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Reformulation of a thermostable broadly protective recombinant vaccine against human papilloma virus

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Summary

The causal relationship between Human Papilloma Virus (HPV) infection and cervical cancer has motivated the development, and further improvement, of prophylactic vaccines against this virus. 70% of cervical cancers, 80% of which in low-resources countries, are associated to HPV16 and HPV18 infection, with 13 additional HPV types, classified as high-risk, responsible for the remaining 30% of tumors. Current vaccines, Cervarix® (GlaxoSmithKline) and Gardasil®(Merk), are based on virus-like particles (VLP) obtained by self-assembly of the major capsid protein L1. Despite their undisputable immunogenicity and safety, the fact that protection afforded by these vaccines is largely limited to the cognate serotypes included in the vaccine (HPV 16 and 18, plus five additional viral types incorporated into a newly licensed nonavalent vaccine) along with high production costs and reduced thermal stability, are pushing the development of 2nd generation HPV vaccines based on minor capsid protein L2. The increase in protection broadness afforded by the use of L2 cross-neutralizing epitopes, plus a marked reduction of production costs due to bacterial expression of the antigens and a considerable increase in thermal stability could strongly enhance vaccine distribution and usage in low-resource countries. Previous studies from our group identified three tandem repeats of the L2 aa. 20-38 peptide as a strongly immunogenic epitope if exposed on the scaffold protein thioredoxin (Trx).

The aim of this thesis work is the improvement of the Trx-L2 vaccine formulation with regard to cross-protection and thermostability, in order to identify an antigen suitable for a phase I clinical trial. By testing Trx from different microorganisms, we selected *P. furiosus* thioredoxin (*Pf*Trx) as the optimal scaffold because of its sustained peptide epitope constraining capacity and striking thermal stability (24 hours at 100°C). Alternative production systems, such as secretory Trx-L2 expression in the yeast *P. pastoris*, have also been set-up and evaluated as possible means to further increase production yields, with a concomitant reduction of production costs.

Limitations in immune-responsiveness caused by MHC class II polymorphisms –as observed, for example, in different mouse strains- have been overcome by introducing promiscuous T-helper (Th) epitopes, e.g., PADRE (Pan DR Epitope), at both ends of *Pf*Trx. This allowed us to obtain fairly strong immune responses even in mice (C57BL/6) normally unresponsive to the basic Trx-L2 vaccine. Cross-protection was not increased, however. I thus designed, produced and tested a novel multi-epitope formulation consisting of 8 and 11 L2(20-38) epitopes derived from different HPV types, tandemly joined into a single thioredoxin molecule ("concatemers").

To try to further increase immunogenicity, I also fused our 8X and 11X *Pf*Trx-L2 concatemers to the N-terminus of an engineered complement-binding protein (C4bp), capable to spontaneously assemble into ordered hepatmeric structures, previously validated as a molecular adjuvant. Fusion to C4bp indeed improved antigen presentation, with a fairly significant increase in both immunogenicity and cross-protection.

Another important issue I addressed, is the reduction of vaccine doses/treatment, which can be achieved by increasing immunogenicity, while also allowing for a delayed release of the antigen. I obtained preliminary, yet quite encouraging results in this direction with the use of a novel, solid-phase vaccine formulation, consisting of the basic *Pf*Trx-L2 vaccine and its C4bp fusion derivative adsorbed to mesoporus silica-rods (MSR).

Sommario

La determinazione della correlazione tra infezione da Papillomavirus (Human Papillomavirus, HPV) e carcinoma cervicale, è stata cruciale per lo sviluppo di strategie profilattiche vaccinali atte alla prevenzione del tumore. Nonostante siano stati classificati 15 sierotipi ad alto rischio, il 70% dei tumori è associato all' infezione da parte di HPV16 e HPV18, la cui incidenza aumenta notevolmente nei paesi in via di sviluppo, dove i trattamenti sanitari sono insufficienti. I vaccini commerciali ad oggi esistenti, Cervarix® (GlaxoSmithKline) e Gardasil® (Merk), sono basati sulle virus like particles (VLP) ottenute mediante self-assemblaggio della proteina capsidica maggiore L1. Nonostante l'elevata immunogenicità e sicurezza, la limitata protezione ai soli sierotipi inclusi nelle formulazioni (HPV 16 and 18, più 5 sierotipi addizionali incorporati nell' ultimo vaccino approvato nonavalente), unita all'elevato costo di produzione, ha spinto la ricerca verso strategie vaccinali di seconda generazione basati sulla proteina capsidica minore L2. L'aumento dello spettro di protezione ottenuto dall'uso di epitopi L2 cross-neutralizzanti, unito ad una riduzione dei costi di produzione, grazie alla possibilità di espressione in batterio, e all'aumento della stabilità termica, possono fortemente incrementare la distribuzione del vaccino nel paesi in via di sviluppo. Studi precedenti del nostro gruppo hanno identificato il tripeptide L2 20-38 come l'epitopo maggiormente immunogenico se inserito in uno scaffold proteico quale la tioredossina (Trx).
Scopo di questa tesi è il miglioramento della formulazione vaccinica Trx-L2 sia in termini di termostabilità che di cross-protezione con l'intento di identificare un antigene da proporre per un trial clinico di fase I. Sottoponendo Trx, derivanti da diversi organismi, a studi di termostabilità, è stato possibile selezionare la Trx di *P. furiosus* (*Pf*Trx) come scaffold ottimale grazie alla sua elevata stabilità termica (100°C per 24h) e il maggiore constraining strutturale. L'uso del sistema di produzione basato sulla secrezione nel lievito *P. pastoris*, progettato e investigato in questa tesi, si è dimostrato un' alternativa valida per aumentare la produzione antigenica con una concomitante riduzione dei costi di produzione. Le limitazioni tra diverse specie dovute all'elevato polimorfismo delle MHC di classe II, sono state superate inserendo epitopi T helper (Th) promiscui, in particolare PADRE (Pan DR Epitope), ad entrambe le estremità della *Pf*Trx. Questo permette di stimolare una forte risposta immunitaria anche in topi (C57BL/6) normalmente non responsivi alla formulazione vaccinica di base Trx-L2 . Se questi epitopi Th permettono di superare i polimorfismi di specie, non consentono però un aumento dello spettro di protezione. A questo scopo, ho progettato, prodotto e testato una nuova formulazione formata da 8 e 11 epitopi L2(20-38) derivati da diversi sierotipi HPV, fusi insieme in una singola molecole all'interno della Trx ("concatenameri").
Per incrementare ulteriormente la risposta immunitaria, ho fuso all'N-terminale dei concatenameri 8X e 11X *Pf*Trx-L2, il dominio ingegnerizzato di oligomerizzazione della complement-binding protein (C4bp), in grado di assemblarsi spontaneamente in una struttura eptamerica, precedentemente valida come adiuvante molecolare. La fusione con C4bp permette un aumentata presentazione antigenica con conseguente incremento dell'immunogenicità e della cross-protezione. Un altro importante aspetto affrontato è la riduzione del numero di somministrazioni, che può essere ottenuto aumentando l'immunogenicità insieme ad rilascio graduale dell'antigene. Dati preliminari incoraggianti sono stati ottenuti con l'uso di nuova formulazione su fase solida, basata sul vaccino di base *Pf*Trx-L2 e la corrispondente versione fusa al C4bp, adsorbite a microparticelle mesoporose di silice (MSR).

1 Introduction

1.1 Impact of Human Papillomavirus in cancer

Infection is one of the main risk factors for cancer development[1].

Cervical carcinoma is the fourth most common cancer in woman, with an approximately 530,000 new cases and 270,000 deaths worldwide in 2012. The majority of cervical cancer new cases and deaths (87%) occurs in the less developed regions (International Agency for Research on Cancer, 2013) (*Fig. 1a and 1b*): in sub-Saharan Africa, 34.8 new cases of cervical cancer are diagnosed per 100,000 women annually, and 22.5 per 100,000 women die from the disease, in contrast to 6.6 and 2.5 per 100,000 women, respectively, in North America. The drastic differences can be explained by the absent of efficient screening programs in lower-resource countries, due to high cost of realization (6.6 billion in U.S.)[2,3], associated to social rejection of these health care practices (pelvic exam for cytological test as Pap smears and HPV DNA testing).

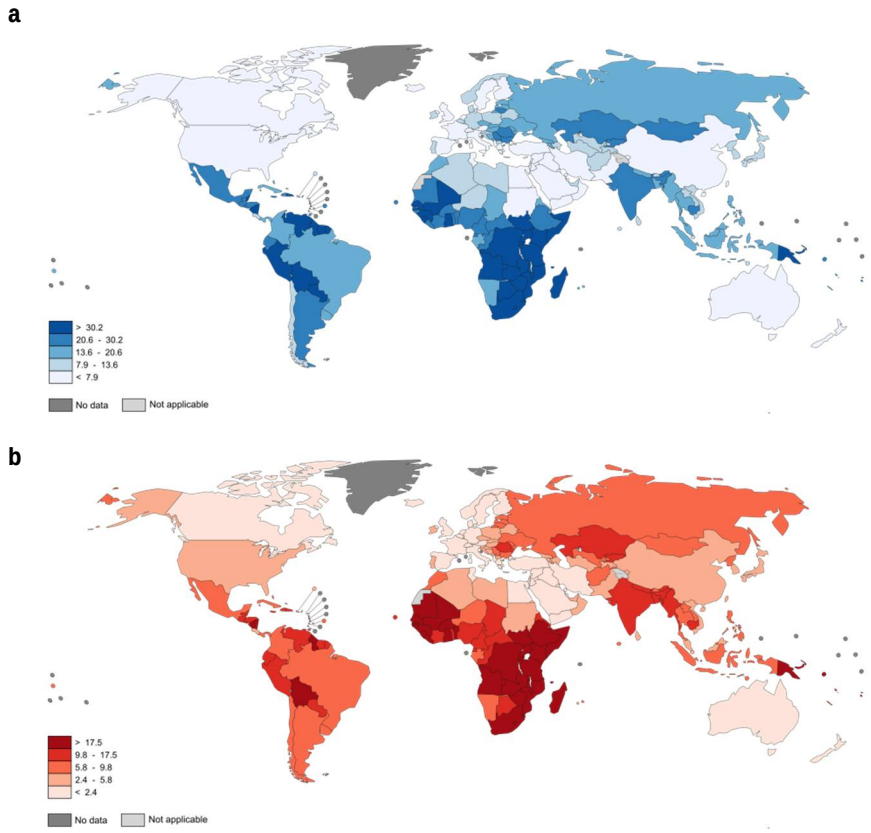

Fig. 1 Estimated cervical cancer incidence and mortality worldwide. *Estimated incidence (a) and mortality (b) of cervical carcinoma worldwide. Mortality varies 18-fold between the different regions of the world, with rates ranging from less than 2 per 100,000 in Western Asia, Western Europe and Australia/New Zealand to more than 20 per 100,000 in Melanesia (20.6), Middle (22.2) and Eastern (27.6) Africa. Estimated age-standardized rates (World) per 100,000.*
Adapted from GLOBOCAN 2012.

Infection with Human Papillomavirus (HPV) is the start point in the cervical cancer development[4,5], even if the only infection cannot be a sufficient cause for all cervical carcinoma: viral, host, and environmental factors may influence the course of HPV infection such as HIV coinfection, smoking, younger mothers and high number of children[6–10]. HPV is considered the most common sexually transmitted infection (STI)[11] but, most of infected people (80-90%) clearing the infections without overt clinical disease in the first 6-24 months after infection[12–18].

Papillomavirus comprises an heterogeneous family of DNA viruses, that are able to infect more than 20 different mammalian species, as well as birds and reptiles[19], in a species-specific manner. Based on nucleotide sequence homology, they are classified in 16 genus, each divided in different species[20]. The 5 genera in which are divided the HPVs (more than 150 types[21]) comprise different types having different life-cycle characteristics and disease associations[19,22–24] (*Fig. 2*).

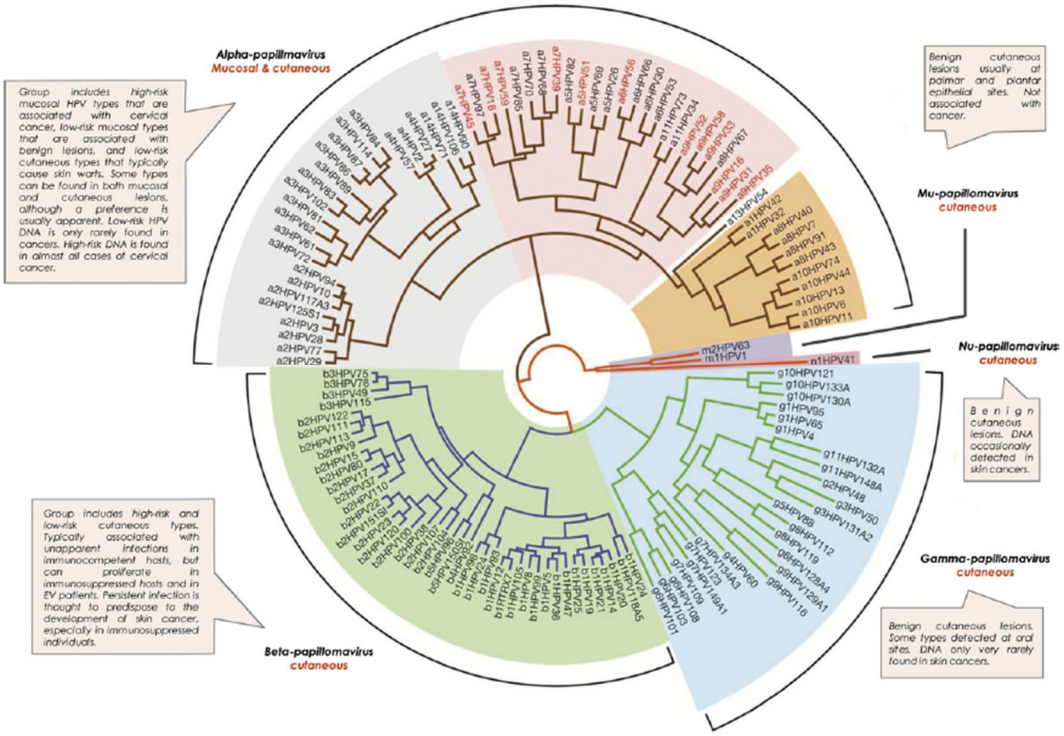

Fig. 2 Classification of HPVs based on sequence homology[69]. *The around 150 HPVs are divided in 5 genera: alpha (pink, violet and orange), beta (green), gamma (light blue), mu (blue) and nu (purple). Carcinogenic mucosal HPVs (pink box) are classified in the alpha papillomavirus genera while the cutaneous types are present in all genera. The Beta papillomavirus comprises the cutaneous types associated to the development of NMSC in immunosuppressed patients.*

The major group, Alpha papillomaviruses, comprises both cutaneous and mucosal types. Cutaneous HPVs are essentially ubiquitous and associated with skin warts while mucosal ones are transmitted by genital contact or rarely by vertically transmission during the birth[25–27] and are associated to the development of different cancers. These HPVs are divided in two category based on their capacity to cause tumors: low-risk HPVs (LR HPVs), such as HPV6 and HPV11, are the major sexually transmitted pathogens and are thought to affect around 1% of the sexually active population causing condylomas acuminata, as condyloma of Buschke-Lowenstein, in patients who have a genetic defect that makes them unable to control or clear these infections[28–33]. They can also infect oral sites where they are generally associated with benign papillomas[34,35] and are considered the etiological agents in respiratory papillomatosis (RRP)[36–39] (where HPV11 is more aggressive than HPV6[40,41]).

In contrast, high-risk HPV (HR HPVs), such as HPV16 and HPV18, cause mucosal lesions that can progress to high-grade neoplasia and cancer[42,43]. Worldwide, HPV16 infection is correlated to 60.6% of cervical cancer and its association with HPV18 covers the 70.8% of these tumor. HPV16 is commonly associated with squamous cell carcinoma, while HPV18 is the predominant type found in adenocarcinomas and neuroendocrine carcinomas[44]. 96% of cervical cancers were attributable to one of 13 HPVs (HPV16, 18, 45, 33, 31, 52, 58, 35, 39, 51, 59, 68 and 56 types) defined carcinogenic by IARC (group 1 and 2A)[45]. Incorporation of seven more types (HPV26, 53, 66, 67, 70, 73 and 82), identified as single infection in rare cervical cancers[46], adds another 2.6% to totalize 98.7% of all HPV-positive cervical cancers (*Fig. 3*).

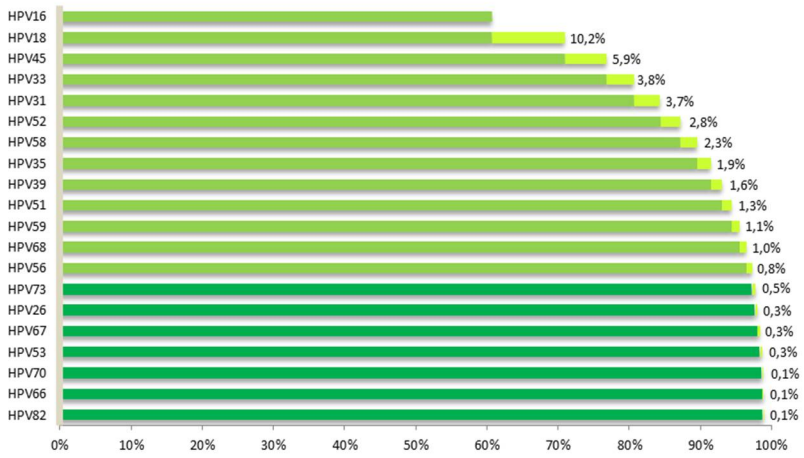

Fig. 3 Prevalence of HPVs types in cervical carcinoma worldwide[45]*. HPV16's infection is present in most cases (60,6%) of cervical carcinoma and with HPV18 covers around 70% of all HPV-positive cervical cancers. Incorporating all carcinogenic HPVs, prevalence arrived to 98%. light green: carcinogenic HPVs based on IARC's classification; dark green: carcinogenic HPVs based on Halec et al., 2014*[46]*; yellow: contribution of each HPVs on HPV16's prevalence.*

HPV types prevalence varies in different geographical areas: HPV16 is the most common types worldwide followed by HPV18, HPV31 and HPV33 in Europe, HPV53, HPV52 and HPV18 in North America, HPV31, HPV18 and HPV53 in Central America, HPV58, HPV18 and HPV45 in South America, HPV52, HPV18 and HPV58 in Africa and HPV52, HPV58 and HPV18 in Asia. In particular, in Africa, the 1% prevalence is estimated for more than the one or two HPV types: in this context a broad-spectrum vaccine is needed[10].

Importantly, these viruses are also associated with cancer in other sites, such as, vagina, vulva, anal transformation zone, head and neck cancers (tonsils, oropharynx and base of tongue) and penile[47–52] (*Fig. 4*). Males play an important role as vectors and/or reservoirs of HR HPVs, even if the progression to penile carcinoma is rare [44]. Several studies have investigated the prevalence of HPV in cancers of the mouth and pharynx defining an average of 23.5% for cancers of the oral cavity and 35.6% for oro-pharyngeal cancers with HPV16 as a predominant type (87% and 68% of HPV-infected oro-pharyngeal cancers and oral cavity, respectively)[53].

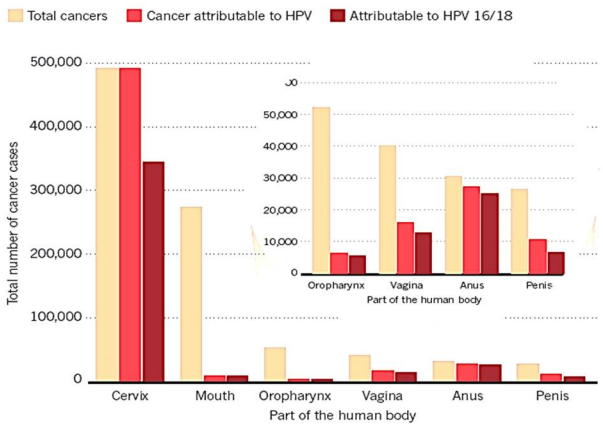

Fig. 4 Prevalence of HPV-associated cancers worldwide*[10]*. *Annual number of reported cancers related to HPV-infection for each indicated bodies site.*

The second major group, known as Beta papillomavirus, includes cutaneous HPVs that cause unapparent or latent infections in the general population; in some patients, such as immune-suppressed individuals or Epidermodyplasia verruciformis (EV)' patients, they can be involved in the development of non-melanoma skin cancer (NMSC), in particular squamous cell carcinoma (SCC)[54–59]. In contrast to cervical carcinoma, no specific HPV subtypes have been associated with NMSC. Several studies identified different β-HPVs correlated with this cancer, in particular the β2-subgroup[60,61]. The high prevalence (90%) of HPV5 and 8 (β1) in SCC, developed in EV patients, permits to define them as "possible carcinogenic HPVs"[59,62,63]. Although a lot of epidemiological

studies, the role of cutaneous β-HPVs as etiological agents in the development of NMSC needs further investigation.

1.2 Human Papillomaviruses' biology and life cycle

Papillomaviruses are small (50nm in diameter), icosahedral and non-enveloped viruses with around 8 kb dsDNA genome[64] that encodes, from same DNA strand and orientation, two types of proteins, based on their temporal expression: early proteins (E1, E2, E4, E5, E6 and E7), that maintain regulatory functions, and late proteins (L1 and L2), that forms the capsid of the virion. There is also a non-coding region, termed the long control region or upstream regulatory region (LCR or URR) that contains most of the regulatory elements involved in viral DNA replication and transcription as well as the viral origin of replication (*Fig. 5a*).

Although the highly conserved structure, there are some differences, in both LCR and coding region, between different genera, in particular beta and alpha HPV. For instance, beta HPV genome is shorter compared to alpha HPV (7.4kb instead 7,7Kb), dues to the reduced size of LCR (400bp compared to 650-700bp in other HPVs). About coding region, most of beta HPVs lack the E5 gene and present a longer E2 ORF (open reading frame). In the other hand, HVP16, HPV18 and HPV31 present an additional early protein, E8^E2C, that represses the expression of E6 and E7 and, consequently, cellular proliferation without proapoptotic activities[65–67].

The life cycle of HPVs is bound to the life cycle of its host cells, the keratinocytes[68,69] (*Fig. 5b*), a specialized cell type that differentiates to form the surface of the skin, as well as the oral and genital mucosa[70]. HPV virions infect the basal cells through damaged areas of the epithelium. The first step of infection requires the interaction between viral major capsid protein L1 and extracellular matrix (ECM) or basal lamina, based on charge-charge and polar interactions with heparin sulphate proteoglycan (HSPG)[71–74], in particular syndecan-1[75], with at least two different HS-binding site[74,76,77]. Recent studies suggest also laminin, in particular the ECM resident laminin 332 (known also as laminin 5), as possible receptor that facilitates attachment of virions that have encountered HS in the upper layers of the epithelium[78,79]. Due to minor structural differences between different virus types (e.g. HPV16, 11, 18 and 35[80]), it is possible that initial interaction/attachment to the ECM and cell surface might be HPV type specific: L1 protein displays structural diversity that could impact to receptor engagement, including binding to attachment receptors as well as putative entry receptors.

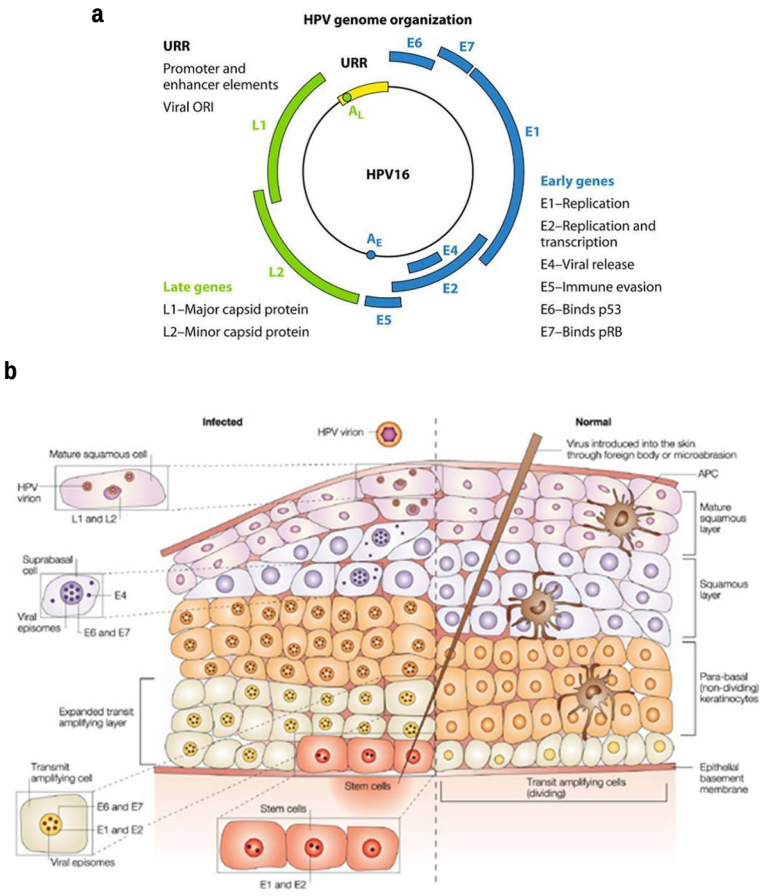

Fig. 5 HPV genome and life-cycle in the cutaneous and mucosal epithelium[134,244]***.*** *a) HPV genome organization. Only one strand of the HPV dsDNA codified for 6 early proteins (E1,E2,E4,E5, E6 and E7) and 2 late proteins (L1 and L2). b) The early end late protein expression in the different epithelia layers. After infection, E1 and E2 are expressed in the first step of cells differentiation and regulate viral replication The following genome amplification is mediated by E4, E5 and also the oncogenic proteins E6 and E7. E4 is also expressed in the last phase of differentiation with L1 and L2 proteins to assemble new virus particles.*

The conformational changes that occurs after these interactions, including partial externalization and cleavage of N-terminal region of minor capsid protein L2 by Cyclophilin B (CyPB) and Furin, respectively[81–85], facilitate the recognition of a non-HSPG receptor on the basal keratinocyte, through cell-surface binding motifs of L2[86]. The nature of this second factor remains controversial[83,87–94], but allows virus internalization (multiple pathways have been identified as for HPV entry[95–101]), virus uncoating and viral genome's transfer to the nucleus in a L2-DNA complex to ubnuclear promyelocytic leukemia protein (PML) bodies [69,83,87,102,103] (*Fig. 6*).

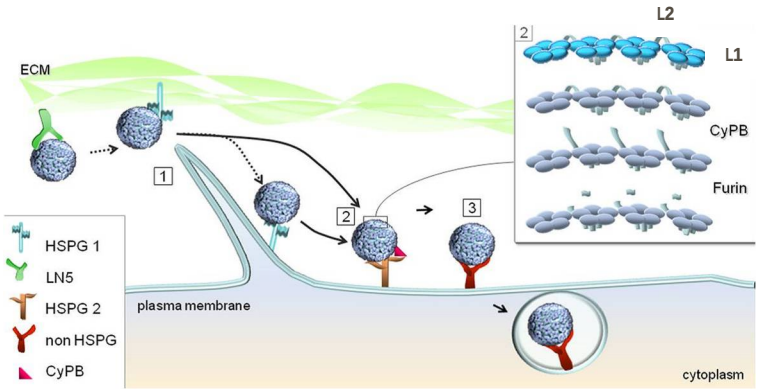

Fig. 6 Model of HPV-entry mechanism[87]***.*** *1) HPVs bind the HSPG on the ECM or on the cell surface; some HPVs, like HPV11, bind ECM-laminin (LM5) and after the HSPG on the cell surface. 2) interaction with the secondo HSPG receptor: HPVs presents at least two binding site for HPSG. The L1-HSPG binding permit some conformational changes in particular the externalization and the cleavage of L2 N-terminal portion by Cyclophilin B and Furin, respectively (panel 2). 3) Binding of a non-HSPG receptor.*

The infection is followed by amplification and maintenance of the viral DNA as episome at low copy number (50-300 copies per cell, in the basal layer[104–107]) (maintenance phase, *Fig. 7*), that replicate in synchrony with the host cellular DNA in a cell cycle dependent manner: this provide a pool of infected basal cells to form the basis of the papilloma[106]. The viral replication proteins E1 and, in particular, E2 are thought to be essential for this initial amplification step, making positive and negative control of the early viral promoter[108,109]. However, they seem to be dispensable once the copy number has stabilized (in basal layer of lesions around 200 copies per cell)[106,110]. During this phase, the oncogenic proteins E6 and E7 are also expressed and, in the upper epithelial layers, they allow the infected cell to re-enter S-phage permitting genome amplification (thousands of copies per cell[111,112]) (vegetative phase, *Fig. 7*). This allows also the increasing number of E1 and E2 proteins necessary for amplification[113]. It is thought that indirect contribution to genome amplification is also given by E4 and E5 protein through cellular environment modification. E4 accumulates to very high levels in cells that support virus synthesis[114,115] and its primary function seems to be in virus release or transmission[116,117]. In the other hand, it is supposed that E5 possesses pore-forming capability, interferes with apoptosis[118] and also being involved in koilocyte formation in cooperation with E6[119].

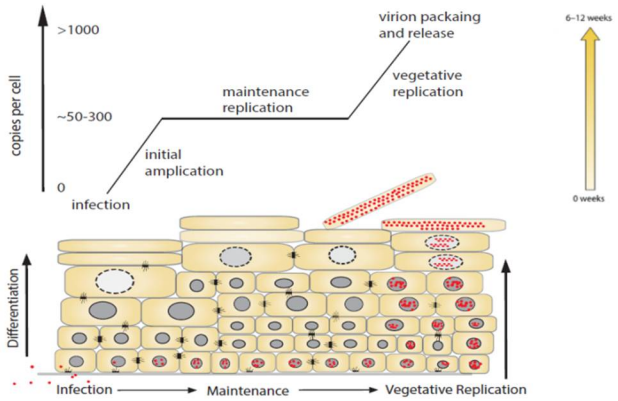

Fig. 7 Phases of HPV DNA replication[105]***.*** *After HPV infection, the maintenance phase is characterized by an initial amplification in which the DNA copies per cell are around 50-300, maintained until the vegetative phase. During the productive HPV infection the copies per cell grow up (>1000) due to an differentiation-dependent HPV DNA amplification (vegetative phase). The virion packaging and release occurs 6-12 weeks after infection.*

The completion of HPV life cycle involves the expression of L2 and L1, within E4, to exit from the cell cycle and allow genome packaging, respectively. Genome encapsidation requires the expression of L1, by recruitment of L2 to replication regions via E2, and the assembly of the icosahedral capsid in the nucleus[120–122]. Virus maturation occurs in oxidizing environment of the most superficial, dying keratinocytes, that permits the production of extremely stable infectious virions by progressive accumulation of disulphide bonds between the L1 proteins[123–125]. Assembled particles contain 360 molecules of L1 arranged into two different pentamers (60 hexavalent and 12 pentavalent pentamers[126]) with a variable number of L2 molecules (generally 5:1 L1:L2 ratio), which occupy the centre of each pentavalent capsomer[124,127]. The L1-L2 interactions seem to involve the residues 113-130 and 247-269 of L1 and the C-terminus of L2 (residues 396-439 in HPV11 L2 and 91-246 in BPV1 L2)[128,129]; X-ray crystallographic studies suggest also that L2 might interact with L1 via N-terminus (residues 24-30 and 260-264)[124,130].

HPVs are non-lytic viruses and the virions release depends on desquamation, a differentiation endpoint in which spontaneously disrupted keratinocytes are shed into the environment. The E4 protein is thought to contribute to this step, assembling into amyloid fibres that disrupt keratin structure and compromise the normal assembly of cornified envelope[115,117,131–133]. As a consequence of epithelial differentiation dependency of the late phase of the HPV's life cycle, the process of virion assembly and release into the environment takes 6-12 weeks to be complete[134].

1.3 From HPV infection to cervical carcinoma

Failure of the immune system to clear persistent HPV infections can lead to the development of cervical cancer[4]. T cell-mediated immune response seems to be directly involved in the clearance of HPV-related diseases as demonstrated in studies of immunocompromised patients, such as organ transplant recipients (OTRs) or HIV-positive individuals, that presents an increase incidence of HPV infection and HPV-related lesions[9,135].

HPV has evolved a strategy to elude both the innate and adaptive natural immune response causing host's immunological ignorance of the infection and virus persistence for many months. The HPV life cycle is organized to coupling the viral late phase, with high level of viral antigen synthesis, to superficial layers of the skin, where immune cell surveillance is restricted[111]. Absence of viremia and spread of the virus by blood represented another immune-response evasion mechanism. The virion release during cells desquamation process prevents both access of virus to lymph nodes, where adaptive immune response is initiated, and cell necrosis or lysis, that contributes to virus persistence, avoiding inflammation[22,136]. In addition to absence of proinflammatory cytokines, HPV down-regulates, trough E6 and E7 proteins, the interferon-induced responses[137–139] and inhibits the migration and activation of epithelial Langerhans cell (LCs) to the site of infection, preventing the total elimination of infected cells and favouring the persistence[140]. E6 and E7 have also been implicated in direct inhibition of TLR9-mediated pathways, by down-regulating the transcription of the TLR9 gene, and in down-regulation of MHC molecules, decreasing the antigen processing and presentation and reducing T cell recognition[139,141,142]. Furthermore, the oncogenic proteins interfere with apoptosis mechanism: E5 inhibits tumor necrosis factor (TNF)-related apoptosis at early stage of infection[143], E6 acts degrading pro-apoptotic proteins such p53 and provides protection from tumor necrosis factor-related apoptosis inducing ligand (TRAIL)-mediated apoptosis in a p53-independent mechanism[144] and, with E7, is able to activate low levels of caspases upon cell differentiation, that is associated to increase levels of anti-apoptotic factors and induction of viral genome amplification[145].

Current research suggests that LR HPVs produce more virions and infect more human hosts whereas HR types are less virulent but more difficult for the immune system to neutralize[15,146,147]. The persistent infection of HR HPV allows the accumulation of additional cellular changes leading to cancer, in particular in the cervical transformation zone that retains the ability to differentiate, important for virion production[148], presents fewer overlaying layers and is susceptible to hormones, that can help both infection and cancer development[149–151].

HPV DNA can be persists in episomal forms, integrated forms or in mixed forms that contain both. In general, the progression of untreated lesions to microinvasive (cervical intraepithelial neoplasia CIN1, CIN2 and CIN3) and invasive cancer is associated with the integration of the HPV genome into the host chromosome, even if there are no apparent chromosomal hotspots for HPV

genome integration[152]. This involves loss or disruption of E2, and subsequent loss of negative-feedback control and up-regulation of E6 and E7 oncogene expression[153] (*Fig. 8*).

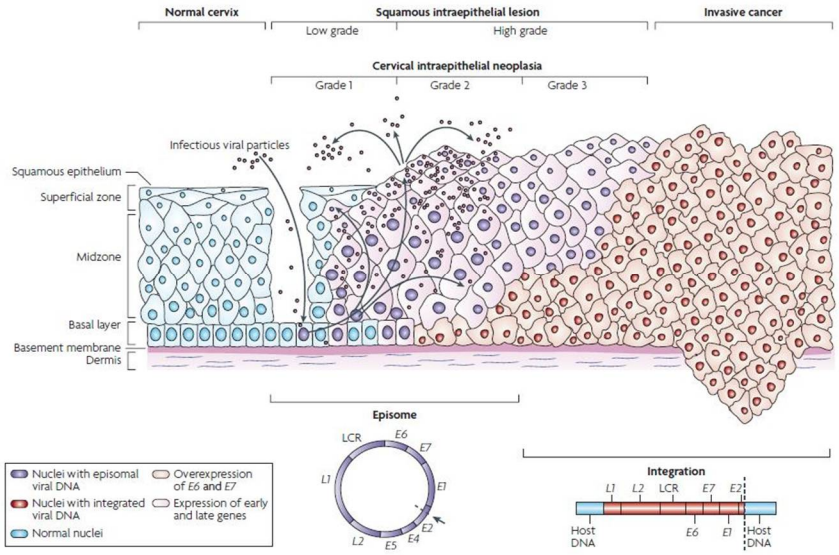

Fig. 8 Progression to cervical cancer mediated by HPV infection[153]***.*** *After infection of basal cells, the early proteins E1, E2, E4, E5, E6 and E7 are expressed and the genome is replicated as episomal form. In the upper layers, the HPV genome is replicated further and, after expression of L1 and L2, new virions are produced and start a new infection. In the cervical intraepithelial neoplasia (CIN) grade 1 e 2 persists the episomal form but the progression through CIN3 to invasive cancer is associated to HPV genome integration into host chromosome whit loss of E2 and subsequent upregulation of E6 and E7.*

The HR HPV oncogenic proteins E6 and E7, with E5, act cooperatively and are the primary mediators of HPV-induced effects. They differ from those of LR HPV because they interact with their host target proteins most efficiently. E5 seems to contribute to early steps of cancer initiation[154] and to enhance the transforming activities of E6 and E7 proteins[143,155,156] even if it is not necessary for maintenance of the transformed phenotype[143,157]. In addition to anti-apoptotic activities, E6 and E7 contribute to malignancy promoting proliferative cell state and genome instability. E7 binds several cellular factors including the pRB tumor suppressor and the related family members p107 e p130, that control the G1-S phase transition by regulating the activity of the transcription factor E2F[158]. The E7 binding targets the Rb family members to proteasomal degradation, trough ubiquitin-dependent pathway[159,160], preventing E2F repression and also abrogating other pRb functions, such as DNA repair and the maintenance of genomic integrity[161,162]. E7 interacts also with key proteins that control cell cycle progression, as p21 (is thought to act as a tumor suppressor in cervical carcinogenesis[163]) and p27: these CDK inhibitors are important regulators of growth arrest during differentiation and are completely neutralized by

E7 binding. One mayor consequence of the E7 activity is an increase in the levels of tumor suppressor p53[164] (*Fig. 9a*). To contrast this, E6 protein can interfere with p53 using different mechanisms: through the complex E6-E6AP, E6 binds p53 and leads it to proteasomal degradation, mediated by ubiquitination. E6 can also bind p53 directly and block transcription activity interfering with its DNA-binding capacity. In another hand, E6 interferes with p53 by binding two histone acetyltransferases, p300 and CREB-binding protein (CBP): this blocks the ability of these factors to acetylate p53 and consequently inhibits the transcription of p53-related genes [165,166] (*Fig. 9b*).

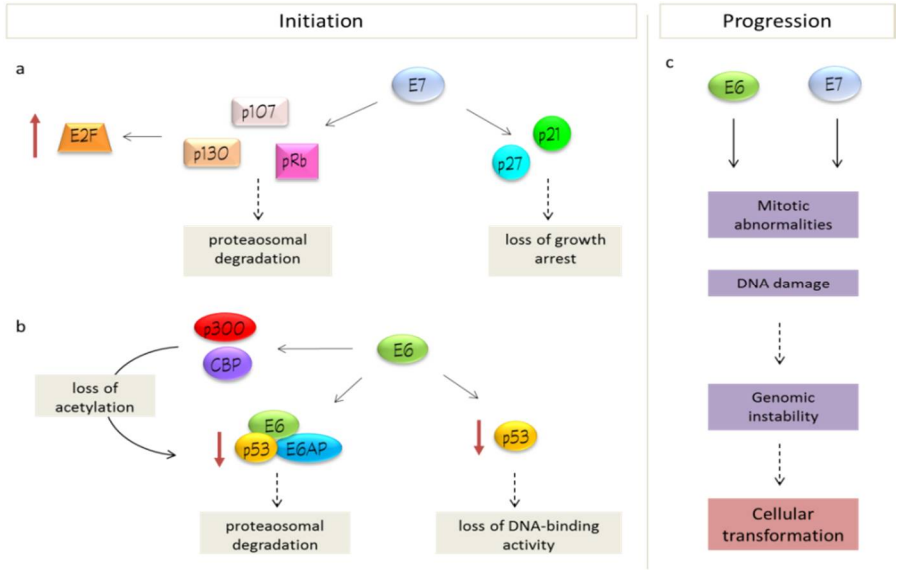

Fig. 9 Schematic representation of HR HPV E6 and E7 activities. *In the upper part (a), E7 block the activities of pRb and related proteins p107 and p130 increasing the E2F activities and promoting the proliferation. The progression is also supported by blocking p21 and p27 proteins. In the lower panel (b), E6 acts interfering with p53 protein with different mechanisms: it acts directly, with or without E6AP proteins or indirectly acting on p300 and CBP (CREB binding protein). The cooperative activities of E6 and E7 lead to genomic instability, the main factor for cellular transformation and cancer progression (c).*

The ability of E6 and E7 to target crucial regulators of proliferation, apoptosis and immortalization, promotes genomic instability, necessary for malignant progression. The expression of E6 and E7 induces mitotic defects, including multipolar mitoses, anaphase bridges and aneuploidy[167]. In addition to centrosome abnormalities[168], E6 and E7 can also induces genomic instability through the induction of DNA damage[169] and increasing the frequency of foreign DNA integration into host genome[170] (*Fig. 9c*).

1.4 The commercial HPV vaccines

Based on correlation between HPV infection and cervical carcinoma, two prophylactic vaccines are currently available, working by induction of virus-neutralizing antibodies. Cervarix® (GlaxoSmithKline; GSK) and Gardasil® (Merck) were approved in the Europe in 2007 and 2006 while in U.S. in 2009 and 2006, respectively. Both vaccines are based on the self-assembly capacity in virus-like particle (VLP) of the major capsid protein L1. Non-infectious L1 VLPs mimic the natural virus structure and present high-density B-cell epitopes, which induce neutralizing antibodies production as vaccination with native virions[171,172]. They also contain intracellular T-cell epitopes, thus inducing both potent humoral and cellular immune response [173,174]. Even if the L1 amino acid sequence is conserved among different HPV, most genotypes seem to be serotypically distinct. Crystal structures of L1 VLPs define that the variability between HPVs is concentrated in the three solvent-exposed loops of L1 to which neutralizing antibodies are directed [175]. For these reason, Cervarix® and Gardasil® permit genotype-specific protection against the serotypes that compose the vaccine: HPV16 and HPV18 for Cervarix® (bivalent) and HPV16, HPV18, HPV6 and HPV11 for Gardasil® (quadrivalent). While there is clinical evidence for cross-protection against the most closely related types (HPV45, HPV31, HPV33 and HPV51[176,177]), the levels of cross-neutralizing antibodies are in general very low and further data to establish the duration of this cross-protection (detectable after 8 years post vaccination) are needed[178,179]. They are both administrated in three doses over 6 months via intramuscular injection but are different in the producer cell and adjuvant used: insect cells with L1-recombinant baculovirus and AS04 adjuvant (Aluminium salt with monophosphoryl lipid A; MPLA) for Cervarix®, and *Saccharomyces cerevisiae* and aluminium salt for Gardasil® (*Fig. 10*).

	Bivalent (Cervarix)	Quadrivalent (Gardasil)	Nonavalent (Gardasil 9)
Manufacturer	GlaxoSmithKline	Merck	Merck
L1 VLP types (dose)	16 (20 µg), 18 (20 µg)	6 (20 µg), 11 (40 µg), 16 (40 µg), 18 (20 µg)	6 (30 µg), 11 (40 µg), 16 (60 µg), 18 (40 µg), 31 (20 µg), 33 (20 µg), 45 (20 µg), 52 (20 µg), 58 (20 µg)
Producer cells	Baculovirus-infected *Trichoplusia ni* insect cell line	*Saccharomyces cerevisiae* (yeast)	*Saccharomyces cerevisiae* (yeast)
Adjuvant	500 µg AS04	225 µg AAHS	500 µg AAHS

AS04=aluminium hydroxide plus 50 µg 3-O-desacyl-4'-monophosphoryl lipid A. AAHS=amorphous aluminium hydroxyphosphate sulfate. L1=major capsid protein. VLP=virus-like particle.

Fig. 10 Features of Cervarix®, Gardasil® and Gardasil 9® HPV L1-VLP vaccines[245]***.*** *In the table, there are the characteristic of the current commercial HPV L1-VLP vaccine. They differ in composition of VLP types, cell producer, amount of each VLP in the formulation and adjuvant but present a similar injection schedule and the same immunization route.*

Probably due to the different adjuvants used, the two vaccines elicit different dominant responses: with Cervarix®, there is an increase of IL-2 and TNF-α, characteristics of a T helper 1 (Th1) response linked to cell mediated immunity, while Gardasil® induced a Th2 response, mediated by IL-4, -10 and -13, correlated to humoral response[180–182].

The main effector of protection is the IgG antibodies, which can, in one hand, move to the site of infection by transudation, and, in the other hand, using exudation, arrive at epithelial sites exposed to infection [48,183]. In term of serum neutralizing response to HPV16 and -18, both vaccines induce a high response, even if, 5 years after the first immunization, there are a 2.3-7.8-fold differences in favor of Cervarix®, depending of the age at which the vaccine was administrated. The immunological duration of the two vaccines is 8.4 years for Cervarix® and 5 years for Gardasil®; the anti-HPV16 and HPV18 are at least 5-10-fold higher, for Gardasil® and Cervarix® respectively, than antibodies levels reached following clearance of natural infection[184,185]. Prediction programs of long-term immunogenicity define that levels of antibodies anti-HPV16 would remain higher in the Cervarix® groups than in Gardasil® ones, even 20 years after the first immunization. The difference of the two vaccinated groups is more pronounced in anti-HPV18 antibodies levels. The protection results longer in the groups vaccinated in the 18-26 years old range, reaching a long-term immunogenicity prediction around 70 years for HPV16 and 40 years for HPV18[185]. This demonstrated the importance of time of vaccination: since it is a sexual transmitted infection and sex is the major factor for propagation, it is important that the vaccination starts before the beginning of sexual activity. The range start from 9 to 25 (Cervarix®) or 26 (Gardasil®) years old and they do not provide protection for HPVs to which a woman has previously been exposed through sexual activities. Studies are ongoing in 9-14 years old girls (ClinicalTrials.gov NCT01462357) and preteen/adolescent girls (ClinicalTrials.gov NCT00877877) to verify the protection obtained, the long-term immunogenicity (until 10 years) and the safety, already demonstrated in adults women[185].

Thanks to the inclusion of HPV6 and HPV11 VLPs, Gardasil® is also able to modulate the RRP, if used as adjuvant therapy[186], as well as prevent genital warts: this is very important to justified men vaccination. Men suffer of HPV-related diseases lower than women, but if only women are vaccinated, there would be little impact on HPV prevalence in men who have sex with men and, they also persist as immune carrier of HPV infection, reducing the impact of women vaccination[187]. Indeed, recent studies forecast an increase of HPV-related cancers, in particular oropharyngeal ones, in the near future with a higher incident in men than women[188].

The high levels of neutralizing antibodies obtained using these two vaccines are just against specific serotypes, in particular HPV16 and HPV18, as described above; this represents the major limitation that we want to overcome. The idea is to extend the cross protection against all the

oncogenic types, in particular to obtain a vaccine suitable for low resource countries in which different serotypes present a prevalence higher than 1%. For this purpose, Merk developed a nonavalent vaccine, Gardasil 9® composed by the four VLPs present in the Gardasil®, plus five additional ones of other oncogenic HPV types (HPV6, 11, 16, 18, 31, 33, 45, 52, and 58) (*Fig. 8*). This new formulation, with similar technology of quadrivalent vaccine, was approved by FDA in 2014 and there is not inferiority of immune response to HPV6, 11, 16 and 18 compared to the Gardasil®, both in young men and women [189,190]. About the protection against HPV31, 33, 45, 52 and 58, first studies defined a prevention of persistent infection and disease related to these types, but the frequency of adverse events is higher using nonavalent than quadrivalent vaccine, maybe due to the higher amount of VLP or adjuvant used[189]. Obviously, further data are needed to verify the additional benefits of the nonavalent vaccine compared to bivalent or quadrivalent ones. If this new formulation could extend the protection against more serotypes, it doesn't go over the cost of production due to expression in eukaryotic cell, to extensive purification protocol, and to the cold chain needed for storage, making the vaccination out of reach for developing country. The funding of the HPV vaccine by GAVI Alliance, stating in the 2012, permits to reduce the price from US $150 to $5 per dose (GAVI Alliance, 2011), increasing the number of countries that had introduced the HPV vaccine into their national immunization programs (57 countries worldwide in 2014) (WHO, 2013).

