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Re-engineering a Nanoparticle Human Papillomavirus Prophylactic Vaccine Antigen Based on the Minor Capsid Protein L2

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List of abbreviations

Abbreviations	Full names
APCs	Antigen-presenting cells
Abs	Absorption
Alum	Aluminum hydroxide
APS	Ammonium persulfate
C4 bp	C4-binding protein
CMV	Cytomegalovirus
CVs	Column volumes
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CD4+	Cluster of differentiation 4
CD8+	Cluster of differentiation 8
CIP	Calf intestinal alkaline phosphatase
CIN	Cervical intraepithelial neoplasia
CIN1	Cervical intraepithelial neoplasia grade 1
CsCl	Cesium chloride
DMEM	Dulbecco modified Eagle's minimal essential medium
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide
DANN	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
ds	Double stranded
DCs	Dendritic cells
DMSO	Dimethyl sulfoxide
E. coli	*Escherichia coli*
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
FCS	Fetal calf serum
FcR	Fc Receptor
GFP	Green fluorescent protein
HLA	Human leukocyte antigen
HEK 293TT	Human embryonic kidney cells 293 with double T antigen
HSPG	Heparin sulfate proteoglycans
HPV	Human papillomavirus
HEPES	Hydroxyethyl-piperazineethane-sulfonic acid buffer
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IFA	Incomplete Freund's adjuvant

List of abbreviation

IL-2	Interleukin-2
IFN	Interferon
IgG	Immunoglobulin G
IC50	Inhibitory concentration 50
LCR	Long non-coding region
LB Luria	Luria-Bertani (lysogeny) broth
LSIL	Low grade squamous intraepithelial lesions
MPLA	Monophosphoryl lipid A
MHC	Major histocompatibility complex
MVA	Modified vaccinia Ankara
MEM	Minimum essential medium
MDSC	Myeloid-derived suppressor cells
NEAAS	Non-essential amino acids
NMSC	Non-melanoma skin cancer
NK	Natural killer cell
NBT	Nitro blue tetrazolium
OD	Optical density
ORF	Open reading frame
Ori	Origin of replication
Pf Trx	*Pyrococcus furiosus* Thioredoxin
PBNA	Pseudovirion-based neutralization assay
PBMCs	Peripheral blood mononuclear cells
PMA	Phorbol myristate acetate
PMSF	Phenylmethylsulfonyl fluoride
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PRK	Protein kinase R
PBS	Phosphate-buffered saline
RNA	Ribonucleic acid
RNase	Ribonuclease
SDS	Sodium dodecyl sulfate
TAE	Tris-acetic acid-EDTA buffer
TE	Tris-EDTA buffer
TEMED	N, N, N', N'-tetramethylethylenediamine
TGS	Tris -glycine-SDS buffer
Th	T helper lymphocyte
TNF-α	Tumor necrosis factor alpha
TLR	Toll-like receptor
Tris	Tris (hydroxymethyl) aminomethane

Pf	*Pyrococcus furiosus*
URR	Upstream regulatory region
VLPs	Virus-like particles

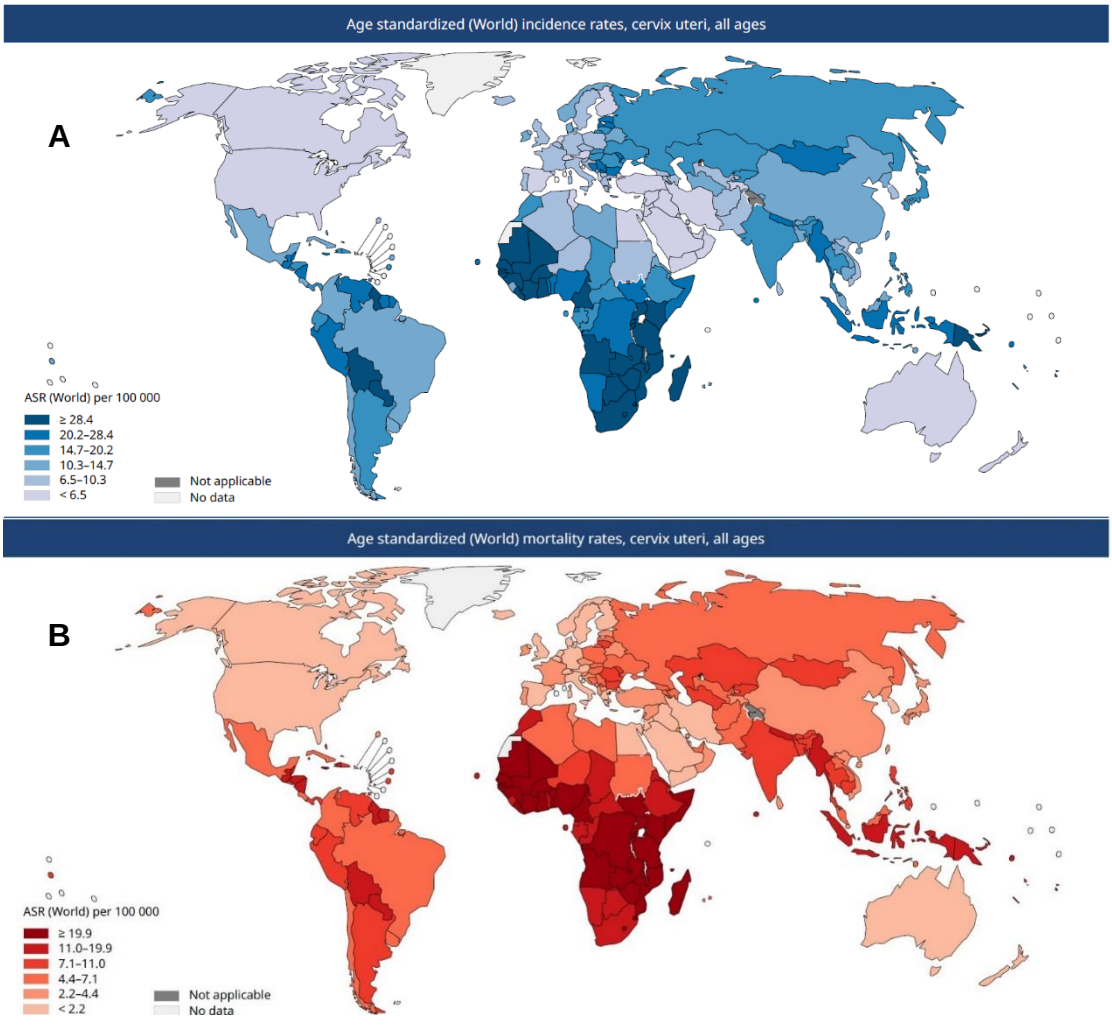
1 Introduction

1.1 Burden of cervical carcinoma and impact of Human Papillomavirus

Cervical cancer is the fourth most common cancer in women, after breast, colorectal, and lung cancer (World Cancer Research Fund American Institue for Cancer Research, 2018). There are over 270,000 mortalities due to cervical cancer, and over 500,000 women are diagnosed every year worldwide (Arbyn et al., 2011; Graham, 2017). Cytology has been widely employed for preventing cervical cancer (Papanicolaou test or Pap smear test) (Hillemanns et al., 2016). Even with a cervical cytology screening system, cervical cancer remains the epidemicity, though the prevalence and motility of cervical cancer are different geographically (figure 1.1) (World Health Organization, 2018). Due to sufficient screening systems and vaccination, cervical cancer lost its predominance in developed countries. However cervical cancer turns out to be the second most common cancer in women in developing countries (Harro et al., 2001; World Cancer Research Fund American Institue for Cancer Research, 2018), where almost 80% of mortality driven by cervical cancer occurs. In South Africa, the estimated age standardized incidence and mortality for cervical carcinoma is 43.1 per 100,000, and 20.0 per 100,000, respectively, in comparison to 6.0 and 1.7 per 100,000 in Australia and New Zealand (figure 1.1) (World Health Organization, 2018). The tremendous difference in cervical cancer incidence and death in different regions may result from the challenges to effectively implement the screening systems and vaccination programs in developing/low-income countries.

Papillomaviruses are ubiquitous and have been found in a wide-range of different species including humans. Over 200 different types of human

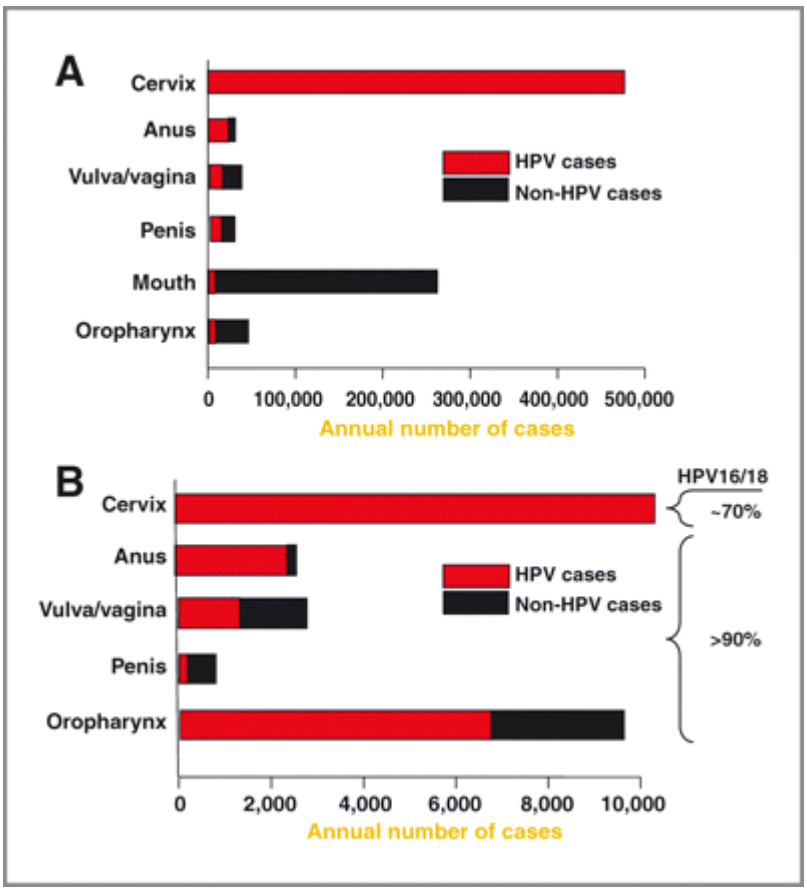
papillomavirus (HPV) have been identified based on their DNA sequences. Infection with HPVs usually causes benign epithelial lesions, such as skin warts, but certain types of HPVs can increase the chances to develop carcinoma, such as cervical cancer (Brescia et al., 1986). Harald zur Hausen firstly demonstrated the link between genital HPV infection and cervical cancer in the early 1980s. Since then it was demonstrated that HPV infection is also linked to anal, penile, vulvar and vaginal carcinoma. In the last decades, HPVs have been found in head and neck cancers as well (Panwar et al., 2014) (figure 1.2).



Update from GLOBOCAN 2018

Figure 1.1 Estimates of incidence and mortality of cervical cancer (World Health Organization, 2018). From world health organization's 2018 data, estimation of incidence A)

and mortality B), there is a significant difference of incidence and mortality of cervical cancer in different regions of the world (IARC Globocan Database, 2018).



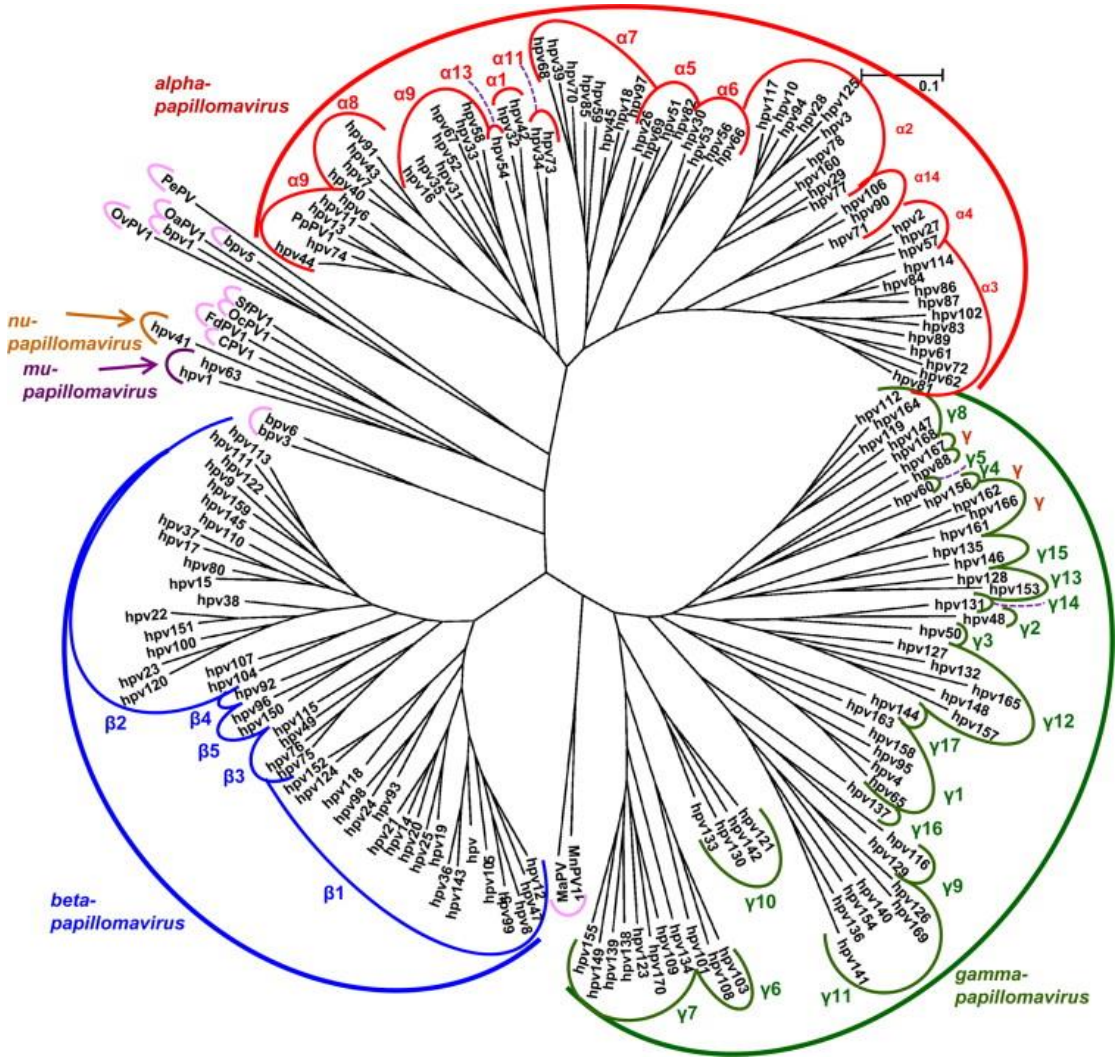
From Lowy, D.R. and J.T. Schiller, Cancer Prev Res (Phila), 2012.

Figure 1.2 Prevalence of HPV-associated cancers (Lowy and Schiller, 2012). A) the prevalence of HPV-associated cancer worldwide; B) the prevalence of HPV-associated cancer in U.S. A reduction in cervical cancer incidence was observed, and meanwhile, the incidences of oropharynx cancers increased.

1.2 HPV classification and HPV- related cancer

Among the currently identified papillomaviruses, 85 types have been characterized, while 120 isolates have not been fully investigated, and defined as potential new genotypes (Bharti et al., 2018). PV phylogenetic classification is based on the differences within the highly conserved L1 gene region between each genotype (a minimum of 10% nucleotide difference distinguishes two

genotypes (Kocjan et al., 2015; Van Doorslaer et al., 2017)). Up to date, five genera have been defined, which are α, β, γ, μ, and v (figure 1.3) (Anonymous, 2003; Doorbar et al., 2012; Graham, 2017; Van Doorslaer et al., 2017). Alternatively, HPVs are also classified according to tissue tropism into cutaneous or mucosal types.

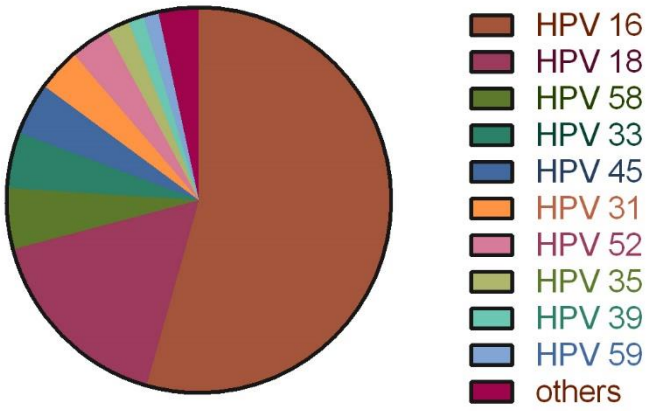


From de Villiers, E.M., Virology, 2013.

Figure 1.3 Phylogenetic tree of PVs based on the L1 sequences of around 150 types of papillomavirus types (de Villiers, 2013).

By analyzing the information of both molecular biology and epidemiology HPVs in the α genus mostly play an important role in genital cancer progression (de

Villiers, 2013) (figure 1.3). Cutaneous HPVs, including those which are related to non-melanoma skin cancer, mostly belong to the genera β and γ (figure 1.3) (Andersson et al., 2013; Fei and de Villiers, 2012; Mackintosh et al., 2009; Massimi et al., 2008). HPV infections do not always lead to apparent diseases, as the infection is transient and benign in most of the cases (Doorbar et al., 2012; Forslund, 2007; Massimi et al., 2008), however, certain genotypes might present increased ability to cause persistent and difficult to treat lesions. Of special concern are the mucosal HPVs of the α genus, which can be subdivided into high-risk and low-risk types according to the oncogenic potential (Bernard et al., 2010). 16 types are highly linked to cervical cancer, and other anogenital cancers or oropharyngeal cancer: these are HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82 (Munoz et al., 2003). Among these high-risk HPVs, different prevalence occur with regard to cervical cancer: 54.4% of cervical cancer cases are caused by HPV16, followed by HPV18, which is responsible for 16.5% of cervical cancers worldwide (figure 1.4) (Crow, 2012). The other types of HPVs responsible for genital warts are called low-risk types (e.g. HPV6, 11, 26, 53, 67 etc.) (Bernard et al., 2010; Hsueh, 2009).

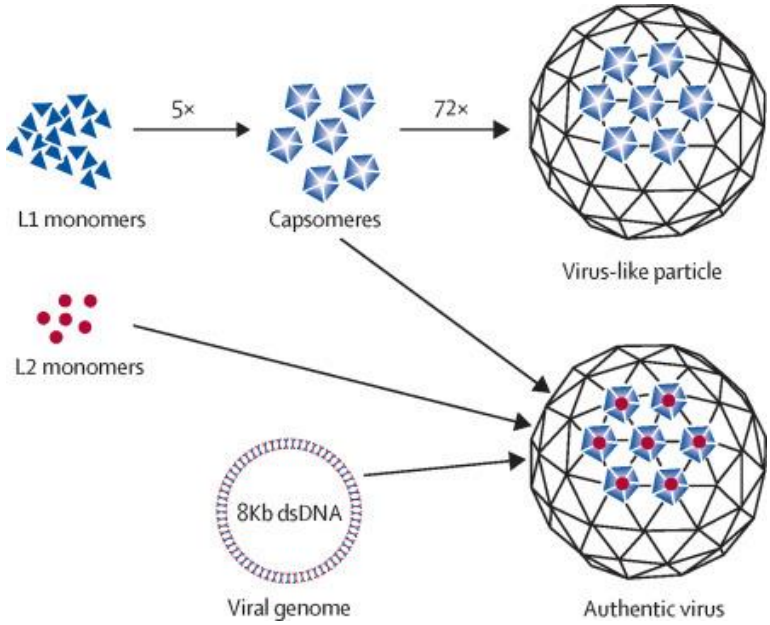


Updated from Crow, J.M., Nature, 2012.

Figure 1.4 Prevalence of HPV types in cervical cancer worldwide (Crow, 2012). The infection by HPV 16 is the most common cause (54.4%) of cervical cancer, followed by HPV18 (16.5%), both of them cause around 70% of cervical carcinoma worldwide. Including other oncogenic HPVs types, the prevalence reaches 98%.

1.3 The Biology of human papillomavirus

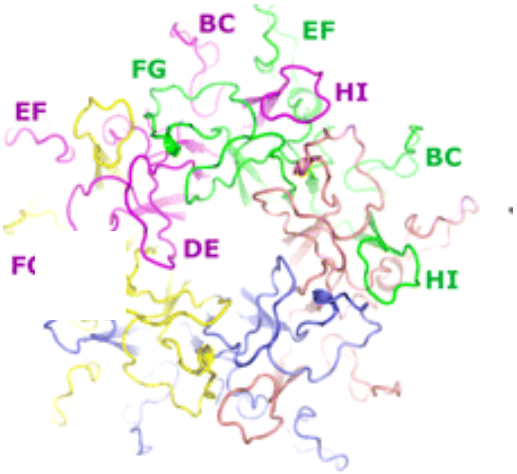
Papillomaviruses are non-enveloped icosahedral viruses with a double-stranded, circular genome which is around eight thousand base pairs in size (McMurray et al., 2001). The HPV genome encodes five early transcribed genes which are *E1, E2, E5, E6* and *E7* encoding non- structure proteins, three late genes, *E4, L1* and *L2* helping to release the virus and encoding the viral capsid proteins, and a long non-coding control region (LCR), also known as the upstream regulatory region (URR) (Doorbar, 2005; Doorbar, 2006). When the virus infects cells, all five early genes start to transcribe as all the early proteins are important for regulating the replication of virus genes and the process of pathogenesis (Gao and Smith, 2016; Tommasino, 2014). Then *E4, L1* and *L2* are expressed during the late phase of the viral life cycle. L1 is the major capsid protein, which is the main structural protein of HPVs. Five L1 monomers spontaneously assemble into a pentamer, which then further assemble into a capsid. During the assembly, the minor capsid protein L2 is positioned at the vertices of virus particle in the center of capsomeres (figure 1.5) (Modis et al., 2002; Schiller and Muller, 2015)



Updated from Schiller and Mueller, The Lancet Oncology, 2015

Figure 1.5 The scheme of HPV capsid protein L1 and L2 assembly (Schiller and Muller, 2015). Five times of L1 monomers format a capsomere, 72 capsomeres assemble on virus-like particle, and 72 capsomeres and L2 protein encapsulated viral genome format an authentic virus.

Eight β strands in the L1 protein form an antiparallel β-sandwich as the core of the capsomere and are labeled as B to I. These β-strands connect with loops BC, DE, FG and HI, which present the most of neutralizing epitopes (figure 1.6) (Bishop et al., 2007; Carter et al., 2003; Ludmerer et al., 1996; Roth et al., 2006; Sapp and Bienkowska-Haba, 2009). Because these loops are not well conserved among different HPVs, anti-L1 neutralizing antibodies are usually type-specific (Bishop et al., 2007). Most of the minor capsid protein L2 is hidden inside of the capsid, only residues from 60-120 at the N-terminus can reach to the surface of the capsid (Sapp and Bienkowska-Haba, 2009; Sapp et al., 1995).



Updated from B. Bishop, The Journal of Biological Chemistry, 2007

Figure 1.6 HPV 16 L1 pentamer crystal structure (Bishop et al., 2007). *Different colors represented separate monomer to indicate the surface loops intertwining.*

1.4 The life cycle of human papillomavirus

HPV infect the proliferating basal keratinocytes through a wound, the virus binds to the extracellular matrix (ECM) which is secreted by keratinocytes through some certain components, as well as heparin sulfate proteoglycans (HSPGs) which are the primary receptors of HPV (Giroglou et al., 2001). This interaction between HPV and their receptors induce conformational changes in both capsid proteins. For L1, the changes are not yet clear, but they seem to enhance the recognition of a neutralizing L1 epitope in the BC loop (Roth et al., 2006; Selinka et al., 2003). Moreover, they may also induce the decrease in affinity of the capsid to HSPG, thereby handing over the virus to the secondary receptor(s). On the other hand, the interaction between the HPV capsid and HSPG leads to another conformational change in the L2 protein whereby the N-terminus of L2 is exposed and consequently cleaved by furin protease. These processing steps seem to be important for the exposure of the L2 cross-neutralization epitopes (Richards et al., 2006; Roden et al., 2000).

The HPV genome is small and the viral DNA replicates independently of the cell cycle. Initially, the infected primitive cells go to transit amplifying proliferation, and the copy number of viral genome does not change. With very few viral genes being expressed, replication factor E1 and transcription factor E2 are the first detected, both in RNA and protein level (Thomas et al., 1999; Wang et al., 2009); E7 will activate the keratinocytes differentiation from phase G1 to S, which is important for later viral genome replication (Graham, 2017; Roman and Munger, 2013), and also plays a critical role in the viral genome replication phase in cells which transfer from mid to the upper layers (figure 1.7) (Graham, 2017).

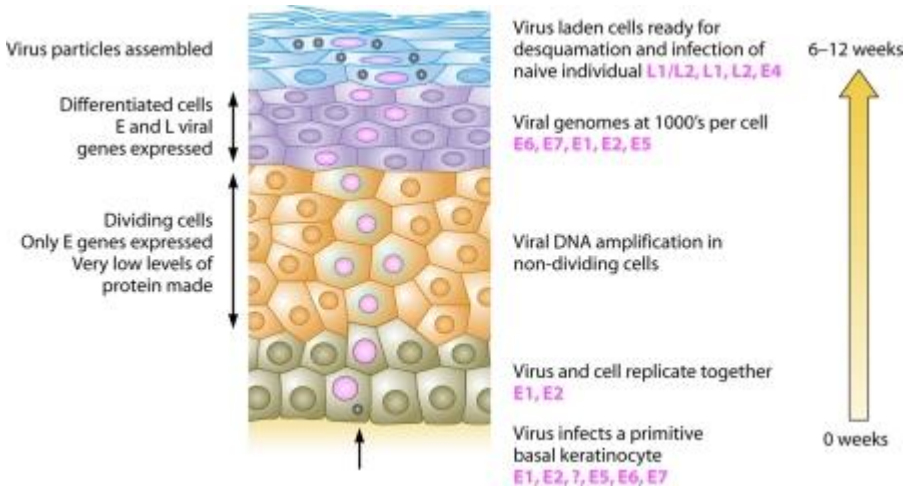
In the later phase of the viral life cycle, not only the expression of *E1* and *E2* increase, but also *E4* and *E5* (Davy et al., 2002; Graham, 2010). For the high-risk HPVs, E4 protein is the most important regulator, and has an greatly effect on differentiated keratinocytes, supporting amplification of the viral genome (Davy et al., 2005), but not for some low-risk HPVs, such as HPV11 (Fang et al., 2006). E5 controls the cell division pathways (Crusius et al., 1997) by stabilizing EGFR and stimulating MARK activity. In the later phase, the infected cells differentiate, exiting the cells in the stratum spinosum, leading to a boost in viral gene expression and DNA replication (figure 1.7) (Doorbar, 2007; Roberts et al., 2018; Stanley, 2012).

To complete the life cycle, the capsid proteins L1 and L2 are synthesized. *L2* expression precedes that of L1 (Becker et al., 2003). The major capsid protein L1 can self assembles into pentameric capsomeres which are transported to the nucleus to encapsulate the virus genome with the help of the minor capsid protein L2 (Nelson et al., 2002; Stauffer et al., 1998). The minor capsid protein L2 is required for the DNA packaging and virion assembly (Stauffer et al., 1998).

E2 also plays a role in the recruitment of the virus genome to the assembly sites during the virus assembling period (Day et al., 1998; Heino et al., 2000).

1.5 Persistence of Human Papillomavirus causes cervical cancer

HPV is the most commonly sexually transmitted infection. Both men and women can transmit HPV (Castellsague et al., 2003). Multiple sexual partners or a sexual partner who had multiple sexual partners, sexual activities at an early age, having children at an early age and having multiple children are risk factors for HPV infections. Moreover, condom use does not help with prevention of HPV infection since HPVs can be transmitted by contacting infected skin, like labial, scrotal and anal tissue (Roden et al., 1997).



From Stanley, M.A., Clin Microbiol Rev, 2012

Figure 1.7 HPV life cycle in the mucosal epithelium (Stanley, 2012). The early and late viral gene expression in the different epithelia layers. After infection, E1 and E2 are the earliest translated proteins, and regulate viral replication, followed by *E5, E6* and *E7* expression, mediating genome amplification. E4 is involved not only in the regulation viral replication but also in cytoskeletal morphogenesis. During the late phase, L1 and L2 proteins are expressed and new virus particles are assembled.

Mostly, HPV infections are transient, 90% of the infections are cleared spontaneously by the immune system (Chua and Hjerpe, 1996; Ostor, 1993; Stoppler et al., 1996).

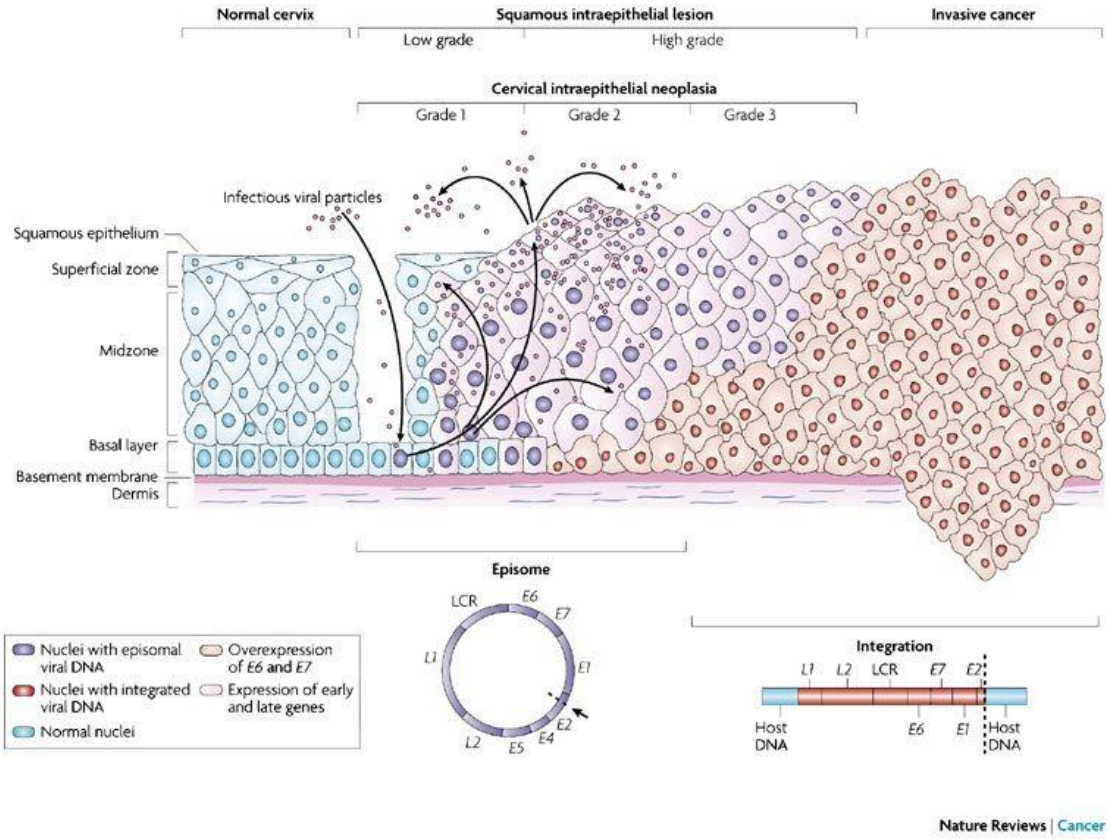
Due to the life cycle of HPV, there is a limited amount of HPV viral synthesis in undifferentiated cells, and a great level of expression of HPV in differentiated cells (Bodily and Laimins, 2011; Scott et al., 2001; zur Hausen, 1996). The infection process normally does not activate the inflammatory response, and suppresses both innate and adaptive immune responses (Bodily and Laimins, 2011). One way to suppress the innate immune response is to interfere with the interferon pathways (Bourgeade et al., 1980). HR HPV E6 and E7 proteins can both repress the transcription of interferon induced genes, such as the transcriptional activator STAT-1 (Bodily and Laimins, 2011; Chang and Laimins, 2000; Nees et al., 2001). Moreover, E6 can block the kinase Tyk-2 and protein kinase R (PKR) which can both act as activators of STAT-1 (Stanley, 2009); E6 also binds to IRF-3 and E7 binds to IRF-1. IRF-3 and IRF-1 are both regulators of interferon pathways (Bodily and Laimins, 2011; Stanley, 2009). HR E6 and E7 can also inhibit toll-like receptor nine (TLR 9), an important sensor of double strand DNA (Ghittoni et al., 2010).

Another way to escape from innate and adaptive immune response is to inhibit the cytokine production in keratinocytes, which limits immune cells infiltration of infected tissue. In HR HPV positive keratinocytes, the expression of IL-1, IL-6, TNF-α, and TGF-β (Alcocer-Gonzalez et al., 2006; Arany and Tyring, 1996) increased, as well as the anti-inflammatory IL-10's expression (Alcocer-Gonzalez et al., 2006; Fichorova and Anderson, 1999). The clearance of the infected cells is dependent on the cell-mediated immune responses, requiring the filtration of CD8+ and CD4+ cells (Stanley, 2008; Stanley, 2009). However, HR HPVs can interrupt the infiltration of Langerhans cells and dendritic cells

into the infected tissue due to the cytokine changes induced by the HR HPV infection (Hubert et al., 1999; Stanley, 2008; Stanley, 2009). Major histocompatibility complex I (MHC I) expression is also downregulated in the course of HR HPV infection, as well as natural killer cells activity. All of these events contribute to an HPV persistent infection (O'Brien and Saveria Campo, 2002; Stanley, 2009).

The persistence of HPV infections leads to larger damage to the tissue and growth of the HPV lesions, which require a lot of nutrients and oxygen. In order to grow, the most important factor is angiogenesis induction which is regulated by the HIF-1 transcription factor which can be activated in hypoxia (Bardos and Ashcroft, 2005; Toussaint-Smith et al., 2004). There is evidence indicating that HR and LR HPV E7 proteins induce the expression of HIF-1 and HIF-1α (Nakamura et al., 2009). E7 protein can bind to pRb which controls the expression of several S phase specific genes and the entry into S phase (Munger et al., 2001). HR E6 can bind to the E3 ubiquitin ligase leading to the degradation of p53 to block apoptosis, and thus increasing the cellular proliferation and maintenance of the viral genome (Howie et al., 2009).

Due to E6 and E7 expression, persistent infection with high-risk HPVs leads to pre-cancerous lesions and ultimately cervical cancer development. Over time, cellular genome changes with E6 and E7 amass lead to oncogenesis. The pre-cancerous lesions are known as low- or high- grade squamous intraepithelial lesions. Low-grade squamous intraepithelial lesions are also referred to as cervical intraepithelial neoplasia grade 1 (CIN1), and high-grade squamous intraepithelial lesions are CIN2 or 3 which are known as the highly proliferative phase (figure 1.8).



From Woodman, C.B., S.I. Collins, and L.S. Young. Nat Rev Cancer, 2007.

Figure 1.8 Persistent infection with HPVs lead to cervical carcinogenesis (Woodman et al., 2007). HPV accesses the basal cells through the microlesions in the cervical epithelium. After the infection, the early genes *E1, E2, E5, E6* and *E7* are expressed and the viral genome replicates. In the upper layers, HPVs genome is replicated further, *L1* and *L2*, late genes are expressed, and form into capsomeres and encapsulate the viral genome in the nucleus. Then mature viruses are released from the dead epithelial cells and initiate a new infection. CIN1 to 2 helps the viral genome replication and the progress to CIN3 or even invasive cancer.

1.6 Current commercial Human Papillomavirus prophylactic vaccines

Three HPV prophylactic vaccines have become available over the last decade, the bivalent (HPV16 and 18), quadrivalent (HPV6, 11, 16, 18) and nonavalent (HPV6, 11, 16, 18, 31, 33, 45, 52, 58) vaccines (table1). They are anticipated

to reduce the morbidity and mortality of cervical cancer (Luckett and Feldman, 2016). The bivalent HPV vaccine (Cervarix, GlaxoSmithKline, Rixensart) can preventcervical cancer caused by HPV16 and HPV18. Quadrivalent vaccine (Gardasil, Merck & Co, Inc.) which targets HPV6, 11, 16 and 18 was approved by the FDA.(Food and Drug Administration) in 2006 and can protect against cervical cancer caused by infection with HPV16 and HPV18, and genital warts caused by infection with HPV6 and HPV11. The nonavalent HPV vaccine (Gardasil 9, Merck & Co, Inc.) targets HPV6, 11, 16, 18, 31, 33, 45, 52, and 58 was proved effective in preventing high grade cervical dysplasia (Joura et al., 2015). All three vaccines show creditable efficacy in the prevention of HPV infections caused by the targeted HPV (Beachler et al., 2016; de Martel et al., 2017).

Table 1 Features of current commercial HPV L1 virus like particle vaccines

	2-valent HPV vaccine	**4-valent HPV vaccine**	**9-valent HPV vaccine**
Company	GlaxoSmithKline	Merck	Merck
Authorized year	2007	2006	2014
Brand Name	Cervarix	Gardasil, Silgard	Gardasil 9
L1 virus-like particle types	HPV16 (20 µg), 18 (20 µg)	HPV6 (20 µg), 11 (40 µg), 16 (40 µg), 18 (20 µg).	HPV6 (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)
Cross-protection	HPV31, 33, 45	HPV31	Unknown
Adjuvant	ASO4 (0.5 mg Aluminum hydroxide and 50 µg 3-O-desacyl-4”-monophosphoryl lipid A [MPL])	0.225 mg Aluminum hydroxyphosphate sulphate	0.5 mg Aluminum hydroxyphosphate sulphate

All three vaccines are based on virus-like particles (VLPs) of the major capsid protein L1. They show high immunogenicity, but are also highly type specific. These VLPs do not contain the viral genome, which makes them non-infectious (Harro et al., 2001), but they can mimic the native viral infection, present B cell epitopes and induce virus neutralizing antibody production. Generally, people should be vaccinated against HPV before engaging in sexual activities (World Health Organization. Electronic address, 2017). Additionally, in order to achieve sufficient neutralizing antibody production against the natural viral infection, the vaccines need to be administrated at least 3 times during a 6 months period which makes vaccine routine difficult to follow due to the age of the individuals

meant to receive the vaccine. Currently, many countries have already approved 2 doses for girls who are younger than 15 years old (Schiller and Muller, 2015). These decisions are made according to the non-inferiority antibodies (Schiller and Muller, 2015). Evidence showed that immunizations with 2 doses of bivalent vaccine induced similar antibody titers after 4 years as if the vaccine was administered 3 times to girls who were in 9-15 years old (Kreimer et al., 2011).

Antibodies are considered to target HPV during infection (Schiller and Lowy, 2012). There are two ways that the anti-HPV antibodies induced by immunizing with VLP HPV vaccines reach the mucosal epithelium at the HPV infection site. At first, the vaccines induce serum IgG (immunoglobulin G) exuding into the genital tract to provide protection against HPV infection. Secondly, serum IgG could transude into cervico-vaginal mucus by interacting with neonatal Fc receptor (Mestecky et al., 2010; Nardelli-Haefliger et al., 2003).

There are several factors that make VLP vaccines highly immunogenic. First of all, the size of VLPs is optimal for trafficking to the lymph nodes after injection, and also enables their recognition and presentation by DCs (dendritic cells) (Jennings and Bachmann, 2008). Secondly, VLPs can induce pro-inflammatory cytokines by interacting with human immunocytes, like monocytes, macrophages and myeloid DCs, resulting in the maturation of DCs in an acute phenotypic and functional manner in order to further promote adaptive immune responses (Lenz et al., 2005; Lenz et al., 2003; Schiller and Lowy, 2012). Thirdly, VLPs can induce MHC II restricted CD4+Th cells which further help the responses of B cells (Schiller and Lowy, 2012).

Despite the high immunogenicity and safety that the current commercial vaccines offer, the VLP vaccines also have limitations. The VLP vaccines are expensive to produce and transport, which makes them unaffordable for most

low income countries which have high incident rates of HPV-linked cancer. Moreover, HPV VLP vaccines are type-specific, with low capacity for cross-protection against other HPVs including some of high-risk HPVs. For examples, the protection induced by the bivalent VLP vaccine, which contains HPV16 and 18 VLPs, is very potent, but the neutralizing antibodies are limited in targeting the other 13 high-risk HPV types which are also responsible for up to 30% cervical cancer. However, there is some cross-protection against very closely related HPVs, like HPV31, HPV33, and HPV45 in some individuals. Another drawback of current VLP vaccines is the need for a continuous cold chain for transport and storage, which is unaffordable in most developing countries. To achieve a certain level of neutralizing antibody, the VLP vaccines require two to three intramuscular injections over six months, which requires patient adherence to the schedule, and also sterile conditions. All of these make the immunization with current VLP vaccines not practical in most developing countries. Finally, current VLP vaccines do not possess a therapeutic function because they do not lead to efficient T cell responses that would regress already established neoplasia.

1.7 The next generation of HPV vaccines

Due to the limitations of current VLP vaccines, efforts are being made to develop a second generation of HPV vaccines. There are certain requirements and expectations for second generation HPV vaccines. Primarily, the vaccine must evoke high immunogenicity and demonstrate a high safety profile, it must also provide long-term protection. Secondly, it should be much cheaper in both production and distribution. Thirdly, this vaccine should have a broader range of protection against high-risk HPV types which are not included in the current VLP vaccines. In addition, protection against low-risk mucosal HPVs and cutaneous HPVs would be beneficial. Last but not the least, it would be

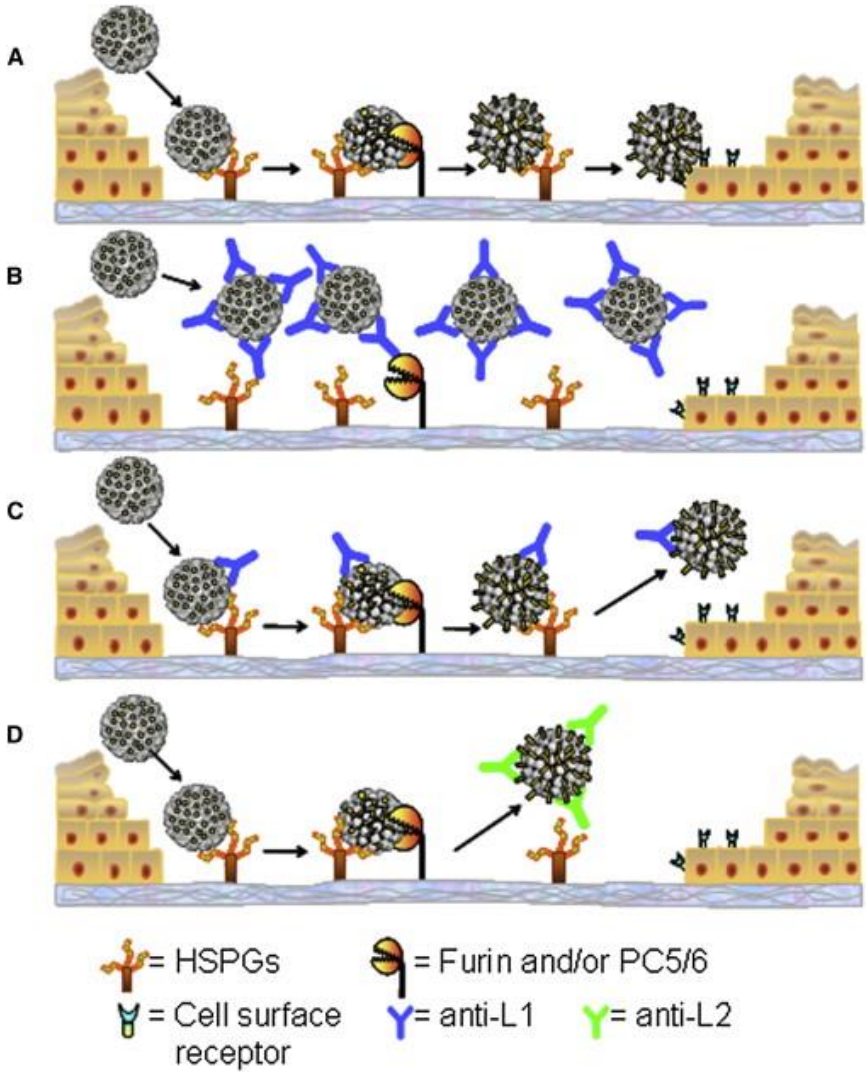
advantageous if the second generation HPV vaccines could also achieve some therapeutic function.

