunogenicity in the case of the HPV 16 L2(20-38) epitope, is quite straightforward and may turn out to be useful as an immunogenicity enhancer in other recombinant peptide epitope contexts.
The reduced multimeric TrxL2 antigens, especially those that were fully reduced at the time of administration, were less effective than the oxidized antigens at eliciting neutralizing responses especially with regard to cross-protection. A very similar behavior was observed for the reduced monomeric TrxL2 antigen, which elicited type-specific titers comparable to those of its oxidized counterpart on HPV 16, but a suboptimal performance in the case of more stringent cross-protection titration analyses carried out on HPV 58 and HPV 33. One could even imagine that the fully reduced antigen at the time of administration may become partially oxidized over time after injection into the mouse, and that this oxidized antigen fraction may be the main contributor to the observed immunogenicity. In fact, the extracellular milieu is extremely oxidizing (15) and hence antigen oxidation after administration seems a likely possibility. Regardless of what may happen in vivo, both the oxidized and the reduced form of TrxL2 elicited potent neutralizing responses. This indicates that the TrxL2 candidate vaccine is quite robust and intrinsically insensitive to uncontrolled changes in oxidation as well as aggregation state.

Ultimately, non-aggregated, fully oxidized monomeric TrxL2, which is what we purify from E. coli and the easiest antigen form to produce with the least batch-to-batch variation, appears to be the best lead for the further development of a broadly cross-protective, low-cost anti-HPV vaccine.
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L2-Oxidation and Multimerization

**Titles and Legends to Figures**

**Figure 1.** Monomeric and multimeric TrxL2 antigens. (A) Thioredoxin scaffolds containing a tripeptide of the HPV 16 20-38 L2 are multimerized and separated by a 15 aa linker (sequence: GAAGPPGGWPRGSH). Intramolecular disulfide bridges (S-S) are mediated between L2 insert and/or scaffold cysteines within one molecule. Intermolecular S-S are mediated between L2 insert and/or scaffold cysteines of separate molecules and lead to aggregation. (B) Expression of mono- and multimers in *E. coli* resulted in soluble products. M = marker in kDa

**Figure 2.** Size-exclusion chromatography reveals aggregation of the soluble multimers. After Ni²⁺-chromatography, the purified TrxL2 proteins were analyzed via size-exclusion chromatography using a Superdex200 column. Except for the monomeric TrxL2, large portions of the multimerized antigens were present as soluble high molecular weight forms with an average of ~ 600 kDa. The aggregate (~600 kDa) and non-aggregate elution peaks are labeled, and the elution volumes are indicated above the peak. mAU = milliabsorbance units

**Figure 3.** Comparable immunogenicities of TrxL2 mono- and multimers determined by *in vitro* neutralization assay. The HPV 16 neutralization titers (IC50) of the 1x-TrxL2, 2x-TrxL2, 3x-TrxL2 and 4x-TrxL2 immune sera are shown. L2 polyclonal sera were titrated in 1:2 steps starting with a 1:50 dilution. Each dot represents one mouse serum with horizontal bars indicating the mean titers. Mice were immunized three times at biweekly intervals with 50 µg antigen adjuvanted with 50% v/v Montanide ISA720. Final sera were collected eight weeks after the third immunization.
**Figure 4.** Aggregate and non-aggregate forms of tetrameric TrxL2 are equally immunogenic.

The HPV 16 neutralization titers for the 4x-TrxL2, 4x-TrxL2 non-aggregate (non-agg) and/or aggregate (agg) immune sera are shown. Sera were serially titrated 1:3 starting with a 1:100 dilution. Each dot represents one mouse serum with horizontal bars indicating the mean titers. Animals were immunized three times at biweekly intervals with 25 µg of antigen formulated with 50% v/v Montanide ISA720. Final sera were collected eight weeks after the third immunization.

**Figure 5.** Oxidation status of purified mono- and multimer TrxL2 proteins. The percentages indicate the degree of reduction as determined via DTNB assay. Untreated TrxL2 purified from *E. coli* is almost completely oxidized. Treatment of the samples with 10-20 mM DTT for different lengths of time and at different temperatures leads to partial or complete reduction.

**Figure 6.** Partial reduction of TrxL2 multimers leads to disappearance of high molecular weight aggregates. The percentage of cysteine residues in reduced state is indicated.

**Figure 7.** Oxidized tetrameric and monomeric TrxL2 elicit similar type-specific titers but cross-protective titers are higher for the latter. Reduced tetrameric TrxL2 is less immunogenic than the oxidized counterpart. TrxL2 monomer and tetramer antigens were fully or partially reduced and then used for mouse immunization. The HPV 16, HPV 58 and HPV 33 neutralization titers were determined. For HPV 16 and HPV 58, sera were serially titrated in 1:3 steps starting with a 1:100 dilution while for HPV 33 1:2 steps starting with a 1:50 dilution were employed. Each dot represents one mouse serum with horizontal bars.
indicating the mean titers. Fifteen mice per group were immunized a total of four times at biweekly intervals with 2 µg antigen adjuvanted with 50% v/v Montanide ISA720. Final sera were collected eight weeks after the fourth immunization.

Figure 8. While HPV16 type-specific titers are similar, the oxidized monomer surpasses its reduced counterpart in terms of cross-protective titers. The Cys → Ser exchange (1xDM-TrxL2) in the Trx scaffold lowers immunogenicity but reduction restores it. Mice were immunized with monomeric TrxL2 proteins which were in an oxidized or reduced state. Shown is a comparison of the induction of neutralizing antibodies by 1x-TrxL2 and 1xDM-TrxL2, a protein in which both scaffold cysteines were exchanged for serines. For HPV 16 and HPV 58, sera were serially titrated in 1:3 steps starting with a 1:100 dilution while for HPV 33 1:2 steps starting with a 1:50 dilution were employed. Each dot represents one mouse serum with horizontal bars indicating the mean titers. Animals were immunized with 2 µg antigen adjuvanted with 50% v/v Montanide ISA720 a total of four times at biweekly intervals. Final sera were collected eight weeks after the fourth immunization.

Figure 9. Binding of cross-neutralizing antibody K18L2-20-38 to a reduced or oxidized target sequence. K18L2-20-38 binding to reduced or oxidized 20-38 L2 epitope of HPV 16 (A) and to a mimotope that deviates from the HPV 16 L2 sequence (B). While reduction of the epitope does not influence K18L2-20-38 to the native sequence, it has a strong impact on recognition of a not-perfect epitope indicating that a disulfide bonds are not an absolute requirement for recognition but contribute to binding. Data points show the mean of duplicates with SD. HPV 16 20-38 L2: KTCKQAGTCPPDIIPKVEG; mimotope ‘18-32 L2’: TTMYCKSTDNCPSDV.
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Literature


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ability to assemble into larger particles correlates with higher immunogenicity. Journal of virology 83:7690-7705.


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