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Development of prophylactic and therapeutic combined vaccines against human papillomaviruses (HPV) using a multimerized thioredoxin (Trx) scaffold

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This thesis is based on research conducted between September 2015 and November 2018 in the group of Tumorvirus-specific Vaccination Strategies at the German Cancer Research Center under supervision of Prof. Dr. Martin Müller.

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Patents

Cutaneous papilloma virus vaccine

Balz, Bolchi, Mariz, Ottonello, **Zhao**, Müller

Application number: PCT/EP2018/076651

Prio date: 01.10.2017

Combined prophylactic and therapeutic vaccine against human papillomavirus

Ottonello, **Zhao**, Müller

Application number: 18 175 218.9

Prio date: 30.05.2018

List of Abbreviations

Abbreviations	Full names
APCs	Antigen-presenting cells
ACT	Adoptive cell transfer
Abs	Absorption
Alum	Aluminum hydroxide
APS	Ammonium persulfate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BCG	Bacillus Calmette-Guérin
BSA	Bovine serum albumin
ChAd63	Chimpanzee adenovirus serotype 63
CTL	Cytotoxic T lymphocytes
CIN	Cervical intraepithelial neoplasia
CIN1	Cervical intraepithelial neoplasia grade 1
CRT	Calreticulin
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
DMEM	Dulbecco modified Eagle's minimal essential medium
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide
DPBS	Dulbecco's phosphate buffered saline
ds	Double stranded
DCs	Dendritic cells
DMSO	Dimethyl sulfoxide
E2F	E2 transcription factor
E6AP	E6-associated protein
EP	Electroporation
E. coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
FCS	Fetal calf serum
FcR	FcReceptor
GFP	Green fluorescent protein
HPV	Human papillomavirus
HSPG	Heparan sulfate proteoglycan
HSV-2	Herpes simplex 2
HIV	Human immunodeficiency virus
HSIL	High grade squamous intraepithelial lesions
Hsp	Heat shock protein
HLA	Human leukocyte antigen
HEPES	Hydroxyethyl-piperazineethane-sulfonic acid buffer
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IFA	Incomplete Freund's adjuvant
IL-2	Interleukin-2
IFN	Interferon
IgG	Immunglobulin G
IC50	Inhibitory concentration 50

kbp	Kilo base pairs
LB	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	Pyrococcus furiosus
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
PBS	Phosphate-buffered saline
PD-1	Programmed cell death protein 1
PP7	RNA bacteriophage PP7
RB	Retinoblastoma
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
SLP	Synthetic long peptide
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 lymphocytes
TNF-α	Tumor necrosis factor alpha
TLR	Toll-like receptor
Tris	Tris (hydroxymethyl) aminomethane
Treg cells	Regulatory T cells
Trx	Thioredoxin
TA-CIN	Tissue antigencervical intraepithelial neoplasia
TEoA	Triethanolamine
TILs	Tumor infiltrating lymphocytes
URR	Upstream regulatory region
v/v	Volume per volume
VLPs	Virus-like particles
VEE	Venezuelan equine encephalitis

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Summary

Cervical cancer is the fourth most common cancer in women worldwide. It is estimated that more than one million women are currently suffering from cervical cancer, and there are 570,000 new cases in 2018. The majority of cases (>80%) occur in less developed region. There are three HPV prophylactic vaccines in the market currently. They are designed to induce L1-specific antibodies blocking the infection of epithelial cells. The therapeutic efficacy was neither observed for Cervarix nor for Gardasil. Therefore, preventive vaccines cannot benefit individuals with already existing viral infections. As a result, a high prevalence of cervical cancer still threatens human life worldwide.

Until now, there are no anti-HPV drugs available, an effective strategy should be the therapeutic vaccination to eliminate HPV-transformed cells by the activated immune system. The objective of my PhD project is to combine prophylactic and therapeutic value in one vaccine. The combined vaccine would ideally resolve productive infections and HPV-related diseases benefitting both uninfected and already infected individuals.

Thioredoxin (Trx) was applied as a scaffold to develop HPV prophylactic vaccine Trx-L2 and Trx-8mer-OVX313 in our lab previously. In my project, we firstly verified that Trx was also able to induce CD8+ cytotoxic T cell responses. Then, we designed our prophylactic and therapeutic combined vaccines based on Trx-L2 and Trx-8mer-OVX313. HPV16 $E7_{49-57}$ was chosen as CTL epitope since it is considered a tumor specific antigen as well as an oncoprotein being expressed throughout the whole HPV life cycle. We developed the combined vaccines PADRE-Trx-L2-flank E7 (monomeric) and Trx-8mer-flank E7-OVX313 (heptameric). The E7-specific T cell responses were compared via IFN-gamma ELISpot between these two vaccines and the data indicates that heptamerization leads to a stronger T cell response. We therefore continued investigating the B cell responses induced by the heptameric antigen. From pseudovirion-based neutralization assay (PBNA), we saw that presence of CD8 T cell epitopes on the antigen does not interfere with the induction of neutralizing antibodies.

In view of the *in vitro* promising results of Trx-8mer-flank E7-OVX313, we were encouraged to evaluate the therapeutic potential of Trx-8mer-flank E7-OVX313 *in vivo*. C57BL/6N mice were administrated with two doses vaccination after TC-1 cells challenge, and a potent antitumor activity was observed. These results demonstrate that antigen Trx-8mer-flank E7-OVX313 is a promising and cost-efficient candidate with both prophylactic and therapeutic effectiveness.

Zusammenfassung

Gebärmutterhalskrebs ist weltweit die vierthäufigste Krebsart bei Frauen. Schätzungen zufolge leiden derzeit mehr als eine Million Frauen an Gebärmutterhalskrebs, und 2018 allein gibt es 570.000 neue Fälle. Die meisten Fälle (>80%) treten in weniger entwickelten Regionen auf. Es gibt derzeit drei prophylaktische HPV-Impfstoffe auf dem Markt. Sie sollen L1-spezifische Antikörper induzieren, die die Infektion von Epithelzellen blockieren. Eine therapeutische Wirksamkeit wurde weder für Cervarix noch für Gardasil beobachtet. Personen mit bereits bestehenden Virusinfektionen können daher nicht von prophylaktischen Impfstoffen profitieren. Infolgedessen bedroht eine hohe Prävalenz von Gebärmutterhalskrebs nach wie vor das menschliche Leben weltweit.

Bis jetzt gibt es noch keine Anti-HPV-Medikamente. Eine therapeutische Impfung stellt eine effektive Strategie dar, um HPV-transformierte Zellen durch das aktivierte Immunsystem zu eliminieren. Ziel meines Promotionsvorhabens ist es, die prophylaktische und therapeutische Wirkung in einem Impfstoff zu kombinieren. Der kombinierte Impfstoff würde idealerweise produktive Infektionen verhindern und von HPV verursachte Krankheiten heilen, von denen sowohl nicht infizierte als auch bereits infizierte Personen profitieren.

Thioredoxin (Trx) wurde bereits als Gerüst zur Entwicklung des prophylaktischen HPV-Impfstoffs Trx-L2 und Trx-8mer-OVX313 in unserem Labor eingesetzt. In meinem Projekt haben wir zunächst verifiziert, dass Trx auch CD8+ zytotoxische T-Zellantworten induzieren konnte. Dann entwickelten wir unsere prophylaktischen und therapeutischen Kombinationsimpfstoffe auf Basis von Trx-L2 und Trx-8mer-OVX313. Als CTL-Epitop wurde HPV16 $E7_{49-57}$ gewählt, da es sowohl als tumorspezifisches Antigen als auch als Onkoprotein gilt, das über den gesamten HPV-Lebenszyklus exprimiert wird. Wir haben die kombinierten Impfstoffe PADRE-Trx-L2-flank E7 (monomer) und Trx-8mer-flank E7-OVX313 (heptamer) entwickelt. Die E7-spezifischen T-Zell-Antworten wurden über IFN-Gamma-ELISpot zwischen diesen beiden Impfstoffen verglichen und die Daten zeigen, dass die Heptamerisierung zu einer stärkeren T-Zell-Antwort führt. Deshalb haben wir die durch das heptamerische Antigen induzierten B-Zell-Antworten weiter untersucht. Aus dem Pseudovirion-basierten Neutralisierungstest (PBNA) haben wir gesehen, dass das Vorhandensein von CD8-T-Zell-Epitopen auf dem Antigen die Induktion von neutralisierenden Antikörpern nicht stört.

Angesichts der vielversprechenden *in vitro* Ergebnisse von Trx-8mer-flank E7-OVX313 haben wir entschieden, das therapeutische Potenzial von Trx-8mer-flank E7-OVX313 *in vivo* zu untersuchen. C57BL/6N-Mäusen wurden zwei Impfdosen nach TC-1-Zellen Injektion verabreicht, und eine starke Antitumor-Aktivität wurde beobachtet. Diese Ergebnisse zeigen, dass das Antigen Trx-8mer-flank E7-OVX313 ein vielversprechender und kostengünstiger Kandidat mit prophylaktischer und therapeutischer Wirkung ist.

1. Introduction

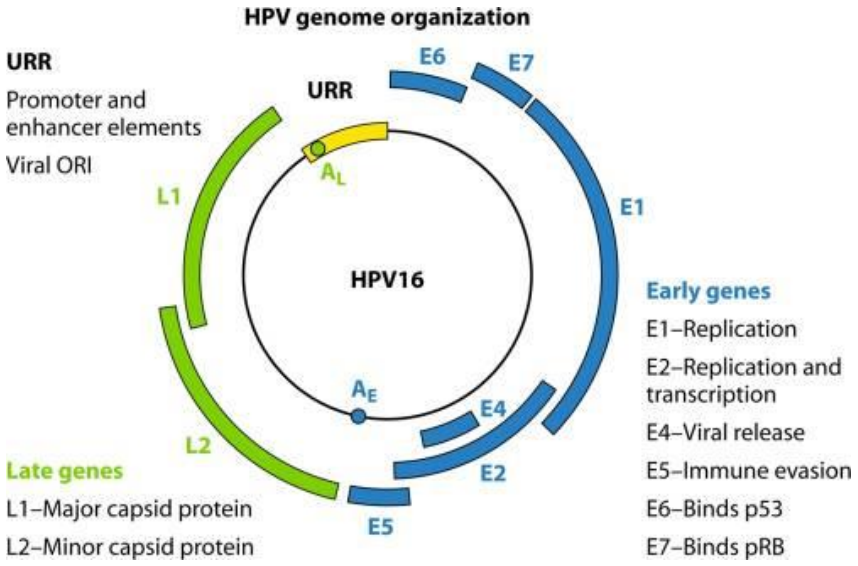
1.1 Impact of human papillomaviruses

Papillomaviruses are ubiquitous and can infect different species including reptiles, birds and mammals (Bernard et al, 2010). More than 350 papillomaviral types have been reported and over 100 types were identified as human papillomaviruses (HPVs) (de Villiers et al, 2004). HPVs are defined as cutaneous and mucosal types due to the infection sites. They cause benign warts or papilloma, while some types are also responsible for malignancies. The cutaneous types HPV5 and 8 are related to skin cancer in patients who suffer from Epidermodysplasia verruciformis (Pfister, 2003). Some types are also described as a co-factor during the pathogenesis of non-melanoma skin cancer (NMSC) (Rollison et al, 2008). The mucosal HPV types have been classified into 'low-risk' and 'high-risk' groups according to their oncogenic capacity. The most common low-risk HPV are HPV 6 and 11, which cause genital warts and recurrent respiratory papillomatosis (Lacey et al, 2006). The high-risk types are directly associated with carcinogenesis, in particular HPV16 and HPV18 are accounting for most of the cervical cancer cases (Bouvard et al, 2009; de Villiers et al, 2004). It has been also reported that tumors of vulvar, vagina, anus, penis, laryngeal and oropharyngeal are linked to high-risk HPV infection (Abramowitz et al, 2011; de Martel et al, 2017; De Vuyst et al, 2009; Herrero et al, 2003; Miralles-Guri et al, 2009; Munoz et al, 2003; St Guily et al, 2011)

Cervical cancer is the fourth most common cancer in women worldwide. It is estimated that more than one million women are currently suffering from cervical cancer, and there are 570,000 new cases in 2018. The majority of cases (>80%) occur in less developed region (WHO, 2018). Nearly all sexually active women will contract at least one type of high risk HPV in their life, while most infections remain asymptomatic and are cleared by immune system. However, in few cases, when the immune system fails to eliminate the viruses, the infection persists over several years and may result in cervical intraepithelial neoplasia (CIN) or even cervical cancer (Ghittoni et al, 2015; Trimble et al, 2005). In total, at least 15 mucosal high-risk HPVs can cause cervical cancer (Doorbar, 2006). HPV16 is responsible for more than 50% of cases and HPV18 for around 20%. The other 13 HPV types including HPV45, 31, 33, 52, 58, 35, 59, 56, 51, 39, 68, 73 and 82, account for 30% of cancer cases (Munoz et al, 2004).

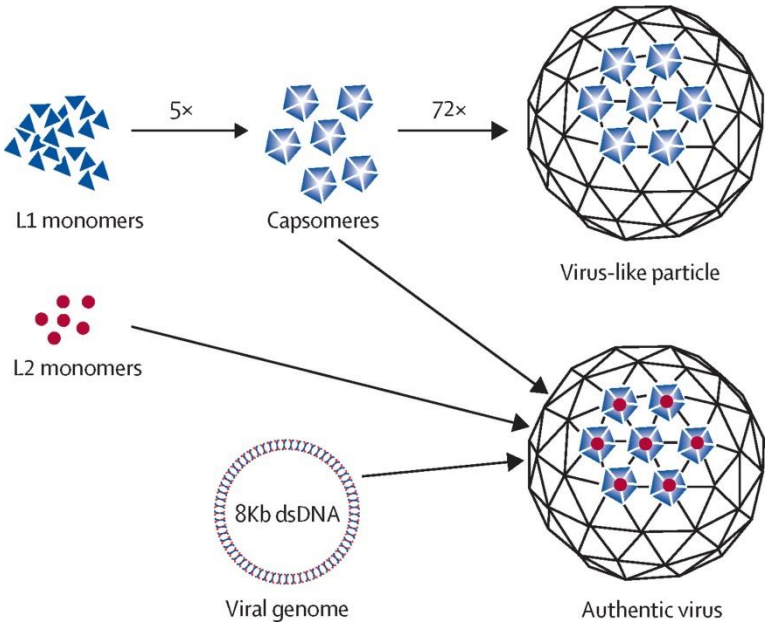
1.2 Papillomaviruses and life cycle

Papillomaviruses are non-enveloped double stranded (ds) DNA viruses. The viral genome is about 8 kbp and encodes eight to ten defined genes which include six “early” genes coding for non-structural proteins E1, E2, E4, E5, E6 and E7 engaged in virus replication, and two “late” genes coding for structural proteins L1 and L2 involved in formation of viral capsid (Figure 1). Different from HPV protein nomination, actually E1, E2, E5, E6 and E7 are expressed in the early stage of infection, while E4, L1 and L2 are expressed in a later phase (Doorbar, 2005). Normally, the major capsid protein L1 assembles into pentameres (capsomeres) spontaneously, and 72 capsomeres together form the HPV capsid in an icosahedral structure. The minor capsid protein L2 is deemed to inlay into the center of capsomeres (Modis et al, 2002). And the HPV viral genome is packaged in the capsid cage formed by L1 and L2 proteins (Figure 2).



from M.A. Stanley, Clinical Microbiology Reviews 2012

Figure 1. HPV16 genome. The ds DNA is about 8kbp and one strand of it encodes all viral genes. There are six “early” genes and two “late” genes. E1 and E2 proteins are responsible for viral genome replication. E6 and E7 are oncoproteins regulating cell cycle. E5 protein is involved in immune evasion while E4 assist virus shedding. L1 and L2 are expressed in the final stage and code for virus structural proteins. The URR (upstream regulatory region) comprises the promoter and enhancer elements.



from J.T Schiller and M. Müller, Lancet Oncol 2015

Figure 2. Assembly of HPV virion particles. Five L1 monomers assemble into pentameric capsomeres and 72 capsomeres together form the HPV capsid. The L2 monomers are deemed to insert into the center of capsomeres. The HPV viral genome is packaged in this icosahedral capsid formed by L1 and L2 proteins.

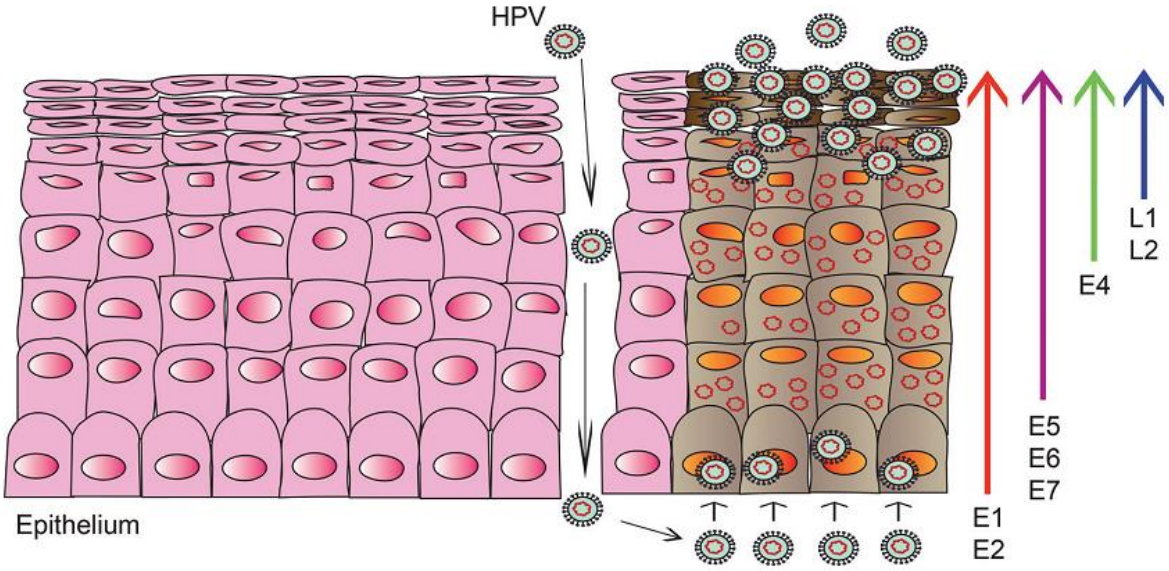
Papillomaviruses infect the skin and mucosa through microtraumas of the epithelium to gain access to the basal layer where the viruses start the life cycle. Their replication is coordinated with the differentiation of the epithelium (Doorbar, 2013b; Doorbar et al, 2012) (Figure 3). Inside the basal cells, the E1 and E2 genes are expressed. E1 is a viral enzyme and mediates episomal DNA replication (Wilson et al, 2002). E2 is a transcription factor regulating viral copy numbers via activation or repression of HPV promoters (Hegde, 2002). But the function of the E2 protein is compromised when the viral genome integrates into the host cell genome along with the migration of infected cells into the superficial layers. This integration derepresses E6 and E7 genes leading to the infected cells in an abnormal state that benefits viral DNA amplification (Boulet et al, 2007; Yim & Park, 2005).

E7 and E6 both interact with regulatory proteins to control the cell cycle. Usually, the tumor-suppressor protein retinoblastoma (RB) sequesters the transcriptional factor E2F. However, if the cells are infected by HPV, E7 binds to RB. This process releases E2F resulting in the promotion of DNA replication (Dyson et al, 1989; Kiyono et al, 1998). The high level of E2F activity and cell cycle progression might cause apoptosis, but the existence of E6 can

overcome this. E6 play an anti-apoptotic role by binding to the ubiquitin ligase E6-associated protein (E6AP) which leads to the ubiquitination and subsequent degradation of tumor-suppressor p53 (Huibregtse et al, 1991; Scheffner et al, 1993). With the inactivation or degradation of tumor suppressors, E7 and E6 are undoubted oncoproteins. The existence of them is critical in the maintenance of cell transformation and the progression to cervical intraepithelial neoplasia (CIN) or even carcinoma. The classification of high-risk and low-risk HPVs is determined by the relative affinity efficiency of E7 and E6 binding to pRB and p53, respectively (Best et al, 2012).

HPVs are nonlytic and the virus release depends on the shedding of apical surface cells (koilocytes) which contain mature viruses. And around 50-100 virion per koilocytes can be released (Paavonen, 2007). It is suggested that E4 not only participates in virus synthesis and amplification, but also facilitates this viral shedding and transmission process (Doorbar, 2013a). The function of E5 is not clearly illustrated. It might stimulate host cell proliferation and inhibits cell differentiation. It is also thought that E5 is involved in the downregulation of MHC I expression on the cell surface (DiMaio & Petti, 2013).

In later phase of the viral life cycle, the infected keratinocytes migrate towards the suprabasal layer and HPV major and minor capsid proteins L1 and L2 are expressed respectively (Figure 2). L1 is expressed after L2, and L1 itself can spontaneously assemble into a 72-capsomeres icosahedral cage (virus-like particle) with L2 located in the center of each capsomere (Buck et al, 2008; Buck et al, 2013). Although L2 is not the major structural protein, it is very important during viral genome packing and viral infection process (Roden et al, 2001; Stauffer et al, 1998). The newly synthesized viral DNA is packaged into the viral capsid and this capsid is stabilized by disulfide bonds between L1 and L2. This stability can aid the virion to resist the cellular “unfavourable” environment after the virus is released from the koilocyte (Buck et al, 2005).



from C. Rosales, Chapter 2 from the book Vaccines, 2017

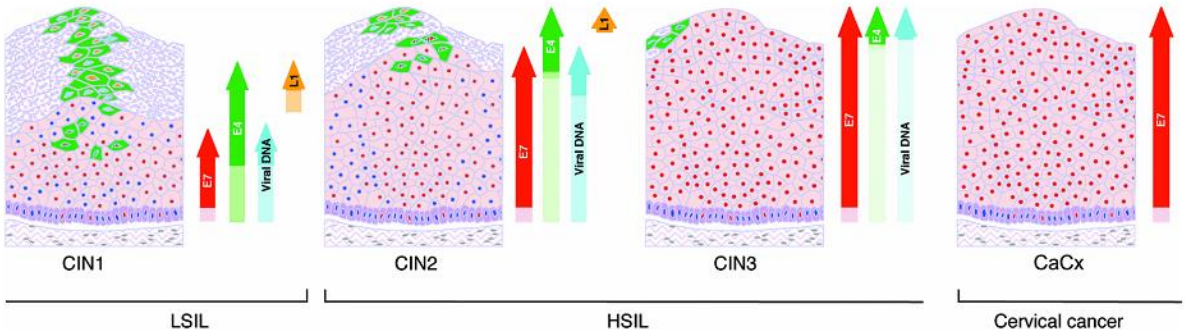
Figure 3. HPV life cycle of infection. Expression of the viral proteins in different epithelial layers is shown at the right. HPVs initiate the infection though microlesions in the basal layers where E1 and E2 are expressed and regulate viral DNA replication and copy numbers. E5, E6 and E7 act by modulating cellular proliferation and hinder cell differentiation to favor viral DNA synthesis. E4 is involved in virion slough and transmission. In the final stage, L1 and L2 are expressed and form the capsid encapsuling viral DNA. At the surface of epithelium, new virions are released along with shedding of koilocytes.

1.3 From persistence to cervical cancer

Genital HPV infections are very common in general, with an 80% life time probability to acquire the infection, but the incidence of cervical cancer is relatively low. This is because the host immune system most of the time can clear HPV infections within 6-18 months (Parkin et al, 2005). Only when the immune system fails to abrogate this, the persistent infection by high-risk HPVs leads to lesions that may progress to cervical intraepithelial neoplasia grade 1 (CIN1), also referred to as low grade squamous intraepithelial lesions (LSIL). The gene expression pattern is similar to the normal viral life cycle and viruses are still in productive infection stage at this time. Approximately 20% of CIN1 lesions develop into to CIN2, and 30% of these can progress to CIN3. CIN2 and CIN3 are defined as high grade squamous intraepithelial lesions (HSIL). In this stage, the viral life cycle cannot be completed due to the low expression of capsid proteins and incomplete viral genome amplification (Doorbar, 2006). But the infected cells are highly proliferative with occasional integration of viral DNA into the host genome, which occurs in some CIN lesions and many cervical cancers

(Huang et al, 2008; Jeon et al, 1995). About 40% of CIN3 eventually progress into cancer (Peto et al, 2004).

During persistent infection with high-risk HPVs, the oncoprotein E7 keeps the infected cells permanently proliferative via continuous S-phase initiation. Furthermore, owing to the interference of the E6 protein with p53-mediated apoptosis and DNA repair, accumulation of mutations can give rise to malignant lesions. In higher grade lesions and cancers, the viral DNA often integrates into the cell genome, which not only stabilize the viral E6 and E7 transcripts, but also interrupt inhibitory regulation of cell proliferation by abolishing E2, E4 and E5 (Choo et al, 1987; Jeon & Lambert, 1995; Klaes et al, 1999). All of these lead to uncontrolled cell proliferation. However, the development of cervical cancer is most likely further advanced by accumulation of additional genetic mutations from for instance, smoking, the long-term taking oral contraceptives and co-infection with other sexually transmitted viruses such as HSV-2 and HIV (Munoz et al, 2006; zur Hausen, 2000). Pre-cancerous lesions can be detected five years after infection. But cervical cancer development is a very long process usually 10-20 years after the initial infection with high-risk HPVs (Woodman et al, 2001). Therefore, cervical carcinogenesis is a long pre-cancerous process and lesions can usually be removed by surgical techniques or ablation at this time. Development of therapeutic vaccines is also an attractive therapy which is based on the high expression of oncoproteins E7 and E6 in CIN lesions and cervical cancers.



from J. Doorbar, Clinical Science 2006

Figure 4. Different viral gene expression patterns in CIN and cervical cancer. In CIN1 the expression pattern is similar to the normal viral life cycle, productive infection. In CIN2 and CIN3, viral production is restricted to extreme upper surface, expression of capsid proteins is reduced strongly and E7 is highly overexpressed. In cervical cancer, the viral DNA is often integrated into host cell genome and transformed cell are proliferated uncontrollably.

1.4 Host immune responses to HPV infection

HPV Infections are often underway unnoticed by the host because the viruses have their own immune escape mechanisms. The protein E5 of high-risk HPV types downregulates MHC I expression on the cell surface while the E6 protein affects Langerhans' cell density (Matthews et al, 2003). More importantly, oncoprotein E7 interferes with IFN signaling pathway which is indispensable in inflammatory reaction and immune responses (Barnard et al, 2000).

Although HPVs develop several ways to evade immune surveillance, the host immune systems have coping strategy to defend themselves against most viral attacks. This is the reason why most immune competent individuals can clean up HPV-related lesions spontaneously (Doorbar et al, 2012; Woodman et al, 2007). Protection is achieved by both innate and adaptive immune responses. HPV infection site is firstly started inflammation reaction attracting neutrophils, macrophages and later lymphocytes. The innate immune cells produce inflammatory cytokines, such as interleukin (IL)-1β, IL-6, IL-8, IL-12, and interferon (IFN)-α, -β- and -γ, as responses to nonspecific viral particles, such as free viral DNA. The stimulated cytokines can attract natural killer (NK) cells (Woodworth, 2002).

Afterwards, when viral proteins are being synthesized, the antigen-presenting cells (APCs), here mostly dendritic cells (DCs) and Langerhans cells, can endocytose these proteins and present small pieces derived from the proteins in context with the major histocompatibility complex (MHC) on the cell surface. The MHC II-peptide complexes stimulate CD4+ T cells and MHC I-peptide structures stimulate CD8+ T cells to initiate an adaptive immune response (Figure 5 and Figure 6).

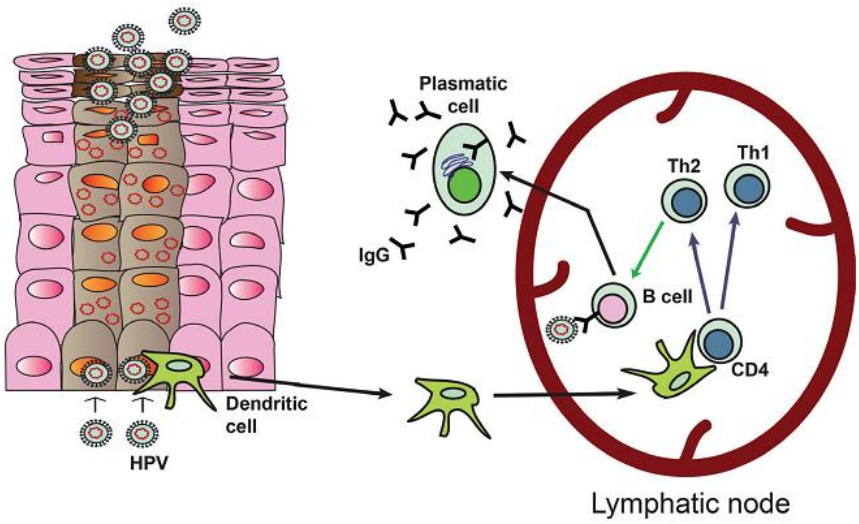
The activated CD4+ T cells can differentiate into Th1, Th2, or Treg/Th3 according to the immune environment. Then, the CD4+ helper T cells assist B cells activation and secretion of anti-virus specific antibodies. Moreover, helper T cells aid CD8+ T cells to differentiate into functional cytotoxic T lymphocytes (CTLs) which produce granzyme, perforin and some proteolytic enzymes (Zhang & Bevan, 2011). CTLs perform as the most potent cell weapon to eliminate HPV-infected cells.

1.4.1 Humoral immune responses

A humoral immune response (Figure 5) can be observed in most patients infected by HPVs. Anti-protein L1, E2, and E4 antibodies are usually detected in the first stage of infection (Dillner et al, 1989; Dillner et al, 1993; Veress et al, 1994). After viral DNA integrates into the host genome, some patients produce antibodies recognizing oncoprotein E6 and E7 (Baay et al, 1997; Fisher et al, 1996; Park et al, 1998). Nevertheless, these antibody responses are weak and neither adequate for shielding individuals from further re-infection, nor effective at clearing HPV infected cells.