There are several attempts to use alternative production systems to generate L1 VLPs. Such as Xiamen Innovax Biotech which produces modified L1 based VLPs in *E. coli*, they already successfully completed a phase I clinical trial, a phase III trial is currently in preparation (Zhao et al., 2014). Despite this success, other companies employ yeast expression to manufacture VLPs (Bazan et al., 2009; Hanumantha Rao et al., 2011; Schiller and Muller, 2015). L1-recommbinant plants expression systems have also been reported with the aim to lower the cost of production. Unfortunately, the yield was low and the VLPs did not assemble properly (Giorgi et al., 2010). Another strategy is employing pentavalent L1 capsomers instead of L1 VLPs, which has the advantage of higher thermostability and low-cost because they can be easily produced in *E. coli.* However the capsomers induce lower neutralizing antibody titers and are less durable than L1 VLPs vaccines (Gersch et al., 2012; Schadlich et al., 2009; Thones et al., 2008). Another approach for L1-based vaccines involves viral or bacterial vectors. These methods may definitely lower the cost and perhaps the number of administrations compared to current commercial vaccines, but safety concerns come into play causing a lot of problems during the clinical trials. Giuseppina Cantarella et al. have combined the HPV16 L1 with the commercial Berna strain of the attenuated measles virus, resulting in high titres of HPV16 neutralizing antibodies, with no influence on measles antibody production (Cantarella et al., 2009). Immunization with this vaccine may, however, lead to conflict with the measles vaccination schedule, since infants usually receive the measles vaccines, but it may also lead to a combined vaccine targeting infants which would offer protection against both measles and HPVs, if the neutralizing antibodies can still provide protection when these infants enter adolescence. Other virus vectors used in academic laboratories are adeno-associated virus

(AAV) and human endogenous retrovirus as genetic vaccines (Lee et al., 2010; Nieto et al., 2012a). The biggest advantage of L1 recombinant AAV-5 or -9 is that it may change the administration method actually making the vaccine needle free (Nieto et al., 2012a).

To achieve broad cross-protection, L2, minor capsid proteins have been studied as another candidate for the second generation of a prophylactic HPV vaccine. L2 can induce cross-protection against different HPVs, but the neutralizing antibody titres are ten times lower than the antibody titres induced by the L1 VLPs (figure 1.9) (Day et al., 2010; Pastrana et al., 2005). One copy of the L2 protein is in each L1 capsomere with a maximum of 72 L2 molecules per capsid (Schiller and Muller, 2015). However, unfortunately, L2 proteins cannot induce neutralizing antibodies efficiently in the natural infections. Different from L1 VLPs, the neutralizing antibodies induced by L2 can recognize linear conserved peptide epitopes, which provide broader cross-protection against homologous and heterologous HPVs (Pastrana et al., 2005; Roden et al., 2000). The ability to induce broad cross-protection with L2 would make the coverage of a range of high-risk and even low-risk mucosal HPVs by a monovalent vaccine possible, maybe also for cutaneous HPVs (Schellenbacher et al., 2013). There is proof that sera transferred from mice immunized with L2 could provide cross-protection against HPVs *in vivo* despite the antibody titres being much lower than VLPs (Day et al., 2012). The reason behind this might be the highly conserved N-terminal region (amino acid 17-36) of HPV L2 (figure 1.10).(Wang and Roden, 2013). RG-1, a monoclonal antibody which binds specially to the 17-36 epitope, can prevent HPV 16 infection *in vivo* and sera of mice that had been immunized with RG-1 can also neutralize HPV4, 6, 16, 18, 31, 45, 52, 58, BPV1 and HPV11 (Gambhira et al., 2007; Wang and Roden, 2013). Moreover, L2 neutralizing epitopes have been inserted into VLPs in order to increase the range of protection, but further evidence needs to be provided to prove if L2

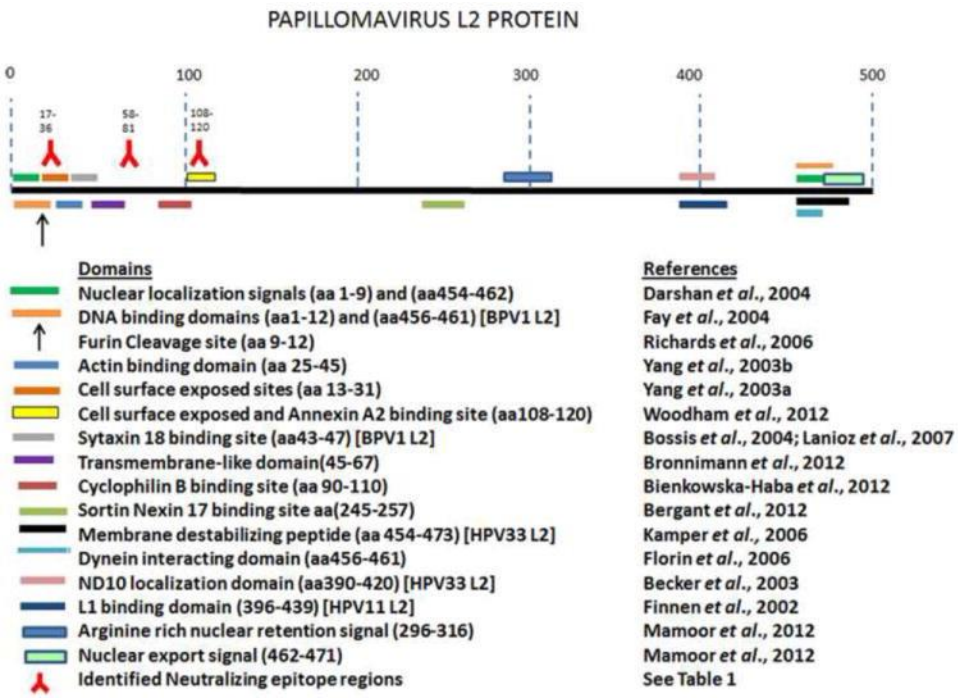
insertion does not interfere with the display of L1 epitopes (Schellenbacher et al., 2013).



From Day, P.M., et al., Cell Host Microbe, 2010.

Figure 1.9 The early stage of HPV infection, binding to HSPGs, and the scheme of how vaccine-induced antibodies interfere with infection (Day et al., 2010). A) HPVs at first reach the basement membrane via microlesions. After HSPGs-HPVs binding, L1 undergoes conformational changes, exposing furin cleavage site at the N-terminus of L2. HPVs are released from HSPGs and move to bind to the cellular entry receptor. B) the first step of infection, binding to HSPG can be blocked by anti-L1 antibodies. C) if the antibody concentration is low, then the HPVs can still bind to HSPGs and be cleaved by furin, but do not

interact further with the cellular entry receptors. D) in the presence of L2-antibodies, binding to cellular entry receptor can also be prevented.



From Wang, J.W. and R.B. Roden, Virology, 2013.

Figure 1.10 Representation of HPV16 L2 neutralization epitopes and protein interaction domains (Wang and Roden, 2013).

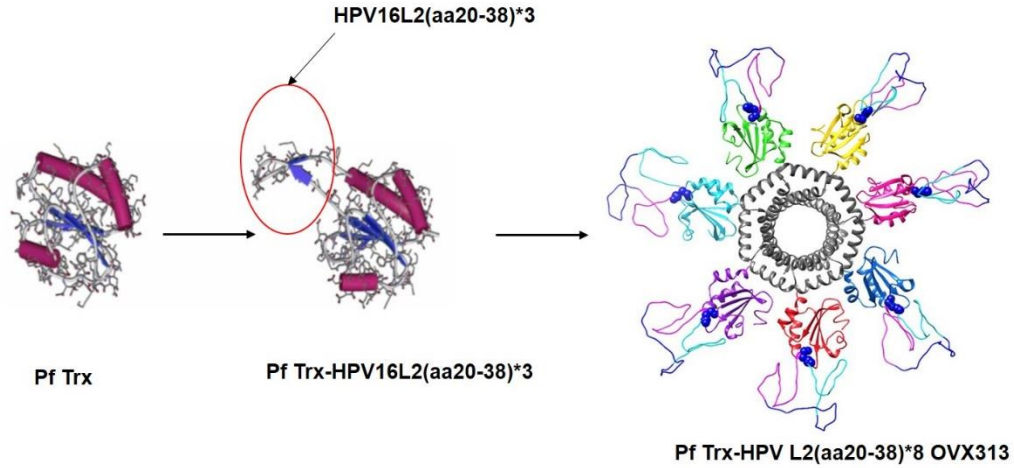
The efficient induction of neutralizing antibodies is still a challenge for prophylactic HPV vaccines based on minor capsid protein L2. Chimeric VLP with L2 epitopes inserted into the surface DE loop of VLPs, with Alum-MPLA as adjuvant were used to immunize mice and rabbits. Cross-neutralizing antibodies were induced against many more HPVs than with the commercial ones, even against some cutaneous HPVs. This was verified by *in vitro* pseudovirus based neutralizing assay and in *in vivo* challenge animal models, and the antibodies could be detected even one year after immunization. Moreover, these chimeric VLPs could also induce cytotoxic T cell response (Schellenbacher et al., 2009). With bacteriophages as a platform vaccine production would be much cheaper (Tumban et al., 2012). To this end AAV

capsid protein was used as a platform to display HPV 16 L2 aa 17-36. The VLPs were found to provide cross-protect against other HPV when tested *in vivo* and *in vitro*, when mice and rabbits were immunized with the help of adjuvant Montanide (Nieto et al., 2012b). However, Montanide is not an approved prophylactic vaccine adjuvant. It has been proven that the AAVLPs vaccines could induce cross-neutralizing antibodies and the antibodies lasted at least 3 months. Surprisingly, the vaccine could also provide long term (6-12 months) protection against HPV16, 31, 39, 45, 58, and 59 on skin challenge rabbit models (Jagu et al., 2015). The HPV16L2-MS2 VLPs, a bacteriophage displaying HPV 16 L2 epitopes, could induce production of L2 driven neutralizing antibodies that lasted three months after immunization. Moreover, this antigen was quite thermal stable, and production was cost efficient (Saboo et al., 2016).

Epitope multiplicity can increase antigen immunogenicity (Puffer et al., 2007; Rubio et al., 2009). Another way to increase immunogenicity of HPV L2 peptides and broaden their cross-protection even more was the creation of a concatenated multitope L2 fusion protein which included 8 L2 epitopes (aa 11-88) from different mucosal HPVs. This antigen was evaluated. The concatenated L2 protein could induce high neutralizing antibody titers against HPV16, 18, 45 and 58, and these antibodies were stable for at least 3 months (Jagu et al., 2009).

In our group, we employed different strategies to not only increase the low immunogenicity of L2 peptides, but to also improve the ability of cross-protection. At first, the L2 epitopes (aa20-38) from HPV16, 31, and 51 were inserted into the active loop of *Pyrococcus furiosus* thioredoxin (Pf Trx), a small, non-toxic and soluble protein. This trivalent protein vaccine was evaluated in mice using Alum-MPLA as adjuvant. The neutralizing antibodies induced by the

trivalent protein could neutralize 12 HPVs *in vitro*, and provided protection against 5 HPVs *in vivo* (Seitz et al., 2015). In order to cover all the high-risk mucosal HPVs, some of the low-risk mucosal HPVs, and even some cutaneous HPVs, a multimerization strategy was further employed to develop antigens based on Pf Trx-L2. Eight L2 epitopes from different HPVs (HPV 16, 18, 31, 33, 35, 6, 51, and 59) were inserted into Pf Trx, and antibodies induced by the polytope antigen could neutralize all 15 high-risk and 2 low-risk mucosal HPVs, but the titers were not as high as those in commercial vaccines. Furthermore, OVX313 was employed, a self-assembling polypeptide providing a secondary platform to display L2 multitopes (figure 1.11).



From Spagnoli, G., et al., Sci Rep, 2017.

Figure 1.11 Development of L2-based antigens (Spagnoli et al., 2017). The predicted structures of Pf Trx, and Pf Trx-HPV16 L2 (aa20-38)*3, and Pf Trx L2 OVX313 fusion protein. On the left panel is the native Pf Trx with the active site; the middle panel is the Pf Trx fused with three copies of HPV16 L2 (aa20-38) epitopes at the active site; the right panel is further fused the OVX313 with Pf Trx assembling into a heptamer and displaying the L2 epitopes.

OVX313 is driven by human C4bp protein, which is a plasma glycoprotein complex acting as an major complement inhibitor and produced in the liver (Hofmeyer et al., 2013). Like Pf Trx, OVX313 also has extraordinary thermodynamic stability and can be produced in bacteria in large scale and

purified by simple thermal treatment. The native C4bp has 4 different isoforms, but mainly contains α chains and β chain formed into a characteristic spider- or octopus- like structures (Dahlback et al., 1983). OVX313 is a positively charged variant developed by OSIVAX, where seven α- chains link together at the C terminus, self-assembling into a heptametrical format, and stabilized by intermolecular disulfide bonds (Hofmeyer et al., 2013). It has been used as a platform for mono or poly peptide epitopes to increase immunogenicity since there is no cross-reactivity with the human C4bp counterpart (Li et al., 2016; Ogun et al., 2008; Spencer et al., 2012), for vaccines for both humans and animals. Pf Trx L2s were fused at the N-terminus of OVX313, and mice and guinea pigs immunized with or without adjuvant and the result was quite exciting. The construct could induce high titers of neutralizing antibody against all 15 high-risk mucosal HPVs, 2 low-risk mucosal HPVs (HPV6 and HPV11, causing 90% of genital warts), and some cutaneous HPVs. Currently, a phase I clinical trial is in preparation (Pouyanfard et al., 2018; Spagnoli et al., 2017).

1.8 Nanoparticles

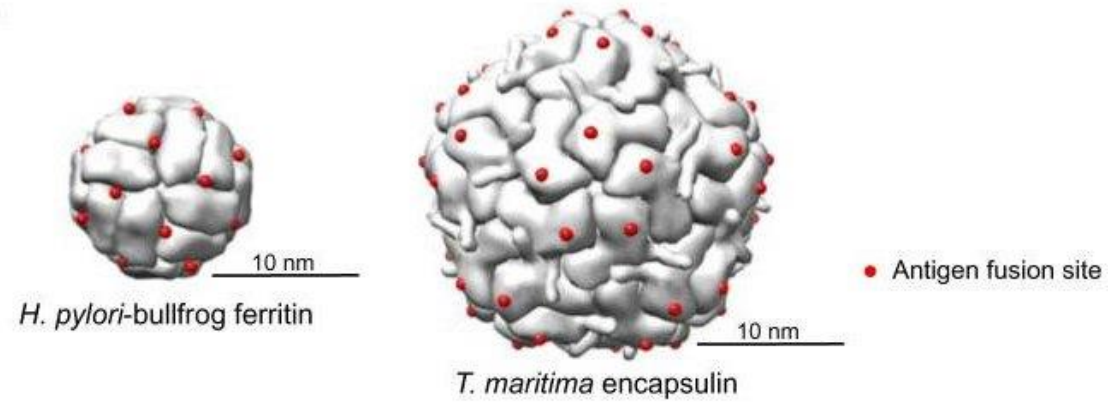
There are a lot of important pathogens for which vaccines are currently not available, such as human immunodeficiency virus, etc. Some vaccines could not elicit a sufficient and durable immune response against the natural pathogens. Therefore, researchers keep searching for a way to boost protection against pathogens induced by vaccines. In the development of vaccines technologies, several aspects need to be considered. Safety is the first concern, followed by avoiding poor immunogenicity, requiring co-formulation with adjuvants and requiring a booster injection for the purpose of maintaining the immunity (Karch and Burkhard, 2016). To promote a sufficient immune response, it is critical that the antigens presented to the immune systems are in the right quality and conformation (Copland et al., 2003; Schijns, 2000).

Nanotechnology has been widely applied in biomedicine in the past decades. A nanoparticle is an organized structure on the scale of 1-1,000 nm, such as gold, polystyrene or VLPs and is used to greatly enhance the immune response against different kinds of pathogens. One type of nanoparticles are polypeptides assemblies which can present several copies of subunits or epitopes of antigens in a well-designed array with organized orientations (Lopez-Sagaseta et al., 2016). By doing so, the nanoparticle antigens can mimic the interaction with immune cells not only due to the similarity of repetitive geometry, but also the size and shape of the natural pathogen (Lopez-Sagaseta et al., 2016). In order to induce an efficient immune response by vaccines, B and T cells need to be stimulated and activated. On the other hand, induction of antibodies secreted by plasma cells relies on the effective cross link between the B cell receptors and how well the epitopes are being displayed by the pathogens for recognition. Therefore, the high immunogenicity, the physicochemical properties, stability and cheap large scale production by *E.coli* or eukaryotic systems put nanoparticle vaccines at the frontline of vaccine development research (Lopez-Sagaseta et al., 2016).

The ferritin family of proteins are detoxification proteins which are involved in iron storage (Yang et al., 2017; Zhao, 2010), may be applied in nanotechnology. Twenty-four subunits self-assemble into a nanoparticle with inner and outer diameters of 8 nm and 12 nm, respectively. The ferritin cage has a high thermal and chemical stability, and it is able to reconstitute through manipulated assembly and dis-assembly (Dominguez-Vera and Colacio, 2003). Drugs, nucleic acids and other materials can be chemically or genetically attached to the modifiable site on the external or internal surface of ferritin (figure 1.12) (de Turris et al., 2017) . All evidence suggests that ferritin could be manipulated easily as an antigen carrier. Moreover, the ferritin family of proteins are relatively easy to produce and isolate, especially *Pyrococcus furiosus* ferritin

which is notably thermally stable (Jacobs et al., 2010). All of these capabilities make ferritin a popular subject to study and use in nanotechnology. Some examples include, the use of ferritin as a bio mineralization scaffold (www.biaqua.com), where *P.furiosus* ferritin is used to prevent water pollution and biofouling (Jacobs et al., 2010), and as Nano devices (Yamashita et al., 2010), medical imaging agents (Jin et al., 2014; Vande Velde et al., 2011), a drug delivery system for cancer treatment (Zhen et al., 2013), and as nanoparticle vaccines (Kanekiyo et al., 2013).

Encapsulins are also a shell-like protein, 60 copies of the monomer assemble into a cage with 24 nm diameter (figure 1.12), or 180 copies of the subunit assemble into a shell cage approximately 80 nm across (McHugh et al., 2014; Sutter et al., 2008). Encapsulins are widespread in bacteria and archaea, but the whole function of encapsulins is not yet clear. However, there is evidence that encapsulins are involved in protecting cells from proteolysis or other challenges by increasing metabolic efficiency (Diekmann and Pereira-Leal, 2013), or like ferritin, are involved in iron storage (Zeth et al., 2016) and in the trafficking and packaging of enzymes (Cannon et al., 2001; McHugh et al., 2014).



Updated from Kanekiyo, M, et al., Cell, 2015

Figure 1.12 Nanoparticle ferritin and encapsulin with antigen fusion site (Kanekiyo et al., 2015). Ferritin with antigen fusion sites (red dot) assembled into 10nm nanoparticles (left), and encapsulin assembled into 24nm nanoparticles. The red dots indicated the engineered insertion sites.

1.9 Adjuvant's role in vaccination

Adjuvants are employed in vaccinations, in order to improve these vaccines (Coffman et al., 2010). Adjuvants have been widely employed in different scenarios, like immunocompromised patients, elderly people, and infants. They can improve the efficiency of vaccines in different ways, such as by reducing the number of administrations (lower vaccination frequency that achieves the same effect), reducing the dosage, improving the immunogenicity and may help stabilize the vaccine formulation (Petrovsky and Aguilar, 2004; Schijns and Lavelle, 2011).

Adjuvants have been classified into different categories based on their immune response interactions. The first generation of adjuvants, as antigen delivery system, increased the antigen uptake at the injection site (Brito and O'Hagan, 2014). These are Aluminum, liposomes, emulsions, and microparticles. The second category of adjuvants are combined adjuvants. They consist of the immune potentiators combined with the first category adjuvants. For example, AS04 which contains aluminum hydroxide as antigen delivery system plus monophosphoryl lipid A (MPL), a TLR4 agonist as an immune potentiator.

Several factors need to be considered in selecting adjuvants. The adjuvant should be safe, compatible with the antigen, well tolerated, easily formulated with the antigens, simple to manufacture and economically feasible, etc. (Brito et al., 2013). Due to differences between vaccines, the choice establishment of the correct adjuvant is challenging.

For a long time, aluminum salts (also called Alum) were the only licensed adjuvants for humans. Alum was used as an antigen delivery system and has interacts in two ways with antigens. First, by electrostatic interactions, which depend on the isoelectric point of the antigen and the type of aluminum salt (al-Shakhshir et al., 1994; Seeber et al., 1991). Second, ligand exchange is another way to enhance antigen immunogenicity, which happens when phosphate groups on the antigen exchange with hydroxide groups on the alum. Another theory implies that alum can induce a humoral immune response by a Th-2 biased response. An important issue for Alum is that it cannot induce cellular immune responses, which are needed against intracellular pathogens, and other pathogens which require a strong cellular response.

In order to overcome the limitations of Alum, it was combined with monophosphoryl lipid A (MPLA), an immune-stimulatory molecule. Studies suggested that alum could enhance MPLA delivery at the injection site and increase the duration of cytokines (Marrack et al., 2009). MPLA is a modified lipopolysaccharide (LPS) and is a much less toxic than the nature LPS and can activate the TLR4 receptors (Casella and Mitchell, 2008). By combining MPLA with alum, vaccines can induce both Th1 and Th2 responses (Garcon et al., 2007). Currently, human papillomavirus vaccine (Cervarix) and hepatitis B (Fendrix) vaccine employ AS04 (Alum hydroxide and MPLA) as adjuvant(Garcon et al., 2007).

Another type of adjuvants used to boost the immune response against vaccines are emulsions. Water-in-oil emulsions are the most common. The earliest emulsion adjuvant was mineral oil-based water-in-oil emulsion called Freund’s adjuvant (Aucouturier et al., 2001; Berg, 2015). The adjuvant was never used for human vaccinations due to the safety concerns (Aucouturier et al., 2001). Right now, there are several commercial vaccines that employ emulsion based

adjuvants. MF59, is a squalene oil-in-water emulsions, and has been used for influenza vaccines (O'Hagan et al., 2013; O'Hagan et al., 2011). The mechanism of MF59 has been studied extensively. The updated theory is that MF59 creates an immune competent environment at the site of injection and leads to the influx of antigen presenting cells and other immune cells. In the meantime, MF59 have been found to also induce the increased production of cytokines and chemokines which can further lead to the recruitment of immune cells to the site of injection. By doing so, the migration of immune cells such as APCs is upregulated as well as the uptake of the antigens (O'Hagan et al., 2012; Seubert et al., 2008).

AddaVax, another emulsion which is similar to MF59, is a squalene-based oil-in-water nano emulsion. In Europe, it has been used in influenza vaccines (Calabro et al., 2013; Drulak et al., 2000). It acts in a similar way to MF59 by inducing both cellular Th1 and humoral Th2 immune responses.

Currently, there are several other kinds of adjuvants employed in vaccinations to improve the immunogenicity of some antigens. Since there is a trend to use recombinant proteins, which normally have low immunogenicity, greater use of adjuvants is inevitable. On the other hand, adjuvants may lead to an extra effort to produce the vaccine, since they may complicate the formulation strategies and increase the cost of the vaccine.

1.10 Objective and aims of PhD project

An ideal vaccine should meet the following criteria: A) it must be safe, elicit immune responses up to a desired level; B) it should be easily prepared, commercially affordable, and C) the vaccine must be chemically and physically stable during production, transport and administration.

The main objective of my thesis is the development of new HPV vaccines with broad antigenicity, high immunogenicity, cheap to produce and no need to cool.

Broad specificity may be achieved by using the conserved region of the epitope of L2 capsid protein (aa20-38) as antigen, which is less specific than the L1-based VLP vaccines.

High immunogenicity shall be achieved by inserting L2 epitopes into nanoparticles formed with *Pyrococcus furiosus* thioredoxin (Pf.Trx). These nanoparticle vaccines can be easily produced and safe to be used since it could not induce auto-immuno responses.

The choice of the adjuvant is critical for the stability and antigenicity of vaccines. Different adjuvants shall therefore be tested. The specific aims include:

a) Designing, producing and characterizing nanoparticle L2 antigens.
b) Selecting the suitable adjuvant for nanoparticle L2 antigens.
c) Understanding the role of Pf thioredoxin on the nanoparticle L2 antigens
d) Selecting the best antigens which can induce high titers of neutralizing antibodies against a large panel of HPVs including high-risk, low-risk mucosal HPVs.
e) Verifying the influence of pre-existing antibodies induced by the immunization with nanoparticle vaccines against the nanoparticle scaffold on induction of anti-L2 antibodies.
f) Checking the cross-reactivity of anti-Pf ferritin antibodies with human ferritin.
g) Determining the effective dose of nanoparticle antigens by understanding the tendency of antibody production.
h) Proving that the antibodies produced by nanoparticle L2 antigens can provide protection in an *in vivo* mouse model challenged with HPV.

2 Materials

2.1 Biological materials**Laboratory animals**

BALB/c mice	6-8 weeks old or 16 weeks old, female, from Charles River, Sulzfeld.
Guinea pig	350- to 400-g female animals, Hartley (Crl:HA),from Charles River, Sulzfeld.

2.1.2 Cell lines

HEK 293TT	Human embryo kidney (HEK) cells containing two gene copies of SV40 large T antigen
HeLaT K4	HeLa cells (human cervical cancer cell line; HPV18 positive) stably transfected with linearized expression plasmid containing SV40 large T antigen under control of the CMV promoter
MCF-10A	Human epithelial breast cancer cell line
CHOΔfurin	Chinese hamster ovary cell line producing secreted furin (gift from David Fitzgerald, NCI Bethesda)
pgsa-745	Chinese hamster ovary cell line deficient for heparan sulfate proteoglycans, cannot produce glycosaminoglycans due to a non-functional xylosyltransferase
Sf9	Clonal isolated from *Spodoptera frugiperda* sf21 cells
TN-High-Five	Ovrian cells of the cabbage looper Trichoplusia ni

2.1.3 Bacterial strains

Strain	**Genotype**	**Company**

E.coli MxDH10α	genotype: F- mcrA Δ(mrr-hsdRMS-mcrBC) ψ80lacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ- rpsL nupG tonA	Invitrogen
E.coli Rosetta™	genotype: F^- ompT hsdSB (rB^-mB^-) gal dcm pRARE (Cam^R) (Merck4Biosciences)	Invitrogen
E.coli SoloPack Gold Competent Cells	genotype: Tet^r Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44thi-1 recA1 gyrA96 relA1 lac Hte [F´ proAB lacIqZΔM15 Tn10 (Tet^R) Amy Cam^R]	Agilent Technologies
DH10 MultiBac^{cre} Electrocompeten *E.coli*	*E.coli* strain harboring DH10MultiBac bacmid	Invitrogen
E.coli MegaX DH10	This strain provides the option of blue/white screening on plates containing either X-Gal or Bluo-Gal. F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZ ΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ - rpsL nupG tonA	Invitrogen
E.coli XL-Blue supercompetant cells	The XL1-Blue strain allows blue-white color screening.recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F´ proAB lacIqZΔM15 Tn10 (Tet^r)]. (Genes listed signify mutant alleles)	Agilent Technologies

2.1.4 Pseudovirions

Designation	Description	Source
HPV16 Gaussia	Co-transfection #988 + #1998	M. Müller

HPV18 Gaussia	Co-transfection #1165 + #1166 + #1998	J. Schiller
HPV31 Gaussia	Co-transfection #1698 + #1998	T. Kanda
HPV33 Gaussia	Co-transfection #2686 + #1998	T. Kanda
HPV35 Gaussia	Co-transfection #2713 + #1998	T. Kanda
HPV39 Gaussia	Co-transfection #2712 + #1998	S. Beddows
HPV45 Gaussia	Co-transfection #2271 + #2273 + #1998	J. Schiller
HPV51 Gaussia	Co-transfection #2466 + #2481 + #1998	H. Seitz / M. Müller
HPV52 Gaussia	Co-transfection #1691 + #1690 + #1998	J. Schiller
HPV56 Gaussia	Co-transfection #3123 + #1998	M. Müller
HPV58 Gaussia	Co-transfection #1694 + #1998	T. Kanda
HPV59 Gaussia	Co-transfection #2714 + #1998	S. Beddows
HPV68 Gaussia	Co-transfection #2687 + #1998	J. Dillner
HPV73 Gaussia	Co-transfection #3122 + #1998	M. Müller
HPV6 Gaussia	Co-transfection #1317 + #1318 + #1998	M. Müller
HPV11 Gaussia	Co-transfection #3973 + #1998	M. Müller
HPV16 Firefly	Co-transfection #988 + #2818	M. Müller
HPV18 Firefly	Co-transfection #1165 + #1166 + #2818	J. Schiller
HPV31 Firefly	Co-transfection #1698 + #2818	T. Kanda
HPV33 Firefly	Co-transfection #2686 + #2818	J. Dillner
HPV35 Firefly	Co-transfection #2713 + #2818	M. Müller

HPV39 Firefly	Co-transfection #2712 + #2818	M. Müller
HPV45 Firefly	Co-transfection #2271 + #2273 + #2818	M. Müller
HPV51 Firefly	Co-transfection #2466 + #2481 + #2818	H. Seitz / M. Müller
HPV56 Firefly	Co-transfection #3123 + #2818	M. Müller
HPV6 Firefly	Co-transfection #1317 + #1318 + #2818	M. Müller
HPV11 Firefly	Co-transfection #3973 + #2818	M. Müller

2.2 Materials for DNA amplification, purification and analysis

2.2.1 Expression plasmids

Designation	Description	Reference
pGF Gaussia-GFP #1998	Gaussia and EGFP gene under control of EF1alpha promoter	C. Buck
pCLucf #2818	Firefly Luciferase under control of CMV promoter. Also encodes GFP under control of SV40 promoter	J. Schiller
HPV16 L1h+L2h #988	pCDNA 4.0 TO vector with humanized HPV16 L1 and L2 linked via IRES under control of CMV promoter and tetracycline-operator, intact SV40-ori	M. Müller
HPV18 L1h #1165	humanized HPV18 L1 under control of CMV promoter	J. Schiller
HPV18 L2h #1166	humanized HPV18 L2 under control of CMV promoter	J. Schiller
HPV31 L1h+L2h #1698	humanized HPV31 L1 and L2 under control of CMV promoter	J. Schiller
HPV33 L1h+L2h #2686	humanized HPV33 L1 and L2 under control of CMV promoter	J. Dillner

HPV35 L1h+L2h #2713	humanized HPV35 L1 and L2 under control of CMV promoter	S. Beddows
HPV39 L1h+L2h #2712	humanized HPV39 L1 and L2 under control of CMV promoter	S. Beddows
HPV45 L1h #2271	humanized HPV45 L1 under control of CMV promoter	S. Beddows
HPV45 L2h #2273	humanized HPV45 L2 under control of CMV promoter	J. Schiller
HPV51 L1h #2466	humanized HPV51 L1 under control of CMV promoter	T. Kanda
HPV51 L2h #2481	humanized HPV51 L2 under control of CMV promoter	T. Kanda
HPV52 L1h #1691	humanized HPV52 L1 under control of CMV promoter	J. Schiller
HPV52 L2h #1690	humanized HPV52 L2 under control of CMV promoter	J. Schiller
HPV56 L1h+L2h #3123	humanized HPV56 L1 and L2 under control of CMV promoter	M. Müller
HPV58 L1h+L2h #1694	humanized HPV58 L1 and L2 under control of CMV promoter	T. Kanda
HPV59 L1h+L2h #2714	humanized HPV59 L1 and L2 under control of CMV promoter	S. Beddows
HPV68 L1h+L2h#2687	humanized HPV68 L1 and L2 under control of CMV promoter	J. Dillner
HPV73 L1h+L2h #3122	humanized HPV73 L1 and L2 under control of CMV promoter	M. Müller
HPV6L1h #1317	humanized HPV6 L1 under control of CMV promoter	M. Müller
HPV6L2h #1319	humanized HPV6 L2 under control of CMV promoter	M. Müller
HPV11L1h+L2h #3973	humanized HPV11 L1 and L2 under control of CMV promoter	M. Müller
Pf encapsulin #3449	Pf encapsulin in MultiBac vector	This thesis

Pf ferritin #3447	Pf ferritin in MultiBac vector	This thesis
Pf encapsulin-Trx-HPV16L2(aa20-38) #3451	Pf encapsulin with Pf Trx and 1 copy HPV16 L2 (aa20-38) in MultiBac vector	This thesis
Pf encapsulin-HPV16L2(aa20-38) #3497	Pf encapsulin with 1 copy HPV16 L2 (aa20-38) in MultiBac vector	This thesis
Pf ferritin-Trx-HPV16L2(aa20-38) #3446	Pf ferritin with Pf Trx and 1 copy HPV16 L2 (aa20-38) in MultiBac vector	This thesis
Pf ferritin-HPV16L2(aa20-38) #3495	Pf ferritin with 1 copy HPV16 L2 (aa20-38) in MultiBac vector	This thesis
Pf encapsulin-Trx-HPV16L2(aa20-38)*3 #3547	Pf encapsulin with Pf Trx and 3 copies HPV16 L2 (aa20-38) in MultiBac vector	This thesis
Pf encapsulin-HPV16L2(aa20-38)*3 #3546	Pf encapsulin with 3 copies HPV16 L2 (aa20-38) in MultiBac vector	This thesis
Pf ferritin-Trx-HPV16L2(aa20-38)*3 #3545	Pf ferritin with Pf Trx and 3 copies HPV16 L2 (aa20-38) in MultiBac vector	This thesis
Pf ferritin-HPV16L2(aa20-38)*3 #3544	Pf ferritin with 3 copies HPV16 L2 (aa20-38) in MultiBac vector	This thesis
Pf encapsulin- Trx-HPVL2(aa20-38)*8 #3502	Pf encapsulin with Pf Trx and 8 copies HPV L2 (aa20-38) from HPV 16, 18, 31, 33, 35, 51, 59, 6 and 11 in MultiBac vector	This thesis

Pf encapsulin-HPVL2(aa20-38)*8 #3501	Pf encapsulin with 8 copies HPV L2 (aa20-38) from HPV 16, 18, 31, 33, 35, 51, 59, 6 and 11 in MultiBac vector	This thesis
Pf ferritin-Trx-HPVL2(aa20-38)*8 #3500	Pf ferritin with Pf Trx and 8 copies HPV L2 (aa20-38) from HPV 16, 18, 31, 33, 35, 51, 59, 6 and 11 in MultiBac vector	This thesis
Pf ferritin-HPVL2(aa20-38)*8 #3499	Pf ferritin with 8 copies HPV L2 (aa20-38) from HPV 16, 18, 31, 33, 35, 51, 59, 6 and 11 in MultiBac vector	This thesis
human-ferritin-GST #3939	gateway clone from homo ferritin to GST-vector	This thesis
Pf ferritin-GST #3504	Pf ferritin GST-vector(#808)	This thesis
Pf thioredoxin-GST #2877	Pf thioredoxin GST-vector(from Parma)	This thesis
human thioredoxin-GST #2902	human thioredoxin GST-vector (from Parma)	This thesis

2.2.2 PCR primers

All oligonucleotide listed, were order and produced at MWG Eurofins in Ebersberg, Germany.

Prime Name	Sequence 5' to 3'
Pf encapsulin Forward Primer	TTTTAAGCTTTTACTCCTGCAGCTCTTTCAGTTTTTTCAGAATCT
Pf encapsulin Reverse Primer	TTTTAAGCTTTTATTCCAGTACGACGATAGCTTCCGGATTC
Pf encapsulin linker CpoI site Forward Primer	TTTTAAGCTTTTACTCCTGCAGCTCTTTCAGTTTTTTCAGAATCT
Pf encapsulin linker CpoI site Reverse Primer	TTTTAAGCTTTTACTCCTGCAGCTCTTTCAGTTTTTTCAGAATCTC

Pf encapsulin Trx HPV16 L2(aa20-38) Forward Primer	TTTTAAGCTTTTACTCCTGCAGCTCTTTCAGTTTTTTCAGAATCT
Pf encapsulin Trx HPV16 L2(aa20-38) Reverse Primer	TTTTAAGCTTTTACGGACCGCCCCCCCCGGAGCTCCCGCCGCCGCTTTCCAGTACG
Pf ferritin Forward Primer	TTTTGGATCCACCATGCTCTGAGCGAACGCATGCTGAAAGCCTTG
Pf ferritin Reverse Primer	GGCCGCAAGCTTTCATTCTCCACCCTGCATC
Pf ferritin linker CpoI site Forward Primer	TTTTGGATCCACCATGGGCGGTCCGATGGAATCACAGGTCCGTCAGAATCTGAG
Pf ferritin linker CpoI site Reverse Primer	GGCCGCAAGCTTTCATTCTCCACCCTGCATC
Pf ferritin Trx HPV16 L2(aa20-38) Forward Primer	TTTTGGATCCACCATGATTATCGAGTATGACGGCGAAATC
Pf ferritin Trx HPV16 L2(aa20-38) Reverse Primer	GGCCGCAAGCTTTCATTCTCCACCCTGCATC

2.2.3 Enzymes

Calf Intestinal Alkaline Phosphatase (CIP)	NEB, Frankfurt
Lysozyme (20 µg/ml in 1 × PBS)	Sigma-Aldrich, Taufkirchen
RNase (1 mg/ml in H_2O)	Roche, Mannheim
T4 DNA ligase	Invitrogen Life Technologies, Darmstadt
QuickLigase	NEB, Frankfurt
Pfu polymerase	Stratagene Agilent Tchnologies, La Jolla, USA
KOD polymerase	Novagen/Merck, Darmstadt

Restriction enzymes	NEB, Frankfurt

2.2.4 Media and solutions for culturing bacteria

LB medium:	10 g Tryptone 5 g Yeast extract 5 g NaCl add up to 1 L with H_2O, pH 7.5, sterilized by autoclaving
LB agar plates:	98% LB-medium (v/v) 1.5% Bacto-agar (w/v) autoclaved, antibiotic added when 40° C is reached

Ampicillin final concentration 100 µg/ml	(Sigma-Aldrich, Taufkirchen)
Kanamycin final concentration 25 µg/ml	(Sigma-Aldrich, Taufkirchen)
Zeocin final concentration 25 µg/ml	(Invitrogen Life Technologies, Darmstadt)
Chloramphenicol final concentration 20 µg/ml	(Sigma-Aldrich, Taufkirchen)
Gentamycin final concentration 10 µg/ml	(Sigma-Aldrich, Steinheim)
Tetracycline final concentration 25 µg/ml	(Sigma-Aldrich, Steinheim)
IPTG final concentration 1 mM	(Applichem, Darmstadt)
Blue gal final concentration 100 µg/µl	(Invitrogen, Karlsruhe)
X-gal final concentration 200 µg/ml	(Sigma-Aldrich, Steinheim)

2.2.5 Solutions and Chemicals for DNA purification and preparation

I. Purification of plasmid DNA

Glucose buffer:	50 mM Glucose

	10 mM EDTA 25 mM Tris in H_2O, pH 8.0
Lysis buffer:	0.5 M NaOH 1% SDS (w/v) in H_2O
Sodium acetate:	3 M Sodium acetate in H_2O, pH 5.3
Phenol mix:	Phenol-CIA mix 1:1 100 µg 8-hydroxyquinoline/ 350 ml Phenol CIA mix solution

II. Agarose gel electrophoresis

1 x TAE:	40 mM Tris 5.71% Acetic acid (v/v) 10% 0.5 M EDTA pH 8 (v/v)
Agarose gel (1 %)	1% Agarose (w/v) 1 µg/ml Ethidium bromide (10 mg/ml ethidium bromide from Roth) in 1 x TAE buffe
Lambda/HindIII marker:	NEB, Frankfurt
100 bp DNA ladder plus:	Fermentas Thermo Scientific, St. Leon Roth
6 × DNA loading buffer	Thermo Scientific

GeneRuler 1 kb DNA ladder	Thermo Scientific

III. Sequencing

DNA was sent to GATC-Biotech (Konstanz) for sequencing.