The difficulty of extending the vaccination programs into the developing countries is also due to the three doses immunization schedule: the reduction of number of doses will have a strong effect both in cost, logistics and efficacy of HPV vaccination programs. Fewer than three doses of Cervarix® are able to induce high level of antibodies: vaccine efficacy for prevention of persistent HPV16 and 18 infection was similar after one, two or three doses[191]. HPV16 and 18 antibody titers in woman receiving one dose or two doses, at least 6 months apart, were non-inferior to the three-doses group, and in the one-dose group the antibodies concentration remains stable over 4 years[192]. Two doses of both vaccine are studied in girls 9-14 years old and, when the doses are separated by 6 months, the immunogenicity is non-inferior to that obtained in 15-26 years old woman immunized thrice[193,194]. Based on these data, some countries introduced the two-dose schedule, reducing the cost and the delay between the immunization of the three doses programs. In 2014, WHO's Strategic Advisory Group of Expert recommended the two-dose vaccination only if it starts before age 15 years (WHO; SAGE); in other cases, in particular for immunocompromised individuals, the three-dose schedule is recommended.

Several second-generation prophylactic vaccines are being developing with the aim to resolving some of the limitations of these two VLP-L1 commercial vaccines in term of cross-protection, cost-production and extension of vaccination programs.

1.5 The second generation HPV vaccines: L1 and L2 approaches

A lot of effort are spending to obtain vaccines able to maintain the high level of neutralizing antibodies and the same long-term protection of commercial ones but also cover the majority of oncogenic HPVs, together with reduction of cost production, administration and distribution, including the increase of thermal stability. Two approaches are possible: use the major capsid protein L1, as in the commercial vaccines, or shift the focus on the minor capsid protein L2. While L1 antibodies are able to prevent both steps of infection as attachment to basal membrane and to keratynocytes, L2 antibodies can interfere only with the second step[83].

∂ L1-based vaccines

Generation of conformational correct L1 VLPs in alternative production system could be a strategy to reduce cost of production. High levels of VLP production is obtained in *E.coli* patented L1 mutant[195,196]. Good results of phase I safety trial of *E.coli* HPV16 and HPV18 VLPs vaccine are obtained in 2014[197] and a phase III efficacy trial of them are ongoing (ClinicalTrials.gov NCT01735006); in parallel there is also an early phase clinical trials of a bivalent HPV6 and HPV11 vaccine, both produced using *E.coli* (ClinicalTrials.gov NCT02405520). Other organisms used to produce VLPs are yeast and plants. Two companies have produced HPV16 and HPV18 VLPs in *Komagataella* (*Pichia) pastoris*[198,199], that are under phase I clinical trial (ClinicalTrials.gov NCT01548118), and *Hansenula polymorpha*[200], both methylotrophic yeast strains that permit to obtain higher yields at low costs than *S. cerevisiae*. Some academic groups have produced L1 VLPs in plants as tobacco[201,202] by transient expression after infection with L1-recombinant plants viruses or infiltration of L1-recombinant *Agrobacterium tumefaciens*. Even if this system theoretically offers a reduction of cost and permit to have a vaccine orally delivered, the yield is low and there are problems in the correct VLP's assembly. It seems that targeting the expression in chloroplast may increase L1 production, important factor for the plants system development[203].

A more thermostable and cost-efficient alternative to VLPs are capsomers. Pentavalent L1 capsomers, that can assemble in VLPs, are easily expressed and purify in high yields in *E.coli*[204]. They are able to induce neutralizing antibodies and, even if the antibodies titers are not so consistent and durable as L1 VLPs[205], protect animal model in experimental challenge[206].

Another platform for L1-based vaccine is represented by virus-based and bacteria-based vectors. Even if the vector-modified microbes require specific monitoring in the early phase clinical trial, that can complicated the system, this approach has potentially advantages in

lowering production costs and in fewer doses needed. The best technique seems to be the introduction of HPV VLP production in vaccine already used as Berna strain attenuated measles virus. The HPV16 L1 recombinant measles virus induces high titers of HPV16 neutralizing antibodies after one dose in mice[207]. In this manner, the vaccinated infant could be protected against measles together with HPV.

Other viral vectors include adeno-associated virus (AAV) and a human endogenous retrovirus[208,209]. An important issue of the L1 AAV-5 and -9 is the possibility of intranasal administration, overcome the needle limit of commercial vaccines[209].
An alternative to virus-based vaccine is the bacterial-based vaccine that present low cost and high-yield of production and more safety than viruses-based one, in particular in immunocompromises patients.
The approach could be analogous to recombinant viruses but using a licensed attenuated bacterial vaccine strain. An example is the L1-recombinant *Salmonella enterica* serotype Typhi, typhoid vaccine live oral Ty21a. This vaccine could be lyophilized and delivered orally or intranasally[210], allowing the increase of distribution in low-resource countries. There are other possible bacterial platforms as *Shigella sonnei*, *Shigella flexneri*, *Listeria monocytogenes*, *Lactococcus lactis*, *Lactobacillus casei* and *Bacillus subtilis*[211]. Sometimes, in this system, the intracellular VLPs from L1 assembly, not acquire the correct structure, reducing the neutralizing antibodies development.

Naked L1 DNA could be another economical alternative to induce neutralizing antibodies, demonstrated in preclinical studies[212]. A potential promoter insertional mutagenesis, together with heterotypic interactions among L1 proteins, reduces the interests on this approach.

∂ L2-based vaccines

The requirement of a cross-protection, a cheaper production and distribution, and needle-free administration, pushed the researches to focus the attention on minor capsid protein L2. This protein, that has up to one copy per L1 capsomer and a maximum of 72 copies per virion, presents epitopes able to induce neutralizing antibodies[213] (*Fig. 11a*) that are exposed only after the virion HSPG-binding to the basement membrane, mediated to L1. The conformational change, occurred after the binding, allows the exposure of N-terminal region (1-120 residues) and its cleavage by furin[214]. Although L2 is a component of viral capsid, natural infection with HPV fails to elicit antibodies against L2, due to the transient exposition on the virus particle surface during the infection process. The L2-directed

neutralizing antibodies, able to recognized linear peptide epitopes, permit a broad cross-protection (against homologous and heterologous HPVs)[215,216], also in passive transfer, probably due to the high conservation of N-terminal L2 region (residues 1-120; *Fig. 11b*)[133,214,217]. Passive immunization with monoclonal antibody against 17-36 L2 epitope, RG-1, protect mice against HPV16 pseudovirion infection, cross-neutralizing also HPV5, 6, 18, 31, 45, 52 and 58 pseudovirions as well as BPV1 and HPV11 virions[133]. Cross-neutralizing activities of L2 antibodies suggest the possibility that a monovalent vaccine might protect against a range of genital or mucosal types and, may be, cutaneous types of HPV, responsible of common warts and squamous cell carcinoma (even if this association need further investigations)[218].

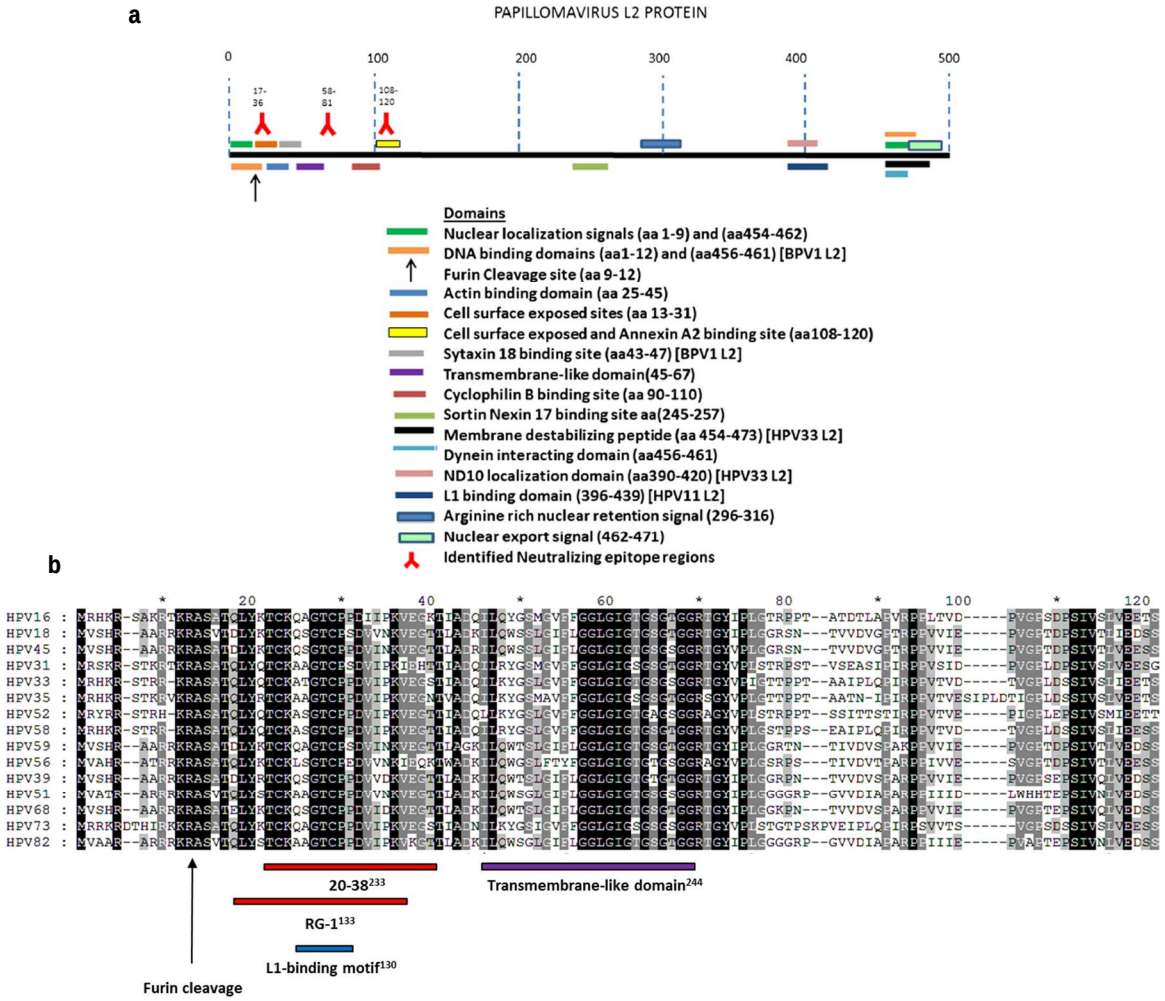

Fig. 11 Structure and high risk sequence alignment of L2. *a) Schematic representation of L2 protein structure[213]. b) Alignment of high risk HPV N-terminus L2 protein (residues 1-120). In red the most neutralizing epitopes, in blue the presumptive L1-binding domain and in violet the conserved transmembrane domain for endosomal penetration[246]. The alignment is performed using ClustalW and the grey scale represents the conservation grade.*

The conservation of neutralizing epitopes in L2 but not in L1 could be explained firstly, by a lack of evolutionary pressure, cause by hidden position of L2 in virion[219] and secondly, by their possible critical role during infection[86,94,220].

The titers induced by L2 peptide against homologous HPV are generally ten times lower than L1 VLP-induced titers, that could be due to the different recognition of peptide or VLP by the immune system. In recent years, numerous efforts have been undertaken to increase the immunogenicity of linear L2 peptide, to insert them in a vaccine context. One approach consist in a concatenated multitypes L2 fusion protein (concatemers) including the L2 11-88 epitope from 5 (5x: HPV1, 5, 6, 16 and 18) to 8 (8x: HPV6, 16, 18, 31, 39, 51, 56 and 73) HPV types[221,222]. These concatemers induce both *in vitro* and *in vivo* cervicovaginal mouse challenge type-specific and cross-neutralizing antibodies[222].

To increase titers and durable response of L2-specific antibodies it is possible to use different platforms as HPV VLP[223,224], adeno-associated virus[209] and tobacco mosaic virus[225]. HPV L1 VLP can be modified to have a surface loop of L1 replaced by an L2 peptide: this approach permit the high L1 response together with a broad cross neutralization conferred by L2 epitope (17-36 amino acids[218]). Same results are obtained using bacteriophage VLP, in which the L2 peptides are included in the surface-exposed constrained loop (the AB-loop) or N-terminus of the coat protein of bacteriophage PP7 or MS2, respectively, without interfere with the VLP assembly[226,227]. Also the HPV16 L1 capsomers were modified for the L2 display introducing L2 peptide (13-47 epitope) from HPV18, 31 and 45[206]: this strategy seems to confer less protection (in particular against heterologous types) than VLP platform even if the L1-response is high and the mouse challenge demonstrated a protection against HPV16 and HPV18 PsV. VLP and capsomers support also insertion of E6, E7 or E2 permitting a possible combination of therapeutic and prophylactic vaccine capacities[228]. A cross-neutralization is obtained also introducing the L2 (17-36) epitope of HPV16 and HPV31 into different position (587 and 453, respectively) of adeno-associated virus 2 (AAV2). The high stability of AAVLP (AAV2 with L2 epitopes) allows lyophilization and rehydration, overcoming the cold chain reaction in vaccine distribution, an important cost factor for developing country[209,229]. The modified tobacco mosaic virus (TMV) represents another possibility to enhance the low immunogenicity of L2 (61-171 L2 peptide of Canine Oral Papillomavirus)[225]. The increasing of immunogenicity could be combined with oral administration: the use of *Lactocossus lactis* displaying amino acids 1-224 of HPV16 L2 elicits systemic IgG and mucosal IgA responses in mice, able to neutralized HPV16, 18, 45 and 58 pseudovirions both *in vivo* and *in vitro* [230]. The complicated immunization schedule (days 0-9, 15-19 and 25-29) is offset by the unnecessary doctor's office visit.

The immunogenicity of L2 peptides is increased also if they are inserted into the active-site loop of thioredoxin, a small, non-toxic and soluble scaffold protein (around 12KDa) able to maintain the constrain of the insert, conferring thermal stability property, and stimulate murine T-cell proliferation[231–234]. This approach, called Thioredoxin Display Multipeptide Immonogens (TDMI)[233–236], using the *E.coli* thioredoxin (*Ec*Trx), permitted to scan the N-terminal region (amino acids residues 1-120) of HPV16 L2, identifying a 19 amino acids L2 peptide (region 20-38) as the most immunogenic and neutralizing epitope, used in our group[235]. The choice of using the tripeptide of HPV16 L2 $(20\text{-}38)_3$ into the *Ec*Trx instead of single peptide epitope insertions rests on a number of previous (largely empirical) observations indicating that multi-epitope antigens are more effectively recognized by antigen presenting cell (APC) [237–239]. This is confirmed in our observations, in which the *Ec*Trx HPV16 L2 $(20\text{-}38)_3$, where a Gly-Gly-Pro linker was interposed between individual epitopes to prevent formation of junctional epitopes, induces the production of polyclonal and monoclonal antibodies able to cross-neutralize different HPV types: HPV18, 45, 58 and HPV31 (even if with lower titer), in addition to HPV16[235,236]. Using Trx as scaffold protein it seem to be also possible to obtain a cyclized epitope by Cys22-Cys28 intramolecular disulfide bonds; this seem to be critical for infectivity of human keratinocytes[240], and permit more reactivity compare to the linear epitope[241].

To increase the cross-neutralization, a three component mix of thioredoxin-L2 antigens (HPV16/31/51), based on the three most high risk divergent types from HPV16, overcomes the coverage of HPV16 L2 alone, permitting protection against HPV16, 18, 45, 31, 33, 52, 58, 35, 59 and 51[242,243]. The only limitation is the use of three components: the possibility to have only one molecule instead of three would be better to reduce the production costs and the complexity of the vaccine.

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2 Aim of the project

Human Papillomavirus (HPV) infection is correlated to the development of cervical cancer, the fourth common cancer in women worldwide. Two commercial vaccines, Gardasil® (Merk) and Cervarix® (GlaxoSmithKline), are based on the major capsid protein L1 of HPV, able to auto-assemble into high immunogenic virus like particles (VLPs). High cost of production and distribution together with a limited protection only against the HPV types whose VLP are present in the formulations (HPV16 and 18 for Cervarix®; HPV16, 18, 6 and 11 for Gardasil®) represent not negligible limits.

General aim of the project is the reformulation, immunological characterization and preclinical testing of an alternative, broadly protective, thermally stable, and low-cost HPV vaccine, based on a cross-neutralizing epitope from minor capsid protein L2 (amino acid positions 20-38)[1] fused to bacterial thioredoxin (Trx) (TDMI approach)[2].

HPV infection and cervical cancer prevalence affect, in particular, low-resources countries such as sub-Saharan region, characterized by high temperature and lack of appropriated medical structure. The first aim is to produce and test thioredoxin polypeptides other than the natural polypeptide from *E. coli* (*Ec*Trx) to identifying a hyper-thermostable scaffold that best minimizes cross-reactivity with mammalian Trxs (in particular mouse and human), yet affords broad cross-protection and allows a cost-effective purification of the Trx-L2 immunogens, using HPV16 L2 $(20\text{-}38)_3$ as antigen. This could be a step forward to obtain a vaccine formulation able to persist at high temperature, reducing cost of production and distribution.

Secondly, the best Trx HPV16 L2$(20\text{-}38)_3$ identified in term of thermostability and lack of cross-reaction, will be express in a methylotrophic yeast *Pichia pastoris*, with the purpose of increasing yield of protein and reduce cost of production. In most cases, this system allows to obtain high pure secretion proteins, easily recovered from medium, free of lipopolysaccharides (LPS), immune system stimulators typically of bacterial expression system, that are not allowed in vaccine preparation.

While needle-free delivery would certainly represent a plus for mass HPV vaccination programs, another significant improvement with respect to the current (three-shots) protocols would be a one-shot vaccination. The idea is to adsorbed Trx-L2 antigen into safe and slow-delivery mesoporous silica rods[3] and test this formulation in mice; this silicate platform is able to self-assemble after injection, permitting, on one hand, the recruitment of immune system cell and, on the other hand, the slowly release of the antigen, allowing a continuous antigen-immune cell interaction.

One of the most important features of a second generation vaccine is the broadly protection, in term of both host range and HPV serotypes. One set-up optimization, aimed at generating an immune response regardless of the specific HLA-DR type, entails the addition of a promiscuous T-cell helper epitopes (PADRE, p25 and p2-p30) to both ends of the Trx-L2 antigen. Furthermore, to increase antigen recognition, a C4bp-derived immuno-stimulating polypeptide, IMX313T, will be fused to the Trx-L2 antigen; the heptameric structure, that spontaneously gives rise, could be better recognized by immune system cells, increasing neutralizing antibodies production.

Knowing the extensive but incomplete cross-protection afforded by a 3X repeated HPV16 L2(20-38) monovalent immunogen[1], design and production of single-molecules concatemers based on L2(20-38) epitope from different HPV types, should extend the protection and overcome, in term of production costs, the mix formulation consisting of equimolar amounts of the monovalent Trx-L2 antigens of the three HPV types (16, 31 and 51)[4,5]. The Trx-L2 polypeptides 11mer and 8mer will include epitopes from 11 and 8 different serotypes, respectively. By including HPV6 L2(20-38) epitope, 8mer is designed to extend the protection to some low risk HPVs. The formulation will include these single-molecules alone or associated to the best promiscuous T helper epitope and the IMX313T polypeptide.

By preclinical testing of all these formulations we hope to identify the best thermostable, broad-protective Trx-L2-based vaccine prototype to be used in a phase I clinical study

1. Rubio I, Bolchi A, Moretto N, et al. Potent anti-HPV immune responses induced by tandem repeats of the HPV16 L2 (20-38) peptide displayed on bacterial thioredoxin. *Vaccine*. 2009;27(13):1949-1956.
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3 Results and Discussion

Section I

P. furiosus thioredoxin: a high thermostable alternative to E.coli thioredoxin

Introduction

Recombinant scaffold proteins (SP) harbouring selected heterologous peptides displayed on their surface are valuable reagents for a number of applications ranging from functional proteomics, to high throughput immune-screenings and peptide-vaccine construction[1–3]. Peptide-SP fusions can be assembled in an end-to-end or an internal grafting configuration. The latter, which mimics the organization of non-terminal (mid-sequence) peptide regions, offers a number of specific advantages such as insertion into a structurally defined site, a superior scaffold-induced constraining and a higher resistance to proteolysis. In the Thioredoxin-Displayed Multipeptide Immunogens approach (TDMI)[4], we have previously exploited internal grafting of selected (multi)peptide epitopes to the disulfide-stabilized, active ("display") site of thioredoxin (Trx) for the construction of recombinant peptide immunogens[4–8]. Thioredoxin (Trx) is a small heat-stable protein, member of oxidoreductase family[9–11], with a key role in the redox regulation of protein activity and redox signaling. It is found in all living cells from Archaea to humans, and the 90% of its residues are folded in secondary structural elements: all the proteins have similar three-dimensional structures despite the large variation in amino acid sequences except for residues in the active site region that are highly conserved[12]. Thioredoxin has a central core of five β-sheet strands enclosed by four α-helices with the active-site disulphide bond localized in a short loop at the N-terminus of the α2-helix. The surface exposed loop (segment 33-36) corresponding to a unique *CpoI* restriction site in the nucleotide sequence, is suitable for insertion of a peptide which can be stabilized by disulphide bond formation[13,14]. *E. coli* thioredoxin (*Ec*Trx) represents the scaffold protein used to develop our vaccine, where epitope, the tripeptide 20-38 of HPV16 L2 protein[6], are inlaid into the active-site loop, permitting a highly soluble and immunogenic recombinant, *Ec*Trx HPV16 L2(20-38)$_3$ (*Fig. 1.1*).

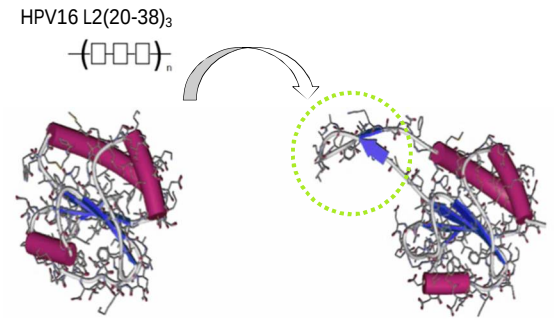

***Fig. 1.1 Prediction of EcTrx HPV16 L2(20-38)$_3$ structure**. In the left, the defined structure of EcTrx (PDB: 2TRX) and, in the right, the inferred structure of EcTrx HPV16 L2(20-38)$_3$ obtained using tertiary structure prediction program CPHmodels (http://www.cbs.dtu.dk/services/CPHmodels/).*

*Ec*Trx presents a numbers of features which make it a particularly suitable scaffold for vaccine development such as reduced dimension (108aa, 11.675kD), solubility, atoxicity, and stimulation capacity of murine T-cell proliferation[15,16]. Although these aspects, the distribution of the HPV L2-vaccine worldwide, in particular in developing countries, needs more thermal stability and low-cost of production. Lack of cross-reactivity of antibodies generated by the Trx scaffold against host (especially human) thioredoxin, represents another important feature.

Taking into account all the above potential improvements, we explored the thermal stability and scaffolding capacity of two archaeabacterial Trx-like proteins, more distantly related to mammalian thioredoxins than *Ec*Trx.

Identification of alternative archaebacterial thioredoxin scaffold proteins

To identify candidate sequences coding for thioredoxins more distantly related to mammalian one and with a higher thermal stability than *Ec*Trx, we used the latter sequence as a query for a BLAST-P search against all sequenced genomes from thermophilic and hyperthermofilic archaeabacteria. Potential homologs ($P \leq 10^{-3}$) were aligned and used to construct a global phylogenetic tree (*Fig. 1.2*), which revealed two major group, among different clusters: one including *Ec*Trx, the only eubacterial sequence in the tree and other phylogenetically related thioredoxins, and another group mainly comprised all far-divergent thioredoxin-like sequences. Sequences identified for further analysis, representative of these two groups, are from the thermophile *Methanosaeta thermophila* (optimal growth temperature~55-60°C: accession N. YP_843141.1) and from the hyperthermophile *Pyrococcus furiosus* (optimal growth temperature ~100°C: accession N. NP_578470.1, hypothetical protein PF0741).

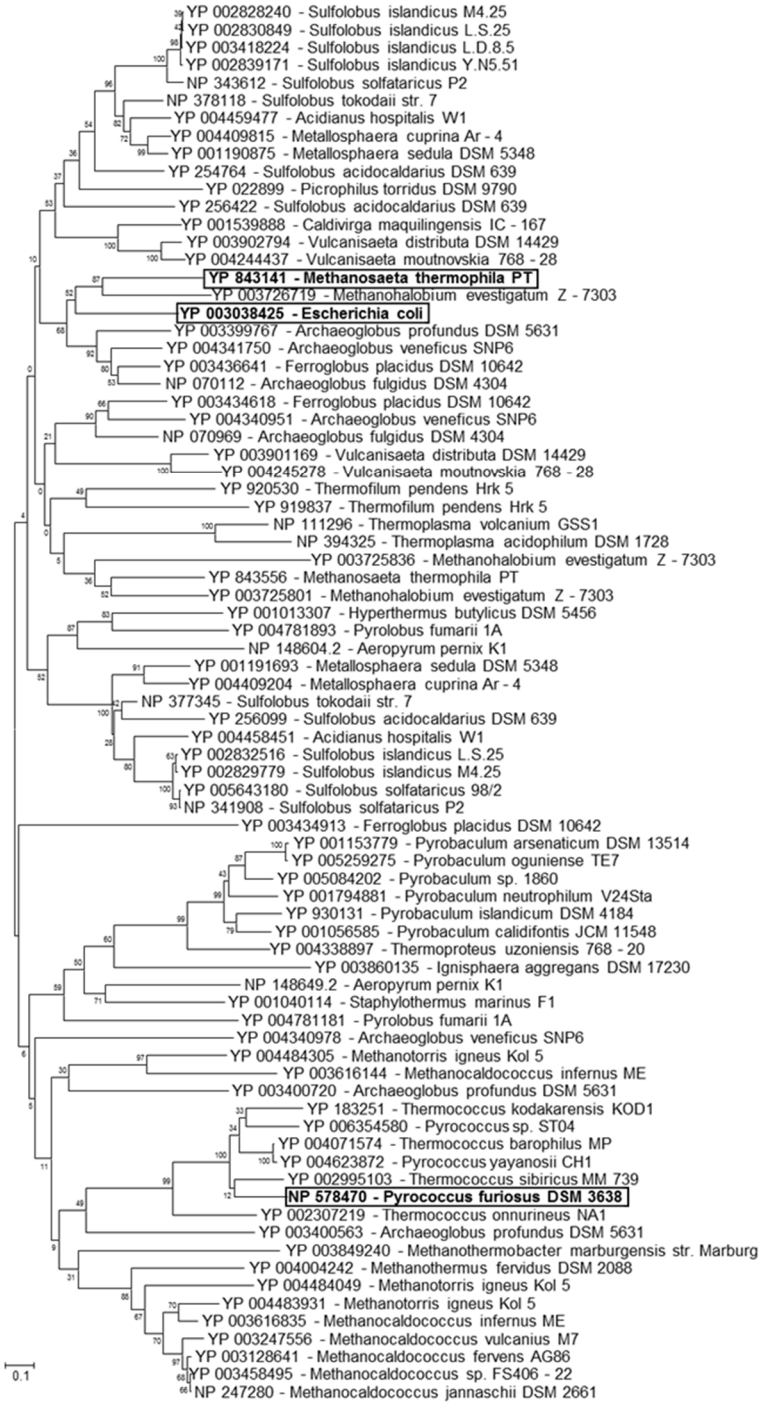

Fig. 1.2 Phylogenetic tree of thermophilic and hyperthermophilic archaebacterial thioredoxins using EcTrx as a eubacterial reference[8]***.*** *E. coli thioredoxin (EcTrx) was aligned with homologous polypeptides from thermophilic and hyperthermophilic archaebacteria. Aligned sequences were used for phylogenetic tree construction using neighbor-joining. Accession number and organism source are shown for each sequence. Bootstrap values for each node are indicated; branch lengths correspond to genetic distances. Thioredoxins from M. thermophila, E. coli and P. furiosus are boxed.*

a

```
H. sapiens     :  ------------------------------MVKQIESKTAFQEALDAAGDKLVVVDFSATWCGPCKMIKP  :  40
M. musculus    :  ------------------------------MVKLIESKEAFQEALAAAGDKLVVVDFSATWCGPCKMIKP  :  40
E. coli        :  ------------------------------MSDKIIHLTDDSFDTDVLKADGAILVDFWAEWCGPCKMIAP  :  41
M. thermophila :  MDELDEIRRKKLEELKRELAARSQGTPTIEYPDRPVLVTDSSIDAGIRQYP-VFVVDCWAPWCGPCRAIAP  :  70
P. furiosus    :  ------------------------------------MIIEYDGEIDFTKGRVVIWFSIPGCPPCRILVER  :  33

H. sapiens     :  FFHSLSEKYS-NVIFLEVDVDDCQDVASECEVKCMPTFQFFKKGQKVGEFSGAN-KEKLEATINELV-  :  105
M. musculus    :  FFHSLCDKYS-NVVFLEVDVDDCQDVAADCEVKCMPTFQFYKKGQKVGEFSGAN-KEKLEASITEYA-  :  105
E. coli        :  ILDEIADEYQGKLTVAKLNIDQNPGTAPKYGIRGIPTLLLFKNGEVAATKVGALSKGQLKEFLDANLA  :  109
M. thermophila :  VIDEMARELKGRVVFGKLNVDQNPLTSRKYGITAIPTLLVFRNGRLVDRLVGAYPKQILMSRVRKYLD  :  138
P. furiosus    :  FMTELSEYGE-DIQIVHINAGKWKNIVDKFNILNVPTLVYLKDGREVGRQNLIRSKEELKKLKELQE  :  100
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b

```
H. sapiens     :  ------------------------------MVKQIESKTAFQEALDAAGDKLVVVDFSA
M. musculus    :  ------------------------------MVKLIESKEAFQEALAAAGDKLVVVDFSA
E. coli        :  ------------------------------MSDKIIHLTDDSFDTDVLKADGAILVDFWA
M. thermophila :  MDELDEIRRKKLEELKRELAARSQGTPTIEYPDRPVLVTDSSIDAGIRQYP-VFVVDCWA
P. furiosus    :  ------------------------------------MIIEYDGEIDFTKGRVVLWFSI

H. sapiens     :  TWCGPCKMIKPFFHSLSEKYS-NVIFLEVDVDDCQDVASECEVKCMPTFQFFKKGQKVGE
M. musculus    :  TWCGPCKMIKPFFHSLCDKYS-NVVFLEVDVDDCQDVAADCEVKCMPTFQFYKKGQKVGE
E. coli        :  EWCGPCKMIAPILDEIADEYQGKLTVAKLNIDQNPGTAPKYGIRGIPTLLLFKNGEVAAT
M. thermophila :  EWCGPCRAIAPVIDEMARELKGRVVFGKLNVDQNPLTSRKYGITAIPTLLVFRNGRLVDR
P. furiosus    :  PGCGPCRLVERFMTELSEYFE-DIQIVHINAGKWKNIVDKFNILNVPTLVYLKDGREVGR

H. sapiens     :  FSGAN-KEKLEATINELV-
M. musculus    :  FSGAN-KEKLEASITEYA-
E. coli        :  KVGALSKGQLKEFLDANLA
M. thermophila :  LVGAYPKQILMSRVRKYLD
P. furiosus    :  QNLIRSKEEILKKLKELQE
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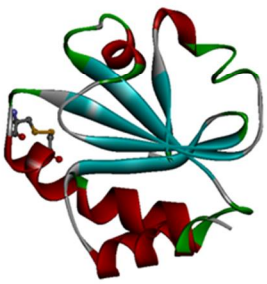

c

	H. sapiens	*M. musculus*	*E. coli*	*M. thermophila*	*P. furiosus*
H. sapiens	100%				
M. musculus	87%	100%			
E. coli	25%	25%	100%		
M. thermophila	17%	18%	35%	100%	
P. furiosus	19%	16%	16%	13%	100%

Fig. 1.3 Alignment of putative archaebacterial thioredoxins with E. coli, mouse and human thioredoxin[8]*. a)Predicted thioredoxin polypeptides from the archaebacteria M. thermophila (Acc. n. YP_843141.1) and P. furiosus (Acc. n. NP_578470.1) are aligned with homologous thioredoxins from E. coli (Acc. n. YP 003038425), M. musculus (Acc. n. NP_035790) and H. sapiens (Acc. n. AY004872). Amino acids that are identical in all or in three out of the five reported sequences are shown on a black or grey background, respectively. The conserved active (display) site is boxed. b) Predicted secondary structures of the five thioredoxin polypeptides; β sheets and α helixes are shown on a blue and a red background, respectively. The 3D structure of E. coli thioredoxin (PDB accession code: 2TRX) is shown on the right with the two Cys residues of the redox active/display site represented in a ball and stick format. c) Amino acid sequence identity values for the five thioredoxin polypeptides of interest.*

Both predicted polypeptides present a predicted structure like the *Ec*Trx with two conserved cysteine residues near the putative active site (*Fig. 1.3a-b*). Interestingly, *M. thermophila* thioredoxin (*Mt*Trx) contains 30 amino acids extension at the N-terminus with two additional predicted α-helices, whereas the Trx-like protein from *P. furiosus* (*Pf*Trx) is 9 amino acids shorter at the N-terminus than *Ec*Trx and lacks one predicted β-stand presents in all the other thioredoxins.

*Mt*Trx is 35% identical to *Ec*Trx and also contains the conserved Gly-Pro dipeptide interposed between the two Cys residues, corresponding on a *CpoI* restriction site. A further reduced sequence identity with *Ec*Trx (16%) characterizes the as yet unannotated thioredoxin-like sequence from *P. fusiosus*, which also diverges from canonical thioredoxins at the level of the active site sequence (-CysPrpProCys-instead of-CysGlyProCys-; *Fig 1.3a*) (*Fig. 1.3c)*. The two thioredoxin-like proteins selected are both distantly related to mouse and human thioredoxins than *Ec*Trx: the anti-Trx antibodies may be less cross-reactive with mammalian thioredoxins compared to *Ec*Trx as described later.

Expression and preliminary evaluation of MtTrx and PfTrx compared to EcTrx

Codon-optimized *Mt*Trx and *Pf*Trx synthetic genes (*Appendix 1)*, with an added unique *CpoI* restriction site in the sequence region corresponding to the display site (the natural display site sequence of *Pf*Trx CPPC was converted into canonical CGPC sequence) commonly used for the insertion of heterologous peptides, were then designed and used for recombinant expression and 6xHis tag-mediated metal-affinity purification of the corresponding polypeptides.

Afterwards the cloning of these sequences into the *NdeI* restriction site of a modified pET28 plasmid in frame with two 6xHis tag sequences, the expression was done in *E.coli* BL21 codon plus (BL21cp) as described in Materials and Methods, using 1mM IPTG as inducer for 3 hours at 37°C. Following cell lysis, the His-tagged proteins were purified by metal-affinity chromatography and dialyzed against phosphate-buffered saline (PBS), the buffer used in the immunization.

Fluorescence spectroscopy ($\lambda_{excitation}$: 280 nm; was $\lambda_{emission}$: measured between 300 and 450 nm) was initially used to determine the apparent melting temperature of the two proteins[17], using His-tagged *Ec*Trx purified in the same way as a reference . Temperature was ramped from 25°C to 95°C in 5°C steps with a 5 min incubation at each temperature, followed by spectral analysis as a function of temperature. These analysis using the intrinsic fluorophore tryptophan and tyrosine respectively for *Ec*Trx and *Mt*Trx revealed conformational changes corresponding to denature state, detected by an increase emission intensity and a red shift from 350 to 360 nm and from 304 to 340 nm for *Ec*Trx and *Mt*Trx, respectively (*Fig. 1.4a-b*)[17].

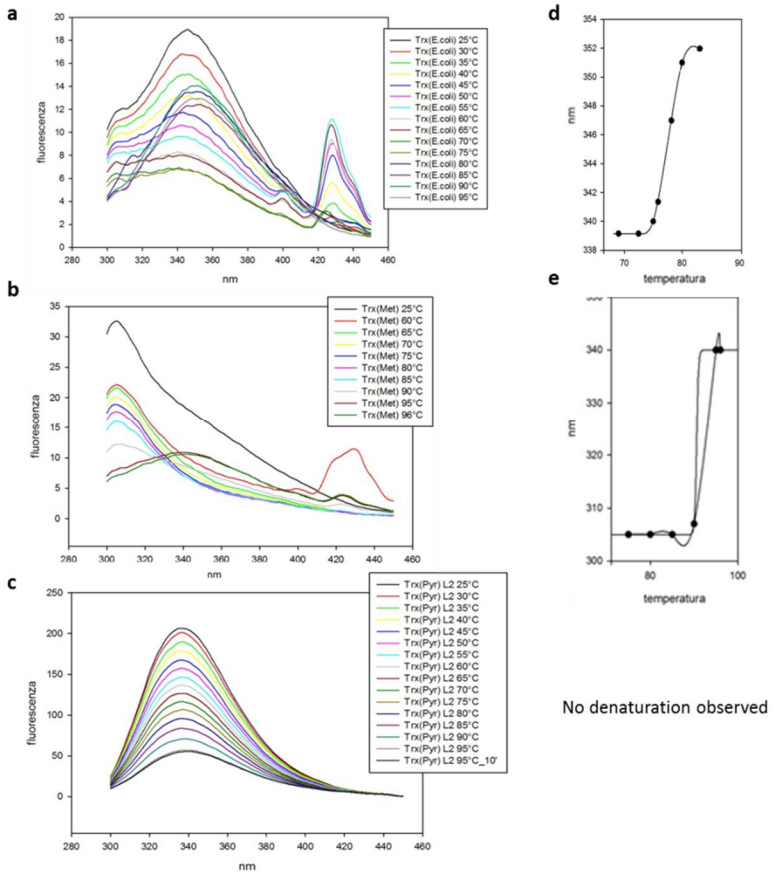

Fig. 1.4 Spectrofluorescence analysis of EcTrx (a), MtTrx (b) and PfTrx (c) during thermal denaturation. *His-tagged EcTrx, MtTrx and Pftrx are analysed using fluorescence spectroscopy during temperature ramp from 25 to 95°C to evaluate the apparent melting temperature, calculated for EcTrx (d) and MtTrx €. Each temperature are indicated in different colors: the PfTrx does not present any spectrum changes in contrast to EcTrx and MtTrx, and melting temperature was not calculated.*

Apparent melting temperatures, calculated as the temperature corresponding to the largest derivative of the sigmoidal curves representing the maximum emission wavelength as a function of temperature, were 80°C for *Ec*Trx (*Fig. 1.4d*) and 92°C for *Mt*Trx (*Fig. 1.4e*). No appreciable spectral change was detected for *Pf*Trx, indicating that this protein did not undergo any appreciable denaturation under these conditions (*Fig. 1.4c*).

Higher temperature conditions (100°C for 1, 13 and 24 h) together with a protein denaturation analysis based on ellipticity (Θ) measurements at 201.5 nm performed by Far-UV circular dichroism (CD) spectroscopy were then used to further characterize the three proteins and to give information on the thermal stability of *Pf*Trx. The analysis in the Far-UV spectral region (190-250

nm), using the peptide bonds as chromophore (absorption at 240 nm), permits to define protein secondary structure[18]. Surprisingly, even after the shortest incubation time, *Mt*Trx formed insoluble aggregates and nearly all of the protein was recovered as an amorphous precipitate, as if thermal denaturation caused the exposure of hydrophobic patches leading to irreversible aggregation. Under the same condition (1 h at 100°C) *Ec*Trx was fully denatured, but remained soluble, whereas an additional 24 h were required to fully denature *Pf*Trx (*Fig. 1.5a*). *Pf*Trx confirms its higher stability also in a longer treatment at lower temperature (50°C), in which completely denaturation occurred after 10 days, in contrast to *Ec*Trx, stable only for 3 days (*Fig. 1.5b*).

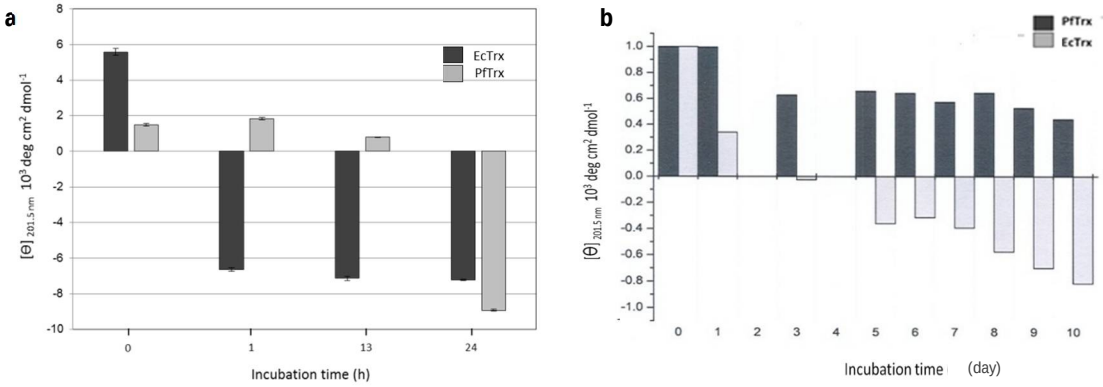

Fig. 1.5 Isothermal denaturation analysis of metal affinity-purified EcTrx and PfTrx[8]. *Molar ellipticity at 201.5 nm was measured by circular dichroism on the EcTrx (black bars) and PfTrx (grey bars) proteins (10 mM in PBS) incubated for (0–24 h) at 100°C (a) and (0-10 days) at 50°C; positive and negative values indicate a folded or unfolded state, respectively. Data are mean ellipticity values ± s.e.m. from three independent experiments.*

Based on the above data, indicating the unfavorable thermal stability properties of *Mt*Trx, we discarded this protein as a potential alternative scaffold. In contrast, *Pf*Trx proved to be an exceptional thermostable protein compared to *Ec*Trx and was subjected to further analysis. With both Trx, antigen stability is higher than VLP that presents a Tm around 45°C, evaluated using Far-UV CD analysis (data not shown).

Thermal stability, solubilization capacity and immunogenicity of PfTrx and EcTrx harbouring an HPV16 L2-derivated multipeptide epitope

To compared the scaffold properties of *Pf*Trx and *Ec*Trx, we inserted the sequence codified for HPV16 L2(20-38)$_3$ epitope[6] inside both Trx. The unique *CpoI* restriction site previously inserted into the sequence coding for the artificial *Pf*Trx protein variant was used for directional cloning of three tandemly repeated copies of HPV16 L2(20-38) peptide, with an interposed GlyGlyPro tripeptide spacer added to minimize junctional epitope formation. Following expression in BL21cp with 1mM IPTG for 3h at 37°C and metal-affinity purification (see Materials and Methods), the *Pf*Trx HPV16 L2(20-18)$_3$ fusion protein and *Pf*Trx without any insert were analyzed by Far-UV circular dichroism at 25°C, using corresponding *Ec*Trx and *Ec*Trx HPV16 L2(20-28)$_3$ as references[6]. Both L2-fusion proteins present a changes in protein secondary structure, in the random coil region (around 200 nm), respect the respectively empty scaffold protein, attributable to the insertion of the HPV16 L2(20-38)$_3$ peptide (*Fig. 1.6 panel a-b*). Thermal unfolding of *Ec*Trx HPV16 L2(20-38)$_3$ (from 25° to 85°C) and its partial refolding (from 85° to 25°C), measured in molar ellipticity [Θ] at 200 nm, yielded midpoint transition temperatures of 52.1°C and 49.9°C for unfolding and refolding, respectively (*Fig. 1.6c*). Under the same conditions, no significant change in spectra, and consequently in molar ellipticity, was observed with *Pf*Trx HPV16 L2(20-38)$_3$ (*Fig.1.6d*), indicating a higher thermal stability and perhaps a stronger insert constraining of the *Pf*Trx scaffold compared to *Ec*Trx, evaluated performing the analysis at 200 nm, probably corresponding to the epitope position. This features is important because could influence peptide epitope presentation and consequently immune response system's recognition.

To evaluate the solubilization capacity of *Pf*Trx, we used the entire N-terminal region of HPV16 L2 that previously proved to be completely insoluble when expressed as an *Ec*Trx fusion protein and required recovery from inclusion bodies under denaturing conditions[6]. The N-terminal region (amino acids 1-120) of HPV16 L2 was inserted into the display site (in the *CpoI* restriction site) of either *Ec*Trx or *Pf*Trx previously cloned into a modified pET28 plasmid. Bacterial cells expressing the two His-tagged Trx HPV16 L2(1-120) proteins were cultured, lysed and the pellet, with unsoluble proteins, and supernatant, containing soluble ones, were loaded into SDS-PAGE to verified the solubilization capacity of the two Trx scaffold proteins (see Materials and Methods). As show in *Fig. 1.7a,* the *Ec*Trx HPV16 L2(1-120) (molecular weight, MW: ~33kDa) is completely unsoluble, as previously described, in contrast to *Pf*Trx HPV16 L2(1-120) (same MW) that was 50% soluble and could be recovered from supernatant after bacterial cell lysis without the use of denaturing agents. This data was confirmed with an immunoblot analysis using a lower amount of the same fractions loaded into the SDS-PAGE and a HPV16 L2(20-38) monoclonal antibody as a primary antibody (*Fig. 1.7b*).