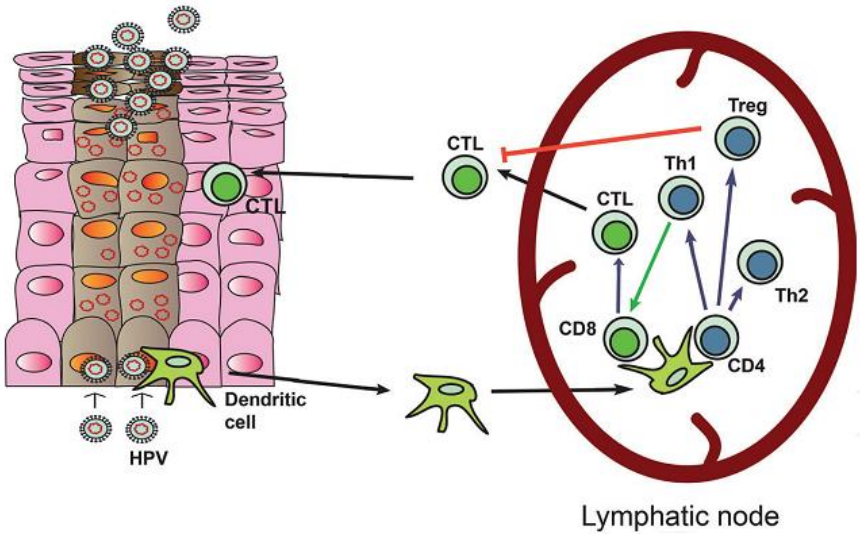
1.4.2 Cellular immune responses

Cellular responses play an essential role for eliminating established HPV lesions (Figure 6). As discussed above, elimimation of lesions is highly correlated with induced CTL performances. For instance, viral specific memory T cell responses are observed in patients who have already cleared HPV16 infections successfully (Welters et al, 2003). And patients with spontaneous regression of vulvar intraepithelial neoplasia grade 3 present robust CD4+ and CD8+ T cell responses (Bourgault Villada et al, 2004). However, deficient T cell responses are found if patients in CIN or cervical cancer stage (de Jong et al, 2004).



from C. Rosales, Chapter 2 from the book Vaccines, 2017

Figure 5. Humoral responses to HPV infection. Dendritic cells (DCs) or Langerhans cells can take up antigens from HPV infected cells. These antigen-presenting cells (APCs) then travel to the lymphatic nodes and activate CD4+ T cells by presenting MHC II-peptide complexes. The stimulated T cells differentiate into Th1 or Th2 cells according to the immune environment. B cells become to plasma cells require native virus stimulation as well as Th2 assistance.



from C. Rosales, Chapter 2 from the book Vaccines, 2017

Figure 6. Cellular responses to HPV infection. DCs or Langerhans cells take up HPV-related antigens and travel to the lymphatic nodes. There, APCs present MHC II-peptide complexes to activate CD4+ T cells and present MHC I-peptide complexes to stimulate CD8+ T cells. The activated CD4+ T cells differentiate into Th1, Th2 or regulatory T (Treg) cells, among which Th1 can help CD8+ T cells differentiate to cytotoxic T lymphocytes (CTLs) while Treg normally negatively regulate CTL activity. CTLs migrate back to infected tissue to perform the cytotoxic function.

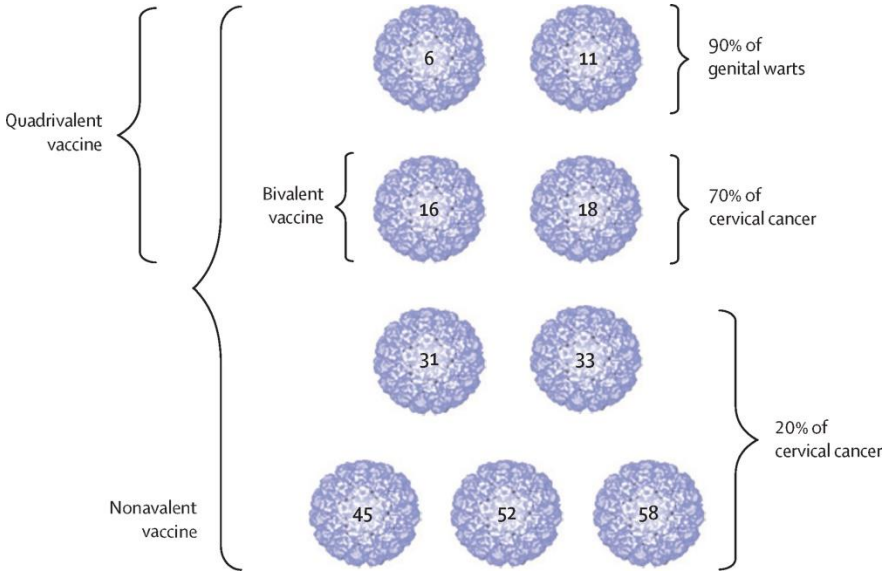
1.5 HPV prophylactic vaccines

As Harald zur Hausen found in 1978 that there is a strong relation between HPV infection and cervical cancer development, it was thought that preventing HPV infections can dispel HPV-associated diseases (zur Hausen, 1991; zur Hausen, 1996; zur Hausen, 2002). Besides, antibodies produced from infected individuals were characterized to recognize the viral capsid proteins. These all inspired the researches to develop HPV prophylactic vaccines based on HPV L1 and L2 proteins.

1.5.1 Current HPV prophylactic vaccines in the market

Three HPV prophylactic vaccines are approved and currently used worldwide. They all take advantage of the major capsid protein L1, which can assemble into virus-like particles (VLPs) spontaneously. Cervarix (manufactured by GlaxoSmithKline (GSK) in Europe) is a bivalent vaccine composed of VLPs from HPV 16 and 18 which cause most HPV-related cancers. The antigen is produced in insect cells and adjuvanted with aluminum salt and monophosphoryl lipid A (MPLA), a TLR-4 agonist. Gardasil (manufactured by Merck in the USA) is a

quadrivalent vaccine containing VLPs not only from HPV 16 and 18, but also from HPV 6 and 11 which are the causative agents for most genital warts. In addition, Merck have developed Gardasil 9 recently by increasing the number of VLP types from four to nine (HPV 6, 11, 16, 18, 31, 33, 45, 52, and 58). This nonavalent vaccine can prevent about 90% occurrence of cervical cancers (Figure 7). Both Gardasil and Gardasil 9 are expressed in yeast and adjuvanted with aluminum salt. Table 1 summarizes the features of three commercial HPV prophylactic vaccines.



from J.T Schiller and M. Müller, Lancet Oncol 2015

Figure 7. HPV VLP types in three commercial prophylactic vaccines. The HPV types and the coverage of HPV-associated diseases in Cervarix, Gardasil and Gardasil 9 are shown.

Table 1. Features of the current HPV prophylactic vaccines in the market

	Cervarix	Gardasil	Gardasil 9
Manufacturer	GSK	Merck	Merck
HPV types included	HPV16 and 18	HPV6, 11,16, 18	HPV6, 11, 16, 18, 31, 33, 45, 52 and 58
Vaccination dose	20 µg HPV16 and 20 µg HPV18	20 µg HPV6, 40 µg HPV11, 40 µg HPV16 and 20 µg HPV18	30 µg HPV6, 40 µg HPV11, 60 µg HPV16, 40 µg HPV18, 20 µg HPV31, 20 µg HPV33, 20 µg HPV45, 20 µg HPV52 and 20 µg HPV58
Producer cells	Trichoplusia ni (Hi 5) cells infected with Baculovirus	Saccharomyces cerevisiae (bread yeast)	Saccharomyces cerevisiae (bread yeast)

Adjuvant	500 µg aluminium hydroxide and 50 µg 3-O-deacylated-4'-monophosphoryl lipid A	225 µg amorphous aluminium hydroxyphosphate sulphate	225 µg amorphous aluminium hydroxyphosphate sulphate
Recommended vaccination schedule	0, 1 and 6 months	0, 2 and 6 months	0, 2 and 6 months

1.5.2 The limitations of current HPV prophylactic vaccines

Despite the success of current commercial HPV vaccines, they still have inherent limitations that restrict the scope of application and potential effects on public health. First, three intramuscular injections are recommended to teenagers during at least 6 months. However, it is difficult for adolescents to complete the three-dose series, since most of them do not routinely access health care. Although a better completion of vaccination can be achieved under school immunization programmes, this is not common, especially in low-income countries where HPV prevention is most needed. Recently, a two-dose immunization strategy has been tried. This is relatively easier and cheaper to achieve and the protection seems to be comparable to three doses (Jit et al, 2015; Kreimer et al, 2015). Second, protection by HPV L1 vaccines is restricted by HPV genotypes, with limited cross-protection for a few additional types which are closely related to HPV 16 and 18 (Schiller et al, 2012). And cross-neutralizing titres are likely to be at two orders of magnitude lower than type-specific neutralizing titres. Hence, cross-protection provided by HPV L1 vaccines might be less efficient and durable. Third, the commercial vaccines are quite expensive to produce and deliver. The current prophylactic vaccines are all produced in eukaryotic cells and require cold-chain transportation which is definitely costly if the vaccines are popularized globally. Fourth, the licensed vaccines offer very limited benefits to the virus infected population, since no cell-mediated immune responses have been observed for these vaccines (Wang & Roden, 2013). As mentioned above, antibodies induced by these vaccines recognize HPV L1 proteins. But once the infection is established, these proteins are only expressed on the very superficial epithelium. Oppositely, a therapeutic vaccine should induce immune responses against proteins that are expressed through the whole lifecycle of infection (Kumar et al, 2015). Lastly, the patents of HPV VLP technology are exclusively possessed by Merck and GSK, which restricts the development of similar vaccines by other

companies. So alternative approaches unrelated to the licensed vaccines are now highly invested.

The discussed considerations provoke the interest for academic research and industry to develop alternative strategies for production of next-generation HPV vaccines.

1.5.3 New prophylactic vaccines

The new HPV vaccines mainly focus on improving the current vaccines from their limitations: type-specific, thermolabile, and costly. Here we will discuss L1-based and L2-based third generation vaccines.

The improvements for L1-based vaccines are in regard to their production system, structure of the vaccine and vector instead of protein immunization. For example, *Escherichia coli* are used by Xiamen Innovax Biotech (China) to achieve L1 mutants in high yield (Zhao et al, 2014). Yeast *Komagataella (Pichia) pastoris* has been applied by Indian Immunologicals (India) (Bazan et al, 2009), Instituto Butantan (Brazil) (Hanumantha Rao et al, 2011) and Shanghai Zerun (China) (NCT01548118) as producer cells. While another India based institute produces VLPs in yeast Hansenula polymorpha. These two yeasts are supposed to generate higher yields of L1 VLPs at a lower cost than *Saccharomyces cerevisiae*. Some groups try to produce L1 VLPs in plants so that the vaccine can be taken orally. This kind of vaccine uptake pattern is especially very important for regions in low medical-resource settings. Unfortunately, the yield of vaccines in plants is normally low and VLP assembly is inefficient. Then, the expression in chloroplasts is proposed and L1 production was improved substantially in this expression system (Giorgi et al, 2010). Other investigators produce simpler L1 capsomeres instead of complex VLPs. Capsomeres are the subunits of the viral VLPs. They share similar immunogenicity as VLP-based vaccines, but have better thermal stability, lower production costs and can also use bacteria as producer systems (Panatto et al, 2015). Vectored vaccines have been assessed as platforms for L1 HPV vaccines. For instance, measles virus, adeno-associated virus and a human endogenous retrovirus have been studied by some researchers. However, application of vectors as vaccines has potential risk of mutation due to genetically modified microbes involved (Schiller & Muller, 2015).The region in the protein L1 for antibody induction is highly specific among different HPV types. In contrast, there are several conserved regions in L2 among most high-risk HPV types which can induce broad cross-neutralizing antibodies; although, L2-induced titres against type

specific virus are at least ten times lower than titres induced by L1 VLPs (Pastrana et al, 2005). The regions of L2 that can be recognized by antibodies are commonly not exposed. Only when the N-terminus of L2 is cleaved by furin, the main L2 neutralization epitopes are uncovered (Day et al, 2010). Cross-neutralizing characteristics of L2 induced antibodies provide feasibility that a monovalent vaccine might protect against a range of mucosal HPV types and even some cutaneous types (Pouyanfard et al, 2018; Schellenbacher et al, 2013). However, unlike L1 VLPs, the L2 protein is not very immunogenic. And several approaches are being tried to improve its immunogenicity. Sanofi Pasteur has developed concatemer vaccine using L2 amino acids 11–88 from five (HPV 6, 16, 18, 31, and 39) or eight (HPV 6, 16, 18, 31, 39, 51, 56, and 73) HPV types of diverse clades. The type-specific and cross protection of these L2 immunogens have been detected in mice (Jagu et al, 2013). Another approach is to generate L1 VLPs with the surface loop replaced by a L2 peptide. This recombinant VLP should possess both advantages of L1 that can induce high type-specific neutralizing titers and of L2 that offer broad cross protection (Schellenbacher et al, 2013). Similarly, other particles can be also employed to display L2 peptides on the surface to enhance L2 immunogenicity. For instance, an inexpensive production is to express L2 peptides on the surface of bacteriophage PP7 (Tumban et al, 2011). *Lactobacillus casei* is also used as an L2 display platform and the recombinant bacteria are possible for oral immunization (Yoon et al, 2012). Our group designed polytopes comprising the L2 amino acids 20-38 from several representative mucosal HPV types. A thioredoxin-derived from thermophile archeabacterium *Pyrococcus furiosus* (*Pf*Trx) is used as a protein scaffold to increase the thermal stability of L2 polytopes. We further apply a heptameric platform OVX313 to repetitively display *Pf*Trx-L2 polytopes seven times in order to achieve a better immunogenicity (Pouyanfard et al, 2018) (Figure 8).

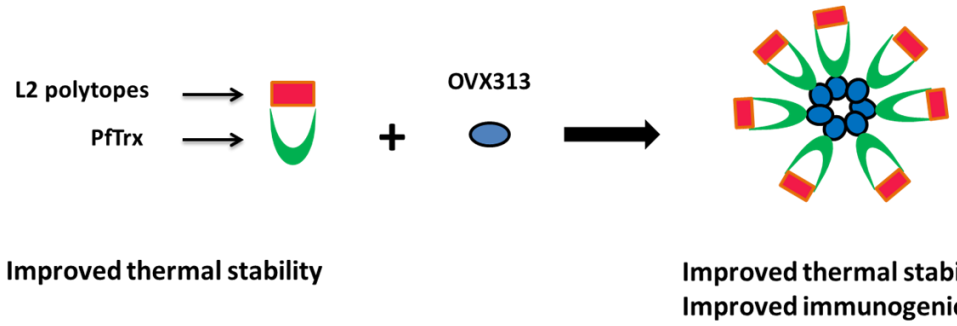


Figure 8. Formation of PfTrx-L2 8mer-OVX313. L2 8mer is the polytopes. The thermal stability and immunogenicity of the vaccine are enhanced by scaffolds PfTrx and OVX313.

1.6 HPV therapeutic vaccines and immunotherapies

Prophylactic vaccines are designed to induce a potent humoral response in which formed antibodies can recognize the viral capsid and block the infection of epithelial cells. These vaccines target extracellular viruses, and are ineffective after viruses have entered the cells. Therefore, preventive vaccines cannot benefit individuals with already existing viral infections. As a result, a high prevalence of cervical cancer still threatens human life worldwide, especially in low-economic countries (Forman et al, 2012; Husain & Ramakrishnan, 2015). Also, despite of the production of effective viral neutralizing antibodies by the prophylactic vaccines, the final prevention of carcinogenesis by these vaccines is still unknown. Since it takes a long time from HPV infection until cancer development, the anti-cancer efficacy of the prophylactic vaccines will be seen from the vaccinated and later viral infected individuals in the future (Schiller et al, 2012). Moreover, there are no anti-HPV drugs available, so an effective strategy should be the therapeutic vaccination to eliminate HPV-transformed cells automatically by activated immune system.

For design of therapeutic vaccines, the viral antigens chosen as immunogens should be expressed in the infected cells throughout life cycle. As mentioned above, the structural proteins L1 and L2 are normally expressed in terminally differentiated keratinocytes at very superficial layers of epithelium (Figure 3). So these proteins are not appropriate for therapeutic design. In contrast, the proteins E1, E2, E6, and E7 are expressed in multiple replication stages throughout infection (Figure 3). Accordingly, they present excellent therapeutic targets.

Until now, different kinds of therapeutic vaccines or immunotherapeutic strategies have been investigated and some even tested in clinical trials. We will discuss some of them in the following sections.

1.6.1 Protein vaccines

The protein vaccines, like the whole protein of E6 and E7, or HPV recombinant proteins have been widely used in early exploitation of therapeutic vaccines (Davidson et al, 2004; de Jong et al, 2002a; Einstein et al, 2007; Frazer et al, 2004). An advantage of this kind of vaccines is that they can be manufactured in large amounts. Moreover, they theoretically cover all possible epitopes of the protein and thus the identification of particular epitopes is not

required. However, the irrelevant peptides derived from the protein may be dominant and drive the immune responses to the unexpected direction rather than desired immunogenicity. Despite this, protein vaccination is still an efficient approach by reason of allowing presentation of both CTL epitopes and T helper epitopes (Brinkman et al, 2007a).

A recombinant protein L1VLPE7 composed of the carboxyl-terminally part of HPV 16 L1 and the amino-terminal part of the HPV 16 E7 can assemble into virus-like particles. L1VLPE7 was administered to patients with HPV-induced CIN 2/3 lesions, but no significant clinical effects were presented (Kaufmann et al, 2007). Another VLP antigen HPV 16 L1(ΔN26)-E7(ΔC38) was proved to induce neutralizing antibodies and some cellular responses in a murine cervical cancer model (Sharma et al, 2012).

A fusion protein HPV16 E6/E7 comprised of HPV E6 and E7 was adjuvanted with ISCOMATRIX for immunization. The antigen was tried in CIN patients and a cellular immune response was detected. However, the regression of lesions was only presented in few patients (Frazer et al, 2004).

Another chimeric protein SGN-00101 formed by HPV 16 E7 protein and a *Mycobacterium bovis*-derived heat shock protein (Hsp) was employed to patients with CIN 3 lesions. The regression was observed in one-third of patients, which was correlated with immune response (Roman et al, 2007).

1.6.2 Peptide vaccines

HPV peptide vaccines are also a good immunization approach due to simple production and safe application (Khallouf et al, 2014). These vaccines employ a small part of E6 or E7 comprising MHCI restricted epitopes. This is also the limitation of short peptide utilization, that the patients have different human leukocyte antigen (HLA) genetic backgrounds so the specific epitope for each HLA has to be defined (Kim et al, 2014a). Another complication is that the exogenously loaded peptides may passively bind onto MHC molecules on cells directly without antigen-presenting process. This may induce immune tolerance instead of stimulation (Melief & van der Burg, 2008).

To overcome limitations of short peptide-based vaccines, synthetic long overlapping peptides were used since they appear to be processed correctly by APCs and encompass all potential CD8+ and CD4+ epitopes (Rosalia et al, 2013). In one clinical trial, a mixture of two

HPV E7 peptides and one T helper peptide was applied. However, there were no induced anti-E7 cytotoxic T cell responses (Ressing et al, 2000).

Another clinical trial was carried out in patients with resected HPV 16-positive cervical cancer. A vaccine (HPV 16-SLP) formed by long peptides from HPV16 oncoproteins E6 and E7 can stimulate HPV16-specific T cell responses but together with regulatory T cells, which indicate that the induced immune responses were not promising (Welters et al, 2008).

In another test, patients with vulvar intraepithelial neoplasia were administered with a mixture of long synthetic peptides derived from E6 and E7 adjuvanted with Montanide ISA-51. The elimination of lesions was pesented in nearly half of vaccinated patients (Kenter et al, 2009).

1.6.3 DNA vaccines

DNA vaccines are another powerful and economical immunization approach. DNAs are easy to be produced, stored and delivered. Usually, a DNA sequence coding for the target antigen is cloned into a bacterial vector, and then the recombinant vector is optimized for expression in mammalian cells.

However, DNA vaccines contain full-length oncogenes E6 and E7 have the inherent risk of bringing about cellular transformation. In order to avoid this hazard, P53 binding site in E6 and Rb binding site in E7 can be mutated. Some groups even shuffled the oncogene sequence to incapacitate carcinogenic function but retain putative HLA epitopes (Brinkman et al, 2007b; Ohlschlager et al, 2006).

DNA vaccines can induce E6 and E7-specific CTLs, and a complete tumor regression was shown in mice (Gomez-Gutierrez et al, 2007; Peng et al, 2004). Unfortunately, no obvious elimination of CIN was observed in humans upon DNA vaccination (Garcia et al, 2004; Trimble et al, 2009). In another experiment of DNA vaccination in mice, a recombinant plasmid CRT/E7 (HPV16 E7 linked with calreticulin (CRT)) was co-injected with another DNA coding for bovine papillomavirus L1 or L2. Both improved CD4+ and CD8+ T cell responses were obtained (Yang et al, 2015).

Recent application of a new delivery method, electroporation (EP), has shown better immune effects (Kim et al, 2014b). EP takes advantage of a panel of short electrical pulses at

the DNA vaccination site resulting in increased DNA uptake and impetus of immune responses. In a phase I trial, DNA vaccine VGX-3100 was injected together with EP pulses, and E6 and E7 specific CTLs were detected in the patients (Bagarazzi et al, 2012). In another phase II study, this preparation was used in patients with HPV16 or 18 associated CIN2-3. The histological regression was presented in 49.5% vaccinated individuals compared to 30% regression in the placebo group (Trimble et al, 2015).

1.6.4 Recombinant viruses

Another therapeutic platform for treatment of HPV-related diseases is the use of recombinant viruses as delivery vehicles, as to activate the immune system.

For example, vaccinia virus, adenovirus, and a modified vaccinia Ankara (MVA) are good carriers for HPV vaccination. They can infect antigen presenting cells effectively, upregulate co-stimulatory molecules and enhance production of cytokines and chemokines, which are important to efficient CTL induction. An adenoviral vaccine was designed containing HPV16 E2, and induced responses showed therapeutic potential resulting in diminished lesions in rabbits (Brandsma et al, 2004). A vaccinia vaccine was developed to induce anti-HPV16 and 18 E6 and E7-specific CTLs, but no clinical responses were found in patients with late-stage cervical cancer (Borysiewicz et al, 1996).

A MVA-based vaccine MVA E2 (E2 protein derived from bovine papilloma virus) has been evaluated in different phases of clinical trials. The vaccine was first tested in patients with CIN 1 to CIN 3 lesions. MVA E2 was injected to patient uterus directly once per week for 6 weeks. The results were optimistic that 94% patients presented complete clearance of precancerous lesions. Moreover, half of the patients eliminated the virus entirely and the rest showed a 90% reduction in HPV DNA load (Corona Gutierrez et al, 2004). Then, the vaccine was assessed in patients with high-grade lesions. In this phase II trial, around 56% patients showed complete lesion regression, and 32% of patients showed lesion reduction by 90–60%. Notably, the induced cytotoxic T cell responses correlated with the clinical results (Garcia-Hernandez et al, 2006). Later in a phase III trial, MVA E2 was administered in female patients with anogenital intraepithelial lesions. About 89% of a total of 1176 patients presented complete clearance of lesions, as well as a specific cytotoxic activity against HPV-transformed cells (Rosales et al, 2014). From above clinical outcome, MVA E2 seems to be a promising vaccine to cure HPV-induced malignancies.

Alphaviruses were designed to express the E6 and E7 mRNAs in the cytosol, which circumvents the potential risk of oncogenes integration into cellular genome. Furthermore, Venezuelan equine encephalitis (VEE) virus has a DC tropism, and most of people do not have preexisting immunity to these viruses (Nishimoto et al, 2007). A study used the VEE platform containing mutated E6 and E7. And the results indicated that the vaccinated mice can be 100% protected from tumor challenge and 90% of established tumors were cleared after vaccination in HLA-A*0201 transgenic mice (Cassetti et al, 2004).

1.6.5 DC vaccines

DCs are major APCs which are able to induce potent cellular immune responses with proper stimulation. As such, DCs are developed as a therapeutic vaccine by loading with HPV antigens and then returning to the same patient (Palucka & Banchereau, 2012).

For instance, a study performed in cervical cancer patients with autologous DCs pulsed with HPV16 or HPV18 E7 proteins. Despite of appearance of HPV-specific CTLs in some patients, clinical outcome has not been promising (Ferrara et al, 2003). A similar study was carried out in late-stage cervical cancer patients. They first received a DC vaccine pulsed with HPV16 and HPV18 E7 proteins and low-doses of IL-2 daily administered for several days after DC vaccination. Although E7-specific CTLs were detected in all patients, there were again no clinical responses (Santin et al, 2006).

In another study, a recombinant protein comprised of anti-human CD40 and HPV16 E6/7 was used to pulse DCs. These activated DCs were proved *in vitro* that they could efficiently stimulate production of E6/7-specific CD8+ T cells which were from the blood of patients with HPV16+ head-and-neck cancer (Yin et al, 2016).

1.6.6 Adoptive cell transfer

Different from DC vaccines, adoptive cell transfer (ACT) means the expansion of antigen specific CTLs instead of APCs *ex vivo* prior to transfer back to a patient. There are some advantages of ACT: CTLs can be manipulated *ex vivo* to acquire a desired activity; CTLs that target specific antigens can be expanded in a large number; the tumor suppressive microenvironment in patients can be controlled, such as elimination of regulatory T cells before cell delivery back.

In one study, HPV16 E6 and E7 specific CTLs were transfused to metastatic cervical cancer patients, and 2 out of 9 patients showed complete regression (Stevanovic et al, 2015). In addition, CTLs can be also engineered to express TCRs against HPV E7 or E6. It was reported that CTLs with E7 TCR had activities toward the target antigen *in vitro* (Scholten et al, 2005). Another study used CTLs with E6 TCR, and HPV+ cells originated from cervical cancer or head and neck cancer cell lines can be killed by these CTLs (Draper et al, 2015).

1.7 Development of HPV prophylactic and therapeutic combined vaccines

Combined vaccines with prophylactic and therapeutic characteristics are principally beneficial to the areas with fewer economical resources, where HPV screening is not prevalent. Such vaccines could be given to both uninfected and already infected population. The combined vaccines would ideally resolve productive infections and HPV-related diseases and would shield the recovered individuals from further HPV re-infections. Moreover, these vaccines could lead to set up herd immunity in the population providing indirect protection for non-vaccinated individuals (Schiller & Muller, 2015).

One approach is to generate fusion proteins with L1 VLPs as frames and E6 or E7 polypeptides incorporating into VLPs (Greenstone et al, 1998; Muller et al, 1997). These chimeric VLPs can not only stimulate high titres of neutralizing antibodies, but also induce CD4+ and CD8+ T cell responses against E6 or E7 epitopes (Greenstone et al, 1998; Peng et al, 1998). However, these VLPs seem to be poor at boosting these responses, either due to the production of anti-L1 antibodies or upregulation of T regulatory responses by the priming. Another clinical trial as mentioned in section 1.6.1, chimeric protein L1VLPE7 was carried out in patients with HPV16+ grade CIN 2 or 3 (Kaufmann et al, 2007). This combined vaccine seems to have some therapeutic effect, but has not been promising enough for conductance of further trials.

Another combined antigen is TA-CIN (tissue antigencervical intraepithelial neoplasia) containing HPV16 L2, E6 and E7, and expressed in *E coli*. A phase II trial (Daayana et al, 2010) was carried out in vulvar intraepithelial neoplasia patients with fusion protein TA-CIN and an immunomodulator, Imiquimod (TLR7 agonist). Three doses of TA-CIN were immunized after 8 weeks administration of Imiquimod. The trial was not placebo-controlled, but lesions

regressed more frequently compared to the observation from previous studies and a local infiltration of CD4+ and CD8+ T cells was observed. The evaluation of humoral responses of this vaccine was hard to conclude due to preexisting neutralizing antibodies in most patients, and the antibody titres did not change considerably after vaccination. Nevertheless, cross-neutralizing antibodies induced by this vaccine were proved in HPV-free mice and macaques (Karanam et al, 2009).

The objective of my PhD project is also to develop HPV prophylactic and therapeutic combined vaccines based on L2 from HPV16, 18, 31, 33, 35, 6, 51 and 59, and E7 from HPV16. The details will be discussed in the results section.

1.8 Aims of PhD project

The main objective of my PhD project is the development of prophylactic and therapeutic vaccines against HPV using a multimerized thioredoxin (PfTrx) scaffold based on L2 and E7 proteins. The specific aims that we pursued in order to produce a combined vaccine which can efficiently induce anti-L2 antibodies and E7 specific T cell responses include:

- Evaluating activity of PfTrx used in induction of CD8+ cytotoxic T cell responses.
- Developing monomeric HPV prophylactic and therapeutic combined vaccines based on PfTrx scaffold and assessing induced B cell and T cell responses *in vitro*.
- Developing heptameric HPV prophylactic and therapeutic combined vaccines based on PfTrx and OVX313 scaffolds and assessing induced B cell and T cell responses *in vitro*.
- Evaluating therapeutic potential of heptameric combined vaccine PfTrx-$L2_{20-38}$8mer-(flank HPV16 $E7_{49-57}$)$_{3X}$-OVX313 in mouse tumor model.

Notes:

$L2_{20-38}$8mer: the amino acid sequence from 20 to 38 of L2 of 8 different HPV types (16-18-31-33-35-6-51-59).

(flank HPV16 $E7_{49-57}$)$_{3X}$: 3 copies of (flank HPV16 $E7_{49-57}$)

flank HPV16 $E7_{49-57}$: five amino acids extension before and after HPV16 E7 H-2D^{b} restricted CTL epitope (QAEPDRAHYNIVTFCCKCD)

PfTrx: Pyrococcus furiosus thioredoxin, a highly thermostable scaffold with a large capacity to accept insertion into its active center.

OVX313: Chimeric version of Avian C4b-binding protein which was shown to lack homology to human C4bp. It can assemble spontaneously into a heptameric structure resulting in displaying fused proteins seven times.

2. Materials

2.1 Biological materials

2.1.1 Bacteria

E.coli MxDH10α	genotype: F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ψ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ(*ara, leu*)7697 *gal*U *gal*K λ- *rps*L *nup*G *ton*A (Invitrogen)
E.coli BL21(DE3)	genotype: F^- ompT hsdS(r_B^- m_B^-) dcm^+ Tetr gal λ(DE3) endA Hte (Stratagene)
E. coli Rosetta	genotype: F^- ompT hsdS(r_B^- m_B^-) dcm^+ gal pRARE (Cam R) (Merck4Biosciences)

2.1.2 Mammalian cell lines

HeLaT K4	HeLa cells, a HPV18 positive cervical cancer cell line, stably express the SV40 Large T-antigen. Used for L1-PBNA.
TC-1	Derived from lung epithelium of C57BL/6 mice, stably transfected with HPV16 E6 and E7 and c-Ha ras. Used for mouse tumor experiment and *in vitro* stimulation of splenocytes in IFN-γ ELISpot.
RMA	Derived from C57BL/6 mice lymphoma cell line (Rauscher virus-induced). Used for *in vitro* stimulation of splenocytes in IFN-γ ELISpot.
RMA/E7	RMA cells, additional stably transfected with HPV 16 E7. Used for *in vitro* stimulation of splenocytes in IFN-γ ELISpot.
EL4	Lymphoma lymphoblast derived from C57BL/6 mice. Used for *in vitro* stimulation of splenocytes in IFN-γ ELISpot.
EG7:	EL4 cells, stably expressing ovalbumin. Used for *in vitro* stimulation of splenocytes in IFN-γ ELISpot.

2.1.3 Pseudovirions

HPV16 Gaussia Co-transfected with plasmid #988 + #1998.

HPV18 Gaussia Co-transfected with #1998 + #1165 + #1166.

- #988: HPV16 L1h+L2h, pCDNA 4.0 TO vector with HPV16 L1 and L2 linked via IRES. It contains CMV promoter, tetracycline-operator and SV40-ori.

  #1998: pGF Gaussia-GFP, Gaussia and EGFP gene controlled by EF1alpha promoter.

  #1165: HPV18 L1h, HPV18 L1 controlled by CMV promoter.

  #1166: HPV18 L2h, HPV18 L2 controlled by CMV promoter.

2.1.4 Laboratory animals

The mice used in IFN-γ ELISpot, intracellular cytokine staining, tetramer staining and streptamer staining are C57BL/6N mice, 6-8 weeks old, female, from Charles River, Sulzfeld, Germany.

The mice used in mouse tumor experiment are C57BL/6N mice, 6 weeks old, female, from Envigo, gannet, France.

The mice used in L1-PBNA are BALB/c mice, 6-8 weeks old, female, from Charles River, Sulzfeld, Germany.