IV. Further chemicals

20 mM dNTP mix	Roche, Mannheim
25 mM $MgCl_2$	Novagen/Merck, Darmstadt
10 mg/ml ethidium bromide	Roth, Karlsruhe

2.3 Materials for protein purification and analysis

2.3.1 Lysis buffer and elution buffer

Lysis buffer (insect cells protein)	5 mM $MgCl_2$ 5 mM $CaCl_2$ 150 mM NaCl 0.5 mM PMSF 0.01% Triton × 100 20 mM HEPES pH 7.4
Lysis buffer (bacterial protein)	200 mM Kaliumacetat 2 mM Magnesiumacetat 1 mM DTT 1 mM PMSF 0.5% Tween 20 20 mM HEPES pH 7.2
Elution buffer(bacterial protein)	10 mM Glutathione in 50 mM Tris, pH 8.0 with HCl

2.3.2 Purification column

1ml GSTrap FF column	Amersha Pharmacia, Uppsala, Schweden

2.3.3 Gradients

Cesium chloride solution	57.7% (w/v) CsCl in Insect cells lysis buffer
Sucrose solution	30% (w/v) Sucrose in Insect cells lysis buffer

2.3.4 Size-exclusion chromatography column

Superdex200 10/300 GL	Amersham GE healthcare, Buckinghamshire, UK

2.3.5 Endotoxin removal

Triton X-114	Sigma-Aldrich, Taufkirchen

2.3.6 Polyacrylamides gels

1x TGS:	2.5 mM Tris 1.45% glycine (w/v) 0.1% SDS (w/v) in H_2O, pH 8.3 with HCl
3 x Protein loading buffer:	30% Glycerol (v/v) 6% SDS (w/v) 15% β-mercaptoethanol (v/v) 0.03% Bromphenol blue (v/v) 187.5 mM Tris in H_2O, pH 6.8 with HCl
Tris buffer pH 8.8:	1 M Tris in H_2O, pH 8.8 with HCl
Tris buffer pH 6.8:	1 M Tris 0.03% bromphenol blue in H_2O, pH 6.8 with HCl

Recipe for 5 polyacrylamide gels:

	15% separating gel	**12.5% separating gel**	**3% stacking gel**
30% Acrylamide	20 ml	18.8 ml	1 ml
1 M Tris buffer	15 ml (pH 8.8)	16.9 ml (pH 8.8)	1.3 ml (pH 6.8)
H_2O	4.2 ml	8.5 ml	7.5 ml
10% SDS	400 µl	450 µl	100 µl
10% APS	400 µl	450 µl	100 µl
TEMED	20 µl	22.5 µl	15 µl

Acrylamide solution	Roth, Karlsruhe
TEMED	Sigma-Aldrich, Taufkirchen
ColorPlus™ Prestained Protein Ladder	NEB, Frankfurt
Coomassie Solution GelCode®	Thermo Scientific, Rockford, USA

2.3.7 Determination of protein concentration

Bradford reagent	BioRad,München
BSA standard (2 µg/µl)	Pierce Thermo Scientific, Rockford, USA

2.4 Materials for immunological methods

2.4.1 Antibodies

Designation	**Description**	**Reference**
K4L220-38	cross-neutralizing mouse monoclonal antibody recognizing the 20-31 HPV L2 epitope; elicited with an *E. coli* thioredoxin-HPV16 L2(20-38)$_3$ antigen	I. Rubion / M. Müller
K18L220-38	cross-neutralizing mouse monoclonal antibody recognizing the 20-31 HPV	I. Rubion / M. Müller

	L2 epitope; elicited with an *E. coli* thioredoxin-HPV16 L2(20-38)$_3$ antigen	
anti thioredoxin	Mouse monoclonal antibody recognizing the Pf thioredoxin, elicited with an E.coli Pf thioredoxin	M.Müller
GAMPO	GAMPO Goat-anti-mouse coupled with HRP,	Dianova, Hamburg,Germany

2.4.2 Adjuvants for immunization

AddaVax	Invivogen
Aluminum hydroxide	Brenntag
Synthetic monophosphoryl lipid-A	AvantiLipids

2.4.3 Animal narcotic

Isoflurane	Baxter, Deerfield, USA

2.5 Materials for pseudovirions infection and neutralization

2.5.1 Pseudovirions production and purification

Brij 58	10% Brij 58 (w/v) in H_2O, filter-sterilized
NaCl	5 M NaCl in H_2O, filter-sterilized
1 × DPBS	Gibco Life Technologies, Paisley, UK

Benzonase (100,000 U/ml)	Merck, Darmstadt
TurboFect™ transfection reagent	Fermentas Thermo Scientific, St. Leon-Roth

RNase A/T1 mix Fermentas	Thermo Scientific, St. Leon-Roth
OptiPrep™ (60% w/v)	Sigma-Aldrich, Taufkirchen

2.5.2 *In vitro* luciferase reporter gene assays

Gaussia GLOW-Juice BIG KIT	PJK, Kleinblittersdorf
Beetle-Juice BIG KIT	PJK, Kleinblittersdorf

2.5.3 *In vivo* cervicovaginal mouse model and firefly luciferase reporter gene assay

CMC (Carboxymethyl cellulose)	4% CMC (w/v) in 65 °C 1 × PBS, add while vortexing, rotate at 37 °C
Medroxyprogesteronacetat	30 mg/ml Depo-Provera (Pharmacia, Ireland) in 1 × PBS
Nonoxynol-9	4% Nonoxynol-9 (Spectrum, USA) in 4% CMC
Beetle luciferin, Potassium salt	15 mg/ml Beetle luciferin (Promega, USA) in DMEM

2.6 Cell culture media and solutions

2.6.1 Full media

DMEM (293TT, HelaT K4)	1% Penicillin/Streptomycin 1% L-glutamine 10% FCS
DMEM (CHOΔfurin, pgsa-745)	1% Penicillin/Streptomycin 200 µM L-proline 10% FCS

DMEM (MCF10A)	5% Horse serum 1% Penicillin/Streptomycin 1% L-glutamine 500 ng/ml Hydrocortisone 10 µg/ml Insulin, human 20 ng/ml EGF, human
DMEM L2 assay medium	10% FCS 1% Antibiotic-Antimycotic 200 µM L-proline

DMEM, RPMI-1640	Sigma-Aldrich, Taufkirchen
DMEM:F12	Genaxxon, Ulm
FCS	PAN Biotec, Aidenbach
FCS (hybridoma)	Gibco Life Technologies, Paisley, UK
Horse serum	Gibco Life Technologies, Paisley, UK
Antibiotic-Antimycotic (100x)	Gibco Life Technologies, Paisley, UK
Penicillin/Streptomycin	Gibco Life Technologies, Paisley, UK
L-glutamine	Genaxxon, Ulm
Hydrocortisone	Calbiochem Merck4Biosciences, Darmstadt
Insulin, human	Roche, Mannheim
EGF, human	PeproTech, Rocky Hill, USA

Lysis buffer	0.5% Triton X-100 (v/v) 20 mM NH_4OH

	in 1 × PBS, pH 7.4, sterilized by filtration
Freezing (cryo) medium	30 ml DMEM or RPMI 15 ml FCS 5 ml DMSO

2.6.2 Further solutions

0.05%, 0.25% Trypsin-EDTA	Gibco Life Technologies, Paisley, UK
Distilled water, sterile	Gibco Life Technologies, Paisley, UK
50 mg/ml Hygromycin B (for selection)	Roche, Mannheim
Heparin (#H4784)	Sigma-Aldrich, Taufkirchen
T-Carrageenan, Type V	Sigma-Aldrich, Taufkirchen
Trypan blue staining solution	Fluka, Neu Ulm

2.7 General buffers and solutions

1 × PBS	140 mM NaCl 2.7 mM KCl 8.1 mM Na_2HPO_4 1.5 mM KH_2PO_4 in H_2O, pH 7.4, autoclaved
1 × PBS-0.3 % Tween (PBST)	0.3% Tween20 (v/v) in 1 × PBS
1 M Tris HCl (pH 8)	1 M Tris in H_2O, pH 8
10 mM Tris HCl (pH 8)	10 mM Tris in H_2O, pH 8
70% Ethanol	70% Ethanol (v/v) in H_2O

BSA-blocking buffer	2% BSA in PBST

Methanol	Sigma-Aldrich, Taufkirchen
Ethanol	VWR, Darmstadt
Butanol	AppliChem, Darmstadt
Isopropanol	VWR, Darmstadt, Fluka, Neu Ulm

2.8 Serology buffer

Multiplex serology blocking buffer	1 mg/ml Casein in 1 × PBS
NTA- Solution	90 g Nitrilotriacetic acid 150 g $NaHCO_3$ in H_2O to 750 ml
Nickel solution	10 g $NiSO_4$ in H_2O to 1000 ml
Serum pre-incubation buffer	2 mg/ml GST-Tag lysate 0.5% (w/v) Polyvinyl alcohol 0.8% (w/v) Polyvinyl pyrrolidone 2.5% (w/v) CBS-K super chemiblock in multiplex serology blocking buffer
Storage buffer	0.05%(w/v) NaN_3 in multiplex serology blocking buffer

2.9 Chemicals

All chemicals, unless noted otherwise, were purchased from Sigma-Aldrich (Taufkirchen), AppliChem (Darmstadt), Merck (Darmstadt), Roth (Karlsruhe), Serva (Heidelberg), Fluka (Neu Ulm), Riedel de Haën (Seelze, Netherlands),

Gerbu (Gaiberg), and Life Technologies (Karlsruhe) in the highest quality available.

2.10 Kits

StrataClone Blunt PCR Clonig Kit	Stratagene Agilent Technologies, La Jolla, USA
QIAGEN Plasmid Plus Maxi Kit	Qiagen, Hilden
QIAprep® Spin Miniprep Kit	Qiagen, Hilden
1 × Multiplex PCR Kit buffer	Qiagen, Hilden
QIAquick Gel extraction kit	Qiagen Hilden, Germany
Gateway® LR Clonase™ II Enzyme mix	Life Technologies Karlsruhe, Germany

2.11 Laboratory equipment

2.11.1 Electrical equipment

Refrigerated tabletop centrifuge 5417R	Eppendorf, Hamburg
Tabletop centrifuge 5417R	Eppendorf, Hamburg
CHRIST Minifuge GL	Heraeus, Hanau
Multifuge 1 S-R	Heraeus, Hanau
Refrigerated centrifuge RC5C	Sorvall, Newton, USA
Refrigerated centrifuge RC-5B	Sorvall, Newton, USA
Ultracentrifuge Sorvall® Discovery 90 SE	Sorvall by HITACHI, Newton, USA
Megafuge 1.0	Heraeus, Hanau
Refrigerated tabletop centrifuge Micromax RF	Thermoquest, Egelsbach
Rotors: SLA 3000, SA-600, VTi65	DuPont, Bad Nauheim

SW41 Ti rotor	Beckmann Coulter, Brea, USA
Vortex Genie 2™	Bender and Hobein, Ismaning
Combimag Red/RET magnetic stirrer	IKA, Staufen
MR 2000/2002 magnetic stirrer	Heidolph, Kelheim
Thermomixer 5436	Eppendorf, Hamburg
Duomax 1030 shaker	Heidolph, Kelheim
Vibramax-VXR	IKA, Staufen
800 W microwave	Bosch, Gerlingen-Schillerhöhe
GFC water bath	Grant Instruments, Cambridge, UK
UC water bath	julabo, Seelbach
CO_2 incubator safe cell UV (cell culture)	Sanyo, Osaka, Japan
Microscope for cell culture, Willovert	Hund, Wetzlar
Microscope for cell culture, Diavert	Leitz, Wetzlar
Refrigerators and freezers	Liebherr, Ochsenhausen
Electrophoresis power supply	Gibco BRL, Eggenstein
Horizontal Gel Electrophoresis Horizon®11.14	Gibco BRL, Eggenstein
Electrophoresis chamber	Roth, Karlsruhe
Electrophoresis gel slides	Roth, Karlsruhe
Function line incubator	Heraeus, Hanau
C1000™ Thermal Cycler	BioRad, München
Mastercycle	Eppendorf, Hamburg
Impulse Sealer	RNS Corp, Taipei, Taiwan
MicroPulser™ Electroporator	BioRad, München

-80°C freezer	New Brunswick Scientific, Hamburg
Ice maker	Hoshizaki, Willich-Münchheide
EconoPump (peristaltic)	BioRad, München
Steril GARD® III Advance cell culture hood	The Baker Company, Sanford, USA
Bio GARD cell culture hood	The Baker Company, Sanford, USA
Multiskan EX (ELISA reader)	Thermo, Waltham, USA
1420 Multilable Counter Victor3	Perkin Elmer, Norwalk, USA
EnVision 2101 Multilabel Reader	Perkin Elmer, Norwalk, USA
Nanodrop spectrophotometer	Peqlab, Erlangen
pH meter	Sartorius, Göttingen
MilliQ ultra-pure water unit Millipore	Merck, Darmstadt
Precision balance	Mettler Toledo, Gießen
Nitrogen tank	Chronos Messer, Krefeld
French press (Emulsi Flex-C5)	Avestin, Ottawa, Canada
Multichannel pipette (1200 µl)	Biozym Scientific, Hessisch Oldendorf
FPLC Amersham Äkta purifier 900	GE Healthcare, Uppsala, Sweden
QIAgility	Qiagen, Hilden
Xenogen IVIS 100 imager	Xenogen/Perkin Elmer, Norwalk, US
BioRad GelDoc™ EZ Imager	BioRad, München
Blood tube rotator SB1	Stuart Scientific, Staffordshire, UK

2.11.2 Common use utensils

96-well flat bottom cell culture plates	Costar, Corning, USA
Single use filter, 0.2/0.4 µm	Renner, Dannstadt
Bottle Top Filter 500 ml, 0.22 µm	Costar, Corning, USA
Dialysis tubing, 0.023 mm	Roth, Karlsruhe
Cryo tubes, 2 ml	Roth, Karlsruhe
Electroporation cuvettes (25 × 2 mm)	Peqlab, Erlangen
ELISA plates, round bottom	BD Falcon, Durham, USA
Whatman filter paper 3MM-paper	Schleicher & Schuell, Dassel
Drying foil for SDS gels	Promega, Madison, USA
10 cm tissue culture plates	Sarstedt, Newton, USA
10 cm petri dishes	Greiner, Frickenhausen
Inoculating loop	Greiner, Frickenhausen
LIA plates, white, 96 K F-Form	Greiner, Frickenhausen
Nitrocellulose membrane	Schleicher & Schuell, Dassel
Parafilm “M”	American National Can, Chicago, USA
Pasteur pipettes, plastic	Greiner, Frickenhausen
Single use pipettes (25 ml, 10 ml, 5 ml), sterile	BD Falcon, Durham, USA
Filter tips (1000 µl, 200 µl, 20 µl, 10µl)	Starlab, Hamburg
Cell scraper	Sarstedt, Newton, USA
50 ml centrifuge tubes	Nalgene, Nunc, Wiesbaden
500 ml centrifuge tubes	Nalgene, Nunc, Wiesbaden
Ultracentrifuge tubes Herolab 13.2 ml	PA Beranek, Weinheim

Quick seal Vti65 centrifuge tubes	Beckmann Coulter, Brea, USA
1000 µl and 200 µl pipette tips	Greiner, Frickenhausen
1.5 ml and 2 ml reaction tubes	Eppendorf, Hamburg
0.5 ml and 1.5ml LoBind Protein tubes	Eppendorf, Hamburg
PCR reaction tubes	Roth, Karlsruhe
15 ml reaction tubes	TPP, Trasadingen, Switzerland
50 ml reaction tubes	Greiner, Frickenhausen
X-ray film	Amersham, München
X-ray	cassette Roth, Karlsruhe
Syringes and needles	BD Falcon, Durham, USA
50 ml reagent reservoir	Costar, Corning, USA
14 ml PP round bottom tubes	BD Falcon, Durham, USA
6- and 12-well test plates	TPP, Trasadingen, Switzerland
25 cm^2, 75 cm^2 and 150 cm^2 tissue flasks	TPP, Trasadingen, Switzerland
1000 µl, 200 µl, 20 µl and 10 µl PIPETMAN Neo®	Gilson, Middleton, USA
1000 µl, 250 µl, 50 µl and 25 µl MICROMAN®	Gilson, Middleton, USA
Nitrile gloves	Freeform®SE, The Hague, Netherland
Latex gloves	Blossom, Madrid, Spain
Disposable scalpel	Feather, Osaka, Japan
Discofix multidirectional stop cock	Braun, Melsungen
1 ml Injekt-F single use syringe	Braun, Melsungen
12-channel Finnpipettes, 5-50 µl, 10-100 µl	Thermo Scientific, Waltham, USA

BioRad columns 1 × 10 cm, 8 ml	BioRad, München
Rotilabo-FEP tubing, inside diameter: 0.8 mm outside diameter:1.58 mm	Roth, Karlsruhe

2.12 Software

Adobe Acrobat Professional X	Adobe, San Jose, USA
Clone Manager 9.0 for Windows	Scientific & Educational Software, Cary, USA
GraphPad Prism 5.0	GraphPad Software, La Jolla, USA
Microsoft Office 2010	Microsoft, Redmond, USA
Microsoft Windows 7	Professional Microsoft, Redmond, USA
Wallac 1420 Workstation	Perkin Elmer, Norwalk, USA
Wallac EnVision Manager	Perkin Elmer, Norwalk, USA
Living Image® 2.50.1	Xenogen/Perkin Elmer, Norwalk, USA
Unicorn 5.0	GE Healthcare, Uppsala, Sweden
ORF script	Agnes Hotz-Wagenblatt, DKFZ, Heidelberg
EndNoteX5	Thomson Reuters, New York, USA
ImageJ 1.40	NIH, Bethesda, USA

3 Methods

3.1 Manipulation of DNA and cultivation of bacteria

3.1.1 Polymerase chain reaction (PCR)

PCR is the main method used to amplify specific DNA fragments exponentially through enzymatic action of a polymerase *in vitro*. A standard PCR protocol was employed to generate the nanoparticles with monomeric or multimeric Trx-L2 constructs by using the KOD HiFi polymerase. The pipetting scheme and cycling parameter were as follows:

	KOD HiFi DANN polymerase PCR
Reaction mix	26.1 µl autoclaved MilliQ H_2O 5 µl 10 × Buffer #2 5 µl dNTPs (final concentration 0.2 mM) 2 µl $MgCl_2$ (final concentration 1 mM) 1 µl ~2 µg/ml template DNA 4 µl fwd primer (5 pmol/µl, final concentration 0.4 µM) 4 µl rev primer (5 pmol/µl, final concentration 0.4 µM) 2.5 µl DMSO (final concentration 5% v/v) 0.4 µl KOD HiFi DNA polymerase (2.5 U/µl) 50 µl total volume

The reaction was prepared on ice, and the cycling parameters were:

Cycling parameters: (1) Denature 15 sec 98 °C
(2) Anneal 2 sec (Tm of primers - 5) °C
(3) Extend 20 sec 72 °C, 25 cycles
(4) 4 °C ∞

3.1.2 Gate-way cloning Polymerase chain reaction (PCR)

Homo ferritin was provided as complete Gateway®-compatible entry-vectors to shuttle into a destination vector with the LR Clonase™ II enzyme mix. All the

reactions were done using the Fast Gateway® LR protocol. It was performed according to the manufacturer´s instructions. The scheme of pipetting for the reaction and cycling parameter were as follows:

Reaction mix	1-7 µl Entry clone 1 µl Destination vector 8 µl TE buffer 2 µl LR ClonaseTM II enzyme mix

The reaction was processed at 25 °C for 1 hr followed by treatment with 1 µl proteinase K at 37 °C for 10 min to stop the reaction. 1 µL of the LR reaction was transformed into 40 µL of electrocompetent bacteria and then resuspended in 500 µL LB, shaken at 37 °C for 1 h, followed by spreading on agar plates with the appropriate antibiotic to obtain colonies at 37 °C overnight. Colonies were picked and cultivated for DNA plasmid extraction, restriction digests and sequencing to confirm correct insertion. Glycerol stocks of the correct colonies were prepared and stored at -80 °C.

3.1.3 Blunt end PCR cloning

Blunt-ended KOD HiFi generated PCR products can be cloned into a vector by combining the activities of topoisomerase I and Cre recombinase. The kit was used according to the manufacturer's instructions with minor modifications.

Reaction mix	3 µl StrataClone Blunt Cloning Buffer 2.5 µl 1:10 diluted PCR product 0.5 µl StrataClone Blunt Vector Mix amp/kan

The solution was gently mixed, incubated for 5-10 minutes at room temperature, then placed on ice before subsequent chemical transformation of SoloPack cells.

3.1.4 Chemical transformation of bacteria

There are StrataClone SoloPack cells in the StrataClone Blunt PCR Cloning kit, the cells were going to be transformed with PCR DNA ligated into the blunt end vector mix. For each transformation, 50 µl of cells were employed. The transformation procedures as following:

The transformation components:	50 µl StrataClone SoloPack cells (thawed on ice) 1 µl cloning reaction

The reaction was gently mixed and incubated on ice for 20 minutes.

In the meantime, LB medium without any antibiotic was pre warmed to 42 °C in water bath.

The transformation reaction was heat-shocked for 45 seconds at 42 °C, then placed n on ice for 2 minutes. Afterwards, 250 µl pre-warmed LB medium was added to the reaction and incubated at 37 °C and 250 rpm for 1 hour.

During the incubation time, the LB-agar plate with ampicillin or kanamycin were spread with 40 µl of 2% X-gal to select blue-white colonies.

Finally, 200 µl of transformation reaction were spread on the plates, and incubated overnight at 37 °C. The white colonies which contain ligated PCR product were picked on the second day.

3.1.5 Transformation of bacteria by electroporation

E. coli MxDH10, Rosseta, and multibac bacteria were transformed by electroporation. The procedure was as follows:

The electroporation cuvettes were chilled on ice, and in the meantime, bacteria were thawed on ice as well; 2 µl of DNA was gently mixed with 40 µl of bacteria and transfer to the chilled electroporation cuvette. The mixture was pulsed once

with 2.5 kV (around 5 mini seconds) and then suspended in 300 µl pre warmed LB medium (37 °C). The transformed bacteria were transfered to a new 2 ml eppendorf tube and incubated on a shaker at 37 °C and 250 rpm for at least 1 hour (for the multibac bacteria the incubation time was extended up to at least 6 hours). Finally, up to 50 µl of bacterial culture were spread onto LB-agar plates containing the appropriate resistance and the plates were incubated at 37 °C overnight.

3.1.6 Electrocompetent bacteria preparation

The electrocompetent bacteria used for transformation were derived from the bacterial strain MegaX DH10 or Rosseta. Before preparing the starting culture, MegaX DH10 were plated from a glycerol stock on a LB agar plate without antibiotics and incubated at 37 °C overnight. A single colony was transferred to 25 ml LB medium and inoculated at 37 °C overnight on a shaker with 200 rpm. After overnight incubation, 5ml of the bacterial culture were transferred to 400 ml of fresh LB medium and inoculated at 37 °C and 200 rpm for 2-4 hours until the culture reached an optical density (OD_{600}) of 0.5-0.6. The bacterial culture was then chilled on ice for 30 min and harvested by centrifugation at 6.000 rpm for 10 min at 4 °C. This pellet was re-suspended in 30 ml ice-cold H_2O and transferred to a dialysis tube and dialyzed against H_2O overnight at 4 °C. The bacterial cells were then harvested by centrifugation at 4,000 rpm for 10 min at 4 ºC and resuspended in 600 µl ice-cold 10% glycerol solution. 10 µl of the bacterial suspension were diluted 1:100 in 990 µl 10% glycerol and the OD_{600} was measured. The obtained OD_{600} was multiplied with the volume of the stock suspension (600 µl) and the calculated value in µl of glycerol was added to the bacterial solution. Aliquots of 40, 80 and 120 µl were prepared and snap-frozen in liquid nitrogen and stored at -80 ºC.

3.1.7 Bacteria cultivation on agar plates and in suspension

Bacteria were cultured in LB medium at 37 °C and 250 rpm, then the bacterial culture was spread onto agar plates. Subsequently the inoculated agar plates were incubated upside down at 37 °C to prevent condensed water from collecting on the colonies. Proper antibiotics were added to liquid LB-agar medium or solid plates to prevent contamination.

3.1.8 Bacterial storage at -80°C

For the purpose of long term storage of bacteria, 1 ml overnight bacteria culture was mixed with 300 µl sterile glycerin in a sterile, screw-cap cryo tube, mixed by pipetting up and down and stored at -80 °C.

3.1.9 Small scale purification of plasmid DNA (Mini-prep)

A bacteria culture was prepared by inoculating a single colony or glycerol stock in 5 ml LB medium with appropriate antibiotic and incubated at 37 °C and 250 rpm overnight. The next day, 2 ml bacteria culture were transferred into a 2 ml reaction tube, and centrifuged at 13,000 rpm for 1 minute at room temperature. The supernatant was discarded and the pellet was re-suspended in 100 µl glucose buffer and the suspension was incubated at room temperature with shaking for 5 minutes. 200 µl lysis buffer was added into the suspension and the reaction was incubated on ice for 5 minutes. Then 150 µl of 3 M sodium acetate (pH 5.3) was added, and incubated on ice for another 5 minutes. 450 µl of phenol-mix was added to remove proteins from the reaction, and vigorously shaken to mix all the components for 5 minutes and then centrifuged at room temperature and 13,000 rpm for 5 minutes Later, 380 µl of aqueous supernatant were transferred into a new reaction tube, and 450 µl isopropanol was added. Incubated at -80 °C for 15 minutes, followed by centrifugation at 13,000 rpm and 0 °C for 30 minutes. The supernatant was removed carefully

and the DNA pellet washed with 500 µl 70% ethanol. Another centrifugation at 13,000 rpm and 0 °C for 5 minutes was carried out. The ethanol was carefully discarded, and the DNA pellet was dried under the hood and later resuspended with 30 µl autoclaved MilliQ water. The DNA was either analyzed by restriction enzyme digest (section 3.1.12) or stored at -20 °C. If the DNA needed to be sent for sequencing, then high quality DNA was required. For this purpose the DNA was purified using the QIAprep Spin Miniprep Kit according to the manufacturer's instructions. The DNA was eluted with autoclaved MilliQ H_2O and 20-30 µl was sent for sequencing.

3.1.10 Large scale purification of plasmid DNA (Maxi-prep)

A bacteria culture was prepared by inoculating a single colony or glycerol stock in 250 ml LB medium with appropriate antibiotic and incubated on the shaker at 37 °C and 250 rpm overnight. The next day, the whole bacteria culture was transferred into a 500 ml centrifuge tube and centrifuged at 5,000 rpm for 10 minutes at room temperature. Then the DNA was purified with the QIAGEN Plasmid Plus Maxi Kit according to the manufacturer's introduction.

3.1.11 DNA concentration and purity determination

For sequencing purpose or for digestion, the DNA concentration and purity needed to be determined. We can use the property that DNA, RNA and protein have specific absorption maxima (DNA at 260 nm and proteins at 280 nm) to calculate a samples purity. A pure DNA solution results in absorption ratio (Abs 260 nm/Abs 280 nm) =1.8, if the value is greater than 1.8, it indicated there was a RNA contamination; while a value lower than 1.8, means the DNA contains proteins. Before each measurement, a blank measurement with 1.5 µl of the solution that the DNA was dissolved in (autoclaved MilliQ H_2O) followed by 1.5 µl of undiluted DNA sample.

3.1.12 Restriction enzyme digestion

DNA was digested by restriction enzymes for two purposes: namely to analyze the plasmid DNA and to digest the plasmid into fragments for further use.. For the different purposes, a different mixture was used. To analyze the plasmid DNA, the components were as follows:

DNA	0.5-1 µg
10 x reaction buffer	2 µl
100 µg/ml Rnase	0.2 µl
restriction enzyme	0.2 µl
	/add autoclaved MilliQ H_2O upto 20 µl

The reaction was incubated at 37 °C for 1-3 hours.

Later, 5 µl of digest reaction was mixed with 1 µl 6 × DNA dye, and loaded onto 1% -2% agarose gel to separate the digested fragments.

After checking the size of fragments, the potential candidates of plasmid were digested in bigger reaction system to prepare DNA. The reaction components were as follows:

DNA	15-50 µg
10 x reaction buffer	20 µl
100 µg/ml Rnase	2 µl
Restriction enzyme	2 µl
	/add autoclaved MilliQ H_2O upto 200 µl

The reaction was incubated at 37 °C overnight.

Then the entire digestion reaction was mixed with 40 µl 6 × DNA dye and loaded onto 1% agarose gel. The DNA fragments of interest were subsequently extracted from the gel.

3.1.13 Horizontal agarose gel electrophoresis

Gel electrophoresis was used to separate nucleic acid fragments by size. Nucleic acids are negative charged because of their phosphate residues and will move in an electric field through an agarose polymer matrix from the negative cathode to the positive anode. The speed at which the nucleic acids will travel not only depends on the size and conformation of the fragments but also the density of the agarose gel matrix. If small fragments are desired, more concentrated gels will be employed since the nucleic acids move slower in them. Generally, smaller molecules of nucleic acids travel faster on the agarose gels, since they are presented with less resistance in the gel matrix. Linear DNA fragments travel slower than the supercoiled ones since they have a larger surface area and are retained in the agarose gel matrix longer than supercoiled DNA. In order to determine the size of the DNA fragments, a DNA marker containing different fragments of DNA with known sizes was applied on the gel with the samples. The DNA fragments were displayed as bands on the gel using ethidium bromide which intercalates into DNA and emits fluorescence when exposed to UV light.

Preparation of agarose gel as following:

Agarose	1 g (1% gel)/2 g (2% gel)
1x TAE buffer	100 ml

The solution was heated in a microwave and allowed to cool down briefly before 6 µl ethidium bromide (1 mg/ml) was added.

Vector DNA (- 15 µg)	1 µl
insert DNA (- 50 µg)	9 µl
5 × ligase buffer	4 µl
MilliQ H_2O	6 µl
T4 ligase	1 µl

3.1.14 DNA extraction from agarose gel

DNA fragments were extracted from agarose gel using the QIAquick Gel Extraction Kit from Qiagen according to manufacturer's instructions.

3.1.15 Dephosphorylation of 5'-DNA ends and ligation

5'-phosphates of DNA vectors were removed by phosphatase to avoid re-circularization of the linear DNA plasmid vector and phosphodiester bonds formations. By dephosphorylating 5'-DNA ends DNA fragments can be ligated into the dephosphorylated vector effectively without re-ligation of themselves.

In order to put the DNA fragment of interest into a suitable vector, DNA ligation was employed. A linear vector plasmid with 5'-ends dephosphorylated was inserted into linear DNA through covalent phosphodiester bonds involving the 3'-hydroxyl end of one nucleotide and the 5'-phosphate of another. T4 DNA ligase (Invitrogen Life Technologies, Darmstadt) was employed. The reaction system was made as follows:

Incubated at 16°C overnight.

The second day, bacteria were transformed via electroporation with the ligated DNA (as described in 3.1.5).

3.2 Protein purification and analysis

3.2.1 Expression of encapsulin and ferritin in *E.coli*

In order to produce the native encapsulin and ferritin nanoparticle for further experiments, the encapsulin and ferritin were transformed into *E.coli* Rosseta, and the expression procedures were as follows:

A single colony was inoculated in 20 ml LB medium overnight at 37 °C, next day, 4 ml of bacteria culture were used to inoculate 400 ml LB medium in a 1 L Erlenmeyer flask at 37 °C and 200 rpm for 4-5 hours. Once the bacteria culture reached OD_{600} 0.5-0.6, it was induced with 1 mM IPTG overnight at 30 °C or at room temperature and 200 rpm. On the third day, the bacterial culture was centrifuged at 5,000 rpm for 10 minutes, the pellet resuspended with 100 ml 1 × PBS, and centrifuged again.The supernatant was discarded and the pellet stored at -20 °C for extraction later.

3.2.2 Protein extraction from *E.coli*

The bacteria pellet was re-suspended in 40 ml lysis buffer, and incubated at room temperature for 10 minutes then another 20 minutes on ice. Afterwards, the bacteria were lysed with an Emulsi Flex-C5 (also called French Press) with three to five cycles at 15,000 psi. Between samples, the French Press was washed thoroughly with 0.5 M NaOH, 10 mM HCl, autoclaved MilliQ H_2O and lysis buffer. Bacteria lysate was centrifuged at 17,000 rpm and 4 °C for 1 hour. The supernatant which contained soluble protein was subsequently purified, and the pellet was discarded.

3.2.3 Purification proteins with GST-tag

The pGex-4T-3 plasmids with native ferritin, encapsulin and human ferritin were transformed in *E.coli* Rosseta bacteria and cultivated in LB medium

supplemented with 100 µl/ml ampicillin and 20 µg/ml chloramphenicol at 37 °C overnight (pre-culture). 50 ml of pre-culture medium was diluted into 1 L LB containing the respective antibodies and grown at 37 °C until OD_{600} reached approximately 0.6. Then the culture was set to rest at 4 °C for 30 minutes, then induced with 250 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). After cultivation at 27 °C overnight, the pellets were collected by centrifugation for 20 minutes at 5,000 rpm at 4 °C in Sorvall GS-3 rotor. The pellets were resuspended in 10 ml GST-lysis buffer containing 0.5 mol complete protease inhibitor cocktail and 2 mM DTT. Then subjected to the French Press and then centrifuged at 14,000 rpm for 1 hour at 4 °C. Finally, the supernatant was mixed with 100% glycerol at 1:1 ratio and stored at -20 °C.

3.2.4 Endotoxin removal

The purified thioredoxin-L2 protein was mixed with 1% Triton X-114 and incubated on ice for 5 minutes followed by 5 minutes at 37 °C. In order to remove the Triton X-114, the samples were centrifuged for 1 minute at 12,000 rpm and 37 °C after which the supernatant was transferred into a new reaction tube. All of the procedures were repeated twice.

3.2.5 SDS- polyacrylamide gel electrophoresis

Proteins were separated by size on SDS polyacrylamide gels. The proteins were mixed with 3 × protein loading buffer which contains SDS that is negatively charged, and the reducing agent β-mercaptoethanol. When the voltage is applied, the proteins gain an overall negative charge and migrate to the positive anode because of SDS. The SDS-polyacrylamide gel has two parts, a stacking and a separating gel. The stacking gel with 3% acrylamide takes up the upper fourth of it, the separating gel with 12.5% acrylamide which is 3/4 of the gel. After the voltage is applied, all proteins start at the same position between the

stacking gel and the separating gel where they move according to size, the larger slower and the smaller faster.

SDS-PAGEs were placed in electrophoresis chambers and filled with 1 × TGS. The proteins were mixed with 3 × protein loading buffer, then denatured at 95 °C for 10 minutes. Afterwards, short centrifugation was carried out before loading the samples onto the gels. Electrophoresis was run at 90 V for 20 minutes, then set to 110 V. After running, the gels were either stained with Coomassie blue staining solution, or moved on to western blot.

3.2.6 Coomassie blue staining of SDS-PAGEs

In order to have better staining, the polyacrylamide gels were rinsed in water at room temperature for 30 minutes, then transferred to Coomassie blue staining solution overnight. The proteins were fixed to the gel matrix by acetic acid which is in Coomassie blue staining solution. Next day, the Coomassie solution was discarded. Then the gels were washed in water several times to reduce the background color. The protein size could be determined by comparing the visible protein bands to the protein marker.

3.2.7 Western blot

For western blot analysis, the proteins were transferred from the SDS-PAGE to the nitrocellulose membrane, where the proteins could be analyzed by specific antibody detection. According to the transfer direction determined by the western blot transfer chamber, the polyacrylamide gel, soaked in 1 × EMBL was placed on top of the nitrocellulose membrane which was previously placed on top of three 1 × EMBL saturated Whatman papers. The stack was covered with another three Whatman papers that had also been immersed in 1 × EMBL. The transfer was then performed at 150 mA/gel for 75 min to ensure an adequate transfer of all proteins to the nitrocellulose membrane. To block remaining

protein binding sites on the nitrocellulose membrane, the membrane was incubated in 5% skim milk dissolved in 1 × PBS-T at RT for 1 hour. Meanwhile, the primary antibody, directed against the protein of interest, was diluted in 5% skim milk PBS-T, according to manufacturer´s instructions. The antibody solution was then applied to the membrane and incubated either at RT for 1 hour or at 4 °C overnight. After incubation with the primary antibody, excess antibody was removed by washing the membrane three times with 1 × PBS-T for 1 min. The corresponding secondary antibody conjugated with horseradish peroxidase (HRP) was diluted 1:10,000 in 5% skimmed milk PBS-T and added to the membrane (room temperature for 1 hour). The washing steps were repeated after incubation of the secondary antibody to remove unbound antibodies from the membrane. To detect the protein solution A, containing luminol plus enhancer and solution B, a peroxide solution of the chemiluminescence kit were mixed in a 1:1 ratio and the membrane was incubated in the substrate solution for 1 min. The position of the bound secondary antibody and therefore indirectly the protein of interest were visualized using light sensitive ECL films exposed to the emitted light of the luminescence reaction.

3.2.8 Chemical reduction of proteins

The proteins which were going to be used in mice or guinea pig immunization, were all treated with 1mM DL-Dithiothreitol (DTT).

3.3 Antigens production in insect cells.

3.3.1 Generation of recombinant baculovirus

The generation of recombinant baculovirus was performed as previously described with some modification (Vinzon et al., 2014). Briefly, recombinant baculovirus was produced by transfecting Sf9 insect cells with recombinant

MultiBac bacmids via calcium phosphate precipitation method. At the time of transfection, the optimal confluency for adherent Sf9 cells was 75% in 25 cm^2 flask. After washing, the medium of the cells was replaced by 1 ml Grace's insect medium supplemented with 1% L-glutamine. The MultiBac bacmid DNA (1 µg) was diluted in 1 ml transfection buffer and addeddropwise to the Sf9 cells. After incubation at 27 °C for 5 hours, the cells were washed twice and then maintained in 10 ml TNM-FH supplemented medium for 6 days. The supernatant was used to generate of a high-titer baculovirus stock. Sf9 cells with a density of 2×10^6 were seeded in a T25 flask and infected with 1 ml supernatant for virus amplification over 6 days. This step was repeated by passaging the cells to increase the virus in the supernatant for at least two weeks (3 ml medium for 1×10^7 Sf9 cells in a 75 cm^2 flask and 5 ml medium for 2.5×10^7 Sf9 cells in 175 cm^2 flask).

3.3.2 Expression test of Recombinant Baculovirus

Sf9 cells in a 25 cm^2 flask were infected with 1 ml supernatant from Passage 2 and incubated for 3 days at 27 °C. Proteins were extracted and the expression of different nanoparticle antigens was analyzed by SDS-PAGE with Coomassie blue staining and western blot.

3.3.3 Nanoparticle antigens production and purification.

TN-High Five cells were cultivated to a density of 2.5×10^6/ml in 250 ml suspension culture. The cells were pelleted and resuspended in 42 ml EX-CELLTM 405 serum free medium and 8ml high titer virus stock. The cells were thereafter shaken at a low speed for 1 hour at room temperature and then incubated within 250 ml final volume of medium for 3 days at 27 °C. Cell pellets were harvested by centrifugation (4,000 rpm, for 10 minutes at 4 °C in Sorval GS-3 rotor) and washed in pre-chilled 1 × PBS twice. Dry pellets were resuspended in 10 ml insect cell extraction buffer containing 200 µl 100 mM

PMSF, and then followed by sonication three times. A two-step gradient consisting of 7 ml of 40% sucrose on the top of 7 ml of CsCl solution (5.8g CsCl in 8ml insect cell lysis buffer) was prepared. Clear lysate was obtained by centrifugation (10,000 rpm for 10 minutes at 4 °C in Sorvall F-28/50 rotor) and carefully loaded onto the top of CsCl. After centrifugation (27,000 rpm for 3 hours at 10 °C in SW-31Ti rotor), the interphase between sucrose and CsCl together with the complete CsCl layer was transferred into a Quickseal tube. The fraction was collected in 1 ml aliquot after 16 hours centrifugation at 48,000 rpm at 20 °C in a Beckman 70Ti rotor and analyzed by SDS PAGE with Coomassie blue staining and western blot. Small aliquots from the fractions with the highest and lowest protein yield were dialyzed against H_2O on a membrane filter and sent for later Electron Microscopy, Size exclusion chromatography and DLS.

3.4 Dynamic Light Scattering (DLS)

The machine was turn on in advance and set at 4-60 °C to let it warm up. The nanoparticle antigens were prepared at concentration of 1 mg protein/ml. The samples were filtered through 0.1 µm filter and placed on ice. A water and buffer baseline was measured before measurement of the samples. Then 30 µl of samples were applied to the cuvette and the cuvette was cleaned properly with lens paper to remove any dust. The cuvette was inserted into the sample holder with the forested side to the left. The samples were equilibrated at least 15 minutes, then tested and repeated it at least 5-10 times.

3.5 Electron microscopy (EM)

The micrographs were taken with Zeiss EM912 and EM910 electron microscopy by Dr. Michelle Neßling or with the help of Dr. Karsten Richer and Dr. Michelle Neßling.

3.5.1 Negative staining

The nanoparticle antigens were visualized by negative staining in electron microscopy. Samples were applied on carbon coated grids and stained with 2% uranyl acetate.

3.5.2 Immune staining

The carbon-coated Formvar grids were activated by glow-discharge at 400 mesh Au and grids placed on the nanoparticle drop to adsorb particles for 10 minutes, then grids washed 3 times by passing through drops of 20 mM Tris. The protein-block PB Aurion blocking agent was used to coat gold conjugates full strength for 15 minutes then incubate with primary antibody anti-L2 antibody in the dilution of 1:50 with 20 mM Tris with protein-block PB for 30 minutes at room temperature. For the control group, the grids went through the same procedure without antibodies. After the incubation, the grids were washed in 20 mM Tris with protein-block 6 times, 3 minutes per wash. They were then incubated with IgG (rabbit anti mouse) in 1:150 dilution for 30 minutes at room temperature and washed 6 times with 20 mM Tris with protein-block. Next, they were incubated with PAG-10nm at 1:50 with 10% protein-block in 20 mM Tris for 30 minutes at room temperature follow 3 washes with protein-blocker and 3 times without protein-blocker. Afterwards, the reporter was fixated with Glutaraldehyde: 1% in 20 mM Tris for 7 minutes, then passed through 20 mM Tris. Finally, the negative staining was conducted.

3.6 Size-exclusion chromatography

Size exclusion chromatograph was carried out with a Superde×200 10/300 GL column using the Aekta purifier 900 FPLC system and operating with Unicorn 5.0. The flow rate was set at 0.5 ml/min, and the maximum pressure was 1.5 MPa. The running buffer was 1 × PBS, and the proteins were eluted with 2

column volumes (48 ml) of running buffer. During the elution, 1 ml fractions were collected, and stored at 4 °C for later analyzed with Bradford assay and SDS-PAGEs.

3.7 Formulation of nanoparticle antigens and immunization of mice and guinea pigs

3.7.1 Formulation of nanoparticle antigens with AddaVax

Antigens were formulated with IFA or AddaVax by v/v 50%. The adjuvanted mixture was vortexed until a homogenous solution formed.

3.7.2 Formulation of nanoparticle antigens with Alum

Antigens were adsorbed by Alum before administration. The Alum used in the underlying work was the Alhydrogel ‘85’ (aluminum hydroxide) from Brenntag which had a concentration of 10 mg/ml. The Alum was added to the nanoparticle antigen in a 1.5 ml reaction tube prepared under rotation on a wheel for 1 hour at room temperature.

3.7.3 Formulation of nanoparticle antigens with Alum- MPLA

For vaccine preparation with Alum-MPLA, the antigen and Alum were adsorbed independently of the Alum and MPLA and only right before administration the 2 adsorption reactions were combined. The MPLA was a synthetic, lyophilized preparation from Avanti Lipids of which we usually ordered 5 mg and prepared a 1 mg/ml solution. The preparation of this 1 mg/ml MPLA solution will be describe first followed by the adsorption of antigen-Alum and Alum-MPLA.

A 0.5% (v/v) solution of Triethanolamine (TEoA) in sterile, Endotoxin-free water was prepared. This solution was filter-sterilized through a 0.22 µm filter. To a 5 mg vial of lyophilized MPLA ~1.5 ml of 0.5% TEoA were added and subsequently heated for 5 minutes in a 65 °C water bath followed by 5 minutes

of sonication in a large water bath. Approximately 6-8 cycles of this heating and sonication were performed until the solution became clear and slightly opalescent. In between each cycle the sample was vortexed vigorously. The 1.5 ml dissolved MPLA were transferred to a sterile 15 ml reaction tube, the volume was filled up to 4.85 ml with 0.5% TEoA, one last cycle of heating and sonication was performed and finally the pH was adjusted to ~7.4 by adding 1 M HCl dropwise. Usually, 30 µl 1 M HCl per 1 ml MPLA-TEoA were required. The MPLA was stored at 4 °C and was stable for at least 6 months.

Mice were usually immunized with 50 µg Alum and 10 µg MPLA. Half of the Alum was added to the nanoparticle antigen while the other half was combined with the MPLA. The separate adsorption reactions were prepared in 2 ml reaction tubes under rotation on a wheel for 1 hour at room temperature. Before intramuscular administration in mice, the two adsorption reactions were combined and briefly mixed by inversion.

3.7.4 Immunization of mice and guinea pigs with nanoparticle antigens.

6-8 weeks old BALB/c female mice were immunized with 20µg antigen in 50 µl of formulated nanoparticle antigens intramuscularly into the left or right caudal thigh muscle. Mice were immunized 3 or 4 times normally, with 2 week intervals. One week after the third immunization, intermediate blood was collected and 4 weeks after last immunization final blood was collected. Mice groups typically contained 8-10 mice.

350-400g female Guinea pigs were immunized with 200 µl of formulated nanoparticle antigens subcutaneously at the left or right flank. They were immunized 3 or 4 times with 2 weeks intervals as well. One week after the third immunization, intermediate blood was collected and 4 weeks after the last immunization final blood was collected. Guinea pig groups typically consisted of 2 animals.

3.7.5 Blood samples in mice and guinea pigs

Intermediate blood samples were collected by the submandibular vein punch at one week after third immunization. Normally, only 200-250 µl of blood could be collected by this way. Four weeks after the final immunization, the final blood sample was collected by cardiac puncture.

Blood samples were incubated at room temperature for 2-3 hours or at 4 °C overnight. They were centrifuged at 4,500 rpm and 4 °C for 15 minutes Cleared serum was transferred to a new sterile 1.5 ml Eppendorf tubes. Two rounds of centrifugation were carried out. Sera were stored at 4 °C or -20 °C for long term preservation.

3.8 Cultivation and manipulation of cells

3.8.1 Cultivation of Mammalian cells

Mammalian cells were grown at 37 °C in an incubator with 5% CO_2 and 95% humidity. HEK 293TT and HeLaT K4 cells were grown in DMEM supplemented with 10% FCS, 1% Penicillin/Streptomycin and 1% L-glutamine. CHOΔfurin and pgsa-745 cells were cultivated in DMEM supplemented with 10% FCS, 1% Penicillin/Streptomycin and 200 µM proline while MCF10A cells were grown in DMEM: F12 medium supplemented with 5% horse serum, 1% Penicillin/Streptomycin, 1% L-glutamine, 500 ng/ml hydrocortisone, 10 µg/ml insuline and 20 ng/ml EGF. The HEK 293TT and HeLaT K4 cells were also selected with Hygromycin B diluted 1:800 and 1:400, respectively. Mammalian cells were cultivated in 150 cm^2 and 75 cm^2 culture flasks with medium volumes of 30 ml and 20 ml, respectively.