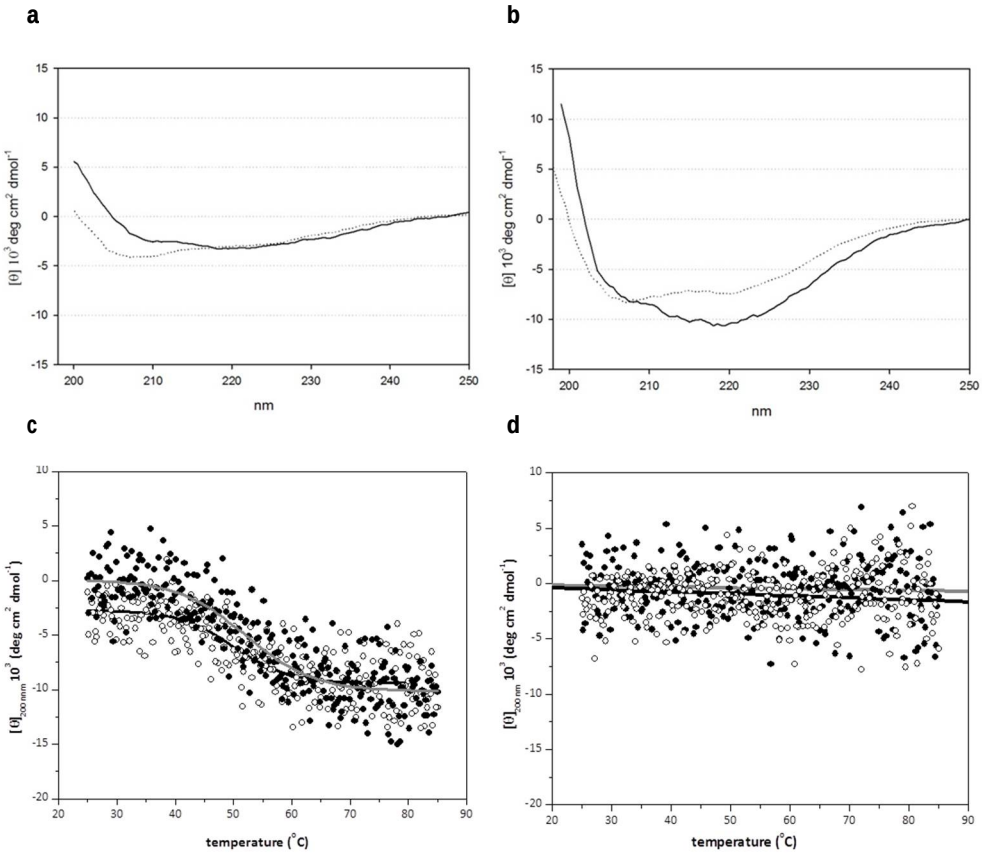

Fig. 1.6 Circular dichroism analysis of empty and L2 tripeptide containing EcTrx and PfTrx*[8]*. *(a) Far-UV CD spectra (recorded at 25°C) of the empty (solid line) and the L2 insert-containing (dotted line) EcTrx protein (10 mM in PBS) purified by metal-affinity chromatography. (b) Same as (a) for PfTrx. Spectra are given as molar ellipticity [Θ] values at different wavelengths (l). Secondary structure alterations produced by the incorporation of theHPV16 L2(20-38)$_3$ tripeptide are apparent. (c) Far-UV CD analysis of metal affinity-purified EcTrx HPV16 L2(20-38)$_3$ (10 mM in PBS). Data are presented as variations of molar ellipticity [Θ] at 200 nm upon unfolding (25–85°C; close dots, gray line) and refolding (85–25°C; open dots, black line). Temperature dependent midpoint transitions at 52.1°C (unfolding) and 49.9°C (refolding) were calculated. (d) Same as in panel C for PfTrx HPV16 L2(20-38)$_3$, for which no changes of [Θ] at 200 nm were observed.*

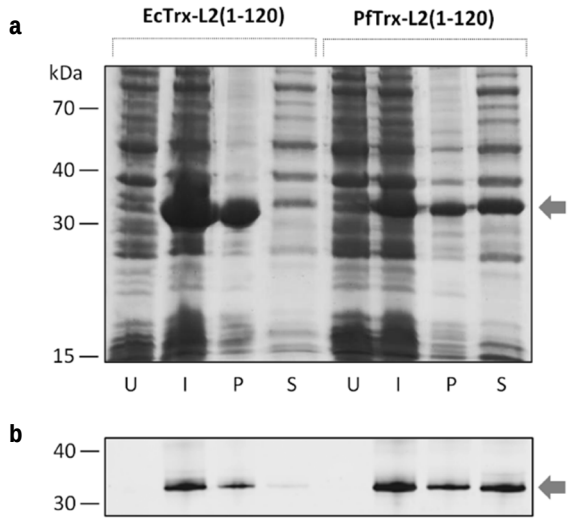

Fig. 1.7 Comparative analysis of the solubilization capacity of PfTrx and EcTrx[8]***.*** *a)The two His-tagged Trx HPV16 L2(1-120) proteins (marked with an arrow) were cultured in parallel (10 ml culture each), transferred to lysis buffer (1 ml), and the resulting lysate (''induced culture'', I) was centrifuged in order to separate pellet-associated, insoluble proteins (P) from soluble proteins recovered in the supernatant (S). An equal volume of the various fractions (15 ml each), including total lysates derived from uninduced bacterial cultures (U), was subjected to SDS-PAGE fractionation. A sizeable fraction (50%) of the PfTrx HPV16 L2(1-120) polypeptide was present in the soluble fraction, whereas EcTrx HPV16 L2(1-120) was almost completely insoluble (cf. lanes 3 and 4 with lanes 7 and 8). b) Results of an immunoblot analysis performed with an anti HPV16 L2(20-38) monoclonal antibody, using scaled-down amounts of the various fractions (0.08 ml equivalents each).*

Following the above *in vitro* results, we performed an immunization experiment aimed to compare the immunogenicity properties of *Pf*Trx and *Ec*Trx, both bearing three copies of the cross-neutralizing HPV16 L2(20-38)$_3$ epitope. Two groups of mice (10 mice per group) were immunized subcutaneously four times with 15 µg of metal affinity-purified *Pf*Trx HPV16 L2(20-38)$_3$ and *Ec*Trx HPV16 L2(20-38)$_3$, mixed with the adjuvant Montanide ISA 720 (50% v/v). The resulting immune-sera, collected eight weeks after the last immunization, were used to determine HPV neutralization titers using L1-Psudovirions-based neutralization assay (L1-PBNA) (see Materials and Methods). Nearly identical neutralization titers, the maximum dilution of immune sera causing 50% pseudovirion neutralization, were elicited by both antigens, indicating a similar immunogenic performance of *Pf*Trx- and *Ec*Trx-based L2(20-38)$_3$ antigens (*Fig. 1.8*), even if less variable immune responses were induced by *Pf*Trx HPV16 L2(20-38)$_3$ conferring a more consistent response.

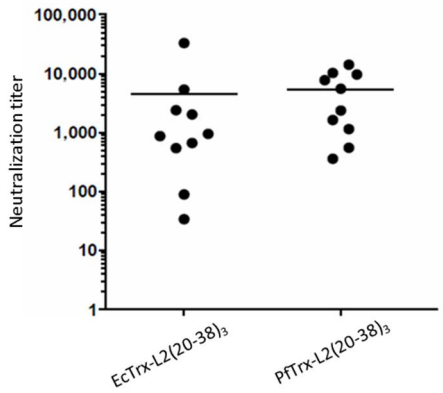

Fig. 1.8 Comparative analysis of the immunogenicity of PfTrx and EcTrx[8]*. HPV16 neutralization titers measured in sera collected after the last immunization (3rd boost) (a) and the first two immunization (b) from mice immunized with either affinity purified EcTrx HPV16 L2(20-38)$_3$ or PfTrx HPV16 L2(20-38)$_3$ adjuvanted with 50% v/v Montanide ISA 720. Dots represent individual neutralization titers of immune sera from the two groups (10 animals each)**;** geometric means of the titers for each group are indicated by horizontal lines.*

Immune cross-reactivity of anti-Trx antibodies

An important aspect for a vaccine is the absence of immune cross-reactivity against self-antigen, as thioredoxin in this specific case. His-tagged *Pf*Trx was used as a test antigen for mouse immunization (see Materials and Methods), with 6xHis *Ec*Trx and mouse thioredoxin (*Mm*Trx) as reference antigens. The anti-Trx (cross)reactivity of the resulting sera was analyzed by ELISA, using *Pf*Trx fused to glutathione S-transferase (GST), together with previously constructed GST-*Ec*Trx, GST-*Mm*Trx and GST-human thioredoxin (*Hs*Trx) fusion proteins, as a capture antigens (see Materials and Methods). As expected, maximal immune-reactivity was observed against each of the parent thioredoxin antigens (*Fig 1.9, left graph in each panel*). A significant cross-reactivity with mammalian thioredoxins was detected with sera from mice immunized with *Ec*Trx, which gave above background signals with both *Mm*Trx and *Hs*Trx (*Fig. 1.9b*). Similarly, sera from mice immunized with *Mm*Trx, not only cross-reacted with *Hs*Trx, but also showed detectable immune-reactivity with *Ec*Trx and *Pf*Trx (*Fig 1.9c*). In contrast, and in keeping with extreme sequence divergence of *Pf*Trx from the other thioredoxins (*Fig. 1.2 and Fig. 1.3*), sera from mice immunized with *Pf*Trx proved to be the least cross-reactive, with practically no cross-reactivity against mouse or human thioredoxin and a minimal, close to background reactivity with *Ec*Trx *(Fig. 1.9a*). Conversely, sera from mice immunized with *Ec*Trx were found to be more cross-reactive with mouse and human thioredoxin than with *Pf*Trx (*Fig. 1.9b*).

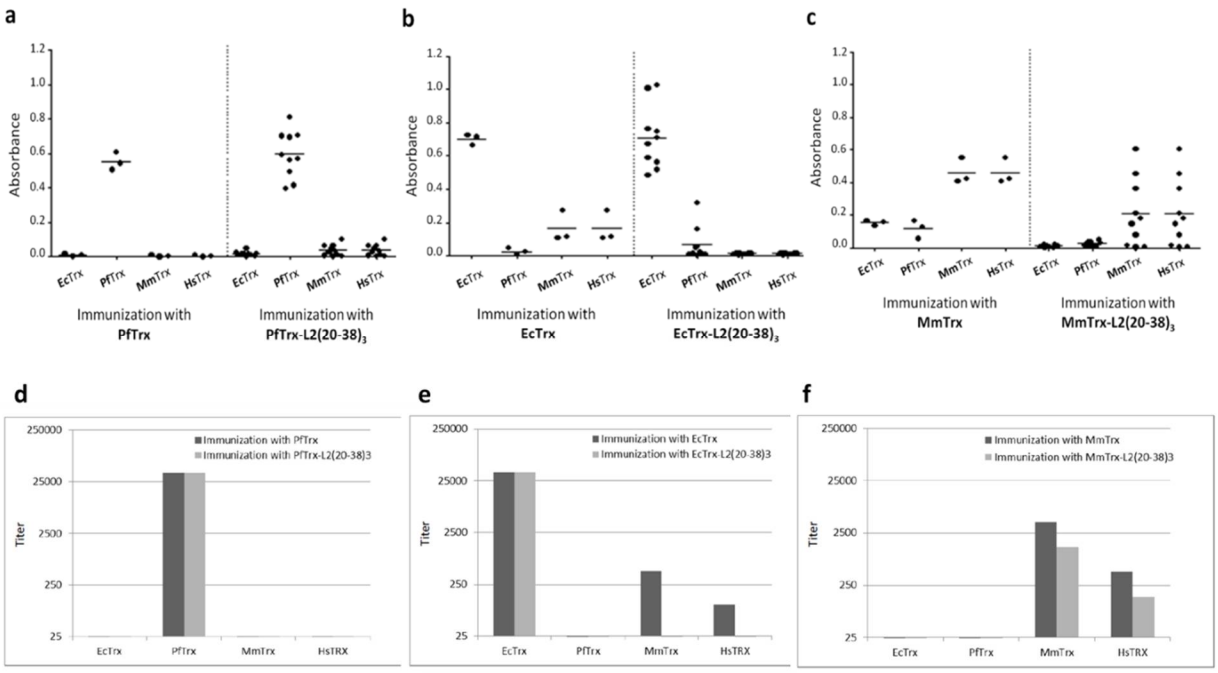

Fig. 1.9 Immune cross-reactivity profiles of anti-PfTrx, anti-EcTrx and anti-MmTrx antibodies[8]*. Sera from mice immunized with empty PfTrx, EcTrx and MmTrx (left graphs in panels a, b and c, respectively) were analyzed by ELISA using the corresponding GST-fusion proteins plus human thioredoxin (GST-HsTrx) as capture antigens. Data obtained with sera from mice immunized with the corresponding HPV16 L2(20-38)$_3$ insert containing antigens are shown in the right graphs (panels a-c, as above). Titration of pooled sera from mice immunized with the two antigen and different trx: PfTrx (d), EcTrx (e) and MmTrx (f). Each dot represents an individual mouse serum; mean absorbance values measured with each of the four capture antigens are indicated by horizontal lines. The immunization protocol was the same as the one utilized for the experiments reported in Fig. 1.8.*

A qualitatively similar picture, albeit with generally reduced cross-reactivity, was obtained with sera from mice immunized with L2 insert bearing, rather than empty thioredoxins. This included *Ec*Trx, where insert addition completely prevented cross-reactivity against human and mouse thioredoxins (*Fig. 1.9, right graph in each panel*). Reduced cross-reactivity upon L2 tripeptide insert addition is likely due to a structural alteration of the trx active site region utilized for peptide display, which represents the most conserved region (*Fig. 1.3*) and probably the major epitope shared by the different thioredoxins. Overall similar results, pointing again to a low cross-reactivity of antibodies elicited by empty *Ec*Trx (but not *Pf*Trx) with mouse and human thioredoxins were obtained by titration of pooled sera from mice immunized with the two antigens (*Fig. 1.9 panel d, e and f).* Therefore, even though cross-reactivity of anti *Ec*Trx antibodies with mammalian thioredoxins is significantly quenched by L2 peptide insertion (*Fig. 1.9b*), *Pf*Trx appears to be the least cross-reactive (it reacts only with itself) and potentially most effective

scaffold protein (*Fig. 1.9a*). As expect, the *Mm*Trx reacts and cross-reacts with itself and *Hs*Trx, respectively (*Fig. 1.9f*). Using an immunoblotting isotyping (see Materials and Methods), we define that antibodies elicited by *Pf*Trx HPV16 L2(20-38)$_3$ also display an IgG1-skewed isotype distribution with no detectable levels of IgG3 and IgM. This differs from isotype profile determined in sera from mice immunized with *Ec*Trx HPV16 L2(20-38)$_3$ which revealed a dissimilar antibody class switching promoting and increased IgG2a, IgG2b and IgG3 levels (*Fig. 1.10*).

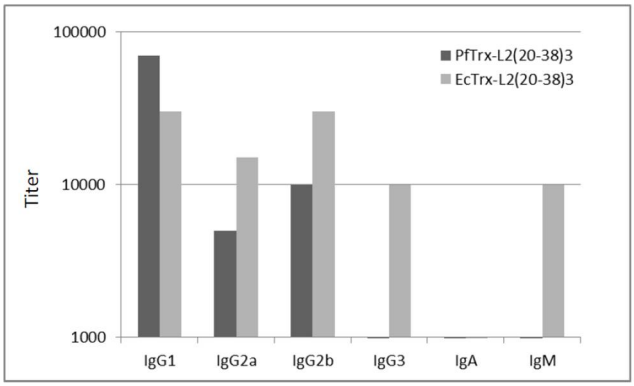

Fig. 1.10 Isotype distribution of antibodies in sera from mice immunized with PfTrx-L2 and EcTrx-L2 antigens[8]. *The bar plot shows the titers of the indicated immunoglobulin types in pooled sera from mice immunized with the two antigens; sera were collected after the last immunization (3rd boost).*

One-step thermal purification and immune performance of a tag-free form of PfTrx HPV16 L2 (20-38)$_3$ antigen

Another improvement for the development of human-use approvable recombinant immonogens suitable for phase I clinical investigations is the absence of foreign sequences commonly used for protein purification. We thus explored the feasibility of purifying a His-tag-free form of the *Pf*Trx HPV16 L2(20-38)$_3$ antigen by exploiting its thermal stability (*Fig. 1.6*) and high relative abundance in crude soluble lysates from overexpressing recombinant *E. coli* (*Fig. 1.7*). To this end, we set up and tested a one-step thermal purification procedure as a means to purify untagged *Pf*Trx HPV16 L2(20-38)$_3$ simply by a 20 min treatment at 70°C, after addition of NaCl (0,25M), followed by centrifugation to remove heat-denatured and insoluble, contaminating bacterial proteins (see Materials and Methods). This procedure allow us to purify the *Pf*Trx HPV16 L2(20-38)$_3$ antigen by more than 90% with yield comparable to that of metal-affinity purification (~20 mg of purified protein/liter of bacterial culture) in less than 1 hour with an extremely low-cost of production (*Fig. 1.11a*).

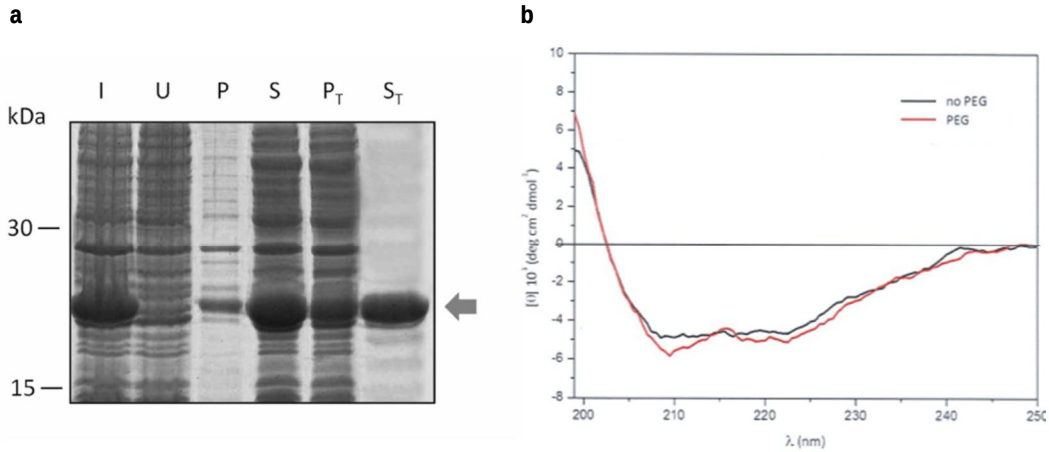

Fig. 1.11 Production of the tag-free, heat-purified form of the PfTrx HPV16 L2(20-38)$_3$ antigen and Far-UV CD analysis. *a) SDS-PAGE analysis of PfTrx HPV16 L2(20-38)$_3$ without His-tag purified with the one-step thermal purification procedure. I: induced bacterial culture; U: uninduced culture; P: post-lysis pellet; S: post-lysis supernatant; PT: heat-treated post-lysis pellet; ST: heat-treated post-lysis supernatant, containing the purified (>90%) PfTrx HPV16 L2(20-38)$_3$ protein (marked with an arrow); b) Far-UV CD analysis of PfTrx HPV16 L2(20-38)$_3$ heat purified with (red line) and without (black line) PEG.*

*Ec*Trx HPV16 L2(20-38)$_3$ could also be purified by heat-treatment, but at variance with the *Pf*Trx HPV16 L2(20-38)$_3$ antigen it require the addition of polyethylene glycol (PEG) 1000 as a protein stabilizer, that had to be removed with dialysis from the final antigen preparation prior to mice immunization. A Far-UV CD analysis at 25°C after purification with and without PEG 1000, demonstrated the maintenance of the same secondary protein structure of *Pf*Trx HPV16 L2(20-38)$_3$ in both procedure, demonstrating another time its higher thermal stability and constraining (*Fig. 1.11b*)

Also, as revealed by the Far-UV CD spectra (*Fig. 1.12a*), while His-tagged *Pf*Trx HPV16 L2(20-38)$_3$ pre-purified by metal-affinity chromatography and exposed to the same treatment conditions utilized for heat-purification (20 min at 70°C) regained exactly the same spectrum of the native, unheated protein, only a partial recovery of the native state CD spectrum was observed in the case of *Ec*Trx HPV16 L2(20-38)$_3$.

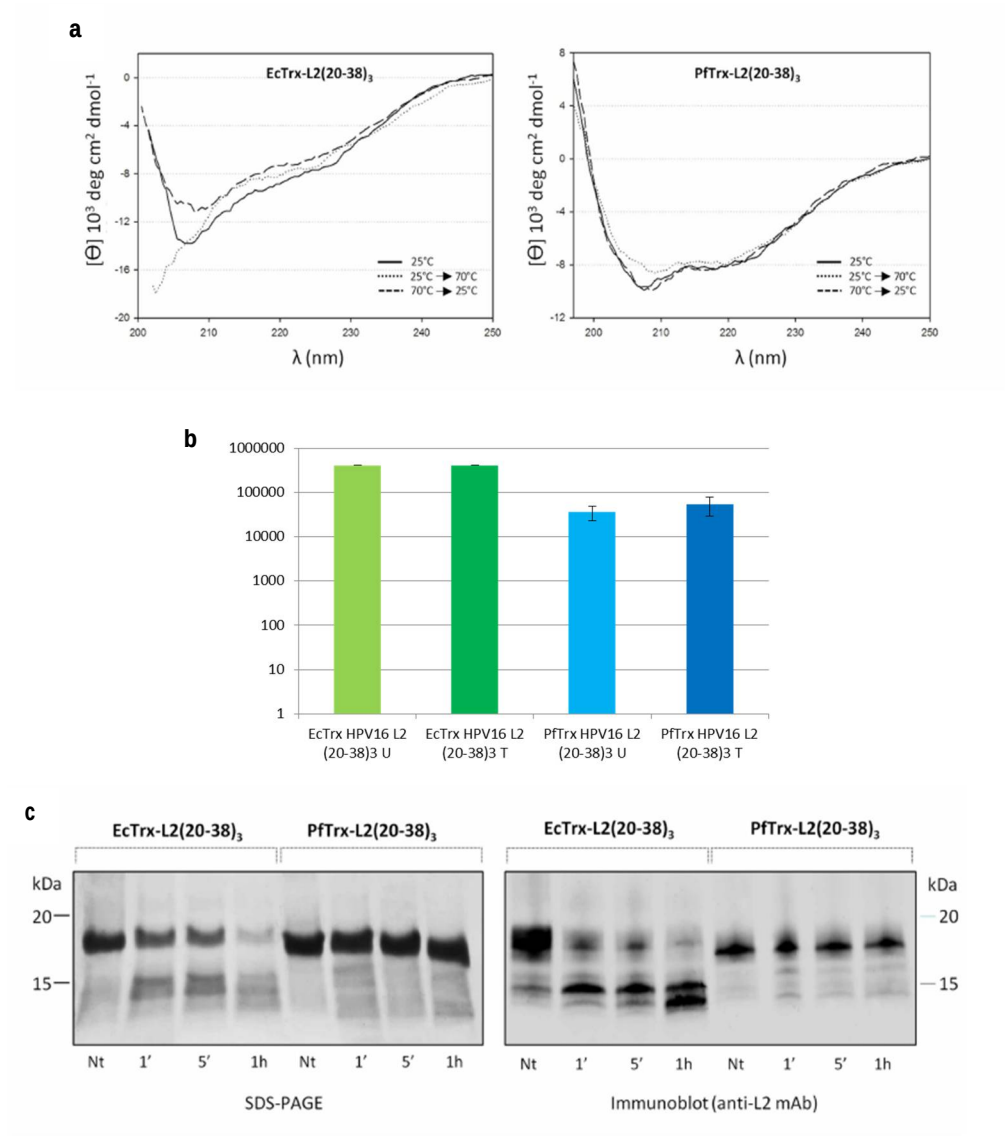

Fig. 1.12 Analysis of the tag-free, heat-purified form of the PfTrx HPV16 L2(20-38)$_3$ antigen[8]***.*** *(a) Far-UV CD analysis of affinity-purified EcTrx HPV16 L2(20-38)$_3$ (left) and PfTrx HPV16 L2(20-38)$_3$ (right) subjected to the same heat-treatment utilized for thermal purification. Spectra of the unheated proteins (25°C), of the proteins incubated under thermal purification conditions (20 min at 70°C), and of the latter samples returned to 25°C are shown as solid, dotted and dashed lines, respectively. (b) L2-capture ELISA. The EcTrx HPV16 L2(20-38)$_3$ and PfTrx HPV16 L2(20-38)$_3$ were treated (T) for 20 min at 70°C (heat-purification condition) to evaluate the epitope recognition capacity of anti-L2 mAb, The corresponding untreated proteins (U) were used as control. (c) Limited proteolysis analysis. Heat-purified EcTrx HPV16 L2(20-38)$_3$ and PfTrx HPV16 L2(20-38)$_3$ were treated for the indicated times with chymotrypsin (enzyme:Trx antigen ratio of 1:100 w/w), fractionated by SDS-PAGE and either stained with Coomassie Blue R-250 (left panel) or subjected to immunoblot analysis with an anti-HPV16 L2(20-38) mAb (right panel). A sample of each heat-purified Trx HPV16 L2(20-38)$_3$ protein not treated with chymotrypsin (Nt) served as control for these experiments*

The HPV16 L2(20-38)$_3$-capture ELISA on *Ec*Trx HPV16 L2(20-38)$_3$ and *Pf*Trx HPV16 L2(20-38)$_3$ treated with the heat-purification conditions, defined a good recognition of epitopes exposed on the different scaffold: the titers were the same of the untreated corresponding proteins (*Fig. 1.12b*). This define that the epitope in the active site loop is well exposed even if the secondary structure of *Ec*Trx HPV16 L2(20-38)$_3$ is not completely regained after heat-treatment. The results confirms the possibility of heat-purification with *Ec*Trx HPV16 L2(20-38)$_3$ even if the PEG 1000 addition increases the costs and time for production.

Further evidence as to a generally more compact structure given by *Pf*Trx scaffold was provided by limited proteolysis analysis, that consists in an incubation of *Pf*Trx HPV16 L2(20-38)$_3$ and *Ec*Trx HPV16 L2(20-38)$_3$ with chymotrypsin at 37°C for different lengths of time (from 1 min to 1 hour) (see Materials and Methods). This revealed a longer-lasting resistance to chymotrypsin of heat-purified *Pf*Trx HPV16 L2(20-38)$_3$ compared to the same antigen based on *E. coli* thioredoxin (*Fig. 1.12c*).

Even if the *in vitro* analysis define a similar behavior of heat-purified *Pf*Trx HPV16 L2(20-38)$_3$ compared with the His-tagged form of the same antigen purified by metal-affinity chromatography, it is important to compare their *in vivo* immune performances. Nearly identical HPV16 neutralization titers were measured in sera from animals (8 mice per group) immunized with 15µg the two antigens Montanide ISA 720-adjuvanted (see Materials and Methods), indicating a comparable immunogenicity of the heat- and metal-affinity purified forms of *Pf*Trx HPV16 L2(20-38)$_3$ antigen (*Fig. 1.13*).

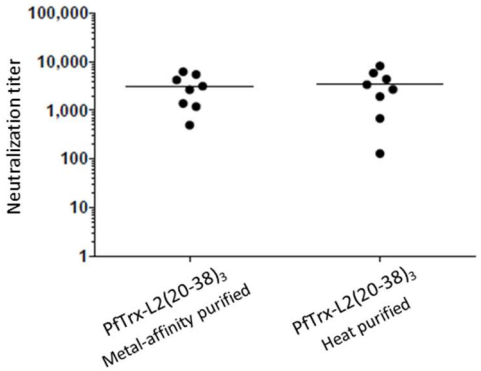

Fig. 1.13 Neutralization capacity given by metal-affinity and heat purified PfTrx HPV16 L2 (20-38)$_3$[8]*.*
HPV16 neutralization capacity of sera collected after the last immunization from mice immunized with the metal affinity-purified or the heat-purified PfTrx HPV16 L2(20-38)$_3$ antigen adjuvanted with 50% v/v Montanide ISA 720. Dots represent neutralization titers measured in sera from individual immunized animals; geometric means of the titers for the two groups (8 animals/each) are indicated by horizontal lines.

Given the possibility that trace amounts of bacterial proteases may contaminate heat-purified preparations thus compromising long-term antigen stability/integrity, we further purified the untagged, heat-purified *Pf*Trx HPV16 L2(20-38)$_3$ antigen on an anion-exchange column and compared the long-term stability of the antigen only subjected to heat-purification with that of the antigen purified by both heat-treatment and anion-exchange chromatography. The treatment consisted in an incubation at 37°C for 7 days. The SDS-PAGE profiles of the two antigen preparation remained essentially the same with no sign of proteins loss due to proteolytic degradation, confirming the quality of heat-purification protocol (*Fig. 1.16*).

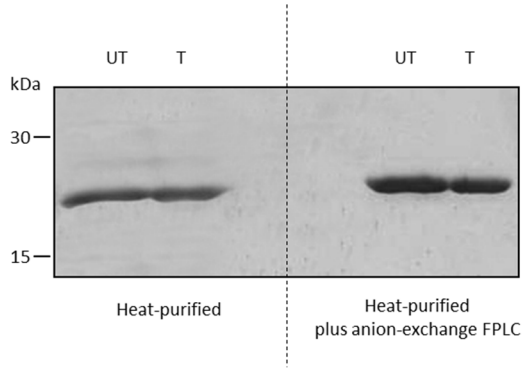

Fig. 1.16 Long-term stability of heat-purified PfTrx HPV16 L2(20-38)$_3$. *PfTrx HPV16 L2(20-38)$_3$ purified by heat treatment and an aliquot of the same protein preparation further purified by anion exchange FPLC were incubated for 7 days at 37°C (treated, T) and their integrity, analyzed by SDS-PAGE, was compared with that of the corresponding proteins kept at –80°C (untreated; UT).*[8]

The high immunogenicity of heat-purified *Pf*Trx HPV16 L2(20-38)$_3$ was also confirmed in rabbits: 300µg of heat-treated antigen, with Allum-MPLA (1:10 ratio) as adjuvant, was administrated subcutaneously at day 1, 28, 42, 60 (see Materials and Methods). High IgG and neutralizing antibody titers against HPV16 and HPV18 PsV were evaluated in ELISA, using L2-GST as a capture antigen, and L1-PBNA assay, respectively (see Materials and Methods) (*Fig. 1.14*).

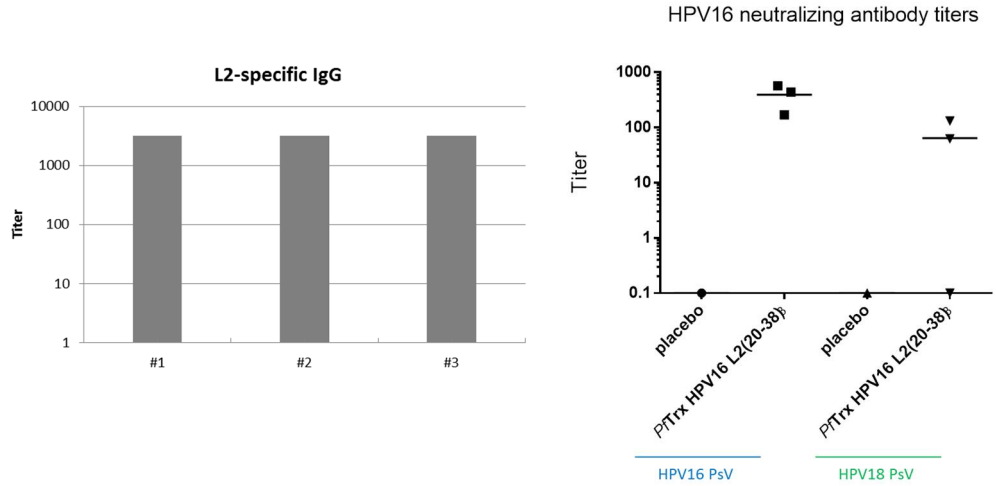

Fig. 1.14 GST-L2 ELISA and L1-PBNA on rabbit's sera. *Sera collected by ear punch from rabbits immunized subcutaneously with 300µg of heat purified PfTrx HPV16 L2(20-38)$_3$ were analyzed by GST-L2 ELISA (a) and L1-PBNA assay against HPV16 PsV (b). # indicate the number of the rabbit.*

Enzyme-linked immunospot (ELISpot) assay permits to confirm the presence of T cell epitopes in *Pf*Trx, giving the ability to stimulate T-cell proliferation, as described for *Ec*Trx[16]. Splenocytes from mice immunized with *Pf*Trx HPV16 L2(20-38)$_3$, are tested one week after the last immunization (see Materials and Methods) against overlapping *Pf*Trx HPV16 L2(20-38)$_3$-epitopes (*Appendix 2*). Two peptides, TELSEYFEDIQIVHINAGKW (B3) and DIQIVHINAGKWKNIVDKFN (C3), were able to induce IFN-γ secretion, with a stronger response with the latter (*Fig. 1.15*). Concanavalin (Con A), a polyclonal T-cell activator, is used as a control of the qualitative assay.

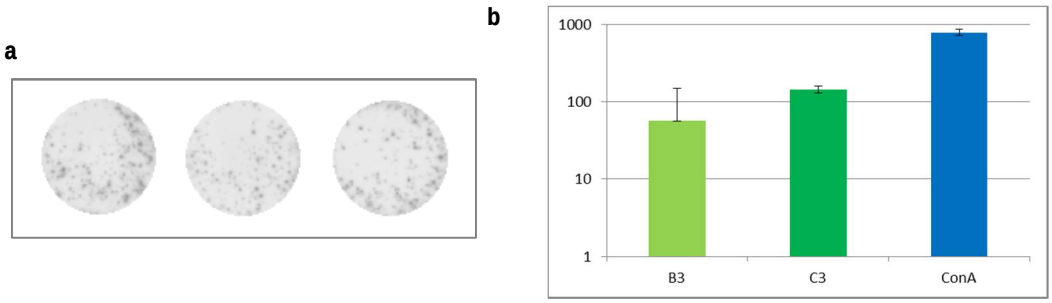

Fig. 1.15 ELISpot assay. *Qualitative ELISpot assay of splenocytes collected 1 week after the last immunization from three mice immunized four times with 20µg of PfTrx HPV16 L2(20-38)$_3$ at biweekly intervals a) Wells with the best response: dots represents the responsive IFN-γ cells. b) Comparison between responses obtained against the peptides B3 and C3: this represent the average of three mice tested in triplicate. Error bars are defined using standard deviation. Concanavalin (Con A) is used as positive control for the assay.*

Conclusion

We identified *P. furiosus* thioredoxin as a superior protein scaffold for the construction of recombinant peptide immunogens. Valuable features of *Pf*Trx compared to the commonly utilized *E. coli* protein are a higher thermal stability and protease resistance, a greater solubilisation capacity, and the complete lack of cross-reactivity of anti-*Pf*Trx antibodies with other thioredoxins, including human, mouse and *E. coli* Trx. Using a HPV L2-derived peptide as a specific case study, we exploited the extreme thermal stability of *Pf*Trx to set up a thermal purification procedure that allowed to purify the *Pf*Trx HPV16 L2(20-38)$_3$ antigen by more than 90% in a single step without the use of foreign sequence tags, nor protein stabilizers. Heat-purified *Pf*Trx HPV16 L2(20-38)$_3$ was found to be significantly more resistant to chymotrypsin digestion than the corresponding *Ec*Trx antigen and could withstand a 7-days incubation at 37°C without any sign of degradation. HPV neutralization titers elicited by untagged, heat-purified *Pf*Trx HPV16 L2(20-38)$_3$ were the same as those of the affinity-purified, His-tagged *Pf*Trx and *Ec*Trx antigens. Therefore, three basic requirements for an improved, human-use approvable vaccine, namely the lack of cross-reactivity with host proteins, a long shelf-life without the need for cold-chain storage and a low-cost of production in the absence of foreign sequence tags, appear to be fully met by the *Pf*Trx-L2 antigen. We thus believe that the superior thermal stability and proteolysis resistance, solubilisation capacity and complete lack of cross-reactivity of *Pf*Trx warrant a more straightforward, trouble-free use of this scaffold protein for a variety of peptide antigens and immunogen formulations. The latter may include poorly soluble multipeptide epitopes, longer and/or less stable than the presently tested 57 aa. L2 tripeptide, as well as further functionalization of the thioredoxin scaffold. For example, the incorporation of exogenous universal T-helper epitopes to enhance immunogenicity in particular hosts such as the C57BL/6 mouse strain, as shown in the next section. Other modifications may entail the addition of (poly)peptide molecular adjuvants as well as specific protein modules to anchor the antigen to solid supports in order to produce particulate immunogens. The above modifications may compromise the solubility and/or stability of the resulting conjugates and can thus benefit from the use of an extremely robust protein scaffold. On a related note, the marked protease resistance of *Pf*Trx may also allow a longer persistence of *Pf*Trx-based antigens at the tissue injection site with a consequently stronger (and more consistent) immune response[19]. This may prove to be particularly important for the development of gastro-protected, orally administrable immunogens that become exposed to the harsh (e.g., protease-rich) environment of the intestine. Another application of recombinant peptide immunogens, in addition to subunit vaccine construction, is their use for the production of diagnostic polyclonal and monoclonal antibodies. This can also benefit from a robust scaffold with a high solubilization capacity and the ability to tolerate harsh purification conditions. Furthermore, the complete lack of immune cross-reactivity with mammalian thioredoxins would result in a strongly reduced background reactivity of the resulting antibodies.

A further ongoing application regards the use of *Pf*Trx as a carrier of overlapping peptide fragments derived from selected oncoproteins to be used as a capture antigens for the immune-screening of sera from HPV-positive patients, with the purpose to develop a new diagnostic (and prognostic) assay of HPV infection. The current diagnostic test, based on PCR, permits to evaluate only HPV presence without any information about the progression of the infection. Time needed to define the infection phase is another limitation of this method; in fact, after the first positive test, the PCR needs to be repeated 4/6 months later and then, the positivity needs to be confirmed by histological analysis (PAP-test in cervical cancer cases).

The oncoproteins E6 and E7 represent good targets to develop a new HPV immune-assay, being highly expressed during HPV proliferation and determining a specific humoral response in the 50% of cervical carcinoma patients.

Luminex Miltiplex (xMAP) seems to be the perfect technology for this purpose, permitting a rapid, quantitative and simultaneously analysis of different specimens, using a little amount of sample volume (20-50 µl). This platform combines microsphere based assays with advanced digital signal processing and identification techniques to perform multi-analytes testing of up to 500 features in real time. The configuration of the xMAP assay consists of a suspension array where specific target proteins are covalently coupled to the surfaces of internally different dyed microspheres (beads). After incubations with a serum sample, the protein-beads, recognized by specific antibody are incubated with a secondary detection antibody. A Luminex analyzer separates and interrogates the beads with two lasers or LEDs: one to identified the analyte (the antibody able to binds the protein) and one for classification of the bead identity (and consequently, the typr of protein fragment covalently bounded on it)[20].

To overcome the difficulties in expression of full length proteins, the E6 and E7 of HPV16 have been divided in overlapping fragments and then cloned into the active site loop (using *CpoI* restriction site) of *Pf*Trx. All the recombinant proteins will be tested with xMAP so as to titrate different (E6 and E7) epitope-specific antibody in sera from HPV-positive patients at different infection phase. The results of this extensive immune-analysis will be used for evaluating the diagnostic/prognostic capacity of this approach.

Materials and Methods

Trx constructs. Codon-usage optimized *Escherichia coli* (*Ec*), *Methanosaeta termophila* (*Mt*), *Pyrococcus furiosus* (*Pf*) and *Mus musculus* (*Mm*) Trx-coding sequences (see Appendix 1) were chemically synthesized (Eurofins MWG Operon) and inserted into the *NdeI* site of a modified pET28 plasmid (Novagen) in frame with two 6xHis tag sequences. The resulting constructs were sequence-verified and transformed into *Escherichia coli* BL21 codon plus (DE3) cells for recombinant protein expression. Glutathione S-transferase (GST) fusions to be used as capture antigens for ELISA analysis were produced from the same sequences, plus *Homo sapiens* (*Hs*) Trx, cloned into the *SmaI* site of the GST expression vector pGEX-4T-2 Trx-L2(20-38)$_3$ antigen constructs containing three tandemly repeated copies of the HPV16 L2 aa 20-38 peptide grafted to the display site of *Ec*Trx and *Pf*Trx as well as the Trx HPV16 L2(1-120) constructs were generated by inserting the corresponding sequences into the Trx *CpoI* sites of pre-assembled pET-*Ec*Trx and pET-*Pf*Trx plasmids as described previously[6]. Untagged derivatives of the above constructs were obtained by subcloning the *Ec*Trx HPV16 L2(20-38)3 and *Pf*Trx HPV16 L2(20-38)$_3$ inserts, without the dual 6xHis-tag sequences, into the *NdeI* site of the His-tag-lacking pET26 plasmid.

Expression and purification of the Trx polypeptides. Recombinant protein expression was induced by adding 1 mM isopropyl-b-D-thiogalactopyranoside (IPTG) to the various *E. coli* trasformants, which were then cultured for 3 hours at 37°C. Following cell lysis, 6xHis-tagged polypeptides (both the empty Trx scaffolds and the Trx-L2 fusion polypeptides) were bound to a metal-affinity resin (Talon, Clontech), purified as per the manufacturer's instructions and dialyzed against phosphate-buffered saline (PBS). Untagged Trx-L2 proteins were purified with a modified heat-treatment procedure[21]. Briefly, following bacterial lysate centrifugation, NaCl (0.25 M) was added to the cleared *Pf*Trx HPV16 L2(20-38)$_3$ supernatant and the resulting solution was incubated for 20 minutes at 70°C, cooled on ice for 10 minutes and centrifuged at 12,000 g for 10 minutes to recover the supernatant containing the purified Trx-L2 protein. A similar procedure, but with the addition of PEG 1000 (12.5%) to the cleared supernatant as stabilizer, was applied to *Ec*Trx HPV16 L2(20-38)$_3$. GST-Trx fusion proteins were affinity-purified on glutathione-sepharose columns (GE Healthcare) according to the manufacturer's instructions. Trx-L2 antigens to be used for mouse immunization experiments (see below) were detoxified by a 3X Triton X-114 (1% v/v) treatment as described[22]; endotoxin/LPS removal (to levels lower than 2 EU/ml) was verified in each sample by the LAL QLC-1000 test (Lonza). The composition and purity of individual protein preparations were assessed by electrophoretic analysis on 11-15% SDS-polyacrylamide gels and MALDI-TOF analysis. Protein concentration was determined by measurement of absorbance at 280 nm using calculated extinction coefficients and with the use of a QubitH 2.0 Fluorometer (Life Technologies).

Melting temperature measurements by fluorescence spectroscopy. Fluorescence measurements were performed with a Jasco FP-6200 Spectrofluorimeter equipped with a Peltier temperature controller, using a fixed 10 mM protein concentration in PBS. Thermal unfolding was carried out in the 25-95°C temperature range with 5°C temperature increments, and samples were allowed to equilibrate for 5 min at each temperature before spectral measurements. Tryptophan and tyrosine residues were excited at 280 nm and fluorescence emission was measured between 300 and 450 nm with a bandwidth of 5 nm, a data-pitch of 1 nm and a scanning speed of 60 nm/min.

Far-UV circular dichroism spectroscopic analyses. Far-UV CD spectra (190–260 nm) were acquired with a Jasco J715 Spectropolarimeter equipped with a Peltier temperature controller, using a 0.1 cm path-length cuvette, a bandwidth of 1 nm, a data pitch of 0.5 nm, and a response time of 4s;

CD spectra were averaged from 4 scans. Protein concentration was 10 mM in PBS for the single spectra recorded at 25°C and 0.1X PBS for thermal stability measurements. Following baseline correction, the measured ellipticity, h (mdeg), was converted to the molar mean residue ellipticity [Θ] (deg.cm^2.dmol^{-1}), using [Θ] 5h/cn$_{res}$l, where Θ is ellipticity, c is the molar concentration of the protein, n$_{res}$ is the number of amino acid residues in the protein and l is the optical path length in centimeters. Thermal unfolding/refolding studies were performed in the 25°C–85°C temperature range; temperature scan was 1°C/min in order to maintain thermal equilibrium. The loss of secondary structure was followed by measuring [Θ] at 200 nm. Tm values were calculated as the inflection point of unfolding curves fitted according to the single-step (two-state) transition model.

Immunoblot analyses. Whole cell extracts or purified proteins were electrophoresed on Criterion TGX precast 4–20% gels (Bio-Rad) in Tris/Glycine buffer. Fractionated samples were electro-transferred to 0.2 mm nitrocellulose membranes (Bio-Rad), that were blocked with 5% skin milk in Tris-buffered saline at 4°C for 12 h. Membranes were then washed with the same buffer and incubated with the anti-L2(20-38) monoclonal antibody K4[7] (diluted 1:2000) at room temperature for 1 h, followed by the addition of an IRDye 680-conjugated, goat anti-mouse secondary antibody (diluted 1:15000) and visualization of immune-reactive bands by near infrared fluorescence (NIRF) with an Odissey (LI-COR) imager.

Limited proteolysis. Heat-purified *Ec*Trx HPV16 L2(20-38)$_3$ and *Pf*Trx HPV16 L2(20-38)$_3$ (5 mg each) were incubated with 0.05 mg of chymotrypsin (Sigma-Aldrich; enzyme: protein ratio of 1:100 w/w) at 37°C for different lengths of time (from 1 min to 1 hour). Reactions, carried out in 50 mM Tris-HCl pH 8.0, 100 mM NaCl, were stopped by adding SDS-containing electrophoresis sample buffer and analyzed by SDS-PAGE (12% acrylamide gels).

Animal immunization and pseudovirion-based neutralization assays. Procedures in animal experiments were approved by the Regierungspräsidium Karlsruhe under permit G246/11 and G19/13. Six- to eight-weeks-old female BALB/c mice (Charles River; Sulzfeld, Germany) were kept in the animal house facility of the German Cancer Research Center under pathogen-free conditions, in compliance with the regulations of the Germany Animal Protection Law. Mice were immunized subcutaneously four times at biweekly intervals with 15 μg of the various detoxified and filter-sterilized Trx-L2 antigens adjuvanted with 50% (v/v) Montanide ISA 720 (Seppic, France). Eight weeks after the last immunization, blood samples were collected by cardiac puncture. Three 6 weeks New Zealand rabbits were immunized subcutaneously four times on days 1, 28, 42, 60 with 300 μg of heat purified *Pf*Trx HPV16 L2(20-38)$_3$ with Alum/MPLA (500 μg and 50 μg, respectively). Blood samples were collected with ear puncture before immunization as pre-immune sera and 1 month after the last immunization. Pseudovirion preparation and neutralization assays were performed as described[23]. Briefly, 50 ml of serially diluted immune sera (or control monoclonal antibodies) were mixed with 50 ml of diluted pseudovirion stocks and incubated at room temperature for 30 min. Next, 50 ml of HeLaT cells (2.5 x 10^5 cells/ml) were added to the pseudovirion-antibody mixture and incubated for 48 h at 37°C under a 5% CO_2 atmosphere. The amount of secreted Gaussia luciferase was then determined in 10 ml of cell culture medium using coelenterazine substrate and Gaussia glow juice (PJK, Germany) according to the manufacturer's instructions. A microplate luminometer (Victor3, PerkinElmer) was used to measure luminescence in the culture medium 15 min after substrate addition.

Immune cross-reactivity analyses. GST–Trx capture ELISAs were carried out in 96-well plates pre-coated with glutathione-casein and subsequently blocked with casein buffer as described previously[24]. Glutathione-casein-coated plates were incubated for 1 h at 37°C with cleared lysates

(diluted to 0.5 mg/ml total protein) containing the different GST–Trx fusion proteins. This was followed by the addition of casein blocking buffer and three washes with Tween-20 (0.3% v/v)-PBS; a GST alone (unfused protein) lysate served as a negative control for these assays. Sera from mice immunized with either *Ec*Trx, *Pf*Trx and *Mm*Trx, with and without the L2 tripeptide insert (10 and 3 animals/group, respectively), were analysed both individually at a fixed 1:300 dilution and by serial dilutions of pooled sera from different immunization groups. Following serum addition and incubation, plates were washed three times as above and incubated with a horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Dianova) previously diluted 1:3000 in 1.5% milk-PBS plus 0.3% Tween-20. Plates were incubated for 1 h at 37°C, washed three times, and developed by adding the ABTS [2,29-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid)] staining solution (1 mg/ml in 100 mM sodium acetate-phosphate buffer pH 4.2, plus 0.015% H_2O_2; 100 ml/well). Absorbance at 405 nm was measured after 40–60 min with an automated plate reader (Titertek, Berthold).