2.2 Materials for DNA operation

2.2.1 Annealing oligonucleotides

Preparation of $OVA_{257-264}$ annealing oligonucleotides to clone $PADRE_{2X}$-PfTrx-$(OVA_{257-264})_{3X}$ and PfTrx-$(OVA_{257-264})_{3X}$-OVX313:

Forward oligonucleotide: GTCCGAGCATTATTAACTTTGAAAAACTGGGCG

Reverse oligonucleotide: GACCGCCCAGTTTTTCAAAGTTAATAATGCTCG

Preparation of $E7_{49-57}$ annealing oligonucleotides to clone $PADRE_{2X}$-PfTrx-(HPV16 $E7_{49-57})_{3X}$:

Forward oligonucleotide: GTCCGCGCGCGCATTATAACATTGTGACCTTTGGCG

Reverse oligonucleotide: GACCGCCAAAGGTCACAATGTTATAATGCGCGCGCG

2.2.2 Plasmids

The following plasmids were cloned in pET-9a vector by GenScript company.

Table 2. Synthesized plasmids from GenScript

Plasmid	Clone number
$PADRE_{2X}$-PfTrx-(flank HPV16 $E7_{49-57})_{3X}$	# 3713
$PADRE_{2X}$-PfTrx-(HPV16 L2 $_{20-38}$-$OVA_{257-264})_{3X}$	# 3753
PfTrx-$L2_{20-38}$8mer-$(OVA_{257-264})_{3X}$-OVX313	# 3754
$PADRE_{2X}$-PfTrx-(HPV 16 L2 $_{20-38}$-flank $E7_{49-57})_{3X}$	# 3843
PfTrx-$L2_{20-38}$ 8mer-(flank $E7_{49-57})_{3X}$ -OVX313	# 3844

2.2.3 Enzymes

KOD polymerase (Novagen, Darmstadt)

Restriction enzymes (NEB, Frankfurt)

T4 DNA ligase (Invitrogen, Darmstadt)

Calf Intestinal Alkaline Phosphatase (CIP) (NEB, Frankfurt)

2.2.4 Chemicals for DNA preparation

- **Chemicals for PCR**

20 mM dNTP mix	Roche (Mannheim)
25 mM $MgCl_2$	Novagen (Darmstadt)

- **Buffer for oligonucleotides annealing**

annealing buffer	10 mM Tris-HCl
	150 mM NaCl
	PH 7.6

- **Buffers and chemicals for plasmid purification**

glucose buffer	50 mM glucose

	25 mM Tris 10 mM EDTA 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	1:1 phenol-CIA mix (CIA, chloroform-isoamyl : alcohol = 24:1) 100 µg 8-hydroxyquinoline in 350 ml solution
100 % isopropanol	
100 % ethanol	

- **Agarose gel electrophoresis**

agarose gel (1 %)	1 % agarose (w/v) 1 µg/ml ethidium bromide (10 mg/ml ethidium bromide from Roth) in 1 x TAE buffe
1 x TAE	40 mM Tris 5.71 % acetic acid (v/v) 10 % 0.5 M EDTA pH 8 (v/v) in H_2O
100 bp DNA ladder plus:	New England Biolabs
GeneRuler 1 kb DNA ladder	Thermo Scientific
6 x DNA loading buffer	Thermo Scientific

- **Sequencing of DNA**

DNA was sequenced by GATC-Biotech (Konstanz).

2.3 Materials for protein expression and purification

2.3.1 Media and solutions for protein expression

LB medium	10 g tryptone
	5 g yeast extract
	5 g NaCl
	in 1 L H_2O
LB agar plates	15 g agar in 1 L LB-medium

ampicillin stock concentration: 100 mg/ml (Sigma-Aldrich)

kanamycin stock concentration: 25 mg/ml (Sigma-Aldrich)

chloramphenicol stock concentration: 20 mg/ml (Sigma-Aldrich)

IPTG stock concentration: 1 M (Applichem, Darmstadt)

2.3.2 Lysis buffer for protein extraction

300 mM NaCl
25 mM Tris
0.16 % Tween20
0.5 mM PMSF
0.1 mg/ml lysozyme
in H_2O, pH 8

lysozyme powder (Sigma-Aldrich, Taufkirchen) (in 1 x PBS with final concentration 20 mg/ml)

2.3.3 Buffers for nickel affinity chromatography

Binding and elution buffers	300 mM NaCl
	25 M Tris
	50 M imidazole (binding buffer)
	100 – 300 mM imidazole (elution buffer)

	in H_2O, pH 7.5
Stripping buffer	300 mM NaCl 25 mM Tris 50 mM EDTA in H_2O, pH 7.5

2.3.4 Purification column for nickel affinity chromatography

HiTrap Chelating HP column 1 ml/ 5 ml (Amersham GE Healthcare, Buckinghamshire, UK)

2.3.5 Buffers for cation exchange chromatography

Binding buffer	200 mM NaCl in 1×PBS, pH 8
Elution buffer	200 mM, 300 mM, 400 mM until 800 mM NaCl in 1×PBS, pH 8

2.3.6 Purification column for cation exchange chromatography

HiTrap SP FF column 1 ml/ 5 ml (GE Healthcare, Uppsala, Sweden)

2.3.7 Endotoxin removal

Triton X-114 (Sigma-Aldrich, Taufkirchen)

2.3.8 Polyacrylamide gels

3 x protein loading buffer	187.5 mM Tris 30 % glycerol (v/v) 6 % SDS (w/v) 15 % β-mercaptoethanol (v/v) 0.03 % bromphenol blue (v/v) in H_2O, pH 6.8
Tris buffer pH 8.8	1 M Tris (pH 8.8)
Tris buffer pH 6.8	1 M Tris (pH 6.8) 0.03 % bromphenol blue (v/v)

1x TGS	2.5 mM Tris 0.1 % SDS (w/v) 1.45 % glycine (w/v) in H_2O, pH 8.3

acrylamide solution (Roth, Karlsruhe)

TEMED (Sigma-Aldrich, Taufkirchen)

2.4 Materials for immunization

2.4.1 Peptides

Peptides were synthesized by GenScript with purity >75%

$OVA_{257-264}$, SIINFEKL, derived from Ovabulmin, H-$2K^b$-restricted CTL epitope

PADRE (pan HLA DR-binding epitope), AKFVAAWTLKAAA

HPV16 $E7_{49-57}$, RAHYNIVTF, derived from HPV16 E7, H-$2D^b$-restricted CTL epitope

HPV16 $E6_{48-57}$, EVYDFAFRDL, derived from HPV16 E6, H-$2D^b$-restricted CTL epitope

OVX313 peptide panel

Table 3. OVX313 overlapping peptide sequences. We designed 20mer-peptide set with 12 amino acids overlap covering the entire OVX313 sequence. There are 9 peptides in total named from I1 to I9.

OVX313 peptide	Sequences
I-1	EVGRQNLIRSKEEILKKLKE
I-2	RSKEEILKKLKELQEGSKKQ
I-3	KLKELQEGSKKQGDADVCGE
I-4	SKKQGDADVCGEVAYIQSVV
I-5	VCGEVAYIQSVVSDCHVPTA
I-6	QSVVSDCHVPTAELRTLLEI
I-7	VPTAELRTLLEIRKLFLEIQ
I-8	LLEIRKLFLEIQKLKVEGRR

I-9	RKLFLEIQKLKVEGRRRRRS

2.4.2 Adjuvants

Incomplete Freund's adjuvant (IFA) (Sigma-Aldrich)

AddaVax (Invivogen)

Aluminum hydroxide (Brenntag)

Synthetic monophosphoryl lipid-A (AvantiLipids)

2.5 Materials for evaluation of humoral immune responses

- **Antibody used in L1-PBNA (pseudovirion-based neutralisation assay)**

K18L2$_{20-38}$: cross-neutralizing monoclonal antibody, recognizing L2$_{20-31}$.

- **Substrate for L1-PBNA**

Gaussia GLOW-Juice BIG KIT (PJK, Kleinblittersdorf)

Beetle-Juice BIG KIT (PJK, Kleinblittersdorf)

2.6 Materials for evaluation of cellular immune responses

2.6.1 Materials for IFN-γ ELISpot

- **Antibody used in IFN-γ ELISpot**

Capture antibody (# 551216, BD Pharmingen)

Biotinylated rat anti-mouse IFN-γ (# 554410, BD Pharmingen)

Streptavidin-AKP (# 554065, BD Pharmingen)

- **Substrate for IFN-γ ELISpot**

BCTP/NBT substrate (# B-1911, Sigma)

2.6.2 Materials for Intracellular cytokine staining

- **Antibody used in Intracellular cytokine staining**

  APC Rat Anti-Mouse IFN-γ (# 562018, BD Pharmingen)

  FITC Rat Anti-Mouse CD4 (# 553047, BD Pharmingen)

  PE Rat Anti-Mouse CD8a (# 553033, BD Pharmingen)

  LIVE/DEAD™ Fixable Yellow Dead Cell Stain Kit (# L34959, Thermo Fisher Scientific)

- **BD Cytofix/Cytoperm Plus kit (# 554715, BD Biosciences Pharmingen)**

  BD Perm/Wash (# 554723)

  BD Cytofix/Cytoperm (# 554722)

  BD GolgiStop (protein transport inhibitor) (# 554724)

2.6.3 Materials for Tetramer and Streptamer staining

- **Tetramer staining:**

iTAg Tetramer/APC-H-2 Kb OVA (SIINFEKL) (# MBL-T03002, MBL)

- **Streptamer staining:**

Strep-Tactin APC for MHC I Streptamer (# 6-5010-001, iba)

MHC I-Strep H-2 D^b HPV 16 $E7_{(49-57)}$ RAHYNIVTF (# 6-7057-001, iba)

- **Anti-CD8 antibody**

CD8-α Antibody (KT15) PE (# sc-53473 PE, Santa Cruz Biotechnology)

2.7 Materials for mouse tumor experiment

Isoflurane for anesthesia (CuraMed, Karlsruhe, Germany)

2.8 Materials for cell culture

2.8.1 Media for cell culture

RPMI (Splenocytes)	10 % FCS
	1 % Penicillin/Streptomycin
	1 % L-glutamine
	1 % HEPES buffer
	1 % sodium pyruvate
RPMI (TC-1 cells)	10 % FCS
	1 % Penicillin/Streptomycin
	0.2 mg/ml hygromycin B
	0.4 mg/ml G418 (Geneticin)
	1mM sodium pyruvate
	1× MEM NEAAS
	50 µM β-mercaptoethanol
RPMI (EL4, RMA cells)	10 % FCS
	1 % Penicillin/Streptomycin
	1 % L-glutamine
RPMI (EG7, RMA/E7 cells)	10 % FCS
	1 % Penicillin/Streptomycin
	1 % L-glutamine
	0.4 % G418 (Geneticin)
DMEM (HeLaT K4)	10 % FCS
	1 % Penicillin/Streptomycin
	1 % L-glutamine
	0.25 % hygromycin B

DMEM, RPMI-1640 (Sigma-Aldrich)

FCS (PAN Biotec, Aidenbach)

Penicillin/Streptomycin (Gibco Life Technologies)

L-glutamine (Genaxxon)

Trypsin-EDTA 0.05% or 0.25% (Gibco Life Technologies)

Hygromycin B 50 mg/ml (Roche)

G418 (Geneticin) 50 mg/ml (Roche)

Sodium Pyruvate 100mM (Gibco)

HEPES buffer 1M (Gibco)

MEM NEAAS 100× (Sigma)

β-Mercaptoethanol 1M (14M stock from Merck)

Trypan blue staining solution (Fluka)

2.8.2 Buffers and solutions

RBC lysis buffer	90 ml solution A (NH_4Cl 8.3 g in 1 l MQ H_2O) plus 10 ml solution B (Tris-Base 20.6 g pH7.6 in l MQ H_2O) mix pH 7.2, Autoclave
FACS buffer	3 % FCS 0.02 % NaN3 in PBS
splenocytes washing buffer	1 ml FCS 0.5 ml Penicillin/Streptomycin in 50 ml PBS
freezing medium	60% DMEM or RPMI 30% FCS 10% DMSO
1 x PBS	140 mM NaCl 2.7 mM KCl

8.1 mM Na_2HPO4

1.5 mM KH_2PO4

Commercial PBS (D8537-500ML, Sigma)

PBST (0.05 % Tween20) 0.5 ml Tween20 in 1 L 1 x PBS

2.9 Kits

QIAprep® Spin Miniprep Kit (Qiagen, Hilden)

QIAGEN Plasmid Plus Maxi Kit (Qiagen, Hilden)

QIAquick® Gel Extraction Kit (Qiagen, Hilden)

2.10 Laboratory equipment

2.10.1 General utensils

6, 12, 24, 48, 96-well plates (cell culture) (Costar, Corning, NY, USA)

Microplate, ps, 96 well, F-bottom (chimney well) white, lumitrac (Greiner)

Petri dishes (Greiner, Frickenhausen, Germany)

Tissue flasks: 25 cm2, 75 cm 2 and 150 cm2 (TPP, Trasadingen, Switzerland)

Parafilm “M” (American National Can, Chicago, USA)

Drying foil for SDS gels (Promega, Madison, USA)

Nitrocellulose membrane (Schleicher & Schuell, Dassel)

Chemiluminescence films (GE Healthcare Limited, Buckinghamshire, UK)

X-ray cassette (Roth, Karlsruhe)

0.2/0.4 µm filter (Renner, Dannstadt)

10 KD dialysis tubing (Roth, Karlsruhe)

Plastic inoculation loops (Neolab, Heidelberg, Germany)

Pasteur pipettes (Greiner, Frickenhausen)

Sterile plastic pipettes (BD Falcon, Durham, USA)

1000 µl, 200 µl, 20 µl and 10 µl PIPETMAN Neo® (Gilson, Middleton, USA)

1000 µl, 200 µl, 20 µl, 10 µl tips (nerbe plus, Germany)

1000 µl, 200 µl, 20 µl, 10 µl filter tips (nerbe plus, Germany)

2 ml cryo vials (Roth, Karlsruhe)

25 x 2 mm electroporation cuvettes (Peqlab, Erlangen)

50 ml centrifuge tubes (SA-600 rotor) (Nalgene, Nunc, Wiesbaden)

500 ml centrifuge tubes (F12 rotor) (Nalgene, Nunc, Wiesbaden)

15 ml, 50 ml falcon tubes (cellstar tubes, Greiner)

1.5 ml and 2 ml Eppendorf tubes (Eppendorf, Hamburg)

0.5 ml and 1.5ml LoBind Protein tubes (Eppendorf, Hamburg)

PCR reaction tubes (Roth, Karlsruhe)

Needles and syringes (BD Falcon, Durham, USA)

50 ml reservoir (Costar, Corning, USA)

Gloves, (Meditrade, Kiefersfelden, Germany)

Disposable scalpel (Feather, Osaka, Japan)

Cell scraper (Sarstedt, Newton, USA)

Cell strainer 70 µm Nylon (FALCON, Corning, USA)

MultiScreen Filter plates (Merck Millipore, Ireland)

2.10.2 Electrical equipment

BD FACSCantoTM II Flow Cytometer (BD Bioscience, San Jose, CA 95131 USA)

Nanodrop spectrophotometer (Peqlab, Erlangen)

pH meter (Sartorius, Göttingen)

AID ELISPOT Reader System (AID, Strassberg, Germany)

Refrigerated tabletop centrifuge 5417R (Eppendorf, Hamburg)

Tabletop centrifuge 5417R (Eppendorf, Hamburg)

Megafuge 1.0 (Heraeus, Hanau)

CHRIST Minifuge GL (Heraeus, Hanau)

Multifuge 1 S-R (Heraeus, Hanau)

Refrigerated centrifuge RC5C (Sorvall, Newton, USA)

Refrigerated centrifuge RC-5B (Sorvall, Newton, USA)

Precision balance (Mettler Toledo, Gießen)

Combimag Red/RET magnetic stirrer (IKA, Staufen)

800 W microwave (Bosch, Gerlingen-Schillerhöhe)

GFC water bath (Grant Instruments, Cambridge, UK)

CO2 incubator safe cell UV (Sanyo, Osaka, Japan)

Steril GARD® III Advance cell culture hood (The Baker Company, Sanford, USA)

Peristaltic EconoPump (BioRad, München)

Microscope for cell culture, Willovert (Hund, Wetzlar)

Refrigerators and freezers (Liebherr, Ochsenhausen)

-80°C freezer (New Brunswick Scientific, Hamburg)

Ice maker (Hoshizaki, Willich-Münchheide)

Nitrogen tank Chronos (Messer, Krefeld)

Electrophoresis power supply (Gibco BRL, Eggenstein)

Horizontal Gel Electrophoresis Horizon®11.14 (Gibco BRL, Eggenstein)

Electrophoresis chamber (Roth, Karlsruhe)

Electrophoresis gel slides (Roth, Karlsruhe)

MicroPulser™ Electroporator (BioRad, München)

Bacteria shakers (Infors AG, Bottmingen, Switzerland)

MilliQ ultra-pure water unit Millipore (Merck, Darmstadt)

French press (Emulsi Flex-C5) (Avestin, Ottawa, Canada)

Multichannel pipette (1200 µl) (Biozym Scientific, Hessisch Oldendorf)

2.11 Software

Clone Manager 9.0 for Windows (Scientific & Educational Software, Cary, USA)

GraphPad Prism 6.0 (GraphPad Software, La Jolla, USA)

Microsoft Windows 7 Professional (Microsoft, Redmond, USA)

Microsoft Office 2010 (Microsoft, Redmond, USA)

Adobe Acrobat Professional X (Adobe, San Jose, USA)

AID ELISPOT Software (AID, Strassberg, Germany)

EndNoteX5 (Thomson Reuters, New York, USA)

3. Methods

3.1 Manipulation of DNA and cultivation of bacteria

3.1.1 CPOI cloning

Firstly, the annealing oligonucleotides were prepared by adding 4 µl (100 pmol) of each oligonucleotide in 32 µl annealing buffer and incubated in the following thermocycler:

5 min 95°C

20 min 72°C

20 min 37°C

Then, the CPOI ligation was performed according to the following components:

1 µl of cleaved (section 3.1.5) and dephosphorylated (section 3.1.6) vector

(7-20 ng/µl-Nanodrop quantification)

1 µl of annealed oligonucleotides

2 µl of 10×T4 liagse buffer (NEB)

1 µl T4 DNA ligase

15 µl ddH_2O

The mixture was incubated at room temperature for 20-40 minutes and 1 µl was used for transformation of MXDH10 electrocompetent bacteria.

3.1.2 Purification of plasmid DNA (without kit)

A single colony was inoculated in 5 ml LB medium with 1:1000 diluted antibiotics. The culture was incubated at 37°C, 250 rpm overnight. The next day, 1.5 - 5 ml of bacterial culture was centrifuged at 12,000 rpm for 2 minutes at room temperature. The bacterial pellet was obtained and resuspended completely in 100 µl glucose buffer. Then, 200 µl lysis buffer were mixed and incubated on ice for 5 minutes. Later, 150 µl of 3 M sodium acetate (pH 5.3) was added with another 5 minutes of ice incubation. To remove proteins from the lysate, 450 µl phenol-mix was added following with a vigorous shaking for 5 minutes. The

aqueous supernatant was obtained by 5 minutes of centrifugation at 12,000 rpm. The supernatant (380 µl) was transferred into a new tube containing 450 µl isopropanol. The new mixture was kept at -80°C for 10 minutes before it was centrifuged for 20-30 minutes at 0°C, 12,000 rpm. Afterwards, the DNA pellet was washed twice with 500 µl 100% ethanol. The DNA was dried in the hood and later was resuspended in 60 µl autoclaved MilliQ water. Restriction enzyme digestion was performed to analyze the purified DNA (section 3.1.5).

If DNA fragment was correct after analysis with enzyme digestion, the DNA was to be purified by using QIAprep® Spin Miniprep Kit instead of above mentioned methods. The DNA was diluted with autoclaved MilliQ H_2O in 20 - 100 ng with 20 µl for sequencing.

3.1.3 Purification of plasmid DNA with a kit (mini prep and Maxi prep)

Preparation of plasmid DNA in a small scale or a large scale was performed by using the Qiagen Plasmid Mini or Maxi Kit according to the manufacturer's instructions. Normally, 2 - 5 ml bacteria were cultured for mini prep while 250 - 300 ml bacteria for maxi prep.

3.1.4 Determination of DNA concentration and purity

The purity of DNA was analyzed by measuring the absorption at 260 nm (DNA) and 280 nm (protein). When the ratio ($Abs_{260\,nm}$/ $Abs_{280\,nm}$) is between 1.8 and 2.0, we consider the DNA pure. A ratio > 2.0 indicates RNA contamination, while a ratio < 1.8 represents a contamination with proteins or organic compounds. For determination of DNA concentration, a blank measurement with 1 µl of H_2O or 1 x TE was performed before 1 µl of the sample was measured.

3.1.5 Restriction enzyme digestion

We normally performed two types of enzyme digests, analytical and preparative digests. For an analytical analysis, 0.5-1 µg DNA was digested for 1 hour at 37°C with 0.1 µl enzyme in 20 µl system. A preparative digest was performed when the digested fragments need to be collected. In this case, 20 - 30 µg DNA were digested with 3 - 5 µl enzyme in 200 µl volume at 37°C overnight. The 200 µl digest was mixed with 40 µl 6x DNA dye, and the entire was loaded into a 1% preparative gel (section 3.1.7). The target fragment was subsequently extracted from the gel (section 3.1.8). The systems for analytical and preparative digests are shown below. Appropriate enzyme buffers were used according to the instructions (NEB).

Table 4. The systems for analytical and preparative digests.

	Analytical digest	preparative digest
DNA	0.5-1 µg	20 - 30 µg
10 X enzyme buffer	2 µl	20 µl
100 µg/ml RNase	0.1 µl	3 µl
restriction enzyme	0.1 µl	3 - 5 µl
	20 µl system	200 µl system

3.1.6 Dephosphorylation of 5'-DNA ends

To prevent religation of linear vector without the integration of the target insert, a phosphatase, calf-intestinal alkaline phosphatase (CIP) was applied to remove the 5'-phosphates from the vector. Therefore, an insert with 5'-terminal phosphates can be more efficiently ligated with a dephosphorylated vector.

After preparative digestion overnight, 2 µl CIP enzyme were added directly into the digestion system and incubated at 37°C for 1 hour. Inactivation of the enzyme (58°C, 10 minutes) was performed before the vector was loaded into an agarose gel for DNA extraction (section 3.1.8).

3.1.7 Agarose gel electrophoresis

DNA fragments are negatively charged because of their phosphate residues. Therefore, DNA moves towards positive anode in an electric field. The separation of DNA depends on their conformation and size. Usually, supercoiled DNA molecules move faster than linear ones, shorter DNA fragments travel faster than longer ones. The density of the agarose gels also decides the separation efficiency. More concentrated gels allow for the separation of smaller sized fragments. The size of specific DNA molecules can be determined by using DNA marker which contains a mix of fragments with known sizes. Ethidium bromide is employed to visualize the DNA under UV radiation.

For preparation of 1% agarose gels, 1 g agarose were dissolved in 100 ml 1 x TAE buffer. The mixture was heated by microwave until a homogeneous solution formed. We applied 6 µl ethidium bromide (1 mg/ml) to 100 ml gel solution.

3.1.8 DNA extraction from agarose gels

The plasmids were digested in a preparative scale (section 3.1.5) and separated by gel electrophoresis (section 3.1.7). The specific fragment was excised by using a clean scalpel. Subsequently, the DNA was extracted from a gel using the QIAquick Gel Extraction Kit following the manufacturer's instructions.

3.1.9 Ligation of DNA fragments

The ligation was performed with a DNA insert and a dephosphorylated DNA vector. Phosphodiester bonds were formed between the insert and vector under the activation of DNA ligase.

T4 DNA ligase was routinely employed and the reaction was carried for 30 to 60 minutes in room temperature, or 16°C overnight. The pipetting scheme is shown below. Afterwards, 1-2 µl of ligated DNA was used for bacteria transformation via electroporation (section 3.1.10).

Table 5. The system (20 µl) for DNA ligation with T4 DNA ligase.

Insert DNA	9 µl
Vector DNA	1 µl
10 X T4 DNA ligase buffer	2 µl
T4 DNA ligase	1 µl
MilliQ H2O	7 µl

3.1.10 Transformation of bacteria by electroporation

E. coli MxDH10, BL21 and Rosetta were transformed via electroporation. For this, 40 - 50 µl electro-competent bacteria were mixed with 100 - 200 ng plasmid DNA or 1-2 µl ligation mix (section 3.1.9) and transferred to a pre-cooled electroporation cuvette. The electroporation was performed with 2.5 kV, 25 µF, 200 Ω. Then, transformed bacteria were resuspended in 300 µl antibiotic-free LB-medium and loaded into a new 1.5 ml Eppendorf tube with shaking at 37 °C for 30-60 min. If plasmid DNA was transformed, 5 - 10 µl bacterial culture were spread onto a agar plate containing the appropriate antibiotics. If DNA was the ligation reaction, 150 - 300 µl of the transformation bacteria were used to a plate. The plates were kept overnight at 37°C.

3.1.11 Preparation of electro-competent bacteria

Bacteria were spread from a glycerol stock onto a LB agar plate and kept at 37°C overnight. The next day, a single bacterial colony was inoculated into 20 ml LB medium. The culture incubated shaking overnight at 37°C. 5 ml of the overnight culture were applied to 400 ml LB and this big culture was allowed to grow until $OD6_{00}$ reached 0.5-0.6. Then, the culture was cooled on ice for 15-30 minutes and transferred into large centrifuge tubes. The culture was centrifuged at 5,000 rpm for 15 min at 4°C, and the obtained pellet was resuspended in 30 ml ice cold MilliQ H_2O. In order to remove the salt, the cell suspension was transferred into a dialysis tube and dialyzed against 2-4 l MilliQ H_2O at 4°C overnight. After exchanges of the H_2O, dialyzed bacteria were harvested into 50 ml Falcon tube at 5,000 rpm, 4°C for 10 min. The bacteria were resuspended in 600 µl 10% glycerol. We diluted 10 µl of this resuspension into 990 µl 10% glycerin, and measured the OD_{600} of it. The measured value was multiplied with 600 to calculate how much more volume (µl) of 10% glycerol still required to add into original 600 µl bacteria. Aliquots of 50 µl or 100µl were shock frozen in liquid nitrogen and stored at -80°C.

3.1.12 Storage of bacteria for long term

Usually, 300 µl autoclaved glycerol were mixed completely with 1 ml overnight culture in a cryo tube. The mixture was stored at -80°C.

3.2 Purification and analysis of protein antigens

3.2.1 Expression and extraction of the antigens in *E. coli*

We inoculated 4 ml *E. coli* BL21 or Rosetta overnight culture into 400 ml LB medium. The bacteria grew at 37°C, 200 rpm for 3 to 4 hours until OD_{600} reached 0.6 - 0.8. At this time, the culture was induced with 1 mM IPTG (final concentration) at room temperature overnight with a lower shaking speed 120 - 140 rpm. Then, the bacterial pellet was harvested after 5,000 rpm, 10 minutes centrifugation and stored at -20°C. If protein extraction was performed afterwards, the pellet was resuspended in 30 ml lysis buffer (300 mM NaCl, 25 mM Tris, 0.16% Tween20, 0.5 mM PMSF, 0.1 mg/ml lysozyme, pH 8) and incubated firstly at room temperature for 10 minutes then 20 minutes on ice. French Press was used to lyse

bacteria with three to four cycles at 1000 ~ 15,000 psi. After finished the lysis, French Press was washed with 0.5 M NaOH, 10 mM HCl, MilliQ H_2O and 20% ethanol. The crude lysate was centrifuged at 4°C, 12,000 rpm for 1 hour and the protein-containing supernatant was collected for subsequent purification (section 3.2.2 and section 3.2.3).

3.2.2 Purification of 'PADRE-PfTrx' based proteins by nickel affinity chromatography

All the 'PADRE-PfTrx' proteins ($PADRE_{2X}$-PfTrx-$(OVA_{257-264})_{3X}$, $PADRE_{2X}$-PfTrx-(HPV16 $E7_{49-57})_{3X}$, $PADRE_{2X}$-PfTrx-(flank HPV16 $E7_{49-57})_{3X}$, $PADRE_{2X}$-PfTrx-(HPV16 L2 $_{20-38}$-$OVA_{257-264})_{3X}$ and $PADRE_{2X}$-PfTrx-(HPV 16 L2 $_{20-38}$-flank $E7_{49-57})_{3X}$) contain dual 6xHis-tag. Therefore, we used nickel affinity chromatography to purify them.

A 1 ml HiTrap Chelating HP column was firstly rinsed with 10 column volumes (CVs) MilliQ H_2O. Next, 5 ml of 100 mM $NiSO_4$ (in H_2O) was employed to the column. After 5 - 10 minutes $NiSO_4$ incubation, the column was washed with 10 CVs of MilliQ H_2O and following 10 CVs of binding buffer (25 mM Tris, 300 mM NaCl, 50 mM imidazole, pH 7.5). The protein-containing supernatant was loaded to the column by peristaltic pump circulating at 0.5 ml/min overnight at 4°C. The next day, 10 CVs binding buffer was applied to the column to remove the unbinding protein and impurities. Then, the target protein was eluted by imidazole elution buffers (25 mM Tris, 300 mM NaCl, pH 7.5, with imidazole 100 mM, 150 mM or 300 mM) and collected 12 fractions with 1 ml per tube. The eluted fractions were analyzed by SDS-PAGE (sections 3.2.6 and section 3.2.7). The fractions with similar protein concentration were pooled together and dialyzed against PBS dialysis buffer (1×PBS with 300 mM NaCl) or Tris dialysis buffer (20 mM Tris with 100 mM NaCl) at 4°C. After dialysis, the sample was centrifuged at 12,000 rpm, 4°C for 15 minutes and the supernatant was measured again before removing endotoxin (section 3.2.5).

HiTrap columns were regenerated by washing with 10 CVs stripping buffer (binding buffer containing 50 mM EDTA to remove the Ni_{2+}). Then, 10 CVs of MilliQ H_2O and 10 CVs of 20% ethanol were used to finally clean the columns. We reserved columns in 20% ethanol at 4°C.

3.2.3 Purification of 'OVX313-PfTrx' based proteins by thermal purification

The 'OVX313-PfTrx'based proteins (PfTrx-$(OVA_{257-264})_{3X}$-OVX313, PfTrx-$L2_{20-38}$8mer-$(OVA_{257-264})_{3X}$-OVX313 and PfTrx-$L2_{20-38}$ 8mer-(flank $E7_{49-57})_{3X}$ -OVX313) are without 6x-HisTag and PfTrx is a thermal stable scaffold, so we used thermal purification method to isolate them.

Afterwards, if the protein was not pure enough for immunization, a further purification was performed by cation exchange chromatography (section 3.2.4).

After the bacterial cells were lysed, the supernatant was obtained by centrifugation and NaCl was added to it in a final concentration of 0.25 M. The standard protocol is 70°C water bath for 20 minutes. But the incubation temperature varies according to different proteins. Usually, we tried from 65°C to 80°C. After thermal incubation, the solution chilled on ice for 15 minutes and centrifuged at 12,000 rpm, 4°C, for 10 minutes. The purity of the protein was detected by SDS-PAGE (section 3.2.6).

3.2.4 Purification of proteins by cation exchange chromatography

If a protein was not pure enough after thermal purification, we continued to perform cation exchange chromatography. The HiTrap SP FF column was washed with 10 CVs MilliQ H_2O and then 10 CVs binding buffer (section 2.3.5) before the sample was applied for overnight binding. Next day, the column was washed with elution buffer containing different concentration of NaCl (section 2.3.5). The samples were collected in 1 ml/tube. After elution, the column was washed with 10 CVs MilliQ H_2O and following by 10 CVs 20% ethanol. We reserved columns in 20% ethanol at 4°C.