Cells were passaged every 3-4 days according to cell density. The medium was aspirated, and the cells were treated with 4 ml of 0.05% or 0.25 % trypsin-EDTA for ~5 minutes to disrupt the intercellular contacts and allow for detachment.

Cells were resuspended in the trypsin-EDTA by gently pipetting up and down before transferring them to 15 ml centrifuge tubes filled with 10 ml medium. The cells were centrifuged at 1,900 rpm and room temperature for 5 minutes. The medium was aspirated and the cell pellet suspended in 10 ml fresh medium of which 1-2 ml were transferred to a cell culture flask with fresh medium. Cells were allowed to grow until further dissemination.

3.8.2 Cultivation of insect cells

Insect cells for the nanoparticle antigens production were grown at 27 °C in an incubator without CO_2 and humidity restriction. Sf9 cells for recombinant baculovirus production were maintained in TNM-FH insect medium supplemented with 10% FCS, 1% Penicillin/Streptomycin, 1% L-glutamine and 0.1% Pluronic F-68. TN-High Five cells for baculovirus expression were cultivated in EX-CELLTM 405 serum free medium supplemented with 1% Penicillin/Streptomycin and 1% L-glutamine.

Adherent cells were detached by cell scraper and passaged at a ratio of 1:3. For cells grown in suspension, 50 ml of medium containing at 1×10^6 viable cells/ml were seeded with 200 ml supplemented medium in the spinner flask and cultivated with constant shaking speed of 125-150 rpm at 27 °C. Cell densities were measured by using a standard trypan blue dye exclusion procedure and counting under the microscope. After two or three days, when cell densities reached 1×10^6 viable cells/ml, cells were seeded into the culture flasks according to different amount requirements.

3.8.3 Determination of cell count and viability

Trypan blue (which stains only dead cells) and a Neubauer counting Chamber were used for determining the cell count and viability. To determine the number of viable cells, 10 µl of a cell suspension were mixed with 10 µl trypan blue. 10

µl of the mix were pipetted into the Neubauer counting chamber and viewed under a light microscope. Viable, unstained cells in 2 quadrants, each with 16 inner squares, were counted, and a mean cell number was calculated. The cell numbers per ml can be calculated as follows:

Cell number/ml= $X \times 2 \times 10^4$ (X: cell number; 2: dilution factor)

3.8.4 Cryo-conservation and thawing of cells

For cryo-conservation, approximately 1×10^7 mammalian cells were harvested by centrifugation, the pellet was resuspended in 1 ml ice-cold freezing medium, and the cells were transferred to 2 ml cryo vials. The cells were slowly cooled to -80 °C overnight in a cryo box. The vials were transferred to the liquid nitrogen tank for long-term storage.

For cryoconservation of insect cells, a cell pellet of approximately 1×10^7 cells was resuspended in 1 ml of Cryo Medium (10% FCS,10% DMSO, 80% TNM-FH supplemented insect medium for Sf9 cells, for High5 cells,10% DMSO and 90% EX-CELLTM 405 supplemented serum free medium) into a cryotube, which was then wrapped in several layers of papers and transferred to -80 °C. For longer storage, cells were transferred to liquid nitrogen after one day.

For re-culturing cells, the cryo vials were placed in a 37 °C water bath until 2/3 of the content was thawed. Using a glass Pasteur pipette the cells were pipetted up and down until complete thawing was achieved and the entire content was transferred to a centrifuge tube containing 10 ml appropriate cell culture medium. After centrifugation at 1,900 rpm and room temperature for 5 minutes, the medium was aspirated and the pellet resuspended in fresh medium and transferred to cell culture flasks.

3.8.5 HPV pseudovirion production and purification

On the first day, 3-4×10^6 293TT cells per 10-cm dish were seeded in 10 ml DMEM, and ten 10-cm dishes were prepared for a standard pseudovirion preparation. Approximately 24 hours after seeding the cells, transfected with 10-15 µg DNA (5 µg reporter plasmid + 5 µg L1 plasmid + 5 µg L2 plasmid) and 2 volumes of TurboFect in 1 ml of unsupplemented DMEM per 10-cm dish The transfection reaction was incubated for 15-20 minutes at room temperature and subsequently added dropwise to the 293TT cells. The cells were incubated for 72 hours at 37 °C and 5% CO_2 after transfection. The 293TT cells were harvested by resuspension and transferred to 50 ml centrifuge tubes. After centrifugation at 1,900 rpm and room temperature for 5 minutes, the supernatant was discarded, the cell pellet resuspended in 1 ml DPBS and transferred to a sterile 1.5 ml reaction tube (#1). After centrifuging in a table-top centrifuge for 5 minutes at 5,000 rpm, the supernatant was discarded and the pellet resuspended in approximately 150 µl of lysis buffer containing 140 µl DBPS, 9 µl 10% Brij58 (w/v) and 1 µl RNase A/T. Cells were rotated overnight at 37 °C to induce pseudovirion maturation. On the next day, the PSV were extracted. The 1.5 ml reaction tubes containing the PSV lysate were placed on ice for 5 minutes before 0.17 volume of 5 M NaCl was added to the samples and another 5 minutes incubation on ice followed. Then, the samples were centrifuged at 10,000 rpm and 4 °C for 10 minutes, the PSV-containing supernatant transferred to a second 1.5 ml reaction tube (#2) and the pellet re-suspended in 300 µl DPBS with 0.8 M NaCl. The samples were centrifuged again at 10,000 rpm and 4 °C for 10 minutes before transferring the supernatant to tube #2. Next, 1 µl benzonase were added to the cleared supernatant and the sample was incubated at 37 °C for 1 hour. The sample was centrifuged once more at 10,000 rpm and 4 °C for 10 minutes before the supernatant was transferred to a sterile 1.5 reaction tube. Approximately 50 µl of this PSV-

containing crude extract were set aside and stored at -80 °C while the rest was immediately purified by OptiPrep.

For the OptiPrep purification three initial solutions with 27%, 33% and 39% iodixanol were prepared by diluting the original iodixanol solution (60%) in DPBS with 0.8 M NaCl. Phenol red was added to the 39% solution (50 µl phenol red to 10 ml solution). In centrifuge tubes for the SW41Ti rotor 3.2-3.3 ml of each solution were added starting with 39% on bottom, 33% in the middle and the 27% on top. The tubes were left at 4 °C overnight to allow softening of the gradient edges. The gradients were prepared on the same day the 293TT cells were harvested and lysed. PSV-containing crude extract was applied on top of the 27% solution and tubes were balanced to 0.00 g using DBPS with 0.8 M NaCl. The gradients were centrifuged for 5 hours at 37,000 rpm and 16 °C in an ultracentrifuge. Approximately eight 500 µl fractions were collected by inserting a sterile needle 1 cm below the 33%-39% interphase thereby allowing the solution to drip out. PSV were collected in 1.5 ml LoBind reaction tubes and stored on ice. The crude extract as well as the purified fractions were analyzed in the *in vitro* neutralization assay, but in this case infection and not neutralization was measured. The crude extract was usually tested in eight serial 1:2 dilutions starting with a 1:500 dilution while the purified fractions were tested at a fixed 1:1,000 dilution.

3.9 *In vitro* and *in vivo* HPV pseudovirion-based neutralization assays

3.9.1 *In vitro* standard (L1) neutralization assay

The neutralization assay is used to detect neutralizing antibodies in sera against human papillomaviruses. In this assay pseudovirions of different HPV types are used to infect HeLaT cells. Due to the infection, the PSV release a plasmid in the cell encoding the reporter Gaussia luciferase. The extent of

infection can be determined by measuring the activity of secreted Gaussia luciferase in the culture medium upon substrate addition. If neutralizing antibodies are present in the sera, pseudovirion infection and in turn the expression of the Gaussia luciferase is prevented (figure 3.1).

First, serum or antibody dilutions were prepared in supplemented DMEM and 50 µl of these were added to each well of a 96-well tissue culture plate (in duplicates). The outside wells of the plate were left free from samples and 150 µl medium or 1 × PBS were applied here. Next, 50 µl of the PSV diluted in DMEM were added to each sample well. While the serum-PSV complex incubated for at least 20 minutes at room temperature, the HeLaT K4 cells were harvested and 50 µl of cell suspension were added to each well (2.5×10^5 cells/ml). The tissue culture plates were lightly wrapped in plastic foil, placed in a moist melamine chamber and incubated at 37 °C and 5% CO_2 for 48 hours.

On the day of the reporter gene assay, 10 µl of cell culture supernatant were transferred from the tissue culture plates to white luminescence plates. For preparation of the substrate, Coelenterazine was combined with the Gaussia glow juice 1 × PBS (100 µl Coelenterazine + 10 ml 1 × PBS) and 100 µl of this mix was added to each well of the luminescence plate already containing 10 µl cell culture supernatant. A microplate luminometer was used to measure the samples 15 minutes after substrate addition.

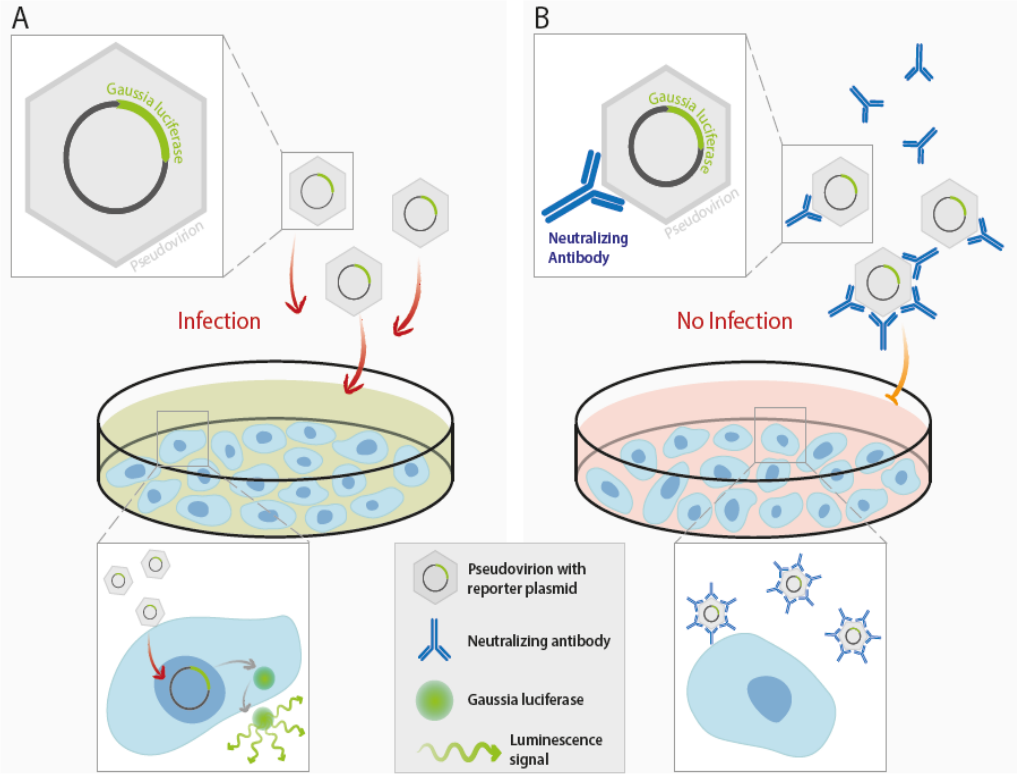


Figure 3.1 The theory of L1-based neutralization assay (Pinto et al., 2018). The assay uses pseudovirions that incorporated the minor capsid protein L2 and the major capsid protein L1, with the reporter gene Gaussia Luciferase. The PSV bind to and enter into HeLa T cells and the reporter plasmid is translocated to the nucleus and expressed A). If neutralizing antibodies are present, PSV infection is blocked with concomitant reduction in reporter signal B).

3.9.2 *In vitro* L2 neutralization assay

The L2 neutralization assay was prepared over the course of 8 days and entailed the production of an extracellular matrix (ECM), furin cleavage of HPV PSV for L2 epitope exposure, antibody incubation and infection of HSPG-deficient target cells. On day 1, 1×10^6 CHOΔfurin cells were seeded in a 75 cm^2 tissue culture flask containing 17 ml medium. These cells were allowed to grow to confluence over the course of four days. On day 3 or the morning of day 4, MCF10A cells were plated on 96-well tissue culture plates with a density of 2×10^4cells/well in 100 µl. On the morning of day 5 the ECM was prepared from

the MCF10A cells. First, the medium was removed from the cells and these were washed 3 times with 100 µl 1 × PBS. Next, 50 µl pre-warmed (room temperature) lysis buffer were added to each well and exactly 5 minutes of incubation at room temperature followed before 100 µl of 1 × PBS were gently added on top of the lysis buffer by steadying the pipette tips along the side walls of the wells. Then, 100 µl of liquid were carefully removed from the cells and fresh 100 µl of 1 × PBS were added. This removing and adding of 100 µl liquid was repeated one more time before all of the liquid was carefully removed and the ECM left behind on the plate was washed two times with 100 µl 1 × PBS. The 1 × PBS from the second wash remained on the plate until the next step; in fact it was of utmost importance to not aspirate the ECM at this time. Next, the cell culture supernatant from the CHOΔfurin cells was decanted into a centrifuge tube, centrifuged at 1,900 rpm and room temperature for 5 minutes and the cleared furin-containing supernatant was set aside. Heparin was added to the supernatant at an end concentration of 8 µg/ml. The 1 × PBS was removed from the ECM and 70 µl of the furin + heparin supernatant was added to each well. The HPV PSV were diluted in L2 assay medium and 50 µl of the dilution were added to each well already containing 70 µl furin + heparin supernatant. The reaction was incubated overnight at 37 °C and 5% CO_2. On day 6 the supernatant-virus mix was removed from the wells, these were rinsed twice with 100 µl 1 × PBS and again, the PBS from the second wash remained on the plate until the next step. The antibody titrations were prepared with L2 assay medium in separate 96-well U-bottom plates to have 120 µl of each dilution. The 1 × PBS was removed from the ECM-virus plate and 100 µl of the antibody dilutions were added to each well. The reaction was incubated for 2-6 hours at 37 °C and 5% CO_2. Finally, the pgsa-745 cells were harvested by trypsinization with 0.05% Trypsin-EDTA and centrifugation at 1,900 rpm and room temperature for 5 minutes. The cell pellet was resuspended in L2 assay

medium, the cell count was adjusted to 1.6×10^5/ml and 50 µl of this cell suspension was added to each well already containing 100 µl serum dilution. Incubation at 37 °C and 5% CO_2 succeeded for 48 hours before the Gaussia luciferase reporter gene assay was performed as described in the last section (figure 3.2).

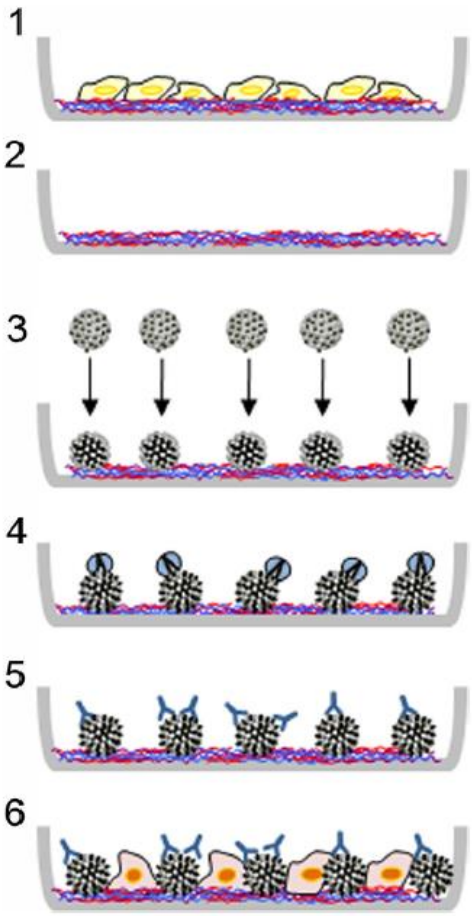


Figure 3.2 Scheme of L2-based neutralization assay (Day et al., 2012). 1, MCF10 cells were plated in a 96 well plates and grown for 24 hours to allow for ECM deposition; 2, the EM remained on the plastic following lysis of the MCF10A cells; 3, Pseudovirus were plated on the ECM, which results in a conformational change in the capsid allowing the N-terminus of L2 expose; 4, exogenous furin which is produced by the CHOΔfurin cells was applied with pseudovirus to allow cleavage of the conserved N-terminal furin cleavage site of L2, which leads to exposure of the major cross neutralizing epitopes in L2; 5, removal the unbound pseudovirus, and antibody titration series is applied to the plate and incubated at 37 °C for 6

hours; 6, HSPG- deficient pgsa-754 cells were plated into the virus/antibody-containing wells. Infection is documented following a 48 hours incubation.

3.9.3 *In vivo* neutralization in cervico-vaginal mouse model

The cervico-vaginal mouse model can be employed to measure the efficiency of L1 or L2-directed antibodies neutralizing *in vivo* transducing HPV PSV. The whole procedure was performed over the course of 8 days (figure 3). On day 1, the cage bedding from males was transferred to the cages of female BALB/c mice to initiate hormonal synchronization of the females. On day 3, 100 µl of a 30 mg/ml Depo-Provera solution was administered subcutaneously to the mice. 100 µl of antibodies or serum (collected from the animals immunized with nanoparticle antigens, and the naïve mice as control) diluted in 1 × PBS were delivered intraperitoneally to each mouse on day 5. On day 5 mice were treated with 4% Nonoxynol-9 (N9) prepared fresh or the day before in 4% CMC in a 50 ml reaction tube. A 20-50 µl positive displacement (PD) MICROMAN® pipette was used to deliver 50 µl N9 intra-vaginally. Approximately 4 hours after N9 treatment HPV PSV was instilled intra-vaginally. For this, 5 µl PSV were mixed with 5 µl 1 × PBS and 10 µl 4% CMC and these 20 µl were applied using a 3-25 µl PD pipette. On day 8 *in vivo* imaging was performed at a Xenogen IVIS imager. Images of the mice were acquired before substrate addition and then 20 µl of 15 mg/ml luciferin substrate were instilled intra-vaginally using a 20 µl pipette. After 3 minutes of incubation an image with 30 seconds - 1 minute of exposure time (and medium binning) was acquired. A region-of-interest (ROI) analysis was performed with the Living Image software and average radiance was selected as the final read-out.

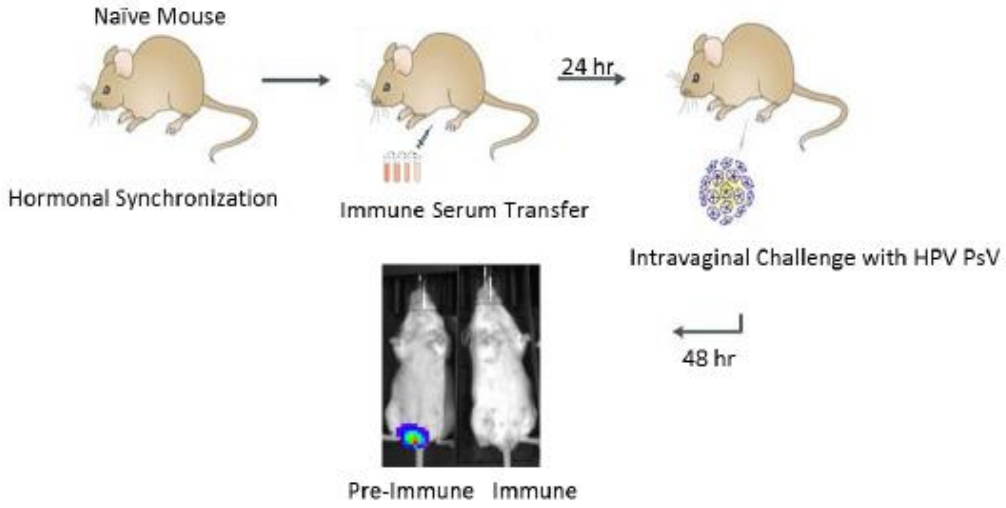


Figure 3.3 *In vivo* cervicovaginal challenge mouse model (Pouyanfard and Muller, 2017). The whole procedure was performed over a course of 8 days. Naïve mice were hormonally synchronized on day 1 by taking bedding from the male cage and subsequently on day 3 by injection with progesterone subcutaneously. On day 5, immune serum (from the animals immunized with nanoparticle antigens, or from the naïve mice as control) are passively transferred intraperitoneally to the naïve mice. On day 6, vaginal epithelium was disrupted by using a surfactant N9 followed by intravaginal administration of HPV PSV encapsulating firefly luciferase 4 hours later. The activity of luciferase was measured on day 8 by IVIS imaging system upon intravaginal instillation of Luciferin.

3.10 Multiplex serology

Multiplex serology was performed with GST-fusion proteins of Pf thioredoxin, human thioredoxin, Pf ferritin and human ferritin, verifying their correct identification. The principle of multiplex serology is illustrated in figure 3.4;

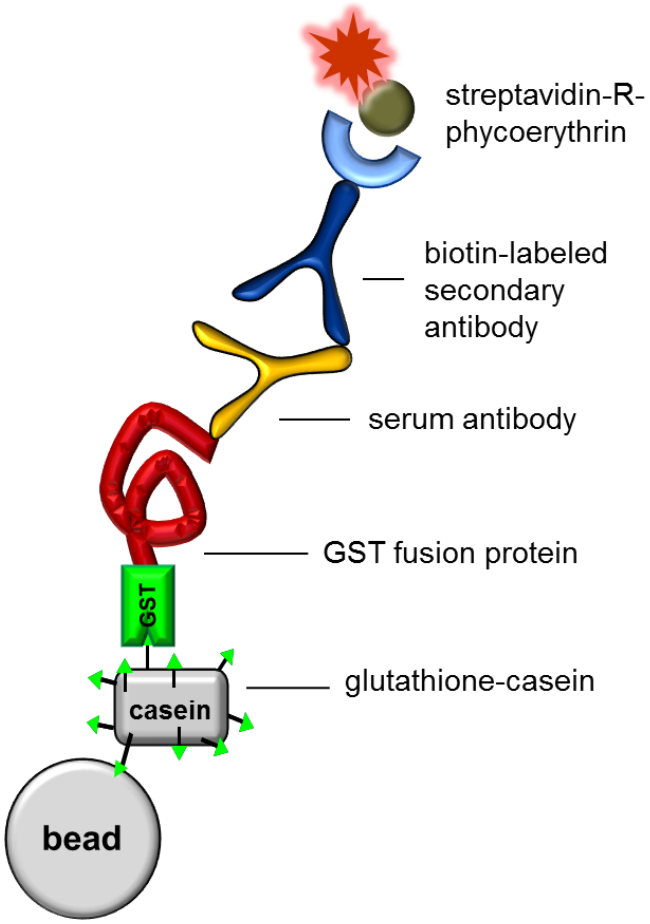


Figure 3.4 Principle of multiplex serology. The proteins were expressed as recombinant GST-fusion proteins and loaded on glutathione-casein coupled spectrally distinct fluorescence-labeled polystyrene beads. Protein loaded beads were mixed and incubated with sera. Sera antibodies bound to protein loaded beads were quantified using a labeled secondary antibody (Hufnagel et al., 2018).

3.10.1 Loading of glutathione-casein beads with GST-fusion proteins.

The Pf thioredoxin, human thioredoxin, Pf ferritin and human ferritin GST-fusion protein lysates were diluted in multiplex serology blocking buffer to a final protein concentration of 1 mg/ml in a total volume of 1 ml. Each lysate dilution was mixed with a specific bead set and incubated for 1 hour at room temperature on a shaker protected from light. Protein-loaded beads were centrifuged at 1,300 rpm for 2 minutes. Then supernatant was discarded and

beads were resuspended in a multiplex serology blocking buffer. This step was repeated three times in order to wash the protein-loaded beads. After the third centrifugation the beads were resuspended in a storage buffer.

3.10.2 Sera pre-incubation

Serum dilutions were prepared in polystyrene flat-bottom plates by diluting serum samples 1:50 in a total volume of 100 µl with serum pre-incubation buffer. Dilutions were incubated at room temperature for 1 hour on a shaker. GST-tag lysate was added to the pre-incubation buffer in order to prevent unspecific binding of antibodies directed against remaining bacterial proteins. In addition PVP and PVA were added to the pre-incubation buffer to suppress unspecific binding of antibodies to the beads.

3.10.3 Equilibration of filter plates

96-well filter plates were incubated with 100 µl multiplex serology blocking buffer per well for 10 minutes at room temperature. Afterwards, the buffer was removed using a vacuum manifold and plates were dried.

3.10.4 Incubation of beads and sera

Protein loaded beads were mixed thoroughly by alternating sonification and vortexing (at least 5 times). All beads were combined in a 100 ml bottle protected from light. 50 µL of the bead mixture were transferred into each well of the equilibrated filter plates. Afterwards 50 µl of 1:50 serum dilution was added to 50 µl bead mix. Plates were incubated on an orbital shaker at 200 rpm for 1 hour at room temperature protected from light.

3.10.5 Incubation of secondary antibody

Serum dilutions were removed from the filter plates using the vacuum manifold. Plates were washed three times with 100 µl multiplex serology blocking buffer

per well and dried afterwards. The secondary antibody was diluted 1:1,000 in multiplex serology blocking buffer, added to the plates and plates were incubated on an orbital shaker at 200 rpm for 1 hour at room temperature protected from light.

3.10.6 Incubation with Strepavidin-R-Phycoerythin (StrepPE)

The secondary antibody was removed from the filter plates by the vacuum manifold. Three washing steps were performed using 100 µl multiplex serology blocking buffer per well and plates were dried afterwards. Strep-PE was diluted 1:750 in multiplex serology blocking buffer and added to the plates. Plates were incubated on an orbital shaker at 200 rpm for 1 hour at room temperature protected from light. Then washed 3 times as described above, followed by the addition of 100 µl of storage buffer per well and plates were stored at 4 °C overnight.

3.10.7 Quantification of serum antibodies using Luminex 200 analyzer

Bead-bound proteins were able to capture serum antibodies. These antibodies were quantified using the Luminex 200 analyzer. The signal obtained by the secondary antibody which bound to the primary serum antibodies, was measured as the median fluorescence intensity (MFI) calculated for each bead set. MFI values were considered to be valid if a minimum of 100 beads per bead set were detected and measured. Net MFI values were generated by subtracting the bead-background and the GST-background. The bead-background was determined by performing a measurement without serum while the GST-background was measured by using one bead set loaded with GST-tag lysate.

4 Results

It is well known that, the commercial HPV VLP vaccines are highly immunogenic and that the neutralizing antibodies induced by the VLPs can last for at least nine years, but the responses are very type-restricted (Herrero et al., 2015). This prompted research on second generation prophylactic HPV vaccines based on the L2 minor capsid protein. L2 can elicit cross neutralizing antibodies, but has low immunogenicity. In recent years, efforts have been made on developing a method to improve the immunogenicity of L2 (Jagu et al., 2009; Jagu et al., 2013; Nieto et al., 2012b; Pouyanfard et al., 2018). In our lab, the immunogenicity of L2 has been improved by employing *Pyrococcus furiosus* thioredoxin (Pf Trx) as a “platform” (Pouyanfard et al., 2018; Seitz et al., 2015; Spagnoli et al., 2017). In the past, we inserted the repetitive HPV16 L2 neutralizing epitope (aa20-33) into the active site loop of Pf thioredoxin, which made the recombinant antigen thermally stable, highly soluble and more immunogenic compared to the small peptide L2 antigen(Seitz et al., 2013), which shows broad protective activity but fails to induce neutralizing antibodies against some of the oncogenic HPV types, such as HPV31, and HPV51. Later, the cross-protective ability of recombinant antigens was improved by replacing 3 copies of HPV16 L2 (aa20-38) with L2 epitopes of eight HPVs (HPV16, 18, 31, 33, 35, 6, 51, and 59), and this antigen was thereby able to cover all the high-risk and two low-risk mucosal HPVs, though the antibody titers were much lower than commercial VLPs vaccines. Thereafter OVX313 was employed as a secondary scaffold, a heptametrical form, in order to further improve the immunogenicity of multimeric antigens, which was proven to be quite successful (Pouyanfard et al., 2018; Spagnoli et al., 2017). In order to efficiently produce neutralizing antibodies, the B cells must be activated and there are certain requirements for B cells activation. At first, the antigens need to bind to

B cell receptors and cross link B cells receptors. Secondly, the same antigens need to activate T helper cells for the co-stimulation. Thirdly, there are cytokines provided by T helper cells which also assist in the activation of B cells. To further optimize the minor capsid protein L2 vaccines, we tried to employ the nanoparticle, *Pyrococcus furiosus* ferritin and encapsulin as the platform to display the L2 epitopes (aa20-38). By using the suitably sized nanoparticles, the cross-link of B cell receptors would not only trigger maximum activation, but also improve uptake by APCs.

4.1 Optimization of L2-based HPV multimeric antigens

Previous studies of our group had already proven that the tripeptide of aa20-38 L2 sequence of HPV16 inserted into the active loop of Pf Trx is more immunogenic than the monomeric L2 antigen, but the multimeric peptide antigen of L2 (aa20-38) epitopes from 8 different HPVs was even better than the tripeptide. Previous research showed that the size of the antigen related to its immunogenicity, like L1 VLPs are more immunogenic than capsomeres and monomeric counter parts (Schadlich et al., 2009; Thones et al., 2008). To display the multimeric peptide antigens on the surface of self-assembling nanoparticles, two potential platforms, ferritin and encapsulin, were employed. Both of them are from the same strain as the primary platform Pf thioredoxin. 24 identical subunits of Pf ferritin form an octahedral cage, and 60 subunits of Pf encapsulin assemble into an octahedral cage. Attachments to the N-terminus of Pf ferritin are then displayed evenly on the nanoparticle surface, and any protein attached on the COOH-terminal of encapsulin subunits is also presented on the nanoparticle surface. The HPVs L2 (aa20-38) epitopes are used in either monomeric (HPV16 L2), trimeric (HPV16 L2*3) or multimeric peptides (HPV16, 18, 31, 33, 35, 6, 51, and 59) and are fused with and without Pf Trx, either on the NH_2-terminus of Pf ferritin or on the COOH-terminus of

encapsulin, respectively (figure 4.1). Antigens were designed with different numbers of L2 epitopes because it was uncertain whether larger insertions would interfere with the nanoparticle assembly.

To understand the assembly abilities of nanoparticle antigens, the baculovirus expression system had been employed here. The insect cell-based system is more complicated than *E. coli* system but it is endotoxin free, and allows superstructure formation. After the production of the recombinant baculovirus, the un-purified nanoparticle antigens were analyzed by SDS-PAGE (figure 4.2).

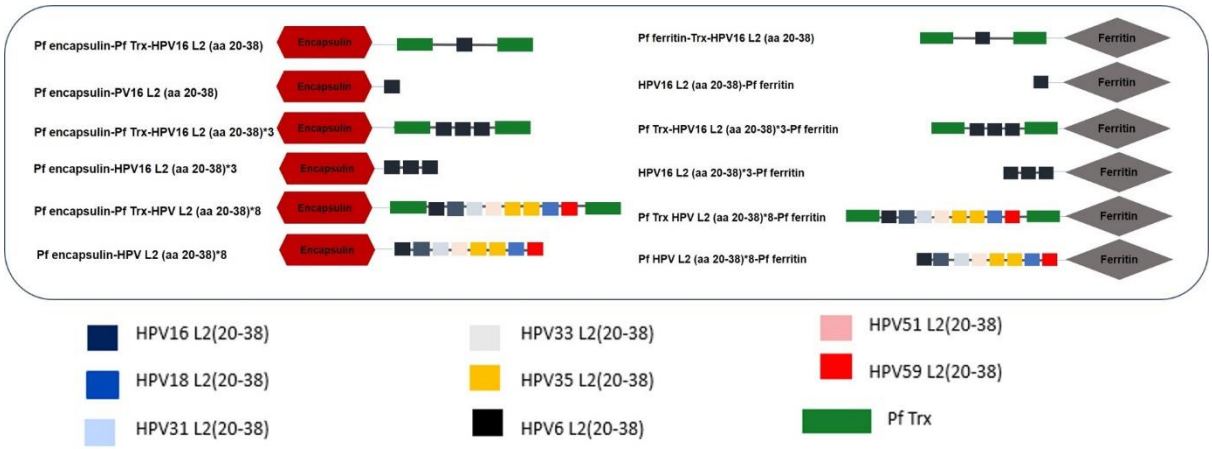


Figure 4.1 Molecule design of L2-based nanoparticle antigens. In the left panel the encapsulin constructs of L2-based HPV antigens are shown with or without Pf Trx. In the right the ferritin analogs are depicted. The construct contained, 1 copy of HPV16 L2 (aa20-38), 3 copies of HPV16 L2 (aa20-38), or the L2 (aa20-38) sequences of HPVs (HPV16, 18, 31, 33, 35, 6, 51, and 59) fused into C-terminus of encapsulin, with or without insertion into the activing loop of Pf Trx,

For the purification, different structures behaved differently. The simplest structures was tried first, such as native Pf encapsulin and Pf ferritin, using a heat purification to enrich for the proteins. It was found that the samples were highly soluble, and stable even when treated at 80 °C. After conducting the heat treatment, the proteins were separated by SDS-PAGE, then analyzed by western blot with K4 or K18 antibody (L2 antibodies that can recognize aa 20-

38 of most HPVs L2 contained in the structure) to verify the L2 epitopes, and with a monoclonal antibody against the Pf Trx scaffold (data not shown). The purity of all the antigen candidates reached 70-80%, the L2 epitopes with L2 antibody, and Pf Trx structure with a monoclonal antibody against the Pf Trx scaffold were confirmed with western blot (data not shown). The purity of all antigen was considered sufficient for the subsequent immunization experiments (figure 4.3).

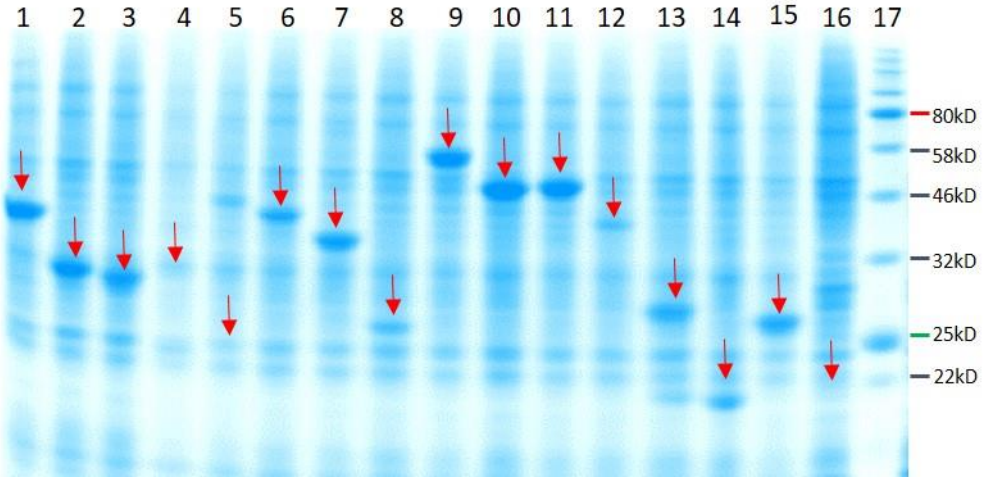


Figure 4.3 SDS-PAGE analyses of nanoparticle antigens. After the production of baculovirus of each potential nanoparticle antigens, small scale of H5 insect cells infections were performed to determine the quality and correctness of the baculovirus constructs, the red arrows point out the target bands. The samples of lanes 1 to 16 are listed in the following table.

Lane	Full name	Abbreviation	Size
1	Pf encapsulin-Pf Trx-HPV16 L2 (aa 20-38)	Pf EnTrx1mer	43.3 kD
2	PfTrx-HPV16 L2 (aa 20-38)-Pf ferritin	Pf FTrx1mer	35 kD
3	Pf encapsulin-PV16 L2 (aa 20-38)	Pf En1mer	32 kD
4	HPV16 L2 (aa 20-38)-Pf ferritin	Pf F1mer	23.6 kD
5	Pf encapsulin-Pf Trx-HPV16 L2 (aa 20-38)*3	Pf EnTrx3mer	48 kD
6	Pf Trx-HPV16 L2 (aa 20-38)*3-Pf ferritin	Pf FTrx3mer	40 kD
7	Pf encapsulin-HPV16 L2 (aa 20-38)*3	Pf En3mer	36 kD
8	HPV16 L2 (aa 20-38)*3-Pf ferritin	Pf F3mer	28 kD
9	Pf encapsulin-Pf Trx-HPV (HPV16, 18, 31, 33, 35, 6, 51, and 59) L2 (aa 20-38)	Pf EnTrx8mer	58.8 kD
10	Pf Trx-HPV(HPV16, 18, 31, 33, 35, 6, 51, and 59) L2 (aa 20-38)-Pf ferritin	Pf FTrx8mer	50.2 kD
11	Pf encapsulin -HPV (HPV16, 18, 31, 33, 35, 6, 51, and 59) L2 (aa 20-38)	Pf En8mer	47 kD
12	HPV(HPV16, 18, 31, 33, 35, 6, 51, and 59) L2 (aa 20-38) –Pf ferritin	Pf F8mer	38.8 kD

13	Pf encapsulin with CpoI site (with RsRII restrict enzyme cutting site)	Pf En-CpoI	29 kD
14	Pf ferritin with CpoI site (with RsRII restrict enzyme cutting site)	Pf F-CpoI	21 kD
15	native Pf encapsulin	Pf En	28 kD
16	native Pf ferritin	Pf F	20 kD
17	Marker		

After heat treatment, samples were characterized by EM, size exclusion chromatography (SEC), and dynamic light scattering (DLS) to determine whether the fusion protein would influence the self-assembling ability of nanoparticles. The nanoparticle structures of Pf EnTrx3mer, Pf FTrx3mer, Pf En3mer, Pf F3mer were observed by electron microscopy (EM), but impurities were detected, such as subunits and aggregations; the size exclusion chromatography and DLS backed up this finding. All analyses suggested these antigens could not assemble properly into nanoparticles after heat treatment, even at lower temperature such as 55°C. Changing the condition of purification and including an assembly step after the purification was not successful. Therefore, for these specific structures, CsCl gradients were employed for the purification. In the end, all L2-based nanoparticles antigens were efficiently produced with 70-80% purity. However, how heat treatment disrupted the assembly ability of theses antigen remains unclear.

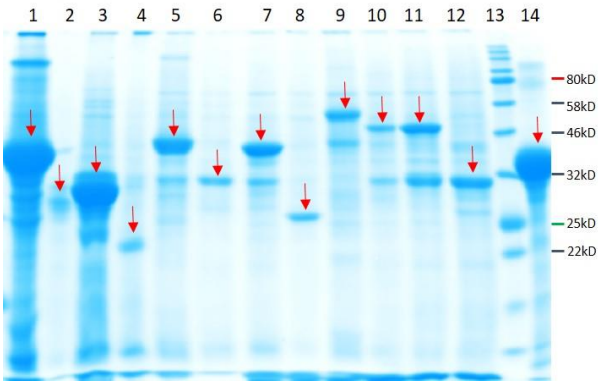


Figure 4.3 Purity checked by SDS-PAGE; Antigens were analyzed by SDS-PAGE with Coomassie blue staining. Samples in lanes 1 to 14 are as follows:

Lane	Full name	Abbreviation	Size
1	Pf encapsulin-Pf Trx-HPV16 L2 (aa 20-38)	Pf EnTrx1mer	43.3 kD
2	Pf Trx-HPV16 L2 (aa 20-38)	Pf FTrx1mer	35 kD

3	Pf encapsulin- HPV16 L2 (aa 20-38)	Pf En1mer	32 kD
4	HPV16 L2 (aa 20-38)-Pf ferritin	Pf F1mer	23.6 kD
5	Pf encapsulin-Pf Trx-HPV16 L2 (aa 20-38)*3	Pf EnTrx3mer	48 kD
6	Pf Trx-HPV16 L2 (aa 20-38)*3-Pf ferritin	Pf FTrx3mer	40 kD
7	Pf encapsulin-HPV16 L2 (aa 20-38)*3	Pf En3mer	36 kD
8	HPV16 L2 (aa 20-38)*3-Pf ferritin	Pf F3mer	28 kD
9	Pf encapsulin-Pf Trx-HPV (HPV16, 18, 31, 33, 35, 6, 51, and 59) L2 (aa 20-38)	Pf EnTrx8mer	58.8 kD
10	Pf Trx-HPV(HPV16, 18, 31, 33, 35, 6, 51, and 59) L2 (aa 20-38)-Pf ferritin	Pf FTrx8mer	50.2 kD
11	Pf encapsulin -HPV (HPV16, 18, 31, 33, 35, 6, 51, and 59) L2 (aa 20-38)	Pf En8mer	47 kD
12	HPV(HPV16, 18, 31, 33, 35, 6, 51, and 59) L2 (aa 20-38) –Pf ferritin	Pf F8mer	38.8 kD
13	marker		
14	HPV(HPV16, 18, 31, 33, 35, 6, 51, and 59) L2 (aa 20-38)-Pf Trx-OVX313	Pf Trx8mer OVX313	36 kD

4.2 Nanoparticle antigens retain their assembly ability after protein fusion.

The delivery systems for the antigens were designed to mimic the properties of the pathogens with the aim of increasing the immunogenicity of the antigens (Jennings and Bachmann, 2007). In this thesis, I tried to use nanoparticles as a delivery system to have the similar dimensions as the HPV virus, such as Pf ferritin and Pf encapsulin.

After purification of the candidates of nanoparticle antigens, SDS-PAGE, negative transmission electron microscopy (TEM), dynamic light scattering (DLS) analysis, and size exclusion chromatography (SEC) analysis were performed.

EM analysis revealed that Pf EnTrx1mer, Pf FTrx1mer, Pf En1mer, Pf F1mer, Pf EnTrx3mer, Pf FTrx3mer, Pf En3mer, Pf F3mer, Pf encapsulin-Cpo, and Pf ferritin-Cpo , the native Pf encapsulin; and the native Pf ferritin after heat treatment, formed smooth spherical cage structures and the size of these nanoparticles in the same antigen sample were uniform (figure 4.4). However

there were also a large number of subunits present as well (figure 4.4). For further investigation and to prove that the L2 epitopes are displayed on the surface of the nanoparticles, an immune transmission electron microscopy (TEM) was performed. The L2 epitopes were indeed displayed on the nanoparticle surface as shown by immune EM with anti-L2 antibody K4, detected with anti-mouse IgG (rabbit) which is coupled with gold (figure 4.5).

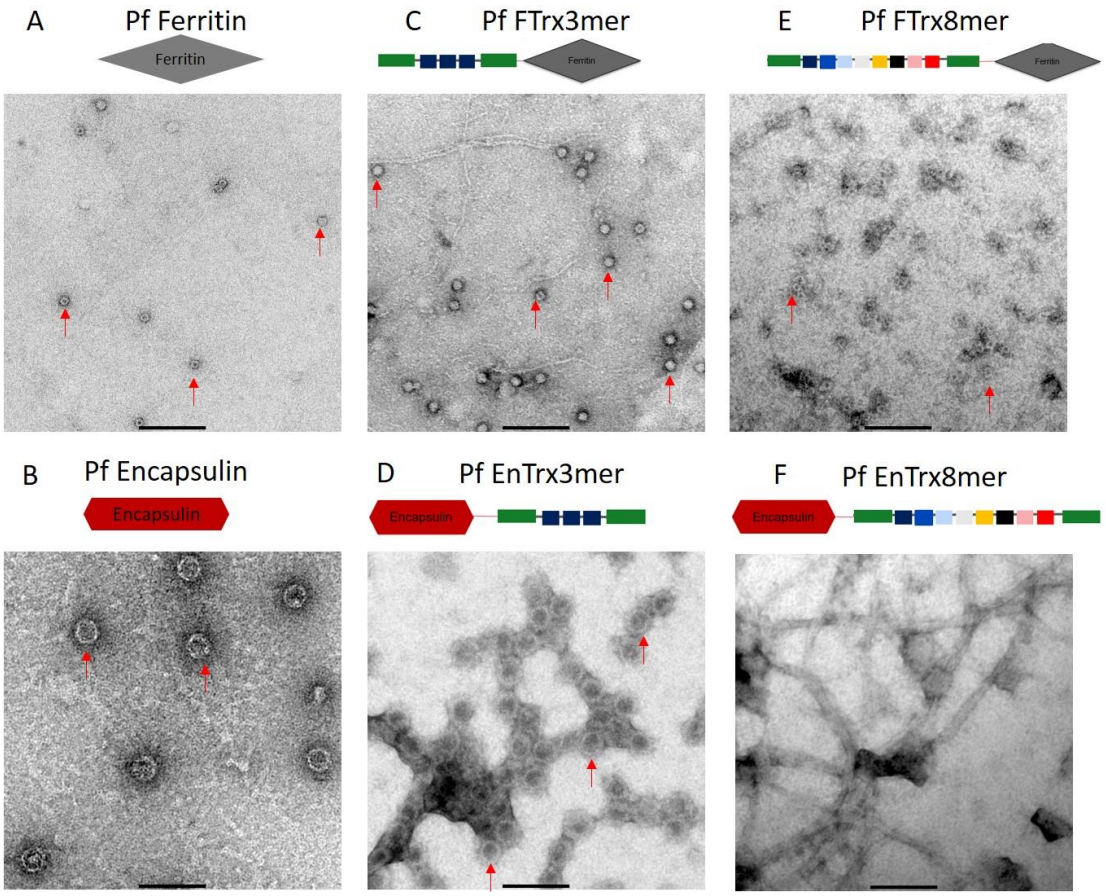


Figure 4.4 The uniform size of nanoparticle antigens was analyzed by EM. The grey bar represents 100 nm, and the red arrows refer to nanoparticles.