ELISpot assay. ELISpot assay was performed in PVDF plate (Millipore). Add 15µl of 35% Ethanol per well and incubate ≤ 1 min until entire well turns dark, decant ethanol and wash plate three times with 200µl 1X PBS. Coating plate was carried out with 100µl/well capture antibody (5 µg/ml in PBS 1X) and incubated over night at 4°C. After removing of antibody solution, the plate was washed 3 times with 200µl/well of sterile 1X PBS (2% FCS, 100U/ml penicillin, 100 µg/ml streptomycin). Blocking was done with 200µl/well of complete RPMI medium (10 mM HEPES, 1 mM Sodium Pyruvate, 10% serum, 100U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine) for at least 2 hours at RT. During the incubation, the splenocytes were prepared. The spleen was remove aseptically, transferred firstly into sterile a 15 ml falcon containing 5 ml of ice cold PBS and after into petri dish and for another washing with 5 ml PBS. Aspirated the PBS and added 5 ml complete RPMI medium, the spleen was smashed via the syringe cap. The cells were passed through the cell strainer and washed with plate with 5 ml complete RPMI medium. Suspension was centrifuged at 1500 rpm for 5 min at 4 °C and the pellet resuspended in 5 ml RBC lysis buffer (170 mM Tris-HCl, 0.16 M NH_4Cl; pH7.2), followed by incubation on ice for 5 min in order to lyse erythrocytes (with frequent shaking). Neutralization of the reaction was performed adding 5 ml PBS containing 2% FCS, 100U/ml penicillin and 100 µg/ml streptomycin (wash buffer), followed by two washing with wash buffer and, finally, resuspended in 5 ml complete RPMI medium. The cells were diluted to 1.0 x 10^7 cells/ml. Send two incubation hours of plate, the blocking medium was remove and 100 µl of cell suspension $3x10^5$ splenocytes per well ($3x10^6$ cell/ml solution) were added. 10 µg/ml Concanavalin (Con A) in 100µl of medium is prepared as a control. The plate was incubated 36 h at 37°C, 5% CO_2. After cells removal, the plate was washed three times with 200 µl PBS/0.05% Tween 20, followed by addition of biotinylated rat anti-mouse IFNγ (100µl/well). The incubation was performed for at least two hours at RT or overnight at 4°C. After three times washing with PBS/0.05% Tween 20 (1 min between each wash), streptavidin-AKP was added (100µl/well) and incubate for 45 min at RT. Using ELISPOT wash buffer (PBST), the plate was washed three times and then two times with PBS 1X without Tween-20. BCTP/NBT substrate (Sigma) was added (100 µl/well) and after 4 min of RT incubation and addition of distillated water to stop reaction, the plate was air dried and the spots were counted using an automated ELISpot reader (AID EliSpot Reader Systems).

Immunoglobulin isotyping. Immunoglobulin subclasses in sera from mice immunized with the different thioredoxin scaffolds were determined by ELISA using a mouse antibody isotyping kit (BD Pharmingen). Briefly, 96-well plates were coated overnight at 4°C with individual isotype-specific (IgG1, IgG2a, IgG2b, IgG3, IgM, IgA) rat anti-mouse monoclonal antibodies (mAb) in 1X PBS. After washing with 0.05% Tween-20 in PBS, plates were blocked with 1% BSA in 1X PBS and incubated at

room temperature for 30 min. Ten sera for each group of mice immunized with the different Trx antigens (*Pf*Trx HPV16 L2(20-38)$_3$, *Ec*Trx HPV16 L2(20-38)$_3$ and *Mm*Trx HPV16 L2(20-38)$_3$) were pooled and an aliquot of each pool was added to the wells at increasing serial dilutions, starting from a 1:5000 dilution. Following incubation at room temperature for 1 h, the plates were washed three times as above and incubated with a HRP-conjugated, rat anti-mouse immunoglobulin mAb previously diluted 1:100 in PBS containing 1% BSA for 1 h at room temperature. After three washes, the reaction was developed by adding the HRP substrate solution and absorbance at 450 nm was read after 10 min with a microtiter plate reader (Bio-Rad).

Bioinformatic and statistical analysis. Sequence alignments were performed with ClustalW and imported into the Molecular Evolutionary Genetics Analysis package (MEGA 4.1)[25]; percentage identity was calculated and visualized with the GeneDoc program (http://www.psc.edu/biomed/genedoc/). Secondary structures were predicted with the YASPIN program (http://www.ibi.vu.nl/programs/yaspinwww/). Statistical significance of neutralization assay results was determined with the nonparametric Mann-Whitney-Wilcoxon test performed with GraphPad Prism 5.00 (GraphPad Software, USA); differences between groups were considered significant at $p<0.05$.

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Section II

Pichia pastoris expression system: yield increase and endotoxin free preparation

Introduction

With the aim of further streamlining *Pf*Trx-L2 production, especially with regard to protein yield, ease of purification and elimination of the endotoxin removal step which is both time- and protein-consuming and requires downstream testing of residual endotoxin levels, we transferred production of our Trx-L2 HPV immunogen to the methylotrophic yeast *Pichia pastoris*.

P. pastoris, currently reclassified as *Komagataella pastoris*[1], has been utilized to produce foreign protein since 1984[2]. This system offers significant advantages over *E. coli*, in particular for proteins that contains multiple disulphide bonds, or requires typical eukaryotic post-translational modifications (glycosylation, phosphorylation). Also proteins that need the absence of an N-terminal methionine or no oligomer formation for the correct assembly, are better expressed in this system. *P. pastoris* metabolism is based on methanol as unique carbon and energy source, initially catalyzed by alcohol oxidase (AOX). *P. pastoris* genome contains two genes encoding two enzymes with AOX activity: AOX1, controlled by a strength promoter and predominantly expressed, and AOX2, under the control of a weaker promoter[3]. Three phenotypes of *P. pastoris* are available: Mut^+, where both AOX genes are active, Mut^S, where AOX1 is knocked out, and Mut^-, which is unable to grow on methanol due to knock-out of both AOX genes[2]. Even if the majority of research was performed in Mut^+ strains, several studies identified Mut^S as the best for recombinant protein production[4,5].

Several factors contribute to make *P. pastoris* an important host organism for production of recombinant proteins[2,6,7]. Firstly, the use of AOX1 promoter to drive the expression of recombinant protein. This is one of the strongest, most regulated promoter, thanks to the possibility to switched it off: non-limiting amounts of carbon source, such as glycerol and glucose, repress AOX1 promoter that could be induce by methanol addition during induction phase, when the biomass has been well established.

Secondly, the possibility to integrate, via homologous recombination, expression plasmids at specific sites in *P. pastoris* genome, in either single or multi-copy, generating genetically stable transformants able to express high amount of recombinant protein without continual selection-based condition, in contrast to episomal plasmid. Multi-copy integration, that could be evaluated using increasing concentration of zeocin[8,9], results in an increase in product yield, even if the relationship is not always linear. In fact, there are some evidences in which high copy number is associated to a reduction in protein amount, in particular in the case of secreted proteins in which the high yield could stress the secretory pathway, upregulating the unfolded protein response (UPR) and resulting in a degradation of the protein[10,11].

Secretion represents another important feature using *P. pastoris* as expression system: cloning the encoding sequence downstream the sequence encoding for prepro α-factor of *S. cerevisiae (*α-MF) permits to obtain chimeric fusion protein that enters the secretion pathway. The α-MF

sequence is processed by two proteases (Ste13 and Kex2) that cleave the amino acid sequence KREAEA at the end of α-MF[12]. Kex2 cleaves efficiently after KR whereas the Ste13 cleavage after the EA repeat may be incomplete, depending on the following amino acids in the protein and the inability of Ste13 to process a large quantity of recombinant protein. This could cause a differential N-terminal ends extensions of the proteins[13]. The Glu-Ala repeats can be omitted but, this can result in a decreased efficiency in the Kex2 cleavage specificity. After the processing, the protein is secreted into the culture media fully intact and with specific modification.

The use of yeast as host organism permits to obtain free of lipopolysaccharides (LPS) polypeptides, characteristic of bacterial preparation, that elicit strong immune response binding the CD14/TLR4/MD2 receptor complex in many cell types[14], and are not admitted in a vaccine preparation. In this context, treatment for LPS removal, such as Triton X-114 extraction, are not necessary, reducing costly and time-consuming of this step.

Construction of the pPICZα-PfTrx vector and expression of PfTrx HPV16 L2(20-38)$_3$ as a secreted non-glycosylated polypeptide in P. pastoris

A general-purpose vector for the production of Trx-displayed peptides in *P. pastoris* was first constructed by insertion of the *Pf*Trx sequence[15] into the *XhoI* site of a pPICZα plasmid, in frame with the α-factor secretion signal peptide sequence (*Fig. 2.1a*). pPICZα-*Pf*Trx contains a unique *CpoI* site corresponding to the display site of *P. furiosus* thioredoxin. This site was used for directional cloning of a DNA sequence coding for three tandem repeats of the HPV16 L2(20-38) peptide (KTCKQAGTCPPDIIPKVEG) separated by a three-amino acid (GGP) spacer in order to avoid junctional epitope formation, thus generating pPICZα-*Pf*Trx HPV16 L2(20-38)$_3$ (*Fig. 2.1a*). The latter construct was transferred into *P. pastoris* KM17H cells (Mut^S strain) by integrative transformation, followed by selection in the presence of increasing concentrations of zeocin[16]. A randomly selected clone, resistant to the highest zeocin concentration (2 mg/ml), was used for a preliminary assessment of *Pf*Trx HPV16 L2(20-38)$_3$ expression. As shown in *Fig. 2.1b*, a polypeptide co-migrating with *E. coli* produced *Pf*Trx HPV16 L2(20-38)$_3$ and recognized by an anti-*Pf*Trx monoclonal antibody was identified in the culture medium of a methanol-induced, small-scale culture of *P. pastoris*. As further shown in *Fig. 2.1b* (cf. Pp-U *vs.* Pp-EndoH), the apparent molecular size of the *Pichia*-produced polypeptide did not change appreciably after endoglycosidase-H treatment. In keeping with the lack of putative glycosylation sites predicted by *in silico* analyses separately conducted on the *Pf*Trx scaffold and on the L2 tripeptide insert, this indicates the lack of co-secretory *Pf*Trx HPV16 L2(20-38)$_3$ glycosylation. The presence of *Pf*Trx HPV16 L2(20-38)$_3$ in the culture medium was further confirmed by MALDI-TOF mass spectroscopy (MS) analysis (*Fig. 2.1c*), which revealed a major polypeptide species with a molecular mass of 18543 Da. The slightly higher

molecular mass yielded by MS analysis (compared to an estimated nominal mass of 18123 Da) is in accordance with the small difference in electrophoretic mobility between the *E. coli*- and the *Pichia*-produced *Pf*Trx HPV16 L2(20-38)$_3$ polypeptide (*Fig. 2.1b-c*) and is explained by the retention of four secretion signal peptide amino acid residues upon *in vivo* N-terminal cleavage by the site-specific Kex2 endoprotease.

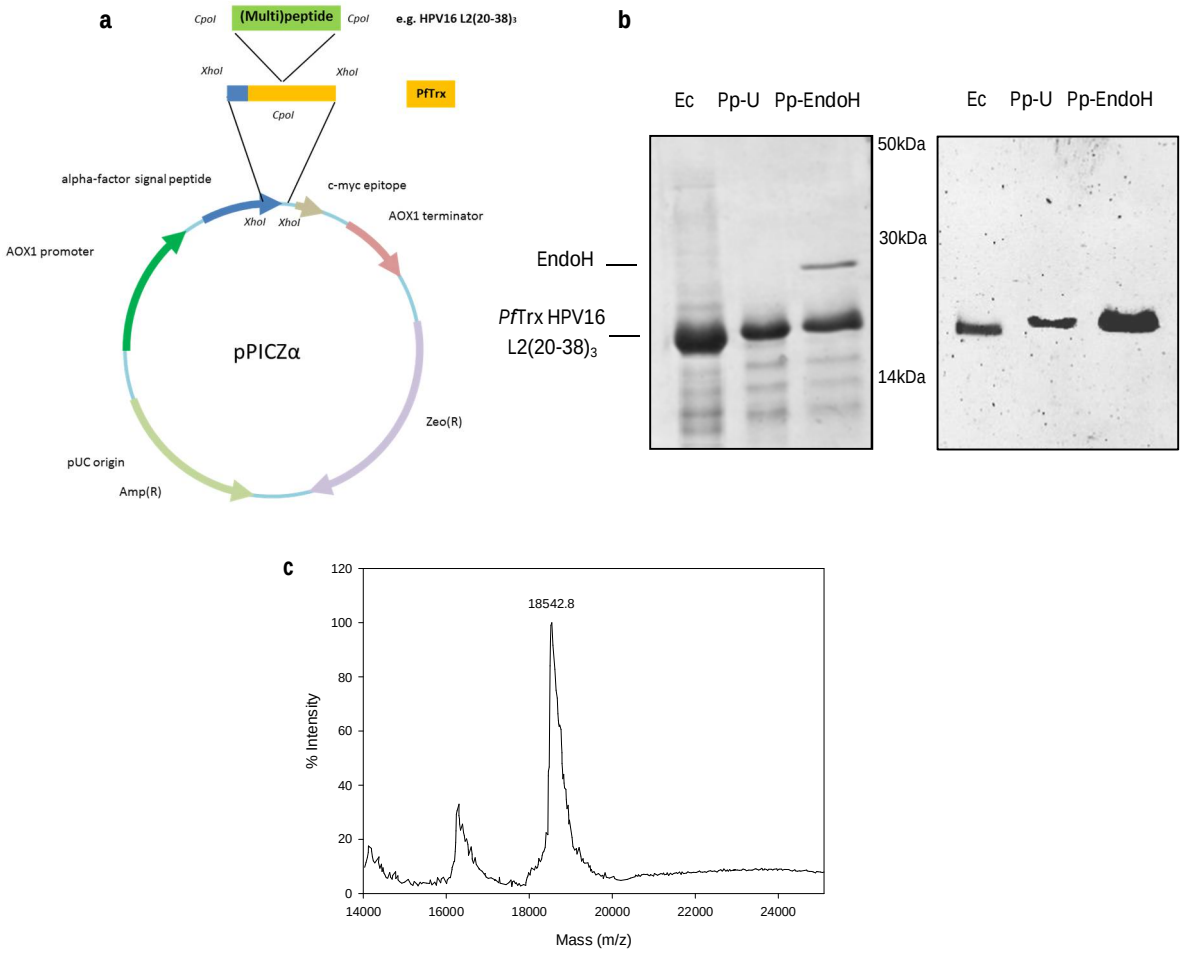

Fig. 2.1 pPICZα-PfTrx vector and its use for the secretory production of the unglycosylated PfTrx HPV16 L2(20-38)$_3$ polypeptide in P. pastoris. *a) Map of the pPICZα-PfTrx vector, including the unique CpoI site used for directional cloning of (multi)peptides within the active ("display") site of PfTrx. b) SDS-PAGE analysis of Pichia-produced untreated (Pp-U) and endoglycosidase-H treated (Pp-EndoH) PfTrx HPV16 L2(20-38)$_3$ recovered from the culture medium of a P. pastoris transformant compared with the same polypeptide purified from E. coli cells (Ec) (left panel). The results of an independent (lower input) immunoblot analysis performed on the same set of samples with an anti-PfTrx mAb are shown in the right panel. The migration positions of molecular size standards ("Precision Plus Protein Unstained Standard" mix) are shown in the middle. c) MALDI-TOF mass spectrum of PfTrx HPV16 L2(20-38)$_3$ recovered from the culture medium (see text for details).*

Optimized production of PfTrx HPV16 L2(20-38)$_3$

Before proceeding with larger-scale production and functional testing of *Pf*Trx HPV16 L2(20-38)$_3$, we investigated some experimental parameters (growth culture pH and temperature, cell suspension:culture flask volume ratio and methanol inducer concentration) known to influence heterologous protein expression in *P. pastoris*. Using the previously selected transformant as producing strain, the highest expression levels (~0.2 mg of *Pf*Trx HPV16 L2(20-38)$_3$ protein/ml of culture medium) were obtained upon growth at 23°C, pH 6.5 with a 1:10 cell suspension:flask volume ratio and a 2% concentration of the methanol inducer (*Table 2.1*).

Condition	pH	Induction temperature	MeOH final concentration	suspension:flask volume ratio
1	5.0	28°C	0.5%	1:5
2	6.0	28°C	0,5%	1:5
3	6.0	23°C	0,5%	1:5
4	6.5	28°C	0,5%	1:5
5	6.5	23°C	0,5%	1:5
6	6.5	23°C	0,5%	1:10
7	6.5	23°C	1.0%	1:10
8	**6.5**	**23°C**	**2.0%**	**1:10**
9	7.0	28°C	0.5%	1:5
10	8.0	28°C	0,5%	1:5

Table 2.1 Induction condition evaluate for PfTrx HPV16 L2 (20-38)$_3$ Pichia-expression. *Different pH, temperature of induction, MeOH concentration and suspension:flask volume ratio evaluate to obtain the higher expression level of PfTrx HPV16 L2 20-38)$_3$ (bold).*

These conditions were then transferred to a 100 ml batch-culture using a multi-induction protocol consisting of multiple cycles of cell growth, methanol inducer addition and growth medium collection, sequentially applied to the same starting culture. As shown in *Fig. 2.2a*, the *Pf*Trx HPV16 L2(20-38)$_3$ polypeptide recovered from the culture medium was approximately 70% pure. A nearly constant production yield (~25 mg protein/100 ml culture/cycle) was obtained in each of the four induction cycles (*Fig. 2.2a*). Altogether, this represents a nearly 20-fold increase with respect to the yield of the same polypeptide produced in *E. coli*. Furthermore, the relatively high purity of *Pf*Trx HPV16 L2(20-38)$_3$ secreted into the culture medium is already sufficient for many downstream applications (including preclinical immunization studies) and allowed to achieve a nearly 100% purity in a single purification step (cation-exchange chromatography on a HiTrap Capto-S column) (*Fig. 2.2b-c*).

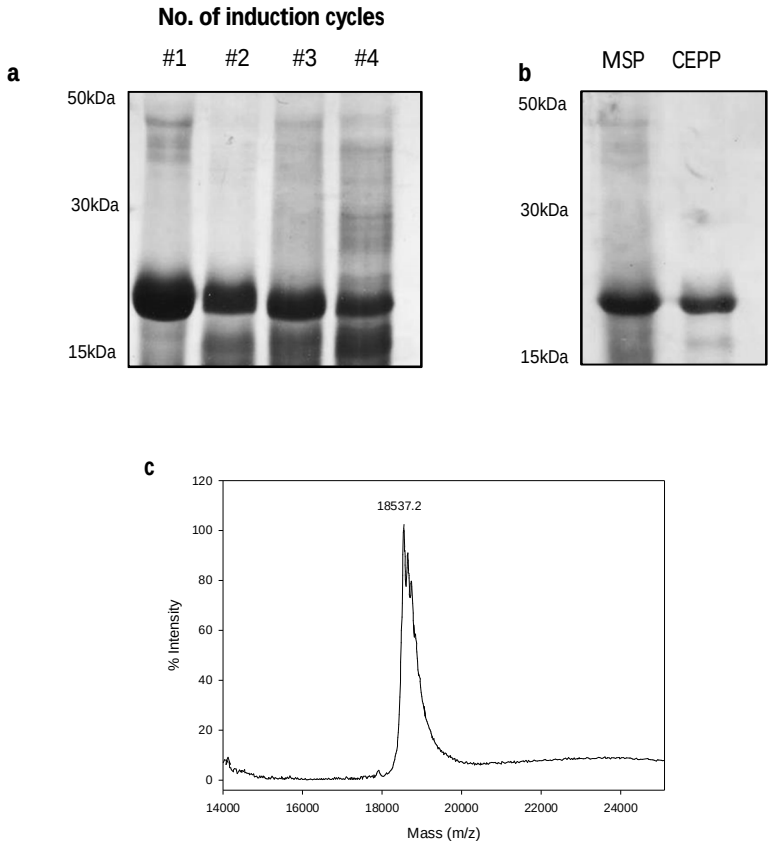

Fig. 2.2 PfTrx HPV16 L2(20-38)$_3$ production by multiple induction cycles and its purification to homogeneity by ion-exchange chromatography. *a) Four cycles of methanol induction (5 days each; indicated as #1-4), each followed by recovery of the PfTrx HPV16 L2(20-38)$_3$ protein secreted into the culture medium, were sequentially applied to the same starting culture (100 ml) of P. pastoris cells grown under optimized culture conditions. b) SDS-PAGE analysis of the medium-secreted PfTrx HPV16 L2(20-38)$_3$ polypeptide (MSP; >70% pure) purified by cation-exchange (CE) chromatography (CEPP; >95% pure). c) MALDI-TOF mass spectrum of CE-purified PfTrx HPV16 L2(20-38)$_3$.*

Biochemical and immunological characterization of the Pichia-produced PfTrx HPV16 L2(20-38)$_3$ antigen

The synthesis of secreted heterologous proteins in *P. pastoris* goes through the endoplasmic reticulum, an oxidizing environment specialized in protein folding and post-translational modification, including disulfide bond formation. Given the presence in *Pf*Trx HPV16 L2(20-38)$_3$ of eight cysteines (two belonging to the Trx scaffold plus two Cys residues for each L2 peptide unit) and our previous observations regarding the spontaneous formation of multiple aggregated species by *E. coli* Trx-L2[17], we initially determined the oxidation (free-SH group content) and

aggregation (monomeric vs. multimeric) state of the *Pichia*-produced polypeptide. As revealed by DTNB titrations, *Pf*Trx HPV16 L2(20-38)$_3$ produced in *Pichia*, contained an average of 0.3 free SH groups/molecule, a value close to that previously determined for the *E. coli*-produced polypeptide[17]. To evaluate the aggregation state of *Pichia*-produced *Pf*Trx HPV16 L2(20-30)$_3$ under native conditions, the purified polypeptide was analyzed by high-resolution gel filtration chromatography. As shown in *Fig. 2.3a*, under non-reducing conditions the protein eluted as a major peak (apparent MW 18600) preceded by a smaller, higher molecular weight peak (apparent MW 32100). A single-peak (MW 18600) elution profile was obtained instead under reducing (β-mercaptoethanol supplementation) conditions, indicating that the major *Pf*Trx HPV16 L2(20-30)$_3$ species in solution is a monomeric form.

Two other points we wished to address are secondary structure and thermal stability of the *Pichia*-produced polypeptide. These were investigated by circular dichroism and as shown in *Fig. 2.3b*, both the alpha-helix content and the heat stability of *Pf*Trx HPV16 L2(20-38)$_3$ produced in *P. pastoris* are the same as those previously determined for the *E. coli*-produced polypeptide[15].

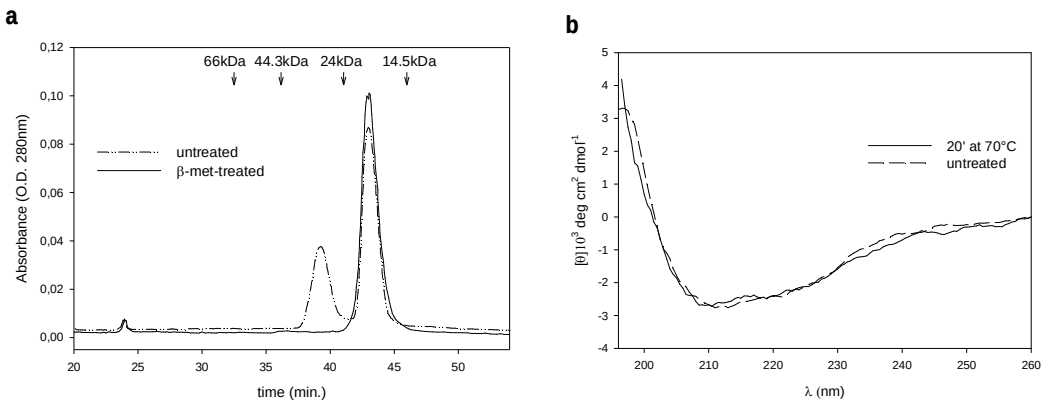

Fig. 2.3 Gel filtration and Circular Dichroism analysis of Pichia-produced PfTrx HPV16 L2(20-38)$_3$. *a) Chromatograms of untreated (dotted line) and β-mercaptoethanol (β-met; 10 mM) pretreated (solid line) PfTrx HPV16 L2(20-38)$_3$ fractionated on a high-resolution gel filtration column; bovine serum albumin (66 kDa), ovalbumin (44.3 kDa), trypsinogen (24 kDa) and lysozyme (14.5 kDa) were utilized as calibration standards. b) CD spectra of untreated PfTrx HPV16 L2(20-38)$_3$ (dotted line) and of the same polypeptide heated at 70°C for 20 min prior to spectrum recording (solid line).*

Finally, we tested the immunogenicity of the *Pichia*-produced *Pf*Trx HPV16 L2(20-38)$_3$ antigen by evaluating both its capacity to elicit the production of L2-recognizing immunoglobulins as well as its specific ability to induce virus-neutralizing antibodies. As shown by the ELISA and the *in vitro* HPV16 neutralization assay data reported in *Fig. 2.4*, fairly high titers

and highly consistent immune responses were observed in both tests with *Pf*Trx HPV16 L2(20-38)$_3$ produced in *P. pastoris*.

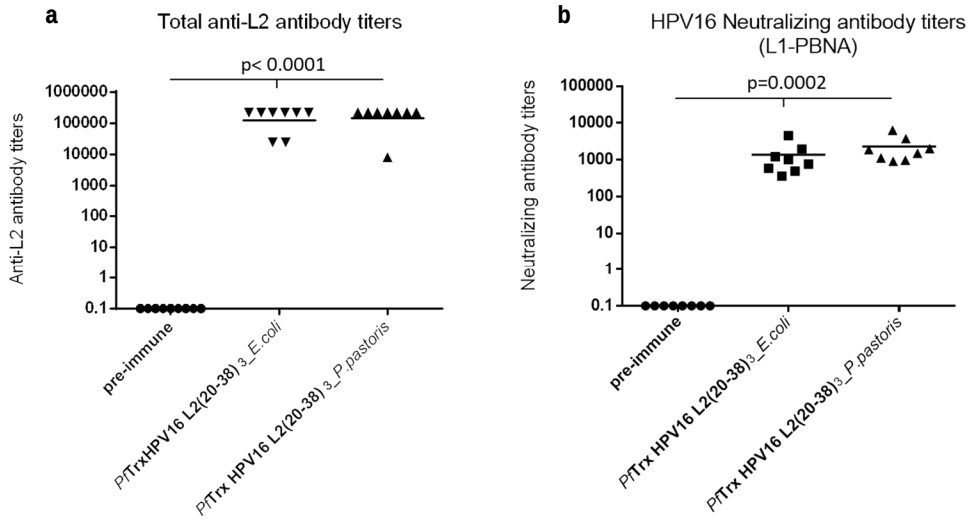

Fig. 2.4 Immunogenicity of the Pichia-produced PfTrx HPV16 L2(20-38)$_3$ antigen*. a) Total anti-L2 antibody titers elicited by immunization with PfTrx HPV16 L2(20-38)$_3$ produced in P. pastoris, measured by ELISA using GST-L2 as capture antigen. b) HPV16 neutralizing antibody titers measured by a pseudovirion-based neutralization assay (L1-PBNA). Antibody titers obtained with the Pichia-produced PfTrx HPV16 L2(20-38)$_3$ antigen are compared with those elicited by the same antigen produced in E. coli, administered with the same immunization protocol. Pre-immune are sera from mice not immunized. Statistical significance (p) are expressed on the graphs.*

Conclusion

Pichia-produced *Pf*Trx HPV16 L2(20-38)$_3$ exhibits all the favorable properties of the same antigen produced in *E. coli*, but with some important advantages. For example, the high production yield (~20-fold higher than in *E. coli*) that can be achieved with the methanol multi-induction protocol, the ease of purification under native conditions without the use of any affinity tag and the lack of endotoxin contamination. The latter option is particularly advantageous for the development and (pre)clinical testing of recombinant peptide antigens to be used as vaccines, where the allowed residual endotoxin (LPS) contamination is strictly regulated. Even in preclinical settings, however, LPS contamination can produce detrimental effects in experimental animals and confound the results obtained with the use of relatively weak (human-compatible) immune-adjuvants and immunogenicity-enhancing polypeptides that need to be evaluated in the absence of any possible endotoxin interference. In this regard, *Pichia* expression allows to eliminate costly

and time-consuming antigen detoxification procedures, thus maximizing batch-to-batch consistency.

The same advantages would apply to the use of the *Pichia Pf*Trx system for the large scale bio-production of endotoxin-free peptides, which upon introduction of appropriate protease cleavage sites in the region surrounding the display site of thioredoxin, could be retrieved from the secreted full-length polypeptide by site-specific protease digestion[18]. The absence of glycosylation we observed in *Pf*Trx HPV16 L2(20-38)$_3$ is in accordance with the lack of putative glycosylation sites in the *Pf*Trx scaffold as well as in the specific L2 peptide we have used as test-sample for this study. In other contexts, however, the high glycosylation capacity of *P. pastoris* could be instrumental to the production of SP-peptide fusions and isolated peptides bearing specific glyco-modifications.

Regarding the specific *Pf*Trx-L2 polypeptide we have used as test-case application, the use of *P. pastoris* as expression host is expected to considerably alleviate the production requirements of our alternative, L2-based HPV vaccine. The main aims of this vaccine are to overcome the type-restricted protection, reduced thermal stability and high production costs of the current L1-VLP-HPV vaccines, which limit accessibility to these vaccines by the populations most in need of HPV protection. Specifically, low-resource countries which bear ~85% of the global cervical cancer burden, often with a prevalence of viral types other than the vaccine types (HPV16 and HPV18) covered by the current VLP vaccines[19]. In fact, a far more complex (likely less stable and even more expensive) nonavalent vaccine (Gardasil® 9/V503) that is produced in yeast cells and targets seven, instead of two, high-risk HPV types[19,20] has been licensed at the end of 2014.

Materials and Methods

***Pf*Trx HPV16 L2(20-38)$_3$ construction and *P. pastoris* transformation.** A chemically synthesized *Pf*Trx coding sequence, previously designed for *E. coli* expression[15], was PCR-mutagenized using the following oligonucleotides (*Pf*Trx-XhoI-Pp) as primers:

Fwd: TAAATATAAA**CTCGAG**AAAAGAGAGGCTGAAGCTATTATCGAGTATGACGGCGAAAT

Rev: AATTATTTTA**CTCGAG**TTACTCCTGCAGCTCTTTCAG

These primers insert a sequence coding for the last six amino acids of the α-factor secretion signal peptide to the 5' end of the amplicon (underlined) and two *XhoI* restriction sites (bold) at both ends of the amplicon. The amplification product was eluted with the Zymoclean DNA gel recovery Kit (Zymo Research), digested with *XhoI* (Takara) and cloned into the XhoI site of the pPICZα plasmid (Invitrogen), using the DNA ligation Mighty Mix kit (Takara), to generate pPICZα-*Pf*Trx. A synthetic DNA fragment coding for the HPV16 L2(20-38)$_3$ tripeptide[15] was then directionally inserted into the unique *CpoI* site of pPICZα-*Pf*Trx to generate pPICZα-*Pf*Trx HPV16 L2(20-38)$_3$. Following transfer into *E. coli* DH10 cells and sequence verification, the latter construct was used for *P. pastoris* transformation. To this end, *P. pastoris* KM17H cells were grown in YPD (1% yeast extract, 2% peptone, 2% glucose) to an OD_{600} of 2.0, sedimented (3000 x g, 5 min), resuspended in one-fifth of the initial culture volume of ice-cold 100 mM Tris-HCl (pH 8.0) containing 10 mM dithiothreitol (DTT), 100 mM lithium acetate and 0.6 M sorbitol, and incubated for 30 min at room temperature. Cells were then washed three times with ice-cold sterile sorbitol (1 M) solution and resuspended in 1 M sorbitol at a 10^{10}cells/ml density prior to electroporation (1500V, 25 μF, 200 Ω; 0.2-cm cuvettes) of an aliquot of cells (80 μl) with 10 μg of *SacI*-digested pPICZα-*Pf*Trx HPV16 L2(20-38)$_3$ using a Gene Pulser (Bio-Rad Laboratories). Immediately after pulsing, 1 ml of ice-cold sorbitol (1 M) was added to the cuvette. Cells were then placed for 2 h at 30°C before plating on YPDS agar (YPD + 18.22% sorbitol) containing different amounts (0.1-2.0 mg/ml) of zeocin (Invivogen) and incubation at 30°C for 3-6 days.

***Pf*Trx HPV16 L2(20-38)$_3$ expression and purification.** A single colony of a *P. pastoris* transformant resistant to the highest zeocin concentration was used for the initial testing of *Pf*Trx HPV16 L2(20-38)$_3$ expression, which was carried out under standard BMMY medium (1% yeast extract, 2% peptone, 0.1M K-phosphate buffer, 1.34% YNB , 0.5% methanol and 4 mg/ml biotin) conditions, i.e., incubation at 30°C with continuous shaking at 200 rpm and 0.5% methanol added every 24h, as specified in the EasySelect Pichia Expression Kit version G.2005 Instruction Manual (Invitrogen). The growth medium of the methanol-induced culture was used for the initial assessment of *Pf*Trx HPV16 L2(20-38)$_3$ expression by SDS-PAGE analysis. This was followed by the systematic examination of different experimental parameters known to affect heterologous protein production in *P. pastoris*, namely culture pH and temperature, cell suspension:culture flask volume ratio and methanol inducer concentration[21]. Growth at 23°C, pH 6.5, with a 1:10 cell suspension:flask volume ratio and a 2% concentration of the methanol inducer were identified as optimal conditions for *Pf*Trx HPV16 L2(20-38)$_3$ expression. These conditions were transferred to a multicycle induction protocol consisting of daily methanol (0.5%) additions to a 100 ml starting culture in BMMY for 5 days (1st cycle), followed by culture medium recovery (3000 x g, 5 min), resuspension of the cell pellet in BMMY (supplemented once with 2% methanol) and culturing for another 5 days (successive cycles), for a total of four cycles. The combined growth media, containing the *Pf*Trx HPV16 L2(20-38)$_3$ polypeptide in a >70% pure form, were exchanged into 25 mM MES (pH 5.8) and loaded onto a 1 ml HiTrap Capto-S column (GE Healthcare), followed by

elution with a 40 ml 0-1 M NaCl linear gradient. An alternative purification procedure consisted of cold-acetone precipitation of the induced growth medium in order to remove small molecular weight contaminants, followed by heat-treatment (70°C for 20 min) as described previously for the *E. coli*-produced *Pf*Trx HPV16 L2(20-38)$_3$ polypeptide.

Biochemical analyses. For enzymatic analysis of the glycosylation state, 1 µl of 10X Glycoprotein Denaturing Buffer (as supplied in the EndoH kit; New Englands BioLabs) was added to 5 µg of *Pf*Trx HPV16 L2(20-38)$_3$ in a final volume of 10 µl and heated for 10 min at 100°C. This was followed by the addition of EndoH (1 µl) plus 10X GlycoBuffer (2 µl), incubation for 1 h at 37°C in a total reaction volume of 20 µl, and SDS-PAGE analysis on 15% polyacrylamide gels stained with Coomassie Blue-R250, using the molecular weight standards provided in the Precision Plus Protein Unstained Standard mix (Bio-Rad). For immuno-blotting analysis, separate (unstained) gels were electrotransferred to a 0.2 µm nitrocellulose membrane (Bio-Rad). After overnight membrane blocking at 4°C with 5% skim milk (Sigma-Aldrich) dissolved in TT buffer (2% Tris-HCl, pH 9.6, 8% NaCl, 0.3% Tween-20) and three 10 min washes with the same buffer, an in-house generated anti-*Pf*Trx monoclonal antibody (A-G4-F5 anti-*Pf*Trx) was added at a final dilution of 1:1000 in TT buffer. The membranes were then kept under stirring conditions for 2 h at room temperature, washed with TT buffer, incubated for 1 h with an IRDye-labeled goat anti-mouse secondary antibody (IgG Odyssey IRDye 680, Li-Cor; diluted 1:5000 in TT buffer), further washed with TT buffer, and visualized with an Odyssey Infrared Imaging analyzer (Li-Cor). Protein concentration was determined with the Coomassie Blue-G250 binding assay[22] as well by absorbance measurements using a calculated OD280 extinction coefficient (15970 M-1 cm-1). Putative glycosylation sites in the *Pf*Trx scaffold and in the L2(20-38)$_3$ tripeptide insert were searched in silico with the NetNGlyc 1.0 program.
MALDI-TOF mass spectroscopic (MS) analyses were performed by mixing 5 µl of purified *Pf*Trx-HPV16 L2(20-38)$_3$ (5 mg/ml) with an equal volume of 1% trifluoroacetic acid (TFA), followed by salt removal on a ZipTip C18 pipette tip (Millipore) prewashed with 0.1% TFA dissolved in acetonitrile and an additional wash with 10 ml of 0.1% TFA. The protein was eluted with 2 ml of the matrix solution containing 10 mg/ml sinapinic acid dissolved in a 55% acetonitrile/water mixture supplemented with 0.1% TFA, spotted on a target plate and dried under a flow of nitrogen prior to MS analysis (Waters-Micromass MALDI mass spectrometer operated in linear positive mode with the following parameters: 15,000 V source voltage, 1,550 V pulse voltage, 1,800 V MCP detector). Data were analyzed with MasslynxTM (version 4.1).
5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) titrations were carried out on purified *Pf*Trx HPV16 L2(20-38)$_3$ (7 µM) as described previously[17]. The aggregation state of *Pf*Trx HPV16 L2(20-38)$_3$ was evaluated on the ion-exchange purified polypeptide by gel-filtration chromatography on a Superdex 200 Increase 10/300 GL column (GE Healthcare), equilibrated in 25 mM Tris-HCl (pH 7.5), 0.15 M NaCl and run at a 0.4 ml/min flow-rate; bovine serum albumin (66 kDa), ovalbumin (44.3 kDa), trypsinogen (24 kDa) and lysozyme (14.5 kDa) were utilized as molecular size calibration standards.
Far-UV circular dichroism (CD) spectra (190–260 nm; average of four scans) were acquired on purified *Pf*Trx HPV16 L2(20-38)$_3$ samples (2 µM protein concentration in 0.1X PBS) using a Jasco J715 Spectropolarimeter (0.1 cm path-length cuvette), equipped with a Peltier temperature controller, with a bandwidth of 1 nm, a data pitch of 0.5 nm and a response time of 4 sec, as described previously[15]. For thermal stability analyses, protein samples were incubated for 20 min at 70°C prior to CD spectrum recording.

Mouse immunization and immune response analyses. Six- to eight-weeks-old female BALB/c mice (Charles River; Sulzfeld, Germany), kept in the animal house facility of the German Cancer

Research Center under pathogen-free conditions in compliance with the regulations of the Germany Animal Protection Law (Regierungspräsidium Karlsruhe, permits G246/11 and G19/13), were immunized subcutaneously (8 animals/treatment group) four times at biweekly intervals with 20 µg of filter-sterilized *Pf*Trx HPV16 L2(20-38)$_3$ produced in *P. pastoris*. Parallel control immunizations were performed with filter-sterilized *Pf*Trx HPV16 L2(20-38)$_3$ produced in *E.coli* and detoxified by Triton X-114 treatment[23] (LPS level determined by the LAL test ≤ 2EU). Both antigens were administered with 50% (v/v) Montanide ISA 720 (Seppic) as immune-adjuvant. Immunological tests (GST-L2 ELISA and pseudovirion-based neutralization assays) were performed on immune sera obtained from blood samples collected by cardiac puncture eight weeks after the last immunization.

Glutathione S-transferase (GST)-L2 capture ELISAs were carried out in 96-well microtiter plates pre-coated overnight with glutathione-casein and blocked with casein buffer (0.2% bovine milk casein dissolved in PBS) as described previously[24]. Glutathione-casein-coated plates were incubated for 1 h a 37°C with purified GST-HPV16 L2 (0.5 mg/ml), followed by three washes with PBS containing 0.3% (v/v) Tween-20. Sera were analyzed by serial dilutions starting from 1:300, using GST alone as negative control. Following serum addition and incubation for 1h at 37°C, plates were washed three times as above and incubated with a peroxidase-conjugated anti-mouse secondary antibody (Sigma-Aldrich) diluted 1:5000 in casein buffer. Following incubation for 1 h at 37°C, plates were washed three times and developed by ABTS [2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)] addition. Absorbance at 405 nm was measured after 30 min at 30°C with an automated microplate reader (iMark, Biorad).

Pseudovirion (PSV) preparation and neutralization assays were performed as described previously[17]. Briefly, 50 µl of serially diluted immune sera (or a control anti-HPV monoclonal antibody) were mixed with 50 µl of a diluted HPV16 pseudovirion stock and incubated at room temperature for 30 min. Next, 50 µl of HeLaT cells (2.5-3x10^5 cells/ml) were added to the PSV-immune serum (or PSV-mAb) mixture and incubated for 48 h at 37°C under a 5% CO2 atmosphere. The amount of secreted Gaussia luciferase was then determined in 10 µl of cell culture medium using the coelenterazine substrate and Gaussia glow juice (PJK) according to the manufacturer's instructions. A microplate luminometer (Victor3, PerkinElmer) was used to measure culture medium-associated luminescence 15 min after substrate addition.

GST-L2 ELISA and neutralization assay data were analyzed statistically with the non-parametric Mann-Whitney-Wilcoxon test performed with GraphPad Prism 5.0 (GraphPad Software); differences between groups were considered significant at $p < 0.05$.

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Section III

Host-range broadness: T-helper epitopes

Introduction

Humoral (antibody) response to protein antigens require specific recognition of the same antigen particle by both B lymphocytes, precursors of antibodies secreting cells, and $CD4^+$ T lymphocytes, that provide help. B cells act as antigen presenting cells (APC) binding, internalizing and degrading the antigen. Some of the peptides obtained by this processing are exposed on the cell surface of B cells bound to class II MHC (Major Histocompatibility Complex) molecules, the only way in which T cells can recognize processed antigen and be activated[1].

In the context of peptide-based vaccine, as *Pf*Trx HPV16 $L2(20\text{-}38)_3$ previously described, the immunogenicity of a given peptide is dependent upon three factors: the generation of the appropriate fragment[2], the presence of an MHC molecule that binds this fragment[3,4] and the presence of T cell capable of recognizing the complex[5]. The lack of appropriated T cell help is in generally due to the extensive genetic polymorphism of MHC or Human Leukocyte Antigen (HLA) in human[6,7], determining different affinities of the processed peptide to the variants of MHC molecules.

Thioredoxin from *P. furiosus*, and other thioredoxin as well, contain intrinsic putative T-cell epitopes, whose functionality is experimentally supported by the ElISpot assay, previously described (see *Section I*), and the fairly strong immune responses elicited by Trx-displayed peptide antigens in various (e.g., BALB/c mice, rabbits, and guinea pigs[8–10] and also previously described) but not all (e.g., C57BL/6 mice[11]) animal models. The aim of this research was to identify a improvement of the basic antigen to make a host-range broadly ("universal") vaccine able to elicit antibody response in different species. In general, T-helper (Th) epitopes reported to be stimulatory in a particular species, are usually MHC-restricted and hence of limited use for outbred populations of that species. Moreover, a broad range of potential universal epitopes, able to be recognized by different variants of HLA and consequently able to increase antigen immunogenicity, have been identified[12–16]. Some Th epitopes have natural origin as p25 (VRRRVNAANKK PEDLTLNDL), from the carboxyl regions of *Plasmodium vivax* circumsporozite protein[17,18], and p2 (QYIKANSKFIGITEL) and p30 (FNNFTVSFWLRVPKVSASHLE), from tetanus toxin. Immunization with tetanus toxin determined p2- and p30-specific T-cell response, indicating that in all individuals these epitopes are generated, bind to a class II molecule and stimulate specific T cells[12]. Based on the residues involved in the MHC-peptide bound, it was possible to design synthetic, non-natural sequences as "universal" Th epitopes, capable of binding with high affinity to most or all MHC alleles. Several studies demonstrated that the Th epitope residues composition, to guarantee an high affinity to most different DR variants (MHC class II cell surface receptor encoded by HLA), needs an aromatic or aliphatic amino acid in position 1, an hydrophilic polar amino acid in position 6 and a little hydrophobic one in position 9[19,20]. From these observations, a totally artificial Th epitope, called Pan DR Epitope (PADRE; AKFVAAWTLKAA) has been proposed. *In vitro* studies

demonstrated that PADRE induces proliferative human T cell response from individuals with diverse HLA-DR (MHC class II cell surface receptor encoded by HLA) alleles and can bind, with high or intermediate affinity, 15 of the 16 common HLA DR types[21]. Even if PADRE was designed to elicit T cell response in human, the cross-reaction with certain mouse class II alleles permits *in vivo* study of its immunogenicity[22], in particular for developing recombinant or synthetic vaccines[6,22–27].

An integrin recognition motif could be another factor to increase epitope recognition and humoral response since it can mediates cell attachment and surface proteins adhesion. This is represented by three specific amino acids (RGD: arginine, glycine and aspartic acid[28,29]) and modification in this short sequence (e.g. alanine instead of glycine) determined a considerable reduction of peptide function[30]. The functionality of RGD motif is also affected by flanking region: a proline immediately after the RGD can silence the recognition[28].

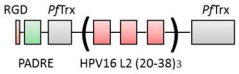
We have decided to apply the above improvements to obtain a host-range broad HPV vaccine, based on TDMI approach using *Pf*Trx.

Design and production of "universal" PfTrx HPV16 L2(20-38)$_3$ antigens

In the spectrum of Th epitopes generally used in recombinant vaccines, we have chosen four of them: PADRE, p25, and a combination of p2 and p30, to cover the most MHC haplotypes[31]. Without knowledge about the best position for epitope recognition within the antigen, we designed different constructs with Th epitopes (PADRE, p25 and p2-p30) plus RGD only at N-terminus or at both N- and C-terminus of *Pf*Trx HPV16 L2(20-38)$_3$ (*Fig. 3.1*). The nucleotide sequences (*Appendix 1*) coding for all proteins were synthetized and cloned into *NdeI* restriction site of pET26b vector. The sequences were designed for maintaining the *CpoI* restriction site at the both ends of HPV16 L2(20-38) tripeptide to have the possibility to substitute it with different epitopes.