3.2.5 Endotoxin removal

Our proteins were all expressed in *E. coli*, so we performed detoxification after protein purification. The purified protein was mixed completely with 1% Triton X-114 and ice incubation for 5 minutes followed by another 5 minutes of incubation at 37°C. To remove Triton X-114, samples were centrifuged at 12,000 rpm, 37°C for 1 minute, and the upper supernatant was transferred to a new Eppendorf tube. This Triton X-114 treatment was performed twice to fully remove the endotoxin.

3.2.6 Denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE is used to separate denatured proteins by their molecular weight. The proteins are denatured and acquire an overall negative charge due to protein loading buffer containing reducing agent β-mercaptoethanol and negatively charged SDS. Thus, the proteins migrate to the positive anode when the gels are in buffer 1 x TGS filled electrophoresis chambers. The gel system is made of two layers, upper gel (stacking gel) and

lower gel (separating gel). The upper gel is used for making the proteins in an equal starting position, while the lower gel has the function of protein separation according to their sizes.

The proteins were mixed with 3 x protein loading buffer and boiled at 95°C for 10 minutes for denaturation. Electrophoresis was performed at 80 - 100 V for stacking gel and 120 - 130 V for separating gel. Subsequently, the gels were coomassie blue stained.

3.2.7 Coomassie blue staining of polyacrylamide gels

The protein gels were washed in water for 15 minutes before coomassie blue staining for 1 hour or overnight. Because of the acetic acid contained in coomassie blue, proteins can be fixed to the gel matrix. After the protein bands were visible, coomassie blue was removed, and the gels were rinsed three times in water to reduce the blue background. The protein marker was employed to determine the protein size.

3.3 Formulation of vaccines and immunization of mice

3.3.1 Formulation of antigens with IFA and AddaVax

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

3.3.2 Formulation of antigens with Alum-MPLA

The Alum used in our work was the Alhydrogel '85' (aluminum hydroxide, 10 mg/ml). The MPLA was synthetic, lyophilized powder (5 mg) and prepared to 1 mg/ml solution. The preparation of MPLA solution was introduced as follows.

Firstly, solution of Triethanolamine (TEoA) was prepared in sterile, Endotoxin-free H_2O at 0.5% (v/v) and sterilized by 0.22 µm filtering. Then, 1.5 ml of 0.5% TEoA were added to 5 mg lyophilized MPLA, and the solution were heated for 5 minutes at 65°C followed by 5 minutes of sonication in water bath until the solution became homogenous and slightly milky white (approximately 6-8 cycles of this treatment). In between each cycle the solution was vigorously vortexed. The dissolved MPLA was transferred to a 15 ml tube, 3.35 ml of 0.5% TEoA was filled in followed by one last cycle of heating and sonication. Lastly, the pH was

adjusted to 7.4 by drop-wise adding 1 M HCl. Normally, 1 ml MPLA-TEoA required 30 µl 1 M HCl. The MPLA was stored at 4°C.

We immunized mice with 50 µg Alum and 10 µg MPLA. Alum-Antigen and Alum-MPLA were prepared separately. Half of the Alum was mixed with the antigen and the other half was mixed with the MPLA. Two tubes rolled for 1 hour at room temperature. Before immunization, the two reactions were mixed by brief vortexing.

3.3.3 Immunization of mice

The 6 to 8 week-old C57BL/6N female mice were used for assessment of cellular immune responses. 20 µg of protein adjuvanted with 50% (v/v) AddaVax or IFA, or 50 µg Alum and 10 µg MPLA was immunized at base of the tail subcutaneously. For peptide administration, the adjuvant formulation and the immunization route were the same as protein vaccination, but the immunized amount was different. Normally, we injected peptide mixture containing 100 µg MHC I restricted peptide and 140 µg MHC II restricted peptide. A maximum volume for subcutaneous administration was 100 µl. For IFN-γ ELISpot, once immunization was enough to detect the efficacy of vaccines. While for ICS, tetramer staining or streptamer staining, twice immunization were applied to present a more obvious result. Seven to ten days later after the last immunization, the splenocytes were obtained and stimulated *in vitro* by corresponding peptide.

The 6 to 8 week-old BALB/c female mice were employed for evaluation of humoral immune responses. 20 µg antigen adjuvanted with 50% (v/v) AddaVax immunized into the caudal thigh muscle intramuscularly. The limit amount for intramuscular immunization was 50 µl. Mice were immunized 4 times at biweekly intervals. Final blood was collected from mice one month after the last immunization and analyzed against HPV 16 and HPV18 pseudovirions using L1-PBNA.

3.3.4 Blood sampling in mice

Intermediate blood samples were taken by puncture of the submandibular vein. Final blood was collected by cardiac puncture. Blood samples were incubated for 2-3 hours at room temperature or at 4°C overnight, allowing for complete clotting, and then centrifuged at 4,000 rpm, 4°C for 30 minutes. Cleared supernatants (sera) were transferred to clean

eppendorf tubes. Two rounds of this centrifugation were performed and sera were stored at 4°C or -20°C for longer storage.

3.3.5 Preparation of splenocytes suspension

All steps were performed in a laminar flow bench under sterile conditions. Single cells were generated by pressing the spleen through a 70 µm cell strainer (section 2.10.1) attached to a 50 mL Falcon tube using the plunger end of a 1 ml-syringe. The strainer was washed by 5 ml PBS and the cell pellet was collected by centrifugation for 5 min at 1,500 rpm. Pellets were resuspended in 5 ml RBC lysis buffer (section 2.8.2) and incubated on ice with frequently gentle shaking for 5 min to lyse erythrocytes. The reaction was neutralized by adding 5 ml complete medium. Then, cells were washed twice by 5 ml splenocytes washing buffer (section 2.8.2) and resuspended in 2-5 ml of RPMI supplemented medium (section 2.8.1) for IFN-γ ELISpot (section 3.5.2), intracellular cytokine staining (section 3.5.4), tetramer staining (section 3.5.3) or streptamer staining (section 3.5.3) assay.

3.4 Manipulation of mammalian cells

3.4.1 Cultivation of mammalian cells

All mammalian cell lines were cultivated in cell culture flasks in their specific media (section 2.8.1). Adherent cells were grown in monolayer and split when confluency was over 80 %. Suspension cells were maintained at a density of $1x10^6$/ml. All cells were cultivated at 37°C in an incubator with 95 % humidity and 5 % CO2. To sub-cultivate the adherent cells, the medium was aspirated and the cells were incubated with 3 - 4 ml 0.25 % (for HeLaT K4) or 0.05% (for TC-1, RMA, RMA/E7 and EL4) trypsin-EDTA for 5 to 15 min at 37°C to detach the cells from the flask. The trypsinization was stopped by adding 6 - 7 ml medium. The cell pellet was obtained by centrifugation at 1,900 rpm for 5 minutes and resuspended by 10 ml fresh medium of which 1 - 2 ml were taken to a flask for a new cultivation. Suspension cells (EG7) were passaged by replacing part of the cell suspension with fresh medium.

3.4.2 Determination of cell count and vitality

Neubauer counting chamber and trypan blue were used to decide the number and viability of the cells. Usually, 50 µl cells were mixed with 50 µl trypan blue and 10 µl of this mixture were applied to the Neubauer counting chamber. Only dead cells can be stained by trypan blue. So we counted the unstained cells in 2 big quadrants and calculated the mean cell number of the two. The cell numbers per ml can be calculated as follows:

Cell number/ml = N $\times 2 \times 10^4$ (N: mean cell number, 2: dilution factor)

3.4.3 Cryo-conservation and thawing of mammalian cells

The cell pellet was harvested by centrifugation and resuspended in ice-cold freezing medium (section 2.8.2). The cell number was adjusted to between $5x10^6$/ml to $10x10^6$/ml by freezing medium of which 1 ml cell suspension was transferred to 2 ml cryo vials. The cells were gradually cooled to -80°C overnight in a cell freezing box. For long-term storage, the vials were transferred to the liquid nitrogen tank.

For re-thawing of cells, the cryo vials were incubated in a 37°C water bath until the content was partially thawed. Cells were then transferred to a Falcon tube containing 5 ml of appropriate medium. After centrifugation at 1,900 rpm for 5 minutes, the cell pellet was resuspended in the specific medium and transferred to a cell culture flask.

3.5 Analysis of humoral and cellular immune responses

3.5.1 L1-pseudovirion based neutralization assay (L1-PBNA)

L1-PBNA is employed to detect neutralizing antibodies titers in sera against human papillomaviruses. We use HPV 16 and HPV18 pseudovirions to infect HeLaT K4 cells. After infection, the plasmid encoding Gaussia luciferase is released by PSV into the host cells. The extent of infection can be detected by measuring the substrate catalytic efficiency of secreted Gaussia luciferase in the medium. If the sera contain neutralizing antibodies, pseudovirion infection is prevented resulting in the less expression of the Gaussia luciferase.

Sera were diluted in supplemented DMEM and 50 µl of this dilution were added to each well in duplicates of a 96-well tissue culture plate. Outer wells of the plate were filled with 150 µl medium or 1 x PBS to prevent evaporation. Then, 50 µl of the PSV dilution prepared in medium were added to the wells. The mixture of serum and PSV was incubated at room temperature for 20 minutes. Next, 50 µl of HeLaT K4 cells (2.5×10^5 cells/ml) were added to each well and the plates were incubated at 37°C humidified incubator for 48 h.

3.5.2 Detection of antigen-specific cytotoxic T-lymphocytes by IFN-γ ELISpot

IFN-γ ELISpot was performed 7 days later after the last immunization. All steps of operating with splenocytes were carried out in a laminar flow bench under sterile conditions. One day before collecting the splenocytes, the 96-well MultiScreen ELISpot plates were activated by adding 15 µl of 35% ethanol per well and washed 3 times by 200 µl PBS before overnight incubation with 100 µl/well of anti-mouse IFNγ capture antibody (5 µg/ml in PBS, # 551216) at 4°C. On the next day, plates were washed 3 times with PBS and blocked with 200 µl/well of splenocytes RPMI supplemented medium (section 2.8.1) for at least 2 h at 37°C. Splenocytes suspension of immunized mice (section 3.3.5) was adjusted to $1x10^7$/ml and seeded 100µl/well in triplicate to capture antibody loaded plates. For experimental groups, the splenocytes were stimulated by 100 ng corresponding peptide ($OVA_{257-264}$, $E7_{49-57}$, PADRE or OVX313 peptide panel) 100 µl/well. The negative control group was stimulated by 100 µl/well supplemented medium. And 5 µg/ml of Concanavalin A (a lectin, universal activating T cells) was used as the positive control. The following scheme is an example for ELISpot working panel.

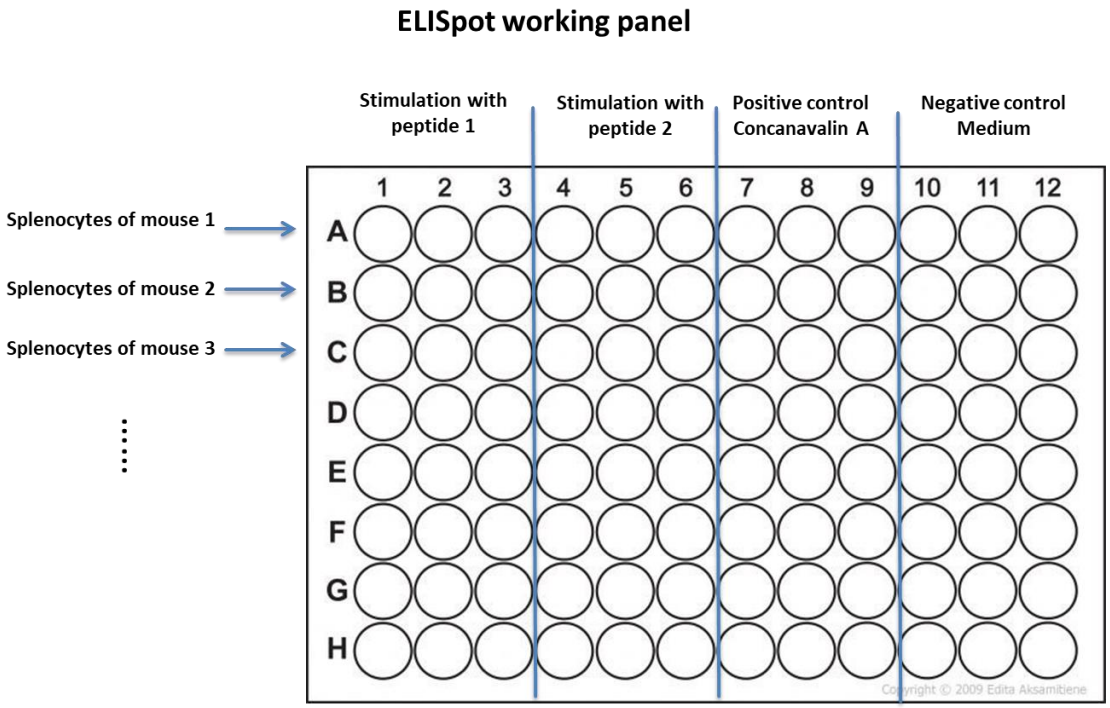


Figure 9. ELISpot working panel. Each row is the splenocytes of different mice. Every three columns have the same stimulation.

After incubation in 37°C humidified incubator for 16 hours (OVX313 peptide panel stimulating for 40 hours), the cells were discarded and washed 3 times with PBST (1×PBS with 0.05% Tween20). The plates were then blocked by 0.1 µg/ well of biotinylated rat-anti mouse IFN-γ antibody (1 µg/ml in PBS, # 554410) for at least 2 h at room temperature or 4°C overnight. Unbound antibody was removed by PBST washing for 3 times with 1 minute PBST incubation during each washing. Then, streptavidin-alkaline phosphatase (1:1000 dilution in PBS, # 554065) were added and incubated for 45 minutes in the dark at room temperature. Subsequently, plates were washed 3 times with PBST and 2 times with PBS. At last, 100 µl/well of the substrate BCTP/NBT (# B-1911) were added for around 4 minutes staining. The reaction was stopped by washing the double sides of plates with water. When the plates were dried, spots were quantified by an ELISpot reader.

3.5.3 Quantification of antigen-specific cytotoxic T-lymphocytes by tetramer or streptamer staining

Tetramer or streptamer staining was carried out 7 days later after the last immunization. The harvested splenocytes were resuspended and counted in 2-5 ml splenocytes washing buffer (section 2.8.2). Around $2x10^6$ cells per well were taken into a 96-well U-bottom plate and

then washed once with FACS buffer (section 2.8.2). The supernatant was discarded by decanting over sink and dry it on the tissue. Afterwards, 100 µl/well FcReceptor (FcR) mix was added to block the unspecific binding between FcR from immune cells and staining antibody. The FcR blocking was incubated at 4°C for 20 minutes. Before tetramer or streptamer staining, cells were washed once by FACS buffer.

Components of FcR mix (preparation for 9 samples and calculation for 10 samples)

5 µl RcR block (50 µl)

1 µl Hamster serum (10 µl)

1 µl Rat serum (10 µl)

93 µl FACS buffer (930 µl)

- **Tetramer staining**

Each sample was incubated with 10 µl iTAg Tetramer/APC-H-2 Kb OVA (SIINFEKL) for 30 minutes at room temperature protected from light. Then 180 µl FACS buffer were added to stop the staining. Cells were washed once by FACS buffer before staining with any additional antibodies.

- **Streptamer staining** (All steps have to be performed at 4°C!)

APC-conjugated H-2Db/ $E7_{49-57}$ streptamer was prepared by incubating 1 µl Strep-Tactin-APC and 0.8 µl MHC I-Strep in a final volume of 10 µl FACS buffer (1 sample amount) at dark for 45 minutes. Then, the splenocytes were suspended in 10 µl of pre-mixed Streptamer for another 45 minutes at dark. Before staining of cell surface marker, cells were washed once by FACS buffer.

CD8-PE antibody (# sc-53473 PE) and Live/dead dye (# L34959) were diluted 1:00 and 1:1000 respectively in FACS buffer and the cells were incubated by the staining solution at 4°C for 40 minutes to 1 hour in the dark. Subsequently, the cells were washed once by FACS buffer and resuspended in 200 µl Fixation buffer at 4°C for at least 1 hour and maximum 24 hours. Flow cytometry was applied to analyze the staining results.

3.5.4 Quantification of antigen-specific cytokine production by intracellular cytokine staining

Intracellular cytokine staining was performed 7 days later after the last immunization. The harvested splenocytes were resuspended and counted in 2-5 ml splenocytes washing buffer (section 2.8.2). The compensation group (single staining) and experimental group (mix staining) were designed as follows. Around $2x10^6$ cells per well were taken into a 96-well U-bottom plate and then washed once with FACS buffer (section 2.8.2).

Intracellular staining working panel

Single staining (compensation)	Splenocytes mix	unstaining	IFN-γ	CD4	CD8	Live/dead
Mix staining	Splenocytes of mouse 1	Medium	PMA and Ionomycin	Peptide		
	Splenocytes of mouse 2	Medium	PMA and Ionomycin	Peptide		
	Splenocytes of mouse 3	Medium	PMA and Ionomycin	Peptide		

Figure 10. Intracellular cytokine staining working panel. The single staining is used for compensation. The mix staining is for experimental groups.

Afterwards, the splenocytes were incubated with 'Golgistop' medium (supplemented medium plus Brefeldin A, # 554724) containing medium only (negative control), PMA/ionomycin (positive control or IFN-γ single staining, red color) or peptide of interest. After 6 hours incubation, cells were washed once by FACS buffer and stained with 1:100 FITC Rat Anti-Mouse CD4 (# 553047), 1:100 PE Rat Anti-Mouse CD8a (# 553033) and 1:1000 Live/dead dye (# L34959) according to the working panel. The staining was kept on ice for 45 minutes protected from light. 150 µl FACS buffer was added to stop staining and followed by 200 µl buffer for washing. Then cells were resuspended in 50 µl fixation buffer (cytofix/cytoperm, # 554722) and incubated in dark at 4°C for 20 minutes or overnight. Before staining with APC Rat Anti-Mouse IFN-γ (1:100 dilution in perm/wash, # 562018), cells were washed twice by 150 µl perm/wash. Subsequently, cells were incubated on ice for 45 minutes to 1 hour and 150 µl perm/wash were added for FACS analysis.

3.5.5 Tumor regression assay

Tumor regression assays were performed to illustrate the therapeutic potentials of prophylactic and therapeutic function combined vaccine PfTrx-$L2_{20-38}$ 8mer-(flank $E7_{49-57})_{3X}$-OVX313.

Tumor inoculation was carried out when mice were under anesthesia by inhalating isoflurane. The 6-week old C57BL/6N mice were first shaved on their right flank. Appropriate amount of TC-1 cells in 100 µl PBS was injected slowly using 27G ½ (0.4×13mm) needle on the place where was shaved. The growth of the tumor was checked three or four days later after tumor inoculation. When tumor showed a clearly measurable size, half of the tumor bearing animals were immunized at the base of the tail subcutaneously with 20 µg PfTrx-$L2_{20-38}$ 8mer-(flank $E7_{49-57})_{3X}$-OVX313 adjuvanted with 50% (v/v) AddaVax and two doses of the vaccine were received with 5 days apart. The rest of tumor mice were used as negative control, either without any vaccination or immunized with protein PfTrx-$L2_{20-38}$ 8mer-OVX313. When the tumor of vaccinated mice was completely regressed, half of the tumor regression animals were re-challenged with more TC-1 cells to detect the memory T cell responses induced by the vaccine. Tumor volume was measured with a digital caliper every 3 or 4 days. Mice were excluded from the experiment when the tumor volume was exceeded cm^3 or the tumor diameter was over 1.5 cm.

3.5.6 Statistical analysis

Statistical significance was calculated with the nonparametric Mann-Whitney test. $P < 0.05$ was considered statistically significant.

4. Results

Persistent infection of high risk HPV types is a causal factor for developing anogenital cancers (Durst et al, 1983; zur Hausen, 1996; zur Hausen, 2009). Especially, almost all cervical cancers are related to HPV infection, mostly HPV16 and HPV18. HPV prophylactic vaccines are available currently, but they are essentially limited for prevention of HPV infection in uninfected population. After population exposure to HPV, prophylactics seem to be powerless (Kumar et al, 2015; Wang & Roden, 2013). Moreover, since there are no anti-HPV drugs available, an effective strategy should be the therapeutic vaccination to eliminate HPV-transformed cells by the activated immune system. Also, given that the coverage of prophylactic HPV vaccines is incomplete worldwide, and the inadequate diagnosis of HPV infection exists in less developed regions, a prophylactic and therapeutic combined vaccine would be a vital requirement. This is also the aim of my PhD project. There are some benefits of a prophylactic/therapeutic combined vaccine over only therapeutic functional vaccine. Firstly, a combined vaccine can be used for both uninfected and infected populations, which means that this kind of vaccine does not require a HPV infection screening when vaccinated. Secondly, a combined vaccine has a unique superiority over only therapeutics in post exposure prophylaxis. In the beginning of the HPV infection, there are not only virus infected cells, but also large amounts of residual viruses. In this scenario, the stimulated production of HPV neutralizing antibodies would be beneficial. Last but not least, a combined vaccine will shield the recovered individuals of HPV-related diseases from further HPV re-infections.

4.1 PfTrx is able to induce CD8+ cytotoxic T cell responses

Pyrococcus furiosus thioredoxin (PfTrx) has been demonstrated to be an excellent carrier for heterologous antigens (Canali et al, 2014; Seitz et al, 2014). It presents a highly thermostable scaffold with a large capacity to accept insertion into its active center. Inserted sequences are restrained by flanking cysteine residues that are forming intramolecular disulfide bonds. We already proved that PfTrx-L2 as an antigen can induce B cells to secrete antibodies neutralizing HPV. Moreover, by PfTrx overlapping peptide-screening (Twenty-four 20mer-peptides in total with 12 amino acid overlap), we found CD4+ T cell epitopes ($H2^d$) within the Trx scaffold. So we supposed that PfTrx-L2 could promote the activation/induction of B cells through APC and T-helper cells (Figure 11).

Can PfTrx also induce CD8+ cytotoxic T cell (CTL) responses? To answer this question, we inserted a well-known CD8 epitope, Ovalbumin CTL epitope (SIINFEKL, $H2K^b$ restricted) into our PfTrx scaffold to test if this '$PADRE_{2X}$-PfTrx-$(OVA_{257-264})_{3X}$' antigen can stimulate CTL responses (Figure 11).

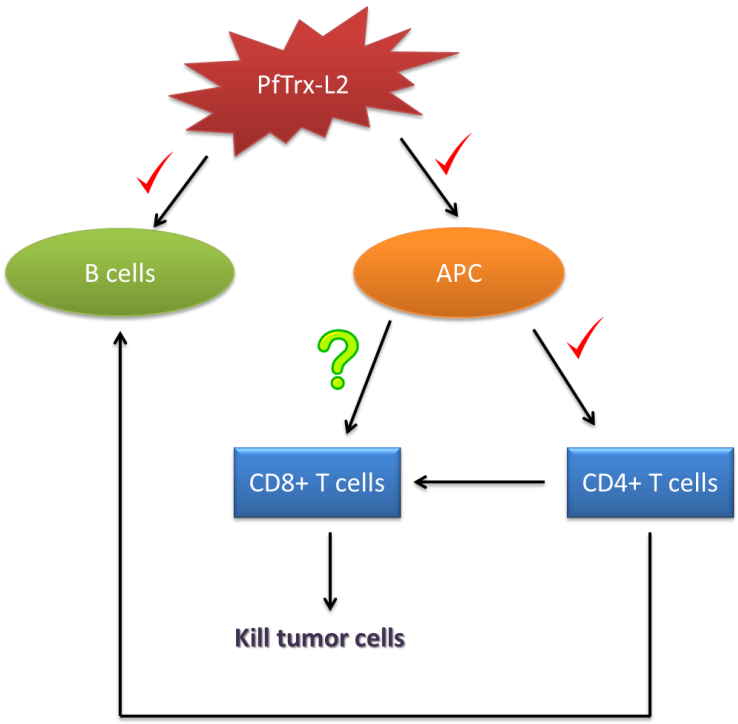


Figure 11. PfTrx-L2 can induce B cell responses. Are CD8+ responses induced as well? Our previous data showed that PfTrx can induce B cell responses. Here we will study if PfTrx can also induce CTL responses.

4.1.1 PfTrx induces CD8+ cytotoxic T cell responses with $OVA_{257-264}$ as a CTL epitope

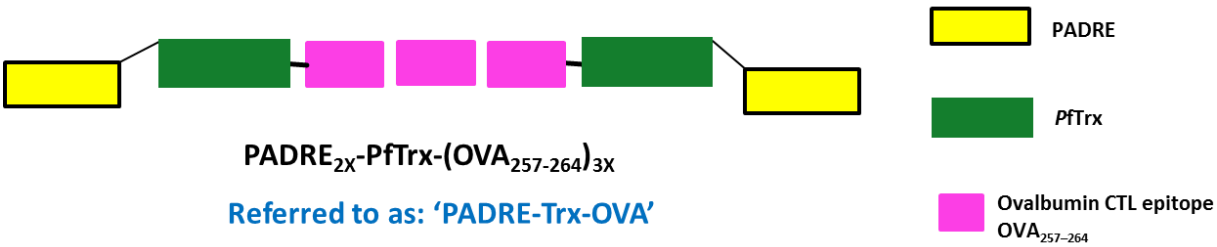


Figure 12. The construct of 'PADRE-Trx-OVA' antigen. PADRE: pan HLA DR-binding epitope. It is a T-helper epitope and recognized by both humans (HLA-DR) and mice ($I\text{-}A^b$) (Alexander et al, 1994; Jemon et al, 2013). PfTrx: the thioredoxin is from *Pyrococcus furiosus* and it is a highly thermostable scaffold with a large capacity to accept insertion into its active center. The thioredoxin we used in all antigens is PfTrx, so we normally write Trx instead of PfTrx as an abbreviation. $OVA_{257–264}$: it is ovalbumin (OVA)-derived CTL epitope (SIINFEKL, $H\text{-}2K^b$ restricted).

PADRE-Trx-OVA antigen was expressed in BL21 and then purified by nickel affinity chromatography. The coomassie blue stained SDS-PAGE (Figure 13) shows the purity and concentration (1.5 mg/ml according to the BSA indication) of the protein. Before immunization, the protein was detoxified by triton X-114.

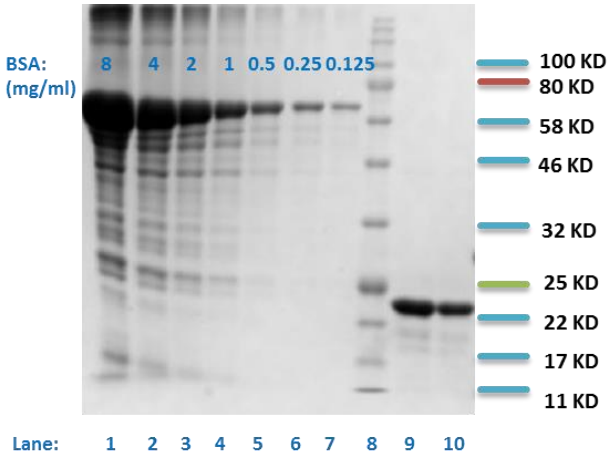


Figure 13. PADRE-Trx-OVA is purified by nickel affinity chromatography. The coomassie blue stained SDS-PAGE shows the purity and concentration of the protein. Before immunization, the protein was detoxified by twice triton X-114 treatment at 37 °C for 5 minutes and following a phase separation. Lane 1 to 7 shows the serial dilution of BSA which is used for determination of protein concentration. Lane 8 is the marker and sizes are indicated on the right side. Lane 9 is protein PADRE-Trx-OVA (22.7 KD) and Lane 10 is 1:2 dilution of it.

IFN-γ ELISpot was used to evaluate CD8+ cytotoxic T cell responses stimulated by Trx. The mice (3 per group) were immunized with either $OVA_{257-264}$ and PADRE peptide mixture, or protein PADRE-Trx-OVA. The splenocytes were later obtained and stimulated by $OVA_{257-264}$ or PADRE peptide. Figure 14 shows that the antigen encompassing Trx as a scaffold induced more IFN-γ secreting OVA specific CD8+ T cells. For CD4+ T cell responses, peptides mixture or protein group stimulated comparable IFN-γ positive PADRE CD4+ T cells. These ELISpot results indicate that Trx can induce CD8+ cytotoxic T cell responses, which emboldened us to involve Trx in the design of prophylactic/therapeutic vaccines.

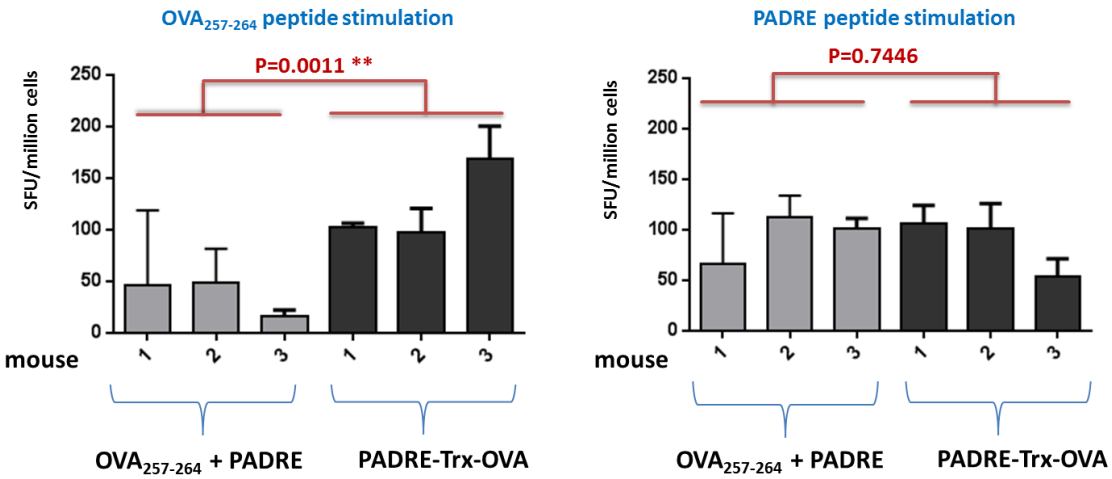


Figure 14. Antigen containing Trx as a scaffold can induce more anti-OVA T cell responses tested by IFN-γ ELISpot. The 6 to 8 week-old C57BL/6N female mice (3 per group) were immunized with 100 μg $OVA_{257-264}$ and 140 μg PADRE peptide mix or 20 μg protein PADRE-Trx-OVA subcutaneously. Both groups were adjuvanted with 50% (v/v) IFA (Incomplete Freund's adjuvant). Seven days later, the splenocytes were obtained and stimulated *in vitro* by $OVA_{257-264}$ or PADRE peptide. Red bar indicates P value.

4.1.2 PADRE-Trx-OVA can be formulated with human compatible adjuvant 'AddaVax'

We have shown that Trx can induce cytotoxic T cells responses when IFA used as adjuvant. Here we would like to study the induced responses when human compatible adjuvant 'AddaVax' applied. Mice were immunized with protein PADRE-Trx-OVA using different adjuvants: IFA, AddaVax, or AlumMPLA. The splenocytes were stimulated *in vitro* with the $OVA_{257-264}$ peptide. We can see from Figure 15 that PADRE-Trx-OVA formulated with AddaVax induces stronger OVA-specific CD8+ T cell responses compared to AlumMPLA. Moreover, AddaVax formulation even works better for induction of CD8+ T cell responses than IFA formulation. Therefore, AddaVax was chosen as adjuvant in our following protein vaccination experiments.