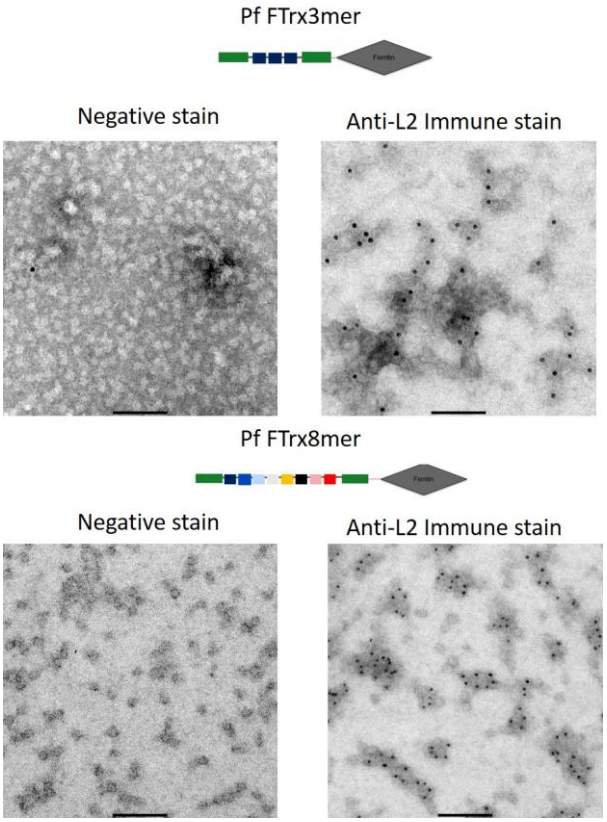


Figure 4.5 L2 epitopes verified by anti-L2 K4 immune EM. The left panel are the TEM image of nanoparticles coupled without anti-L2 K4 antibody linked anti-mouse IgG (rabbit) with immune-geld labeling. The right panel are the nanoparticle TEM coupled with anti-L2 K4 antibody linked anti-mouse IgG (rabbit) with immune-gold labeling. (Electron dense particles in right panel). The grey bar represented 100 nm.

To further determine the formation of the nanoparticles, size exclusion chromatography (SEC) was performed. Results of SEC provided further evidence that there were un-identified structures in some samples after thermal purification, such as subunits of nanoparticles, or miss-assembled particles. For these samples, an alternative purification strategy was employed, using a two-step density sucrose and CsCl gradient ultracentrifugation. More uniformly sized nanoparticle structures were found in these samples, especially for the Pf F3mer. There was only one structure detected in Pf EnTrx3mer, Pf FTrx3mer, and Pf FTrx8mer samples by SEC. However, as we observed with TEM, there

were several structures of different sizes in Pf EnTrx8mer and Pf En8mer preparations (figure 4.6). Pf FTrx8mer was purified by the heat treatment protocol, and the TEM images confirmed uniform size distribution and a smooth spherical cage, DLS showed the Pf FTrx8mer nanoparticles had a limited size distribution, which was consistent with the TEM results (figure 4.5). The SEC and DLS also confirmed there were neither subunits nor misshapen particles in these samples (figure 4.6 and 4.7). However, any nanoparticle for the Pf EnTrx8mer, or the Pf En8mer could not be found.

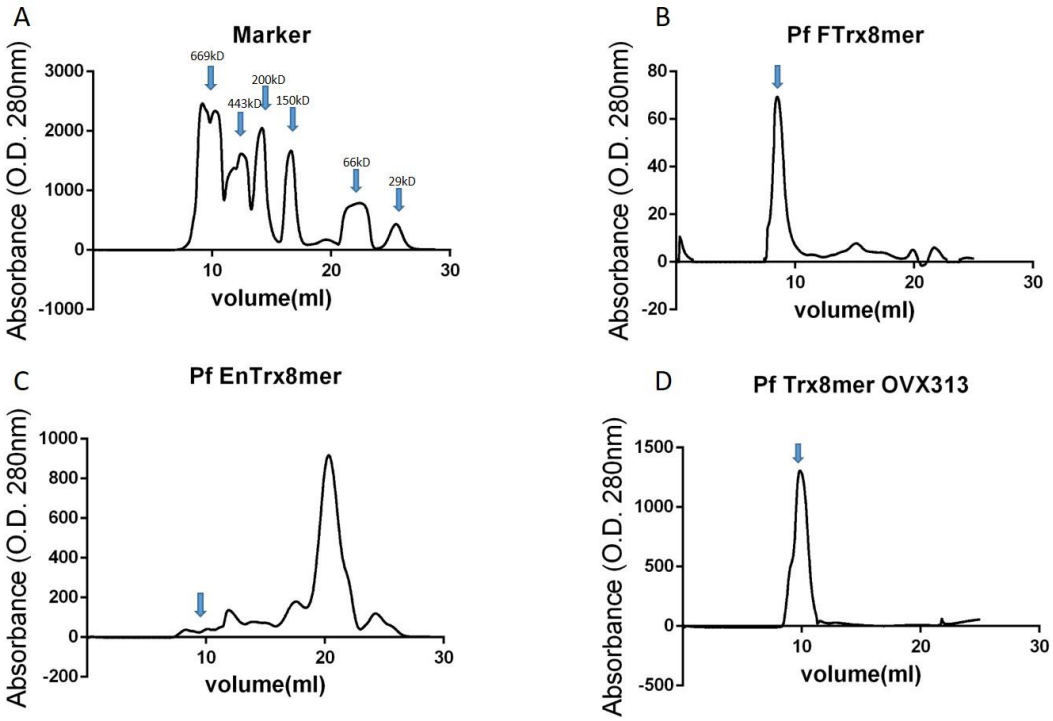


Figure 4.6 Size exclusion chromatography (SEC) analysis of Pf FTrx8mer, Pf EnTrx8mer, and Pf Trx8mer OVX313. Chromatograms of Pf FTrx8mer, Pf EnTrx8mer, and Pf Trx8mer OVX313. A), a marker fractionated on a high resolution gel filtration column; thyroglobulin (669 kD); APO ferritin (443 kD), β-amylase (200 kD), dehydrogenase (150 kD), albumin (66 kD), anhydrase (29 kD) were utilized as calibration standards; B)-D), Pf FTrx8mer, Pf EnTrx8mer, and Pf Trx8mer OVX313 fractionated on the column, respectively. The blue arrows on Marker SEC indicated the positions of proteins in different sizes. The blue arrows in B; C, D indicated the positions of nanoparticles antigens.

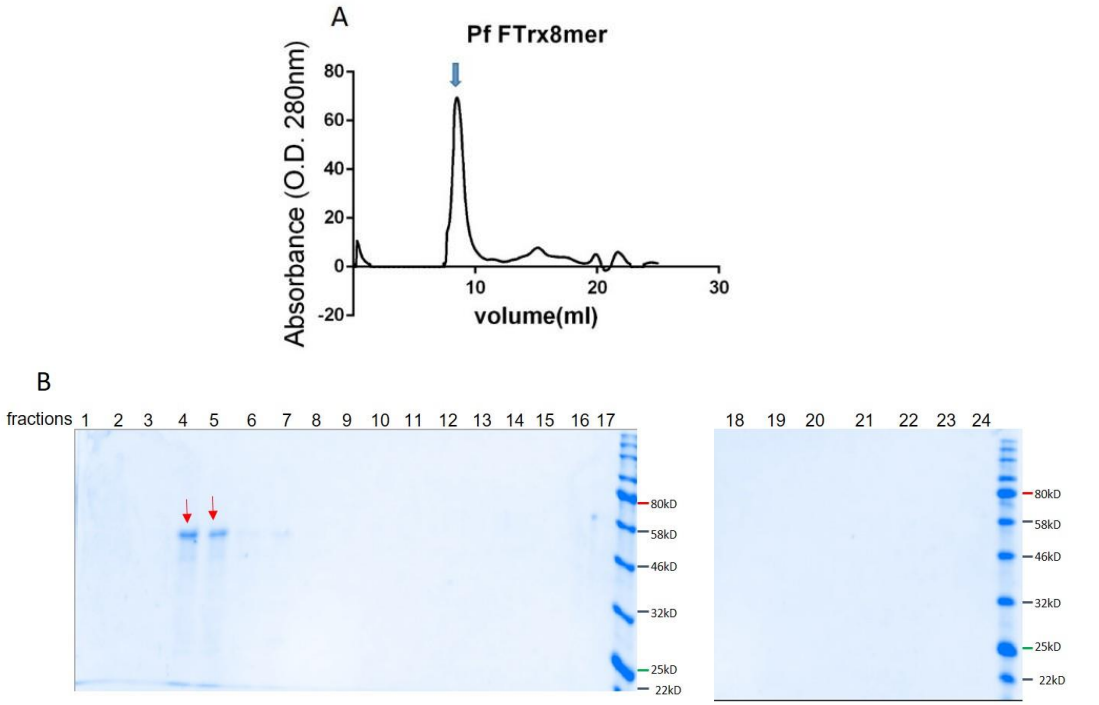


Figure 4.7 Size exclusion chromatograph (SEC) analysis of Pf FTrx8mer and SDS-PAGE verified fraction 1-24. A) Chromatograms of Pf FTrx8mer, fractionated on the column, the blue arrow pointed the fractions of nanoparticles of Pf FTrx8mer, B) Fractions analyzed by SDS-PAGE staining with Coomassie blue. The major peak represents the nanoparticle Pf FTrx8mer.

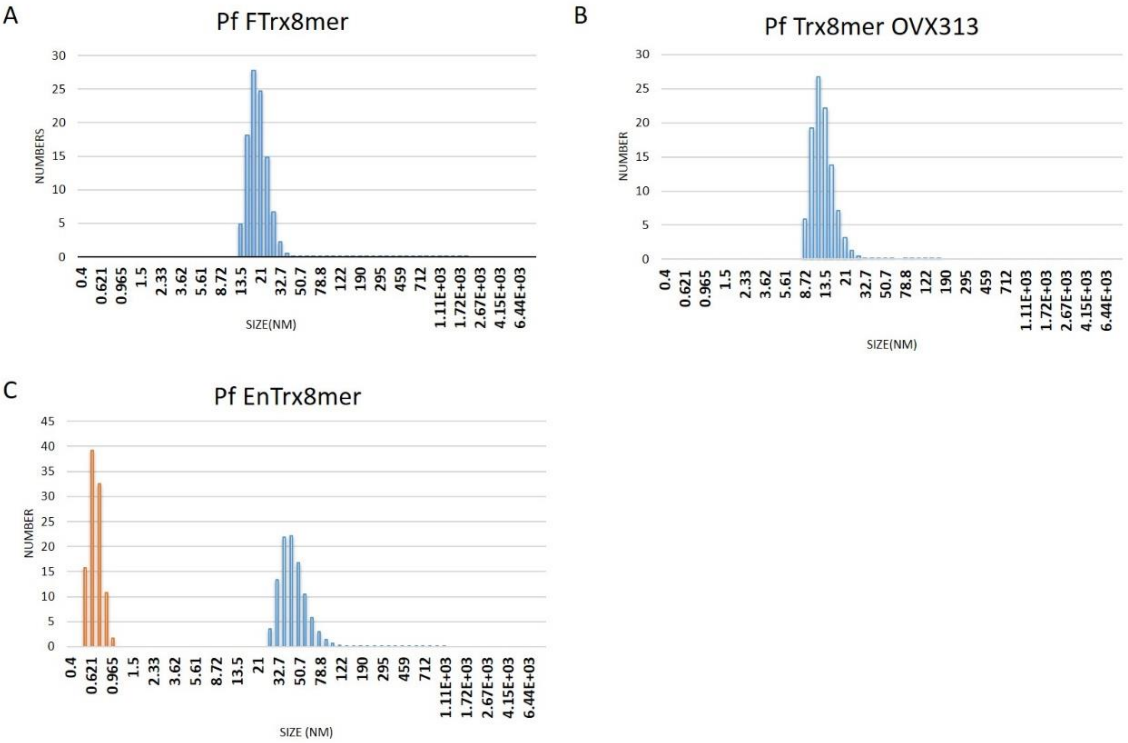


Figure 4.8 Dynamic light scattering (DLS) reveals a homogeneous structure of nanoparticle antigens. Pf FTrx8mer A) and Pf Trx8mer OVX313, B) were found in uniform size, which are around 20nm and 12nm, respectively. But when analyzing Pf EnTrx8mer, which the nanoparticle of Pf EnTrx8mer should be bigger than 25 nm, there are two peaks indicating that this antigen preparation contains subunits or misassembled structures C), , indicating this antigen preparation contains subunits or miss-assembled structures.

4.3 Immunogenicity of L2-based nanoparticle antigens is adjuvant dependent.

Adjuvants are already extensively used in vaccines, however, there are properties of adjuvants to be considered. On the one hand adjuvants help to improve the immunogenicity of antigens, but on the other hand, using adjuvants may cause problems in antigen formulation and administration. To determine whether L2-based nanoparticle antigens require the use of adjuvants. The best immune stimulating adjuvants were investigated with HPV16 trimer in context of L2-based nanoparticle antigens had been investigated. The candidates included: AddaVax, a squalene-based oil-in-water emulsion, stimulating both Th1 and Th2 immune responses, Aluminum hydroxide (Alum), inducing humoral immune responses and Alum in combination with monophosphoryl lipid A (Alum-MPLA), a TLR-4 agonist enhancing IL-10 production.

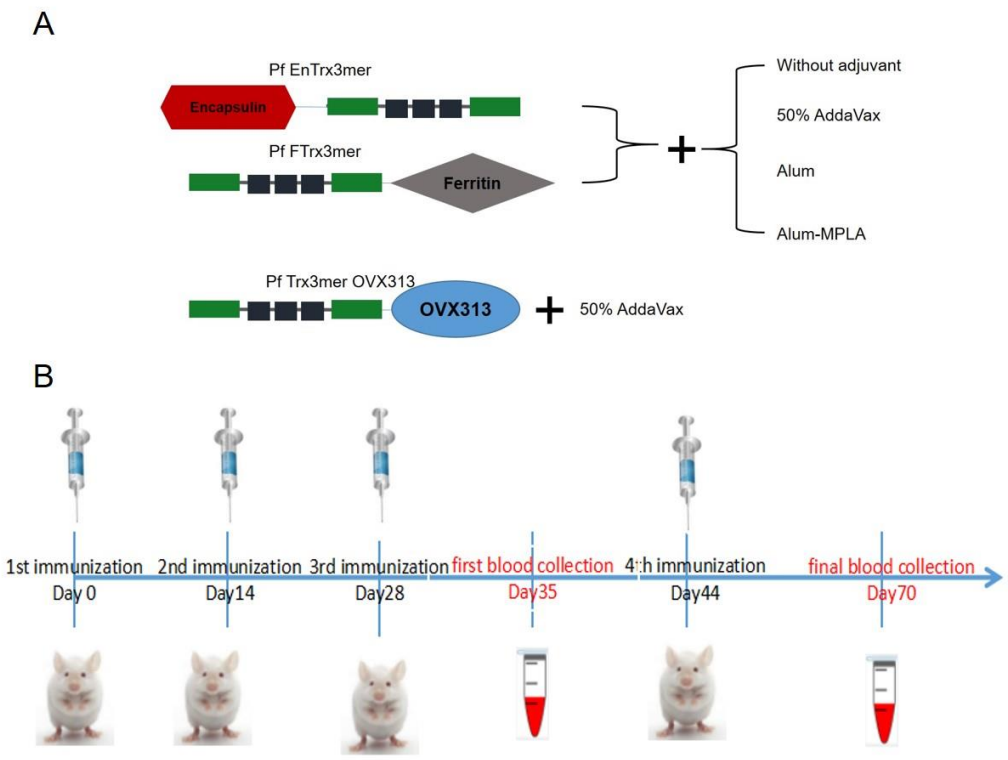
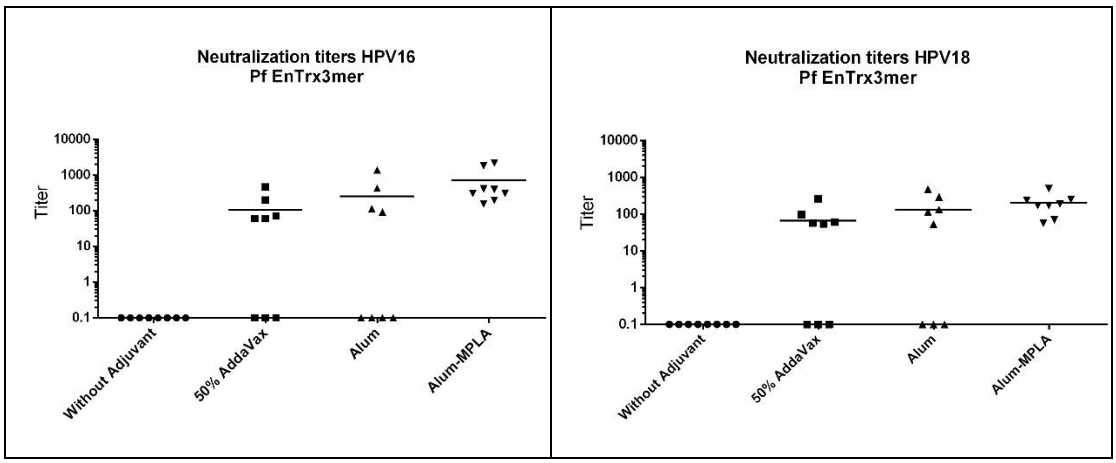


Figure 4.9 The immunization scheme for electing the adjuvants for L2-based nanoparticle antigens. Mice were immunized with 20 µg of Pf EnTrx3mer, Pf FTrx3mer and Pf Trx3mer OVX313, non-adjuvanted or adjuvanted 50% (v/v) AddaVax, 50 µg Alum, 50 µg Alum+ 10 µg MPLA, respectively A). The mice were immunized four times at biweekly intervals. Four weeks after the last immunization, sera were collected for L1 PBNA B).

The animals were immunized with 20 µg of the L2-based nanoparticle antigens Pf EnTrx3mer and Pf FTrx3mer, formulated with either (1) no adjuvant, (2) 50% (v/v) AddaVax, (3) 50 µg Alum, or (4) 50 µg Alum + 10 µg MPLA (figure 4.9). As a comparison, Pf Trx-HPV16 L2 (aa20-38)*3-OVX313 (Pf Trx3mer OVX313) with 50% (v/v) AddaVax as adjuvants was used for the immunizations. Mice were immunized four times, once every two weeks and blood was collected by cardiac puncture four weeks after last immunization. The sera were analyzed by L1 PBNA to evaluate neutralizing antibody titers against HPV16, 18, 31, 39, 45 and 51 PSV. The results indicated that the immune reaction to Pf Trx- L2

fused to nanoparticle Pf encapsulin was adjuvant dependent and in combination with Alum-MPLA elicited the highest immune responses (figure 4.10). However, the antigen could not induce cross-protection against HPV31, 39, 45, and 51. Pf FTrx3mer adjuvanted with 50% AddaVax not only induced the highest neutralizing antibodies against HPV16, HPV18, compared to the other two adjuvants, but also induced neutralizing antibodies titers against HPV45. No neutralizing antibodies against HPV31, 39, 51 were detected (figure 4.11). Moreover, even without adjuvant, Pf FTrx3mer stimulated neutralizing antibody production, and provided protection against HPV16 and 18. To better understand which nanoparticle platform was able to induce the strongest immune response against HPV, the sera from the mice immunized with Pf EnTrx3mer formulated with Alum-MPLA, Pf FTrx3mer formulated with AddaVax and Pf Trx3mer OVX313 adjuvanted with AddaVax were tested by L1 PBNA side by side. Mice responded to Pf FTrx3mer and Pf Trx3mer OVX313 better than to Pf EnTrx3mer; the antibody responses against Pf FTrx3mer and Pf Trx3mer OVX313 were, however, comparable (figure 4.12).



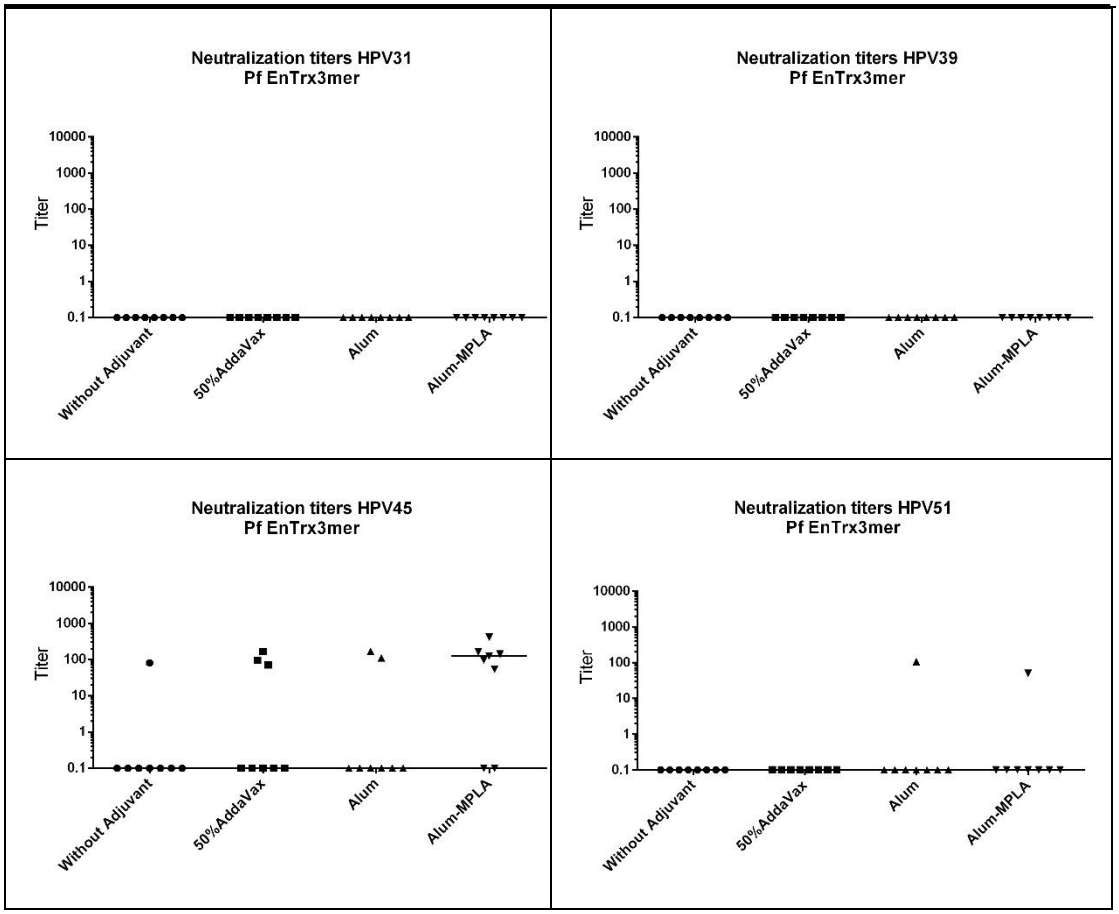
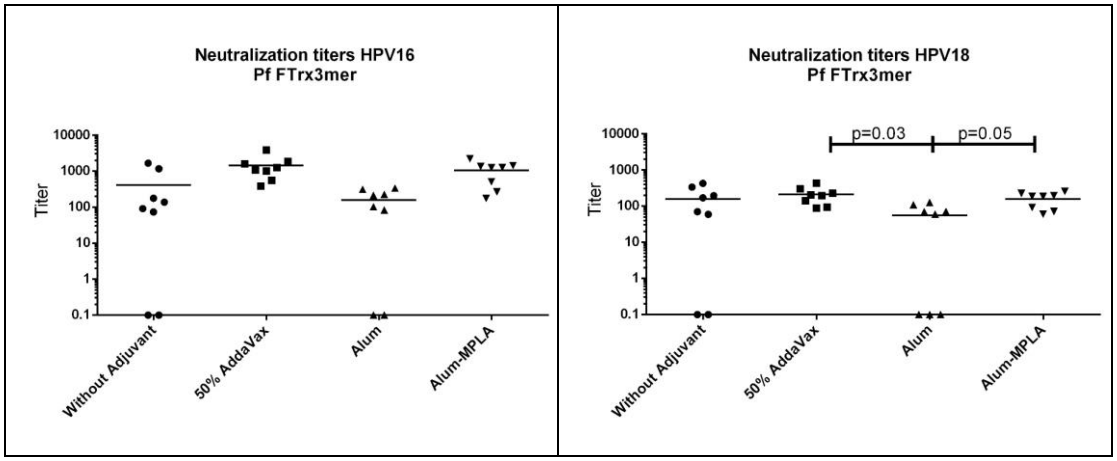


Figure 4.10 Pf EnTrx3mer formulated with Alum-MPLA induced highest neutralizing antibody titers against HPV16 and HPV18. Mice immunized Pf EnTrx3mer without adjuvant or adjuvanted with Addavax, Alum, and Alum-MPLA, and sera were analyzed with L1 PBNA 4 weeks after last immunization. The symbols represent the neutralizing titers of individual mice. The means of the titers for each group are indicated by horizontal lines. The y axis displays EC50 titers. And a *p* value of ≤ 0.05 was considered significant.



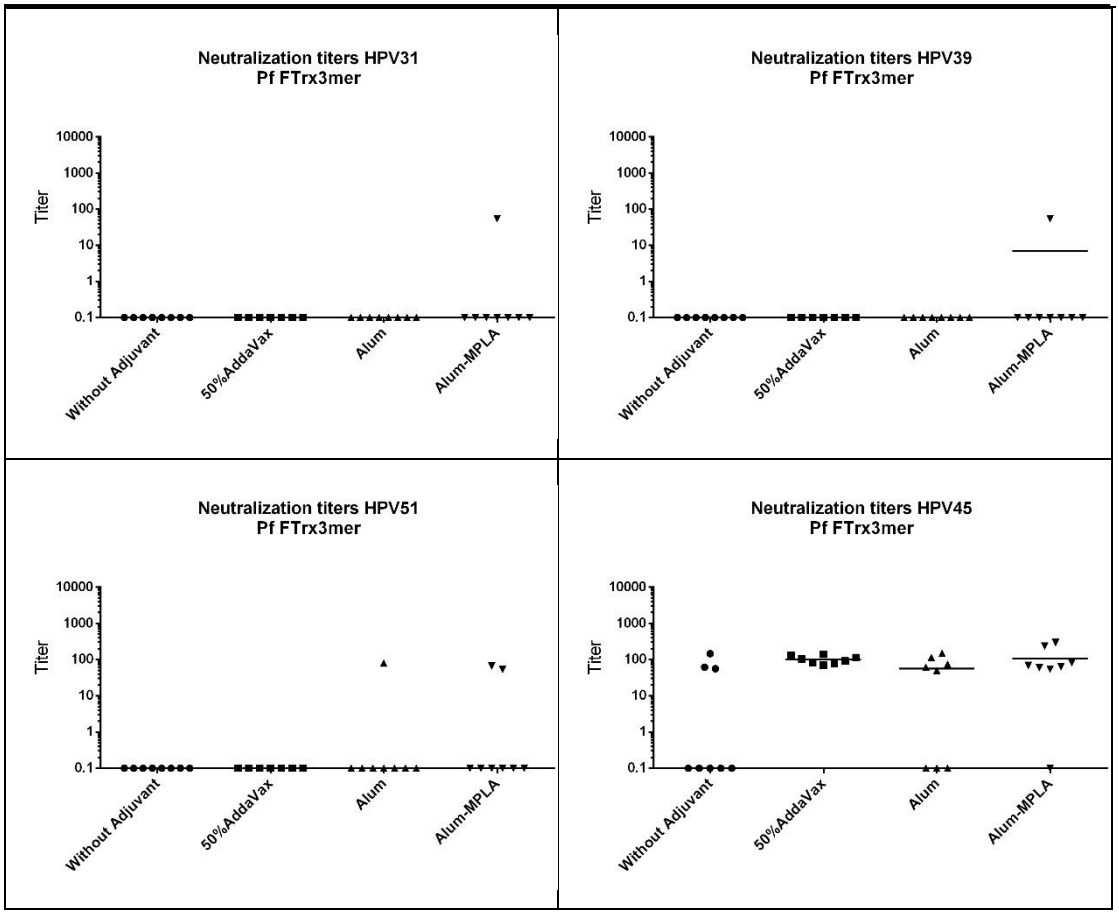
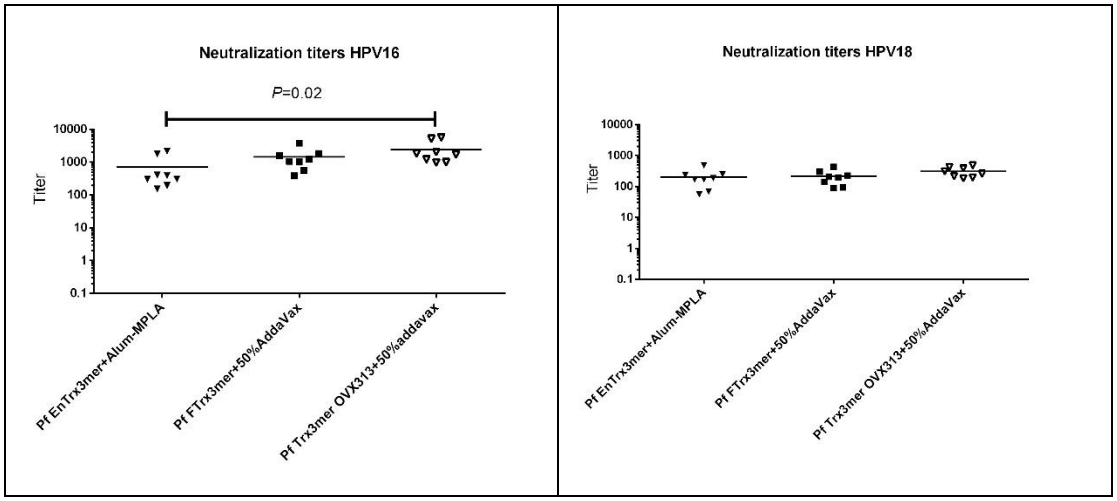


Figure 4.11 Pf FTrx3mer formulated with 50% AddaVax induced highest neutralizing antibody titers against HPV16 and HPV18. Mice were immunized Pf FTrx3mer without adjuvant or adjuvanted with AddaVax, Alum, and Alum-MPLA, and sera were analyzed with L1 PBNA four weeks after the last immunization. The symbols represent the neutralizing titers of individual mice. The means of the titers for each group are indicated by horizontal lines. The y axis displays EC50 titers. And a *p* value of ≤0.05 was considered significant.



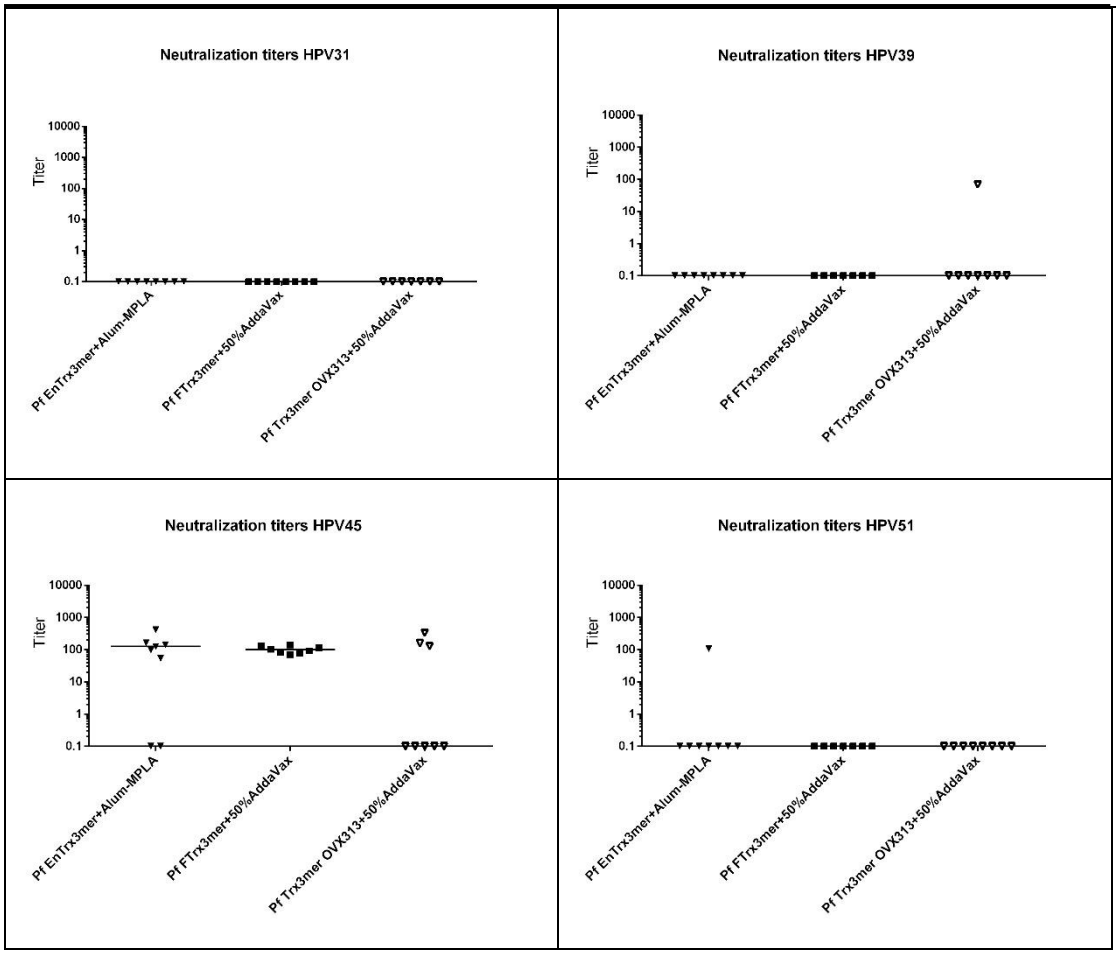


Figure 4.12 Pf FTrx3mer formulated with 50% AddaVax induced highest neutralizing antibody titers compared to Pf EnTrx3mer formulated with Alum-MPLA and Pf Trx3mer OVX313. Mice were immunized with Pf FTrx3mer formulated with AddaVax, Pf EnTrx3mer formulated with Alum-MPLA or with Pf Trx3mer OVX313 formulated with AddaVax. Sera were analyzed by L1-based PBNA four weeks after the last immunization. The symbols represent the neutralizing titers of individual mice. The means of the titers for each group are indicated by horizontal lines. The y axis displays EC50 titers. And a *p* value of ≤ 0.05 was considered significant.

4.4 Pf thioredoxin is essential for L2-based nanoparticle antigens.

In order to increase the immunogenicity of L2-based HPV prophylactic vaccines, the following criteria should be considered: (1) the size of the vaccine, which

will influence the antigen uptake by dendritic cells (DC)s and cellular processing, (2) how the antigen is transported to the target immune organs, such as lymph nodes; (3) whether the antigen targets and activates B cells efficiently to produce the antibodies against the antigen; (4) whether the antigen is also sufficiently targeted by the DCs (Bachmann and Jennings, 2010). In this thesis, nanoparticles consisting of either Pf encapsulin or Pf ferritin were employed as the delivery platform to transport and display the L2 epitopes. Both structures are theoretically in the suitable size range and thus possess properties for increasing the antigen immunogenicity. The antigen structures described above all carry the L2 neutralization epitopes inserted into bacterial thioredoxin as a primary scaffold raising the question whether there is actually a need for thioredoxin for epitope presentation in context of ferritin and encapsulin nanoparticles? To answer this question, mice were immunized with Pf EnTrx8mer, Pf En8mer adjuvanted with Alum-MPLA (figure 4.14); Pf FTrx3mer without any adjuvant and Pf F3mer with/without AddaVax adjuvant (figure 4.13).

The repetitive L2 epitopes increase the immunogenicity of the nanoparticle vaccines, presumably by causing a hyper-activation of B cell receptors. A Pf ferritin nanoparticle theoretically presents 24 copies of either Pf Trx-L2 or L2 on its surface. An encapsulin nanoparticle presents 60 copies. Both of them would sufficiently activate B cells to produce neutralizing antibodies due to the size and repetitive epitope presenting pattern. To answer the question of thioredoxin's necessity for the nanoparticles, Neutralizing antibody titers against HPV16, 18, and 51 were measured using in the standard L1 PBNA. The data demonstrated that the presence of Pf Trx in the nanoparticle vaccines confers significantly stronger immunogenicity on the L2 epitopes even competing with Pf F3mer adjuvanted with AddaVax (figure 4.13).

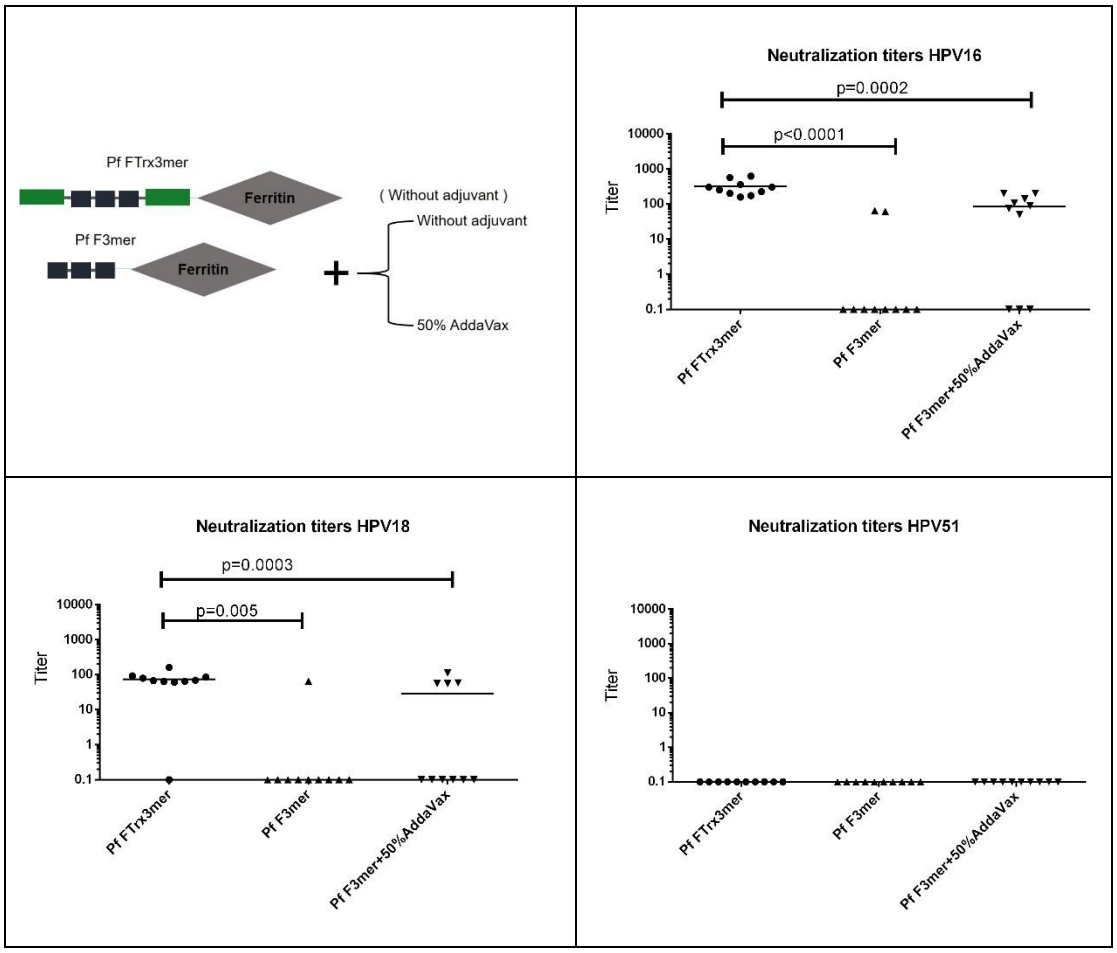
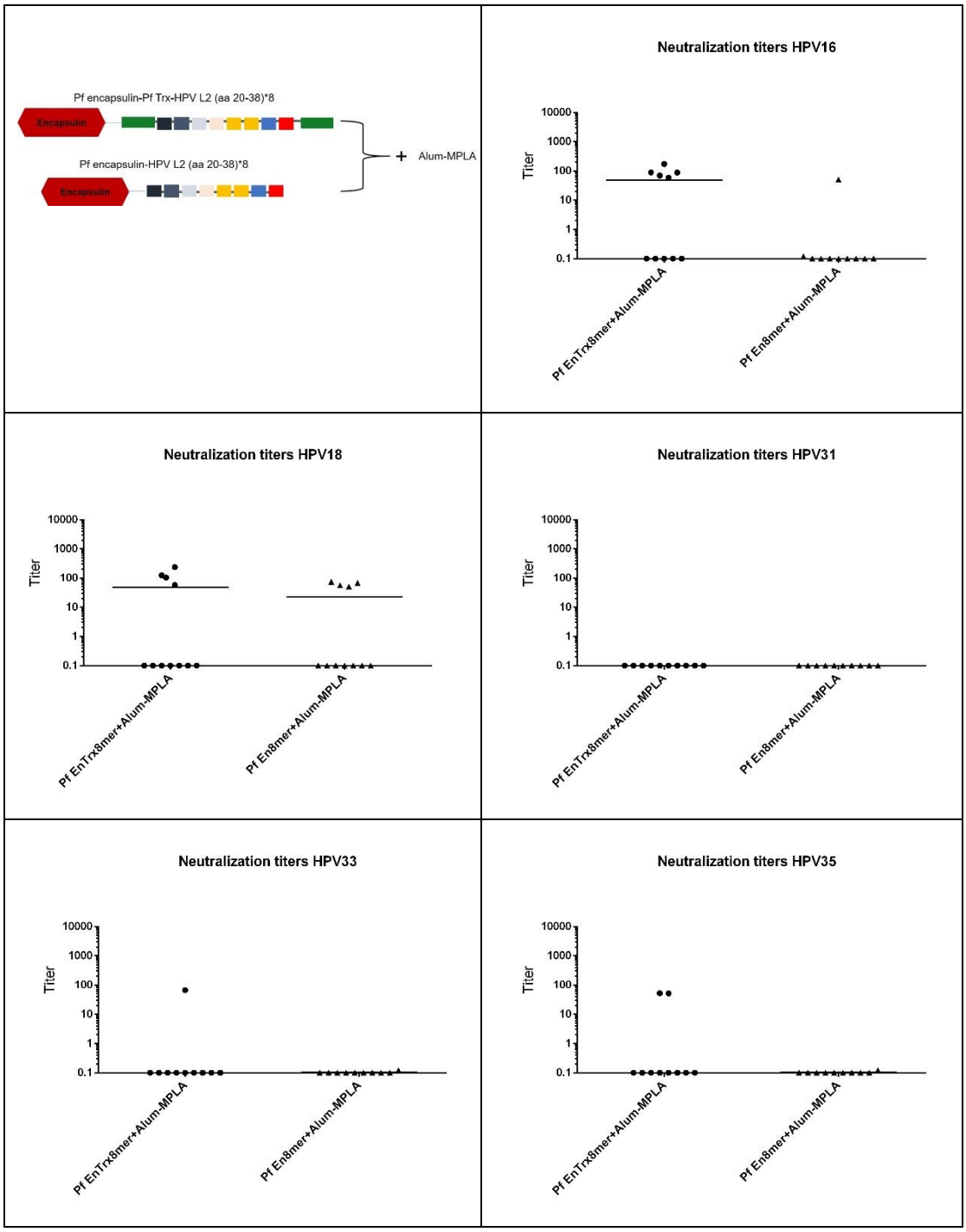


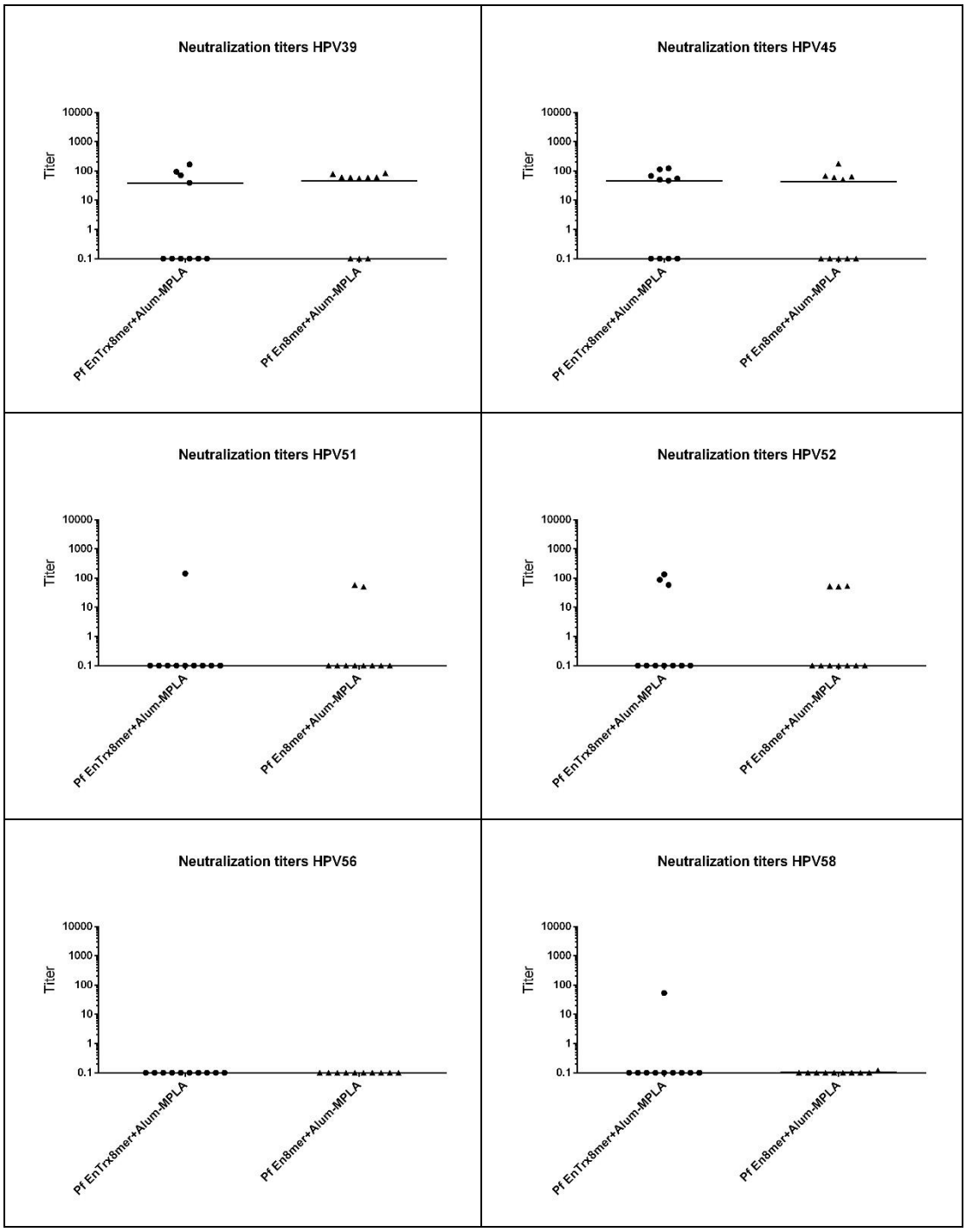
Figure 4.13 Pf thioredoxin is critical in displaying L2 epitopes on Pf ferritin nanoparticles. Mice were immunized with 20 µg Pf FTrx3mer and Pf F3mer, with or without AddaVax, for four times at biweekly intervals. Four weeks after the last immunization, sera were collected and analyzed by L1 PBNA. The neutralizing antibody titers induced by Pf FTrx3mer without adjuvant are significantly higher than those induced by the Pf F3mer, even with the help of the adjuvant AddaVax. The symbols represent the neutralizing titers of individual mice. The means of the titers for each group are indicated by horizontal lines. The y axis displays EC50 titers. A *p* value of ≤0.05 was considered significant.