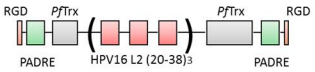
PADRE$_{1x}$ *Pf*Trx HPV16 L2 (20-38)$_3$: RGD-PADRE-*Pf*Trx L2

HMRGDGAKFVAAWTLKAAAGPGPGMIIEYDGEIDFTKGRVVLWFSIPGCGPKTCKQAGTCPPDIIPKVEGGGPKTCKQAGTCPPDIIPKVEGGGPKTCKQAGTCPPDIIPKVEGGGPCRLV
ERFMTELSEYFEDIQIVHINAGKWKNIVDKFNILNVPTLVYLKDGREVGRQNLIRSKEEILKKLKELQE



PADRE$_{2x}$ *Pf*Trx HPV16 L2 (20-38)$_3$: RGD-PADRE-*Pf*Trx L2-PADRE-RGD

HMRGDGAKFVAAWTLKAAAGPGPGMIIEYDGEIDFTKGRVVLWFSIPGCGPKTCKQAGTCPPDIIPKVEGGGPKTCKQAGTCPPDIIPKVEGGGPKTCKQAGTCPPDIIPKVEGGGPCRLV
ERFMTELSEYFEDIQIVHINAGKWKNIVDKFNILNVPTLVYLKDGREVGRQNLIRSKEEILKKLKELQEGPGPGAKFVAAWTLKAAAGRGD



p25$_{1x}$ *Pf*Trx HPV16 L2 (20-38)$_3$: RGD-p25-*Pf*Trx L2

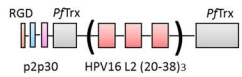
MRGDGKLIPNASLIENCTKAELGPGPGMIIEYDGEIDFTKGRVVLWFSIPGCGPKTCKQAGTCPPDIIPKVEGGGPKTCKQAGTCPPDIIPKVEGGGPKTCKQAGTCPPDIIPKVEGGGPCRL
VERFMTELSEYFEDIQIVHINAGKWKNIVDKFNILNVPTLVYLKDGREVGRQNLIRSKEEILKKLKELQE

p25$_{2x}$ *Pf*Trx HPV16 L2 (20-38)$_3$: RGD-p25-*Pf*Trx L2-p25-RGD

MRGDGKLIPNASLIENCTKAELGPGPGMIIEYDGEIDFTKGRVVLWFSIPGCGPKTCKQAGTCPPDIIPKVEGGGPKTCKQAGTCPPDIIPKVEGGGPKTCKQAGTCPPDIIPKVEGGGPCRL
VERFMTELSEYFEDIQIVHINAGKWKNIVDKFNILNVPTLVYLKDGREVGRQNLIRSKEEILKKLKELQEGPGPGKLIPNASLIENCTKAELGRGD

p2p30$_{1x}$ *Pf*Trx HPV16 L2 (20-38)$_3$: RGD-p2-p30-*Pf*Trx L2

MRGDGQYIKANSKFIGITELGPGFNNFTVSFWLRVPKVSASHLEGPGPGMIIEYDGEIDFTKGRVVLWFSIPGCGPKTCKQAGTCPPDIIPKVEGGGPKTCKQAGTCPPDIIPKVEGGGPKTC
KQAGTCPPDIIPKVEGGGPCRLVERFMTELSEYFEDIQIVHINAGKWKNIVDKFNILNVPTLVYLKDGREVGRQNLIRSKEEILKKLKELQE



p2p30$_{2x}$ *Pf*Trx HPV16 L2 (20-38)$_3$: RGD-p2-p30-*Pf*Trx L2-p2-p30-RGD

MRGDGQYIKANSKFIGITELGPGFNNFTVSFWLRVPKVSASHLEGPGPGMIIEYDGEIDFTKGRVVLWFSIPGCGPKTCKQAGTCPPDIIPKVEGGGPKTCKQAGTCPPDIIPKVEGGGPKTC
KQAGTCPPDIIPKVEGGGPCRLVERFMTELSEYFEDIQIVHINAGKWKNIVDKFNILNVPTLVYLKDGREVGRQNLIRSKEEILKKLKELQEGPGPGQYIKANSKFIGITELGPGFNNFTVSFWL
RVPKVSASHLEGRGD

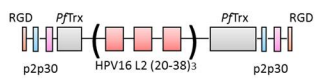


Fig. 3.1 Amino acid sequences and structures of PfTrx HPV16 L2(20-38)$_3$-Th epitopes constructs. *Amino acid sequences of PfTrx HPV16 L2 (20-38)$_3$-Th epitopes constructs and corresponding graphic representations.*

All the constructs were used to transform *E. coli* BL21 codon plus and the proteins were expressed for three hours at 37°C with 1mM IPTG as inducer. After cell lysis by sonication, the proteins $p25_{1x}$ *Pf*Trx HPV16 $L2(20\text{-}38)_3$, $p25_{2x}$ *Pf*Trx HPV16 $L2(20\text{-}38)_3$, $PADRE_{1x}$ *Pf*Trx HPV16 $L2(20\text{-}38)_3$ and $PADRE_{2x}$ *Pf*Trx HPV16 $L2(20\text{-}38)_3$ appeared soluble and were purified follow thermal purification protocol (see Materials and Methods), confirming their thermal stability conferring by *Pf*Trx scaffold, although the presence of Th epitopes. In *Fig. 3.2,* it is represented the $PADRE_{2x}$ *Pf*Trx HPV16 $L2(20\text{-}38)_3$ as reference for the procedure adopted. The proteins $p2\text{-}p30_{1x}$ *Pf*Trx HPV16 $L2(20\text{-}38)_3$ and $p2\text{-}p30_{2x}$ *Pf*Trx HPV16 $L2(20\text{-}38)_3$, conversely, were completely unsoluble, even in different induction conditions (e.g. 20°C or 4°C overnight) and different *E. coli* strains, including those generally used to increased solubility (e.g. SoluBL21). Consequently of their insolubility, these two proteins were discarded from further analysis.

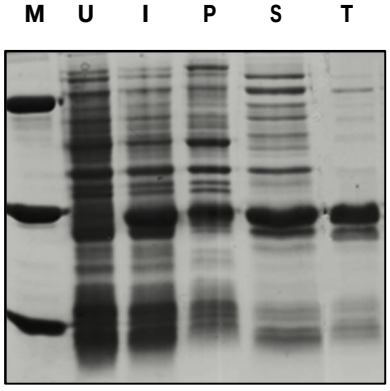


Fig. 3.2 Induction and thermal purification of $PADRE_{2x}$ PfTrx HPV16 $L2(20\text{-}38)_3$. *The induction of the protein (3h at 37°C with 1mM IPTG) and its solubility (after cell lysis with sonication) are analyzed by 11% SDS-APGE; U: uninduced; I: induced; P: pellet after cell lysis; S: supernatant after cell lysis. The protein is purified using thermal purification protocol (12,5% PEG 1000; 0,25M NaCl) and its purity analyzed by SDS-PAGE; T: thermal treatment. The protein is soluble and thermal stable (MW:~23 kDa) .*

Immune performance of Th epitopes-PfTrx HPV16 $L2(20\text{-}38)_3$ antigens

All the antigens, expressed and detoxified by bacterial LPS with Triton X-114 extraction (see Materials and Methods), were tested *in vivo* to evaluate immune performance in the presence of one or two copies of Th epitopes. After four subcutaneous immunizations, adding Montanide ISA 720 as adjuvant, and blood collection by heart puncture four weeks after the last immunization, the neutralization titers were defined with L1-PBNA assay (See Materials and Methods). To evaluate the host-range broadness of our antigens, the immunizations, using 20 µg of antigens, were done in two mouse strains characterized by different variants of MHC class II molecules: BALB/c and C57BL/6 mice.

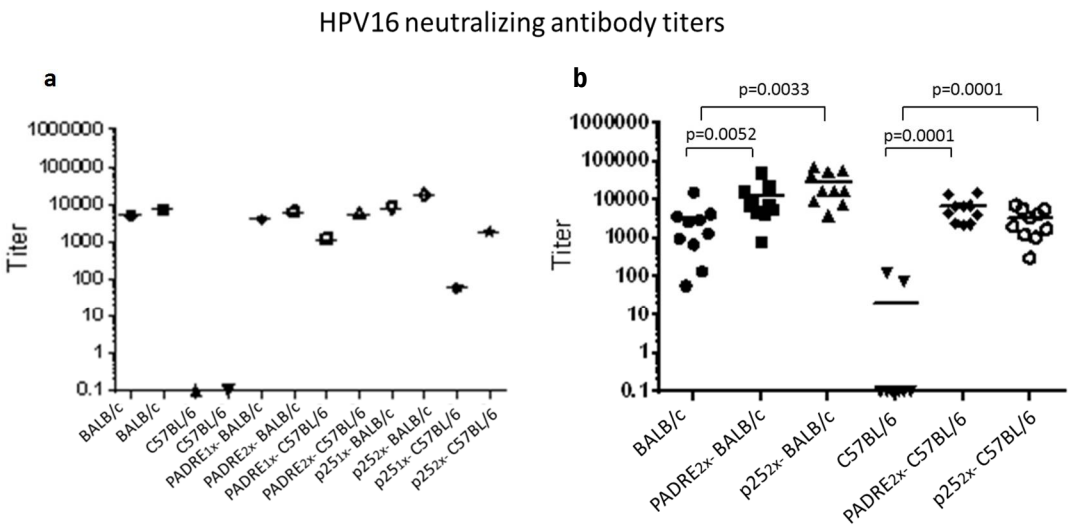


Fig. 3.3 HPV16 L1-PBNA titers using PfTrx HPV16 L2(20-38)$_3$ with one or two copies of PADRE or p25 in BALB/c and C57BL/6 mice. *10 mice per group were immunized with 20 µg of antigen with 50% (v/v) Montanide ISA 720 for 4 times at biweekly intervals; final blood was obtained by heart puncture 4 weeks after the last immunization. a) Comparison between one or two copies. The dots represent a pool of sera from each group. b) Analysis of single neutralization titer of mice (BALB/c and C57BL/6) immunized with antigen added of two copies of Th epitopes. Each dot represent a mouse. BALB/c and C57BL/6 are control group immunized with PfTrx HPV16 L2(20-38)$_3$ without any Th epitopes.*

The absence of any additional Th epitopes in the antigen did not permit a response in C57BL/6 mice, in contrast to the high neutralization titers obtained in BALB/c mice; this confirmed the high polymorphic differences in MHC class II alleles in these two mouse strains (*Fig 3.3a*).

In the context of BALB/c mice, the presence of PADRE epitope in one or two copies did not significantly change neutralizing antibody titers, while the presence of two copies of p25 increase the response. In contrast, in C57BL/6 mice the addition of Th epitopes is a critical factor to obtain a significant response: two copies of PADRE confer the higher HPV16 neutralizing antibody titers (*Fig. 3.3a*), according to previously studies in which it has been demonstrated the capacity of this strain to generate a strong helper T cell response to PADRE[21]. Based on this first comparison, the presence of Th epitopes at both ends of our antigen confers the best immune response, permitting high HPV16 neutralization titers also in C57BL/6 mice (*Fig. 3.3b*), further highlighted in cross-neutralization assays against HPV18 and HPV45 (*Fig. 3.4a*).

The lower immune response in both mice strains against HPV31 and HPV51 (*Fig. 3.4b*) is most likely due to the difference in amino acid sequence of L2(20-38) region between this HPV types and HPV16 one *(Fig. 3.4c).*

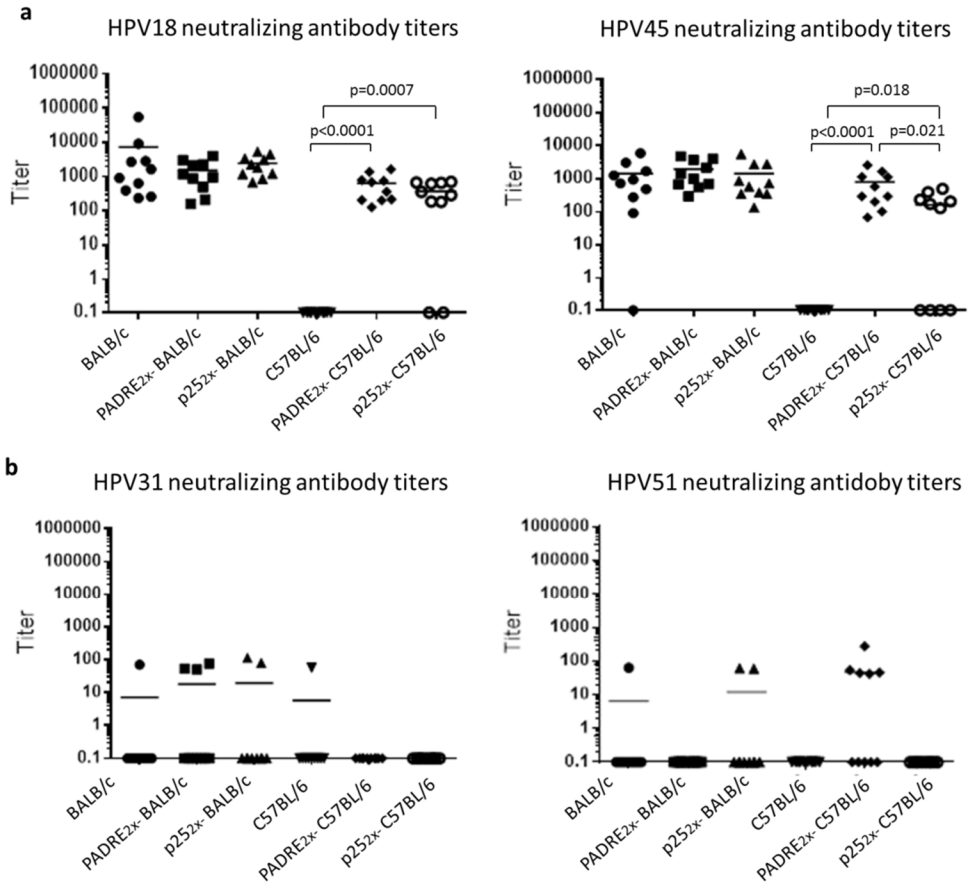


Fig. 3.4 Cross-neutralization against HPV18, HPV45, HPV31 and HPV51 in BALB/c and C57BL/6 mice. *10 mice per group were immunized with 20 µg of antigen PfTrx HPV16 L2(20-38)$_3$ with PADRE (1x/2x) or p25 (1x/2x) with 50% (v/v) Montanide ISA 720 for 4 times at biweekly intervals; final blood was obtained by heart puncture 4 weeks after the last immunization. a) HPV18 and HPV45 cross-neutralization. All the Th epitopes-antigens permit high neutralization titers against HPV18 and HPV45. b) HPV31 and HPV51 cross-neutralization. There is no response also in presence of Th epitope Each dot represents a mouse. BALB/c and C57BL/6 are control group immunized with PfTrx HPV16 L2(20-38)$_3$ without any Th epitope. c) Alignment of L2(20-38) amino acid region of HPV16, 18, 45, 31 and 51. The difference in sequence determined a bad cross-neutralization against HPV31 and HPV51. Alignment obtained using ClustalW; green; different amino acids compared to HPV16; yellow: different amino acids but with the same chemical properties compared to HPV16.*

Comparing the neutralization and cross-neutralization titers, both Th epitopes induce high neutralizing titers, but PADRE formulations permit a better Th response, in particular in C57BL/6 mice, allowing us to choose this Th epitope for our further formulation. As shown in *Fig. 3.5*, the thermal stability of $PADRE_{2x}$ *Pf*TRx HPV16 $L2(20\text{-}38)_3$ is a little bit reduced (Tm~80°C) compared with the same antigen without Th epitope[32], even if it remains higher than VLPs (Tm~50°) and enough for thermal purification (as described previously).

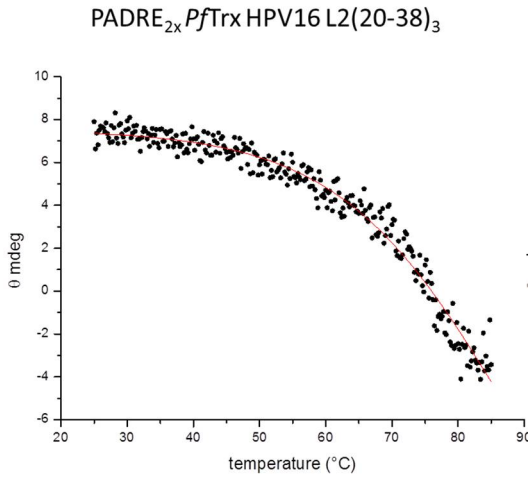


Fig. 3.5 Far-UV Circular dichroism spectra of $PADRE_{2x}$ PfTrx HPV16 $L2(20\text{-}38)_3$ in thermal denaturation condition. *Far-UV CD analysis at 200 nm of heat purified $PADRE_{2x}$PfTrx HPV16 $L2(20\text{-}38)_3$ upon unfolding (25-85°C). Midpoint transition is around 80°C.*

Conclusion

Antigen recognition is the first step to obtain a high and specific immune response. Extensive MHC polymorphism between species implies difficulties in a broadly cross-protective vaccine's development. Identification of Th epitopes, able to stimulate T cell activation, and a RGD motif for cell-cell interaction, represents a progress even if they are in general specie-specifics. This limit, together with MHC-restriction, is overcome in our HPV vaccine context adding p25 or PADRE, Th epitopes with natural and artificial origin, respectively. The insolubility of *Pf*Trx HPV16 $L2(20\text{-}38)_3$ conferred by the addition of p2-p30, two natural promiscuous Th epitopes, did not permit analysis of its immune stimulation capacity, even if they are associated with multiple HLA class II alleles[12]. In our analysis, we added Th epitopes (PADRE and p25), with the RGD motif, at one end (N-terminus) or at both ones of our antigen *Pf*Trx HPV16 $L2(20\text{-}38)_3$. Although the best immune response is obtained adding Th epitopes at both ends, the presence of Th epitope only at N-

terminus permitted a response high enough to consider this antigen modification sufficient for our purposes. Such an antigen, maintaining a free C-terminus, could be useful for addition of other immune-enhancing elements (e.g. IMX313T, described later).

By comparing the response to the same antigen in BALB/c and C57BL/6 mice, the importance of Th epitopes was easily assessable. Without Th epitopes, C57BL/6 mice were not able to recognize L2 epitope and induce neutralizing antibodies, in contrast to BALB/c mice. This confirms the different MHC haplotype that characterizes this two mouse strains: BALB/c mice's MHC haplotype antigens is d (H-2K^{d},H-2D^{d}, H-2L^{d}, I-A^{d}, and I-E^{d}), whereas C57BL/6 mice presents the b haplotype (H-2K^{b},H-2D^{b},and I-A^{b}). Evaluating neutralizing antibody titers obtained, in particular in C57BL/6 mice that are not responding in the absence of Th epitopes, we have chosen PADRE as the best Th epitopes for our antigen.

Materials and Methods

T helper epitopes-*Pf*Trx HPV16 L2(20-38)$_3$ constructs and proteins expression. T helper epitopes are designed to be only at N-terminus and at both ends of *Pf*Trx HPV16 L2(20-38)$_3$-coding sequence. All the sequences (see *Appendix 1*) were chemically synthesized (Eurofins MWG Operon), putting a linker between Trx-scaffold and Th epitope (GPGPG) and *NdeI* restriction site to clone it into a modified pET26 plasmid (Novagen). The resulting constructs were sequence-verified and transformed into *Escherichia coli* BL21 codon plus (DE3) cells for recombinant proteins expression, induced by adding 1 mM isopropyl-b-D-thiogalactopyranoside (IPTG) to the various *E. coli* trasformants, which were then cultured at 30°C overnight. Following cell lysis, Th epitopes-*Pf*Trx HPV16 L2(20-38)$_3$ proteins were purified with a modified heat-treatment procedure[36]. Briefly, following bacterial lysate centrifugation, NaCl (0.25 M) and PEG 1000 (12.5%) were added to the cleared Th epitopes-*Pf*Trx HPV16 L2(20-38)$_3$ supernatant and the resulting solution was incubated for 20 min at 70°C, cooled on ice for 10 min and centrifuged at 12,000 g for 10 min to recover the supernatant containing the purified Trx-L2 proteins. All Th epitope-*Pf*Trx-L2 antigens to be used for mouse immunization experiments (see below) were firstly dialyzed against PBS 1X to remove PEG 1000 using an Amicon Stirred Cell 8200 (Millipore) and detoxified by a 3X Triton X-114 (1% v/v) treatment as described[37]; endotoxin/LPS removal (to levels lower than 2 EU/ml) was verified in each sample by the LAL QLC-1000 test (Lonza).

Animal immunization and pseudovirion-based neutralization assays. Procedures in animal experiments were approved by the Regierungspräsidium Karlsruhe under permit G246/11 and G19/13. Six- to eight-weeks-old female BALB/c mice (Charles River; Sulzfeld, Germany) were kept in the animal house facility of the German Cancer Research Center under pathogen-free conditions, in compliance with the regulations of the Germany Animal Protection Law. Mice were immunized subcutaneously four times at biweekly intervals with 20 μg of the various detoxified and filter-sterilized Th epitopes *Pf*Trx HPV16 L2(20-38)$_3$ antigens adjuvanted with 50% (v/v) Montanide ISA 720 (Seppic, France). Four weeks after the last immunization, blood samples were collected by cardiac puncture. Pseudovirion preparation and neutralization assays were performed as described[38]. Briefly, 50 ml of serially diluted immune sera (or control monoclonal antibodies) were mixed with 50 ml of diluted pseudovirion stocks and incubated at room temperature for 30 min. Next, 50 ml of HeLaT cells (2.5 x 10^5 cells/ml) were added to the pseudovirion-antibody mixture and incubated for 48 h at 37°C under a 5% CO_2 atmosphere. The amount of secreted Gaussia luciferase was then determined in 10 ml of cell culture medium using coelenterazine substrate and Gaussia glow juice (PJK, Germany) according to the manufacturer's instructions. A microplate luminometer (Victor3, PerkinElmer) was used to measure luminescence in the culture medium 15 min after substrate addition. Immune cross-reactivity analyses.

Far-UV circular dichroism spectroscopic analyses. Far-UV CD spectra (190–260 nm) were acquired with a Jasco J715 Spectropolarimeter equipped with a Peltier temperature controller, using a 0.1 cm path-length cuvette, a bandwidth of 1 nm, a data pitch of 0.5 nm, and a response time of 4s; CD spectra were averaged from 4 scans. Protein concentration was 10 mM in PBS for the single spectra recorded at 25°C and 0.1X PBS for thermal stability measurements. Following baseline correction, the measured ellipticity, h (mdeg), was converted to the molar mean residue ellipticity [Θ] (deg.cm^2.dmol^{-1}), using [Θ] 5h/cn$_{res}$l, where Θ is ellipticity, c is the molar concentration of the protein, n$_{res}$ is the number of amino acid residues in the protein and l is the optical path length in centimeters. Thermal unfolding/refolding studies were performed in the 25°C–85°C temperature range; temperature scan was 1°C/min in order to maintain thermal equilibrium. The loss of

secondary structure was followed by measuring [Θ] at 200 nm. Tm values were calculated as the inflection point of unfolding curves fitted according to the single-step (two-state) transition model.

Sequence alignment and statistical analysis. Amino acids sequence were aligned using ClustalW and statistical significance of neutralization assay results was determined with the nonparametric Mann-Whitney-Wilcoxon test performed with GraphPad Prism 5.00 (GraphPad Software, USA); differences between groups were considered significant at $p<0.05$.

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Section IV

Supramolecular PfTrx-L2 derivative

Introduction

The induction of higher level of T cell responses may be crucial in achieving vaccine efficacy and strategies to increase this levels could have a substantial impact in neutralization antibodies production. Several studies demonstrated an increase in both B and T cell responses by fusing the antigen to the oligomerization domain encoded by the last exon of the complement 4 binding protein (C4bp) α-chain (e.g. $MSP1_{19}$ for malaria vaccine)[1,2]. Full-length native C4bp is an inhibitor of the classical and lectin pathways of complement activation. It consists of seven α-chains, one of which may be replaced by a β-chain in human, which are linked together by an heptamerization domain located at the C-terminus of the α-chains[3]. This oligomerization domain includes 57 amino acids residues in human and 54 ones in mouse; it presents a short helix H1 and a second ring forming helix H2, necessary and sufficient for heptamerization[4,5]. Each monomer is linked via disulfide bridges to the adjacent two monomers on either side of H1, determining a continuous ring wrapped around the assembles ring of H2 helices. The stability of the complex is maintaining by a combination of disulfide and hydrogen bonds, salt bridges, and hydrophobic interactions, creating a three internal non-covalent interaction layers[5].

a

Human C4bp	ETPEG**C**EQVLTGKRLM-Q**C**LPNP**EDVK**MALEVYKLSLEIEQLELQRDSARQSTLDKEL
1st chicken C4bp	GDADV**C**GEVAYIQSVVSD**C**HVPT**EDVK**TLLEIRKLFLEIQKLKVELQGLSKEFLEHILH
2st chicken C4bp	KKQKTFYV**R**KKIDQIKETF-D**C**GLPL**AELR**TLLEVQKLYLEIQKLEKELGAKGSRWWP
IMX313	KKQGDADV**C**GEVAYIQSVVSD**C**HVPT**AELR**TLLEIRKLFLEIQKLKVELQGLSKE

b

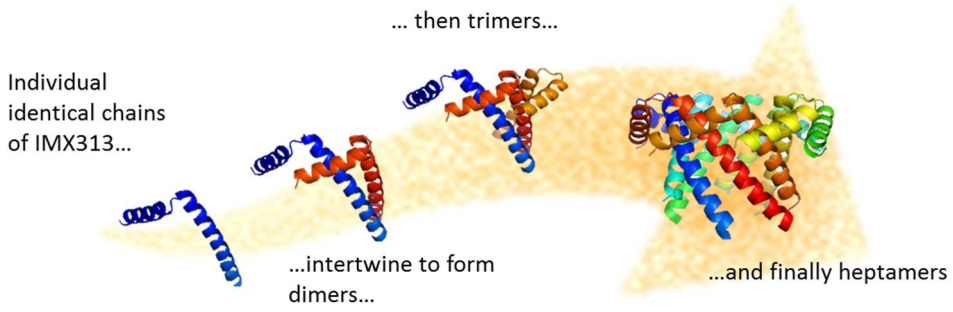

Fig. 4.1 Sequence of IMX313 and spontaneous heptameric conformation process. *a) Sequences of human and chickens C4bp oligomerization domain: based on these it was designed IMX313 to reduce auto-antibody induction in particular in human (upper panel). b) schematic representation of heptameric process of IMX313.*

The most immunogenic form, termed IMX313, is a hybrid derived from the oligomerization domains of the two chicken orthologues of C4bp α-chain. It was designed to minimize similarity to mammalian C4bp α-chain domain and has less than 20% amino acids identity to human C4bp (*Fig. 4.1*). IMX313 is an antigen re-engineering technology to develop vaccines and immunotherapies designed by IMAXIO, a French biotech company. Several studies show the increase in T and B cell immune response conferred by this technology platform, covalently linked at the C-terminus of the antigen[2,6,7]. Supramolecular structure formation due to oligomerization, allowing better antigen recognition by immune system, is a key factor in the adjuvanticity of this structural motif, even if the increased antibody titers is not observed for all fused antigens[6]. However, antigen heptamerization is not the unique mechanism explaining the B and T cell higher responses. The central core domain of C4bp has been reported to contribute to binding CD40 on B cells[8], which could be responsible for making the antigen a better target for both phagocytosis and cross-presentation[9]; this may potentially explain the ability of these domains to enhance also antigen-specific T cell responses.

PfTrx HPV16 L2(20-38)$_3$-IMX313T and HPV16 L2(20-38)$_3$-IMX313T design, production and purification

In collaboration with the IMAXIO company, we decided to evaluate the IMX313 adjuvant capacity in our HPV vaccine context. In this case, the IMX313 amino acids sequence was modified (IMX313T) introducing a GS linker, to separate the oligomerization domain from our antigen and arginine-rich sequence for protein purification, instead of C-terminal sequence LQGLSKE (*Fig. 4.2*).

IMX313

KKQGDADVCGEVAYIQSVVSDCHVPTAELRTLLEIRKLFLEIQKLKVELQGLSKE

IMX313T

GSKKQGDADVCGEVAYIQSVVSDCHVPTAELRTLLEIRKLFLEIQKLKVEGRRRRRS

Fig. 4.2 Sequence of IMX313 and IMX313T. *Amino acid sequence of IMX313 and the corresponding modified IMX313T. The modified form presents two additional motif at the N-terminus and different sequence at the C-terminus where the sequence LQGLSKE was replaced by GRRRRRS .Yellow: GS linker; light blue: arginine-rich motif.*

The antigens chosen for this purpose were our standard *Pf*Trx HPV16 L2(20-38)$_3$ and the same antigen without *Pf*Trx, namely HPV16 L2(20-38)$_3$, the latter to evaluate the immune stimulatory contribution of our scaffold. The *NdeI* restriction site is used to cloned the synthetic genes, with IMX313T sequence at the C-terminus of both proteins (*Appendix 1*), into a pET26b vector and the

corresponding recombinant proteins were expressed into *E. coli* BL21cp adding 1 mM IPTG at 30°C overnight. Cell lysis was performed by sonication and both proteins were soluble (*Fig. 4.3*) (see Materials and Methods).

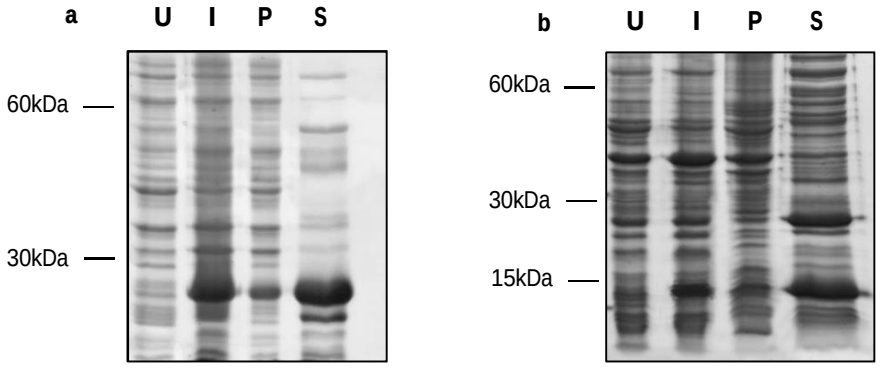

Fig. 4.3 PfTrx HPV16 L2(20-38)$_3$-IMX313T and HPV16 L2(20-38)$_3$-IMX313T are expressed and soluble. *The expression and solubility of PfTrx HPV16 L2(20-38)$_3$-IMX313T (a: MW:~25kDa) and HPV16 L2(20-38)$_3$-IMX313T (b; MW: ~13kDa) were analyzed by SDS-PAGE 11%: both proteins were highly expressed and presented a good solubility ratio. U:uninduced; I:induced; P: pellet post cell lysis; S: supernatant post cell lysis.*

Even if heptameric C4bp-fused antigens showed no significant change in secondary structure in a 35°C-95°C gradient[5], the antigens *Pf*Trx HPV16 L2(20-38)$_3$-IMX313T and L2(20-38)$_3$-IMX313T reduced their thermal stability: aliquots of supernatant after cell lysis were incubated for 20' at different temperatures (70°C, 80°C and 95°C) and solubility analyzed by SDS-PAGE, with *Pf*Trx HPV16 L2(20-38)$_3$ as a control. Both proteins resisted at the lower temperature (70°C) but precipitated at 80°C and 95°C conditions (*Fig. 4.4*), indicating a lowering of thermal stability conferred by IMX313T, not allowing a thermal purification protocol.

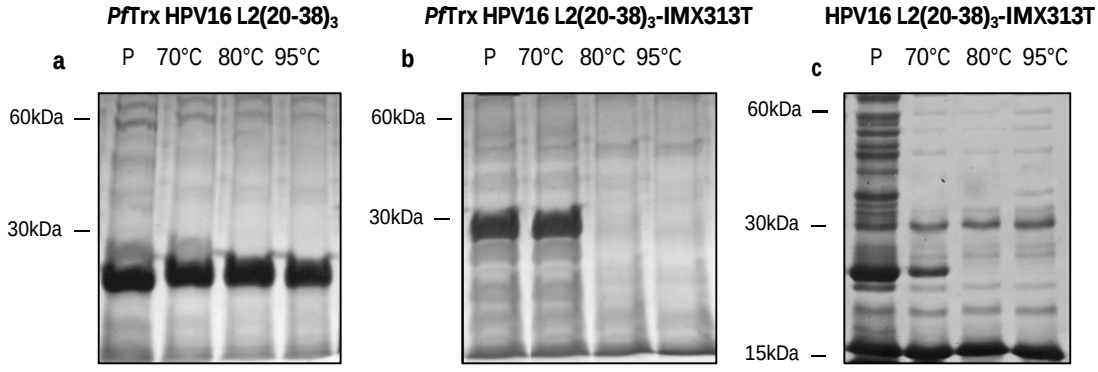

Fig. 4.4 Thermal stability of PfTrx HPV16 L2(20-38)$_3$-IMX313T and HPV16 L2(20-38)$_3$-IMX313T. *The supernatant after cell lysis corresponding to PfTrx HPV16 L2(20-38)$_3$ (MW: ~19kDa) (a), PfTrx HPV16 L2(20-38)$_3$-IMX313T (MW:~25kDa) (b) and HPV16 L2(20-38)$_3$-IMX313T (MW: ~13kDa) (c) expression were analyzed by SDS-PAGE 11% after heat treatment at 70°C, 80°C and 95°C: both proteins were stable only after 70°C treatment in contrast to the control protein stable at 95°C (a). P:supernatant pre-treatments.*

The presence of an arginine-rich motif at the C-terminus of IMX313T allowed the purification of the proteins by a single step heparin chromatography (see Materials and Methods).

For *Pf*Trx HPV16 L2(20-38)$_3$-IMX313T, two major peaks were detected, the first one was eluted around 0.3 M NaCl and containing about 80% of the protein, and the second one at approximately 0.5 M NaCl and containing the rest of the protein. A single peak (at about 0.8 M NaCl) was obtained for the protein HPV16 L2(20-38)$_3$-IMX313T under the same condition.

Each fraction of both proteins was subjected to SDS-PAGE under reducing and non-reducing conditions. Under reducing conditions, both peaks of *Pf*Trx HPV16 L2(20-38)$_3$-IMX313T contained an almost pure protein, with expected monomer MW (~25kDa) (*Fig. 4.5a*). Without β-mercaptoethanol (non-reducing conditions), two major bands were detected: one corresponding to the expected MW for an heptameric form (˜180kDa) and one with an higher MW (˜290kDa), probably an oligomeric form of the heptamer (*Fig. 4.5b*), already identified in other IMX313T-recombinant proteins[6]. Also with HPV16 L2(20-38)$_3$-IMX313T fractions, the SDS-PAGE under reducing condition shown an almost pure protein with the expected monomer MW (˜13kDa) whereas in non-reducing condition two major bands were detected, one of the expected size for the heptameric form (~90KDa) and one with higher size (˜140kDa), probably formed by covalent oligomerization of the heptameric protein (*Fig. 4.4c and d*).

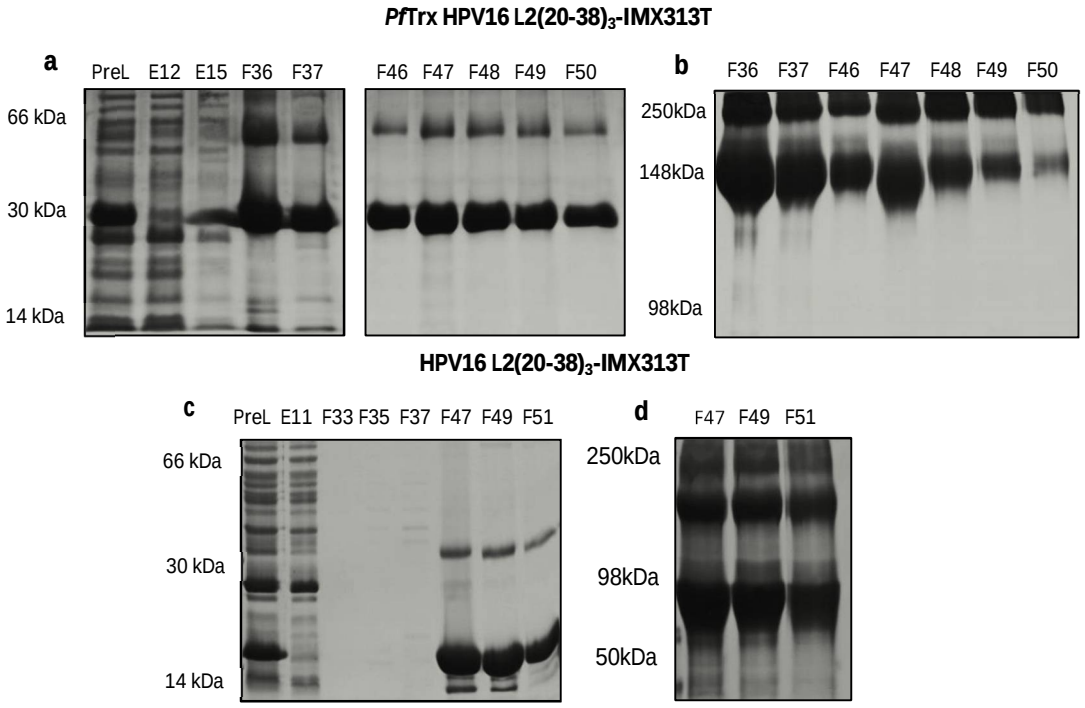

Fig. 4.5 Heparin-purification and reducing/non reducing analysis of PfTrx HPV16 L2(20-38)$_3$-IMX313T and HPV16 L2(20-38)$_3$-IMX313T. *PfTrx HPV16 L2(20-38)$_3$-IMX313T heparin purified analyzed in reducing (a) and non-reducing (b) conditions. HPV16 L2(20-38)$_3$-IMX313T heparin purified analyzed in reducing (c) and non-reducing (d) conditions. PreL: supernatant from bacterial lysate before purification; E (excluded volume), contaminants removed in the flow-through; F (fraction) fractions eluted from the column.*

After heparin chromatography, both proteins were subjected to gel filtration chromatography (see Materials and Methods) to separate the heptameric conformation from the multimeric form. In agreement with non-denaturing SDS-PAGE, the chromatography elution profile highlighted the presence of two major peaks for both proteins *Pf*Trx HPV16 L2(20-38)$_3$-IMX313T and HPV16 L2(20-38)$_3$, one corresponding to the heptameric form of the protein and one of higher MW, eluted in the volume excluded (Ve) (*Fig. 4.6a-b*).

The separation of the two oligomeric species for both proteins were verified by non-denaturing SDS-PAGE after gel filtration chromatography (*Fig. 4.6c-d)*.

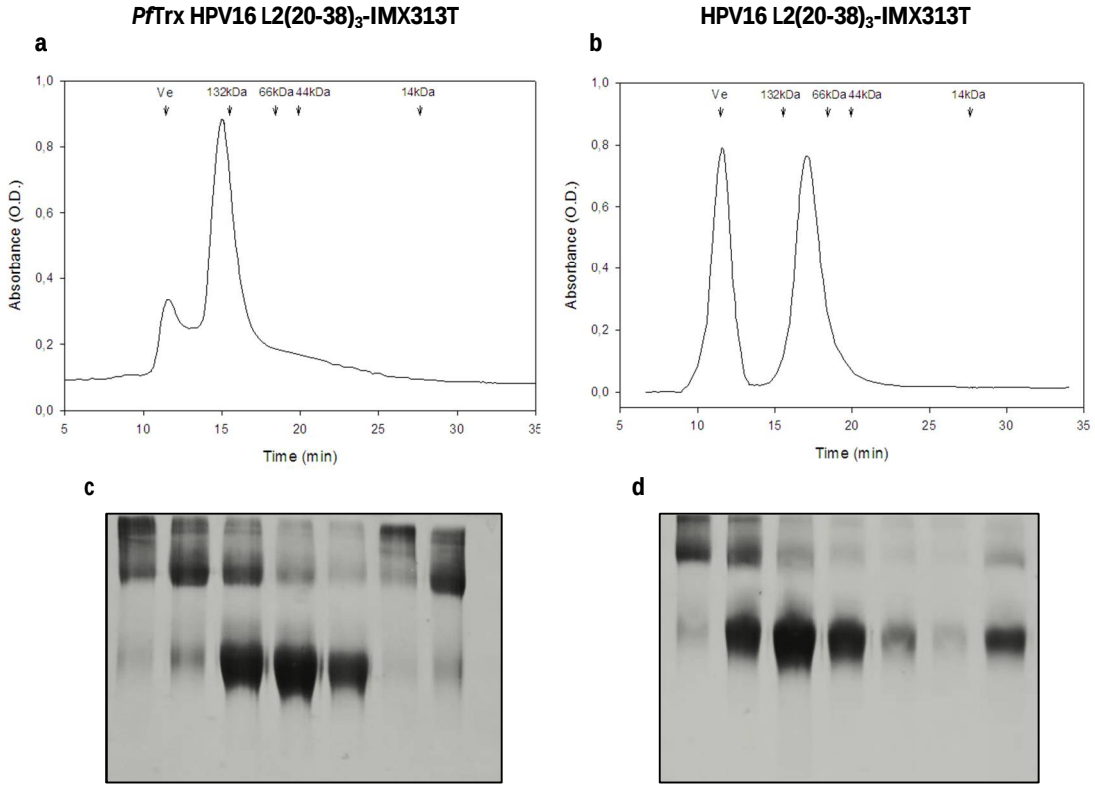

Fig. 4.6 Gel filtration profiles and non-denaturing SDS-PAGE of PfTrx HPV16 L2(20-38)$_3$-IMX313T and HPV16 L2(20-38)$_3$-IMX313T. *After heparin chromatography, PfTrx HPV16 L2(20-38)$_3$-IMX313T (a) and HPV16 L2(20-38)$_3$-IMX313T (b) were subjected to gel filtration chromatography to separate the oligomeric components. In both proteins, there were two peaks corresponding to heptameric and more complex conformations. The separation of the different species was confirmed by non-denaturing SDS-PAGE c) PfTrx HPV16 L2(20-38)$_3$-IMX313T, d) HPV16 L2(20-38)$_3$-IMX313T . Ve; excluded volume. Bovine serum albumin (66 kDa), ovalbumin (44.3 kDa) and lysozyme (14.5 kDa) were utilized as calibration standards.*

IMX313T increases antigen immunogenicity

To measure the neutralizing antibodies stimulating capacity, *Pf*Trx HPV16 L2(20-38)$_3$-IMX313T and HPV16 L2(20-38)$_3$-IMX313T were detoxified and injected intramuscularly in mice with the adjuvant Alum/MPLA. *Pf*Trx HPV16 L2(20-38)$_3$ was used in parallel as a control (see Materials and Methods). All the antigens prove to be immunogenic and elicit neutralization antibodies against HPV16 and HPV18 PsV as demonstrated in L1-PBNA assay (see Materials and Methods) (*Fig. 4.7a*).

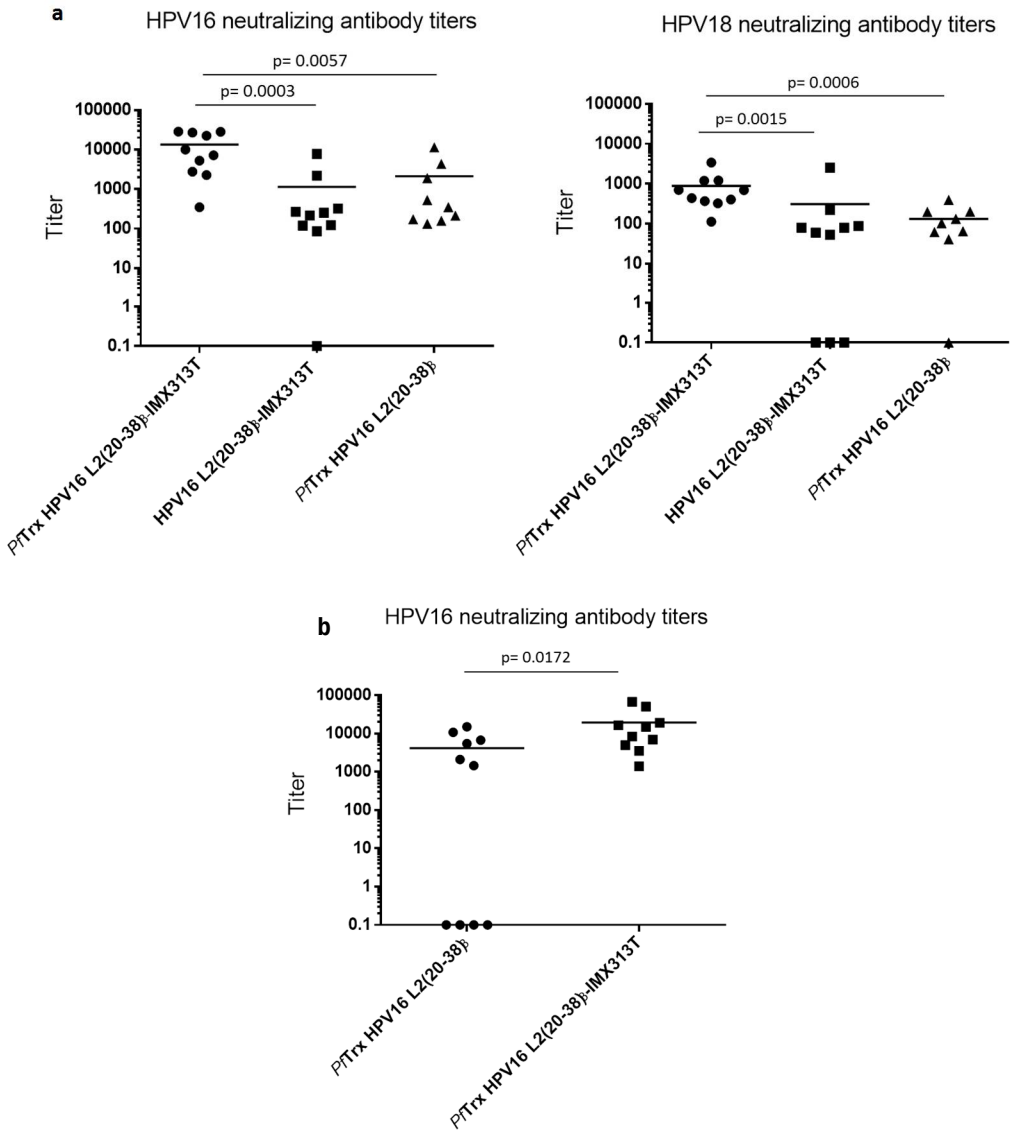

Fig. 4.7 Neutralization antibody titers obtained with IMX313T L2-derivative antigens. *a) HPV16 and HPV18 L1-PBNA and b) HPV16 L2-PBNA assays from sera collected 1 month after the last immunization from mice immunized intramuscularly with PfTrx HPV16 L2(20-38)$_3$-IMX313T and HPV16 L2(20-38)$_3$-IMX313T adjuvanted with Alum/MPLA. PfTrx HPV16 L2(20-38)$_3$ is used as a control. Dots represent individual neutralization titers of immune sera; geometric means of the titers for each group are indicated by horizontal lines. Statistical significance (p) is indicated in the graph.*

The presence of *Pf*Trx as scaffold protein is confirmed essential to obtain high and specific humoral immune response and IMX313T confers higher titers (10-fold increase) when compare to the control without the C4bp heptamerization domain. (*Fig. 4.7a*). This increase in neutralization titers was also established using the same sera by HPV16 L2-PBNA assay (see Materials and Methods)(*Fig. 4.7b*)

No statistical significance in neutralizing antibody titers are observed comparing mice injected with gel filtrated *Pf*Trx HPV16 L2(20-38)$_3$-IMX313T and with the same antigen containing the multimers of heptamers (not gel filtrated recombinant proteins; see *Fig. 4.5*) (*Fig. 4.8*), allowing to exclude that formation of aggregate of heptamers, during protein expression, could increase the immune response.

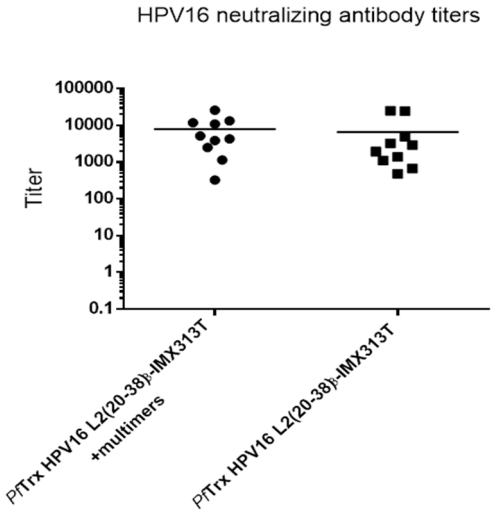

Fig. 4.8 Immune response obtained with PfTrx HPV16 L2(20-38)$_3$-IMX313T with and without gel filtration purification. *HPV16 neutralization titers measured in sera collected 1 month after the last immunization from mice immunized with PfTrx HPV16 L2(20-38)$_3$-IMX313T and PfTrx HPV16 L2(20-38)$_3$-IMX313T+multimers (without gel filtration treatment) adjuvanted with Alum/MPLA. Dots represent individual neutralization titers of immune sera; geometric means of the titers for each group are indicated by horizontal lines.*

Consistent with neutralization antibody response, ELISpot assay demonstrated an increase in *Pf*Trx T-cell epitope recognition (B3 and C3 peptides; see *Section I* and *Appendix 2*) using IMX313T-antigen compared to the control, with a significant difference in C3 peptide-IFN-γ producing cells (*Fig. 4.9*).

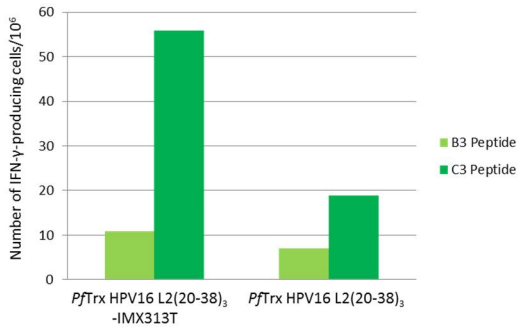

Fig. 4.9 ElISpot assay. *Qualitative ElISpot assay of splenocytes collected 1 week after the last immunization from 5 mice immunized four times with 20 µg of PfTrx HPV16 L2(20-38)$_3$ and PfTrx HPV16 L2(20-38)3-IMX313T at biweekly intervals. Histogram represents the number of cell producing IFN-γ stimulated in response to peptide designed on PfTrx HPV16 L2(20-38)$_3$. B3 and C3 were the positive peptide identified and IMX313T permitted an higher T-cell response.*

Adjuvants in the IMX313T-context

Adjuvants are compounds injected along with the antigens, able to increase/modulate immune response to antigens, by different mechanisms as depot or immune-stimulator depending on their molecular composition and structure (*Fig. 4.9*). Adjuvants are in general request to obtain potent and persistent response using less antigen and fewer immunizations[10,11].