We compared the T cell responses induced by $OVA_{257-264}$ and PADRE peptide mix and antigen PADRE-Trx-OVA, with AddaVax adjuvant (Figure 16). Firstly, we employed IFN-γ ELISpot to test CD8+ cytotoxic T cell responses. Results shown in figure 16 (a) demonstrate that PADRE-Trx-OVA vaccination induces much more OVA specific CD8+ T cells than peptide mix, when AddaVax is utilized as an adjuvant. To further confirm this result, intracellular cytokine staining was following performed. The mice (3 per group) were immunized twice with $OVA_{257-264}$ and PADRE peptide mix, or PADRE-Trx-OVA. Afterwards, the activated splenocytes

were collected and stimulated by $OVA_{257-264}$ peptide, PMA/ionomycin (positive control) or only medium (negative control). The stimulated cells were later stained by fluorescence-conjugated monoclonal antibodies (anti-mouse-CD8+, anti-mouse-CD4+ and anti-mouse-IFN γ) and analyzed by flow cytometry. The percentage of IFN-γ secreting CD8+ T cells is shown in figure 16 (b). It is obvious that PADRE-Trx-OVA immunized mice generate more IFN-γ positive CD8+ T cells compared to peptide immunization.

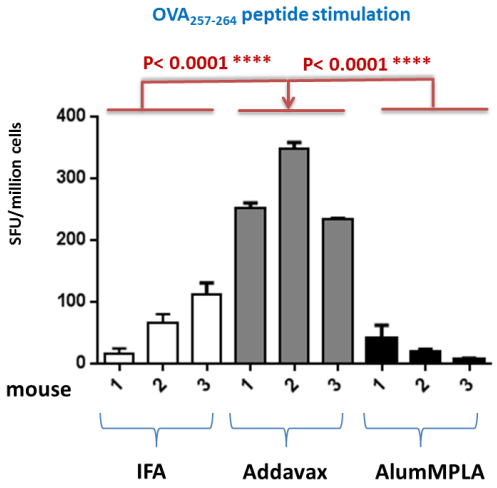


Figure 15. PADRE-Trx-OVA formulated with AddaVax induces stronger anti-OVA T cell responses compared to AlumMPLA adjuvanted. The 6 to 8 week-old C57BL/6N female mice (3 per group) were vaccinated with 20 μg PADRE-Trx-OVA formulated with 50% (v/v) IFA, AddaVax or AlumMPLA. Seven days later, the splenocytes were obtained, and IFN-γ ELISpot was performed with *in vitro* stimulation by $OVA_{257-264}$ peptide. Red bar indicates P value.

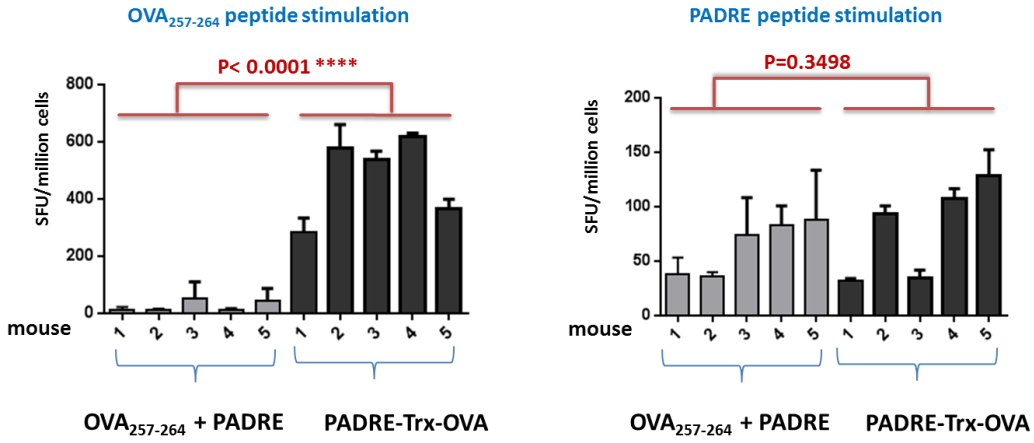


Figure 16 (a). Antigen comprising Trx and adjuvanted with AddaVax can induce a potent anti-OVA T cell response. The mice (5 per group) were immunized with 100 μg $OVA_{257-264}$ and 140 μg PADRE peptide mix or 20 μg protein PADRE-Trx-OVA. Both groups were adjuvant with AddaVax 50% (v/v). The splenocytes were *in vitro* stimulated by $OVA_{257-264}$ or PADRE peptide. Red bar indicates P value.

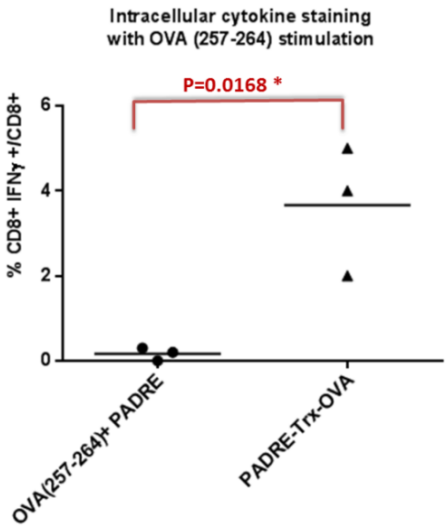


Figure 16 (b). Antigen comprising Trx and adjuvanted by AddaVax can induce more IFN-γ secreting CD8+ T cells shown by intracellular cytokine staining. The mice (3 per group) were immunized twice at biweekly intervals with 100 µg $OVA_{257-264}$ and 140 µg PADRE peptide mix, or 20 µg PADRE-Trx-OVA at the base of the tail subcutaneously. Both were adjuvanted with AddaVax 50% (v/v). Seven days later after the second immunization, the splenocytes were incubated with 'Golgistop' medium (supplemented medium plus Brefeldin A) containing $OVA_{257-264}$ peptide, PMA/ionomycin (positive control) or only medium (negative control). After 6 hours incubation, the cells were stained by fluorescence-conjugated monoclonal antibodies (anti-mouse-CD8+, anti-mouse-CD4+ and anti-mouse-IFN γ). The percentage of IFN-γ secreting CD8+ T cells was determined by flow cytometry. Each dot represents one mouse with the mean percentage (%CD8+ IFNγ+ / CD8+) showing by horizontal bars. P value is indicated in the figure.

4.1.3 PfTrx carrying the HPV $E7_{49-57}$ CTL epitope induces weak CD8+ cytotoxic T cell responses

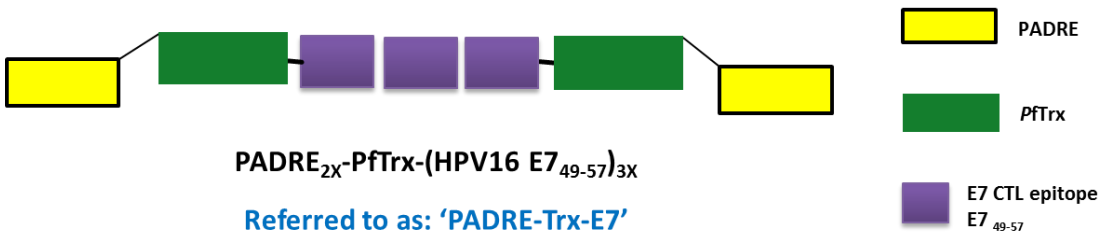


Figure 17. The 'PADRE-Trx-E7' antigen. HPV16 $E7_{49-57}$: it is HPV16 E7 derived CTL epitope (RAHYNIVTF, $H2D^b$ restricted) (Feltkamp et al, 1993).

Figure 18 demonstrates the purity of PADRE-Trx-E7 in coomassie blue stained SDS-PAGE. The protein was expressed in *E.coli* and purified by nickel affinity chromatography. Before immunization, the protein was detoxified by triton X-114 treatment.

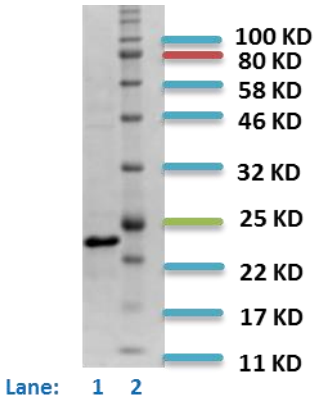
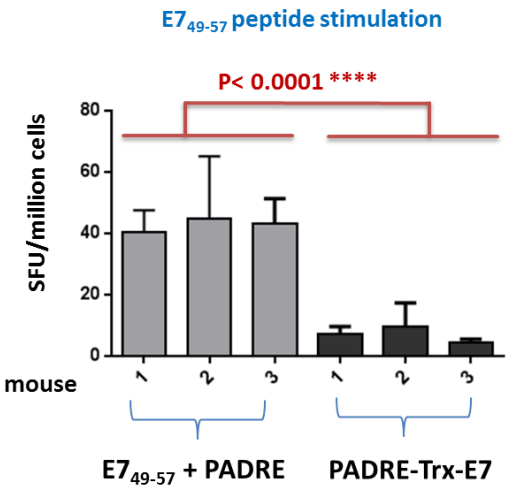


Figure 18. PADRE-Trx-E7 is purified by nickel affinity chromatography. Lane 1 indicates the purified protein (23.2 KD) shown in coomassie blue stained SDS-PAGE gel. Lane 2 is the marker and sizes are presented on the right.

Our previous data showed that cytotoxic T cell responses can be induced when famous CTL epitope $OVA_{257-264}$ was incorporated into Trx scaffold. Here we wanted to study T cell responses when human related CTL epitope HPV16 $E7_{49-57}$ was used instead of $OVA_{257-264}$. IFN-γ ELISpot was performed to evaluate E7-specific CD8+ cytotoxic T cell responses induced by PADRE-Trx-E7. The mice were immunized with HPV16 $E7_{49-57}$ and PADRE peptide mix, or PADRE-Trx-E7. The antigens were either formulated with IFA (Figure 19 (a)), or AddaVax (Figure 19 (b)). The splenocytes were stimulated with the HPV16 $E7_{49-57}$ peptide. As shown in figure 19 (a) and (b), the PADRE-Trx-E7 antigen cannot induce a significant E7-specific CD8+ T cell response. For peptide immunization, a weak but specific response was observed. This result inspired us to explore an appropriate HPV16 E7 CTL epitope.

(a) IFA as adjuvant



(b) AddaVax as adjuvant

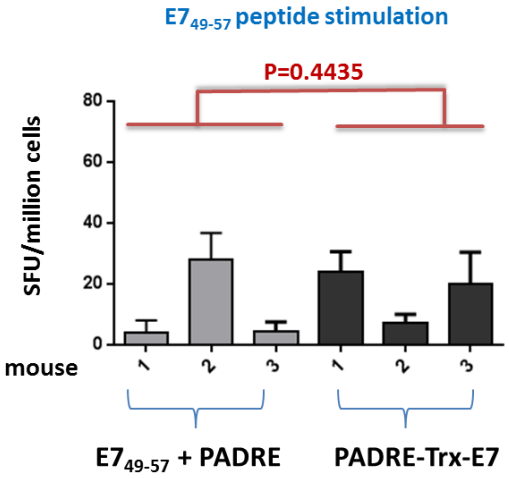


Figure 19. Antigen encompassing PfTrx as a scaffold and $E7_{49-57}$ as a CTL epitope cannot induce a significant E7-specific CD8+ T cell response as tested by IFN-γ ELISpot. The mice (3 per group) were immunized with 100 µg HPV16 $E7_{49-57}$ and 140 µg PADRE peptide mix, or 20 µg PADRE-Trx-E7. The antigens were either formulated with 50% (v/v) IFA (a), or 50% (v/v) AddaVax (b). The splenocytes were later stimulated with the HPV16 $E7_{49-57}$ peptide.

4.1.4 CD8+ cytotoxic T cell response is significantly enhanced if $E7_{49-57}$ is flanked by five E7-derived amino acids (QAEPDRAHYNIVTFCCKCD)

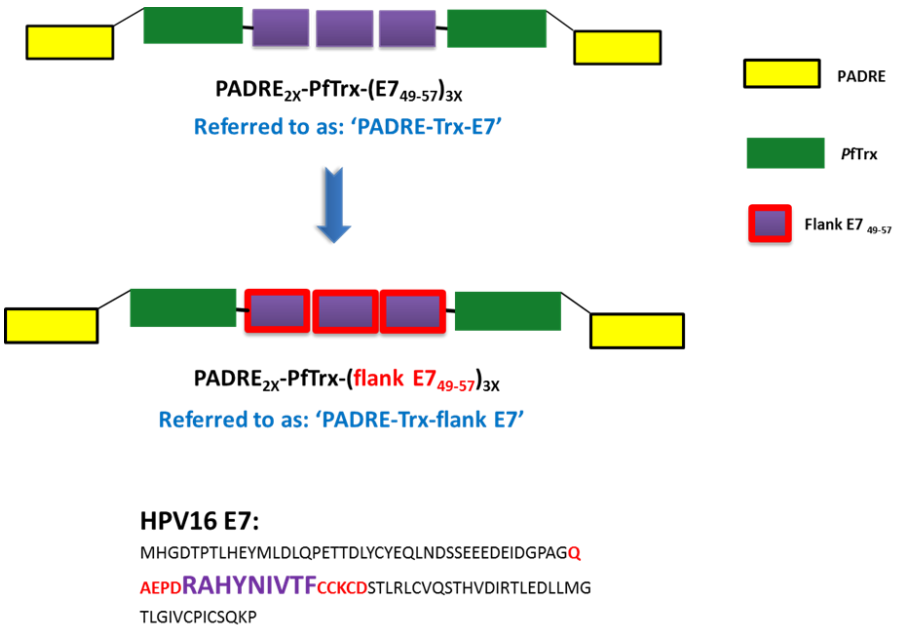


Figure 20. The construct of 'PADRE-Trx-flank E7' antigen. Flank $E7_{49-57}$: HPV16 $E7_{49-57}$ is flanked by 5 amino acids on both sides. The sequence RAHYNIVTF is extended to QAEPDRAHYNIVTFCCKCD.

Figure 21 demonstrates the purity of PADRE-Trx-flank E7 in coomassie blue stained SDS-PAGE. The operation of the protein (expression, purification and detoxification) is the same as that of PADRE-Trx-E7.

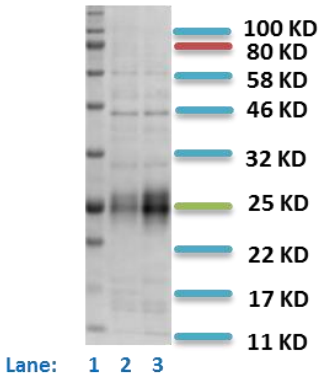


Figure 21. PADRE-Trx-flank E7 is purified by nickel affinity chromatography. The purity of the protein is shown in coomassie blue stained SDS-PAGE. Lane 1 demonstrates the marker and sizes are on the right side. Lane 2 and 3 are two fractions of protein PADRE-Trx-flank E7 (26.5 KD).

Since the T cell responses induced by PADRE-Trx-E7 were not significant, we extended the HPV16 E7 CTL epitope $E7_{49\text{-}57}$ (RAHYNIVTF) to flank $E7_{49\text{-}57}$ (QAEPDRAHYNIVTFCCKCD) to enhance the intracellular processing and presentation of the E7 T-cell epitope. And the immunogenicity of PADRE-Trx-E7 and PADRE-Trx-flank E7 was compared by IFN-γ ELISpot. Mice were immunized with HPV16 $E7_{49\text{-}57}$ and PADRE peptides adjuvanted with IFA (control group), or protein PADRE-Trx-E7 adjuvanted with AddaVax, or protein PADRE-Trx-flank E7 adjuvanted with AddaVax. The splenocytes were stimulated by HPV16 $E7_{49\text{-}57}$ peptide. We can see from figure 22 that CD8+ cytotoxic T cell response is significantly enhanced if flank $E7_{49\text{-}57}$ is used in Trx scaffold. Based on this conclusion, the epitope flank $E7_{49\text{-}57}$ instead of $E7_{49\text{-}57}$ is always applied in our subsequent vaccine designs.

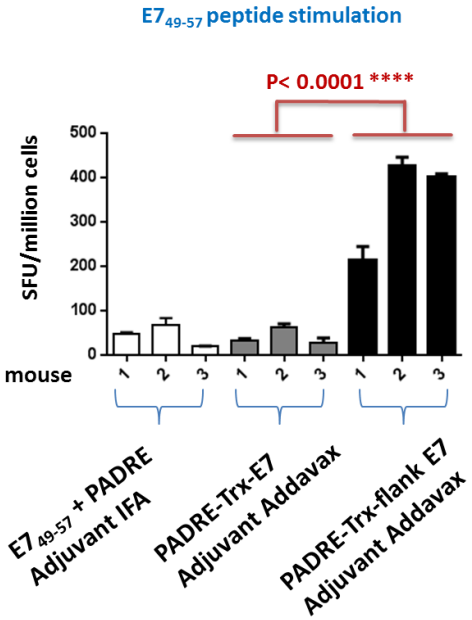
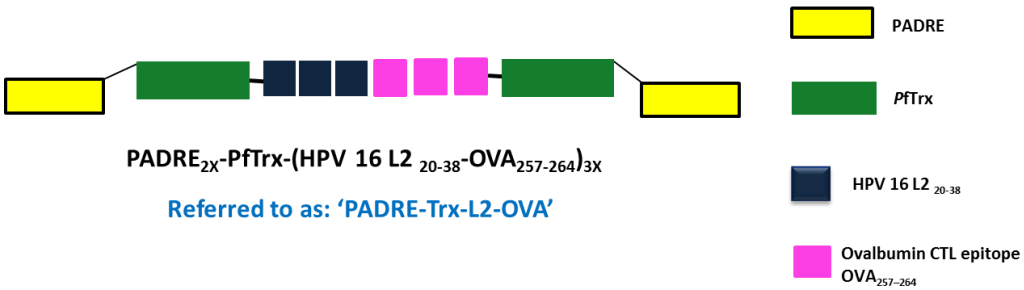


Figure 22. CD8+ cytotoxic T cell response is significantly enhanced if flank $E7_{49-57}$ used in PfTrx scaffold. The mice (3 per group) were immunized with 100 µg HPV16 $E7_{49-57}$ and 140 µg PADRE peptides formulated with 50% (v/v) IFA, or 20 µg PADRE-Trx-E7 formulated with 50% (v/v) AddaVax, or 20 µg PADRE-Trx-flank E7 formulated with 50% (v/v) AddaVax. Seven days later, the splenocytes were stimulated by HPV16 $E7_{49-57}$ peptide. Red bar indicates P value.

4.2 HPV prophylactic and therapeutic combined vaccines based on PfTrx scaffold

4.2.1 A monomeric vaccine contains B-cell epitope: L2 and T-cell epitope: OVA



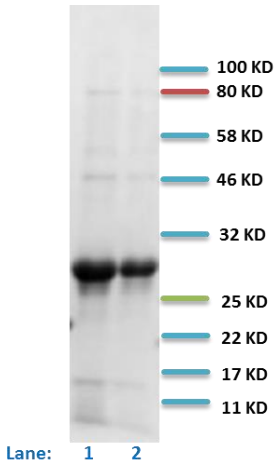


Figure 23. The design and purification of 'PADRE-Trx-L2-OVA' antigen. HPV16 L2 $_{20\text{-}38}$: the amino acid sequence is from 20 to 38 of HPV 16 L2 minor capsid protein, and our previous work proved that this part of L2 can induce HPV neutralizing antibodies efficiently. The protein is purified by nickel affinity chromatography and shown in coomassie blue stained SDS-PAGE. Lane 1 displays protein PADRE-Trx-L2-OVA (29.3 KD) and Lane 2 is one time dilution of it. The size is indicated by the marker on the right side.

As shown in the last section that Trx can be used in induction of cytotoxic T cell responses, and our previous work proved that Trx can stimulate antibody production. Here we want to incorporate both CTL epitope and B cell epitope into Trx scaffold to develop a combined vaccine. Figure 24 shows the strategy to study the T cell and B cell responses of the combined vaccine PADRE-Trx-L2-OVA. We have designed comparison antigens which contain either only T cell epitope (PADRE-Trx-OVA) or only B cell epitope (PADRE-Trx-L2). By comparison, we can determine if the existence of B cell epitope HPV16 L2 $_{20\text{-}38}$ influences anti-OVA T cell response or not, and vice versa.

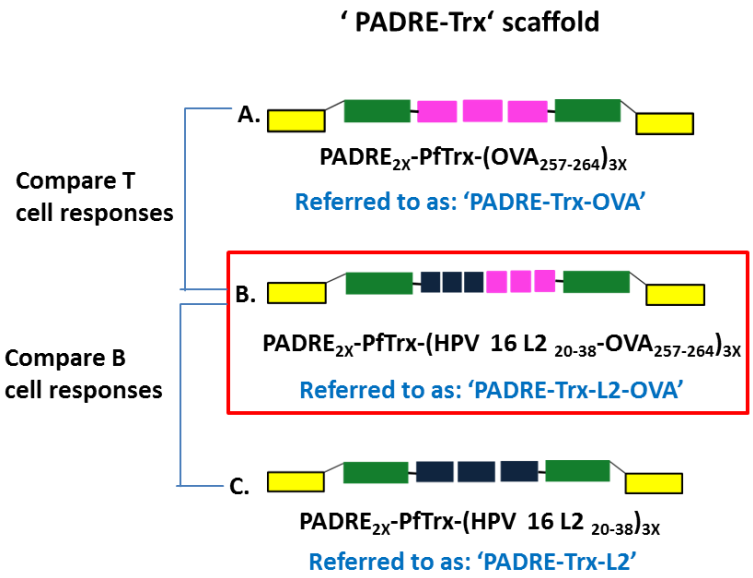
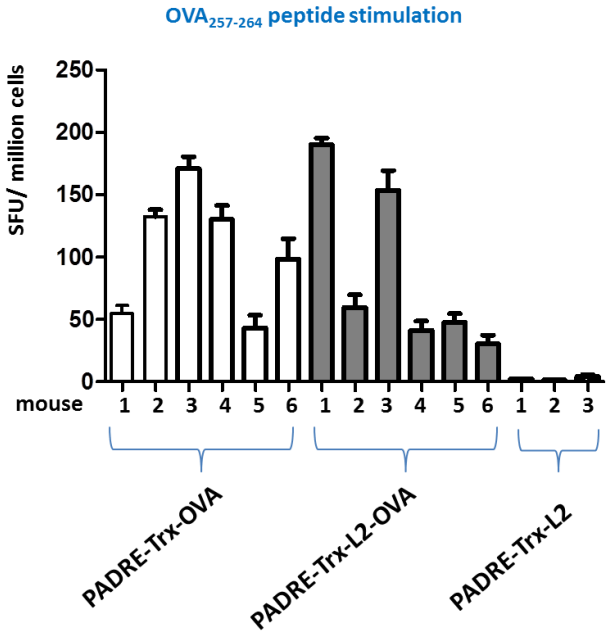


Figure 24. Strategy to study the T cell and B cell responses of combined vaccine PADRE-Trx-L2-OVA. Group A. Antigen PADRE-Trx-OVA contains only T cell epitope $OVA_{257-264}$. **Group B.** Antigen PADRE-Trx-L2-OVA contains both T cell epitope $OVA_{257-264}$ and B cell epitope HPV16 $L2_{20-38}$. **Group C.** Antigen PADRE-Trx-L2 contains only B cell epitope HPV16 $L2_{20-38}$. By Group A and B comparison, we can see the efficacy of T cell responses induced by combined vaccine. By Group B and C comparison, the B cell responses of combined vaccine can be explored.

- **PADRE-Trx-L2-OVA induces comparable anti-OVA T cell responses as PADRE-Trx-OVA**

In order to test the anti-OVA T cell responses induced by the combined vaccine, we compared the number of IFN-γ secreting T cells of PADRE-Trx-L2-OVA and PADRE-Trx-OVA through IFN-γ ELISpot (Figure 25). Mice were injected with PADRE-Trx-OVA, PADRE-Trx-L2-OVA or PADRE-Trx-L2 (negative control). The collected splenocytes were stimulated by $OVA_{257-264}$ peptide. Figure 25 (a) shows the anti-OVA T cell responses of each mouse after different antigens vaccination. Figure 25 (b) represents the average T cell responses of each vaccination group. We can see from Figure 25 (a) and (b) that the stimulated anti-OVA T cell responses of PADRE-Trx-L2-OVA are comparable to the T cell responses induced by PADRE-Trx-OVA, which means that the B cell epitope HPV16 $L2_{20-38}$ has no significant influence on anti-OVA T cell responses induced by the combined vaccine.

(a)



(b)

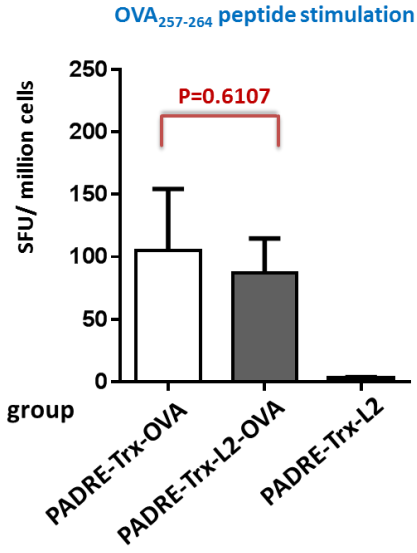
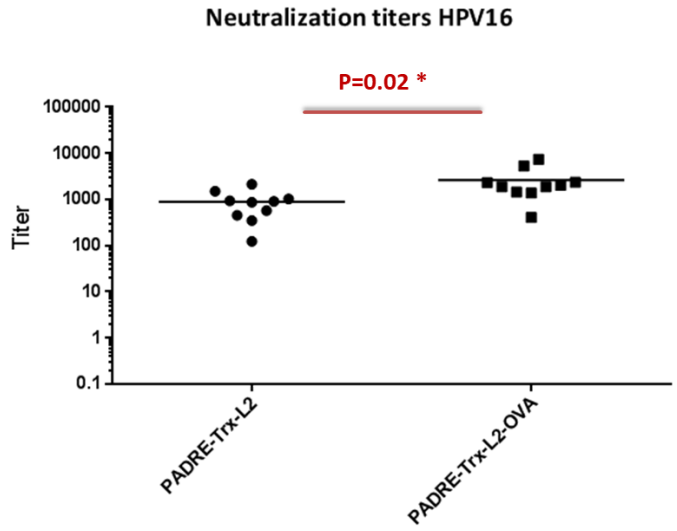


Figure 25. T cell and B cell epitope-combined antigen PADRE-Trx-L2-OVA can induce comparable anti-OVA T cell responses as PADRE-Trx-OVA. IFN-γ ELISpot was performed to compare the anti-OVA T cell responses induced by PADRE-Trx-OVA and PADRE-Trx-L2-OVA. We use antigen PADRE-Trx-L2 as the negative control. The mice (6 or 3 per group) were immunized with 20μg antigen PADRE-Trx-OVA, PADRE-Trx-L2-OVA or PADRE-Trx-L2. The splenocytes were stimulated by $OVA_{257-264}$ peptide. (a) shows the anti-OVA T cell responses of each mouse after immunization. (b) represents the average T cell responses of each vaccination group. Red bar indicates P value.

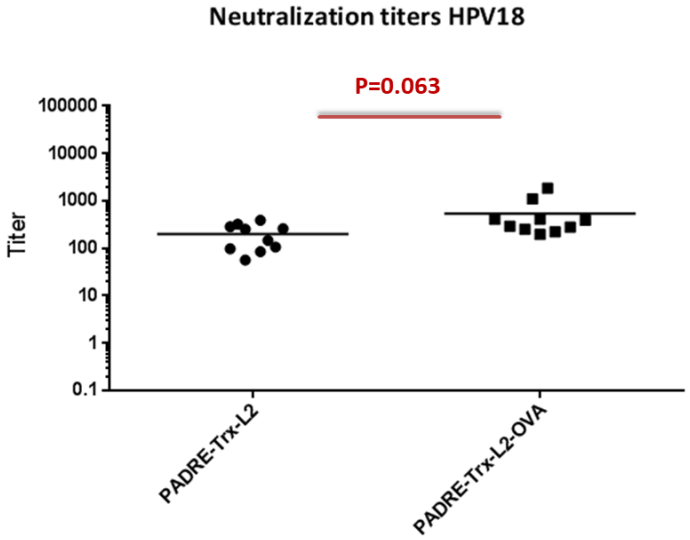
- **PADRE-Trx-L2-OVA induces comparable anti-HPV16 and anti-HPV18 neutralizing antibodies compared to the PADRE-Trx-L2**

We performed L1-PBNA (pseudovirion-based neutralization assay) against HPV 16 and HPV 18 pseudovirions to evaluate the B cell responses aroused by the combined vaccine. The BALB/c mice were injected 4 times with PADRE-Trx-L2 or PADRE-Trx-L2-OVA. Sera were collected one month after the last immunization and analyzed with L1-PBNA. The results (Figure 26) show that comparable antibody titers against HPV16 (P value 0.02) and 18 (P value 0.023) were induced no matter T cell epitope $OVA_{257-264}$ exists or not. This data indicates that $OVA_{257-264}$ has no negative impact on B cell responses induced by HPV16 L2 $_{20-38}$.

(a)


Neutralization titers HPV16
P=0.02 *
Titer
100000
10000
1000
100
10
1
0.1
PADRE-Trx-L2
PADRE-Trx-L2-OVA

(b)


Neutralization titers HPV18
P=0.063
Titer
100000
10000
1000
100
10
1
0.1
PADRE-Trx-L2
PADRE-Trx-L2-OVA

(C)

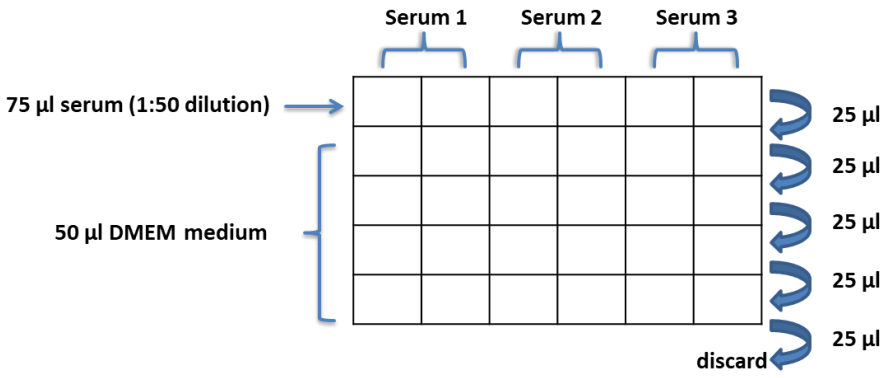

Serum 1
Serum 2
Serum 3
75 µl serum (1:50 dilution)
50 µl DMEM medium
25 µl
25 µl
25 µl
25 µl
25 µl
discard

Figure 26. Antigen PADRE-Trx-L2-OVA involving both B-cell and T-cell epitopes produces comparable anti-HPV16 and anti-HPV18 neutralizing antibody titers compared to PADRE-Trx-L2. Ten BALB/c mice per group were immunized intramuscularly 4 times at biweekly intervals with 20 µg of PADRE-Trx-L2 or PADRE-Trx-L2-OVA formulated with 50% (v/v) AddaVax. Sera were collected from mice one month after the last immunization and analyzed against HPV 16 and HPV18 pseudovirions using the L1-PBNA. The sera were started with a 1:50 dilution and then titrated in 1:3 series as an example in (C). The neutralization titer is defined as the reciprocal of the maximum dilution in which case 50% pseudovirions can be still neutralized (IC50). The HPV16 and HPV18 neutralization titers from group PADRE-Trx-L2 and PADRE-Trx-L2-OVA are shown in (a) and (b), respectively**.** Each dot indicates one mouse with the mean titers showing by horizontal bars. P value is indicated in the figure.