Theoretically, Pf encapsulin would be the more suitable platform for the epitope presentation, considering the size of the particle which is 24 nm and also the higher repetition of subunits (60 subunits) per particle. However, the results do not support this hypothesis. A comprehensive analysis of the immunogenicity

was carried out by analyzing neutralizing antibodies against 15 HPV (14 high-risk HPVs and HPV6). In most cases, only some of the mice responded to the vaccine, and limited cross-protection was detected. Neutralizing antibodies against HPV31, 33, 51, 56, 58 and 6 could not be detected.

My former colleagues already confirmed that Guinea pigs showed stronger responses against PfTrx-L2-OVX313 antigens than mice (Pouyanfard et al., 2018). To verify the results of L1 PBNA obtained with the mice sera, the guinea pigs were immunized with Pf EnTrx8mer and Pf En8mer adjuvanted with Alum-MPLA 4 times every two weeks. One month after the last immunization, the guinea pigs were sacrificed and sera were collected. At first, the sera were tested by L1 PBNA, the results showed Pf EnTrx8mer induced higher titers than Pf En8mer. However, the sera failed to neutralize HPV31, 52, and 56 in the standard neutralization assay. Therefore a more sensitive neutralization assay L2-PBNA was employed. Again, no neutralizing antibodies were detected against HPV31 for both Pf encapsulin nanoparticle antigens. At the same time, the antibody titers against other HPVs were higher than the titers detected in the L1 PBNA confirming the higher sensitivity of the assay (data not shown). To sum up all the data, the results showed Pf En8mer failed as a suitable nanoparticle platform for presenting L2 epitopes, but Pf EnTrx8mer could induce cross-protection against 11 high-risk HPVs and 2 low-risk HPVs in some animals (figure 4.14).


Pf encapsulin-Pf Trx-HPV L2 (aa 20-38)*8
Encapsulin
Pf encapsulin-HPV L2 (aa 20-38)*8
Encapsulin
+ Alum-MPLA
Neutralization titers HPV16
Titer
10000
1000
100
10
1
0.1
Pf EnTrx8mer+Alum-MPLA
Pf En8mer+Alum-MPLA
Neutralization titers HPV18
Titer
10000
1000
100
10
1
0.1
Pf EnTrx8mer+Alum-MPLA
Pf En8mer+Alum-MPLA
Neutralization titers HPV31
Titer
10000
1000
100
10
1
0.1
Pf EnTrx8mer+Alum-MPLA
Pf En8mer+Alum-MPLA
Neutralization titers HPV33
Titer
10000
1000
100
10
1
0.1
Pf EnTrx8mer+Alum-MPLA
Pf En8mer+Alum-MPLA
Neutralization titers HPV35
Titer
10000
1000
100
10
1
0.1
Pf EnTrx8mer+Alum-MPLA
Pf En8mer+Alum-MPLA


Neutralization titers HPV39
Titer
10000
1000
100
10
1
0.1
Pf EnTrx8mer+Alum-MPLA
Pf En8mer+Alum-MPLA
Neutralization titers HPV45
Titer
10000
1000
100
10
1
0.1
Pf EnTrx8mer+Alum-MPLA
Pf En8mer+Alum-MPLA
Neutralization titers HPV51
Titer
10000
1000
100
10
1
0.1
Pf EnTrx8mer+Alum-MPLA
Pf En8mer+Alum-MPLA
Neutralization titers HPV52
Titer
10000
1000
100
10
1
0.1
Pf EnTrx8mer+Alum-MPLA
Pf En8mer+Alum-MPLA
Neutralization titers HPV56
Titer
10000
1000
100
10
1
0.1
Pf EnTrx8mer+Alum-MPLA
Pf En8mer+Alum-MPLA
Neutralization titers HPV58
Titer
10000
1000
100
10
1
0.1
Pf EnTrx8mer+Alum-MPLA
Pf En8mer+Alum-MPLA

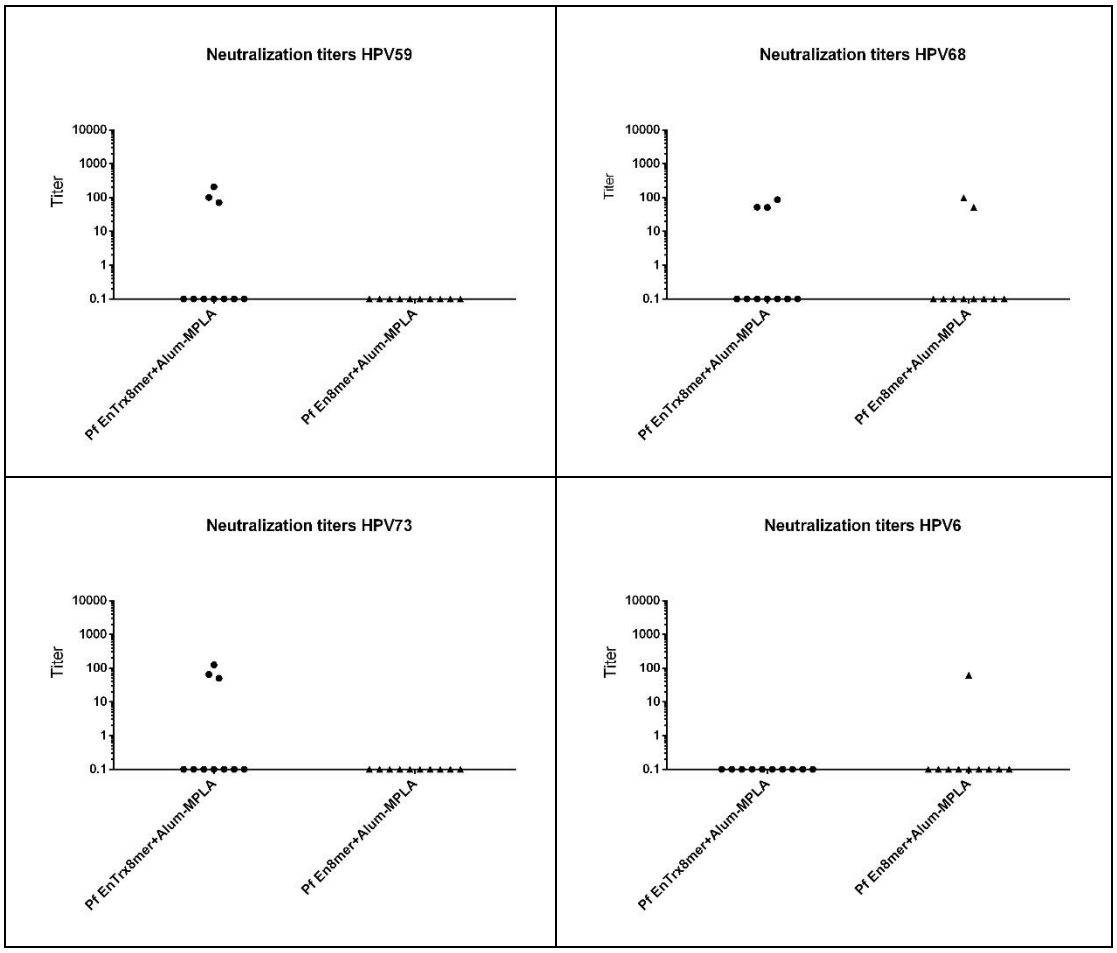
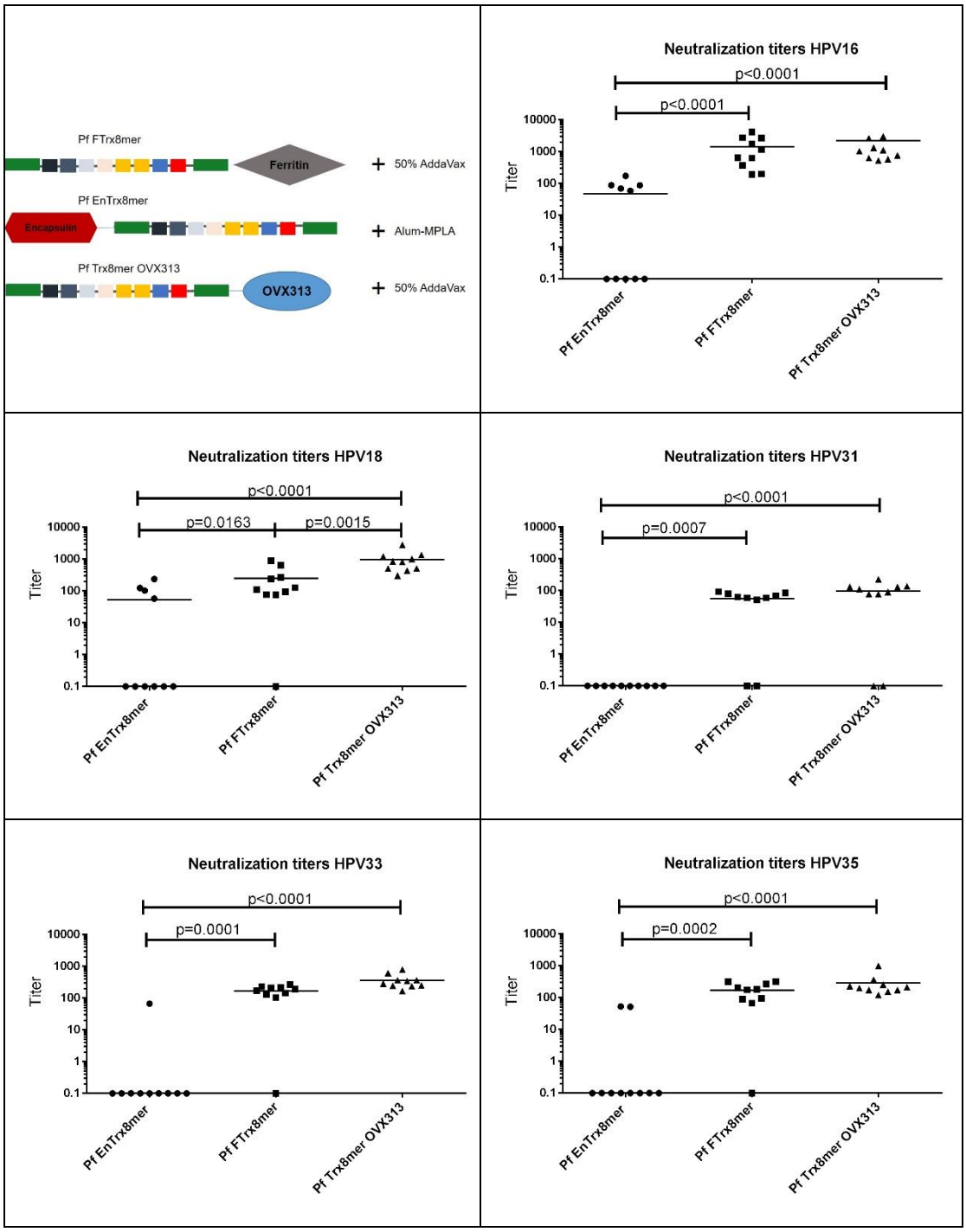
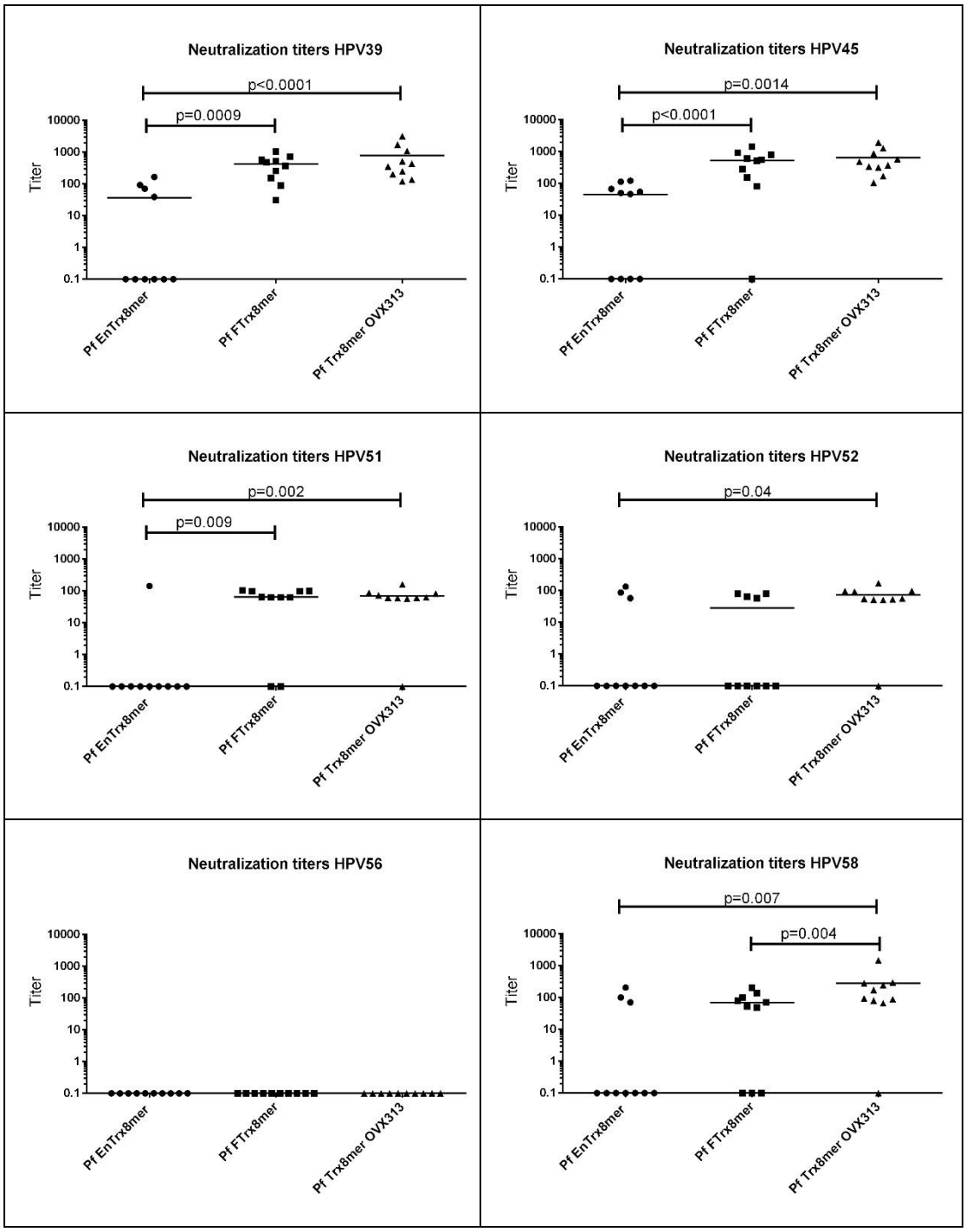


Figure 4.14 Inclusion of Pf thioredoxin on Pf encapsulin nanoparticles displaying L2 epitopes increases immunogenicity of the nanoparticle antigens. Mice were immunized with 20 µg Pf EnTrx8mer, Pf En8mer, with Alum-MPLA as adjuvant, four times at two weeks intervals. Four weeks after the last immunization, sera were collected for L1 PBNA. The titers of neutralizing antibodies induced by Pf EnTrx8mer are higher than those induced by the Pf En8mer. The symbols represent the neutralizing titers of individual mice. The means of the titers for each group are indicated by horizontal lines. The y axis displays EC50 titers.

4.5 The Pf FTrx8mer nanoparticle antigen induces neutralizing antibody responses against 14 high-risk and one low-risk of HPV types.

After selecting the adjuvant and determining the role of Pf Thioredoxin, we wanted to identify the best nanoparticle vaccine and determine the full broadness of protection. To allow side by side comparison, mice or guinea pigs were immunized with the nanoparticle antigens, and the sera were tested for reaction against pseudovirions of 15 HPV types by the L1 PBNA. Additionally, the more sensitive L2 PBNA was employed for HPV52, 56 and 31, since responses against these types which are generally difficult to detect by the standard L1 PBNA. The animals were immunized with either the Pf EnTrx8mer with Alum-MPLA as adjuvant, the Pf FTrx8mer with AddaVax, or Pf Trx8mer OVX313 with AddaVax as control. Using the insensitive L1 PBNA, the antibodies induced by the Pf FTrx8mer and Pf Trx8mer OVX313 were able to neutralize 13 high-risk HPVs, and one low-risk HPV. As for HPV56, none of the three antigens could induce measurable immune responses as judged by the L1 PBNA. The immune responses of mice inoculated by Pf FTrx8mer and Pf Trx8mer OVX313 were comparable, but there were some significant differences for certain HPVs, like HPV18, HPV58, and HPV59. For these types, higher antibody titers induced by Pf Trx8mer OVX313 than by Pf FTrx8mer were detected (figure 4.15). For the guinea pigs, a similar pattern was observed, no neutralizing antibodies against HPV31, 52 and 56 were detected using the L1 PBNA. In the L2 PBNA, however, neutralizing antibodies against HPV31 induced by Pf FTrx8mer and Pf Trx8mer OVX313 could be detected (figure 4.16). Unfortunately, L2 PBNA analyses to detect neutralizing antibodies against HPV52 and HPV56 could not be performed due to the lack of available HPV52 and HPV56 pseudovirions.


Pf FTrx8mer
Ferritin
+ 50% AddaVax
Pf EnTrx8mer
Encapsulin
+ Alum-MPLA
Pf Trx8mer OVX313
OVX313
+ 50% AddaVax
Neutralization titers HPV16
p<0.0001
p<0.0001
Titer
10000
1000
100
10
1
0.1
Pf EnTrx8mer
Pf FTrx8mer
Pf Trx8mer OVX313
Neutralization titers HPV18
p<0.0001
p=0.0163
p=0.0015
Titer
Pf EnTrx8mer
Pf FTrx8mer
Pf Trx8mer OVX313
Neutralization titers HPV31
p<0.0001
p=0.0007
Titer
Pf EnTrx8mer
Pf FTrx8mer
Pf Trx8mer OVX313
Neutralization titers HPV33
p<0.0001
p=0.0001
Titer
Pf EnTrx8mer
Pf FTrx8mer
Pf Trx8mer OVX313
Neutralization titers HPV35
p<0.0001
p=0.0002
Titer
Pf EnTrx8mer
Pf FTrx8mer
Pf Trx8mer OVX313


Neutralization titers HPV39
p<0.0001
p=0.0009
Titer
10000
1000
100
10
1
0.1
Pf EnTrx8mer
Pf FTrx8mer
Pf Trx8mer OVX313
Neutralization titers HPV45
p=0.0014
p<0.0001
Titer
Pf EnTrx8mer
Pf FTrx8mer
Pf Trx8mer OVX313
Neutralization titers HPV51
p=0.002
p=0.009
Titer
Pf EnTrx8mer
Pf FTrx8mer
Pf Trx8mer OVX313
Neutralization titers HPV52
p=0.04
Titer
Pf EnTrx8mer
Pf FTrx8mer
Pf Trx8mer OVX313
Neutralization titers HPV56
Titer
Pf EnTrx8mer
Pf FTrx8mer
Pf Trx8mer OVX313
Neutralization titers HPV58
p=0.007
p=0.004
Titer
Pf EnTrx8mer
Pf FTrx8mer
Pf Trx8mer OVX313

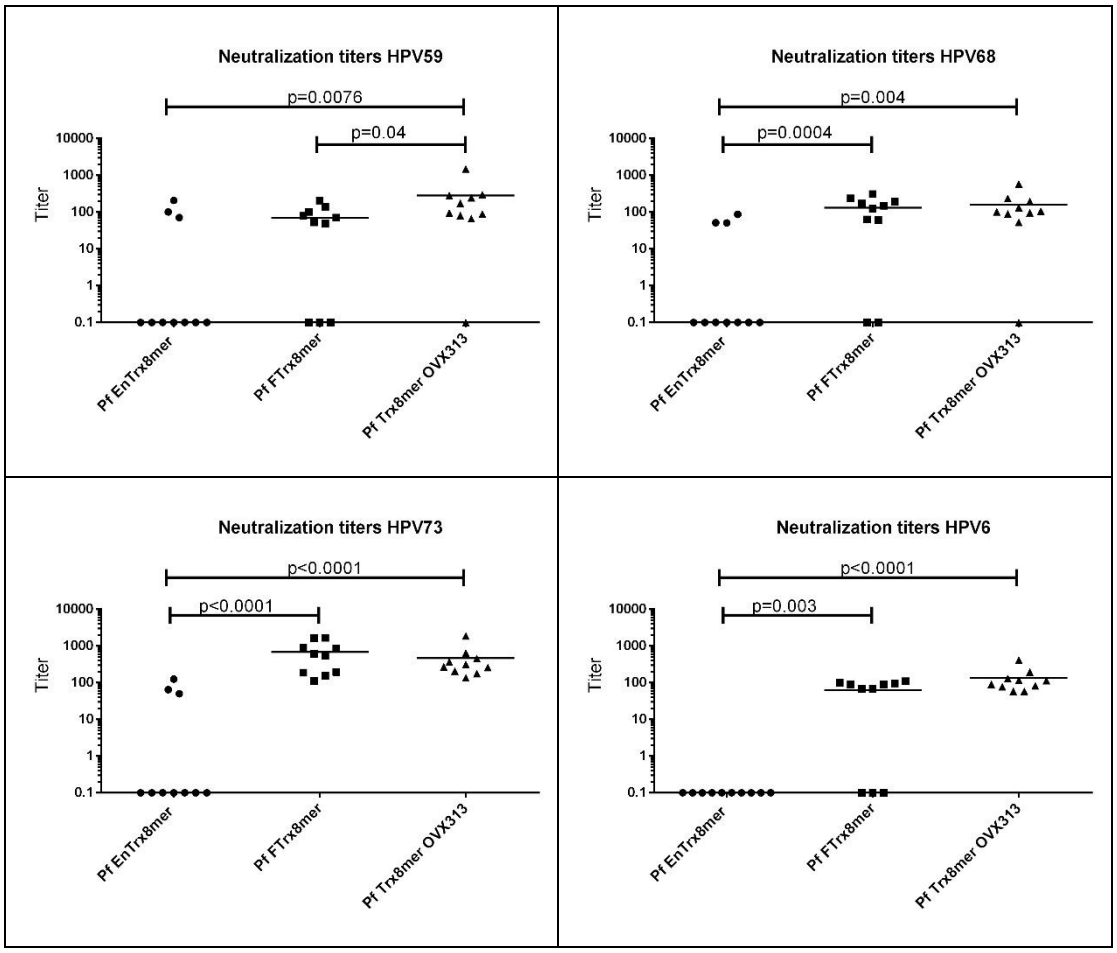


Figure 4.15 Pf FTrx8mer induced higher titers of neutralizing antibodies than Pf EnTrx8mer, and provided broad protection. Mice were immunized with 20 µg Pf EnTrx8mer with Alum-MPLA as adjuvant, Pf FTrx8mer with AddaVax as adjuvant, and Pf Trx8mer OVX313 with AddaVax as adjuvant as control four times at two weeks intervals. Four weeks after the last immunization, sera were collected and analyzed by L1 PBNA. Pf FTrx8mer was highly immunogenic, comparable to Pf Trx8mer OVX313. Symbols represent individual neutralizing titers of mice for the HPV types indicated. The means of the titers for each group are indicated by horizontal lines. The y axis displays EC50 titers. A *p* value of ≤0.05 was considered significant.

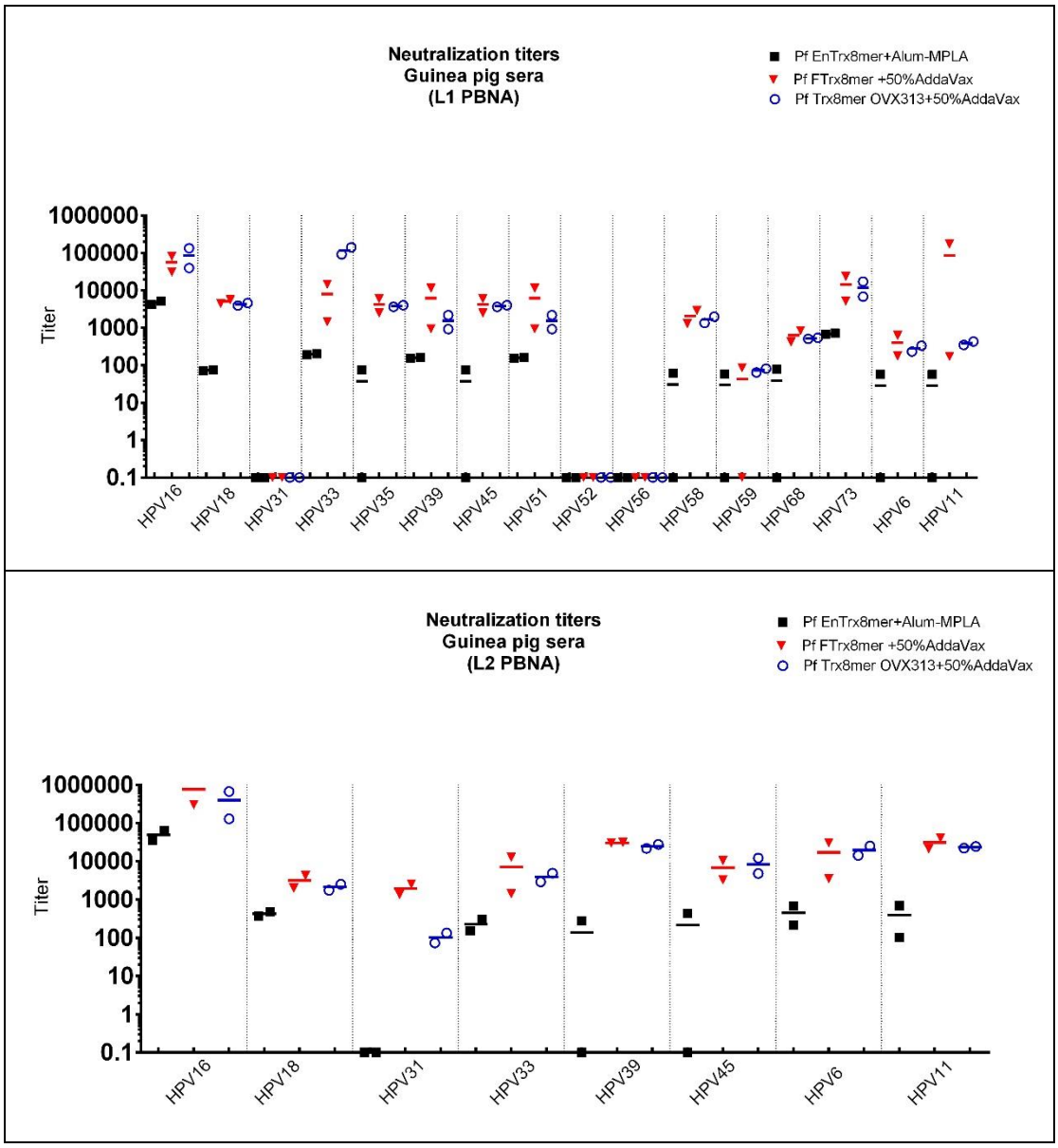


Figure 4.16 Pf FTrx8mer with AddaVax as adjuvant induced high titers of neutralizing antibodies, and provided broad protection. Guinea pigs were immunized with 50 µg Pf EnTrx8mer with Alum-MPLA as adjuvant, Pf FTrx8mer with AddaVax as adjuvant, and Pf Trx8mer OVX313 with AddaVax as adjuvant as control for four times at two weeks intervals. Four weeks after the last immunization, sera were collected and analyzed by L1 PBNA and L2 PBNA. Pf FTrx8mer was highly immunogenic, and comparable to Pf Trx8mer OVX313. Symbols represent individual neutralizing titers of guinea pigs for the corresponding HPV types.

The means of the titers for each group are indicated by horizontal lines. The y axis displays EC50 titers.

4.6 Time course of the development of neutralizing antibodies.

Early analysis of sera in some of the immunization experiments shown above indicated that the Pf FTrx8mer induced higher titers than the control Pf Trx8mer OVX313 antigen, but these difference disappeared after the completion of the full immunization schedule. These results promoted me to further investigate the kinetics of antibody responses. Mice were immunized with Pf FTrx8mer and Pf Trx8mer OVX313 both with AddaVax as adjuvant and blood was collected one week after every immunization and one day before the next immunization (figure 4.17 A and B). The final blood was collected on day 70, one month after the last immunization. The sera were examined for neutralizing antibodies against HPV16 pseudovirions by L1 PBNA. Interestingly, there was a robust boost in titers after each immunization with Pf Trx8mer OVX313, however, there was a different trend for the mice which were immunized with PfFTrx8mer was antibody titers increased during the first three immunizations, however, the antibodies decreased after the fourth immunization (figure 4.17C). Moreover, the neutralizing antibody titers induced by Pf FTrx8mer were higher than those induced by Pf Trx8mer OVX313 on day 35 and 41. Afterwards, this superiority was lost.

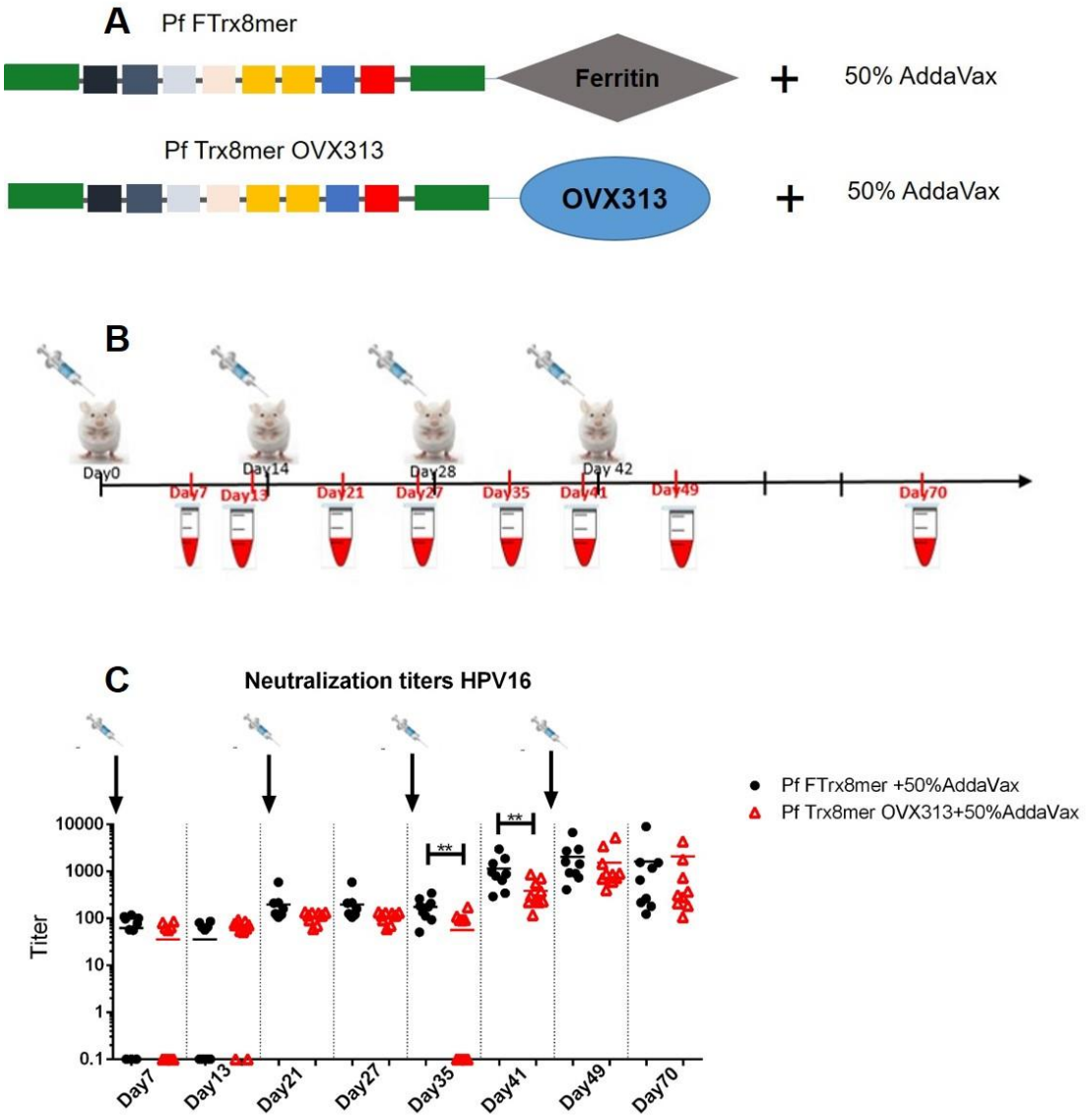
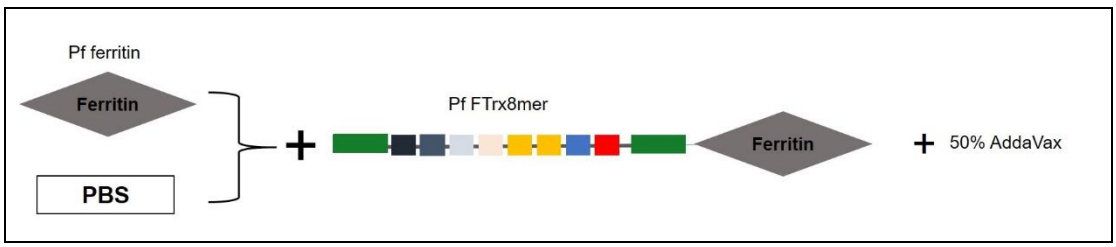


Figure 4.17 Time course of neutralizing antibody development. A) Mice were immunized four times at two weeks intervals with 20 µg Pf FTrx8mer and Pf Trx8mer OVX313 with AddaVax as adjuvant. B) On Day 7, 13, 21, 27, 35, 41, and 49, intermediate blood was collected, and on day 70, the final blood was collected for the HPV16 L1 PBNA. C) the neutralizing antibody titers induced by Pf FTrx8mer were higher than those induced by Pf Trx8mer OVX313 on day 35 and day 41. After this, the differences were no longer observed. Symbols represent individual neutralizing titer of mice for HPVs, respectively. The means of the titers for each group are indicated by horizontal lines. The y axis displays EC50 titers. A p value of ≤0.05 was considered significant. **, *p* value <0.01.

4.7 Pre-immunization with Pf ferritin interferes with the neutralizing antibody response induced by the Pf FTrx8mer.

In the previous experiment, a decreasing or even negative effect of booster immunization using the antigens based on the Pf ferritin scaffold was observed and I hypothesized that this might be due to antibodies evoked against the scaffold itself. To verify this, mice were immunized with Pf ferritin (without L2 epitopes) for three times every two weeks to induce an antibody response against the empty ferritin scaffold. A control group was injected with PBS. After the pre-immunization with Pf ferritin (pre-immunized group) or PBS (non-pre-immunized group), the mice were immunized with Pf FTrx8mer with adjuvant another 3 times. One month after the last immunization, the mice were sacrificed and the sera were collected and analyzed by L1 PBNA (figure 4.18). The results showed that the neutralizing antibody titers of the mice that received the pre-immunization led to a reduced immune response against the L2, of both HPV16 and 18. This suggests that the pre-immunization with Pf ferritin nanoparticle antigen interfered with neutralizing antibody production against the Pf FTrx8mer which might pose a limitation of the use of the Pf ferritin platform in the future.



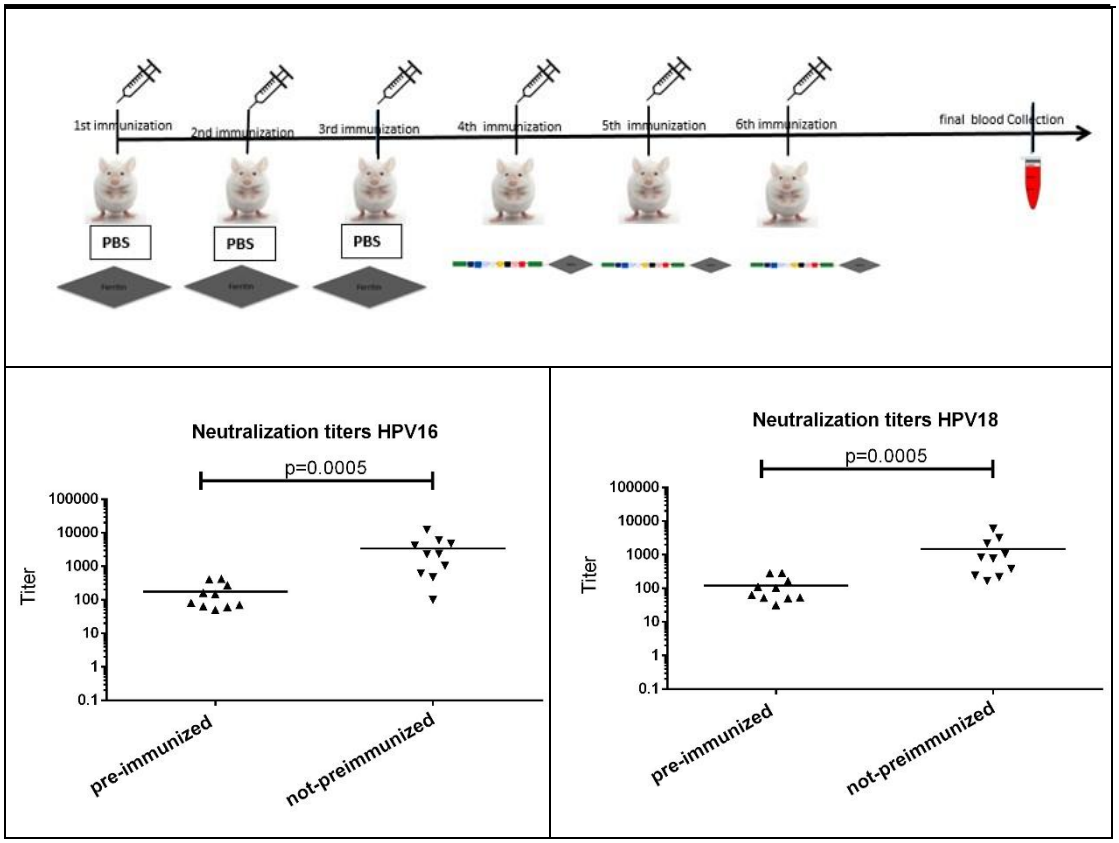
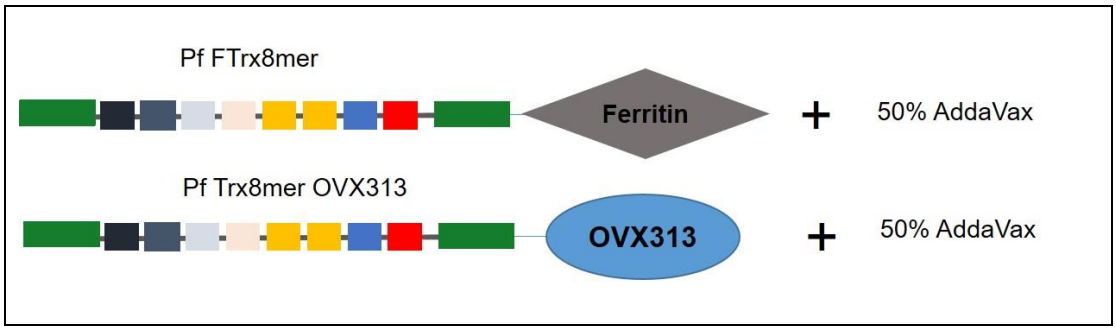


Figure 4.18 Pre-immunization against the ferritin scaffold interfered with the induction of neutralizing antibodies. Mice were immunized three times with PBS or Pf ferritin. Then animals were immunized with Pf FTrx8mer. All the immunizations were with 50% AddaVax as adjuvant. One month after the last immunization, the sera were collected and antibody titers against HPV16 and HPV18 were analyzed by the L1 PBNA. The symbols represent the neutralizing titers of individual mice. The means of the titers for each group are indicated by horizontal lines. The y axis displays EC50 titers. A *p* value of ≤0.05 was considered significant.