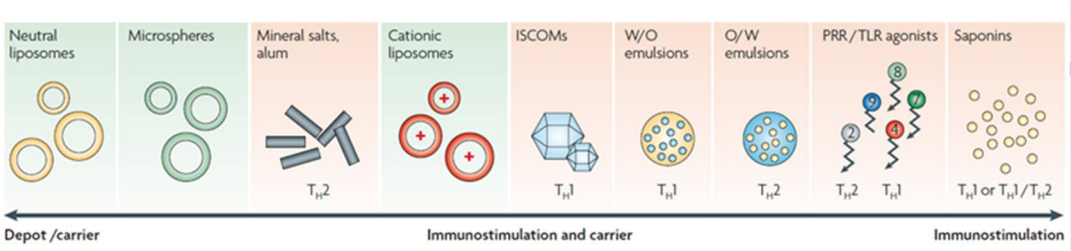

Fig. 4.9 Adjuvants properties*[11]*. *Different types of adjuvants present different characteristics as depot or immunostimulatory capacity: some compounds present both properties some others only one. ISCOMs, immunostimulating complexes; O/W, oil-in-water emulsion; PRR, pattern-recognition receptor; TLR, Toll-like receptor; W/O, water-in-oil emulsion.*

Our previous studies demonstrated the potent immunogenicity of first generation HPV16 Trx-L2 antigens using Freund's adjuvant[12] (water-in-oil adjuvant); however, its use has been associated with adverse reactions and, consequently, limited to the research only, in particular for antibodies production.

In attempt to identified a clinical trial formulation and the best adjuvant human-approved in the IMX313T context, the *Pf*Trx HPV16 L2 $(20\text{-}38)_3$-IMX313T was injected with different adjuvants: Montanide ISA 720, Alum/MPLA and AddaVax™. Montanide ISA 720 is a squalene-based oil-in-water (70:30 oil:water ratio)[13] formulation not human approved yet for prophylactic uses; nevertheless, phase I and phase II clinical trials (for several malaria and HIV vaccines) demonstrated the well tolerance of the product[14]. Squalene is an oil more readily metabolized than the paraffin oil used in Freund's adjuvants and our previous studies demonstrated a very good immune response enhancement when used in combination with different antigens reaching titers closed to the ones achieved by Freund's adjuvant addition[15,16]. Since it cannot be used in our human prophylactic vaccine formulation, we included it in the analysis as a positive reference. Alum/MPLA is a two based component adjuvant: aluminium hydroxide (alum) and monophosphoryl lipid A (MPLA), a TLR-4 agonist enhancing IL-10 production derivative of lipid A from *Salmonella minnesota* R595 lipopolysaccharide (LPS). Aluminium salts adjuvants, with different adsorption capacities depending on aluminium salt type, are the most common adjuvants for human and veterinary vaccines, used also in HPV commercial vaccines (AS04 in Cervarix® and Aluminium in Gardasil®). Mainly, they act through the formation of an antigen depot at the injection site that enhance antigen availability and uptake by immune cells[17]. It was also tested with our antigens in previous studies, in which it elicits a strong antibodies production[18], although not as robust as the one obtained by Montanide ISA 720 addition. Furthermore, the differences in the protocol formulation of alum-adjuvanted vaccines between different laboratories, contribute to a lack of reproducible results. AddaVax™ is squalene-based oil-in-water emulsion with a formulation similar to the human-licensed MF59®[19], which is included in some European influenza virus vaccine formulations[20].

*Pf*Trx HPV16 L2$(20\text{-}38)_3$ and *Pf*Trx HPV16 L2$(20\text{-}38)_3$-IMX313T were prepared in three different adjuvant formulations and injected in BALB/c mice four times at biweekly intervals subcutaneously for Montanide ISA 720 and intramuscularly for Alum/MPLA and AddaVax™. Sera collected one month after the last immunization were analysed L1-PBNA assay to evaluate neutralizing antibodies titers against HPV16 PsV (see Materials and Methods). As shown in *Fig. 4.10*, in the absence of IMX313T, the adjuvant represents an important component to obtain the best response and, as expect, Montanide ISA 720 was the stronger adjuvant; however no significance difference was observed between Alum/MPLA and AddaVax™.

The higher neutralizing antibodies titers elicited by IMX313T-constructs seemed to be adjuvant-independent: all the titers are comparable, raising the same values of Montanide ISA 720 adjuvanted-antigens and confirming the better antigen recognition mediated by heptameric structure. This response could also imply a "saturation" of the immune system by IMX313T-constructs, whose antibody titers induction cannot increase further even adding a strong adjuvant as Montanide ISA 720. We cannot exclude the possibility that this extreme antibody titer derives

from a combination of IMX313T supramolecular structure affect and less potent adjuvanticity, as Alum/MPLA or AddaVax™.

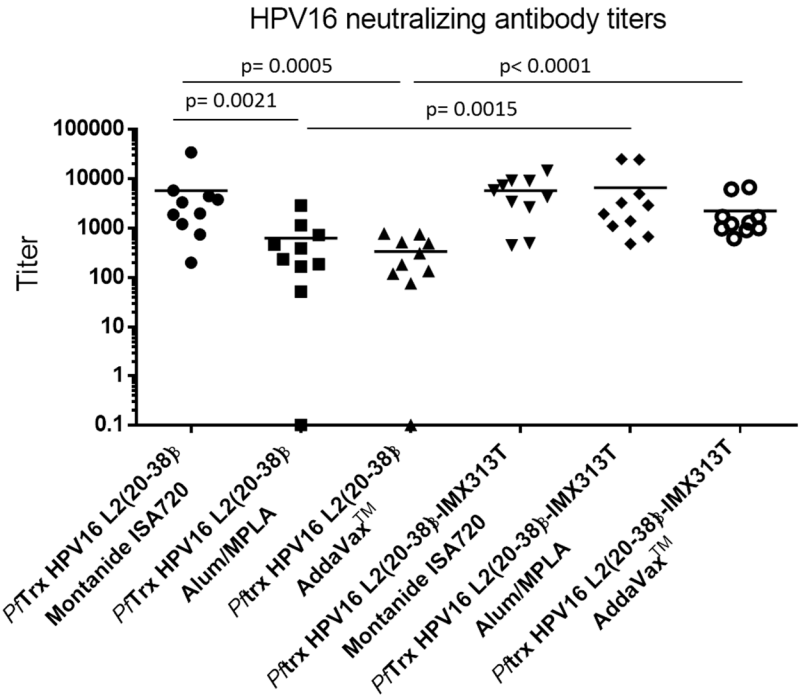

Fig. 4.10 Neutralizing antibodies titers against HPV16 in different adjuvant formulations. *HPV16 L1-PBNA on sera collected 1 month after the last immunization from 10 mice immunized four times with PfTrx HPV16 L2(20-38)$_3$ and PfTrx HPV16 L2(20-38)$_3$-IMX313T adjuvanted with Montanide ISA 720 (subcutaneous injection), Alum/MPLA and AddaVax™ (intramuscular injection). Dots represent individual neutralization titer; geometric means of the titers for each group are indicated by horizontal lines. Statistical significance (p) is indicated in the graph.*

Observing these results, Alum-MPLA and AddaVax™ could be possible alternative candidate adjuvants for trial clinical experiments in the IMX313T L2-derivative, since they are currently used under the trademarked name of AS04 and MF59®. The different interactions involved in antigen binding to Alum/MPLA and AddaVax™, which depend on their different composition, could be an important features for the final choice. Hydrophobic and electrostatic interaction are the main forces involved for antigen absorption onto Aluminium salts adjuvants, importantly for obtain maximum adjuvant effect; for the latter type of binding, the efficiency of adsorption to this adjuvant can be very different depending on the ion charges of the protein at physiological. On the other hand, oil-in-water emulsions, as AddaVax™ (and MF59®), with both depot forming and immunostimulatory properties, are simply mixed with antigen without any adsorption-charge based process; it was also demonstrated that administration of adjuvant 24 hours before or 1 hour

after the antigen elicit the same antibody responses as administered the mixture adjuvant-antigen, making unnecessary the direct association of antigen to the emulsion droplets[21]. This property makes AddaVax™ more suitable for different ion charge antigens, eliminating also the adsorption protocol needed for Alum/MPLA antigen adsorption.

Conclusion

As revealed by this study, supramolecular L2-derivative could be an improvement of *Pf*Trx HPV16 L2(20-38)$_3$ antigen. The induction of higher level antibody responses may be achieved introducing a modified oligomerization domain of C4bp, IMX313T, to obtain a spontaneously heptameric structure of corresponding recombinant protein. 10-fold increase of HPV16 neutralizing antibody titers was attained in the presence of both *Pf*Trx and IMX313T, underlined the importance of the scaffold protein, while not permit a thermal purification procedure. Multimers of heptameric structure, that could be formed during protein production and could be removed with a gel filtration step, did not contribute to elicit neutralizing antibodies. Adjuvants are crucial in achieving vaccine efficacy. In this study, three different adjuvants were tested to evaluate the best adjuvant-antigen combination: both Alum/MPLA and AddaVax™ permitted to obtain the same high titers using *Pf*Trx HPV16 L2(20-38)$_3$-IMX313T than Montanide ISA 720, a very strong adjuvant. While Montanide ISA 720 is not human approved in particular for prophylactic vaccine, Alum/MPLA and AddaVax™ (in the trademarked AS04 and MF59®, respectively) could be use in human clinical trial. Limitations in human use availability of some adjuvants, give raise the idea to design delivery platforms as protein which do not need adjuvant addition. IMX313T, forming heptameric antigens, may be present intrinsic adjuvant properties: this will be tested comparing the neutralizing antibody titers inducing by IMX313T L2-derivatived in the presence and absence of adjuvant.

Another important issue is the structural study of heptameric *Pf*Trx HPV16 L2(20-38)$_3$-IMX313T to define the epitope arrangement in this complex. Under different buffer conditions, some crystals appeared after three months and X-ray crystallography data (higher resolution limit 1,5Å) will be analyzed soon. Knowing IMX313T structure and using the *Ec*Trx one as reference, it will be possible to define the structure of *Pf*Trx and the HPV16 L2(20-38)$_3$ epitope, not determined so far.

Other supramolecular L2-derivative could increase the immunogenicity of our L2-based vaccine. Encapsulin[22] and ferritin[23] could create self-assembling nanoparticles with an icosahedral (60 identical units) and octahedral (24 subunits) cage, respectively. As Shown in *Fig. 4.11*, both encapsulin and ferritin present higher dimension than IMX313T heptamer, reaching a similar

dimension of VLP, in particular for encapsulin; this could optimize the presentation and recognition of bound antigen as *Pf*Trx HPV16 L2(20-38)$_3$ and increase the immune response, may be without adjuvant requirement.

Encapsulin and ferritin from *P. furiosus* will be cloned at N- and C-terminus of the monomer *Pf*Trx HPV16 L2(20-38), respectively. Recombinant proteins will be injected in mice and the neutralizing antibody responses will be compared to those elicit by IMX313T-constructs, also in presence and absence of adjuvant. This could be another important step further to increase antigen recognition and consequently increase neutralizing and cross-neutralizing antibody titers to cover the most prevalence HPV types.

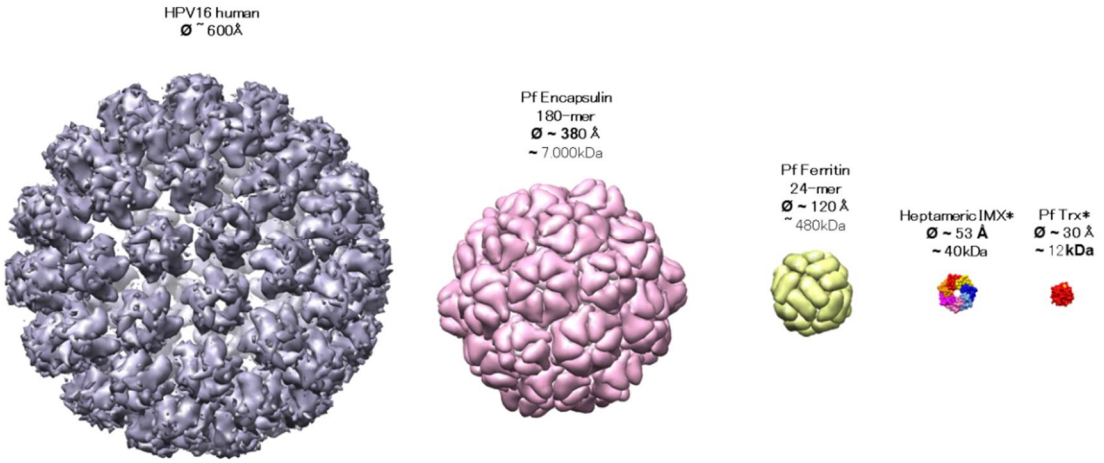

Fig. 4.11 Structure of different protein delivery platforms. *Realistic proportion of multimers of encapsolin and ferritin of P. furiosus, compared with VLP, IMX313T and PfTrx. The high density of this proteins could increase antigen recognition and neutralizing antibody titers as VLP. Structures obtained using Chimera based on PDB database.*

Materials and Methods

IMX313T constructs, proteins expression and purification. Sequence codified for *Pf*Trx HPV16 L2(20-38)$_3$ or HPV16 L2(20-38)$_3$ both with IMX313T sequence at C-terminus (see Appendix 1) were chemically synthesized (Eurofins MWG Operon) and inserted into the *NdeI* site of a modified pET26 plasmid (Novagen), without any tag-seqeunces. The resulting constructs were sequence-verified and transformed into *Escherichia coli* BL21 codon plus (DE3) cells for recombinant proteins expression.
Induction was performed over night at 30°C, adding 1mM isopropyl-b-D-thiogalactopyranoside (IPTG), followed by sonication to lyse cell. The soluble proteins were then purified with single step Heparin chromatography (Hi Trap heparin column, GE Healthcare), allowed by arginine-rich motif at the C-terminus of IMX313T. 50ml of supernatant from 500 ml of bacterial lysate was loaded onto the column previously equilibrated in buffer A (25 mM Tris/HCl pH 7.5, NaCl 100 mM), at a flow of 1.0 ml/min. Elution of the proteins was carried out with 30ml NaCl linear gradient to 2M in the same buffer. After heparin chromatography, both proteins were subjected to gel filtration chromatography on a Superdex 200 column (24 ml, GE Healthcare), equilibrated in 25 mM Tris/HCl, 150 mM NaCl, at 0.7 ml/min flow, to separate the heptameric conformation from the aggregate form. Bovine serum albumin (66 kDa), ovalbumin (44.3 kDa) and lysozyme (14.5 kDa) were used as calibration standards. The purity of the proteins were checked into SDS-PAGE.

MPLA preparation and antigen formulation with Alum/MPLA. A 0.5% (v/v) solution of Triethanolamine (TEoA) was prepared in sterile, endotoxin-free water and then filter-sterilized (0.22µm filter). To a 5 mg of lyophilized MPLA, 1.5 ml of TEoA were added and subsequently heated for 5 min at 65°C followed by 5 min of sonication in a water bath. Approximately 6-8 cycles of heating and sonication were needed until the solution became clear and slightly opalescent. Between each cycle the sample was vortexed vigorously. 1.5 ml of dissolved MPLA were transferred to a new tube and filled up to 4.85 ml with 0.5% TEoA. Another heating and sonication cycle was performed and pH was adjusted to 7.4. MPLA was stored at 4°C where is stable for 6 months. For Alum/MPLA formulation, half volume of Alum (Alhydrogel "85"; Brenntag) was added to antigen while the other half was combined to MPLA. The separated adsorption reaction were prepared under rotation for 1 h at room temperature. Before administration, the two reaction were united and mix by inversion.

Animal immunization. Procedures in animal experiments were approved by the Regierungspräsidium Karlsruhe under permit G246/11 and G19/13. Six- to eight-weeks-old female BALB/c mice (Charles River; Sulzfeld, Germany) were kept in the animal house facility of the German Cancer Research Center under pathogen-free conditions, in compliance with the regulations of the Germany Animal Protection Law. Mice were immunized four times at biweekly intervals with 20 µg of the various detoxified and filter-sterilized IMX313T-HPV16 L2 antigens adjuvanted with 50% (v/v) Montanide ISA 720 (Seppic, France) (subcutaneous injection), Alum/MPLA (50 µg and 5 µg, respectively) or 50% (v/v) AddaVax (intramuscolary injection). Four weeks after the last immunization, blood samples were collected by cardiac puncture and sera recovered after 2 hours at RT followed by centrifugation at 4500 rpm for 10 min.

***In vitro* standard (L1) pseudovirion-based neutralization assay (L1-PBNA)**. Pseudovirion preparation and L1-PBNA were performed as described[15]. Briefly, 50 ml of serially diluted immune sera (or control monoclonal antibodies) were mixed with 50 ml of diluted pseudovirion stocks and incubated at room temperature for 30 min. Next, 50 ml of HeLaT cells (2.5 x 10^5 cells/ml) were

added to the pseudovirion-antibody mixture and incubated for 48 h at 37°C under a 5% CO_2 atmosphere. The amount of secreted Gaussia luciferase was then determined in 10 ml of cell culture medium using coelenterazine substrate and Gaussia glow juice (PJK, Germany) according to the manufacturer's instructions. A microplate luminometer (Victor3, PerkinElmer) was used to measure luminescence in the culture medium 15 min after substrate addition.

***In vitro* (L2) pseudovirion-based neutralization assay (L2-PBNA).** MCF10A cells were plated at a concentration of 2 x 10^4/well in a 96-well assay plate in a volume of 100 µl of growth medium and propagated for 24 hours. Following this incubation, which allows for deposition of the ECM, the cells were washed twice with PBS 1X and lysed by incubation for 5 min at 37°C with 50 µl of pre-warmed lysis buffer (PBS containing 0.5% [v/v] Triton X-100–20 mM NH_4OH). Following lysis, 100 µl of PBS 1X was added to each well, and a 100 µl of volume was removed. This gentle washing was repeated two more times. Then the entire remaining volume was gently removed. The ECM containing wells were then washed twice further with 100 µl of PBS 1X. A pseudovirion solution, prepared in conditioned medium from CHO furin cells in a total volume of 120 µl/well, was added to the prepared ECM-containing wells following removal of the final PBS 1X wash. This pseudovirion solution also contained 5 µg/ml of heparin (Sigma H-4784). This virus-furin-heparin mixture was incubated overnight at 37°C. The next day, the medium was removed and the wells were washed twice with PBS 1X. The final wash was replaced with an antibody dilution series made in pgsa-745 growth medium at a volume of 100 µl/well. The plate was then incubated at 37°C for 6 h to allow efficient antibody binding to target epitopes. Following this incubation, pgsa-745 cells were added directly to the antibody-containing wells at a concentration of 8x10^3/well in a volume of 50 µl. For all experiments, 3-fold dilution series of antisera were made starting with an initial dilution of 1/50. Data were analyzed, and 50% inhibitory concentrations (IC50s) were determined using the GraphPad Prism software program.

ELISpot assay. ELISpot assay was performed in PVDF plate (Millipore). Add 15µl of 35% Ethanol per well and incubate ≤ 1 min until entire well turns dark, decant ethanol and wash plate three times with 200µl 1X PBS. Coating plate was carried out with 100µl/well capture antibody (5 µg/ml in PBS 1X) and incubated over night at 4°C. After removing of antibody solution, the plate was washed 3 times with 200µl/well of sterile 1X PBS (2% FCS, 100U/ml penicillin, 100 µg/ml streptomycin). Blocking was done with 200µl/well of complete RPMI medium (10mM HEPES, 1mM Sodium Pyruvate, 10% serum, 100U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine) for at least 2 hours at RT. During the incubation, the splenocytes were prepared. The spleen was remove aseptically, transferred firstly into sterile a 15 ml falcon containing 5 ml of ice cold PBS and after into petri dish and for another washing with 5 ml PBS. Aspirated the PBS and added 5 ml complete RPMI medium, the spleen was smashed via the syringe cap. The cells were passed through the cell strainer and washed with plate with 5 ml complete RPMI medium. Suspension was centrifuged at 1500 rpm for 5 min at 4 °C and the pellet resuspended in 5 ml RBC lysis buffer (170 mM Tris-HCl, 0.16 M NH_4Cl; pH7.2), followed by incubation on ice for 5 min in order to lyse erythrocytes (with frequent shaking). Neutralization of the reaction was performed adding 5 ml PBS containing 2% FCS, 100U/ml penicillin and 100 µg/ml streptomycin (wash buffer), followed by two washing with wash buffer and, finally, resuspended in 5 ml complete RPMI medium. The cells were diluted to 1.0 x 10^7 cells/ml. Send two incubation hours of plate, the blocking medium was remove and 100 µl of cell suspension 3x10^5 splenocytes per well (3x10^6 cell/ml solution) were added. 10 µg/ml Concanavalin (Con A) in 100 µl of medium is prepared as a control. The plate was incubated 36 hours at 37°C, 5% CO_2. After cells removal, the plate was washed three times with 200 µl PBS/0.05% Tween 20, followed by addition of biotinylated rat anti-mouse IFNγ (100µl/well). The incubation was performed for at least two hours at RT or overnight at 4°C. After three times

washing with PBS/0.05% Tween 20 (1 min between each wash), streptavidin-AKP was added (100µl/well) and incubate for 45 minutes at RT. Using ELISPOT wash buffer (PBST), the plate was washed three times and then two times with PBS 1X without Tween-20. BCTP/NBT substrate (Sigma) was added (100 µl/well) and after four min of RT incubation and addition of distillated water to stop reaction, the plate was air dried and the spots were counted using an automated ELIspot reader (AID EliSpot Reader Systems).

Statistical analysis. Statistical significance of neutralization assay results was determined with the nonparametric Mann-Whitney-Wilcoxon test performed with GraphPad Prism 5.00 (GraphPad Software, USA); differences between groups were considered significant at $p<0.05$.

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Section V

One shot immunization

Introduction

The standard vaccination schedule used with the commercial HPV vaccines (Cervarix® and Gardasil®) requires three immunizations in order to achieve optimal protection, even though recent data[1] indicate that apparently satisfactory antibody titers can be reached with only two immunizations, or even a single immunization, at least in young females (9-14 years old)[2–5]. The latter shortened schedule, however, would not be recommended for all subjects. For example, immunocompromised as well as malnourished or otherwise not completely healthy subject will likely require the standard three-doses regimen. This, along with cold-chain maintenance but also economical, sociocultural and health system problems, represents one of the barriers that limit massive HPV vaccine implementation in low- resource countries, which bear the highest incidence of HPV-related cervical cancers worldwide (see *Fig. 1* in *Introduction*).

Three doses vaccine program requires precise time schedule of immunization to induce the higher immune response activation and not appropriated health systems, together with cultural rejection, could compromise the vaccine program. Consequently, one-shot immunization could be an improve to extend the vaccination program in those population who live far from medical centers and in those areas in which medical structures are not always present.

Biomaterials are recently intensively investigated to identify new material properties that could enhance vaccine efficacy[6–8]. Vaccine carrier materials have to respect several requisites including biocompatibility, high drug loading and control release, fundamental for a one-shot immunization vaccine. Silica materials enclose all the properties described and are used in biomedical applications[9,10], drugs release (as antibiotics)[11–13] and drug delivery system to increase biocompatibility[14,15].

Mesoporous silica rods (MSRs), presenting a good biocompatibility[16,17], are recently identified as a potent vaccine platform to induce adaptive immune response[18]. The MSRs, 88 μm in length and 4.5 μm in diameter, are able to spontaneously form a 3D microenvironment into injection site (*Fig. 5.1*).

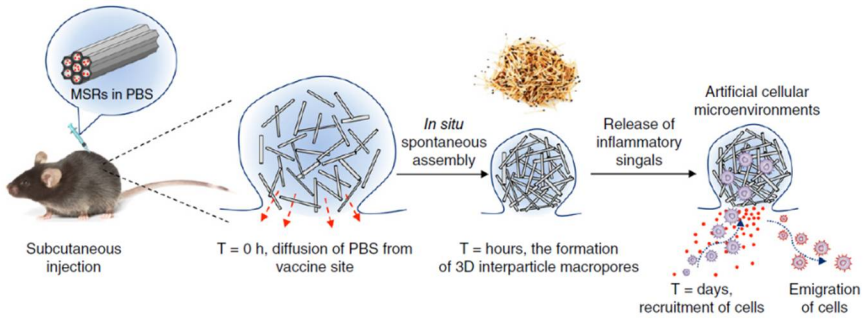

Fig. 5.1 Schematic representation of MSRs in vivo auto-assembly[18]*. After PBS diffusion, the 3D microenvironment from MSRs assembly recruits host immune cells and slowly releases the absorbed antigen.*

This structure, degraded *in vivo* some days after injection, is capable, on one hand, to recruit cells of host immune system, as dendritic cells, B cell and monocytes and, on the other hand, to slowly release loaded antigen generating a potent immune response, including T_H1 (IgG2a) and T_H2 (IgG1) ones (*Fig. 5.1*).

The biodegradation and safety of MSRs approach should be considered for potential medical applications even if the bump formation in the injection site (not observed in our study) may not be accepted in a prophylactic context where there is not a danger for the patient. As a proof of concept to obtain a one-shot vaccination, the two antigens *Pf*Trx HPV16 L2(20-38)$_3$ and *Pf*Trx HPV16 L2(20-38)$_3$-IMX313T, previously described, were adsorbed to the MSRs.

Production of MSRs and preliminary evaluation of MSRs-antigens interaction

Mesoporous silica rods was produced as described by Kim et al., 2014[18]. The conformational properties were analyzed with N_2 absorption/desorption (see Materials and Methods), demonstrating the mesoporous characteristic of the MSRs. The Brunauer-Emmett-Teller (BET) surface area (see Materials and Methods) was 650 m^2g^{-1}, comparable to those described for the same particles[18].

The antigens *Pf*Trx HPV16 L2(20-38)$_3$ and *Pf*Trx HPV16 L2(20-38)$_3$-IMX313T were produced as described previously (see *Section IV*). Briefly, the expression of both proteins was done at 30°C overnight and the purification was performed using cationic exchange for *Pf*Trx HPV16 L2(20-38)$_3$ and a single step heparin chromatography for *Pf*Trx HPV16 L2(20-38)$_3$-IMX313T (see Materials and Methods).

The interaction between MSRs and antigens is essential to take advantage of the MSRs characteristics in term of recruitment of cells and slow antigen release.

Firstly, the loading capacity of MSRs was evaluated; different amounts of *Pf*Trx HPV16 L2(20-38)$_3$ (50 μg, 150 μg, 300 μg and 600 μg) were incubated with 5 mg of MSRs at room temperature in vigorous shaking for 12 h (as described in Kim et al., 2014[18]). The samples was then centrifuged and, as shown in *Fig. 5.2a*, the antigens is always present into the pellet, demonstrating a complete adsorption to MRSs. 5 mg of MSRs are able to load up to 600 μg of antigen. Despite the higher dimension due to the heptameric conformation, *Pf*Trx HPV16 L2(20-38)$_3$-IMX313T was also completely adsorbed to MSRs (assay performed with 150 μg of antigen, *Fig 5.2b*).

The adsorption of the antigens is mediated by electrostatic interactions between the negative charge of SiO_4^- and the positives ones on the antigens, at pH condition of the binding buffer.

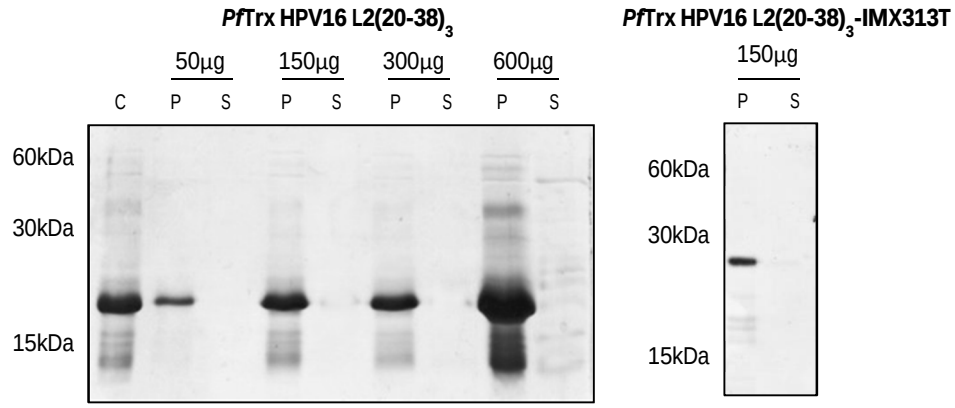

Fig. 5.2 SDS-PAGE of antigens loading on MSRs*. a) Different amounts of PfTrx HPV16 L2(20-38)$_3$ incubated with 5 mg of MSRs at RT for 12h in vigorous shaking. After centrifugation, pellet (P) and supernatants (S) were loaded into SDS-PAGE to verified the adsorption. 600 µg of antigens was completely loaded into 5 mg of MSRs. b) same treatment with 150 µg of PfTrx HPV16 L2(20-38)$_3$-IMX313T.*

Due the strong forces involved in the MSRs-antigen interaction and the high association rate, the loading time could be reduced to few minutes as compared to the 12 h described in the reference paper[18]. As shown in *Fig. 5.3,* after 15 min, 300 µg of *Pf*Trx HPV16 L2(20-38)$_3$ were completely loaded on MSRs, confirming the tight interactions between proteins and MSRs.

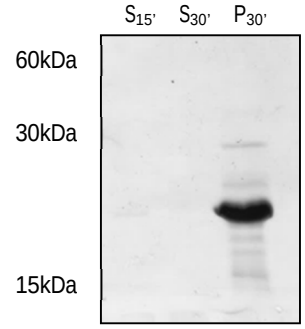

Fig. 5.3 MSRs-antigen loading time. *PfTrx HPV16 L2(20-38)$_3$ (300 µg) was incubated with 5 mg of MSRs for 15 min and 30 min to evaluated the time needed for loading. After 15 min, the sample was centrifuged and an aliquot of supernatant was collected ($S_{15'}$); sample was then resuspended and incubated for other 15 min, followed by another centrifugation. An aliquot of supernatant ($S_{30'}$) and pellet ($P_{30'}$) were loaded into an SDS-PAGE to evaluate the presence of the antigen.*

Antigen release is another important property, but its study is more complex because of both the low amount of proteins released in a short time and the difficulties of an *in vivo* environment reproduction, in which bloodstream shifts the balance of the adsorption toward release.

The simplest way is evaluate the protein accumulation during a period of time in the buffer used for particulate antigen resuspension; the MSRs-*Pf*Trx HPV16 L2(20-38)$_3$ complex was incubated in a moderate shaking at 37°C and, after centrifugation, aliquots of supernatant at different times, were loaded into a SDS-PAGE (*Fig. 5.4*).

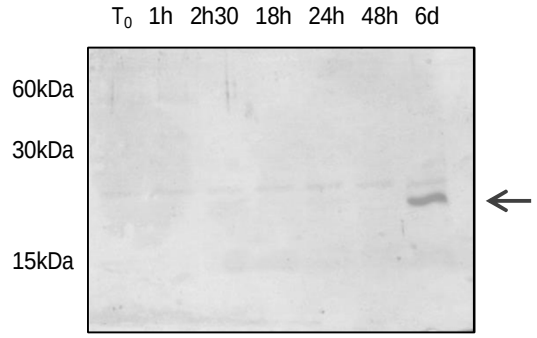

Fig. 5.4 PfTrx HPV16 L2(20-38)$_3$ release. *Accumulation in the supernatant of PfTrx HPV16 L2(20-38)$_3$ (19kDa), previously loaded into MSRs. the system was incubated in moderate shaking at 37°C for one week and at different time (1 h, 2 h 30, 18 h, 24 h, 48 h and 6 d) the sample was centrifuged and an aliquot of supernatant recovered to evaluated the presence of the protein in a SDS-PAGE. T_0 represents the supernatant after the loading time.*

The protein was detectable only after 6 days of incubation, underlined the slow release of the system (calculated in 0,007 µM of antigen/h), also confirmed by parallel studies with GFP as reference protein (data not shown). *In vivo*, the bloodstream could increase the release, but the high interaction observed between silicate and antigen permits that complete release occurs at least one month after injection. This low antigen clearance should get immune system to be steadily stimulated and, theoretically, it permits to obtain the same antibody titers reducing administrations number.

In vivo immune performance of MSRs-antigen complex

Even if the *in vitro* analysis define a high interaction and slow release of the antigen, it is important to define the *in vivo* immune performance of the MSRs system, comparing it with the corresponding antigen administered following the classical schedule. 150 µg of *Pf*Trx HPV16 L2(20-38)$_3$ and *Pf*Trx HPV16 L2(20-28)$_3$-IMX313T were adsorbed to 5 mg of MSRs, as described in Kim et al. 2014[18], with the addition of the cytokine GM-CSF and the oligonucleotide CpG-ODN (see Materials and Methods). Both these two additional components could help to increase the immune stimulation according to Kim et al., 2014[18]. In contrast with these authors, any attempt to determine the amount of CpG-ODN adsorbed to MSRs system revealed the absence of it onto the particulate-antigens, even using components such as $MgCl_2$ or spermidine, known to help DNA binding to negative substrate (*Fig. 5.5*).

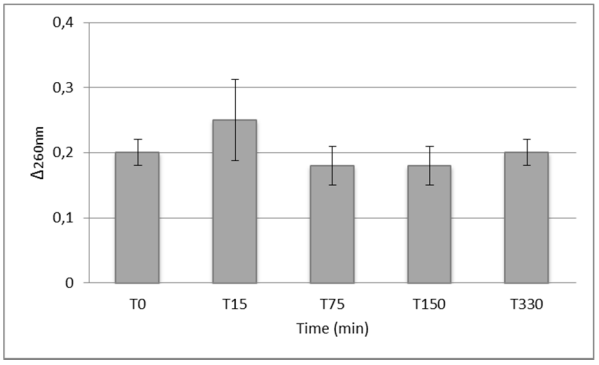

Fig. 5.5 Absence of CpG absorption. *Representation of Δ_{260nm} emission of supernatant after different incubation times with MSRs: no difference were observed compared with T0, value obtained without incubation. Δ_{260nm} is calculated as difference between the absolute 260 nm value and the value of the start point at 260 nm.*

Two groups of mice were immunized with the two MSRs-antigens. In each group, half of the animals received MSR-antigen plus GM-CSF and CpG-ODN, whereas the other half were immunized with the same mixture without the CpG component. Testing these two formulations with both antigens has the aim to define whether CpG-ODN helps or not to increase the response. The administrations were performed subcutaneously two times (day 0 and day 40) with the above mentioned formulations which were prepared, after absorption step, by their lyophilization, followed by their resuspension with PBS (see Materials and Methods). In parallel as a controls, two groups of mice were immunized subcutaneously four times at biweekly intervals, with the antigen *Pf*Trx HPV16 L2(20-38)$_3$ and *Pf*Trx HPV16 L2(20-38)$_3$-IMX313T with 50% (v/v) Montanide ISA 720. Intermediated blood was collected after the third immunization of the controls corresponding to 35 days after the first immunization of MSRs groups and final blood were collected one month

after the last immunization for all groups (*Fig. 5.6a*). IgG antibody titers and neutralizing antibody titers were evaluated in GST-L2 ELISA and L1-PBNA assay, respectively (see Materials and Methods) (*Fig. 5.6b-c*).

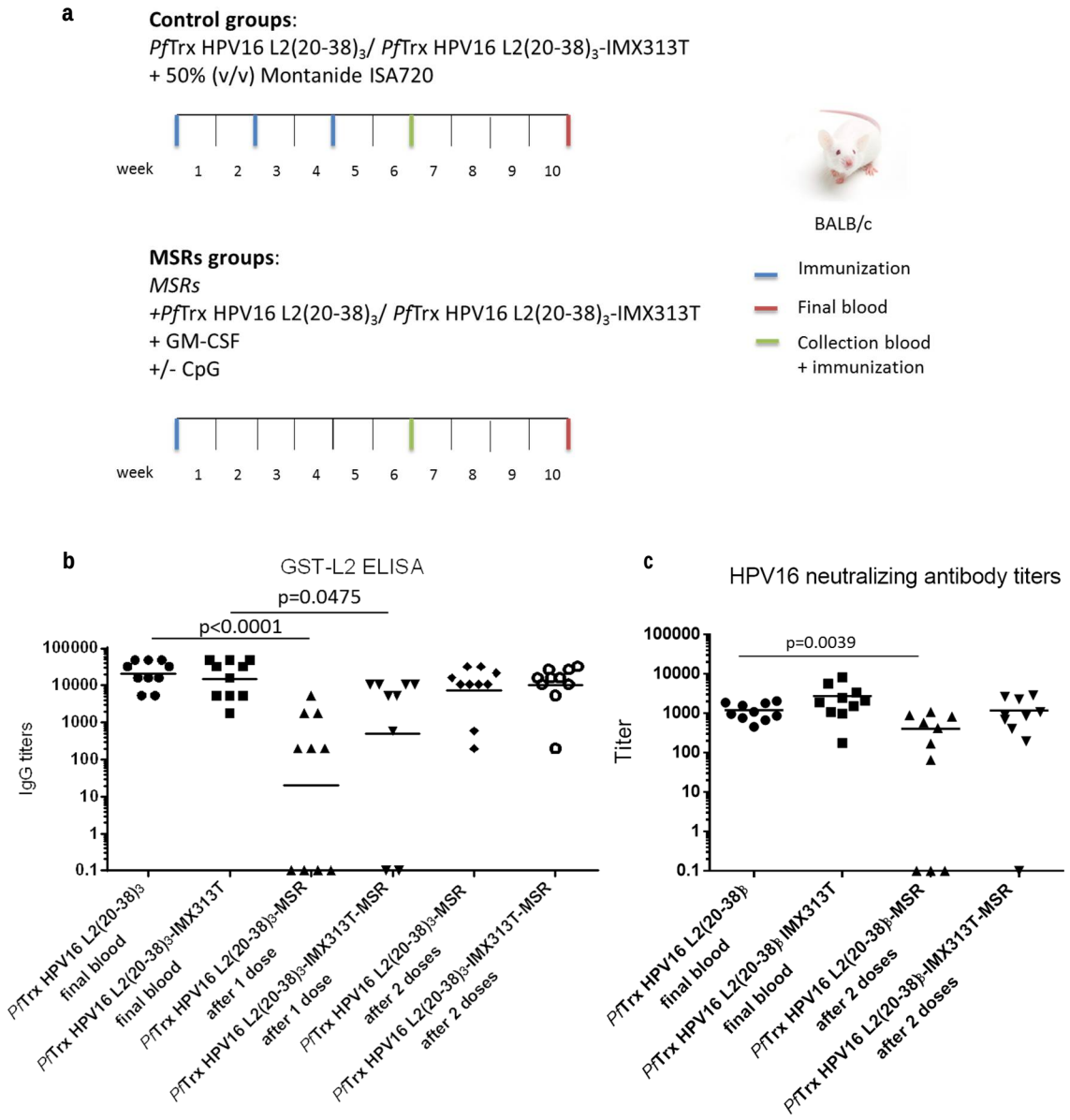

Fig. 5.6 Schematic representation of immunization schedule and in vivo immune performance of MSRs-antigens. *a) Representation of immunization schedule for control groups immunized with PfTrx HPV16 L2(20-38)$_3$ and PfTrx HPV16 L2(20-38)$_3$-IMX313T with 50% (v/v) Montanide ISA 720 and for MSRs groups with the same antigens with GM-CSF and CpG-ODN in half group. b) GST-L2 ELISA with L2 as a capture antigen on control sera, sera after 1 and 2 doses of MSRs-antigen complexes. c) HPV16 L1-PBNA assay only with the control group and the sera after the second dose of MSRs–antigen complexes. Statistical significances (p) are explicated on the graphs.*

After one dose of *Pf*Trx HPV16 L2(20-38)$_3$-MSR and *Pf*Trx HPV16 L2(20-38)$_3$-IMX313T-MSR, the IgG titers in GST-L2 ELISA were lower than the control groups, even if this difference became not significant after a second administration (*Fig. 5.6b*). Significant difference after two doses between control and *Pf*Trx HPV16 L2(20-38)$_3$-MSR was also observed in L1-PBNA assay. Conversely, IMX313T-construct with MSRs, administrated twice, permits to achieve the same neutralizing antibody titers as the corresponding control (*Fig. 5.6c*). Moreover, the addition of CpG-ODN to the formulation did not shown any enhancement in neutralizing titers.

The requirement of two doses instead of one to achieve comparable control titers could explain by difficulties found during the first administration, due to the uncomplete solubility of MSRs-antigen solution. However, the number of immunizations were reduced, underlined the unquestionable MSRs properties described[18], even if there were not information about the long term protection.

Conclusion

HPV infection affects in particular low resource countries, where economic and cultural aspects limits the screening and vaccination programs. Commercial vaccines (Gardasil® and Cervarix®) request three immunizations for the best immune protection, but reduction of doses could increase the vaccine distribution, also in areas where medical structures are not constantly present. Mesoporous silica rods were identified for this purpose as safe biomaterial able to absorbed antigen[18]; the capacity to both retrieve immune cells and release slowly the absorbed antigen, represent the most important features that could reduce the doses number, through a continually stimulation of the immune system. *Pf*Trx HPV16 L2(20-38)$_3$ and *Pf*Trx HPV16 L2(20-38)$_3$-IMX313T were used as proof of concept to test the MSR system. The complete adsorption of both antigens needed only few minutes incubation, reaching a limit loading capacity of 600 µg per 5 mg of MSRs. *In vitro* release study confirmed the shifting of interaction balance to the binding, conferring a very slowly release of the antigens. *In vivo* experiments shown the need of two doses of *Pf*Trx HPV16 L2(20-38)$_3$-MSR and *Pf*Trx HPV16 L2(20-38)$_3$-IMX313T-MSR to achieve comparable titers of the same antigen without MSRs, administrated following the classical schedule of four immunization at biweekly intervals. Problems encountered during the first MSR administration, due to the not complete solubility of MSRs-antigens, might have compromised the results. We believe that in further tests with MSR system, by improving the injection protocol, it could be possible to reach comparable immunity levels soluble antigens even with one-dose administration. The possibility to lyophilize the complex MSRs-antigens permits an higher stability of vaccine, that could be resuspended just before injection, improving its shelf-life even without cold chain storage. Knowledge about the long term response could also help to consider the MSRs approach as a good alternative to classical immunization schedule, in particular in low resource countries.

Materials and Methods

Synthesis and characterization of mesoporous silica rods (MSRs). Mesoporous silica rods were prepared as described in Kim et al., 2014[18]. To synthesize high-aspect-ratio MSRs (88 μm × 4.5 μm), typically, 4 g of P123 surfactant (average Mn ~5,800, Aldrich) and 46 mg of ammonium fluoride (NH_4F, 98%, Sigma-Aldrich) were dissolved in 150 g of 1.6 M HCl solution and were stirred with 8.6 g of tetraethyl orthosilicate (TEOS, 98%, Aldrich) at 40 °C for 20 h, followed by aging at 100 °C for 24 h. To extract the surfactant, the as-synthesized particles were refluxed for 10 h in 1% HCl in ethanol. The resulting MSR particles were then filtered, washed with ethanol and dried. Surface area were analyzed using N2 adsorption/desorption isotherms. The pore-filled MSRs were prepared following a previous report with a slight modification[19]. One gram of high-aspect-ratio MSRs was impregnated with 1.4 ml of TEOS under gentle agitation. Aqueous HCl (pH 1) was added dropwise and mixed with MSRs. The mixture powder was aged at 40°C for 3 h and water and ethanol formed during hydrolysis were removed by evaporation at 80°C. The impregnation procedure was repeated four times. The monolith-type MSRs were prepared by pressing 5 mg of high-aspect-ratio MSRs in a mold of 8-mm diameter using a laboratory press.

Antigen expression and purification. *Pf*Trx HPV16 L2(20-38)$_3$ and *Pf*Trx HPV16 L2(20-38)$_3$-IMX313T were expressed and purified as described in Section IV. Briefly, after cloning the corresponding sequence (Appendix 1) in pET26 expression vector, the induction of both proteins was performed at 30°C overnight. After cell lysis through sonication, the soluble proteins were purified using cationic exchange for *Pf*Trx HPV16 L2(20-38)$_3$ and a single step of heparin chromatography for *Pf*Trx HPV16 L2(20-38)$_3$-IMX313T. For cationic chromatography, 50 ml of supernatant were loaded onto a column (Hi Trap Capto, GE Healthcare), previously equilibrated in buffer A (MES 25 mM pH 5.8, NaCl 0 M). at a flow of 1.0 ml/min. the gradient was performed with buffer B (MES 25 mM pH 5.8 NaCl 1 M). In heparin chromatography (Hi Trap Heparin, GE Healthcare), 50 ml of supernatant was loaded onto the column, previously equilibrated in buffer A (25 mM Tris/HCl pH 7.5, NaCl 100mM), at a flow of 1.0 ml/min. Elution of the proteins was carried out with 30 ml NaCl linear gradient to 2 M in the same buffer. Proteins were then treated with Triton X-114 for endotoxins removal[20] and quantified with Bradford assay[21].

***In vitro* studies: antigen loading capacity, antigen loading time and antigen release**. Antigen loading capacity was performed loading 50 μg, 150 μg, 300 μg and 600 μg of *Pf*Trx HPV16 L2(20-38)$_3$ and 150 μg of *Pf*Trx HPV16 L2(20-38)$_3$-IMX313T on 5 mg of MSR (150 μl final volume with PBS 1X) for 12 h at room temperature under vigorous shaking. The mixtures were then centrifuged at 14000 rpm for 15 min and then 15 μl of supernatant and pellet, previously resuspended in 150 μl of PBS 1X, were loaded into SDS-PAGE to evaluated the adsorption. To verify the time needed for the complete adsorption, 5 mg of MSRs were loaded with 300 μg of *Pf*Trx HPV16 L2(20-38)$_3$ and incubated at room temperature under vigorous shacking for 15 min and 30 min: after centrifugation at 14000 rpm for 15 min, an aliquot of pellet and supernatant were loaded into SDS-PAGE to verified the presence of the antigen. For the antigen release study, firstly 150 μg of *Pf*Trx HPV16 L2(20-38)$_3$ were loaded on MSRs in a final volume of 150 μl and then centrifuged and resuspended in 300 μl to create a more similar *in vivo* environment. The system was subjected a moderate shacking at 37°C for different time and aliquots of release media was recovered after centrifugation at different times (0, 1 h, 2 h 3 min, 18 h, 24 h, 48 h, 6 days) and loaded into a SDS-PAGE to evaluate antigen presence.

***In vitro* study of CpG-ODN loading.** To test the capacity of MSR to load CpG-ODN, they were incubated at room temperature for different times (15 min, 75 min, 150 min and 330 min) under

vigorous shaking. Emission at 260 nm of aliquots of supernatant after centrifugation permitted to evaluate the presence of CpG-ODN. This analysis was performed using only CpG-ODN or adding spermidine or 8 mM $MgCl_2$ that should promote the DNA adsorption.

Preparation of MSR vaccine and immunization. 5 mg of MSRs were loaded with 1 µg of GM-CSF, 100 µg of CpG-ODN (only in half preparation) and 150 µg of antigen (*Pf*Trx HPV16 L2(20-38)$_3$ and *Pf*Trx HPV16 L2(20-38)$_3$-IMX313T) for 4 h at room temperature under vigorous shaking. The particles were then lyophilized, resuspended in cold PBS (150 µl) and injected subcutaneously in BALB/c mice (10 mice per group). A second immunization was performed 40 days after the first dose. Control groups (10 mice per group) were immunized subcutaneously four times at biweekly intervals with the only antigen (*Pf*Trx HPV16 L2(20-38)$_3$ or *Pf*Trx HPV16 L2(20-38)$_3$-IMX313T) with 50% (v/v) Montanide ISA 720. Intermediated blood was collected from submandibular vein at day 35 and final blood was collected by heart puncture one month after last immunization.