4.2.2 A heptameric vaccine contains B-cell epitope: 8mer and T-cell epitope: OVA

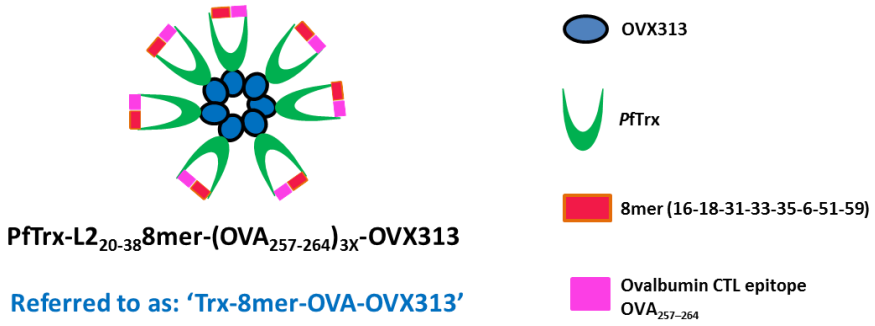


Figure 27. The design and purification of 'Trx-8mer-OVA-OVX313' antigen. OVX313: Chimeric version of Avian C4b-binding protein which was shown to lack homology to human C4bp. It assembles spontaneously into a heptameric structure resulting in displaying the fused antigens seven times. $L2_{20-38}$ 8mer: the amino acid sequences are from 20 to 38 of L2 of 8 different HPV types (16-18-31-33-35-6-51-59).

Figure 28 shows the purity of Trx-8mer-OVA-OVX313 in coomassie blue stained SDS-PAGE. The protein was isolated by thermal purification and detoxified by TritonX-114.

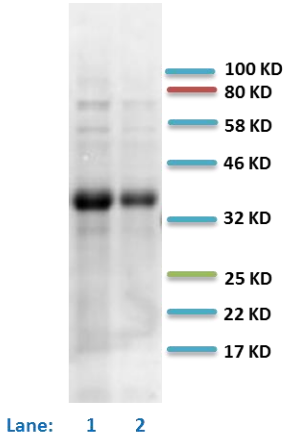


Figure 28. Trx-8mer-OVA-OVX313 is isolated by thermal purification. The protein was expressed in BL21 and isolated by thermal purification with 0.5M sodium chloride in 70°C for 40min. Lane 1 is Trx-8mer-OVA-OVX313 (39 KD) shown in coomassie blue stained SDS-PAGE and Lane 2 is 1:2 dilution of it. The size is indicated by marker on the right.

We have developed monomeric T-cell epitope and B-cell epitope combined vaccine. Here we used a heptameric platform OVX313 expecting an improved immunogenicity. As controls (Figure 29) we designed two antigens which contain either only T cell epitope Trx-OVA-OVX313 or only B cell epitope Trx-8mer-OVX313 to indicate the effectiveness of T cell and B cell responses derived from combined vaccine Trx-8mer-OVA-OVX313.

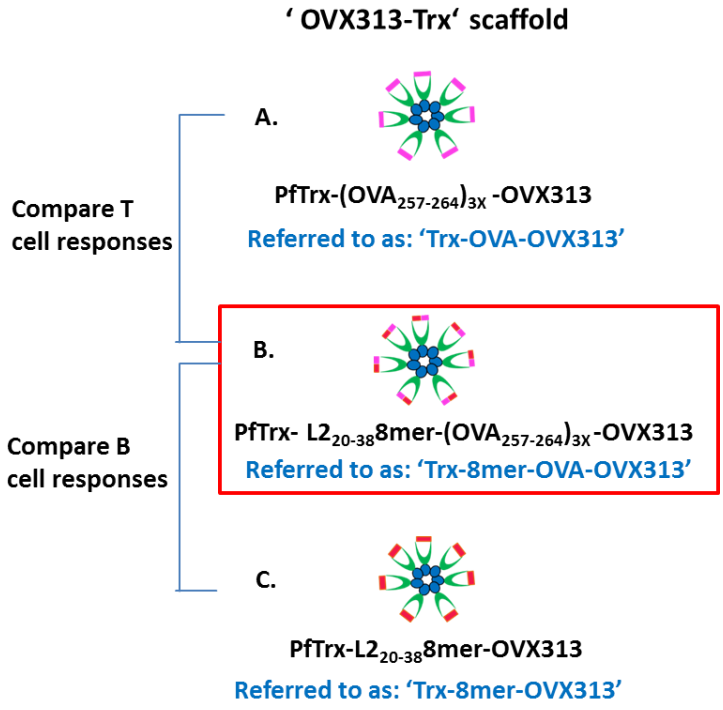


Figure 29. Antigen design to determine the T cell and B cell responses induced by combined vaccine Trx-8mer-OVA-OVX313. Group A. Trx-OVA-OVX313 antigen contains only the T cell epitope $OVA_{257-264}$. **Group B.** Trx-8mer-OVA-OVX313 antigen contains both the T cell epitope $OVA_{257-264}$ and the B cell epitope L2 $_{20-38}$8mer. **Group C.** Trx-8mer-OVX313 antigen contains only the B cell epitope L2 $_{20-38}$8mer. By Group A and B comparison, Group B and C comparison, we can acquire the cellular and humoral immunogenicity of the combined heptameric vaccine.

- **Trx-8mer-OVA-OVX313 induces stronger anti-OVA T cell responses compared to Trx-OVA-OVX313**

The induced anti-OVA T cell responses of the combined vaccine can be illustrated by comparison with Trx-OVA-OVX313 in IFN-γ ELISpot (Figure 30). Trx-8mer-OVX313 was used as a negative control. Mice were immunized once and 7 days later the splenocytes were stimulated by $OVA_{257-264}$ peptide. Figure 30 shows that the combined vaccine Trx-8mer-OVA-OVX313 induces three fold OVA-specific T cell responses compared to Trx-OVA-OVX313. This result indicates that the B cell epitope $L2_{20-38}$8mer has a positive influence on the OVA-specific T cell responses induced by the combined vaccine.

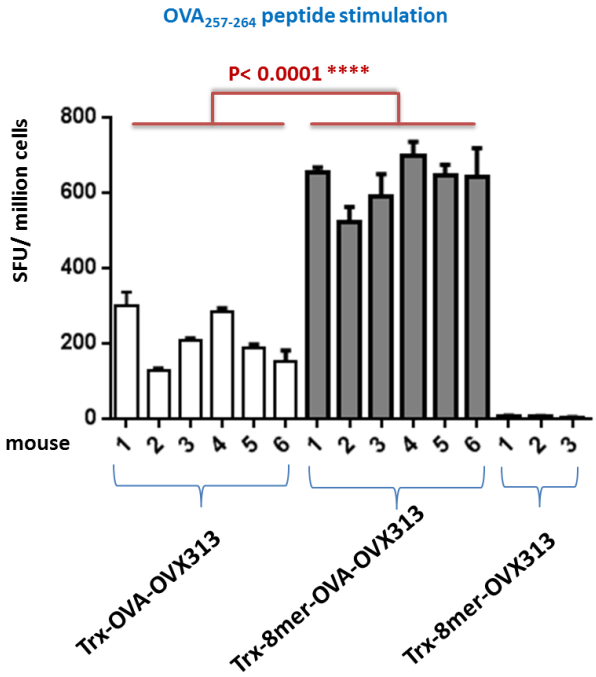


Figure 30. T cell and B cell epitope-combined vaccine Trx-8mer-OVA-OVX313 induces stronger anti-OVA T cell responses compared to Trx-OVA-OVX313. IFN-γ ELISpot was performed to compare the anti-OVA T cell responses induced by Trx-8mer-OVA-OVX313 and Trx-OVA-OVX313. Trx-8mer-OVX313 works as a negative control. The mice (6 or 3 per group) were immunized with 20 μg antigen Trx-OVA-OVX313, Trx-8mer-OVA-OVX313 or Trx-8mer-OVX313, and 7 days later the splenocytes were stimulated with $OVA_{257-264}$ peptide. Red bar indicates P value.

We not only used the $OVA_{257-264}$ peptide to stimulate splenocytes *in vitro*, EG7 cells (OVA expressing derivatives of the EL4 cell line) were also applied to evaluate anti-OVA T cell responses activated by combined vaccine Trx-8mer-OVA-OVX313 in IFN-γ ELISpot. Three mice were immunized once with the combined vaccine. The splenocytes were stimulated by either EG7 cells or EL4 cells (as the negative control). We can see from Figure 31 that the IFN-

γ was secreted when the splenocytes were stimulated with EG7 cells compared to the results after EL4 stimulation. The data further shows that the anti-OVA T cell responses are effectively induced when mice were vaccinated with the Trx-8mer-OVA-OVX313 antigen.

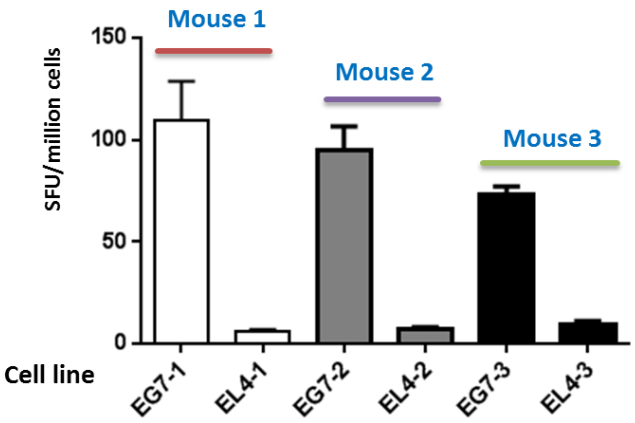
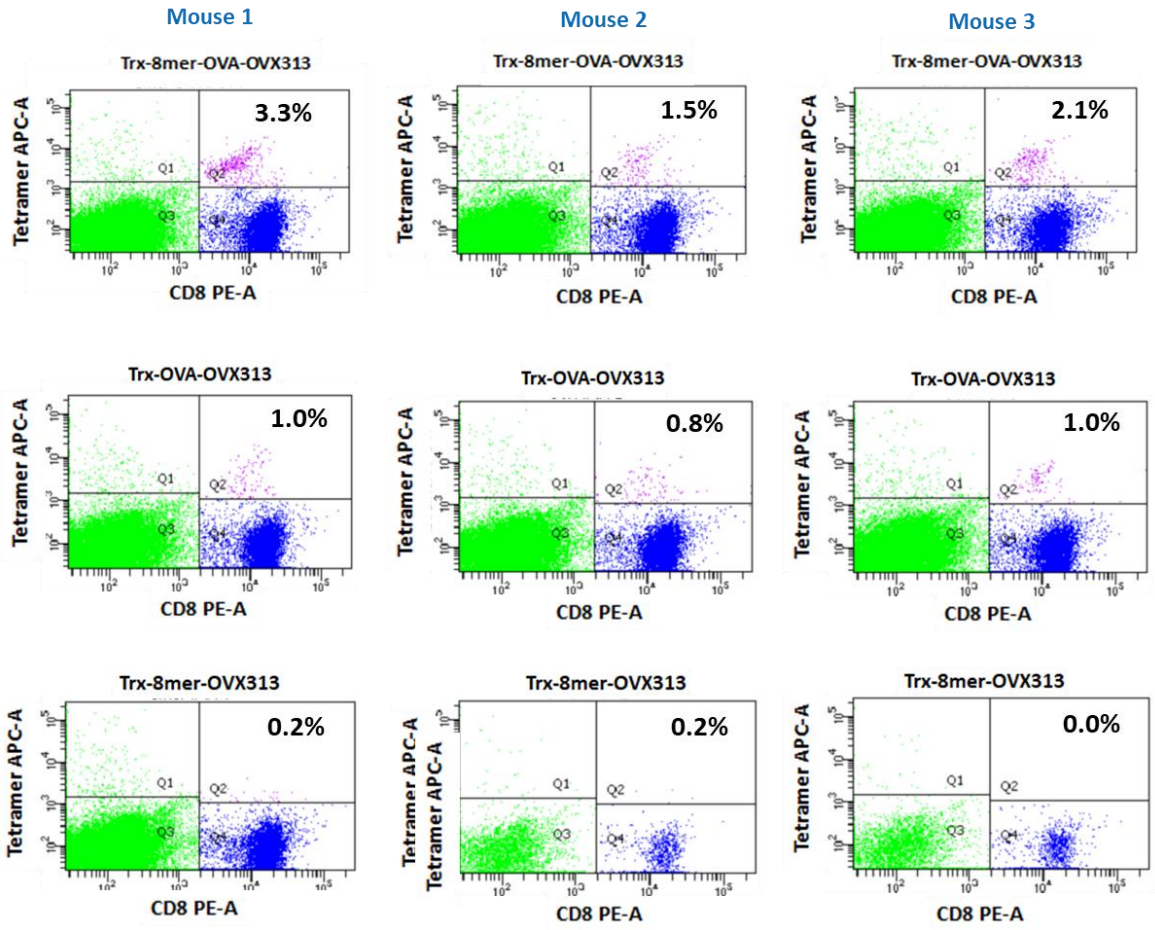


Figure 31. Induced OVA-specific T cells can recognize EG7 cells. EG7 cells or EL4 cells (as the negative control) were used to stimulate the splenocytes *in vitro* in IFN-γ ELISpot.

To further evaluate the immunogenicity of the antigen Trx-8mer-OVA-OVX313, we quantified OVA-specific CD8+ T cells by flow cytometry using H-2Kb/$OVA_{257-264}$ (SIINFEKL) tetramers. We immunized three mice per group twice with Trx-8mer-OVA-OVX313, Trx-OVA-OVX313 or Trx-8mer-OVX313 (as negative control). One week after the last immunization, the splenocytes were stained with anti-CD8-PE and APC-conjugated H-2Kb/$OVA_{257-264}$ tetramer. We can see that after Trx-8mer-OVA-OVX313 immunization, 3.3%, 1.5% and 2.1% OVA-specific CD8 T cells were detected among the total CD8 T cell population, respectively, compared to 1%, 0.8% and 1% of Trx-OVA-OVX313 immunized mice (Figure 32 (a)). The percentage of OVA-specific CD8 T cells from combined vaccine stimulation is around two to three folds of only T cell epitope comprising vaccine (Figure 32 (b)). This result is consistent with the data from IFN-γ ELISpot.

(a)



(b)

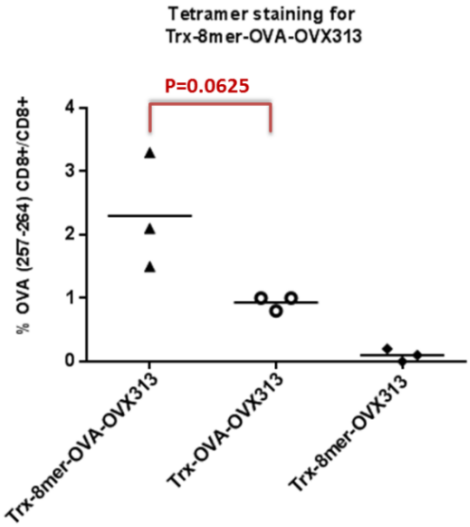


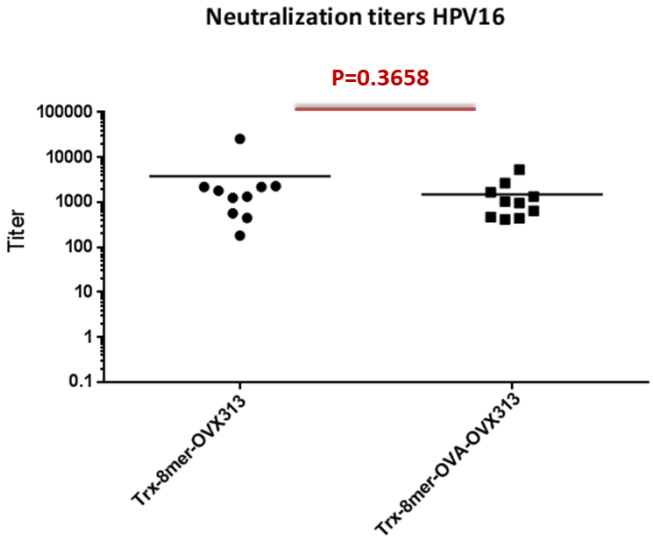
Figure 32. Trx-8mer-OVA-OVX313 induces more OVA-specific CD8 T cells than Trx-OVA-OVX313 does. We evaluated OVA-specific CD8+ T cells by flow cytometry using H-2Kb/$OVA_{257-264}$ (SIINFEKL) tetramers. We immunized three mice per group twice at weekly intervals with 20μg Trx-8mer-OVA-

OVX313, Trx-OVA-OVX313 or Trx-8mer-OVX313 (as negative control). One week after the last immunization, the splenocytes were stained with anti-CD8-PE and APC-conjugated H-2Kb/$OVA_{257-264}$ tetramer. The FACS data are shown in (a). The purple dots represent the OVA-specific CD8+ T cells. The analyzed data are represented in (b). Each dot (triangles, circles or diamonds) indicates one mouse, with the mean percentage (OVA (257-264)-CD8+/CD8+) showing by horizontal bars.

- **Trx-8mer-OVA-OVX313 induces comparable anti-HPV16 and anti-HPV18 neutralizing antibodies compared to the Trx-8mer-OVX313**

We performed L1-PBNA against HPV 16 and HPV 18 pseudovirions to evaluate the anti-L2 B cell responses stimulated by the combined vaccine (Figure 33). BALB/c mice were injected 4 times with Trx-8mer-OVX313 and Trx-8mer-OVA-OVX313. Sera were collected one month later after the last immunization and analyzed for presence of HPV 16 and HPV18-specific neutralizing antibodies using the L1-PBNA. Figure 33 illustrates that the combined vaccine Trx-8mer-OVA-OVX313 performs almost as efficient as vaccine Trx-8mer-OVX313 regarding the produced antibodies against HPV16 (P value 0.5288) and 18 (P value 0.1431). This also indicates that concerning the heptameric OVX313 scaffold, T cell epitope $OVA_{257-264}$ has little negative influence on B cell responses induced by $L2_{20-38}$8mer.

(a)



(b)

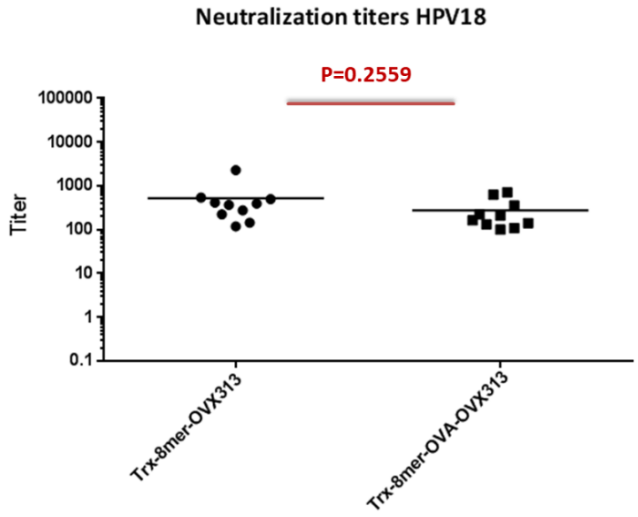


Figure 33. Heptameric antigen Trx-8mer-OVA-OVX313 produces comparable anti-HPV16 and anti-HPV18 neutralizing antibodies compared to the antigen Trx-8mer-OVX313. Ten BALB/c mice per group were immunized intramuscularly 4 times at biweekly intervals with 20 μg of Trx-8mer-OVX313 and Trx-8mer-OVA-OVX313 formulated with 50% (v/v) AddaVax. Sera were collected from mice one month after the last immunization and analyzed against HPV 16 and HPV18 pseudovirions using the L1-PBNA. The sera were started with a 1:50 dilution and then titrated in 1:3 series. The neutralization titer is defined as the reciprocal of the maximum dilution in which case 50% pseudovirions can be still neutralized (IC50). The HPV16 and HPV18 neutralization titers from Trx-8mer-OVX313 and Trx-8mer-OVA-OVX313 immune sera are shown in (a) and (b)**.** Each dot indicates one mouse with the mean titers showing by horizontal bars. P value is indicated in the figure.

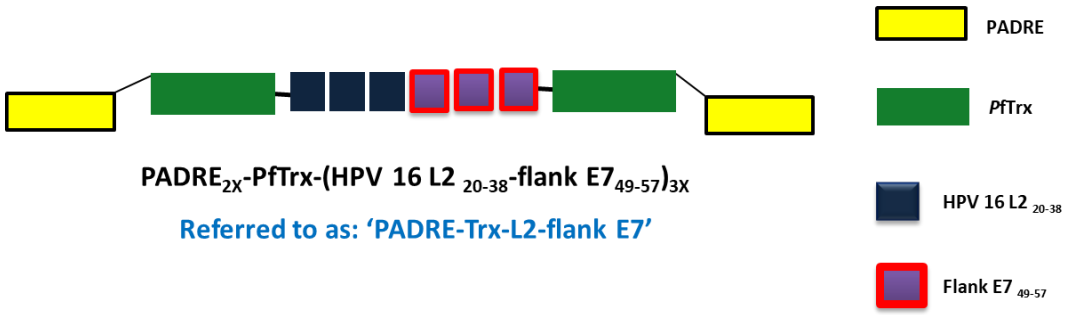
We successfully developed B cell and T cell responses combined monomeric vaccine PADRE-Trx-L2-OVA and heptameric vaccine Trx-8mer-OVA-OVX313. These results encouraged us to further develop the HPV prophylactic and therapeutic combined vaccines based on HPV $L2_{20-38}$ and flank $E7_{49-57}$.

4.2.3 Development of monomeric and heptameric vaccines comprising human related epitopes: 8mer and flank E7

Based on the promising results from PADRE-Trx-L2-OVA and Trx-8mer-OVA-OVX313, we further designed monomeric vaccine PADRE-Trx-L2-flank E7 and heptameric vaccine Trx-8mer-flank E7-OVX313 comprising human related epitopes $L2_{20-38}$ and flank $E7_{49-57}$. Both proteins were expressed in BL21. PADRE-Trx-L2-flank E7 was purified by nickel affinity

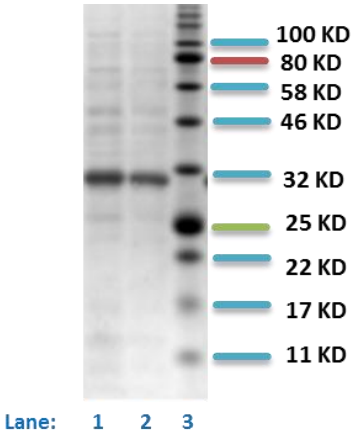
chromatography, and Trx-8mer-flank E7-OVX313 was isolated by thermal purification. Both antigens were detoxified before immunization.

(a)

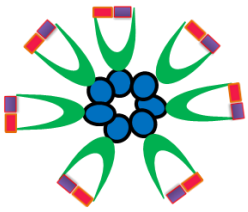


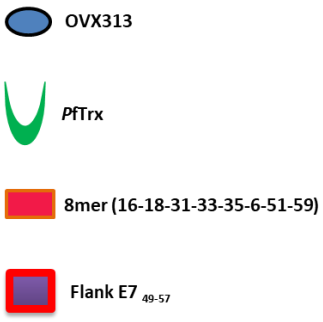
$PADRE_{2X}$-PfTrx-(HPV 16 $L2_{20-38}$-flank $E7_{49-57})_{3X}$

Referred to as: 'PADRE-Trx-L2-flank E7'



(b)





PfTrx-$L2_{20-38}$8mer-(flank $E7_{49-57})_{3X}$ -OVX313

Referred to as: 'Trx-8mer-flank E7-OVX313'

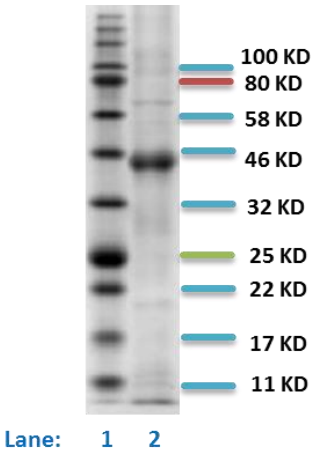
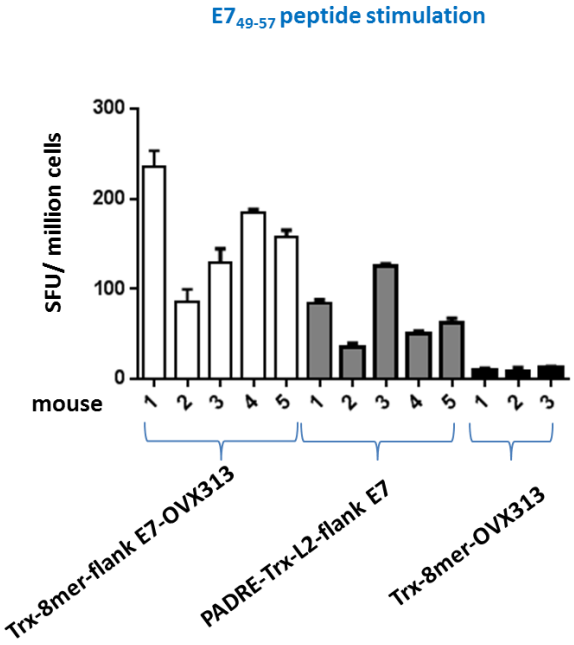


Figure 34. The design and purification of vaccine PADRE-Trx-L2-flank E7 and Trx-8mer-flank E7-OVX313. The proteins are shown in coomassie blue stained SDS-PAGE. (a) shows the structure and purity of PADRE-Trx-L2-flank E7. Lane 1 and 2 are two fractions of the protein (33 KD). The marker is in Lane 3 with the indication of sizes on the right. (b) presents the structure and purity of Trx-8mer-flank E7-OVX313. Lane 1 is the marker and Lane 2 is the protein (42.8 KD).

- **Heptameric antigen Trx-8mer-flank E7-OVX313 induces stronger anti-E7 T cell responses compared to monomeric antigen PADRE-Trx-L2-flank E7**

We compared the anti-E7 T cell responses induced by PADRE-Trx-L2-flank E7 and Trx-8mer-flank E7 -OVX313 by IFN-γ ELISpot (Figure 35). The mice were immunized once with Trx-8mer-flank E7-OVX313, PADRE-Trx-L2-flank E7 or Trx-8mer-OVX313 (as negative control). The splenocytes were stimulated with $E7_{49-57}$ peptide. Figure 35 (a) indicates the anti-E7 T cell responses of each mouse after different antigens vaccination. Figure 35 (b) represents the average T cell responses of each vaccination group. We can see that the heptameric antigen induces stronger T cell responses than the monomeric antigen (Figure 35 (a) (b)). As a next step we therefore investigated the B cell responses of the heptameric one.

(a)



(b)

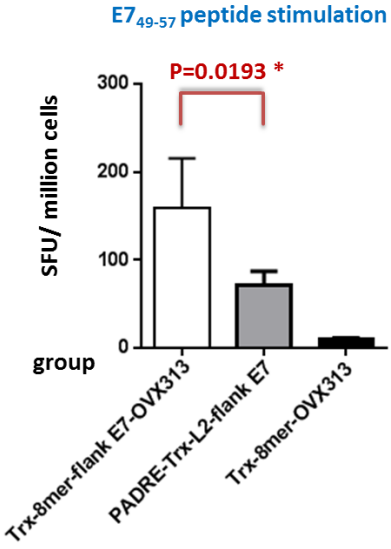


Figure 35. Heptameric antigen Trx-8mer-flank E7-OVX313 induces stronger anti-E7 T cell responses than monomeric antigen PADRE-Trx-L2-flank E7. IFN-γ ELISpot was performed to compare the anti-E7 T cell responses induced by Trx-8mer-flank E7 -OVX313, PADRE-Trx-L2-flank E7 or Trx-8mer-OVX313 (negative control). The mice (5 or 3 per group, respectively) were immunized once with 20 μg antigens. Seven days later, the splenocytes were obtained and stimulated with $E7_{49-57}$ peptide. (a) indicates the anti-E7 T cell responses of each mouse after three antigens immunization. (b) represents the average T cell responses of each vaccination group. Red bar indicates P value.

Similarly like EG7 *in vitro* stimulation of splenocytes, here we employed TC-1 cells (derived from lung epithelium of C57BL/6 mice, with HPV E6 and E7 epitopes expression) and RMA/E7 cells (derived from RMA cell line, with HPV E7 epitopes expression) as target cells to assess anti-E7 T cell responses induced by combined vaccine by Trx-8mer-flank E7 -OVX313. Mice were immunized twice with the vaccine. Activated splenocytes were stimulated by either TC-1 cells, RMA/E7 cells or RMA cells (as the negative control of RMA/E7). Figure 36 demonstrates that with TC-1 or RMA/E7 stimulation, the IFN-γ was obviously produced compared to the responses with RMA stimulation. The results additionally demonstrate that a potent anti-E7 T cell responses are induced when Trx-8mer-flank E7 -OVX313 vaccinated.

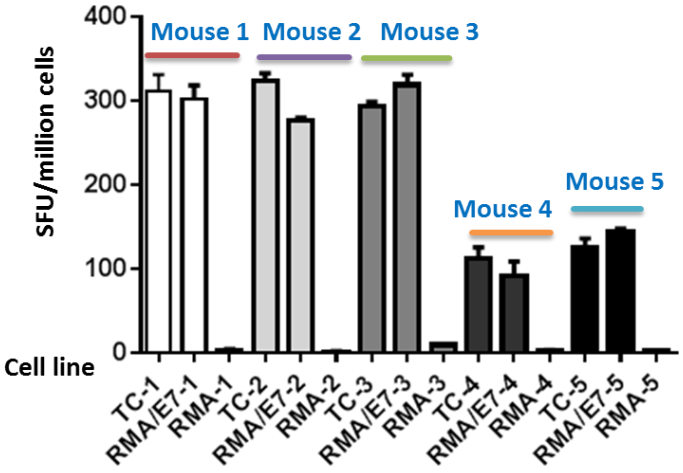
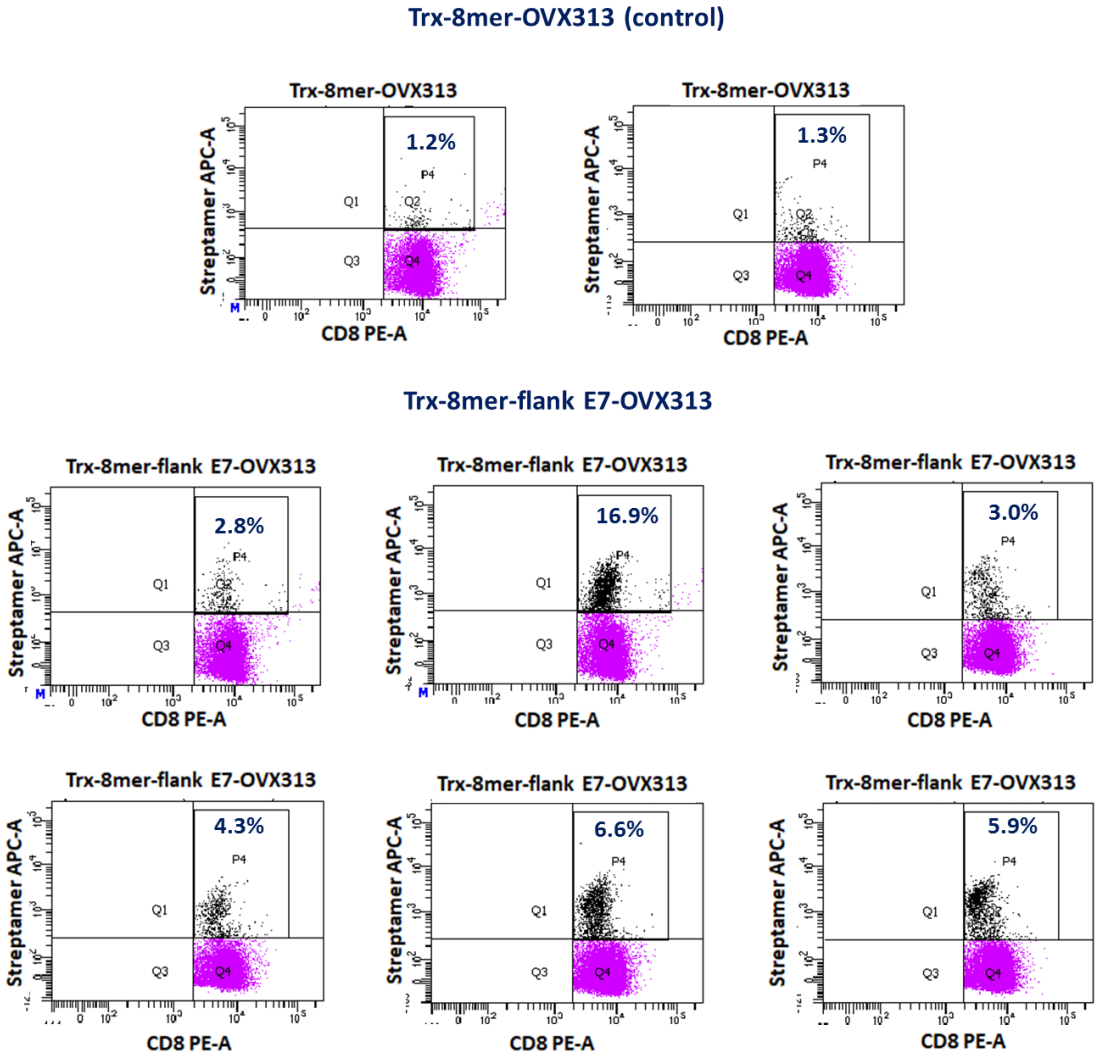


Figure 36. Induced E7-specific T cells can recognize TC-1 and RMA/E7 cells. Analysis was performed using the IFN-γ ELISpot assay. Five mice were immunized twice at 5 days as intervals with the vaccine. Seven days after the last immunization, the splenocytes were stimulated by either TC-1 cells, RMA/E7 cells or RMA cells (as the negative control of RMA/E7).