4.8 Neutralizing antibodies induced by the L2-based nanoparticle antigen Pf FTrx8mer are stable over 1 year after immunization.

The HPV VLP vaccines induce a high level of neutralizing antibodies with high avidity, which can leak into the mucosal surfaces. This is how HPV vaccines provide protection against natural infection (Artemchuk et al., 2019; Petaja et al., 2011; Schwarz et al., 2010). The duration of the immune response induced

by the vaccines determines the timing of the boost doses in order to achieve the strongest immune protection to cover the vulnerable time span of infection during the whole life, or at least the time of life in which most of the HPV transmissions occur. For HPV vaccines, several studies indicate that the antibody titers induced by the HPV VLP vaccines can last for at least eight years (Ferris et al., 2014; Nygard et al., 2015). In order to determine the duration of immunity induced by the best L2-based nanoparticle candidate antigen, mice were immunized with Pf FTrx8mer and compared to mice immunized with Pf Trx8mer OVX313. The mice were immunized three times every two weeks, one month after the last immunization intermediate blood samples were collected and one year after the last immunization the final blood samples were taken. All sera were tested by L1 PBNA. The neutralizing antibody titers induced by Pf FTrx8mer and Pf Trx8mer OVX313 were robust and comparable, which was consistent with former results. The antibodies decreased over time after one year the titers were around 1/10 of values after one month. However, one year later, the neutralizing antibodies against HPV16 were still detected in nine out of ten mice of both groups; and antibodies against HPV18 were detected in seven out of ten mice which were immunized with Pf FTrx8mer and 6 out of 10 mice which were immunized with Pf Trx8mer OVX313 (figure 4.19).



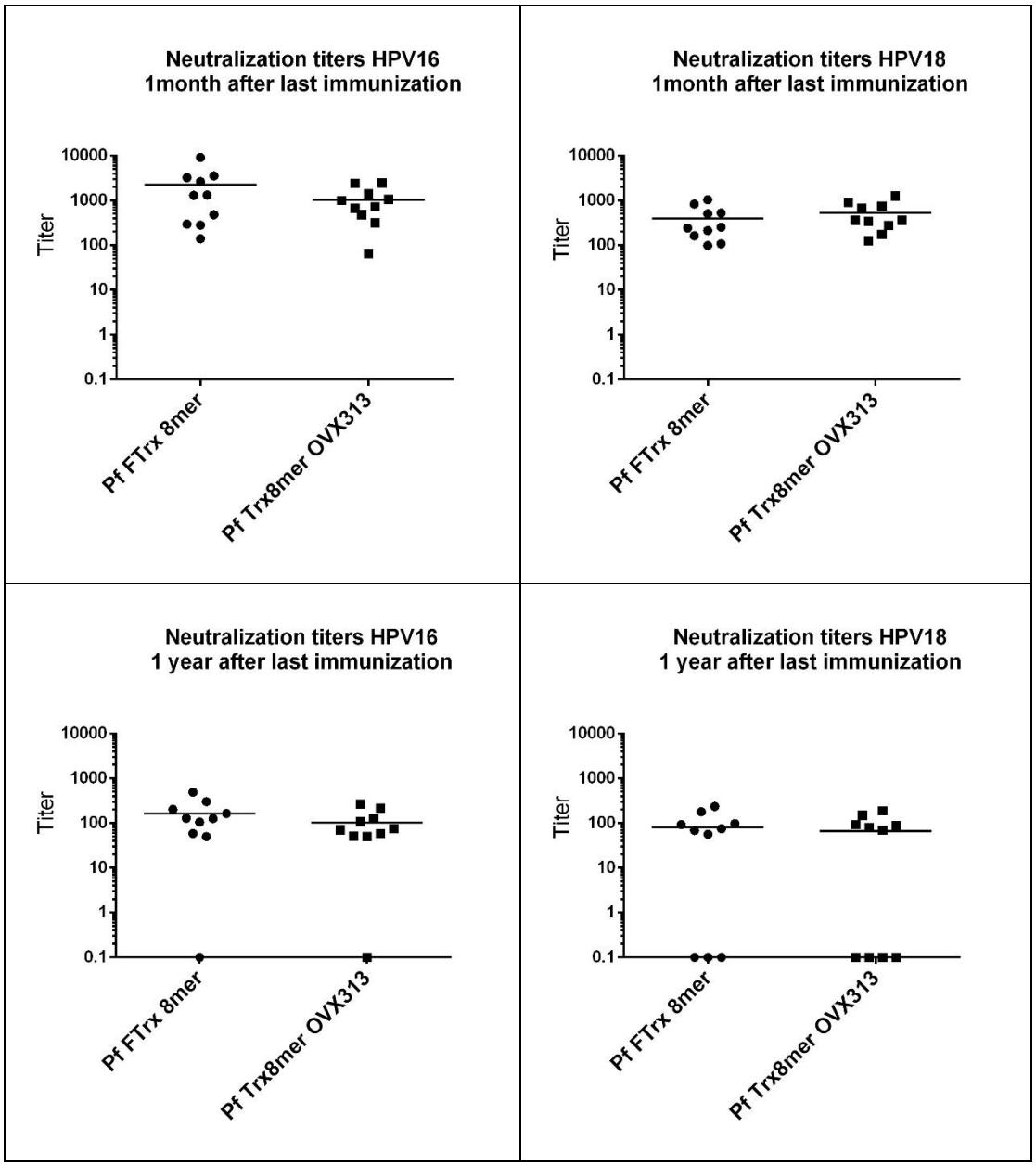
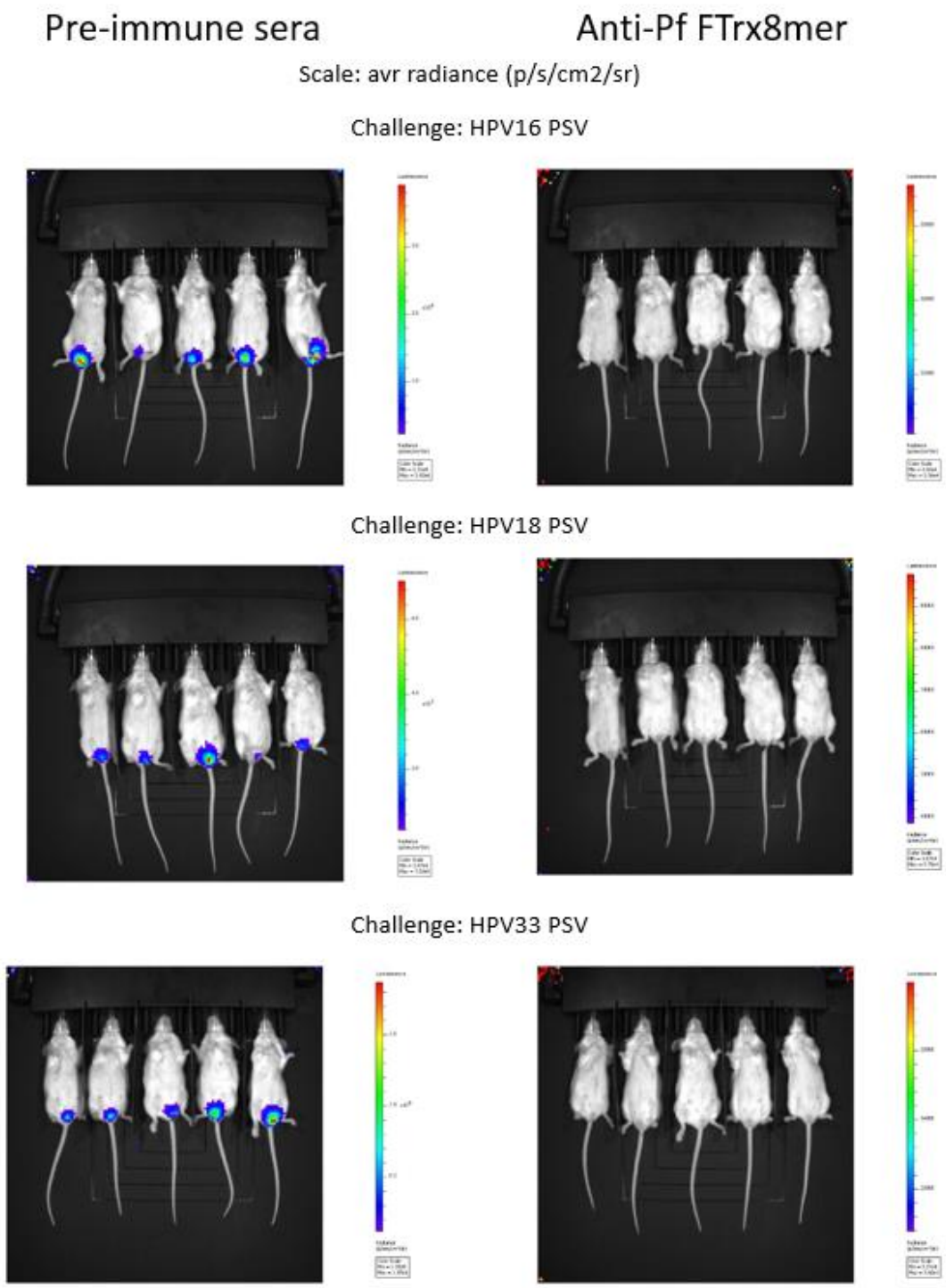
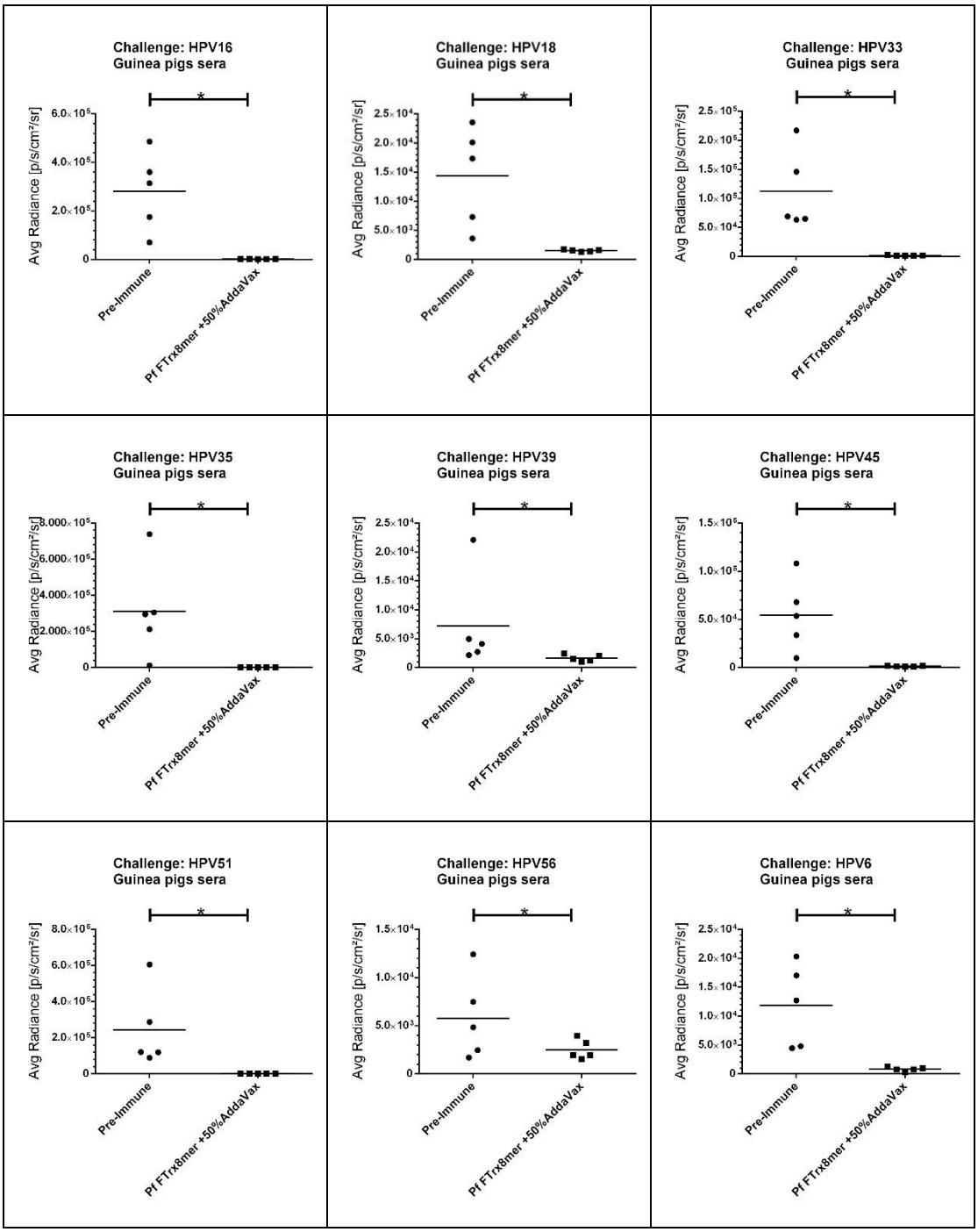


Figure 4.19 L2-based Pf ferritin nanoparticle antigen can induce long term protection against HPV16 and HPV18. Mice were immunized with Pf FTrx8mer or with Pf Trx8mer OVX313 with 50% AddaVax as adjuvant three times every two weeks. One month and one year after the third immunization, the intermediate and final blood sample was collected for L1 PBNA. The symbols represent the neutralizing titers of individual mice. The means titers for each group are indicated by horizontal lines. The y axis displays EC50 titers.

4.9 Neutralizing antibodies induced by L2-based nanoparticle antigen Pf FTrx8mer can provide cross-protection against HPVs infection in a cervico-vaginal challenge model.

The data presented above provide evidence in both mice and guinea pigs that proves that neutralizing antibodies induced by the Pf FTrx8mer nanoparticle antigens are produced in high titers and are cross protective against several HPV according to the L1 PBNA and L2 PBNA assays. To evaluate the efficacy of Pf FTrx8mer nanoparticle antigens in cervico-vaginal challenge models, passive transfer of immune sera from guinea pigs, immunized with the Pf FTrx8mer nanoparticle antigens, to naïve mice was performed. Sera from two immunized guinea pigs were pooled and passively transferred into mice intraperitoneally followed by a challenge with HPV16, 18, 33, 39, 45, 51, 56, 6, and 11 pseudovirions, respectively. Pre-immune sera were used as control. The degree of infection was determined by luciferase activity two days after the HPV pseudovirions instillation. The luminescence signals were measured *in vivo* by IVIS. Passively transferred sera from guinea pigs immunized with the Pf FTrx8mer nanoparticle antigens provided protection against all the HPVs we tested in a 1:40 dilution (figure 4.20).


Pre-immune sera
Anti-Pf FTrx8mer
Scale: avr radiance (p/s/cm2/sr)
Challenge: HPV16 PSV
Challenge: HPV18 PSV
Challenge: HPV33 PSV


Challenge: HPV16
Guinea pigs sera
Challenge: HPV18
Guinea pigs sera
Challenge: HPV33
Guinea pigs sera
Challenge: HPV35
Guinea pigs sera
Challenge: HPV39
Guinea pigs sera
Challenge: HPV45
Guinea pigs sera
Challenge: HPV51
Guinea pigs sera
Challenge: HPV56
Guinea pigs sera
Challenge: HPV6
Guinea pigs sera
Avg Radiance [p/s/cm²/sr]
Pre-Immune
Pf FTrx8mer +50%AddaVax
*

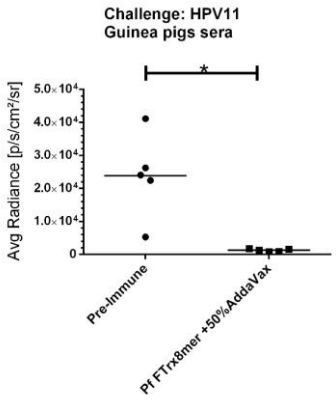
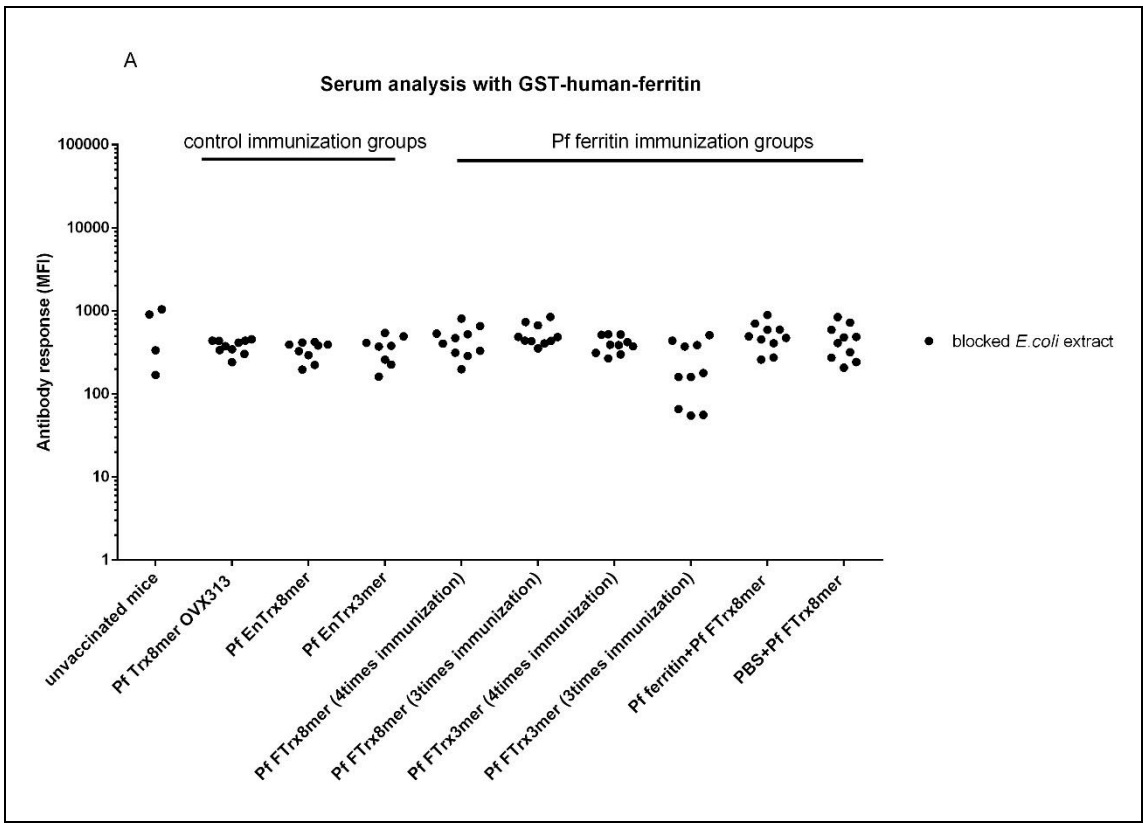


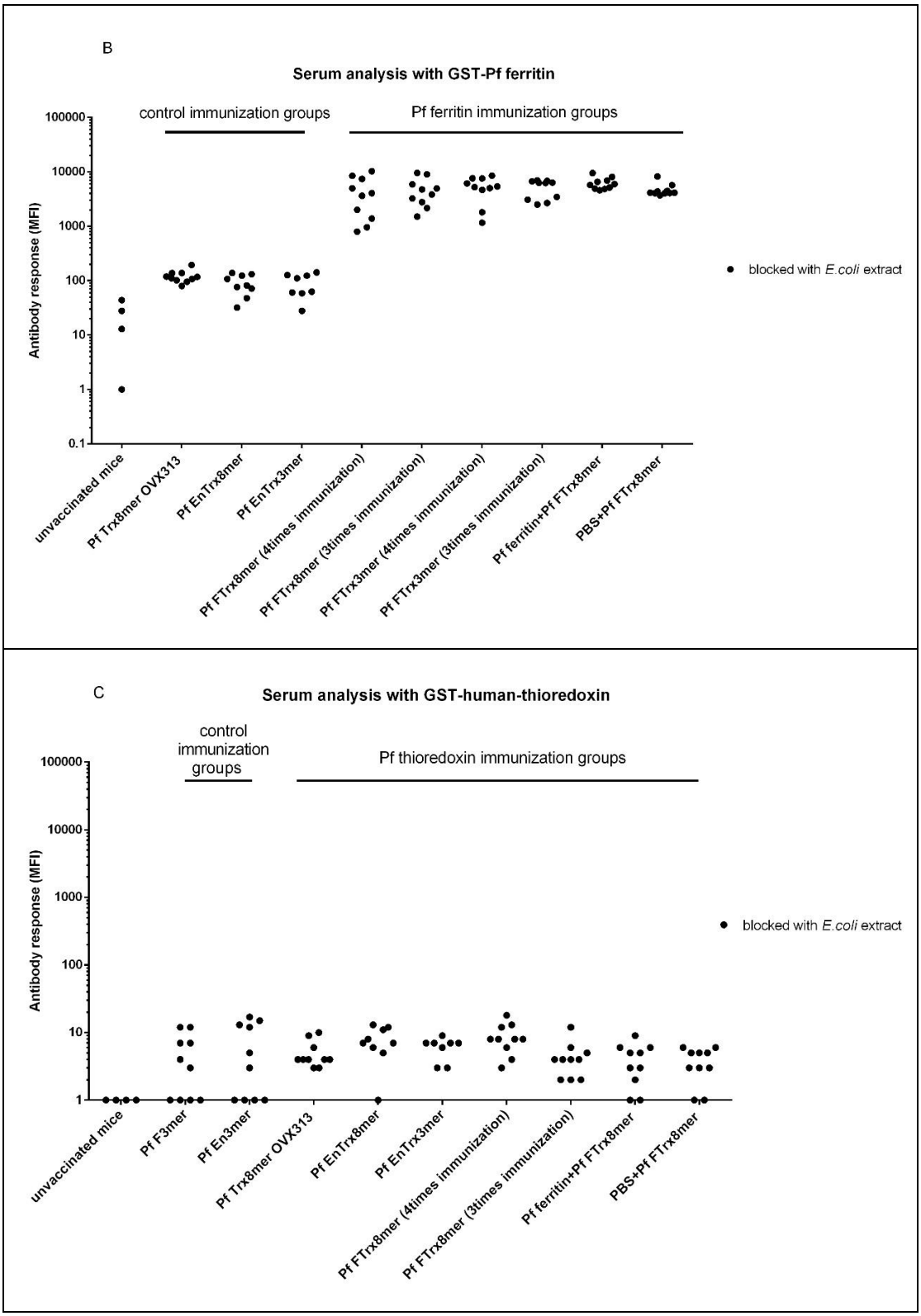
Figure 4.20 Transfer of sera of guinea pigs immunized with Pf FTrx8mer can provide protection against HPV infection *in vivo*. A pool of sera from 2 guinea pigs which were immunized with the Pf FTrx8mer and the sera of mice which were not immunized as control was passively transferred into mice which were then infected with HPV PSV intravaginally. The antibodies elicited by Pf FTrx8mer afforded broad protection against the HPV PSV infection compared to sera from pre-immunized mice. The y axis displays the average radiance measured as photons per second per square centimeter per steradian. The representative images showed the magnitude of vaginal infections. The color scale shown on the right indicates the intensity of luciferase expression, an ROI analysis for average radiance (photons per second per square centimeter per steradian) was performed with Living Image 2.50.1 software. Due to the different *in vivo* transduction activities of the various HPV pseudovirions types, different scales were used. A p value of < 0.05 was considered significant. *.

4.10 Antibodies induced by Pf FTrx8mer do not cross-react with human ferritin.

Given that the sequence homology between Pf ferritin and human ferritin is 26.7%, and between the thioredoxin is, I was concerned that using either Pf ferritin or Pf thioredoxin could possibly cause an autoimmune reaction. To verify this, multiplex serology, a high-throughput bead-based fluorescent method to detect antibodies simultaneously against multiple antigens in one reaction vessel (Brenner et al., 2018; Waterboer et al., 2005), was employed. The

human ferritin heavy chain was selected for the development and validation of multiplex serology assays. Pf ferritin and human ferritin were expressed as recombinant GST-fusion proteins. The mice were immunized four times with antigens fused either with or without Pf ferritin, such as Pf Trx8mer OVX313 or Pf Trx8mer ferritin, etc.; or fused with or without thioredoxin, such as Pf F3mer or Pf FTrx3mer, etc. One month after the last immunization, the mice were sacrificed and the sera were either used without prior treatment or after blocking with *E. coli* lysate. The results indicated there is no difference between the antibody reactions against human ferritin in the sera of all the mice. But there is a significant difference in the antigenicity against Pf ferritin in sera of the mice immunized with antigens containing Pf ferritin and antigens containing other platforms. Similar results were found for human thioredoxin and Pf thioredoxin. The results indicated that the antibodies induced by the Pf FTrx8mer are targeting neither human ferritin nor human thioredoxin (figure 4.21).




B
Serum analysis with GST-Pf ferritin
control immunization groups
Pf ferritin immunization groups
Antibody response (MFI)
100000
10000
1000
100
10
1
0.1
blocked with E.coli extract
unvaccinated mice
Pf Trx8mer OVX313
Pf EnTrx8mer
Pf EnTrx3mer
Pf FTrx8mer (4times immunization)
Pf FTrx8mer (3times immunization)
Pf FTrx3mer (4times immunization)
Pf FTrx3mer (3times immunization)
Pf ferritin+Pf FTrx8mer
PBS+Pf FTrx8mer
C
Serum analysis with GST-human-thioredoxin
control immunization groups
Pf thioredoxin immunization groups
Antibody response (MFI)
100000
10000
1000
100
10
1
blocked with E.coli extract
unvaccinated mice
Pf F3mer
Pf En3mer
Pf Trx8mer OVX313
Pf EnTrx8mer
Pf EnTrx3mer
Pf FTrx8mer (4times immunization)
Pf FTrx8mer (3times immunization)
Pf ferritin+Pf FTrx8mer
PBS+Pf FTrx8mer

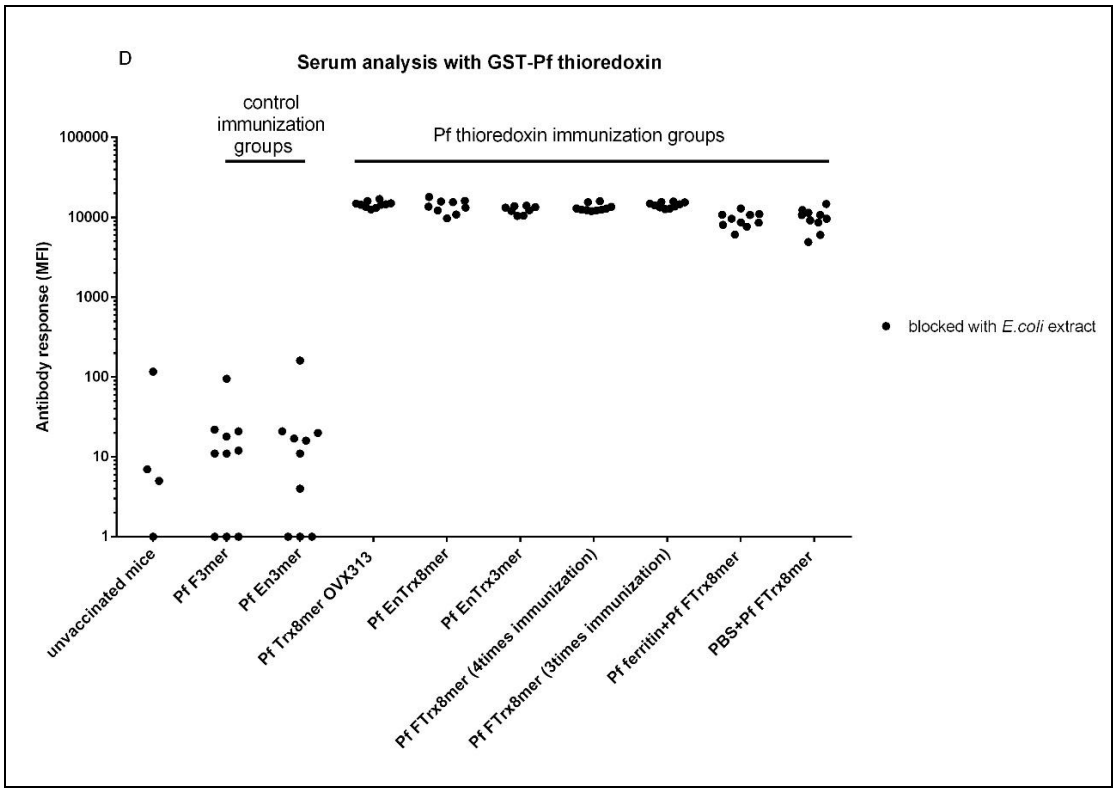


Figure 4.21 Quantitative antibody measurements (MFI) with reference to sero status in ferritin and thioredoxin species-specific monoplex serology. A), Pf ferritin was loaded on the beads; B), human ferritin was loaded on the beads; C), human thioredoxin was loaded on the beads; D), Pf thioredoxin was loaded on the beads. The beads were either not pretreated or blocked with *E. coli*. Although the immunization with Pf ferritin or Pf thioredoxin induced strong responses against these Pf proteins, the antibodies did not cross-react with the human counterparts.

5 Discussion

5.1 The current status of commercial vaccines and the need for second generation prophylactic HPV vaccines.

Unquestionably, current commercial HPV virus like particles (VLP) vaccines are immensely powerful strategies to prevent HPV-associated cancers. After implementation of HPV vaccinations in the last decade, the morbidity and mortality of HPV-related diseases has dramatically decreased (Hall et al., 2019). Currently, there are three prophylactic HPV vaccines on the market (a 2-valent HPV vaccine (HPV16/18) called Cervarix; a 4-valent HPV vaccine (HPV6, 11, 16 and 18) called Gardasil/Silgard, and a nonavalent HPV vaccine (HPV6, 11, 16, 18, 31, 33, 45, 52, and 58) called Gardasil 9, which are all HPV VLPs vaccines). All of them have been proven safe and highly immunogenic. They can provide protection against HPVs that are responsible for almost 90% of cervical cancers. Moreover, the 4-valent and nonavalent HPV vaccines contain VLPs of HPV6 and HPV11, which are the two main types mainly causing benign genital warts. There is also research illustrating that the neutralizing antibodies induced by Cervarix and Gardasil can stably protect from infection up to at least 8 years (De Carvalho et al., 2010). However, the cross-protection against the HPVs not included in the vaccines is limited. The bivalent VLPs vaccine can provide restricted cross-protection against HPV31, 33 and 45 (Brown et al., 2009; Wheeler et al., 2009), but the cross-protection induced by the 4-valent vaccine against HPV31, 33 and 45 is even lower. It is still not clear whether the nonavalent vaccine can also induce cross-protection against the HPVs not included in the vaccine.

As of the 4th October, 2018, there are 91 countries that have included the HPV vaccine in their national immunization program, which also means that more than half of the countries in the world failed to do so (Sterbenc et al., 2018).

There are several reasons that may cause this unfortunate situation. First of all, the high cost of HPV vaccination is the main reason why most developing countries can still not benefit from it. This is such an important challenge to overcome since the majority of cervical cancers occurs in these developing countries. Secondly, it is difficult to reach the target population and make the vaccine schedule endurable. According to World Health Organization guidelines, the HPV vaccine needs to be administered in at least 2 doses to the population which is younger than 15 years and 3 doses to those who are over 15 years of age in order to induce efficient immune responses against possible HPV infections later in life. The multiple immunizations over a long period hinder the compliance of patients (Meites et al., 2016). Thirdly, in many developing countries, the healthcare infrastructure including the vaccination system is not well established for adolescent vaccinations (Meites et al., 2016; Sterbenc et al., 2018).

Despite the success of the current commercial vaccines, the drawbacks drive a lot of efforts on developing second generation HPV vaccines that provide protection against more HPVs and push the use of HPV vaccines in developing countries. Different strategies have been used for this development. One of the strategies is trying to produce the VLPs in an *E. coli* system which lowers the cost of manufacture and speeds up the cycle of production. There is a trivalent HPV VLPs vaccine generated in an *E.coli* system currently being studied in a *rhesus macaque* model for immunogenicity and dosing (Yin et al., 2017). This trivalent prophylactic vaccine contains three different types of VLPs: HPV16, 18, and 58. The reason HPV58 was included might be that it’s the third most prevalent HPV related to cervical cancer in Asia, but is hardly found it in other regions. Therefore, the vaccine is targeting the Asian markets, such China, Japan and Korea (Yin et al., 2017).

Research has revealed that the minor capsid protein L2 is less type specific and can therefore induce broader immune responses than the L1 capsid protein (Day et al., 2012). However, the immunogenicity of L2 is far lower than that of L1 VLPs due to the linear nature of the conserved epitopes which is used to elicit the cross-neutralizing antibodies. There is a lot of research going on to optimize the presentation of L2 epitopes to the immune system in order to increase their immunogenicity without compromising the cross-protection. The most well-known region of L2 cross-neutralizing epitope is aa17-36, which is located at the L2 N-terminus and is conserved among several HPVs (Gambhira et al., 2007). Efforts have been made focusing on the presentation of this region of L2 to B cells repetitively, to evoke sensitivity and stronger immunogenicity. One approach was by the attempt to insert the L2 epitopes into the DE loop of L1 VLPs of HPV16 in order to increase cross-protection (Schellenbacher et al., 2013; Schellenbacher et al., 2009) without losing the high immunogenicity of L1 VLPs. Other authors used the capsomeres which contain both L1 and L2 epitopes. They can be produced in *E.coli* reducing the cost of production and their thermal stability is higher than that of VLPs, which makes the distribution easier, and more cost efficient. Unfortunately, this method compromised the cross-protective ability (Wu et al., 2011). Other approaches are displaying the L2 epitopes on adeno-associated virus particles (Jagu et al., 2015), or using bacteriophages to present the L2 epitopes (Tumban et al., 2011), or simply conjugating a longer portion of L2 which contains the cross protective regions from different HPVs (Jagu et al., 2009; Jagu et al., 2013).

In our group, we focus on how to improve the immunogenicity of L2 epitopes without compromising the ability to elicit cross-protection. We already proved that using Pf thioredoxin as a platform to display the L2 epitopes (aa20-38) increases the immunogenicity (Rubio et al., 2011) and multimerizing the L2 epitopes, using aa20-38 from different HPVs can induce broad cross-protection

among oncogenic HPVs (Seitz et al., 2014). For further improvement, we employed the heptamerization platform OVX313 as the second platform, and instead of one type of L2 epitope, we conjugated the L2 (aa20-38) epitopes of eight different HPVs to Pf Trx. We achieved efficient protection against fourteen high-risk HPVs and two low-risk HPVs.

The work of this PhD thesis focuses on further developing second generation prophylactic vaccines based on the minor capsid protein L2 by employing nanoparticle platform. Nanoparticles are used to present L2 (aa20-38) epitopes to improve L2 immunogenicity and obtain broad protection against all of the high-risk and two low-risk (HPV6 and HPV11) HPVs. An additional aim was that the vaccine would be cost-effective, and thermally stable.

5.2 *Pyrococcus furiosus* ferritin as a promising nanoparticle to present L2 epitopes.

The protection provided by prophylactic vaccines depends on IgG expression or production and the classical development of prophylactic vaccine optimizes for the B cell responses. In order to induce efficient protective responses. The various steps are involved. With the aim of activating both T cell and B cell responses, the principle of vaccine design is trying to have (1) a similar size to the native pathogen, (2) repetitive epitopes to stimulate B cells responses, and (3) the vaccine needs to be recognized by antigen presenting cells and activates T helper cell to help B cells to produce antibodies.

5.2.1 The effects of the nanoparticle size of on the immunogenicity of L2-based antigens

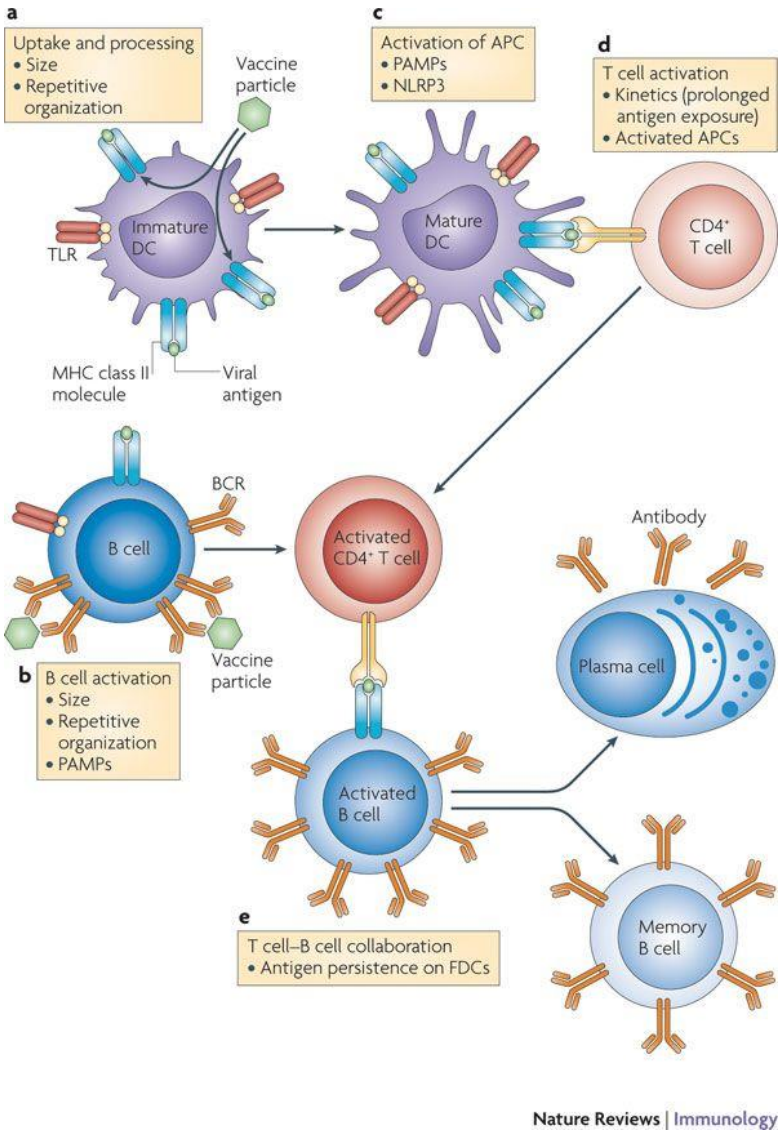
In order to stimulate immune responses, the antigens size has several effects:

i. The suitable size of the antigen helps the uptake and processing by antigen presenting cells.

There are two ways to elicit immune response by vaccination. One is activating B cells directly by interaction with the B cell receptors, the other is the vaccine antigen uptake by antigen presenting cells (APCs), which is then degraded into peptides, and further presented by MCH II that induce CD4+ T helper cell responses, which finally induce B cells to produce antibodies against the infection (figure 5.1). To stimulate T cell responses, the antigen needs to be taken up by APCs, mainly dendritic cells (DCs), these then travel to the lymph nodes and trigger DC maturation to generate potent immune responses. It has been proven that APCs cannot take up and present small soluble proteins efficiently. In contrast, particles which have similar sizes as the pathogens can be taken up and presented by APCs efficiently (Kovacsovics-Bankowski et al., 1993; Swartz et al., 1996; Unanue, 1981).

ii. The antigen with the right size can be transported efficiently to secondary lymphoid organs.

The general methods of vaccine administration are injections either subcutaneously or intramuscularly. Then in order to activate the immune responses, the antigen needs to be transported to lymphoid organ through lymphatic system. There are several factors that affect the efficiency of the entry of antigens into the lymphatic vessels, one of the important factors is the size of the antigen. The optimal size of the antigen which can efficiently enter into the lymphatic system is ~40 nm (Oussoren et al., 1997; Reddy et al., 2006; Swartz, 2001). If the antigen is too small (<10 nm), it will directly enter the blood circulation system after injection; if the antigen is too big (>200 nm) it cannot efficiently enter the lymphatic system since a special carrier is needed to help do so, and thus the antigen takes more time to reach the lymph nodes(Reddy et al., 2007).



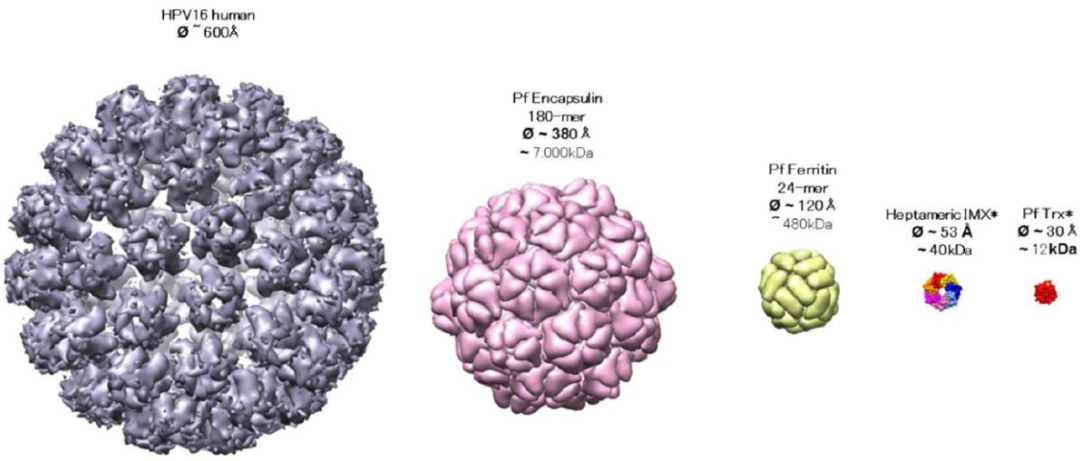
From Bachmann, M.F. and G.T. Jennings, Nat Rev Immunol, 2010.

Figure 5.1 The process of the protective immune responses induced by antigens (Bachmann and Jennings, 2010). In order to generate efficient immune responses, there are steps and critical factors of the antigen that affect the procedure. a) the size and the epitopes repetitive organization would affect the efficiency of antigens uptake by DCs; b) to activate B cells, the antigens' size and the repetitive epitope displayed on the surface of antigens, and the pathogen-associated molecules pattern (PAMPs) influence the efficiency of direct activation of B cells by the antigens; c) Toll-like receptors (TLRs) and other receptors which are also used for pattern-recognition of the antigen PAMPs for APCs activation; d) T helper cells activation is

influenced by the prolonged presence of antigens; e) T helper cells play an essential role in the activation of B cells for antibody generation.

iii. The size of antigen directly affects B cell activation.

There are three critical points for the B cells activation. At first, the antigen needs to stay in its native configuration when the B cell follicles access it in the lymph nodes. Second, a repetitive structure can facilitate the cross-linking of B cell receptors, which is a strong signal for B cells activation (Bachmann et al., 1993; Chackerian et al., 2001; Leadbetter et al., 2002). Third, Toll-like receptor ligands can improve antibody production (Eckl-Dorna and Batista, 2009; Leadbetter et al., 2002; Pasare and Medzhitov, 2005).