Glutathione S-transferase (GST)-L2 capture ELISA. GST-L2 ELISA were carried out in 96-well microtiter plates pre-coated overnight with glutathione-casein and blocked with casein buffer (0.2% bovine milk casein dissolved in PBS) as described previously[22]. Glutathione-casein-coated plates were incubated for 1 h a 37°C with purified GST-HPV16 L2 (0.5 mg/ml), followed by three washes with PBS containing 0.3% (v/v) Tween-20. Sera were analyzed by serial dilutions starting from 1:300, using GST alone as negative control. Following serum addition and incubation for 1 h at 37°C, plates were washed three times as above and incubated with a peroxidase-conjugated anti-mouse secondary antibody (Sigma-Aldrich) diluted 1:5000 in casein buffer. Following incubation for 1 h at 37°C, plates were washed three times and developed by ABTS [2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)] addition. Absorbance at 405 nm was measured after 30 min at 30°C with an automated microplate reader (iMark, Biorad).

***In vitro* standard (L1) pseudovirion-based neutralization assay (L1-PBNA)**. Pseudovirion preparation and L1-PBNA were performed as described[23]. Briefly, 50 ml of serially diluted immune sera (or control monoclonal antibodies) were mixed with 50 ml of diluted pseudovirion stocks and incubated at room temperature for 30 min. Next, 50 ml of HeLaT cells (2.5 x 10^5 cells/ml) were added to the pseudovirion-antibody mixture and incubated for 48 h at 37°C under a 5% CO_2 atmosphere. The amount of secreted Gaussia luciferase was then determined in 10 ml of cell culture medium using coelenterazine substrate and Gaussia glow juice (PJK, Germany) according to the manufacturer's instructions. A microplate luminometer (Victor3, PerkinElmer) was used to measure luminescence in the culture medium 15 min after substrate addition.

Statistical analysis. GST-L2 ELISA and L1-PBNA data were analyzed statistically with the non-parametric Mann-Whitney-Wilcoxon test performed with GraphPad Prism 5.0 (GraphPad Software); differences between groups were considered significant at $p < 0.05$.

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Section VI

Concatemers: single-molecules with broad-spectrum protection

Introduction

L1 virus-like particles (VLPs) are the main component of the HPV commercial prophylactic vaccines (Gardasil® and Cervarix®)[1], able to elicit a very high immune response and a long-term protection[2,3]. Unfortunately, current production and storage costs limit their distribution in low resource countries, where HPV infection incidence is higher. Protection is only directed to HPV16 and HPV18 (and HPV6 and HPV11 for Gardasil®), the most common high risk HPV types found in cervical carcinoma (~70%), excluding other types involved in the remaining ~30% of cervical cancer cases[4]. Gardasil 9®, the last Merk proposal with nine different VLPs, was developed to overcome types-restriction protection of the other commercial vaccines, even if it covers only the oncogenic HPV types within the α9 and α7 species,[5,6]. However, this increase broadly protection is not associated with a reduction of production costs.

Minor capsid protein L2, without auto-assembling in VLPs, seem to be a good alternative to L1: it is easily produced in bacteria[7–10] and is able to induce a broadly immune protection, due to the cross-neutralizing epitopes present in the N-terminus region[11]. Identified the tripeptide 20-38 as the best cross-neutralizing HPV16 L2 epitope[8], the main challenge is to overcome the intrinsically low immunogenicity of the L2 protein; as previously described (see *Section I*), a scaffold protein, as *Pyrococcus furiosus* thioredoxin (*Pf*Trx), permits a better antigen recognition together with a high thermostability[10,12]. Exploiting the TDMI approach, a three component mix with monovalent L2-antigen (HPV16, HPV31 and HPV51) conferred broader and more robust cross-protection compared to the type-specific monovalent and the L1-VLPs[13,14].

Despite this broader extension, the possibility of a single, pan-oncogenic protein would reduce the complexity of the vaccine. Based on the idea that multi-epitopes antigens are more recognized by antigen presenting cell (APC) and B-cell receptors[15–17], concatenated multi-type L2 fusion proteins may be the step further to obtain a simple and easily purified broadly spectrum vaccine also against α5, α6, and α11 papillomaviruses. High neutralizing antibody titers are obtained using a protein contains L2 residues 11 to 88 of 5 (HPV6, 16, 18, 31 and 39) or 8 (HPV6, 16, 18, 31, 39, 51, 56, 73) different HPVs[17–19]. Both vaccines, containing two key neutralization epitopes (residues 17-36 and 65-81), induced comparable titers of HPV16-neutralizing antibodies, suggesting absence of immunologic competition between the different units[19].

In the wake of this data, we transferred the base concept of multi-type fusion protein on our 20-38 L2 epitope[8], exploiting the *Pf*Trx-TDMI approach: this could be a cheaper alternative to the mix of HPV16, 31 and 51, eliciting the same immune response.

PfTrx-L2 concatemer: design, cloning and expression

Amino acids region 20 to 38 of minor capsid protein L2, the best neutralizing epitope identified in our previous studies[8,9], is the base of our multi-types L2 fusion proteins. Each monomeric epitope from different HPV types was concatenated with a Gly-Pro-Gly linker, to allow antigen correct folding and prevent junctional epitopes formation. Identification of the serotypes to include in the formulation implied the chemical properties analysis of 15 high risk HPV type L2 20 to 38 regions; this approach allowed to cluster them in eleven different groups (*Fig. 6.1*).

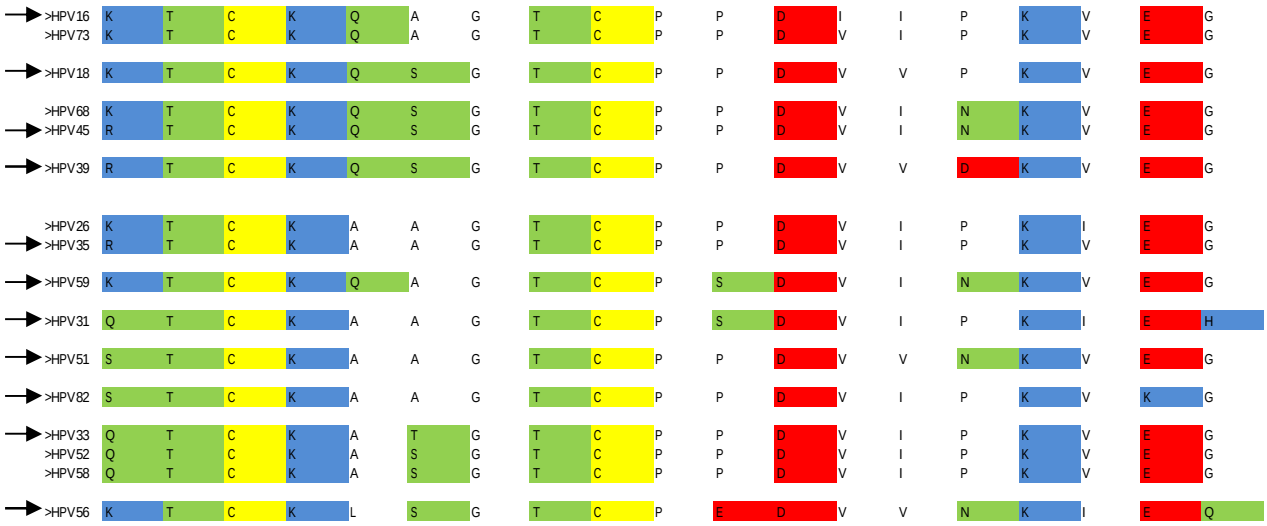

Fig. 6.1 Analysis of 20-38 amino acids region of 15 high risk HPVs. *HPV types were divided in different groups based on chemical properties of amino acids present in region L2 20-38 of 15 HPVs. Green: polar amino acids; blue: basic amino acids; white: non polar amino acids; red: acidic amino acids; yellow: cysteine conserved in all HPV types. Indicator shows the 11 HPV types selected for concatemer.*

Based on the higher prevalence worldwide (see *Fig. 3* in *Introduction*), one HPV type for each cluster was selected for the concatemer formulation (11mer): HPV16, 18, 31, 33, 35, 39, 45, 51, 56, 59 and 82, excluding consequently HPV26, 52, 58, 68 and 73. Not knowing the contribution of the position for epitope recognition, the types order followed the numeric one.

Sequence coding for 11mer (*Appendix 1*) was chemical synthesized and cloned into pET26-*Pf*Trx expression vector into *CpoI* restriction site, within the *Pf*Trx coding sequence (*Pf*Trx 11mer; see Materials and Methods). After sequencing to verify the cloning, *E. coli* BL21 codon plus strain was transformed for protein expression, induced at 37°C for 3 h. Protein was then purified follow thermal purification protocol, thanks to solubilization capacity and thermal stability of the *Pf*Trx (see *Section I*[10]) (*Fig. 6.2*). After endotoxin removal, *Pf*Trx 11mer was injected subcutaneously in BALB/c mice four times at biweekly intervals (see Materials and Methods) and sera were tested to

evaluate neutralizing and cross-neutralizing antibody titers in L1-PBNA assay (see Materials and Methods) against 13 oncogenic serotypes.

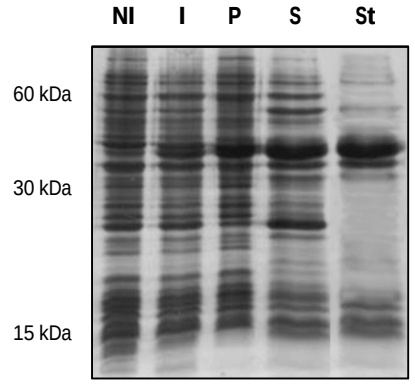

Fig. 6.2 Induction and thermal purification of PfTrx 11mer. *Induction of PfTrx 11mer (around 40 kDa) was performed at 37°C for 3h with 1mM IPTG. After sonication, the protein was purified follow a thermal purification protocol (70°C for 20 min). U: uninduced culture; I: induced culture; P: pellet after sonication; S:supernatant after sonication; St: supernatant after thermal treatment.*

As show in *Table 6.1* and *Fig. 6.3*, high titers were elicited by *Pf*Trx 11mer conferring protection against HPV16, 18, 33, 35, 39, 45 and 59 presented in the formulation and HPV58, 68 and 73 not inserted in the concatemer; this suggested that it is not necessary to include 20-38 L2 epitope from each high risk serotypes, thanks to the high conservation of this amino acids region (see *Fig. 11*, *Introduction*).

The substantial broadly protection reached is essential for a worldwide distribution of the vaccine: firstly, HPV types prevalence varies in different geographic areas and secondly, infection of relatively uncommon HPV types (less than 1% prevalence) increases after type-specific vaccination (e.g. commercial vaccines), in particular in immunocompromised patients (e.g. HIV-positive women)[20].

	*Pf*Trx 11mer									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
HPV16	14840	1420	6770	390	1760	1850	3500	26400	4480	12570
HPV18	11030	3790	10630	880	2670	4820	10430	1190	1610	14680
HPV31	210	-	130	40	20	220	120	80	90	50
HPV33	2720	390	1350	210	380	1990	1740	270	100	170
HPV35	1050	460	1170	120	350	340	660	450	220	810
HPV39	150	500	1490	140	130	250	480	160	-	420
HPV45	2160	190	2320	390	200	630	2150	500	-	1420
HPV51	-	60	-	50	50	50	90	-	80	-
HPV56	-	-	-	-	-	-	-	-	-	210
HPV58	11870	11430	13930	1250	3860	7350	13950	2100	2900	22070
HPV59	1490	250	570	90	230	180	90	60	-	150
HPV68	1320	210	770	200	200	400	730	160	-	1250
HPV73	3920	1220	6100	460	380	470	1700	2050	580	3760

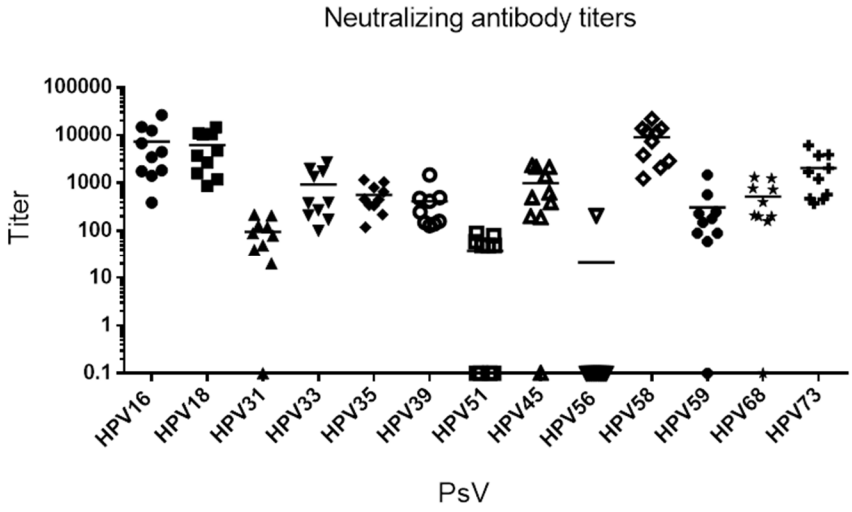

Table 6.1 and Fig. 6.3 L1-PBNA titers against 13 different HPVs types from PfTrx 11mer vaccinated mice. *Sera from mice immunized with 20µg of PfTrx 11mer with 50% (v/v) Montanide ISA 720 subcutaneously four times with biweekly intervals were tested in L1-PBNA against 13 different HPV types. Each value is indicated in the table and in the graph each dot represents a mouse and geometric means of the titers for each group are indicated by horizontal lines.*

The protection achieved with the single molecule *Pf*Trx 11mer was comparable, and in most cases higher, to the mix with three-fold repeated L2(20-38) peptide from HPV16, HPV31 and HPV51 and to the monovalent *Pf*Trx HPV16 L2(20-38)$_3$, used as a control[21] (*Table 6.2* and *Fig. 6.4*); the possibility to achieve a higher cross-reactivity using a single-molecule antigen is an important issue to reduce the cost of production.

	*Pf*Trx 11mer	*Pf*Trx HPV16 L2[14]	*Pf*Trx L2 mix[14]
	average	average	average
HPV16	7398	3196	3177
HPV18	6173	473	556
HPV31	96	-	627
HPV33	932	155	295
HPV35	563	129	240
HPV39	372	82	147
HPV45	996	317	526
HPV51	38	-	53
HPV56	-	-	-
HPV58	9071	1368	2651
HPV59	311	-	222
HPV68	524	149	183

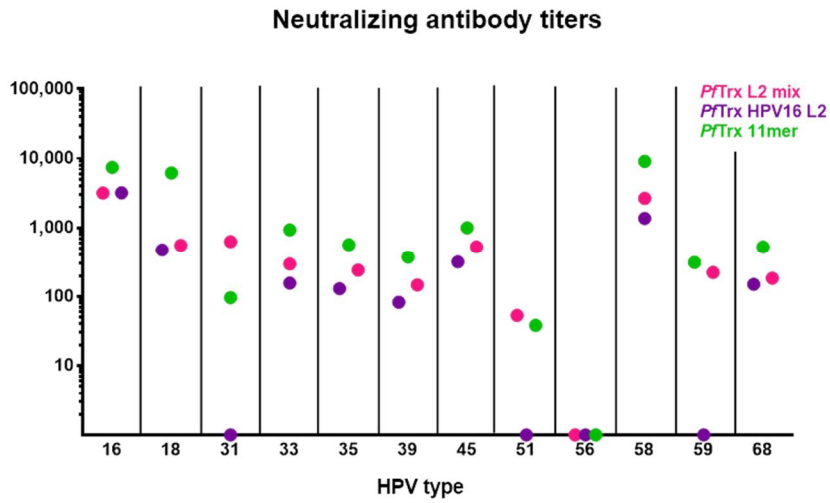

Table 6.2 and Fig. 6.4 L1-PBNA titers against 12 different HPVs types from PfTrx 11mer, PfTrx HPV16 L2 and PfTrx L2 mix vaccinated mice. *In table 6.2 average of titers from L1-PBNA assay with sera from mice (10 pour group) immunized subcutaneously four times at biweekly intervals with 20µg of PfTrx 11mer (green), PfTrx HPV16 L2(20-38)$_3$ (violet) and the mix of PfTrx HPV16 L2(20-38)$_3$, PfTrx HPV31 L2(20-38)$_3$ and PfTrx HPV51 L2(20-38)$_3$ (fuchsia), against 13 different HPV types. Data from PfTrx HPV16 L2(20-38)$_3$ and PfTrx L2 mix were from Seitz et al., 2015[14]. Fig. 6.4 is the corresponded graphical representation. Each dot represents the titer average from 10 sera.*

Even if inserted in both formulations, *Pf*Trx L2 mix and *Pf*Trx 11mer did not elicit considerable cross-protection against HPV31 and HPV51 (*Fig. 6.1* and *6.2*): higher response induce by *Pf*Trx L2 mix against HPV31 compare to the *Pf*Trx 11mer could be explain by a possible epitope competitions or reduced exposition of HPV31 epitope within the multi-type L2 derivative. However, comparable low titers against HPV51 excluded this explanation. L2-PBNA assay and *in vivo* assays (vaginal challenge and passive transfer) demonstrated the immune performance of

*Pf*Trx L2 mix also against this two serotypes[14]: the antibodies neutralization capacity could be underestimated using *in vitro* L1-PBNA assay, in which some mechanisms, as conformational changes during pseudovirions cell attachment and enhance antibody-mediated neutralization, are not reproduced. A limited access of the antibodies to L2, may be due to strong L1/L2 interaction in HPV31 and HPV51 pseudovirions, could explain the need of an *in vivo* environment or L2-PBNA assay in which furin cleavage mimics the L2 presentation during natural infection.

Knowing the importance of immune response evaluation in different animal models, parallel immunizations with *Pf*Trx 11mer and *Pf*Trx L2 mix were performed in guinea pig (see Materials and Methods): even if the neutralization antibody titers against HPV16 were comparable, *Pf*Trx 11mer was able to elicit a higher cross-neutralization titers against seven of eight other HPV types tested in L1-PBNA (*Table 6.3 and Fig. 6.5*).

	*Pf*Trx 11mer		*Pf*Trx L2 mix	
	#1	*#2*	*#1*	*#2*
HPV16	32343	48325	108792	8125
HPV18	7384	8589	1853	1218
HPV33	2418	819.9	158.7	-
HPV35	2099	869.5	18479	124.2
HPV45	7715	3812	721.4	351.8
HPV51	453.7	203.2	587.5	-
HPV56	792.0	-	-	-
HPV58	10687	4182	901	160.1
HPV59	343.7	-	-	-

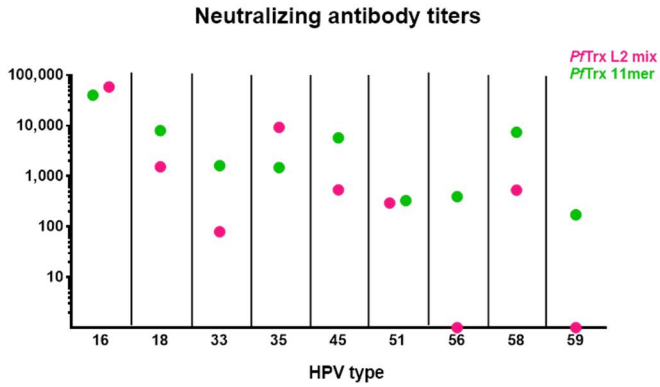

Table 6.3 and Fig. 6.5 L1-PBNA titers from PfTrx 11mer and PfTrx L2 mix vaccinated guinea pigs. *Two guinea pigs per group were immunized 4 times subcutaneously with 50 µg of PfTrx 11mer (green) and PfTrx L2 mix (fuchsia) adjuvanted with 300 µg of Alum and 20 µg of MPLA. In table 6.3 each value from L1-PBNA assay against nine high risk HPV types is expressed. # indicates the number of guinea pig. Fig. 6.5 is the corresponded graphical representation of the table 6.3. Each dot represents the titer average from 2 sera.*

Only HPV35 seems to be better neutralized by *Pf*Trx L2 mix (*Fig. 6.3*). However, evaluating the single values in *Table 6.3*, it was appreciable the higher consistency of *Pf*Trx 11mer induced responses, not evaluable from the mean value.

Both antigens formulation, in both animal models, failed to neutralize HPV56 infection. Conversely, immunization of BALB/c with the tripeptide 20 to 38 of HPV56 L2 (*Pf*Trx HPV56 L2(20-38)$_3$) (see Materials and Methods) was the only antigen able to induce a specific immune response (*Fig. 6.6*), compared also with other HPV56 L2 epitopes (28-43 and 66-83 amino acids region). *Pf*Trx L2 mix supplemented with *Pf*Trx HPV56 L2(20-38)$_3$ (*Pf*Trx L2 mix + HPV56) as well as *Pf*Trx 11mer, which contains the HPV56 epitope, did not elicit a significant HPV56 neutralization

response. Epitope competition within concatemer or a too low amount of antigen in the *Pf*Trx L2 mix + HPV56 could be the reason for unappropriated antigen recognition.

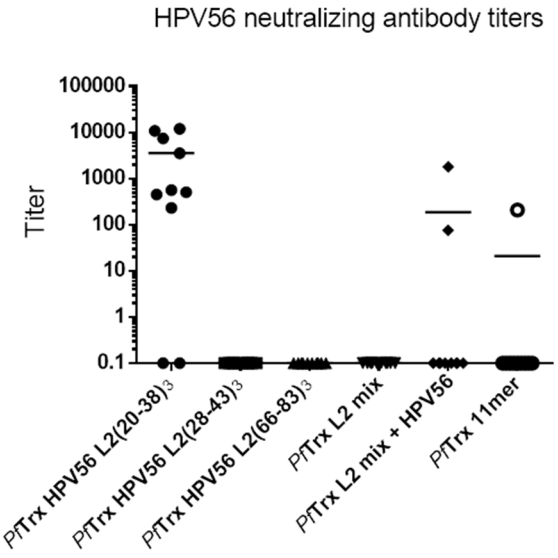

Fig. 6.6 HPV56 neutralizing antibody titers in L1-PBNA assay. *Mice (10 per group) were immunized with subcutaneously with 20 µg of different antigens with 50% (v/v) Montanide ISA 720. Among three different epitopes of HPV56 L2 tested, only the region 20 to 38 was able to induce neutralizing epitope. The same antigen did not elicit immune response inside the PfTrx 11mer or in combination with the PfTrx L2 mix. Each dot represents a mouse and geometric means of the titers for each group are indicated by horizontal lines.*

PfTrx 8mer: optimization of the L2-concatemer

Considering the cross-protection induced by the L2-concatemer against HPVs types whose epitope 20 to 38 was not inserted in the formulation, a further reduction of included serotypes could decrease possible epitopes competition and permit a better antigen exposure with a consequent increase of neutralization antibodies production.

Identification of the minimum epitopes number needed for higher broadly protection was established by chemical properties analysis of L2 epitope amino acids, considering only the region 20 to 31, which is specifically bound by the monoclonal cross-neutralizing antibodies $K4L2_{20–38}$ and $K18L2_{20–38}$[9] (*Fig. 6.7*).

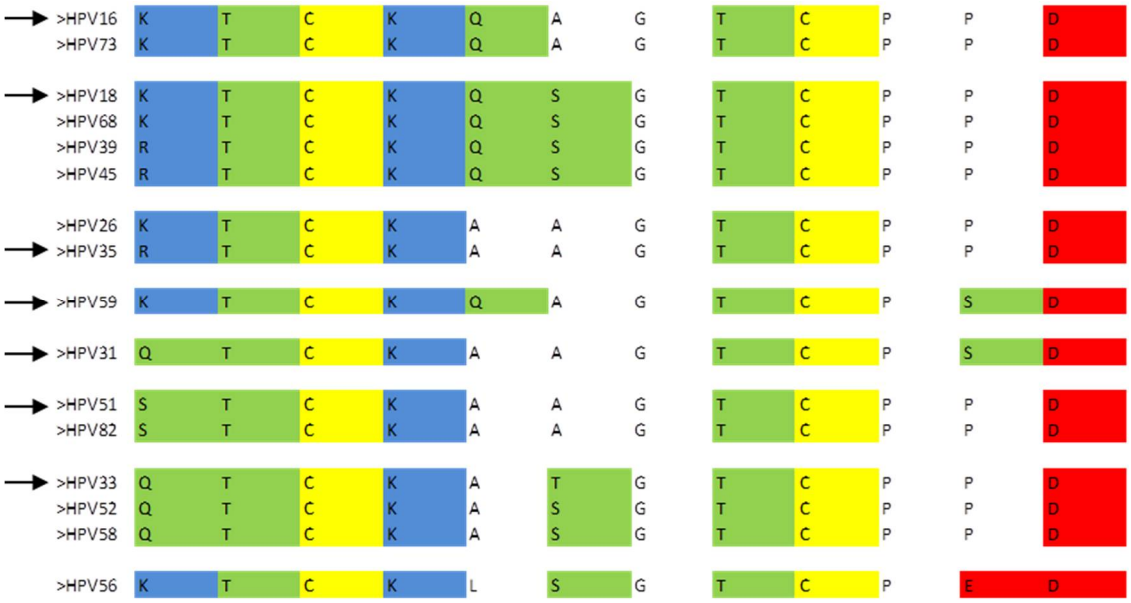

Fig. 6.7 Analysis of 20-31 amino acids region of 15 high risk HPVs. *HPV types were divided in 8 groups based on chemical properties of amino acids present in region L2 20-31 of 15 high risk HPVs. Green: polar amino acids; blue: basic amino acids; white: non polar amino acids; red: acidic amino acids; yellow: cysteine conserved in all HPV types. Indicator shows the 8 HPV types selected for concatemer.*

Based on 20-31 region, HPVs were divided in eight different clusters; the most prevalence HPV of each group (HPV16, 18, 31, 33, 35, 51 and 59) was selected and the corresponding L2(20-38) epitope was then introduced in a new construct. HPV56 was excluded due to the absent of specific response obtained with *Pf*Trx 11mer, in which the epitope was present.

To extend the protection also against low risk HPVs, which are correlated to the development of condylomas, HPV6 L2 (20-38) was included in this formulation, raising the serotypes number to eight: 8mer. The 11mer types order was maintained and HPV6 takes the place of HPV39.

11mer: HPV16, 18, 31, 33, 35, 39, 45, 51, 56, 59, 82
8mer: HPV16, 18, 31, 33, 35, 6, 51, 59

As previously described for 11mer, sequence coding for the 8mer (*Appendix 1*) was synthetically produced and cloned in the unique *CpoI* restriction site in a pET26-*Pf*Trx for protein expression (*Pf*Trx 8mer; see Materials and Methods). Induction was performed using *E. coli* BL21 codon plus transformed with pET-*Pf*Trx 8mer for three hours at 37°C adding 1mM IPTG. *Pf*Trx 8mer, as *Pf*Trx 11mer, was soluble after sonication and could be purified.

Identification of the best antigen

To identify the best formulation to propose for clinical trial, the immune performance of the two antigens (*Pf*Trx 11mer and *Pf*Trx 8mer) were compared after a further implementation consisting of the introduction of PADRE or IMX313T. In fact, based on the previous results, we knew these additional sequences could enhance the immune response, also in different mouse strains, thanks to T-helper cell activation and better antigen recognition. Sequence encoding for 11mer and 8mer were both cloned into two different vectors at *CpoI* restriction site (see Materials and Methods): pET26-PADRE$_{2x}$*Pf*Trx (see *Section III*) and pET26-*Pf*Trx-IMX313T (see *Section IV*).

E. coli BL21 codon plus were then transformed with the sequenced plasmids for protein expression, performed with 1mM IPTG at 30°C overnight; all the proteins appeared soluble after sonication. Even if *Pf*Trx 11mer, *Pf*Trx 8mer and the corresponding PADRE-constructs presented high stability suitable for a thermal purification system, with the aim of be consistent in antigen preparation with IMX313T-constructs that needed heparin chromatography, all the proteins were purified cationic exchange chromatography (see Materials and Methods).

To verify and confirm the heptamerization of *Pf*Trx 8mer-IMX313T and *Pf*Trx 11mer-IMX313T, essential for high immune response activation, the proteins were loaded in non-denaturing SDS-PAGE, that maintaining disulfide bridges, responsible for heptamerization stability (*Fig. 6.8*).

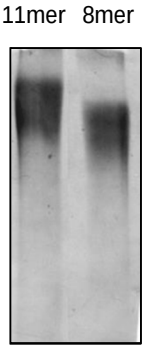

Fig. 6.8 Non-denaturing SDS-PAGE of PfTrx 11mer-IMX313T and PfTrx 8mer-IMX313T. *An aliquot of purified proteins were loaded into 6% SDS-PAGE without β-mercaptoethanol to evaluate the heptamer formation, based on disulfide briges.*

Groups of BABL/c mice were then immunized with $PADRE_{2x}$ *Pf*Trx 11mer, $PADRE_{2x}$ *Pf*Trx 8mer, *Pf*Trx 11mer-IMX313T and *Pf*Trx 8mer-IMX313T by intramuscularly administration with AddaVax™ as adjuvant (see Materials and Methods). Knowing that the response in BALB/c is not affected by PADRE Th epitope introduction, in this study the two PADRE-concatemers constructs have been used as references instead of the corresponding ones without any additional sequences. As term for comparison, immunization with *Pf*Trx HPV16 $L2(20\text{-}38)_3$, *Pf*Trx HPV16 $L2(20\text{-}38)_3$-IMX313T, *Pf*Trx L2 mix and $PADRE_{2x}$ *Pf*Trx L2 mix were done in parallel: in the case of $PADRE_{2x}$ *Pf*Trx L2 mix, all the three mix component presented the PADRE epitope at both N- and C-terminus (see Materials and Methods).

Sera collected one month after the last immunization were analyzed in L1-PBNA assay to evaluate neutralizing antibody titers against 5 HPV types (*Fig. 6.9*). In this experiments, may be due to the use of AddaVax™, the titers induced by *Pf*Trx L2 mix were lower than in previously studies (see *Fig. 6.4*), in both neutralization and cross-neutralization. Addition of PADRE to the *Pf*Trx L2 mix increased neutralization antibody titers even if the immune response was always lower than those induced by the monomer *Pf*Trx PV16 $L2(20\text{-}38)_3$, with and without IMX313T, and by the concatemers *Pf*Trx 8mer and *Pf*Trx 11mer, with PADRE or IMX313T. For this reason, sera from this groups were not included in subsequent analysis.

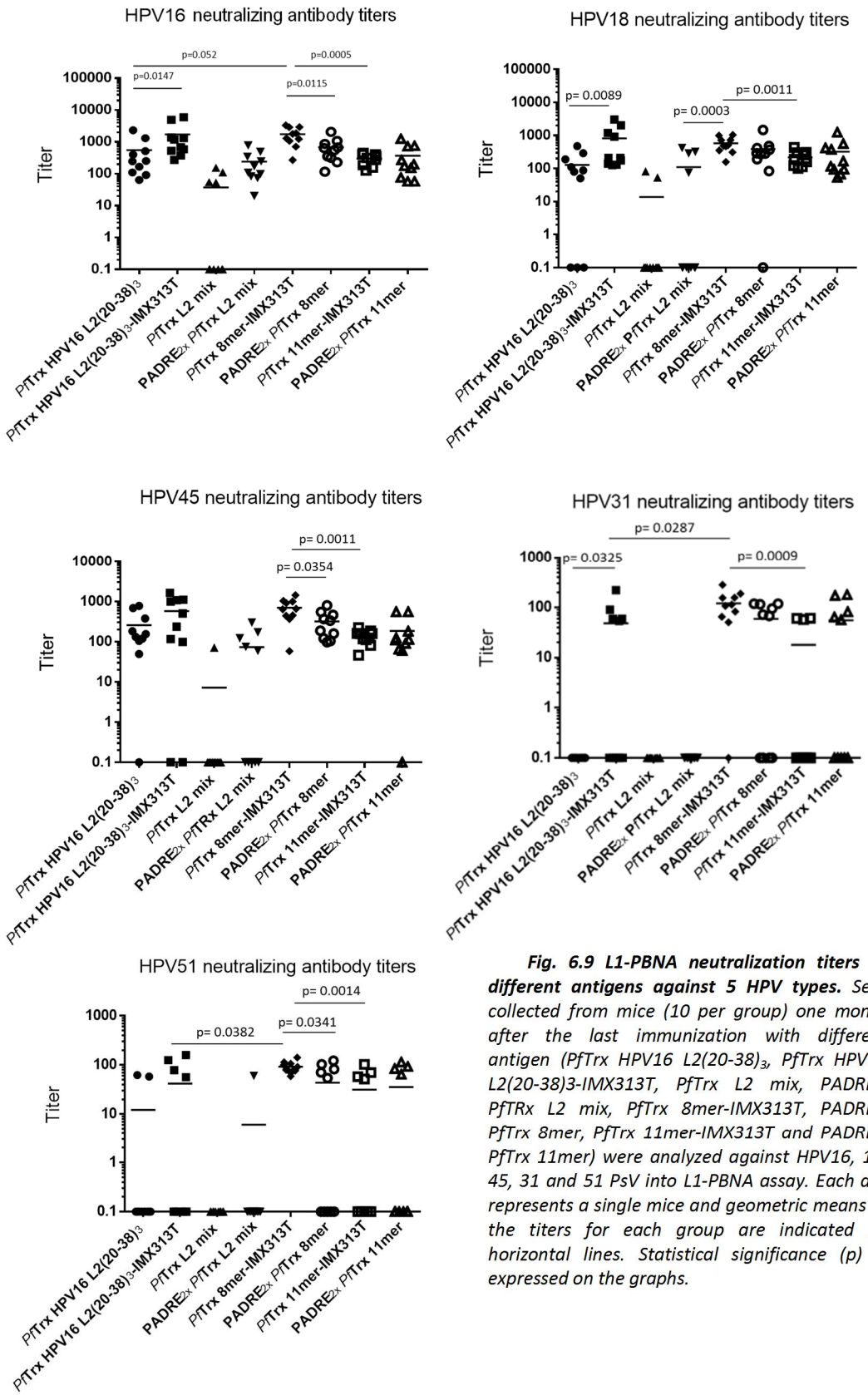

Fig. 6.9 L1-PBNA neutralization titers of different antigens against 5 HPV types. *Sera collected from mice (10 per group) one month after the last immunization with different antigen (PfTrx HPV16 L2(20-38)$_3$, PfTrx HPV16 L2(20-38)3-IMX313T, PfTrx L2 mix, PADRE$_{2x}$ PfTRx L2 mix, PfTrx 8mer-IMX313T, PADRE$_{2x}$ PfTrx 8mer, PfTrx 11mer-IMX313T and PADRE$_{2x}$ PfTrx 11mer) were analyzed against HPV16, 18, 45, 31 and 51 PsV into L1-PBNA assay. Each dot represents a single mice and geometric means of the titers for each group are indicated by horizontal lines. Statistical significance (p) is expressed on the graphs.*

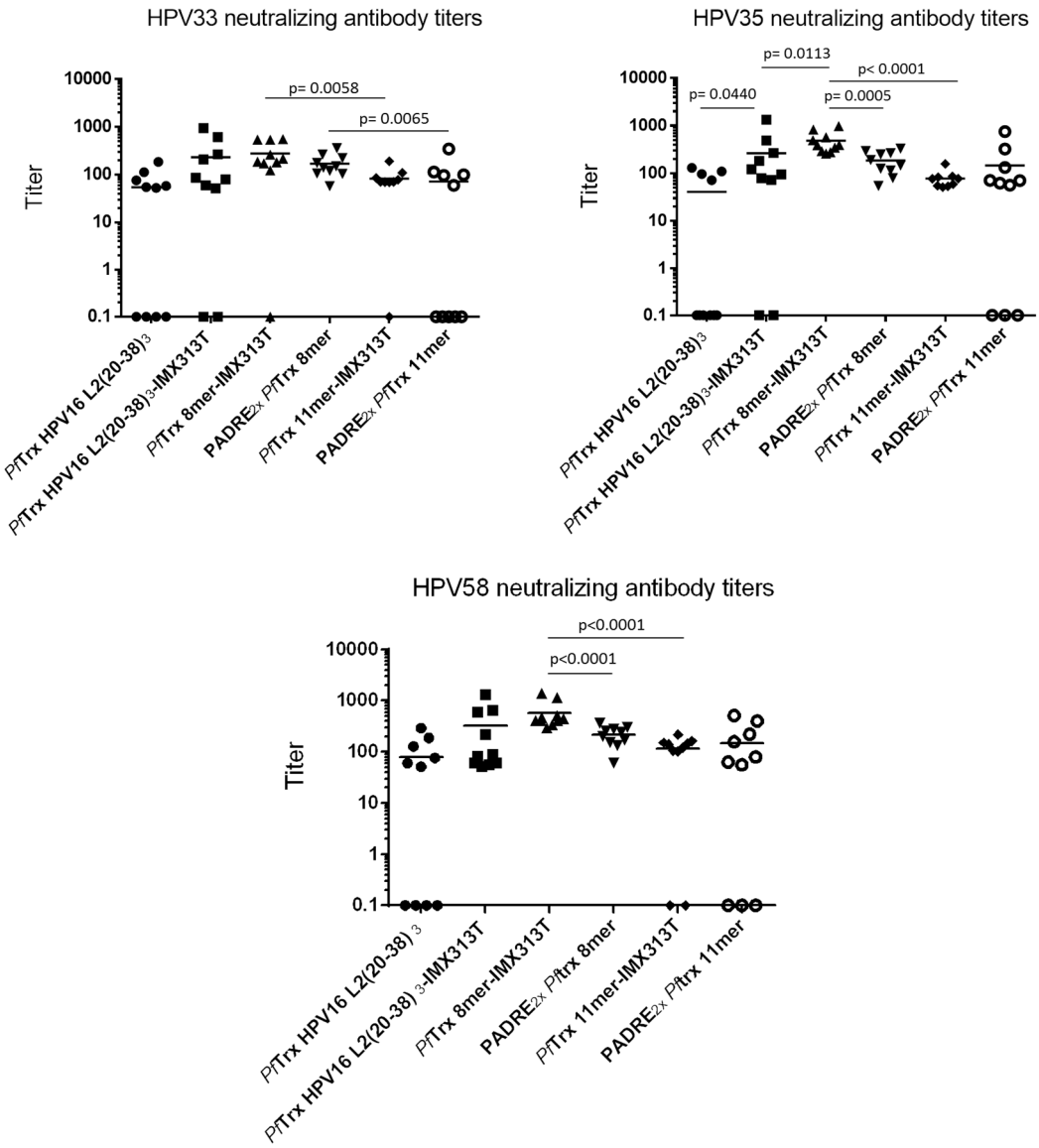

Fig. 6.10 Cross-neutralization in L1-PBNA assay of sera from immunized mice. *Sera from mice immunized with PfTrx HPV16 L2(20-38)$_3$, PfTrx HPV16 L2(20-38)3-IMX313T, PfTrx 8mer-IMX313T, PADRE$_{2x}$ PfTrx 8mer, PfTrx 11mer-IMX313T and PADRE$_{2x}$ PfTrx 11mer, four times intramuscularly with 50% (v/v) AddaVax™ as adjuvant, were tested in L1-PBNA against HPV33, 35 and 58. Each dot represents a single mice and geometric means of the titers for each group are indicated by horizontal lines. Statistical significance (p) is expressed on the graphs.*

*Pf*Trx 8mer-IMX313T was the best antigen in all the L1-PBNA assay, also against HPV types as HPV33, 35 and 58 (*Fig 6.10*): the responses were always consistent and statistical significant higher than *Pf*Trx 11mer-IMX313T. The superiority of protection generated by *Pf*Trx 8mer-IMX313T is evident especially against HPV types which are present in both concatemers, as HPV16, as HPV31, 51 and 35: the neutralization antibody titers corresponding to *Pf*Trx 8mer-IMX313T were higher and more consistent. The same antigen was able to elicit also neutralizing antibodies against HPV types not included in the formulation, but present in the 11mer, such as HPV39 (*Fig. 6.11a*). This higher response obtained using lower complex concatemer (8mer) suggested a possible better exposition of each epitope within the antigen.

*Pf*Trx 8mer-IMX313T conferred also consistent protection against the low-risk HPV6 type evaluated in L2-PBNA assay (see Materials and Methods) (*Fig. 6.11b*): a lower response obtained also using HPV16 monomers suggested a possible role of HPV16 L2(20-38)$_3$ in cross-protection, even if the insertion of the specific HPV6 epitope increased the neutralizing antibody titers. Since HPV11 has a L2 20 to 38 amino acids sequence identical to HPV6 one (QTCKLTGTCPPDVIPKVEH), we aspect for it similar protection results.

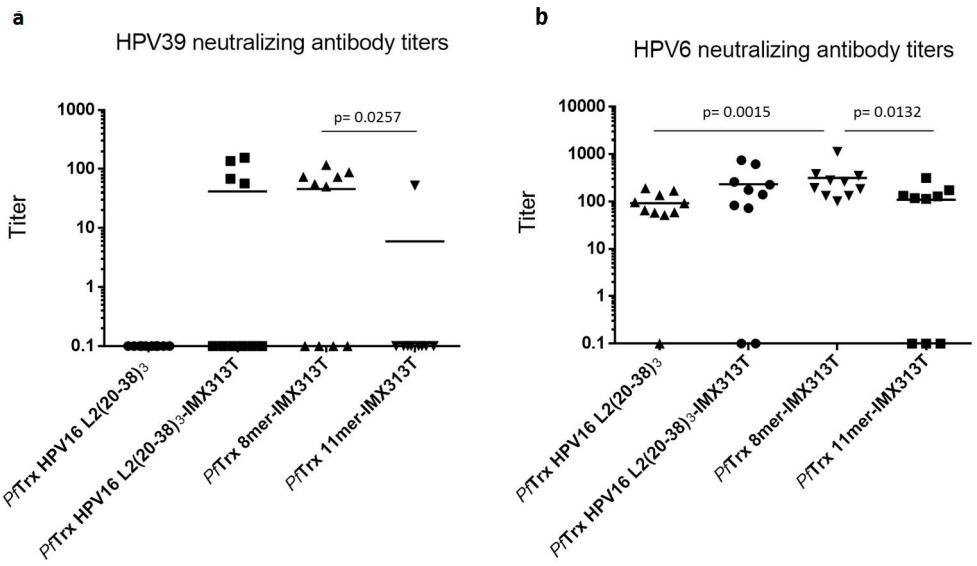

Fig. 6.11 Cross-neutralization titers against HPV39 and HPV6. *Sera from mice immunized with PfTrx HPV16 L2(20-38)$_3$, PfTrx HPV16 L2(20-38)$_3$-IMX313T, PfTrx 8mer-IMX313T and PfTrx 11mer-IMX313T four times intramuscularly with 50% (v/v) AddaVax™ as adjuvant, were tested in L1-PBNA assay against HPV39 (a) and L2-PBNA against HPV6. Each dot represents a single mice and geometric means of the titers for each group are indicated by horizontal lines. Statistical significance (p) is expressed on the graphs.*

Concatemers

All the results described are summarized in *Table 6.4* and *Fig. 6.12*: even if this data representation permits a simpler comparison among tested antigens, geometric mean hides the mouse variability and the response consistence is not appreciable.

	*Pf*Trx HPV16 L2	*Pf*Trx L2-IMX313T	*Pf*Trx L2 mix	PADRE$_{2x}$*Pf*Trx L2 mix	*Pf*Trx 8mer-IMX313T	PADRE$_{2x}$*Pf*Trx 8mer	*Pf*Trx 11mer-IMX313T	PADRE$_{2x}$*Pf*Trx 11mer
HPV16	549,58	1725,45	37,31	241,89	1743,03	691,66	300,15	371,37
HPV18	128,04	817,01	13,65	110,49	576,94	387,23	217,68	320,11
HPV31	-	48,45	-	-	120,43	58,95	17,94	55,76
HPV33	54,13	231,63	/	/	277,82	170,62	82,61	71,81
HPV35	40,87	264,55	/	/	482,89	185,06	77,84	146,67
HPV39	-	41,79	/	/	45,82	/	5,95	/
HPV45	257,88	575,55	7,18	72,95	699,33	318,20	136,81	185,27
HPV51	12,13	41,99	-	6,12	92,07	43,34	31,39	35,72
HPV58	79,44	320,28	/	/	580,68	217,49	114,60	149,16
HPV6	92,06	231,68	/	/	314,87	/	109,11	/

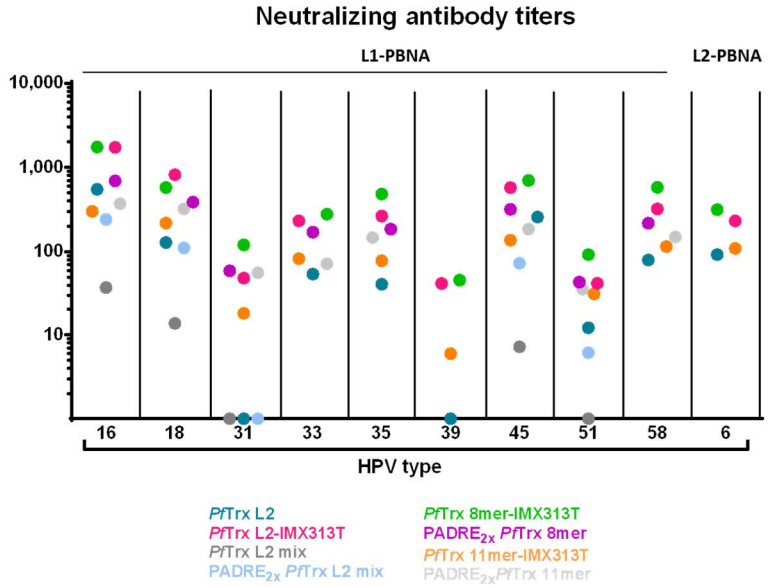

Table 6.4 and Fig. 6.12 Comparison in L1 and L2-PBNA of cross-neutralization titers elicited by different antigen. *Sera from mice immunized with PfTrx HPV16 L2(20-38)$_3$ (PfTrx L2; blue), PfTrx HPV16 L2(20-38)$_3$-IMX313T (PfTrx L2-IMX313T; fuchsia), PfTrx L2 mix (grey), PADRE$_{2x}$ PfTrx L2 mix (light blue), PfTrx 8mer-IMX313T (green), PADRE$_{2x}$ PfTrx 8mer (violet), PfTrx 11mer-IMX313T (orange) and PADRE$_{2x}$ PfTrx 11mer (light grey) four times intramuscularly with 50% (v/v) AddaVax™ as adjuvant, were tested in L1-PBNA and L2-PBNA (for HPV6). Table and graph reported the geometric mean values from 10 mice per group. /: group was not tested for the corresponding HPV PsV; -: absence of neutralizing antibodies.*

Comparing IMX313T and PADRE constructs, the first ones presented a higher immunogenicity respect antigens with the universal T helper epitope, especially the *Pf*Trx 8mer-based antigen (*Figs. 6.9, 6.10 and 6.11*): this could be due to the MHC polymorphism of BALB/c strain able to recognize and process this antigen, inducing a good antibody production without extra T-helper epitope need.