To evaluate the frequency of E7-specific CD8+ T cells induced by Trx-8mer-flank E7-OVX313, we quantified E7-specific CD8+ T cells by flow cytometry using H-2D^{b}/ $E7_{49-57}$ (RAHYNIVTF) streptamer. Mice were injected with Trx-8mer-flank E7-OVX313 or Trx-8mer-OVX313 (negative control). Afterwards, the stimulated splenocytes were stained by anti-CD8-PE and APC-conjugated H-2Db/ $E7_{49-57}$ streptamer. The flow cytometry was used to determine the percentage of E7-specific CD8+ T cells. The FACS data are shown in Figure 37(a). The black dots represent the E7-specific CD8+ T cells. The data are analyzed and represented in Figure 37(b). The average frequency of E7-specific T cells among the total CD8+ T cell population induced by Trx-8mer-flank E7-OVX313 is around 7 %.

(a)


Trx-8mer-OVX313 (control)
Trx-8mer-OVX313
1.2%
1.3%
Streptamer APC-A
CD8 PE-A
Trx-8mer-flank E7-OVX313
2.8%
16.9%
3.0%
4.3%
6.6%
5.9%
P4
Q1
Q2
Q3
Q4

(b)

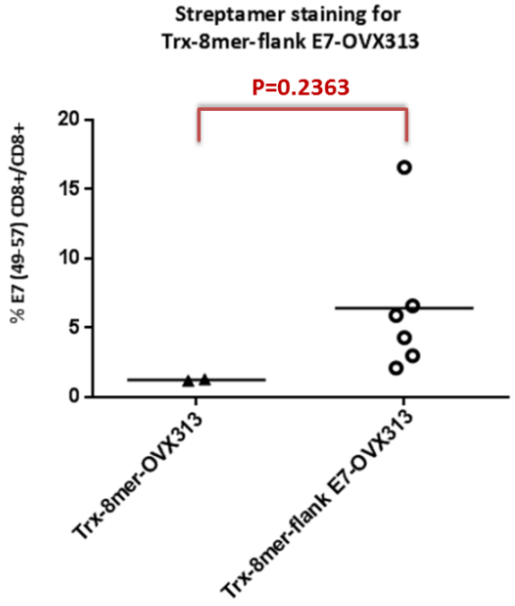
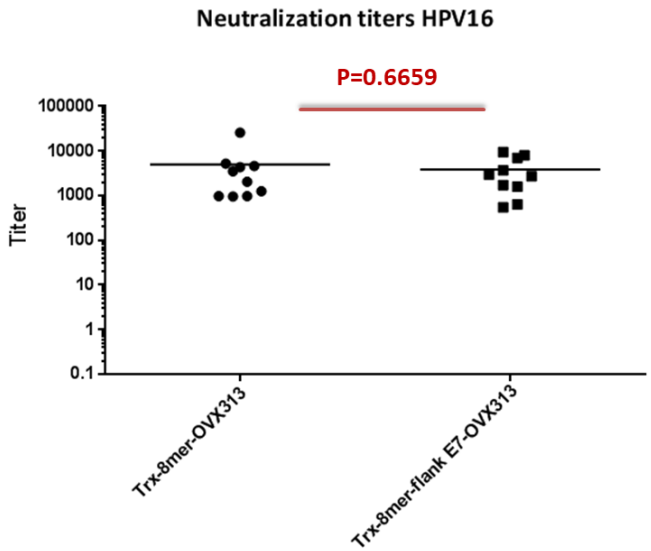

Streptamer staining for
Trx-8mer-flank E7-OVX313
P=0.2363
% E7 (49-57) CD8+/CD8+
20
15
10
5
0
Trx-8mer-OVX313
Trx-8mer-flank E7-OVX313

Figure 37. E7-specific T cells can be induced by Trx-8mer-flank E7-OVX313 measured via streptamer staining. We immunized mice (2 or 6 per group) twice at a 5 days interval with 20μg Trx-8mer-OVX313 (negative control) or Trx-8mer-flank E7-OVX313. One week later after last immunization, the splenocytes were stained by anti-CD8-PE and APC-conjugated H-2Db/ $E7_{49-57}$ streptamer. The flow cytometry was used to analyze the percentage of E7-specific CD8+ T cells. The FACS data are shown in (a). The black dots represent the E7-specific CD8+ T cells. The analyzed data are represented in (b). Each dot (triangle or blank circle) represents one mouse, with the mean percentage (E7(49-57)-specific CD8+ T cells/total CD8+ T cells) showing by horizontal bars.

- **Trx-8mer-flank E7-OVX313 induces comparable neutralizing antibody titers compared to Trx-8mer-OVX313**

L1-PBNA against HPV 16 and HPV 18 pseudovirions was used to evaluate the anti-L2 antibody production of the combined vaccine Trx-8mer-flank E7-OVX313 (Figure 38). BALB/c mice were immunized 4 times with Trx-8mer-OVX313 or Trx-8mer-flank E7-OVX313. Sera were collected one month later after the last immunization and analyzed by L1-PBNA. Figure 38 demonstrates that there is no significant difference regarding to the antibody titer (against both HPV16 (P value 0.9765) and HPV18 (P value 0.4813)) produced by the combined vaccine Trx-8mer-flank E7-OVX313 and Trx-8mer-OVX313, which illustrates that T cell epitope flank $E7_{49-57}$ dose not interfere with the induction of B cell responses against the L2 epitopes.

(a)



(b)

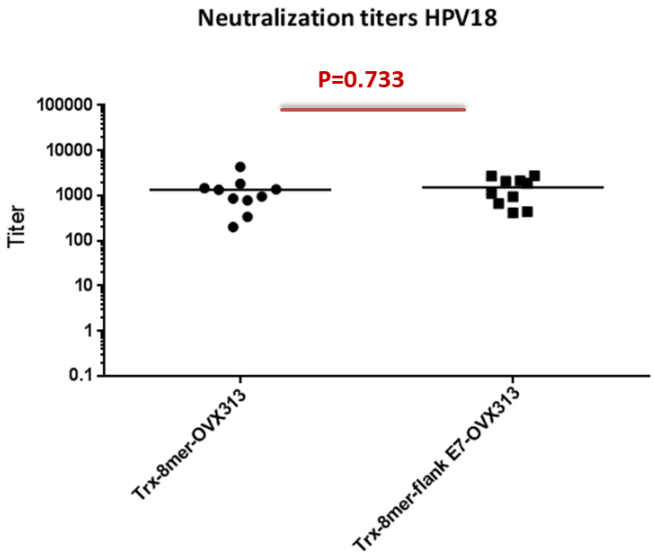


Figure 38. Heptameric antigen Trx-8mer-flank E7-OVX313 induces comparable anti-HPV16 and anti-HPV18 neutralizing antibodies compared to Trx-8mer-OVX313. Ten BALB/c mice per group were immunized intramuscularly 4 times at biweekly intervals with 20 μg of Trx-8mer-OVX313 and Trx-8mer-flank E7-OVX313 formulated with 50% (v/v) AddaVax. Sera were collected from mice one month after the last immunization and analyzed against HPV 16 and HPV18 pseudovirions using the L1-PBNA. The sera were started with a 1:50 dilution and then titrated in 1:3 series. The neutralization titer is defined as the reciprocal of the maximum dilution in which case 50% pseudovirions can be still neutralized (IC50). The HPV16 and HPV18 neutralizing titers induced by Trx-8mer-OVX313 and Trx-8mer-flank E7-OVX313 are shown in (a) and (b). Each dot indicates one mouse with the mean titers showing by horizontal bars. P value is indicated in the figure.

4.2.4 A T-helper epitope is found in OVX313 scaffold

From the comparison of T cell responses induced by PADRE-Trx-L2-flank E7 and Trx-8mer-flank E7-OVX313, we can see that stronger anti-E7 T cell responses were induced by OVX313-PfTrx scaffold. This is consistent with the results of anti-OVA T cell responses induced by PADRE-Trx-L2-OVA and Trx-8mer-OVA-OVX313. Except some very potent CD8+ epitopes, the induction of cytotoxic T cell responses requires the activation of T-helper pathway. It was previously proved in our lab that there is no T-helper epitope within the PfTrx protein recognized in C57BL/6 mice. So here we are ignited to explore the T-helper epitopes from OVX313. We designed 20mer-peptide set with 12 amino acids overlap covering the entire OVX313 sequence (Table 3 in section 2.4.1). Three mice were immunized once and three mice were vaccinated twice with Trx-8mer-flank E7-OVX313. The splenocytes were stimulated with the peptide panel derived from OVX313 protein sequence. We can observe a T cell response induced by OVX313-I5 peptide in IFN-γ ELISpot, and the responses

are stronger with two immunizations than only one (Figure 39). But this response could not be identified as being CD8+ or CD4+ T cell-specific by intracellular cytokine staining. Even though, we assumed that this is a T helper responses due to the long size (20 amino acids) of the stimulating peptide. Other assays need to be further established to confirm the epitope OVX313-I5. As aforementioned, we supposed that the CD8+ T cell responses induced by the antigens comprising OVX313-PfTrx could partially benefit from T-helper activation from OVX313-I5 peptide.

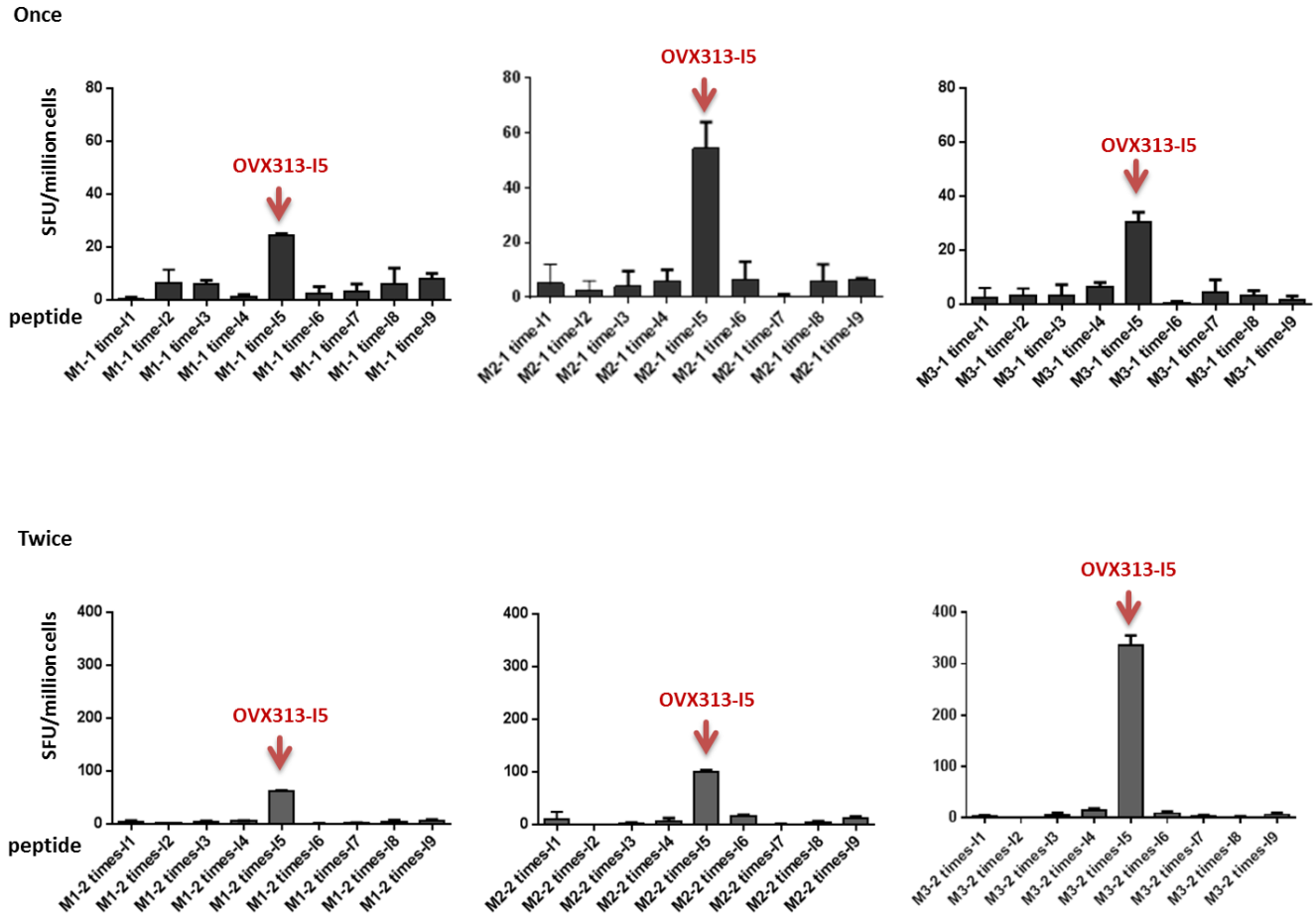


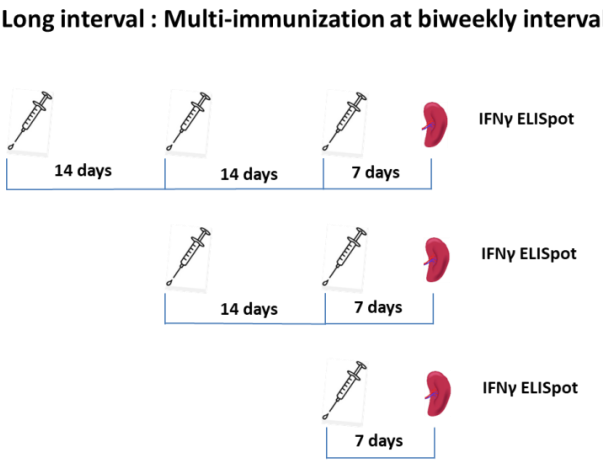
Figure 39. A T-helper response is induced by OVX313-I5 peptide. Mice were separated into two groups. One group (3 mice per group) was immunized once and the other group was immunized twice at 5 days intervals with 20 µg Trx-8mer-flank E7-OVX313. Seven days after the last immunization, the splenocytes were stimulated with the peptide panel derived from OVX313 protein sequence. The T cell responses induced by the nine peptides (I1 to I9) are shown.

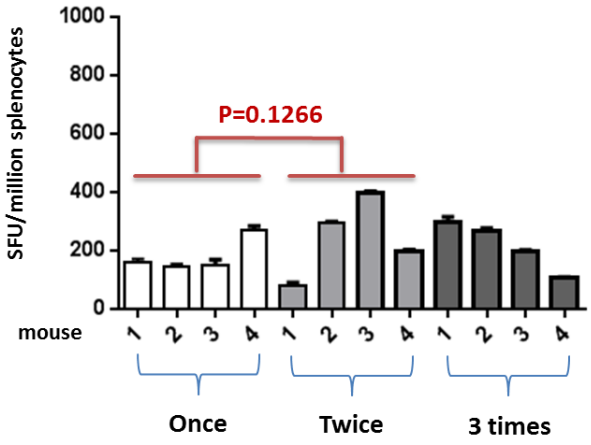
4.3 The prophylactic and therapeutic combined vaccine Trx-8mer-flank E7-OVX313 is effective in TC-1 tumor therapy.

4.3.1 Two vaccinations with short intervals induce robust and stable T cell responses.

Before we start tumor therapy with Trx-8mer-flank E7-OVX313 vaccine, we wanted to determine how many doses immunization and how long immunization intervals can induce the robust and stable T cell responses by IFN-γ ELISpot. We designed the immunization strategy as shown in Figure 40 (a) and (b). The mice were immunized either at long immunization intervals (14 days) or at short intervals (5 days). The number of doses ranged from one to four. It is evidently represented in the figure that a stronger anti-E7 T cell responses is produced with short-interval immunization than long-interval. Moreover, the peak of T cell responses is achieved with two immunizations at 5 days interval. So we used short-interval immunization and two doses in the mouse tumor assays.

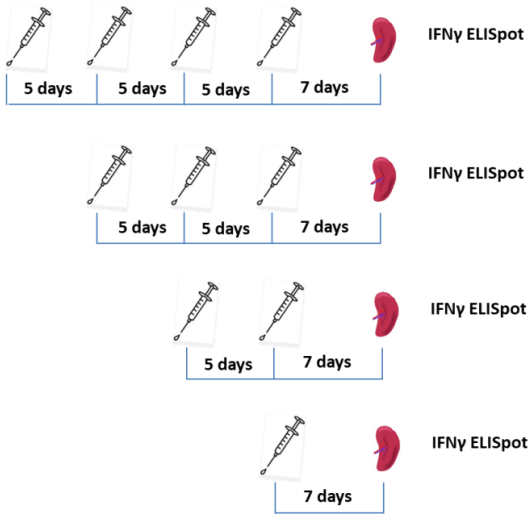
(a)





(b)

Short interval: Multi-immunization at 5 days intervals



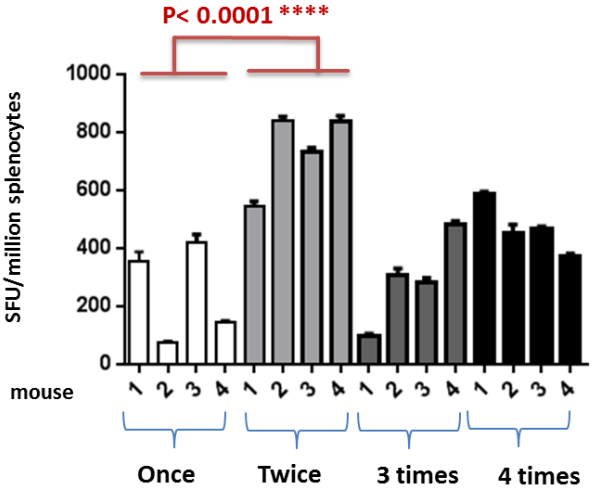


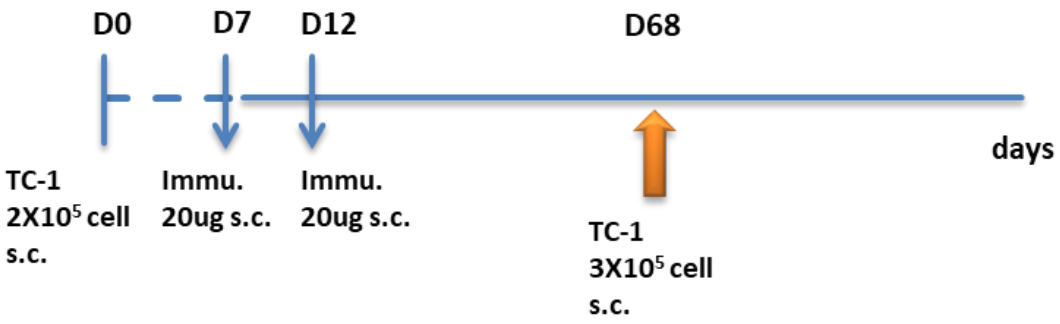
Figure 40. The strongest anti-E7 T cell responses are induced when mice are immunized twice at 5 days interval. IFN-γ ELISpot was performed to decipher the most applicable immunization strategy with Trx-8mer-flank E7-OVX313 for mouse tumor assays. Mice were immunized either at long immunization intervals (14 days) or at short intervals (5 days). The number of doses ranged from one to four. (a) indicates the long-interval immunization and the results of ELISpot. (b) gives the short-interval immunization and the analysis of T cell responses.

4.3.2 The vaccine Trx-8mer-flank E7-OVX313 can induce TC-1 tumor regression.

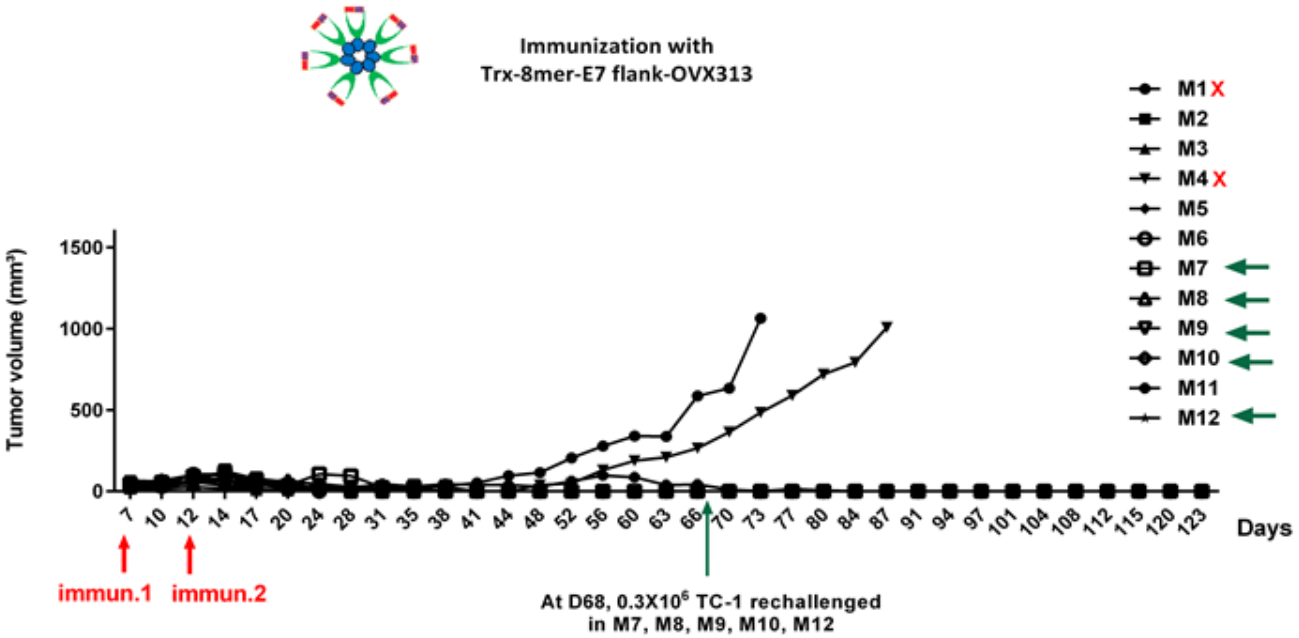
- **Trx-8mer-flank E7-OVX313 has therapeutic efficacy when 200,000 TC-1 cells were implanted and 300,000 TC-1 cells rechallenged.**

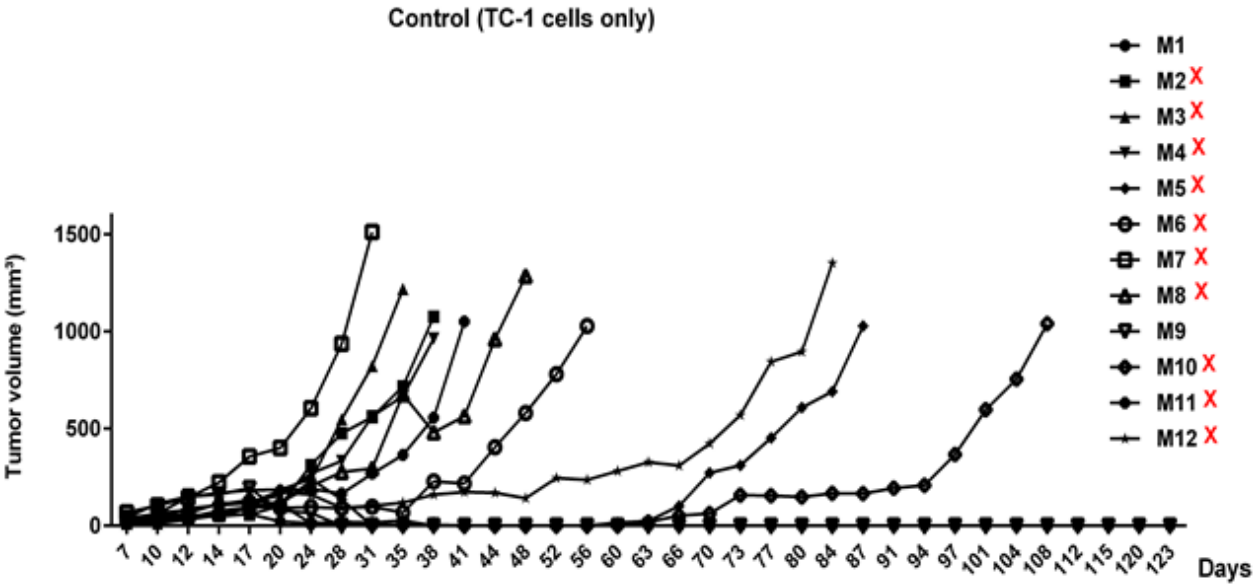
The mice were inoculated with $0.2X10^6$ TC-1 tumor cells. Half of the animals were started vaccination one week after inoculation (when tumor size was between 3-5mm diameter), and two doses of Trx-8mer-flank E7-OVX313 were applied 5 days apart (Figure 41 (a)). The other 12 mice were left without any treatment. 68 days after inoculation of tumor cells, 5 vaccinated mice (tumors were totally regressed) were rechallenged with $0.3X10^6$ TC-1 cells. Besides, 5 naïve mice at the similar age were also implanted with $0.3X10^6$ TC-1 cells as comparison. The tumor growth curves are shown in Figure 41 (b). The animal survival curves are presented in Figure 41 (c). It is apparent that the vaccination with antigen Trx-8mer-flank E7-OVX313 slows the growth of TC-1 tumors and improves the survival rate in mice. The zoomed curves after tumor rechallenging are represented in Figure 41 (d). We can see that the memory T cell responses induced by Trx-8mer-flank E7-OVX313 still exist after 4 months. In addition, until the end of the experiment, tumor growth has not been observed in the vaccinated mice.