From Spagnoli, G. Reformulation of a thermostable broadly protective recombinant vaccine against human papilloma virus. 2016.

Figure 5.2 Different structures of platforms to present HPV antigen (Spagnoli, 2016). The realistic size of different epitopes presenters.

To optimize the L2-based antigen, the nanoparticles Pf encapsulin and ferritin are employed in this thesis, the sizes of these nanoparticles are 24 nm and 10 nm in diameter, respectively (figure 5.2). Pf encapsulin we used in this thesis is assembled by 60 copies of the subunits, and 24 copies of the subunit assemble

into one Pf ferritin nanoparticle. The OVX313, a heptamerization domain, has been used for vaccines and immunotherapies, here all antigens are in the optimum dimensions in order to target adaptive immune responses. The HPV L2 epitopes conjugated with or without Pf thioredoxin were inserted at the C-terminus of Pf encapsulin and N-terminus of Pf ferritin. Theoretically, there are 60 copies and 24 copies of L2 epitopes displayed on the surface of nanoparticles, respectively. One of the reasons behind the high immunogenicity of HPV VLP vaccines is that the size of VLPs resembles that of authentic native HPV viruses, which is 60 nm in diameter, and can thus be taken up by APCs, transported to the lymph node and activate B cells. Encapsulin with Pf Trx-HPV L2 (aa20-38) insertions assembles into a larger structure than that of the native encapsulin (section results 4.2), reaching a similar dimension of VLPs, which can improve the recognition and presentation of L2 epitopes. Pf ferritin is smaller than Pf encapsulin with a diameter of 10 nm and with the L2 epitopes its size is also slightly bigger than native Pf ferritin. The heptamerized Trx-L2 antigen is smaller than Pf ferritin and presents eight copies of L2 epitopes (section 4.2). According to the structural prediction, the OVX313 supermolecule is much smaller than Pf ferritin, Pf encapsulin and VLPs (figure 5.2). Surprisingly, the DLS and SEC shows a different picture. The DLS results indicated the diameter of Pf Trx8mer OVX313 to be 12 nm, which is much bigger than the native OVX313 which is around 5 nm, which may be due to the Pf Trx8mer attachments, and the results of SEC supported this. As for Pf encapsulin, nicely assembled nanoparticles of Pf EnTrx1mer and Pf EnTrx3mer were found by TEM, but subunit structures were also observed and the results were verified by SEC and DLS. Unfortunately, nicely assembled nanoparticles of Pf EnTrx8mer could not be found by TEM. SEC and DLS also showed there were un-uniform structures in the antigens. With Pf ferritin, nicely assembled nanoparticles with all the constructs were produced. The size of nanoparticles

was uniform, such as the diameter of Pf FTrx8mer being 20nm, which is twice as big as the native Pf ferritin. Theoretically, all the nanoparticle antigens, except the Pf EnTrx8mer, are in the right size for uptake and processing by APCs, trafficking to lymph nodes, and interacting with B cells directly to elicit adaptive immune responses.

5.2.2 The highly organized and repetitive nature of the L2 epitopes helps recognition by B cells and T cells.

72 capsomeres assemble into one HPV VLP. These consist of L1 in a highly organized and repetitive manner, similar to that of the native HPV virus. It can efficiently activate B cells and T helper cells since both of them can recognize this significant structure. To activate B cells, the highly repetitive patterns of the VLPs surface play a very important role (Bachmann and Zinkernagel, 1997).

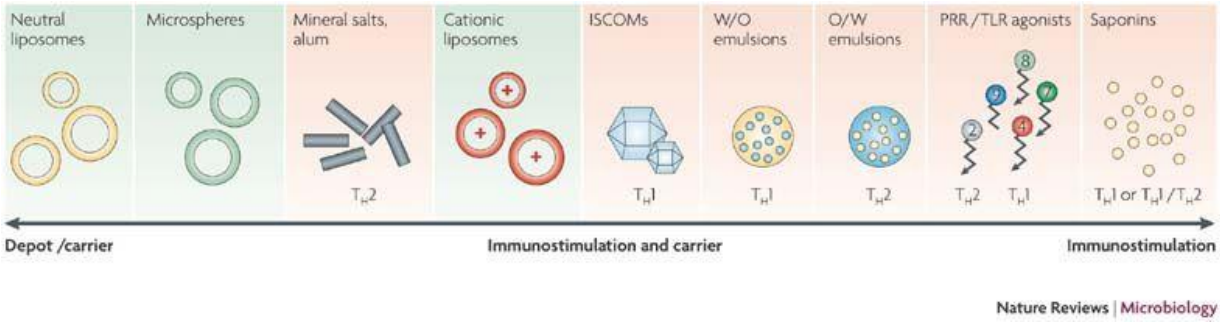
They can activate the cross link of B cell receptors, passing the strong signal to B cells in order to activate them. The research shows that in order to activate B cells, the ideal geometry are the 15-20 hapten molecules which are spaced 5-10 nm apart (Vogelstein et al., 1982). VLPs share a similar geometry to the HPV viral capsid proteins which are in the average space to activate B cells (Bachmann and Jennings, 2010). This is another explanation of the high immunogenicity of VLP vaccines. With this in mind, I designed the nanoparticles displaying L2 epitopes in the 5-10 nm space to optimize the antibody responses. Pf encapsulin nanoparticles present 60 copies of L2 epitopes on their surface. The geometry of the L2-based Pf encapsulin nanoparticle antigens are similar to VLPs. Theoretically, they could efficiently cross-link the B cell receptors to activate B cells. Pf ferritin only contains 24 subunits, and displays 24 copies of L2 epitopes. It is smaller than Pf encapsulin and VLPs, but I presumed it should be better than Pf Trx8mer OVX313 which only presents eight copies of L2 epitopes.

However, the immunogenicity of L2-based Pf encapsulin antigen did not show the superiority over the other smaller particles. Mice were immunized with three different constructs displaying Pf Trx-HPV16 L2 (aa20-38)*3 and the immune responses were evaluated by L1 PBNA (section 4.3). The results showed that Pf Trx3mer OVX313 induced a significantly higher response against HPV16 than Pf EnTrx3mer did. Moreover, Pf FTrx3mer provided more cross-protection against HPV45 than Pf EnTrx3mer (section 4.3). The size of all three particles is within 10-50 nm and all in an efficient range, and it would be expected that the encapsulin construct would be more immunogenic than the other two if one would only consider the impact of size, since bigger antigens can be recognized by APCs more efficiently. A potential explanation of this surprising outcome is that the different immune responses induced by the three particles are influenced by the orientation of the Pf Trx-HPV16 (aa20-38)*3 insertion, which determines how the epitopes are being displayed on the surface of the particle and the density of packing, especially between nanoparticle Pf encapsulin and ferritin. This may influence the epitopes accessibility, how they are approached by B cell receptors and how they present to the T helper cells after processing by DCs. The distance of L2 epitopes on Pf ferritin and OVX313 may allow better cross linking of B cells receptors to further increase the stimulation of B cells. Another explanation of why the immunogenicity of Pf Trx3mer OVX313 is high even as a small antigen is that the central core domain of OVX313 has been proven to contribute to binding of the antigen to CD40 on B cells (Brodeur et al., 2003).

5.3 The L2-based prophylactic nanoparticle vaccines are adjuvant dependent.

Adjuvants have been used to increase the immunogenicity of vaccines for more than 100 years. One function of adjuvant employment during the vaccination is

to create an antigen depot, which means it can prolong the antigens exposure to the immune system. As mentioned before, the IgG responses induced by antigens are T helper cells dependent, so the adjuvant role here is to boost T helper cells responses by acting as an antigen depot rather than stimulate B cells directly (Zinkernagel, 2003). There are many kinds of adjuvants employed in vaccines, they all have different applications as immune-stimulators or antigen depots (figure 5.3).



From Guy, B., Nat Rev Microbiol, 2007.

Figure 5.3 Different properties of adjuvants (Guy, 2007). The main properties of adjuvants are antigens depots, carriers and immunostimulators. Those that can stimulate T helper responses are shown with red background. ISCOMs, immunostimulating complexes; O/W; oil-in-water emulsion; PRR, pattern-recognition receptor; TLR, Toll-like receptor: W/O, water-in-oil emulsion.

Despite the boosting effect that adjuvants provide, there are also some limitations or complications that the adjuvants may cause in vaccination. The adjuvant may complicate the vaccine formulation procedure, which makes the vaccine more expensive, or may cause acute reactogenicity and toxicity issues (Guy, 2007).

In this thesis, in order to find out whether the nanoparticle antigens would still require adjuvants to help reach efficient immune responses, three adjuvants were tested with L2-based nanoparticle antigens in comparison to plain antigens. Alum, acts as an antigen depot and can also stimulate T helper 2 cell

responses (HogenEsch, 2002). Alum-MPLA, besides the benefit Alum provides, can agitate the TLR4 receptors which can induce both Th1 and Th2 responses (Iwasaki and Medzhitov, 2004). The commercial HPV vaccine Cervarix employs Alum-MPLA as an adjuvant to further increase the immunogenicity. AddaVax is an oil-in-water nano emulsion, generating a pro-inflammatory environment to facilitate further immune responses (Tritto et al., 2009). The adjuvants tested with nanoparticle antigens revealed the following results. Pf EnTrx3mer with Alum-MPLA as adjuvant induced the highest neutralizing antibodies against HPV 16 and HPV18, and limited cross-protection against HPV45. Pf FTrx3mer with 50% AddaVax induced the highest titers of neutralizing antibody against HPV16, 18 and 45 (section 4.3). Interestingly, mice responded to Pf FTrx3mer without any adjuvant. It is possible that the nanoparticle Pf ferritin itself works as an antigen depot, and that the T helper epitope, which Pf thioredoxin has, can stimulate T helper cells responses, making it self adjuvanted. Later, I compared different adjuvants with Pf FTrx8mer, verifying that Pf FTrx8mer was able to induce neutralizing antibodies against HPV16 and 18, but the titers induced by Pf FTrx8mer with Alum, Alum-MPLA or without any adjuvant were lower than with 50% AddaVax, and could not induce cross-protection against the HPVs not included in the antigen. Comparing the two nanoparticles, the reasons why Alum-MPLA is the best adjuvant to Pf encapsulin, but not for Pf ferritin, might be the antigen pH and the isoelectric point. Moreover, ferritin with AddaVax's help creates an immune competent environment which drove more antigen presenting cells to the injection site and induced the higher production of cytokines and chemokines, which also helps B cells to be stimulated by the antigen and produce antibodies.

5.4 Comprehensive analysis of immunogenicity of L2-based nanoparticle prophylactic HPV vaccines

5.4.1 Nanoparticles cannot fully substitute for Pf Trx.

In order to investigate the role of Pf Trx in the nanoparticle platform, the mice and guinea pigs were immunized with Pf EnTrx8mer and Pf En8mer with Alum-MPLA, or Pf FTrx3mer and Pf F3mer with 50% AddaVax. Then the immunogenicity of antigens was investigated by using L1 and L2 PBNA. Our data indicated that the nanoparticle platforms fused with L2 epitopes in the context of Pf Trx scaffold had improved immunogenicity in comparison to the L2s displayed on the nanoparticle directly (section 4.4). This finding is consistent with our previous findings, which confirms that Pf Trx is an efficient platform to present L2 neutralizing epitopes (Pouyanfard et al., 2018). As mentioned before, the reason for this meaningful immune response being evoked might be the size of the antigens. Without Pf Trx, the size of nanoparticles would be smaller, and also Pf Trx contains T cell epitopes, which are also important in activating B cells to produce neutralizing antibodies. Moreover, the optimal scenario is that the insertion of the active site of the L2 does not change the structure of Pf thioredoxin, nor the insertion itself, which is the likely case here. Despite all the advantages that Pf thioredoxin provides, another aspect might be that without Pf thioredoxin as the primary platform to display the L2 multimeric epitopes, the orientation of epitopes and how they are displayed on the nanoparticles might be changed, which makes some epitopes were more difficult to reach and thus affected the immunogenicity of the nanoparticle antigens.

5.4.2 Pf ferritin fused with Pf Trx 8mer is the most immunogenic candidate.

To determine the most immunogenic antigen, the sera from the animals which were immunized with Pf EnTrx8mer with Alum-MPLA as adjuvant, Pf FTrx8mer

with 50% AddaVax, and Pf Trx8mer OVX313 with 50% AddaVax were analysed in cell culture and *in vivo* assays.

The cell culture assays are L1 and L2 pseudovirion-based neutralization assays (PBNA). The L1 PBNA is the standard PBNA, but shows lower sensitivity. The L2 assay was developed to detect L2-specific antibodies of which titers are very low in L1 assays. Neutralizing antibody titers in L2 PBNA are 10-20 times higher than those in the L1 PBNA. The L2 PBNA tries to mimic the natural HPV infection, but the extensive furin cleavage and prolonged exposure of L2 epitope may not happen during the natural HPV infection in humans. The L1 and L2 PBNA can only give an indication of the immunogenicity of the antigens and the results should be viewed side by side.

In this thesis, the *in vivo* challenge assay was also employed to evaluate the antigen candidates. The sera of guinea pigs which were immunized with the antigen candidates were transferred into naïve mice and these were transduced with pseudovirions one day later. This assay is a sensitive and stringent measurement of antibody-mediated protection, since neither L1 nor L2 PBNA can reflect the natural neutralization of HPVs *in vivo* in humans, as Joura et al. determined. They found undetectable HPV18 L1 antibody in L1 PBNA can still neutralize HPV18 *in vivo* (Joura et al., 2008).

The mice and guinea pigs sera were tested with thirteen high-risk and one low-risk HPVs. The antibody titers are displayed on the heat map (table 5.1). Pf EnTrx8mer could not induce sufficient antibody production. The reason might be that the antigen I immunized the mice with did not form nanoparticles, since I could not produce well assembled nanoparticle Pf EnTrx8mer. Thus the L2 epitopes were not exposed and could not be presented well to activate B cells causing the low immunogenicity of Pf EnTrx8mer. However, the results of Pf FTrx8mer and Pf Trx8mer OVX313 were comparable with the commonality that

none of the mice produced antibodies against HPV56. The reason might be that HPV 56 aa20-38 sequences are different from the HPVs included in the 8mer. Also, in 6 out of 10 mice, efficient antibodies induced by Pf FTrx8mer against HPV 52 could not be detected, but further L2-based neutralization assay may reveal the truth of antibodies against HPV52. Additionally it seems like one mouse in the Pf FTrx8mer group did not respond well since the mouse could not induce antibodies against several HPVs.

The inbred animal models like BALB/c mouse I employed cannot accurately reflect the immune responses to the antigens in humans. Additionally, the T helper epitopes and antigen uptake efficiency are also different in different animals. To gain a better understanding of how the antigen would behave in humans, another animal model, guinea pigs, was also employed. Moreover, the guinea pig sera were also tested with thirteen high-risk and two low-risk HPVs by L1 and L2 PBNA. The results were consistent with the data from the mice experiments. Pf EnTrx8mer still could not elicit efficient neutralizing antibody production. For HPV31, I could not detect neutralizing antibodies by the L1 PBNA, but there were high titers of neutralizing antibody detected in the L2 PBNA, and the titers of the antibodies induced by Pf FTrx8mer was ten times higher than the titers induced by Pf Trx8mer OVX313. But I could not detect antibodies against HPV52 and 56 by the L1 PBNA and I could not perform L2 PBNA due to our supply constraints of HPV52 and 56 pseudovirions. The antibody titers in guinea pigs sera are much higher than those in of mice sera and in the same sera, the titers detected by L2 PBNA are much higher than those analysed in the L1 PBNA.

Table5.1 Heatmap of the titers of neutralizing antibodies in mice immunized with Pf EnTrx8mer, Pf FTrx8mer, and Pf Trx8mer OVX313 four times at two weeks intervals formulated with Alum-MPLA, 50% AddaVax, and 50% AddaVax respectively. Final sera were collected one month after the last immunization and titrated in the standard L1 PBNA.

		Neutralization titer (L1 PBNA)													
	animal (mice)	HPV16	HPV18	HPV31	HPV33	HPV35	HPV39	HPV45	HPV51	HPV52	HPV56	HPV58	HPV68	HPV73	HPV6
Pf EnTrx8mer +Alum-MPLA	1	70	57.54	0.1	0.1	0.1	71.4	54.82	0.1	0.1	0.1	0.1	0.1	0.1	0.1
	2	0.1	0.1	0.1	0.1	0.1	39.15	46.68	0.1	86.9	0.1	0.1	0.1	0.1	0.1
	3	88.39	103.4	0.1	0.1	0.1	93.86	67.89	0.1	0.1	0.1	0.1	0.1	50.18	0.1
	4	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
	5	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	58.07	0.1	209.2	209.2	0.1	0.1
	6	58.87	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	101.8	101.8	0.1	0.1
	7	174.2	238.7	0.1	67.2	53.04	0.1	122.7	0.1	0.1	0.1	0.1	0.1	64.91	0.1
	8	87.79	124.1	0.1	0.1	51.69	167.8	114.3	143.4	133.9	0.1	70.98	70.98	125.6	0.1
	9	0.1	0.1	0.1	0.1	0.1	0.1	50.27	0.1	0.1	0.1	0.1	0.1	0.1	0.1
	10	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Pf FTrx8mer +50% AddaVax	1	1164	129	69.95	172.1	176.4	371.3	524.7	65.84	0.1	0.1	49.39	49.39	111.9	88.81
	2	4123	0.1	92.23	219.3	273.2	728.7	921.8	97.44	0.1	0.1	79.05	79.05	1633	96.96
	3	661.8	94.8	85.57	233.9	179.9	528.1	566.3	104.7	80.65	0.1	140.2	140.2	858.7	99.26
	4	201	110.5	62.98	130.7	96.07	263.4	279.7	63.02	0.1	0.1	53.23	53.23	622.1	0.1
	5	625.9	660.4	0.1	103.6	91.05	90.72	82.15	63.36	57.53	0.1	0.1	0.1	154.1	0.1
	6	366.7	266.1	59.1	144	67.18	155.7	156	98.44	80.86	0.1	0.1	0.1	187.5	68.7
	7	2707	901.9	79.75	211.2	319.2	1060	1442	99.44	65.57	0.1	209.2	209.2	537.6	67.48
	8	1789	242.9	51.17	190.4	203.8	484.1	618.1	62.95	0.1	0.1	101.8	101.8	1676	89.42
	9	192.6	77.31	0.1	0.1	0.1	31.21	0.1	0.1	0.1	0.1	0.1	0.1	884.2	0.1
	10	2766	76.56	59.51	271.1	323.5	571.4	800.8	0.1	0.1	0.1	70.98	70.98	195.8	110.8
Pf Trx8mer OVX313+50% AddaVax	1	2651	830.3	131.2	248.1	175.4	1097	588.5	60.04	51.03	0.1	1502	1502	261.4	82.34
	2	1343	512.8	77.73	369.7	154.9	250	378	61.58	55.77	0.1	67.11	67.11	177.4	130
	3	636.6	304.5	0.1	166.6	121	121.6	106.7	0.1	0.1	0.1	0.1	0.1	135.5	58
	4	3065	846.6	130.8	367	256.9	3199	1280	60.36	55.11	0.1	244.1	244.1	370.1	113.3
	5	585.1	1026	0.1	289	214.6	136.9	169.8	62.95	52.07	0.1	93.78	93.78	315.6	78.25
	6	1030	510.2	78.15	237.7	175.6	203	323.4	73.29	96.97	0.1	175.3	175.3	456.5	91.72
	7	531.6	1218	90.87	345.6	230.4	523.3	872.8	83.03	96.51	0.1	283.7	283.7	640.7	116.9
	8	1125	1371	111.5	613.3	366.4	438.3	477.9	57.8	52.61	0.1	89.31	89.31	269.3	197
	9	770.6	438	138.2	252.4	198.8	358.2	344.2	87.95	92.08	0.1	81.18	81.18	206.8	58.72
	10	10689	2808	228.1	794.1	1005	1747	1940	160.3	172.7	0.1	299.4	299.4	1898	412.1

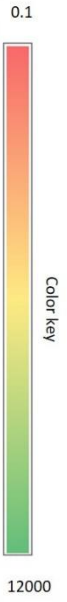


Table 5.2 Heatmap of the titers of neutralizing antibodies of guinea pigs immunized with Pf EnTrx8mer, Pf FTrx8mer, and Pf Trx8mer OVX313 four times at two weeks intervals formulated with Alum-MPLA, 50% AddaVax, and 50% AddaVax, respectively. Final sera were collected one month after last immunization and titrated in the standard L1 PBNA and L2 PBNA.

		neutralization antibody titers															
		HPV16		HPV18		HPV31		HPV33		HPV35		HPV39		HPV45		HPV51	
	animal (guinea pig)	L1 PBNA	L2 PBNA	L1 PBNA	L2 PBNA	L1 PBNA	L2 PBNA	L1 PBNA	L2 PBNA	L1 PBNA	L2 PBNA	L1 PBNA	L2 PBNA	L1 PBNA	L2 PBNA	L1 PBNA	L2 PBNA
Pf EnTrx8mer +Alum-MPLA	1	5196	35142	76.63	477.2	0.1	0.1	191.7	298.1	75.45	/	155.3	273.1	75.45	434.3	155.3	/
	2	4270	63157	71.43	366.6	0.1	0.1	202.9	153.2	0.1	/	162.1	0.1	0.1	0.1	162.1	/
Pf FTrx8mer +50% AddaVax	1	31237	303946	4486	1986	0.1	1344	1459	1421	2484	/	944.2	30219	2484	3249	944.2	/
	2	80766	1228000	5641	4266	0.1	2541	14677	12715	6021	/	11502	31088	6021	10400	11502	/
Pf Trx8mer OVX313 +50% AddaVax	1	39981	128474	3962	2520	0.1	133.4	92122	4874	4037	/	2185	21625	4037	4780	2185	/
	2	134706	669213	4600	1754	0.1	73.09	142069	2915	3637	/	928.4	27658	3637	12098	928.4	/
		HPV52		HPV56		HPV58		HPV59		HPV68		HPV73		HPV6		HPV11	
	animal (guinea pig)	L1 PBNA	L2 PBNA	L1 PBNA	L2 PBNA	L1 PBNA	L2 PBNA	L1 PBNA	L2 PBNA	L1 PBNA	L2 PBNA	L1 PBNA	L2 PBNA	L1 PBNA	L2 PBNA	L1 PBNA	L2 PBNA
Pf EnTrx8mer +Alum-MPLA	1	0.1	/	0.1	/	61.79	/	59.28	/	77.24	/	720.9	/	56.84	683.8	56.84	696
	2	0.1	/	0.1	/	0.1	/	0.1	/	0.1	/	658	/	0.1	214.6	0.1	100.7
Pf FTrx8mer +50% AddaVax	1	0.1	/	0.1	/	1297	/	0.1	/	418.7	/	5214	/	177.2	3531	171.4	21184
	2	0.1	/	0.1	/	2895	/	85.78	/	841.6	/	23724	/	622.5	30498	1737.19	40863
Pf Trx8mer OVX313 +50% AddaVax	1	0.1	/	0.1	/	1358	/	81.67	/	546.1	/	6815	/	232.8	14327	429.3	24479
	2	0.1	/	0.1	/	1983	/	64.21	/	505.4	/	17192	/	334.4	25091	350.7	22097

In order to verify the neutralizing antibodies in guinea pigs induced by the Pf FTrx8mer cervico-vaginal challenge *in vivo* experiments were performed. Guinea pig sera were passively transferred to naïve mice intraperitoneally, then the mice were challenged with HPV16, 18, 33, 35, 39, 45, 51, 56, 6, and 11, respectively. Guinea pig sera provided clear protection against all HPV infections. Even against HPV56 where the neutralizing antibody could not be detected by the L1 PBNA, sera from guinea pigs immunized with Pf FTrx8mer were able to provide protection against infection *in vivo*. However, these results should be retested in large scale experiments, since there is a variability of the infection efficiency in the mice. This inspired another question: at what scale

can titer tested *in vitro* give us an expectation about whether it is sufficient to provide protection against natural infection?

5.4.3 Pf FTrx 8mer could provide long-term protection against HPVs

The HPV vaccines are mostly administered to young adolescent girls. However, women face the risk of HPV infection throughout their whole life, which makes the duration of the protection critical to the effectiveness of HPV prophylactic vaccines. There is a proof that the current HPV VLPs vaccines can provide protection against HPV infection for 8 years after administration of the vaccines. Another experiment had to be performed to evaluate if the nanoparticle vaccine could offer a similar long term protection. The mice were immunized three times with Pf FTrx8mer and Pf Trx8mer OVX313, with 50% AddaVax at two week intervals, then the antibody responses checked1 month after the last immunization. One year after the last the immunization, the mice were sacrificed and the sera were collected for antibody detection. The results showed both antigens provide long-term protection against HPV16 and 18 *in vitro*, and the Pf FTrx8mer showed a slight advantage over Pf Trx8mer OVX313, but the difference was not significant (figure 4.19). There are several factors which influence long-term protection induced by vaccination. In humans, the peak of antibody responses induced by vaccination is 1 month after the last immunization then the rates decay. The proliferation rate of B cells, the immunologic memory of B cells, individual variability in the immunity response are all factors that influence the duration of effectiveness of vaccines (De Vincenzo et al., 2014). In order to provide long term protection, one important aspect of the vaccine is the ability of immune memory development, which also means inducing long lived memory immune cells, such as memory B cells, that should be activated when an individual is re-exposed to the antigen in a relative vigorous infection (Stanley, 2010). Different scenarios are then possible, one

can induce memory lymphocytes which may not generate high titers of serum antibodies, such as hepatitis B vaccine (Ward et al., 2008); or one could continue to provide high titers of neutralizing antibodies by generating long-lived plasma cells, with the induction of memory B cells, and then supply antibody-secreting cell pools, such as HPV VLP vaccines (Stanley, 2010). In the long term experiments when the antibody titers were tested one month after the three-dose series, all the mice showed high levels of specific anti-HPV antibody production, which means the antigens efficiently activate B cells to produce anti- antigen antibodies. But one year later, the neutralizing antibodies against HPV16 were still detected, which may indicate that these two antigens induced the production of long-lived plasma cells, and maybe the production of memory B cells, which would need to be tested by a fourth dose. In addition, the results of the long-term experiments when gave us further evidence of whether another booster would be required, in this case the 3-time dosage proved sufficient.

5.5 Pf ferritin induced pre-existing anti-ferritin antibodies that interfered with anti-L2 antibody production.

During the experiments, I found that the titers of L2-antibodies tested one week after immunizing the mice with Pf FTrx8mer were higher than those of Pf Trx8mer OVX313, but one month later this particular advantage disappeared. Then the kinetics of the antibody levels had been investigated because the fourth booster of Pf FTrx8mer negated the former advantage of this antigen. There was a hypothesis that pre-existing antibodies against Pf ferritin would interfered with L2-specific antibody production. To test this, two groups of mice were immunized 3 times with either Pf ferritin or 1 × PBS with a biweekly interval, then both groups were immunized with Pf FTrx8mer. The mice were sacrificed and sera were investigated by L1 PBNA for neutralizing antibody detection. The results confirmed our hypothesis. The pre-existing anti-ferritin antibodies

interfered with the neutralizing antibody production against the HPV L2. As discussed before, in order to activate B cells to produce antibodies against specific antigens, not only do the antigens need to bear the appropriate B cell epitopes to directly activate the B cells, but the epitopes also have to be presented to MHC II to stimulate T helper cells activation and maturation in order to further activate B cells to produce neutralizing antibodies (Okada et al., 2005; Parker, 1993; Ruprecht and Lanzavecchia, 2006). The point here would be that the epitopes which were recognized by B cells directly, should be the same ones to present to T helper cells. Otherwise, the immune response to the protein presenter will dominate over the target antigen (Peeters et al., 1991; Pobre et al., 2014; Schutze et al., 1985). I think that it is possible that Pf ferritin epitopes are also presented to T helper cells, which then interfered with the L2 specific B cell activation. On the other hand, the primary purpose of the immune system is to identify foreign substances, such as antigens. Then the immune response induced by the foreign antigens develops a defence against it. The whole procedure normally includes antibody production by activating B cells, and T cells in order to eliminate the foreign antigens. The influence of the antibodies against Pf ferritin may involve both. One reason might be that the anti-Pf ferritin hinder the binding of neutralizing antibody, the other possibility might be the T cell responses induced by the antigens eliminate themselves during further immunization, which makes the boost inefficient.

5.6 Auto immune responses against human ferritin and human thioredoxin

Safety is always the first concern in vaccine development. Despite the toxicity of antigens, there are rising concerns regarding molecular mimicry. Molecular mimicry can occur when there is significant similarity between human proteins and the proteins contained in vaccines (Kanduc, 2010; Kanduc et al., 2008;

Oldstone, 1998). This homology between antigens and self-proteins might lead to immune cross reactivity, which means that the antibodies produced by the vaccines mean to target the pathogens, they may target the human proteins as well, which may cause autoimmune diseases (Agmon-Levin et al., 2009). In an attempt to understand the potential ability for vaccine driven autoimmune responses, I compared the sequence of human ferritin and *Pyrococcus furiosus* ferritin and it is 27.749% homologous is compared (figure 5.4) Multiplex serology was used to detect if the antibodies induced by the antigens recognise human ferritin or thioredoxin (section 4.10). The antibodies induced by the antigens, especially Pf FTrx8mer did not target human ferritin, and meanwhile, all antigens containing Pf Trx induced antibodies that did not target the human thioredoxin either. There is no doubt that the relationship between human and their disease-inducing micro-organisms is complicated, but this result affirms our hopes to further investigate nanoparticles as promising vaccines.

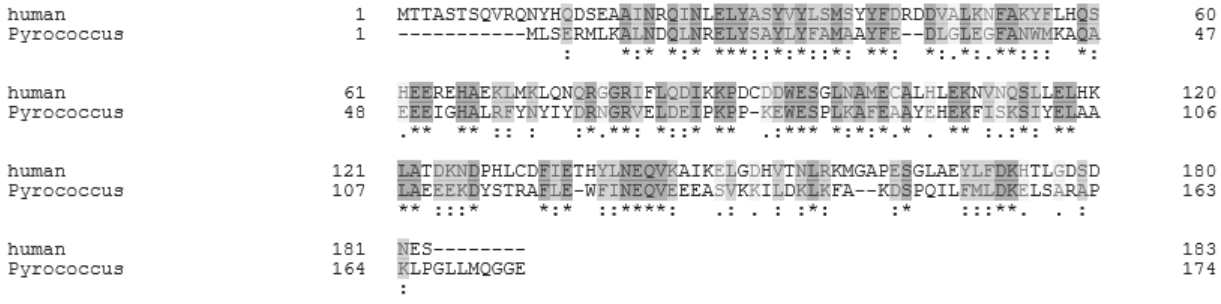


Figure 5.4 Alignment of *Pyrococcus furiosus* ferritin and human ferritin. Predicted ferritin polypeptides from humans are aligned with *Pyrococcus furiosus*. Amino acids that are identical in both are shown with a dark background with '*', closely similar sequences are shown in grey with ':'.

5.7 Conclusions & Outlook

Despite the success of current commercial HPV VLP vaccines, their limitations cannot be ignored. These limitation include low cross-protection, cost

inefficiency, and need for multiple administrations over a long time period causing poor compliance of patients. The L2-based second generation prophylactic HPV vaccine approach provides new perspectives to overcome these limitations. We fused the Pf Trx with different copies of HPV L2 epitopes complexes to the NH_2- or COOH- terminus of Pf ferritin or encapsulin, purified and characterized the antigens with different methods. We produced nicely assembled nanoparticles except of the Pf EnTrx8mer, which may be due to the different charges between Pf Trx 8mer and the Pf EnTrx8mer protein resulting in the inability to assemble into native-like nanoparticles properly. Separate immunizations were carried out in mice and guinea pigs, the cell culture based neutralization L1 and L2 PBNA, and animal *in vivo* infection assays were employed to investigate the neutralizing antibodies induced by the antigens. The best adjuvant for each platform was determined. With nicely assembled particles, the animal responses to Pf EnTrx3mer were not as good as to Pf FTrx3mer or to Pf Trx3mer OVX313. This observation highlights the importance of adequate antigen presentation, such as how well the conformational authenticity of functional domains, in this case the HPV L2 epitopes, referenced the quality of immune response. The diameter of Pf FTrx8mer and Pf EnTrx8mer nanoparticles are between 10-50 nm, and are therefore within the efficient range to traffic through lymph cords to lymph nodes, which means the capacity of trafficking should be the same as that of the natural virus. Once they reach the lymph nodes, they would be taken up by DCs and macrophages to activate B cells, here the Pf encapsulin platform should be more advantageous, but it is not One potential explanation for the surprising difference in immunogenicity is the orientation of L2 epitopes displayed by Pf encapsulin relative to the surface of the particle, as well as the packing density. These may affect how efficiently the L2 epitopes are accessed by the immune receptors, which results in different responses of animals.

If the nanoparticles contained ferritin instead of encapsulin as in the case of Pf FTrx8mer these showed a lot of strengths. The antibodies induced by Pf FTrx8mer could neutralize a large panel of high-risk oncogenic HPVs, and 1-2 low-risk HPVs, both *in vitro* and *in vivo*. I believe that this nanoparticle antigen comprised of HPV L2 epitopes can provide broad cross-protection against oncogenic and other disease related HPV types.

However, there are certain limitations about this nanoparticle antigen: a), the antigens were produced in H5 insect cell systems, which makes them more expensive than the antigens produced in *E.coli* or other systems, but there is evidence that the same nanoparticle antigen construct can also be produced successfully in an *E.coli* system. b), there is a big concern about the pre-existing anti ferritin antibodies inferring with the production of neutralizing antibodies, further investigation about heterologous immunization may give us the opportunity to overcome it. Since nicely assembled Pf encapsulin nanoparticles with Pf Trx8mer could not be produced up to now, there are several strategies that could be employed to solve this problem, such as spytag-spycatcher, and streptomycin avidin-avitag technologies. In addition, the opportunity to combine the nanoparticle with E7 epitopes and/or E2 epitopes, which are HPV onco-proteins, would produce a combined vaccine with prophylactic and therapeutic benefits.

6 Summary

Over 200 different types of human papillomaviruses (HPV) have been identified based on their DNA sequences. Some of them are highly associated with the development of cancer, such as HPV 16 and 18 which are responsible for 70% of cervical cancer in the world. Thirteen other oncogenic HPVs are also related of cervical cancer. HPV infections have also been proven highly related to anal cancer, penile cancer and others (such as head and neck cancers). There are three HPV prophylactic vaccines currently available, all composed of virus-like particles (VLPs) based on the HPV major capsid protein L1. They have been proven safe and highly immunogenic, but they are HPV type specific. They all need at least two to three administrations which results in low medicine compliance. Moreover, they are not cost efficient, which means that most developing countries could not benefit from them. All of these shortcomings pushed the development of a second generation of HPV prophylactic vaccines. The minor capsid protein L2, in contrast to the major capsid protein L1, contains cross neutralization epitopes which can elicit cross-protective responses, but the immunogenicity of L2 is low since the cross-neutralizing epitope is linear. Former colleagues showed that inserting the cross-neutralization epitope (aa20-38) of L2 into *Pyrococcus furiosus* thioredoxin (Pf Trx) greatly enhanced the immunogenicity of L2 and could induce the production of neutralizing antibodies. The objective of this thesis is the development of cross-protective L2-based HPV nanoparticle vaccines. To this final goal, I 1) verified the best adjuvant for the nanoparticle antigens; 2) identified Pf Trx's role in the nanoparticle antigenicity; 3) determined the best nanoparticle antigen candidate; 4) determined the influence of the antibodies induced by the nanoparticle platform on the production of neutralizing anti-HPV antibodies; 5) defined the effective nanoparticle antigen dose; 6) demonstrated the

immunogenicity of nanoparticle antigens *in vivo*; and, 7) assessed the immune-relationship between Pf ferritin and human ferritin.

Pf ferritin and encapsulin were employed as nanoparticle platforms to carry and display the trimeric and multimeric L2 epitopes with/without thioredoxin. Correctly assembled and uniformed nanoparticle antigens were produced with all platforms except with the exception of the multimeric L2 epitopes inserted into Pf encapsulin.

The best adjuvant for Pf encapsulin is Alum-MPLA, and the best adjuvant for Pf ferritin is AddaVax. However, high titer antibodies against HPV16 induced by Pf ferritin even in the absence of an adjuvant were detected, which indicated that Pf ferritin based L2 prophylactic vaccine work in a self-adjuvanted way. And the nanoparticle antigens with Pf thioredoxin elicited much higher immunological responses in mice compared to constructs that did not contain Pf Trx. The nanoparticle antigen consisting of Pf ferritin fused with eight different HPV L2 epitopes displayed by Pf Trx (Pf ferritin-Trx-HPVL2(aa20-38)*8) was identified as the best candidate as it could induce high titers of neutralizing antibodies against 13 types of high-risk HPVs and HPV6 tested by Pseudovirus based neutralization assay (PBNA). Moreover, the capacity of the neutralizing antibodies induced by the nanoparticle antigen Pf ferritin-Trx-HPVL2(aa20-38)*8 was verified in an *in vivo* cervico-vaginal challenge animal model with HPV16, 18, 33, 35, 39, 45, 51, 56, 6, and 11. By tracking the antibody levels during the course of the immunizations I found that three doses would be efficient to induce high titers of antibodies.

Experiments testing the influence of the Pf ferritin platform on the immune responses revealed that pre-existing anti-Pf ferritin antibodies interfered with the induction of L2-specific neutralizing antibody production. Serology

experiments verified that the antibodies produced by the nanoparticle L2 antigens containing Pf ferritin could not target the human ferritin.

All in all, Pf ferritin Trx-HPVL2(aa20-38)*8 proved to be an excellent candidate for the presentation of epitopes that could elicit high titers of neutralizing antibodies that were effective *in vitro* and *in vivo.*

7 Zusammenfassung

Über 200 verschiedene Arten von humanen Papillomaviren (HPV) wurden anhand ihrer DNA-Sequenzen identifiziert. Einige von ihnen sind in hohem Maße mit der Entstehung von Krebs verbunden, wie HPV 16 und 18, die für 70% der Gebärmutterhalskrebse auf der Welt verantwortlich sind. Dreizehn weitere onkogene HPVs sind ebenfalls mit Gebärmutterhalskrebs verbunden. HPV-Infektionen sind auch in hohem Maße mit Analkrebs, Peniskrebs und anderen Krankheiten (z.B. Kopf- und Halskrebs) assoziiert. Es gibt derzeit drei prophylaktische HPV-Impfstoffe, die alle aus virus-ähnlichen Partikeln (VLPs) auf Basis des HPV major capsid protein L1 bestehen. Sie sind nachweislich sicher und hoch immunogen, aber sie sind HPV-typenspezifisch. Sie benötigen alle mindestens zwei order drei Verabreichungen, was zu einer geringen Impfkonformitat führt. Außerdem sind die Impfstoffe teuer, was bedeutet, dass die meisten Entwicklungsländer nur schwer von ihnen profitieren können. Alle diese Mängel trieben die Entwicklung der zweiten Generation eines prophylaktischen HPV-Impfstoffs voran. Das Nebenkapsidprotein L2 enthält im Gegensatz zum major capsid protein L1 Kreuzneutralisationsepitope, die Kreuzschutzreaktionen gegen andere HPV Spezies hervorrufen können, aber die Immunogenität von L2 ist gering, da das L2 Epitope linear ist. Ehemalige Kollegen zeigten, dass die Einführung des hockkonservierten Epitops (aa20-38) von L2 in *Pyrococcus furiosus* Thioredoxin (Pf Trx) die Immunogenität von L2 stark erhöht und die Produktion von neutralisierenden Antikörpern induzieren kann. Das Ziel meiner Arbeit war die Entwicklung von kreuzschützenden L2-basierten HPV-Nanopartikel-Impfstoffen. Zu diesem Zweck haben ich 1) das beste Adjuvans für die Nanopartikelantigene überprüft; 2) die Rolle von Pf Trx im Nanopartikelantigen identifiziert; 3) den besten Nanopartikelantigenkandidaten bestimmt; 4) den Einfluss der durch die Nanopartikelplattform induzierten Antikörper auf die Produktion

neutralisierender anti-HPV Antikörper bestimmt; 5) die effektive Nanopartikel-Antigendosis definiert; 6) die Immunogenität von Nanopartikel-Antigenen *in vivo* nachgewiesen; und (7) die Immunrelation zwischen Pf-Ferritin und menschlichem Ferritin bewertet.

Pyrococcus furiosus Ferritin und Encapsulin wurden als Nanopartikelplattformen eingesetzt, um die trimeren und multimeren L2-Epitope mit oder ohne Thioredoxin zu inkoroprieren und zu präsentieren. Mit alle Platformen wurden korrekt zusammengesetzte und uniformierte Nanopartikelantigene hergestellt, mit Ausnahme der multimeren L2-Epitope, die mit Pf-Encapsulin zusammengesetzt eingesetzt wurden.

Das beste Adjuvans für Pf-Encapsulin ist Alum-MPLA, das beste Adjuvans für Pf-ferritin ist AddaVax, aber ich konnte auch ohne Adjuvans nachweisen, dass Pf-Ferritin-basierte L2-e-Impfstoffe selbst-adjuvant wirken und effektive Antikörper produziert wurden. Nanopartikel-Antigene die Pf Thioredoxin enthielten riefen bei Mäusen eine viel höhere Reaktionen hervor als Konstrukte ohne Pf Thioredoxin. Somit is Pf Thioredoxin ein wichtiges Immunstimulans. Das Nanopartikel Konstrukt mit Pf-Ferritin, das mit acht verschiedenen durch Pf Trx präsentierten HPV L2-Epitopen (Pf-ferritin-Trx-HPVL2(aa20-38)*8) fusioniert ist, wurde als bester Kandidat identifiziert. Es konnte führte zu hohen Titer von neutralisierenden Antikörpern gegen 13 hoch riskante HPVs und HPV6, wie ich in derPseudovirus-basierter Neutralisationstest zeigen konnte. Darüber hinaus wurde die Kapazität der neutralisierenden Antikörper, die durch das Nanopartikelantigen Pf ferritin-Trx-HPVL2(aa20-38)*8 induziert wurden, in einem *in vivo* zervikal-vaginalen Challenge-Tiermodell überprüft. Durch Überprüfung der Antikörpertiter während der Impfungen fanden ich heraus, dassdrei Dosen effizient sein würden, um hohe Titer von Antikörpern zu induzieren.

Experimente, die den Einfluss der Pf-Ferritin-Plattform auf die Immunantwort untersuchten, zeigten, dass nach der ersten Impfung gebildete Anti-Pf-Ferritin-Antikörper die Induktion der L2-spezifischen neutralisierenden Antikörperproduktion behinderten. Serologische Experimente bestätigten, dass die von den Pf Ferritin-Nanopartikel-L2-Antigenen produzierten Antikörper das menschliche ferritin nicht erkennen konnten.

Insgesamt erwies sich Pf Ferritin-Trx-HPVL2(aa20-38)*8 als hervorragender Kandidat für die Präsentation von Epitopen, die hohe Titer neutralisierender Antikörper hervorrufen konnten, die *in vitro* und *in vivo* wirksam waren.

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zur Hausen, H. (1996). **Papillomavirus infections--a major cause of human cancers**. Biochim Biophys Acta *1288*, F55-78.

9 Publications

1. **Minor Capsid Protein L2 Polytope Induces Broad Protection against Oncogenic and Mucosal Human Papillomaviruses;** Somayeh Pouyanfard, Gloria Spagnoli,b Lorenzo Bulli, Kathrin Balz, **Fan Yang**, Caroline Odenwald, Hanna Seitz, Filipe C. Mariz, Angelo Bolchi, Simone Ottonello, Martin Müller,(2017)

2. Re-engineering nanoparticle HPV prophylactic vaccine based on minor capsid protein L2. (in preparation)

10 Curriculum Vitae

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12 Thesis Declaration

Declaration according to §8 of the doctoral degree regulations

I hereby declare that I have written the submitted dissertation independently and have not used other sources and materials than those particularly indicated.

Further, I declare that I have not applied to be examined at any other institution, nor have I presented this dissertation to any other faculty, nor have I used this dissertation in any form in another examination.

Heidelberg, Germany Fan Yang