IMX313T constructs were also able to induce neutralizing antibody production in C57BL/6 mice, although the absence of any extra Th epitope generally needed for this mouse strain (see *Section III*). As shown in L1-PBNA assay (*Fig. 6.13*), the responses elicited by *Pf*Trx 8mer-IMX313T and *Pf*Trx 11mer-IMX313T were comparable and the same immune performance was observed between the corresponding PADRE constructs. Despite titer geometric means achieved by PADRE derivative were higher than IMX313T ones, data did not show any significant difference between these two groups. The high dimension due to the heptamerization of the antigen or the presence of Th epitopes in the C4bp oligomerization domain could explain these analogous immune responses. Keeping in mind the overall *in vivo* antigen performances in both mouse strains, *Pf*Trx 8mer-IMX313T represents our possible candidate antigen for a phase I clinical trial, even if in C57BL/6 mice it was not predominant respect the other formulations. Further neutralization assays against other high and low risk HPV types are needed to validate this choice. Combination of PADRE and IMX313T could be the final approach to achieve the same titers obtained in the BALB/c mice with *Pf*Trx 8mer-IMX313T (*Fig. 6.13*, *control of the assay*). Possible antigen combinations to test could be a construct with one copy of PADRE Th epitope fuse at the N-terminus (PADRE *Pf*Trx 8mer-IMX313T) or with two copies of it inserted at the N-terminus and between 8mer and IMX313T regions respectively (PADRE *Pf*Trx 8mer PADRE-IMX313T).

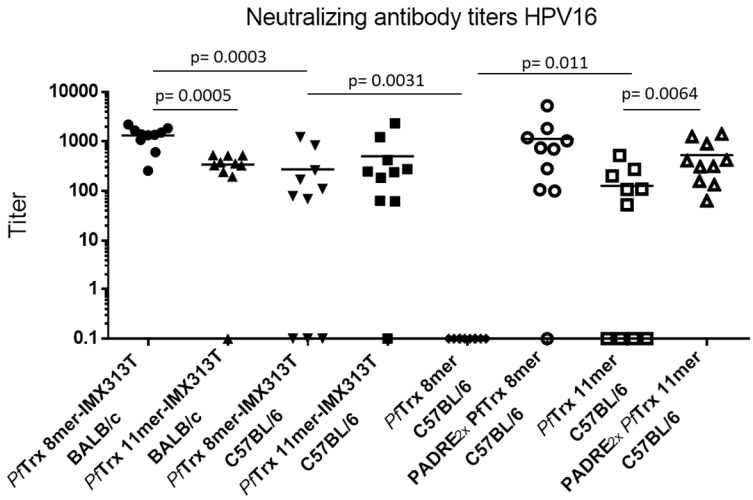

Fig. 6.13 L1-PBNA HPV16 neutralization titers of sera from C57BL/6 mice immunized with different antigens. *Mice (10 per group) were immunized intramuscularly with 20 µg of different antigens with 50% (v/v) AddaVax™. PfTrx 8mer-IMX313T and PfTrx 11mer-IMX313T in BALB/c are used as control of the assay and the higher titers previously obtained. Each dot represents a single serum and geometric mean is represented by horizontal bar. Statistical significance (p) is expressed in the graph.*

Conclusion

Since fifteen HPV types were associated, with different prevalence, to cervical carcinoma development, multivalence is one of most important feature for a prophylactic HPV vaccine. In contrast to L1-commercial vaccines, L2-based ones seem to enlarge the protection range thanks to the cross-neutralizing epitopes present in L2 N-terminus region. Previous studies identify the tripeptide L2 20 to 38, structural controlled and exposed by Trx (*Ec*Trx or *Pf*Trx), as the best neutralizing epitope[8,9]. Based on that, a mix of three different monovalent antigens containing the epitope 20-38 of HPV16, 31 and 51 (*Pf*Trx L2 mix) represented a first strategy to protect against the high risk HPV types[13,14]. The high production costs of *Pf*Trx L2 mix, due to the three antigens preparation and the lack of a sufficient cross-protection against all high risk HPV types, gave raise to another approach as a multi-type fusion proteins (concatemers) in which L2 20-38 of different HPV types were fused together. 11mer and 8mer (composed by 11 or 8 different L2 20-38 epitopes respectively) were exposed by *Pf*Trx and fused also with the universal T helper epitope PADRE and IMX313T heptamerization domain. Administration in BALB/c and C57BL/6 mice permitted to identify *Pf*Trx 8mer-IMX313T as our best candidate for a phase I clinical trial, thanks to the host-range broadness and cross-protection elicited. The supremacy of *Pf*Trx 8mer-IMX313T was evident in term of cross-protection and consistence of neutralization titers, in particular in BALB/c: it was able to elicit an higher immune response also against HPV types not presented in the formulation. Moreover, the presence of HPV6 L2 20-38 epitope in the 8mer extended the protection to some low risk HPV types as well. Knowing that *Pf*Trx HPV16 L2$(20\text{-}38)_3$ was also able to induce significant L1-PBNA titers against the dermatotropic α-type HPV5 (data not shown), we can presume that concatemers, which contains HPV16 L2 epitope, could similarly protect against cutaneous HPV types.

Since IMX313T was sufficient to permit antigen recognition in C57BL/6 mice at comparable level obtained with the extra universal Th epitope PADRE, this could indicate the presence of possible Th epitopes within IMX313T domain. A subsequent improvement such as fusion of both PADRE and IMX313T with *Pf*Trx 8mer could combine two important elements and further increase antigen immune performances.

Materials and Methods

Cloning, proteins expression and purification. Sequence codified for *Pf*Trx 11mer or 8mer (see *Appendix 1*) were chemically synthesized (Eurofins MWG Operon) and inserted into the *CpoI* restriction site of modified pET26 plasmids (Novagen): pET26 *Pf*Trx, pET26 $PADRE_{2x}$ *Pf*Trx (described in *Section III*) and pET26 *Pf*Trx-IMX313T (described in *Section IV*), without any tag-sequences. The same cloning procedure was performed to insert the HPV16 $L2(20\text{-}38)_3$, HPV31 $L2(20\text{-}38)_3$ and HPV51 $L2(20\text{-}38)_3$ antigens into pET26 $PADRE_{2x}$ *Pf*Trx plasmid. The resulting constructs were sequence-verified and transformed into *Escherichia coli* BL21 codon plus (DE3) cells for recombinant proteins expression.
Induction was performed three hours at 37°C or overnight at 30°C, adding 1mM isopropyl-b-D-thiogalactopyranoside (IPTG), followed by sonication to lyse cell. The soluble proteins were then purified following different procedure. Thermal purification protocol, used for *Pf*Trx 11mer, consists in the addition of NaCl (0.25 M) and PEG 1000 (12.5%) to the cleared supernatant and the resulting solution was incubated for 20 minutes at 70°C, cooled on ice for 10 min and centrifuged at 12,000 g for 10 min to recover the supernatant containing the purified Trx-L2 protein. To compared all the proteins, the purification procedure were based on chromatography: *Pf*Trx 11mer, *Pf*Trx 8mer and PADRE-constructs [*Pf*Trx 8mer, *Pf*Trx11mer, *Pf*Trx HPV16 $L2(20\text{-}38)_3$, *Pf*Trx HPV31 $L2(20\text{-}38)_3$ and *Pf*Trx HPV51 $L2(20\text{-}38)_3$] were purified with a cationic exchange chromatography: 50 ml of supernatant were loaded onto a column (Hi Trap Capto, GE Healthcare), previously equilibrated in buffer A (MES 25 mM pH 5.8, NaCl 0M). at a flow of 1.0 ml/min. the gradient was perfomed with buffer B (MES 25 mM pH 5.8 NaCl 1M). IMX313T-constructs (*Pf*Trx 8mer and *Pf*Trx 11mer) were purified with single step Heparin chromatography (Hi Trap Heparin column; GE Healthcare), allowed by arginine-rich motif at the C-terminus of IMX313T. 50 ml of supernatant from 500 ml of bacterial lysate was loaded onto the column, previously equilibrated in buffer A (25 mM Tris/HCl pH 7.5, NaCl 100 mM), at a flow of 1.0ml/min. Elution of the proteins was carried out with NaCl linear gradient to 2 M in the same buffer. The purity of the proteins were checked into SDS-PAGE.

Animal immunization. Procedures in animal experiments were approved by the Regierungspräsidium Karlsruhe under permit G246/11 and G19/13. Six- to eight-weeks-old female BALB/c and C57BL/6 mice (Charles River; Sulzfeld, Germany) were kept in the animal house facility of the German Cancer Research Center under pathogen-free conditions, in compliance with the regulations of the Germany Animal Protection Law. Mice were immunized four times at biweekly intervals with 20 μg of the various detoxified and filter-sterilized antigens adjuvanted with 50% (v/v) Montanide ISA 720 (Seppic, France; subcutaneous injection) or AddaVax™ (Invivogen; intramuscularly injection). Guinea pigs were subcutaneously immunized with 50 μg of *Pf*Trx 11mer or *Pf*Trx L2 mix adjuvanted with 300 μg of Alum and 20 μg of MPLA.
Four weeks after the last immunization, blood samples were collected by cardiac puncture and sera recovered after 2 hours at RT followed by centrifugation at 4500 rpm for 10 min.

***In vitro* standard (L1) pseudovirion-based neutralization assay (L1-PBNA)**. Pseudovirion preparation and L1-PBNA were performed as described[22]. Briefly, 50 ml of serially diluted immune sera (or control monoclonal antibodies) were mixed with 50 ml of diluted pseudovirion stocks and incubated at room temperature for 30 min. Next, 50 ml of HeLaT cells (2.5×10^5 cells/ml) were added to the pseudovirion-antibody mixture and incubated for 48 h at 37°C under a 5% CO_2 atmosphere. The amount of secreted Gaussia luciferase was then determined in 10 ml of cell culture medium using coelenterazine substrate and Gaussia glow juice (PJK, Germany) according to

the manufacturer's instructions. A microplate luminometer (Victor3, PerkinElmer) was used to measure luminescence in the culture medium 15 min after substrate addition.

***In vitro* (L2) pseudovirion-based neutralization assay (L2-PBNA).** MCF10A cells were plated at a concentration of 2 x 10^4/well in a 96-well assay plate in a volume of 100 µl of growth medium and propagated for 24 hours. Following this incubation, which allows for deposition of the ECM, the cells were washed twice with PBS 1X and lysed by incubation for 5 min at 37°C with 50 µl of pre-warmed lysis buffer (PBS containing 0.5% [v/v] Triton X-100–20 mM NH_4OH). Following lysis, 100 µl of PBS 1X was added to each well, and a 100 µl of volume was removed. This gentle washing was repeated two more times. Then the entire remaining volume was gently removed. The ECM containing wells were then washed twice further with 100 µl of PBS 1X. A pseudovirion solution, prepared in conditioned medium from CHO furin cells in a total volume of 120 µl/well, was added to the prepared ECM-containing wells following removal of the final PBS 1X wash. This pseudovirion solution also contained 5 µg/ml of heparin (Sigma H-4784). This virus-furin-heparin mixture was incubated overnight at 37°C. The next day, the medium was removed and the wells were washed twice with PBS 1X. The final wash was replaced with an antibody dilution series made in pgsa-745 growth medium at a volume of 100 µl/well. The plate was then incubated at 37°C for 6 h to allow efficient antibody binding to target epitopes. Following this incubation, pgsa-745 cells were added directly to the antibody-containing wells at a concentration of 8x10^3/well in a volume of 50 µl. For all experiments, 3-fold dilution series of antisera were made starting with an initial dilution of 1/50. Data were analyzed, and 50% inhibitory concentrations (IC50s) were determined using the GraphPad Prism software program.

Statistical analysis. L2-PBNA and L1-PBNA data were analyzed statistically with the non-parametric Mann-Whitney-Wilcoxon test performed with GraphPad Prism 5.0 (GraphPad Software); differences between groups were considered significant at $p < 0.05$.

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4 Conclusion

Correlation between HPV infection and cervical carcinoma development has prompted research to identify prevention strategies. Despite the high immunogenicity of the two currently VLP-based commercial vaccines, Cervarix® (GloxoSmithKline) and Gardasil® (Merk), cross-protection limits and high production costs pushed the development of second generation vaccines. Thanks to the presence of cross-neutralizing epitope and the possibility of an easy bacterial expression, the minor capsid protein L2 represents a suitable candidate for this purpose. In previous study, the insertion of L2 into the *E. coli* thioredoxin display site, which is the basis of our TDMI (Thioredoxin-Displayed Multipeptide Immunogens) approach, allowed us to increase its extremely low immunogenicity and to identify the tripeptide of L2 20 to 38 region as the best immunogenic epitope

In this study, we found out that *P. furiosus* thioredoxin (*Pf*Trx) is a superior protein scaffold, for the construction of recombinant peptide immunogens, compared to the *E. coli* one, thanks to higher thermal stability (100°C for 24 hours), superior protease resistance, greater solubilisation capacity, and the complete lack of cross-reactivity of anti-*Pf*Trx antibodies against other thioredoxins. *Pf*Trx HPV16 L2(20-38)$_3$, which represents our based formulation, could be produced also in *P. pastoris*, exhibiting all the favorable properties of the same antigen produced in *E. coli*, but with some important advantages: the high production yield, the ease of purification under native conditions without the use of any affinity tag and the lack of endotoxin contaminations. The possibility to a slow release of this antigen inducted by its adsorption to mesoporous silica rods (MSR). with a consequent reduction of administration doses, could increase vaccine distribution in low resources countries where the HPV infection incidence is higher and medical structures are inadequate. This tested approach represented a proof of concept knowing the difficulties of introducing silica use in prophylactic treatments.

The reformulation of our based antigen had the goal to obtained the higher neutralization titers with an increase in host-range broadness and cross-protection. The self-antigen heptamerization obtained fusing the C4bp oligomerization domain (IMX313T) at the C-terminus of *Pf*Trx HPV16 L2(20-38)$_3$ represents a valid approach to permit an higher antigen recognition and consequently an increase in neutralization titers.

The specificity of host responses conferred by MHC class II polymorphism has been overcome adding universal T helper (Th) epitopes, which are able to stimulate T cell activation. Pan DR Epitope (PADRE) permitted the highest antigen recognition in mice strains C57BL/6, where immune response was absent without Th epitope. PADRE was so selected as a possible element to improve our antigen formulation.

Cross-protection is a central target in vaccine development. Multi-types fusion proteins (concatemers), consisting in different HPV types L2 20 to 38 epitopes fused together and exposed by *Pf*Trx, represented an alternative to the mix of three monovalent antigens (HPV16, 31 and 51)

previously proposed. This strategy permitted to reduced cost of production since it is composed only by one protein without loss in cross-protection. Two concatemers were tested, 11mer (HPV16, 18, 31, 33, 35, 39, 45, 51, 56, 59 and 82) and 8mer (HPV16, 18, 31, 33, 35, 6, 51 and 59), designed based on chemical properties of amino acids composing L2 20-38 region of all 15 high risk HPV types. The 8mer presents also the HPV6 epitope so as to extend the protection to some low risk serotypes. Since the final goal is identified a candidate antigens for a phase I clinical trial, PADRE and IMX313T were also fused to both concatemers and tested in different mice strains. Considering the higher protection in terms of titers and cross-neutralization, IMX313T-constructs, in particular 8mer, represents our best antigen candidate.

Appendix

Nucleotide sequences

EcTrx

ATGGGCGATAAAATTATTCACCTGACTGACGACAGTTTTGACACGGATGTACTCAAAGCGGACGGGG
CGATCCTCGTCGATTTCTGGGCAGAGTGGTGCGGTCCGTGCAAAATGATCGCCCCGATTCTGGATGA
AATCGCTGACGAATATCAGGGCAAACTGACCGTTGCAAAACTGAACATCGATCAAAACCCTGGCACT
GCGCCGAAATATGGCATCCGTGGTATCCCGACTCTGCTGCTGTTCAAAAACGGTGAAGTGGCGGCAA
CCAAAGTGGGTGCACTGTCTAAAGGTCAGTTGAAAGAGTTCCTCGACGCTAACCTGGCGTGA

MtTrx

ATGGACGAGCTGGACGAAATCCGCCGTAAAAAACTGGAAGAACTGAAACGTGAACTGGCTGCCCGTA
GTCAAGGAACACCGACGATCGAGTATCCTGACCGCCCTGTACTGGTTACTGATTCTAGCATTGATGC
CGGGATCCGCCAATATCCTGTCTTTGTGGTGGACTGTTGGGCTGAATGGTGCGGTCCGTGTCGTGCT
ATTGCTCCGGTGATCGATGAAATGGCCCGTGAGCTGAAAGGACGTGTGGTATTCGGGAAACTGAACG
TGGACCAAAATCCGCTGACGAGTCGTAAATATGGCATTACCGCCATCCCTACACTGCTGGTTTTCCG
TAACGGTCGTCTGGTTGATCGCCTGGTTGGTGCTTATCCGAAACAAATTCTGATGTCTCGTGTCCGT
AAATATCTGGACTAG

PfTrx

ATGATTATCGAGTATGACGGCGAAATCGACTTCACCAAAGGTCGTGTTGTACTGTGGTTTAGCATTC
CGGGATGCGGTCCGTGTCGTCTGGTTGAACGCTTCATGACCGAACTGAGCGAGTATTTTGAGGATAT
CCAAATTGTCCATATCAATGCCGGCAAATGGAAAAACATCGTAGACAAATTCAATATTCTGAACGTG
CCGACCCTGGTATATCTGAAAGATGGCCGTGAGGTTGGACGCCAAAACCTGATTCGTTCTAAAGAAG
AGATTCTGAAAAAACTGAAAGAGCTGCAGGAGTAA

MmTrx

ATGGTGAAGCTGATCGAGAGCAAGGAAGCTTTTCAGGAGGCCCTGGCCGCCGCGGGAGACAAGCTTG
TCGTGGTGGACTTCTCTGCTACGTGGTGCGGTCCGTGCAAAATGATCAAGCCCTTCTTCCATTCCCT
CTGTGACAAGTATTCCAATGTGGTGTTCCTTGAAGTGGATGTGGATGACTGCCAGGATGTTGCTGCA
GACTGTGAAGTCAAATGCATGCCGACCTTCCAGTTTTATAAAAAGGGTCAAAAGGTGGGGGAGTTCT
CCGGTGCTAACAAGGAAAAGCTTGAAGCCTCTATTACTGAATATGCCTAA

HsTrx

ATGGTGAAGCAGATCGAGAGCAAGACTGCTTTTCAGGAAGCCTTGGACGCTGCAGGTGATAAACTTG
TAGTAGTTGACTTCTCAGCCACGTGGTGTGGGCCTTGCAAAATGATCAAGCCTTTCTTTCATTCCCT
CTCTGAAAAGTATTCCAACGTGATATTCCTTGAAGTAGATGTGGATGACTGTCAGGATGTTGCTTCA
GAGTGTGAAGTCAAATGCATGCCAACATTCCAGTTTTTTAAGAAGGGACAAAAGGTGGGTGAATTTT
CTGGAGCCAATAAGGAAAAGCTTGAAGCCACCATTAATGAATTAGTCTAA

EcTrx HPV16 L2(20-38)₃

ATGGGCGATAAAATTATTCACCTGACTGACGACAGTTTTGACACGGATGTACTCAAAGCGGACGGGG
CGATCCTCGTCGATTTCTGGGCAGAGTGGTGCGgtccg**aaaacctgcaaacaagcaggtacttgtcc**
gccggatatcatcccgaaggtcgagggtggtggtccgaagacttgtaagcaggcgggcacctgtcca
ccagacattattccaaaagtggaaggcggcggcccaaaaacatgtaaacaggccgggacttgccctc
ctgacatcatccctaaagttgaagggggcgGTCCGTGCAAAATGATCGCCCCGATTCTGGATGAAAT
CGCTGACGAATATCAGGGCAAACTGACCGTTGCAAAACTGAACATCGATCAAAACCCTGGCACTGCG
CCGAAATATGGCATCCGTGGTATCCCGACTCTGCTGCTGTTCAAAAACGGTGAAGTGGCGGCAACCA
AAGTGGGTGCACTGTCTAAAGGTCAGTTGAAAGAGTTCCTCGACGCTAACCTGGCGTGA

PfTrx HPV16 L2(20-38)$_3$

ATTATCGAGTATGACGGCGAAATCGACTTCACCAAAGGTCGTGTTGTACTGTGGTTTAGCATTCCGG
GATGCGgtccg**aaaacctgcaaacaagcaggtacttgtccgccggatatcatcccgaaggtcgaggg**
tggtggtccgaagacttgtaagcaggcgggcacctgtccaccagacattattccaaaagtggaaggc
ggcggcccaaaaacatgtaaacaggccgggacttgccctcctgacatcatccctaaagttgaagggg
gcgGTCCGTGTCGTCTGGTTGAACGCTTCATGACCGAACTGAGCGAGTATTTTGAGGATATCCAAAT
TGTCCATATCAATGCCGGCAAATGGAAAAACATCGTAGACAAATTCAATATTCTGAACGTGCCGACC
CTGGTATATCTGAAAGATGGCCGTGAGGTTGGACGCCAAAACCTGATTCGTTCTAAAGAAGAGATTC
TGAAAAAACTGAAAGAGCTGCAGGAGTAA

PADRE$_{1x}$ PfTrx HPV16 L2(20-38)$_3$

CGCGGCGATggcGCCAAATTCGTTGCGGCATGGACCCTGAAAGCGGCAGCAggccctggaccgggtA
TGATTATCGAGTATGACGGCGAAATCGACTTCACCAAAGGTCGTGTTGTACTGTGGTTTAGCATTCC
GGGATGCG**gtccg**aaaacctgcaaacaagcaggtacttgtccgccggatatcatcccgaaggtcgag
ggtggtggtccgaagacttgtaagcaggcgggcacctgtccaccagacattattccaaaagtggaag
gcggcggcccaaaaacatgtaaacaggccgggacttgccctcctgacatcatccctaaagttgaagg
gggcgGTCCGTGTCGTCTGGTTGAACGCTTCATGACCGAACTGAGCGAGTATTTTGAGGATATCCAA
ATTGTCCATATCAATGCCGGCAAATGGAAAAACATCGTAGACAAATTCAATATTCTGAACGTGCCGA
CCCTGGTATATCTGAAAGATGGCCGTGAGGTTGGACGCCAAAACCTGATTCGTTCTAAAGAAGAGAT
TCTGAAAAAACTGAAAGAGCTGCAGGAGTAA

PADRE$_{2x}$ PfTrx HPV16 L2(20-38)$_3$

CGCGGCGATggcGCCAAATTCGTTGCGGCATGGACCCTGAAAGCGGCAgcaggccctggaccgggtA
TGATTATCGAGTATGACGGCGAAATCGACTTCACCAAAGGTCGTGTTGTACTGTGGTTTAGCATTCC
GGGATGCG**gtccg**aaaacctgcaaacaagcaggtacttgtccgccggatatcatcccgaaggtcgag
ggtggtggtccgaagacttgtaagcaggcgggcacctgtccaccagacattattccaaaagtggaag
gcggcggcccaaaaacatgtaaacaggccgggacttgccctcctgacatcatccctaaagttgaagg
gggcgGTCCGTGTCGTCTGGTTGAACGCTTCATGACCGAACTGAGCGAGTATTTTGAGGATATCCAA
ATTGTCCATATCAATGCCGGCAAATGGAAAAACATCGTAGACAAATTCAATATTCTGAACGTGCCGA
CCCTGGTATATCTGAAAGATGGCCGTGAGGTTGGACGCCAAAACCTGATTCGTTCTAAAGAAGAGAT
TCTGAAAAAACTGAAAGAGCTGCAGGAGggtccgggtccagggGCGAAGTTTGTGGCCGCTTGGACG
TTAAAAGCCGCTGCGgggCGTGGGGACTAA

p2p30$_{1x}$ PfTrx HPV16 L2(20-38)$_3$

CGCGGAGATggcCAATACATCAAAGCGAATTCCAAATTCATCGGCATTACGGAACTGggtccaggcT
TTAACAACTTTACCGTGAGCTTTTGGTTGCGCGTACCGAAAGTGTCTGCTTCACACCTGGAAggtcc
gggtccaggaATGATTATCGAGTATGACGGCGAAATCGACTTCACCAAAGGTCGTGTTGTACTGTGG
TTTAGCATTCCGGGATGCG**gtccg**aaaacctgcaaacaagcaggtacttgtccgccggatatcatcc
cgaaggtcgagggtggtggtccgaagacttgtaagcaggcgggcacctgtccaccagacattattcc
aaaagtggaaggcggcggcccaaaaacatgtaaacaggccgggacttgccctcctgacatcatccct
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AGGATATCCAAATTGTCCATATCAATGCCGGCAAATGGAAAAACATCGTAGACAAATTCAATATTCT
GAACGTGCCGACCCTGGTATATCTGAAAGATGGCCGTGAGGTTGGACGCCAAAACCTGATTCGTTCT
AAAGAAGAGATTCTGAAAAAACTGAAAGAGCTGCAGGAGTAA

p2p30$_{2x}$ PfTrx HPV16 L2(20-38)$_3$

CGCGGAGATggcCAATACATCAAAGCGAATTCCAAATTCATCGGCATTACGGAACTGggtccaggcT
TTAACAACTTTACCGTGAGCTTTTGGTTGCGCGTACCGAAAGTGTCTGCTTCACACCTGGAAggtcc
gggtccaggaATGATTATCGAGTATGACGGCGAAATCGACTTCACCAAAGGTCGTGTTGTACTGTGG
TTTAGCATTCCGGGATGCG**gtccgaaaacctgcaaacaagcaggtacttgtccgccggatatcatcc**
cgaaggtcgagggtggtggtccgaagacttgtaagcaggcgggcacctgtccaccagacattattcc
aaaagtggaaggcggcggcccaaaaacatgtaaacaggccgggacttgccctcctgacatcatccct
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AGGATATCCAAATTGTCCATATCAATGCCGGCAAATGGAAAAACATCGTAGACAAATTCAATATTCT
GAACGTGCCGACCCTGGTATATCTGAAAGATGGCCGTGAGGTTGGACGCCAAAACCTGATTCGTTCT
AAAGAAGAGATTCTGAAAAAACTGAAAGAGCTGCAGGAGgggcctggtccgggcCAGTATATCAAAG
CCAATAGCAAGTTCATTGGCATTACCGAGTTAggtcccggcTTTAATAACTTCACTGTCTCGTTTTG
GCTGCGTGTTCCGAAAGTTTCGGCAAGTCATCTCGAAggcCGTGGGGACTAA

p25$_{1x}$ PfTrx HPV16 L2(20-38)$_3$

CGTGGTGATggcAAACTGATTCCGAATGCGAGTTTGATCGAGAATTGCACCAAAGCTGAACTTggtc
caggcccaggcATGATTATCGAGTATGACGGCGAAATCGACTTCACCAAAGGTCGTGTTGTACTGTG
GTTTAGCATTCCGGGATGCG**gtccgaaaacctgcaaacaagcaggtacttgtccgccggatatcatc**
ccgaaggtcgagggtggtggtccgaagacttgtaagcaggcgggcacctgtccaccagacattattc
caaaagtggaaggcggcggcccaaaaacatgtaaacaggccgggacttgccctcctgacatcatccc
taaagttgaaggggcgGTCCGTGTCGTCTGGTTGAACGCTTCATGACCGAACTGAGCGAGTATTTT
GAGGATATCCAAATTGTCCATATCAATGCCGGCAAATGGAAAAACATCGTAGACAAATTCAATATTC
TGAACGTGCCGACCCTGGTATATCTGAAAGATGGCCGTGAGGTTGGACGCCAAAACCTGATTCGTTC
TAAAGAAGAGATTCTGAAAAAACTGAAAGAGCTGCAGGAGTAA

p25$_{2x}$ PfTrx HPV16 L2(20-38)$_3$

CGTGGTGATggcAAACTGATTCCGAATGCGAGTTTGATCGAGAATTGCACCAAAGCTGAACTTggtc
caggcccaggcATGATTATCGAGTATGACGGCGAAATCGACTTCACCAAAGGTCGTGTTGTACTGTG
GTTTAGCATTCCGGGATGCG**gtccgaaaacctgcaaacaagcaggtacttgtccgccggatatcatc**
ccgaaggtcgagggtggtggtccgaagacttgtaagcaggcgggcacctgtccaccagacattattc
caaaagtggaaggcggcggcccaaaaacatgtaaacaggccgggacttgccctcctgacatcatccc
taaagttgaaggggcgGTCCGTGTCGTCTGGTTGAACGCTTCATGACCGAACTGAGCGAGTATTTT
GAGGATATCCAAATTGTCCATATCAATGCCGGCAAATGGAAAAACATCGTAGACAAATTCAATATTC
TGAACGTGCCGACCCTGGTATATCTGAAAGATGGCCGTGAGGTTGGACGCCAAAACCTGATTCGTTC
TAAAGAAGAGATTCTGAAAAAACTGAAAGAGCTGCAGGAGgggccaggtccgggcAAACTGATCCCG
AATGCCTCTCTGATCGAAAACTGCACCAAAGCGGAATTGggtCGTGGTGATTAA

PADRE$_{2x}$ PfTrx HPV31 L2(20-38)$_3$

CGCGGCGATggcGCCAAATTCGTTGCGGCATGGACCCTGAAAGCGGCAgcaggccctggaccgggtA
TGATTATCGAGTATGACGGCGAAATCGACTTCACCAAAGGTCGTGTTGTACTGTGGTTTAGCATTCC
GGGATGCG**gtccg**gcaaacctgtaaggcagccgggacctgcccgagtgatgtcatcccgaaaattga
acatggtgggccacagacatgtaaagccgcgggcacttgtccttccgatgttatccctaagatcgag
catgggggccctcagacttgcaaagcggcaggtacttgcccatctgacgtgattccaaaaatcgagc
acggcgGTCCGTGTCGTCTGGTTGAACGCTTCATGACCGAACTGAGCGAGTATTTTGAGGATATCCA
AATTGTCCATATCAATGCCGGCAAATGGAAAAACATCGTAGACAAATTCAATATTCTGAACGTGCCG
ACCCTGGTATATCTGAAAGATGGCCGTGAGGTTGGACGCCAAAACCTGATTCGTTCTAAAGAAGAGA
TTCTGAAAAAACTGAAAGAGCTGCAGGAGggtccgggtccagggGCGAAGTTTGTGGCCGCTTGGAC
GTTAAAAGCCGCTGCGgggCGTGGGGACTAA

PADRE$_{2x}$ PfTrx HPV51 L2(20-38)$_3$

CGCGGCGATggcGCCAAATTCGTTGCGGCATGGACCCTGAAAGCGGCAgcaggccctggaccgggtA
TGATTATCGAGTATGACGGCGAAATCGACTTCACCAAAGGTCGTGTTGTACTGTGGTTTAGCATTCC
GGGATGCG**gtccg**gagtacctgcaaagcagcgggcacttgcccgcctgacgtcgttaataaagtcga
gggtgggggcccttctacttgtaaggcggccgggacttgtccaccggatgtggtcaacaaggtggag
ggcggtgggccgtccacatgcaaggccgcaggtacctgccctccagatgttgtgaataaagttgaag
gggcgGTCCGTGTCGTCTGGTTGAACGCTTCATGACCGAACTGAGCGAGTATTTTGAGGATATCCAA
ATTGTCCATATCAATGCCGGCAAATGGAAAAACATCGTAGACAAATTCAATATTCTGAACGTGCCGA
CCCTGGTATATCTGAAAGATGGCCGTGAGGTTGGACGCCAAAACCTGATTCGTTCTAAAGAAGAGAT
TCTGAAAAAACTGAAAGAGCTGCAGGAGggtccgggtccagggGCGAAGTTTGTGGCCGCTTGGACG
TTAAAAGCCGCTGCGgggCGTGGGGACTAA

PfTrx HPV16 L2(20-38)$_3$-IMX313T

ATTATCGAGTATGATGGCGAGATTGACTTCACCAAAGGTCGCGTCGTACTGTGGTTTAGCATTCCCG
GTTGCG**gtccg**aaaacgtgcaaacaggcagggacctgtccgccggatattatcccgaaggttgaagg
aggtggtccgaaaacgtgtaaacaagccggtacgtgccctccagacattattccgaaagtcgaagga
ggcgggcctaaaacctgtaaacaagctggcacttgtccgccagatatcatcccgaaagtggaaggcg
gcgGTCCGTGCCGTCTTGTGGAACGGTTTATGACCGAGTTATCCGAATACTTCGAGGACATTCAGAT
CGTGCACATTAATGCGGGCAAATGGAAGAACATCGTTGACAAATTCAACATCCTCAATGTCCCTACC
CTGGTTTACCTCAAAGATGGTCGCGAAGTTGGGCGCCAGAACTTGATTCGCAGCAAAGAAGAGATTC
TGAAGAAACTGAAAGAATTGCAAGAAGGCTCGAAGAAACAGGGCGATGCCGATGTATGCGGAGAAGT
GGCGTATATCCAGTCTGTGGTCAGTGATTGCCATGTGCCGACAGCGGAATTACGCACTCTTCTGGAA
ATTCGCAAACTGTTTCTGGAAATTCAGAAACTGAAGGTAGAGGGTCGTCGTCGTCGCCGTTCATAAT
AA

PfTrx HPV16 L2(20-38)$_1$-IMX313T

ATTATCGAATACGATGGCGAAATTGACTTTACCAAGGGGCGTGTTGTGCTTTGGTTCAGCATTCCGG
GTTGCG**gtccg**aaaacgtgcaaacaagctggcacatgtccaccggatatcattcccaaagttgaggg
aggcgGTCCGTGTCGCTTAGTCGAACGGTTTATGACCGAACTGTCAGAATACTTTGAGGACATTCAG
ATCGTGCACATTAATGCAGGCAAATGGAAGAACATCGTCGACAAATTCAACATCCTGAATGTGCCGA
CGTTAGTGTATCTGAAAGATGGTCGCGAAGTTGGACGCCAAAACCTGATTCGCTCCAAAGAAGAGAT
TCTCAAGAAACTGAAAGAGCTTCAGGAAGGCAGCAAGAAACAGGGTGATGCGGATGTATGCGGGGAA
GTAGCGTATATCCAGAGTGTCGTTTCGGATTGCCATGTGCCAACTGCCGAATTGCGTACCCTGTTGG

AGATTCGCAAACTCTTCCTGGAAATCCAGAAACTGAAAGTGGAAGGTCGTCGTCGTCGCCGCTCTTA
ATAA

HPV16 L2(20-38)$_3$-IMX313T

ggcG**gtccg**aaaacgtgcaagcaagcgggtacttgcccgccagacatcattccgaaagttgaaggtg
gagggccgaaaacgtgcaaacaggctgggacctgtcctccggatattatccccaaagtggaaggtgg
tggcccgaaaacctgtaaacaggcaggcacctgtccgccagacattatcccgaaagtggaaggaggc
gGTCCGGGTTCCAAGAAACAAGGCGATGCCGATGTATGTGGCGAAGTGGCGTATATCCAGAGCGTTG
TCTCGGATTGCCATGTACCAACAGCCGAATTGCGCACCTTACTGGAGATTCGGAAACTGTTTCTGGA
GATTCAGAAGCTCAAAGTCGAAGGCCGTCGTCGTCGCCGCAGTTAATAA

PfTrx 11mer

ATTATCGAGTATGACGGCGAAATCGACTTCACCAAAGGTCGTGTTGTACTGTGGTTTAGCATTCCGG
GATGCGgtccg**aagacgtgcaaacaagcgggcacctgtccgcccgatattatcccgaaagtcgaggg
tggtgggccgaaaacgtgcaaacagtctggaacatgcccgccggatgtggtgccgaaagtggaagga
ggaggtccgcaaacgtgcaaagcagcagggacctgtccgtcagatgtgattccgaagattgaacatg
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tggccctcgtacgtgcaaagctgcgggcacatgccctccggatgttattccgaaagtagaaggcggc
ggcccacgcacttgcaaacagagtggtacctgcccgccggacgtcgtggataaagttgaaggcggtg
gtcctcgcacgtgcaagcaaagcggcacatgcccacccgacgtaatcaataaggtcgaaggcggtgg
gccatcgacttgtaaggcggccgggacttgtccgccagatgtggttaacaaagtggaaggcggcgga
cctaaaacttgcaaactgagtggaacctgtccggaggatgtagtcaacaaaatcgaacagggcggcc
cgaaaacctgtaaacaagcaggcacctgtccatcggatgtgattaacaaagttgaaggtggtggtcc
gagcacgtgtaaagccgctggtacatgccctcccgatgttatccccaaagttaaaggcggcgGTCCG**
TGTCGTCTGGTTGAACGCTTCATGACCGAACTGAGCGAGTATTTTGAGGATATCCAAATTGTCCATA
TCAATGCCGGCAAATGGAAAAACATCGTAGACAAATTCAATATTCTGAACGTGCCGACCCTGGTATA
TCTGAAAGATGGCCGTGAGGTTGGACGCCAAAACCTGATTCGTTCTAAAGAAGAGATTCTGAAAAAA
CTGAAAGAGCTGCAGGAGTAA

PfTrx 8mer

ATTATCGAGTATGACGGCGAAATCGACTTCACCAAAGGTCGTGTTGTACTGTGGTTTAGCATTCCGG
GATGCGgtccg**aagacctgtaaacaggccgggacttgcccaccggatattattccaaaggtagaagg
tggtggacccaaaacgtgcaaacaaagcgggacgtgcccaccagacgtggtgcccaaagttgaaggc
ggcgggccgcaaacgtgtaaggccgctggtacgtgcccgagtgatgttattccgaaaattgaacatg
gtggtccacagacctgtaaagcgaccggcacatgcccgccggatgtgattcctaaagtggaaggtgg
aggccctcgtacatgcaaggcggctggtacatgcccgcctgatgtcatcccgaaagtcgaaggtggc
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gcccgtctacgtgcaaagcagcagggacctgtccgcctgatgtcgtaaacaaagtcgagggtggcgg
tcccaaaacctgtaaacaagcgggaacttgtccgtcagacgtcatcaacaaagtagaaggcggcgGT
CCG**TGTCGTCTGGTTGAACGCTTCATGACCGAACTGAGCGAGTATTTTGAGGATATCCAAATTGTCC
ATATCAATGCCGGCAAATGGAAAAACATCGTAGACAAATTCAATATTCTGAACGTGCCGACCCTGGT
ATATCTGAAAGATGGCCGTGAGGTTGGACGCCAAAACCTGATTCGTTCTAAAGAAGAGATTCTGAAA
AAACTGAAAGAGCTGCAGGAGTAA

PADRE$_{2x}$ PfTrx 11mer

CGCGGCGATggcGCCAAATTCGTTGCGGCATGGACCCTGAAAGCGGCAgcaggccctggaccgggtA
TGATTATCGAGTATGACGGCGAAATCGACTTCACCAAAGGTCGTGTTGTACTGTGGTTTAGCATTCC
GGGATGCG**gtccgaagacgtgcaaacaagcgggcacctgtccgcccgatattatcccgaaagtcgag**
ggtggtgggccgaaaacgtgcaaacagtctggaacatgcccgccggatgtggtgccgaaagtggaag
gaggaggtccgcaaacgtgcaaagcagcagggacctgtccgtcagatgtgattccgaagattgaaca
tggtgggccacagacctgtaaagccaccggcacgtgtccgccagacgtaatccctaaagtcgaaggt
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gcggcccacgcacttgcaaacagagtggtacctgcccgccggacgtcgtggataaagttgaaggcgg
tggtcctcgcacgtgcaagcaaagcggcacatgcccacccgacgtaatcaataaggtcgaaggcggt
gggccatcgacttgtaaggcggccgggacttgtccgccagatgtggttaacaaagtggaaggcggcg
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CGTGTCGTCTGGTTGAACGCTTCATGACCGAACTGAGCGAGTATTTTGAGGATATCCAAATTGTCCA
TATCAATGCCGGCAAATGGAAAAACATCGTAGACAAATTCAATATTCTGAACGTGCCGACCCTGGTA
TATCTGAAAGATGGCCGTGAGGTTGGACGCCAAAACCTGATTCGTTCTAAAGAAGAGATTCTGAAAA
AACTGAAAGAGCTGCAGGAGggtccgggtccagggGCGAAGTTTGTGGCCGCTTGGACGTTAAAAGC
CGCTGCGgggCGTGGGGACTAA

PADRE$_{2x}$ PfTrx 8mer

CGCGGCGATggcGCCAAATTCGTTGCGGCATGGACCCTGAAAGCGGCAgcaggccctggaccgggtA
TGATTATCGAGTATGACGGCGAAATCGACTTCACCAAAGGTCGTGTTGTACTGTGGTTTAGCATTCC
GGGATGCG**gtccgaagacctgtaaacaggccgggacttgcccaccggatattattccaaaggtagaa**
ggtggtggacccaaaacgtgcaaacaaagcgggacgtgcccaccagacgtggtgcccaaagttgaag
gcggcgggccgcaaacgtgtaaggccgctggtacgtgcccgagtgatgttattccgaaaattgaaca
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ggaggccctcgtacatgcaaggcggctggtacatgcccgcctgatgtcatcccgaaagtcgaaggtg
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cggcccgtctacgtgcaaagcagcagggacctgtccgcctgatgtcgtaaacaaagtcgagggtggc
ggtcccaaaacctgtaaacaagcgggaacttgtccgtcagacgtcatcaacaaagtagaaggcggcg
GTCCGTGTCGTCTGGTTGAACGCTTCATGACCGAACTGAGCGAGTATTTTGAGGATATCCAAATTGT
CCATATCAATGCCGGCAAATGGAAAAACATCGTAGACAAATTCAATATTCTGAACGTGCCGACCCTG
GTATATCTGAAAGATGGCCGTGAGGTTGGACGCCAAAACCTGATTCGTTCTAAAGAAGAGATTCTGA
AAAAACTGAAAGAGCTGCAGGAGggtccgggtccagggGCGAAGTTTGTGGCCGCTTGGACGTTAAA
AGCCGCTGCGgggCGTGGGGACTAA

PfTrx 11mer-IMX313T

ATTATCGAGTATGATGGCGAGATTGACTTCACCAAAGGTCGCGTCGTACTGTGGTTTAGCATTCCCG
GTTGCG**gtccg**aagacgtgcaaacaagcgggcacctgtccgcccgatattatcccgaaagtcgaggg
tggtgggccgaaaacgtgcaaacagtctggaacatgcccgccggatgtggtgccgaaagtggaagga
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gccatcgacttgtaaggcggccgggacttgtccgccagatgtggttaacaaagtggaaggcggcgga
cctaaaacttgcaaactgagtggaacctgtccggaggatgtagtcaacaaaatcgaacagggcggcc
cgaaaacctgtaaacaagcaggcacctgtccatcggatgtgattaacaaagttgaaggtggtggtcc
gagcacgtgtaaagccgctggtacatgccctcccgatgttatccccaaagttaaaggcggcgGTCCG
TGCCGTCTTGTGGAACGGTTTATGACCGAGTTATCCGAATACTTCGAGGACATTCAGATCGTGCACA
TTAATGCGGGCAAATGGAAGAACATCGTTGACAAATTCAACATCCTCAATGTCCCTACCCTGGTTTA
CCTCAAAGATGGTCGCGAAGTTGGGCGCCAGAACTTGATTCGCAGCAAAGAAGAGATTCTGAAGAAA
CTGAAAGAATTGCAAGAAGGCTCGAAGAAACAGGGCGATGCCGATGTATGCGGAGAAGTGGCGTATA
TCCAGTCTGTGGTCAGTGATTGCCATGTGCCGACAGCGGAATTACGCACTCTTCTGGAAATTCGCAA
ACTGTTTCTGGAAATTCAGAAACTGAAGGTAGAGGGTCGTCGTCGTCGCCGTTCATAATAA

PfTrx 8mer-IMX313T

ATTATCGAGTATGATGGCGAGATTGACTTCACCAAAGGTCGCGTCGTACTGTGGTTTAGCATTCCCG
GTTGCG**gtccg**aagacctgtaaacaggccgggacttgcccaccggatattattccaaaggtagaagg
tggtggacccaaaacgtgcaaacaaagcgggacgtgcccaccagacgtggtgcccaaagttgaaggc
ggcgggccgcaaacgtgtaaggccgctggtacgtgcccgagtgatgttattccgaaaattgaacatg
gtggtccacagacctgtaaagcgaccggcacatgcccgccggatgtgattcctaaagtggaaggtgg
aggccctcgtacatgcaaggcggctggtacatgcccgcctgatgtcatcccgaaagtcgaaggtggc
gggccgcagacgtgcaagttgaccggcacctgtccgccggatgttatcccgaaagttgagcatggcg
gcccgtctacgtgcaaagcagcagggacctgtccgcctgatgtcgtaaacaaagtcgagggtggcgg
tcccaaaacctgtaaacaagcgggaacttgtccgtcagacgtcatcaacaaagtagaaggcggcgGT
CCGTGCCGTCTTGTGGAACGGTTTATGACCGAGTTATCCGAATACTTCGAGGACATTCAGATCGTGC
ACATTAATGCGGGCAAATGGAAGAACATCGTTGACAAATTCAACATCCTCAATGTCCCTACCCTGGT
TTACCTCAAAGATGGTCGCGAAGTTGGGCGCCAGAACTTGATTCGCAGCAAAGAAGAGATTCTGAAG
AAACTGAAAGAATTGCAAGAAGGCTCGAAGAAACAGGGCGATGCCGATGTATGCGGAGAAGTGGCGT
ATATCCAGTCTGTGGTCAGTGATTGCCATGTGCCGACAGCGGAATTACGCACTCTTCTGGAAATTCG
CAAACTGTTTCTGGAAATTCAGAAACTGAAGGTAGAGGGTCGTCGTCGTCGCCGTTCATAATAA

Nucleotide sequences of the antigens used in this work. The epitopes (written in bold lowercase) was cloned into CpoI restriction site (grey) of Trx. Sequence codified for RGD (solid line), PADRE (double solid line), p2 (dotter line), p30 (bold solid line), p25 (dashed line), IMX313T (wavy line) are underlined.

Overlapping PfTrx HPV16 L2(20-38)$_3$-peptides

A1> MGSSHHHHHHSSGLIEKKIA

B1> HHSSGLIEKKIAKMEKASSV

C1> KKIAKMEKASSVFNVVNSHM

D1> ASSVFNVVNSHMIIEYDGEI

E1> NSHMIIEYDGEIDFTKGRVV

F1> DGEIDFTKGRVVLWFSIPGC

G1> GRVVLWFSIPGCGPKTCKQA

H1> IPGCGPKTCKQAGTCPPDII

A2> CKQAGTCPPDIIPKVEGGGP

B2> PDIIPKVEGGGPKTCKQAGT

C2> GGGPKTCKQAGTCPPDIIPK

D2> QAGTCPPDIIPKVEGGGPKT

E2> IIPKVEGGGPKTCKQAGTCP

F2> GPKTCKQAGTCPPDIIPKVE

G2> GTCPPDIIPKVEGGGPCRLV

H2> PKVEGGGPCRLVERFMTELS

A3> CRLVERFMTELSEYFEDIQI

B3> TELSEYFEDIQIVHINAGKW

C3> DIQIVHINAGKWKNIVDKFN

D3> AGKWKNIVDKFNILNVPTLV

E3> DKFNILNVPTLVYLKDGREV

F3> PTLVYLKDGREVGRQNLIRS

G3> GREVGRQNLIRSKEEILKKL

H3> LIRSKEEILKKLKELQEGPP

A4> LKKLKELQEGPPGRLEHHHH

B4> KLKELQEGPPGRLEHHHHHH

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Canali E, Bolchi A, Spagnoli G, Seitz H, Rubio I, Pertinhez T, Müller M, Ottonello S. **A high-performance thioredoxin-based scaffold for peptide immunogen construction: proof-of-concept testing with a human papillomavirus epitope.** *Sci Rep* 2014, **4**:4729.

Bolchi A, Canali E, Santoni A, Spagnoli G, Viarisio D, Accardi R, Tommasino M, Muller M, Ottonello S. **Thioredoxin-Displayed Multipeptide Immunogens**. *Methods Mol Biol* 2015, **1348**:137–151.

Spagnoli G, Bolchi A, Cavazzini D, Pouyanfard S, Müller M, Ottonello S. **High-yield secretory production of thioredoxin-displayed multipeptides in *Pichia pastoris*: application testing to a thermostable human papillomavirus L2 vaccine.**
Submitted