(a)

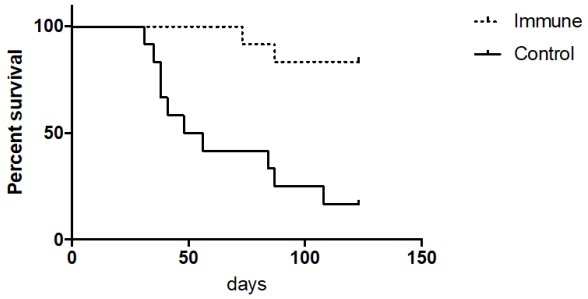


(b)


Immunization with
Trx-8mer-E7 flank-OVX313
M1 X
M2
M3
M4 X
M5
M6
M7
M8
M9
M10
M11
M12
Tumor volume (mm³)
1500
1000
500
0
7 10 12 14 17 20 24 28 31 35 38 41 44 48 52 56 60 63 66 70 73 77 80 84 87 91 94 97 101 104 108 112 115 120 123
Days
immun.1
immun.2
At D68, 0.3X10⁶ TC-1 rechallenged
in M7, M8, M9, M10, M12


Control (TC-1 cells only)
M1
M2 X
M3 X
M4 X
M5 X
M6 X
M7 X
M8 X
M9
M10 X
M11 X
M12 X
Tumor volume (mm³)
1500
1000
500
0
7 10 12 14 17 20 24 28 31 35 38 41 44 48 52 56 60 63 66 70 73 77 80 84 87 91 94 97 101 104 108 112 115 120 123
Days

(c)


Percent survival
100
50
0
0
50
100
150
days
Immune
Control

p=0.0005 ***

(d)

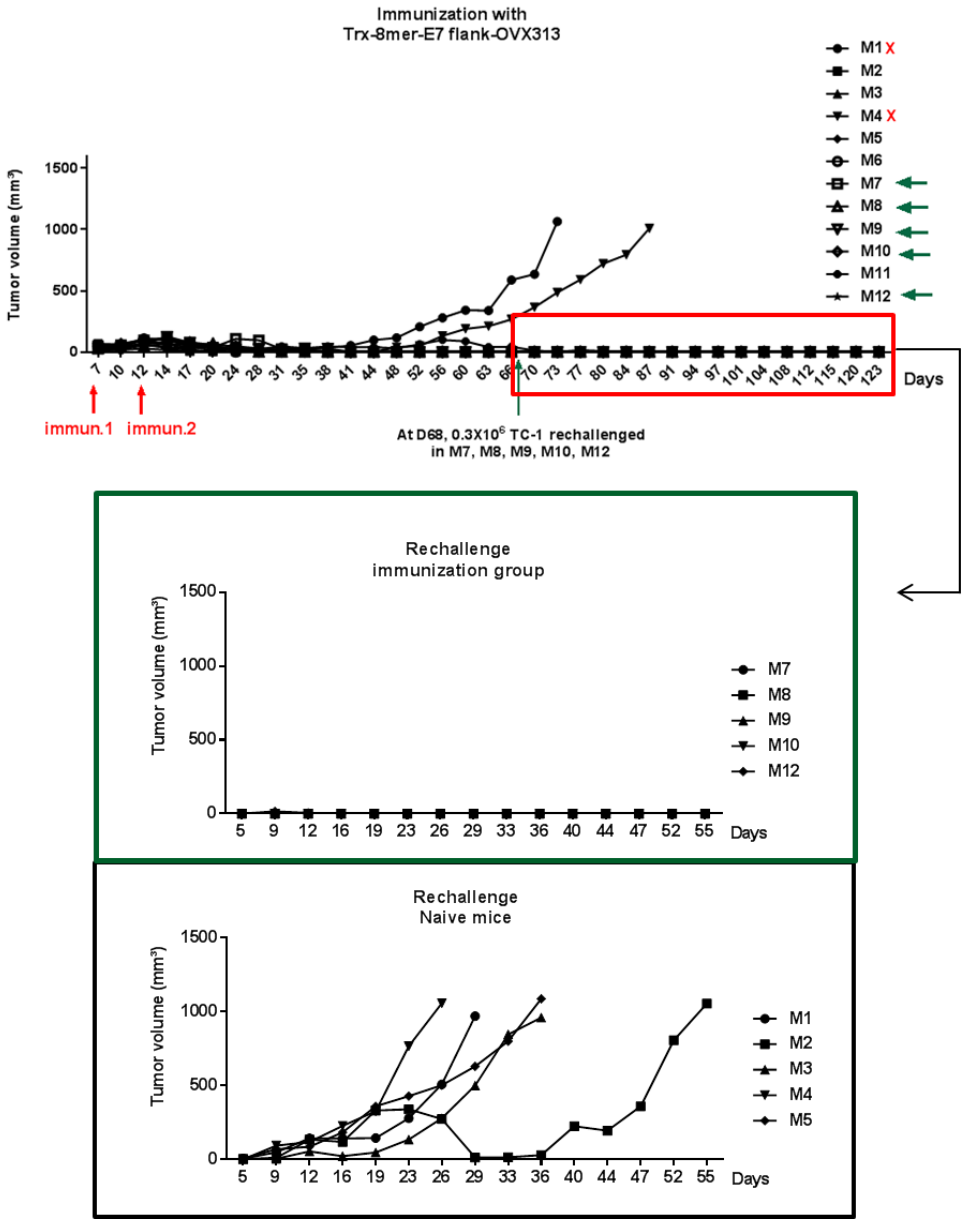

Immunization with
Trx-8mer-E7 flank-OVX313
Tumor volume (mm³)
1500
1000
500
0
M1 X
M2
M3
M4 X
M5
M6
M7
M8
M9
M10
M11
M12
Days
immun.1
immun.2
At D68, 0.3X10⁶ TC-1 rechallenged
in M7, M8, M9, M10, M12
Rechallenge
immunization group
M7
M8
M9
M10
M12
Rechallenge
Naive mice
M1
M2
M3
M4
M5

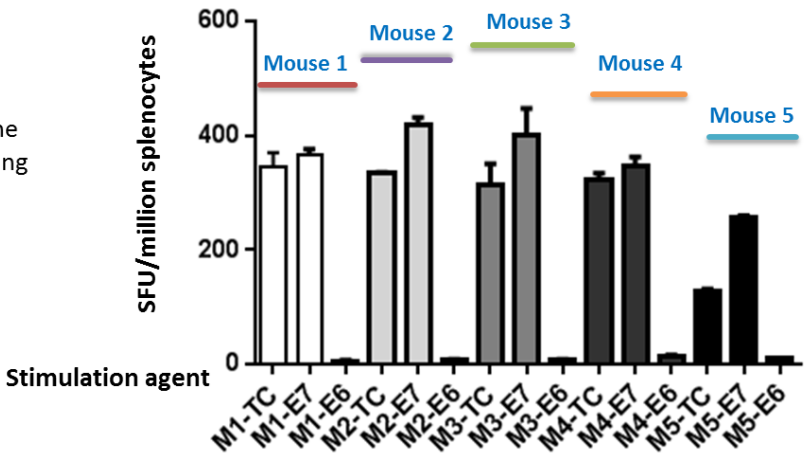
Figure 41. Trx-8mer-flank E7-OVX313 vaccination can inhibit the growth of TC-1 tumor, and the subsequent memory T cell responses can constrain tumor occurrence. The vaccination regime started one week after implantation of $0.2X10^6$ TC-1 tumor cells (a). Half of the tumor animals (12 out of 24 mice) received two doses of Trx-8mer-flank E7-OVX313 5 days apart. Twelve tumor mice were without any treatment. The illustration of the experimental design is shown in (a). The tumor growth curves are represented in (b). The animal survival rate is shown by Kaplan-Meier curves (c) and the Log-rank test indicates a significant difference in survival (p=0.0005). 68 days after inoculation of tumor cells, 5 vaccinated mice (with green arrows pointed) were rechallenged with $0.3X10^6$ TC-1 cells. Besides, 5 naïve mice at the similar age were also implanted with $0.3X10^6$ TC-1 cells as comparison. (d) gives the tumor growth kinetics after rechallenging. Tumor size was measured with a caliper every 3 or 4 days. Mice were sacrificed when the tumor volume exceeded $1cm^3$ or the tumor diameter exceeded 1.5cm. The red crosses mark the sacrificed mice due to the tumor burden.

- **Potent T cell responses are induced in mice that have a regressed tumor.**

Tumor regression was observed in vaccinated mice of initial tumor challenging as well as the rechallenged mice (Figure 41 (b) (d)). We wanted to determine how potent the T cell responses were generated, and if epitope spreading occurred after the TC-1 tumor was destroyed and eradicated. IFN-γ ELISpot was performed to evaluate the T cell responses of tumor regressed mice. The splenocytes were stimulated with TC-1 cells, $E7_{49\text{-}57}$ peptide or $E6_{48\text{-}57}$ peptide *in vitro*. The IFN-γ elicitation is observed with either TC-1 cells or $E7_{49\text{-}57}$ peptide stimulation, but not with $E6_{48\text{-}57}$. And the T cell responses of tumor rechallenged mice are apparently stronger than the mice with only tumor implanted in the beginning (Figure 42 (a) (b)). The potent T cell responses correlated with tumor protection upon rechallenge. However, no epitope spreading is detected in the tumor regressed mice.

(a)

Tumor regressed in the beginning and following a rechallenge.



(b)

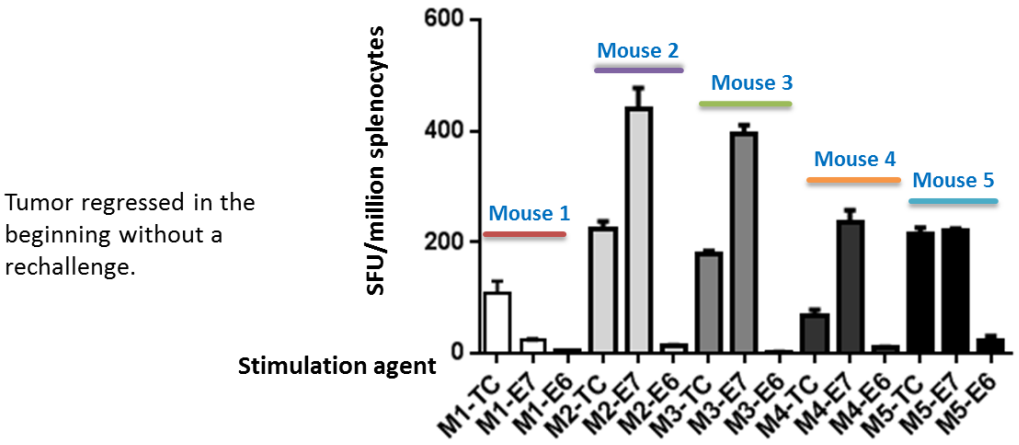
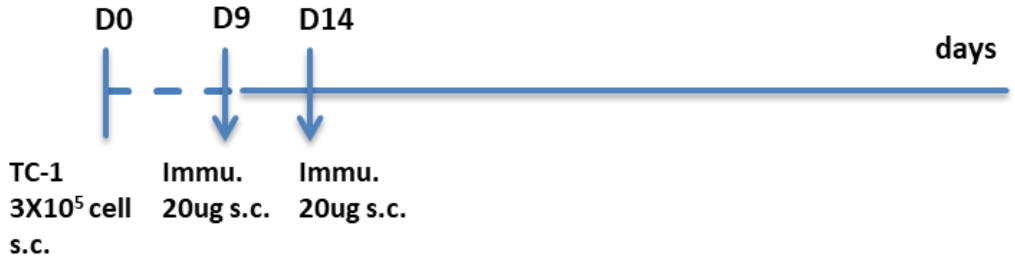


Figure 42. Potent T cell responses are induced in tumor regressed mice. IFN-γ ELISpot was performed to evaluate the T cell responses of tumor regressed mice. The splenocytes were stimulated by TC-1 cells, $E7_{49-57}$ peptide or $E6_{48-57}$ peptide *in vitro*. T cell responses of tumor rechallenged mice are indicated in (a). T cell responses of the mice with only tumor challenged in the beginning are illustrated in (b).

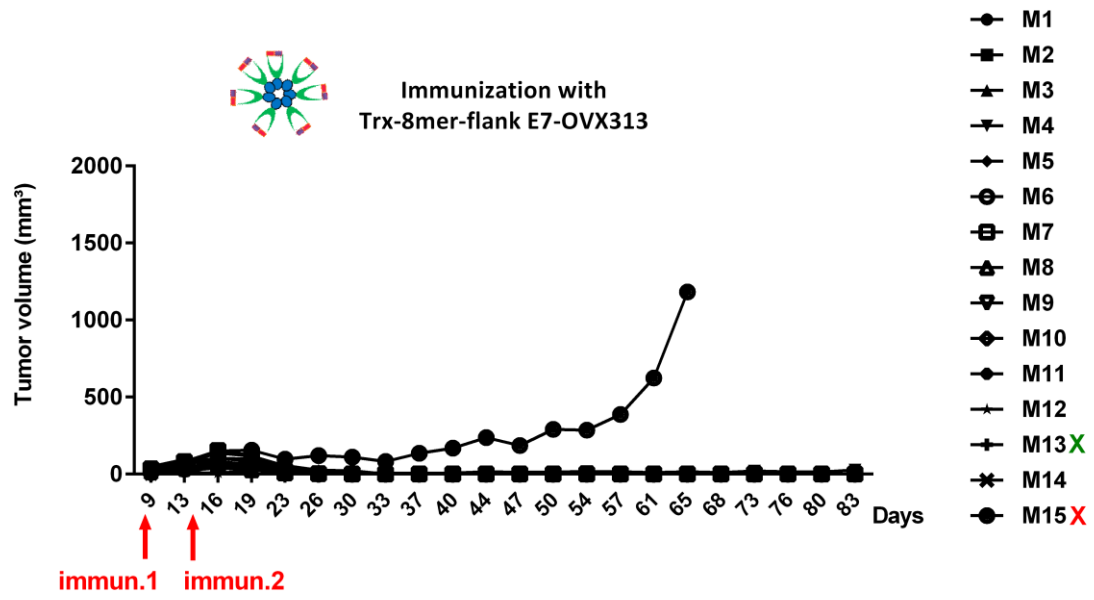
- **Trx-8mer-flank E7-OVX313 is effective to induce tumor regression when mice receive more tumor cells and later vaccination.**

The vaccine Trx-8mer-flank E7-OVX313 is effective when $0.2X10^6$ TC-1 cells are implanted. Here we would like to know if the vaccine is still efficient with more TC-1 cells ($0.3X10^6$) inoculated, and with a later vaccination time. The mice were inoculated with $0.3X10^6$ of TC-1 tumor cells. Nine days after inoculation, when the tumor size was between 4-6mm diameters, half of the animals received Trx-8mer-flank E7-OVX313 and half of the animals were immunized with Trx-8mer-OVX313 (as a control). All mice received two doses vaccine 5 days apart. The tumor growth kinetics is shown in Figure 43 (b) (c) and the survival curves of mice are presented in Figure 43 (d). We can see that the tumor growth is highly impeded and the animal survival rate is apparently improved when mice are immunized with Trx-8mer-flank E7-OVX313 compared to mice that obtained Trx-8mer-OVX313 vaccination. This indicates that Trx-8mer-flank E7-OVX313 is still effective to induce tumor regression when the mice receive more tumor cells implantation and later vaccination. And the tumor regression results from the epitope flank E7 of antigen Trx-8mer-flank E7-OVX313, not because of Trx-8mer-OVX313 part.

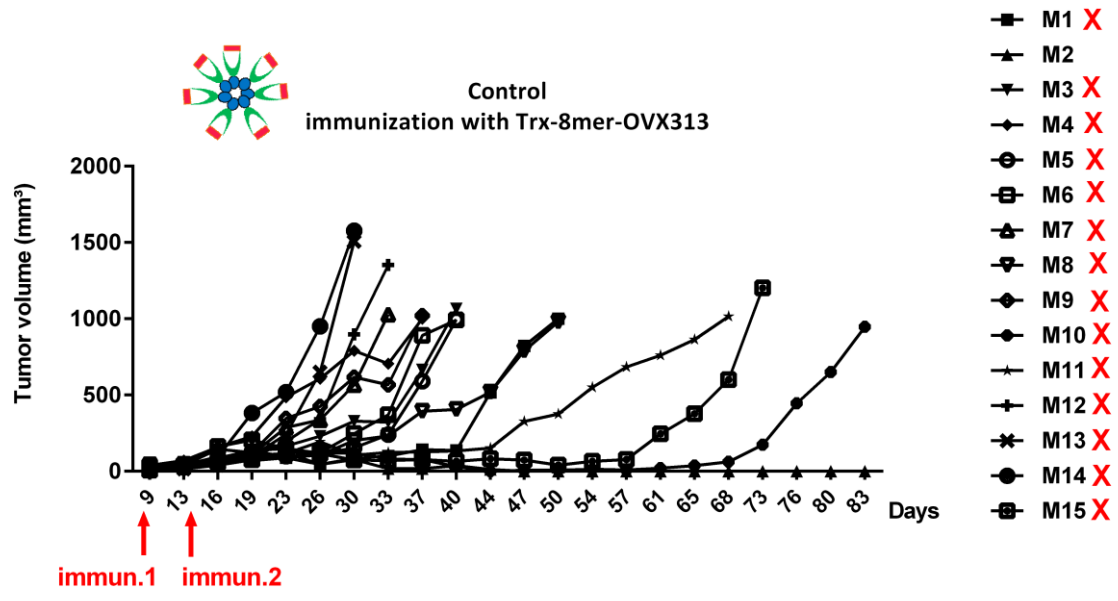
(a)


D0
D9
D14
days
TC-1
3X10⁵ cell
s.c.
Immu.
20ug s.c.
Immu.
20ug s.c.

(b)


Immunization with
Trx-8mer-flank E7-OVX313
Tumor volume (mm³)
2000
1500
1000
500
0
9 13 16 19 23 26 30 33 37 40 44 47 50 54 57 61 65 68 73 76 80 83
Days
immun.1
immun.2
M1
M2
M3
M4
M5
M6
M7
M8
M9
M10
M11
M12
M13 X
M14
M15 X

(c)


Control
immunization with Trx-8mer-OVX313
Tumor volume (mm³)
2000
1500
1000
500
0
9 13 16 19 23 26 30 33 37 40 44 47 50 54 57 61 65 68 73 76 80 83
Days
immun.1
immun.2
M1 X
M2
M3 X
M4 X
M5 X
M6 X
M7 X
M8 X
M9 X
M10 X
M11 X
M12 X
M13 X
M14 X
M15 X

(d)

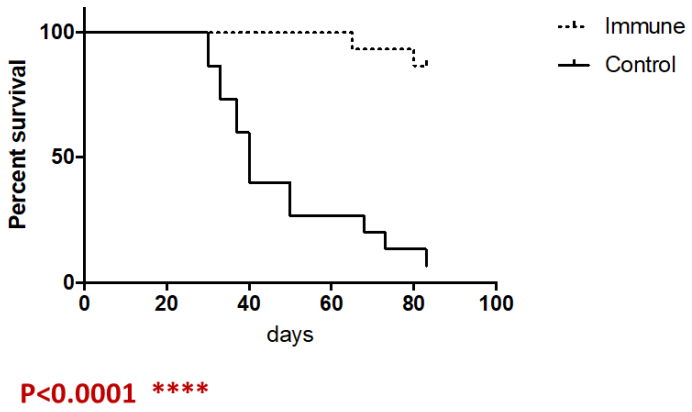


Figure 43. Trx-8mer-flank E7-OVX313 is effective to induce tumor regression when the mice receive more tumor cells and later vaccination. The illustration of the experimental design is shown in (a). The mice were inoculated with $0.3X10^6$ TC-1 tumor cells. Nine days after inoculation, half of the animals (15 out of 30 mice) were received Trx- 8mer-flank E7-OVX313. The tumor growth curve is shown in (b). The rest half were immunized with Trx-8mer-OVX313 as a control. The tumor growth curve of control mice is represented in (c). All mice received two doses vaccine 5 days apart. The animal survival rate is shown by Kaplan-Meier curves (d) and the Log-rank test indicates a significant difference in survival ($p<0.0001$). Tumor size was measured with a caliper every 3 or 4 days. Mice were sacrificed when the tumor volume reached more than $1cm^3$ or the tumor diameter exceeded 1.5 cm. The red crosses mark the sacrificed mice due to the tumor burden. The mouse indicated by the green cross in (b) was sacrificed owing to skin lesions irrelevant to the tumor.

5. Discussion

5.1 What challenges are faced by current HPV vaccines and what are the possible solutions for a better therapeutic achievement?

5.1.1 Challenges for immunotherapies of HPV-associated diseases

Due to the detection of anti-L1 T cell responses in animal models and various clinical trials (Passmore et al, 2006; Steele et al, 2005; Williams et al, 2002), it was surmised that the commercial prophylactic HPV L1 vaccines would also play a role in therapy of HPV-related lesions. However, the therapeutic efficacy was neither observed for Cervarix nor for Gardasil (2007; Hildesheim et al, 2007). The exact reasons are unknown but there are two speculations. First, the Alum adjuvants applied in the commercial vaccines drive the T cell responses to a Th2 direction mainly related to CD4 T cell responses, which could result in suppression of CD8+ T cell response (Liu et al, 2003). Second, the expression of L1 is normally in the superficial epithelium at the late stage, thus L1-presenting cells are hardly perceived by the immune system. Owing to the penurious therapeutic feasibility of current preventive vaccines, the development of therapeutic vaccine has provoked a strong enthusiasm in academic research as well as pharmaceutical industry.

As aforementioned in the introduction, different types of therapeutic vaccines have been tested in clinical trials for their potential of activation cell-mediated responses and so far variable results were obtained. However, they are effective mostly in early stage with low grade CIN, or used as an adjuvant therapy after surgery. They are likely not as successful as the clinical trial tested prophylactic vaccines. It still remains that the treatment of high grade HPV positive lesions and cervical cancer through therapeutic vaccines falls short of expectations. This is due to HPV various immune escape mechanisms and the increased immunosuppressive microenvironment as HPV-disease progresses.

HPVs take advantage of their own immune escape mechanisms to evade host immune surveillance (Kanodia et al, 2007). The protein E5 of high-risk HPV types downregulates MHC I expression on the cell surface via inhibition of their Golgi apparatus transportation (Ashrafi et al, 2006). The E6 protein affects Langerhans' cell density to prevent immune responses (Matthews et al, 2003). More importantly, the oncoprotein E7 interferes with IFN signaling

pathway which is indispensable in inflammatory reaction and immune responses (Barnard et al, 2000). All these mechanisms may set barriers for immunotherapy. With the progression of lesions, the immune microenvironment is more suppressive, including the enhanced expression of PD-1 (on T cells) and PDL-1 (on APCs) (Yang et al, 2013); distorted equilibrium of Th1 cells and Th2 cells (Bais et al, 2005); reduced Th1 cytokines (IL-2, TNF-α and IL-12) and increased Th2 cytokines (Peghini et al, 2012; Scott et al, 2013; Yang et al, 2013); increased immunosuppressive cells, such as Tregs (Curiel et al, 2004; Lukesova et al, 2014) and MDSC (myeloid-derived suppressor cells) (Nagaraj et al, 2007; Stone et al, 2014). The adverse tumor microenvironment may prevent T cell-mediated antitumor reactivity in more aggressive lesions.

Facing these challenges, current therapeutic vaccines applied alone in clinical are not the complete solution. The combination strategies may give us an opportunity to overcome the immune barriers.

5.1.2 Possible solutions for the immunotherapy challenges

From the previous studies reported by a large number of investigations we learned that single-pronged treatment seems to be not sufficient for eradicating lesions in patients with advanced-staged diseases. This is due to HPV multiple immune escape mechanisms and some unknown reasons. Therefore, efforts should be made on modulation of the tumor microenvironment and promotion of systemic as well as local immune responses. The combinational mode is likely to be the key to battle HPV-associated diseases successfully (Ma et al, 2017).

- Prime-boost regimens

Heterologous prime-boost strategies can be applied to promote vaccine efficacy. One option is to utilize different vaccine forms. For example, a protein vaccine can be administered firstly then followed by a DNA or virus-based vaccination. In a phase II trial, patients with anogenital intraepithelial neoplasia were primed with the TA-CIN protein and boosted with recombinant vaccinia virus TA-HPV. Five out of 29 individuals showed an enhanced T cell response (Fiander et al, 2006; Smyth et al, 2004). An alternative possibility is to perform prime-boost in different immunization sites. Prime in the deltoid muscle is able to induce

systemic immune responses, and a boost can be in cervix prone to elicit local responses speculated a better lesion-elimination.

- Vaccination-chemotherapy regimens

Pairing-use of vaccine candidates with various therapeutic approaches such as chemotherapy is another attractive regimen, which has been also implemented in clinical trials. For instance, an ongoing phase I/II study (NCT02128126) was carried out in 2014 in cervical cancer patients. The vaccine ISA101/ISA101b (a HPV16 vaccine composed of synthetic long peptides) was synergistically employed with chemicals carboplatin and paclitaxel, and the HPV-specific immune responses and the safety will be evaluated.

- Vaccination-checkpoint inhibitors regimens

Immune checkpoint inhibitors, such as antibodies against CTLA-4, PD-1 or PD-L1, can prevent negative modulation of T cells resulting in improved CTL cytotoxicity (Blank & Mackensen, 2007; La-Beck et al, 2015; Peggs et al, 2006). A study was performed to detect the expression level of PD-L1 in cervical intraepithelial neoplasia and cervical cancers. It was found that 95% CIN patients (20 out of 21) had an increased expression of PD-L1 and 80% patients with cervical squamous cell cancer (56 out of 70) showed obviously upgraded PD-L1 (Mezache et al, 2015). This suggests that PD-L1 and PD-1 blockade may play a role in cervical cancer treatment.

A research group used an Ad5 viral vaccine (containing HPV 16 E6 and E7) together with PD-L1 blockade in mice loaded with HPV+ tumor. An improved antitumor activity and increased survival rate were observed in mice obtained combination therapy compared to the mice with vaccination alone. Accordingly, the number of CD8+ TILs (tumor infiltrating lymphocytes) were increased but with a smaller fraction showing PD-1+, and a reduced expression of PD-L1 was observed on tumor cells after combination treatment. This indicates that synergistic use of a therapeutic vaccine and checkpoint inhibitors benefits tumor microenvironment and favors tumor eradication. A phase 2 clinical trial was carried out with ISA101/ISA101b and nivolumab (a PD-1 blockade) in patients with incurable HPV16+ cancer. The combination therapy showed a better clinical outcome compared to application of PD-1 blockade alone, with the overall response rate to treatment of 33% and the median overall survival of 17.5 months (Massarelli et al, 2018). It seems that utilization of therapeutic HPV vaccines or

checkpoint inhibitors alone is not as effective as pairing both therapies. It would be very interesting to see the therapeutic potential of synergistic use of antigen PfTrx-L2 8mer-flank E7-OVX313 with checkpoint inhibitors. This combination should offer a targeted immune attack induced by the vaccine, as well as an improved overall immune level generated by checkpoint inhibitors, both of which are required in the invasive HPV+ tumors.

5.2 The benefits and limitations of our HPV prophylactic and therapeutic combined vaccine

5.2.1 The benefits of the vaccine

Due to availability of commercial prophylactic vaccines, HPV-related morbidity and mortality can be reduced. However, given that the coverage of these vaccines is incomplete worldwide and the inadequate diagnosis of HPV infection exists in less developed regions, a prophylactic vaccine additionally comprising a therapeutic function would be significantly helpful. It means that such combined vaccines could be given to both uninfected and already infected populations without a requirement of HPV-infection screening. Especially, a combined vaccine is very beneficial in post exposure prophylaxis. In the beginning of the HPV infection, there are not only virus infected cells, but also large amounts of residual viruses. In this scenario, both B cell responses and T cell responses are required. The combined vaccines would ideally resolve productive infections in the early stage, as well as HPV-related diseases in a later HPV-infected phase because of the combined B-cell and T-cell epitopes. Moreover, such combined vaccines would shield the recovered individuals from further HPV re-infections. The discussed benefits and the scope of the application embolden us to develop the HPV prophylactic and therapeutic combined vaccines.

Except for the collective values of prophylaxis and therapeutics, our combined vaccine has unique superiorities. Cost-effectiveness always has to be considered before licensing a vaccine. The development of our vaccine stands on reducing the economic burden on global health. For starters, our vaccine PfTrx-L2 8mer-flank E7-OVX313 was produced in *E. coli* which is a cost-effective and easy-operating protein producer. Secondly, PfTrx scaffold used in our antigen is from the hyperthermophile *Pyrococcus furiosus*. It presents a highly thermostable protein with a large capacity to accept insertion into its active center. With the

help of this scaffold, our vaccine does not depend on cold-chains for transportation, which cut costs and makes it applicable for many regions worldwide.

In addition, it was reported that T helper responses against E7 are considerably correlated with self-regression of HPV-induced lesions in healthy individuals (Kadish et al, 2002; Koskimaa et al, 2017). We assume that our vaccine used to the individuals with early-stage HPV infection can benefit from E7-specific T helper responses which 'kick up' the immune system resulting in clearing HPV-transformed cells.

5.2.2 The limitations of the vaccine

The antigen PfTrx-L2 8mer-flank E7-OVX313 comprising eight L2 $_{(20\text{-}38)}$ epitopes from HPV16-18-31-33-35-6-51-59 can induce cross-neutralizing antibodies, even against some cutaneous HPV types (Pouyanfard et al, 2018). The vaccine thus provides cross-protection in prophylaxis. However, for the therapeutic side of the vaccine, the function could be constrained. On the one hand, only the E7 epitope of HPV16 was incorporated in our combined vaccine. On the other hand, the E7 proteins derived from different high-risk HPV types are not conserved as shown in the following graph (Figure 44). Therefore, we expected the induced T cell responses could only against rather limited HPV types. With the purpose of broadening the therapeutic characteristics, various HPV E7 epitopes should be included in the vaccine.

```
HPV16     MHGDTPTLHEYMLDLQPET---TDLYCYEQLSDSSE--EEDEIDG-------PAGQA 45
HPV31     MRGETPTLQDYVLDLQPEA---TDLHCYEQLPDSSD--EEDVIDS-------PAGQA 45
HPV35     MHGEITTLQDYVLDLEPEA---TDLHCYEQLCDSSEE-EEDTIDG-------PAGQA 46
HPV52     MRGDKATIKDYILDLQPET---TDLHCYEQLGDSSDEEDTDGVDR-------PDGQA 47
HPV33     MRGHKPTLKEYVLDLYPEP---TDLYCYEQLSDSSDE-D-EGLDR-------PDGQA 45
HPV58     MRGNNPTLREYILDLHPEP---TDLFCYEQLCDSSDE-DEIGLDG-------PDGQA 46
HPV39     MRGPKPTLQEIVLDLCPYNEIQPVDLVCHEQLGESED--EIDEPDHAVNH-QHQLLARRE 57
HPV68     MHGPKPTVQEIVLELCPCNEIEPVDLVCHEQLGDSDD--EIDEPDHAVNHHQHQLLARRD 58
HPV59     MHGPKATLCDIVLDLEPHN-FEEVDLVCYEQLPDSDSENEKDEPDG---VNHPLLLARRA 56
HPV18     MHGPKATVQDIVLHLEPQNEI-PVDLLCHEQLSDSE---EENDEIDG---VNHQHLPARRA 54
HPV45     MHGPQATLQEIVLHLEPQNELDPVDLLCYEQLSESE---EENDEADG---VSHAQLPARRA 55
HPV51     MRGNVPQLKDVVLHLTPQTE---IDLQCYEQFDSSEEEDE-V---DN---MRD-QLPERRA 50
HPV56     MHGKVPTLQDVVLELTPQTE---IDLQCNEQLDSSEDEDE-DEVDH---LQERPQQARQA 53
HPV66     MHGKVPTLQEVILELAPQTE---IDLQCNEQLDSSEDEDE-DEIDH---LLERPQQARQA 53
          *:*    : : :*.* *       ** * **: .*.   :    *            :

HPV16     EPDRAHYNIVTFCCKCDSTLRLCVQSTHVDIRTLEDLLMGTLGIVCPICSQKP-      98
HPV31     KPDTSNYNIVTFCCQCESTLRLCVQSTQVDIRILQELLMGSFGIVCPNCSTRL-      98
HPV35     KPDTSNYNIVTSCCKCEATLRLCVQSTHIDIRKLEDLLMGTFGIVCPGCSQRA-      99
HPV52     EQATSNYYIVTYCHSCDSTLRLCINSTATDLRTLQQMLLGTLQVVCPGCARL—       99
HPV33     QPATADYYIVTCCHTCNTTVRLCVNSTASDLRTIQQLLMGTVNIVCPTCAQQ—       97
HPV58     QPATANYYIVTCCYTCDATVRLCINSTATEVRTLQQLLMGTCTIVCPSCAQQ—       98
HPV39     EPQR—HTIQCSCCKCNNTLQLVVEASRDTLRQLQQLFMDSLGFVCPWCATANQ      109
HPV68     EQQR—HRIQCMCCKCNNPLQLVVEASRENLRKLQLLFMDSLNFVCPWCATETQ      110
HPV59     EPQR—HNIVCVCCKCNNQLQLVVETSQDGLRALQQLFMDALSFVCPLCAANQ-      107
HPV18     EPQR—HTLLCMCCKCEARIELVVESSADDLRAFQQLFLNTLSFVCPWCASQQ-      105
HPV45     EPQR—HKILCVCCKCDGRIELTVESSADDLRTLQQLFLSTLSFVCPWCATNQ-      106
HPV51     GQAT-CYRIEAPCCRCSSVVQLAVESSGDTLRVVQQMLMGELSLVCPCCANN—      101
HPV56     KQHT-CYLIHVPCCECKFVVQLDIQSTKEDLRVVQQLLMGALTVTCPLCASSN-      105
HPV66     EQHK-CYLIHVPCCKCELVVQLDIQSTKEELRVVQQLLMGALTVTCPLCASSK-      105
               : :   *  *.  :.* ::::   :*.: :::.   ..** *:
```

Figure 44. The alignment of E7 protein sequences from high-risk HPV types. Asterisk (*) indicates the position containing the fully conserved residue. Colon (:) indicates strongly similarity of the residue between different groups. Period (.) indicates weakly similarity of the residue between different groups. Therefore, the degree of conservation indicated by these symbols is Asterisk (*)>Colon (:)>Period (.).

5.3 Exploring the efficiency of OVX313-based vaccines

5.3.1 Efficiency derived from OVX313 multimerization

Oligomerization is utilized in many vaccine-designs to improve immunogen stability, uptake, binding affinity and immunogenicity (Engel & Kammerer, 2000). Heptameric scaffold OVX313 (old name: IMX313, 55 amino acid) is developed from the complement C4-binding protein (C4 bp) which is involved in complement system inhibition. It is designed from the avian C4 bp and shows less than 20% similarity to human C4bp to minimize auto-antibody induction. It contains an amphipathic α-helix region, which is necessary and sufficient for heptamerization, as well as two cysteine residues which stabilize the structure (Kask et al, 2002). Antigens of interest can be fused with the N-terminus of α-chains resulting in repetitively displayed immunogens. Several vaccines developed have been employed OVX313 platform to enhance immune responses (Li et al, 2016b; Spencer et al, 2012; Tomusange et al, 2016).

Tomusange, K. *et al.* have designed an HIV DNA vaccine by fusing HIV Tat protein (involved in viral replication) to OVX313 and as a result, improved antigen-specific IgG and cellular-mediated immune responses were observed in mice (Tomusange et al, 2016). Another group has developed DNA and MVA (modified vaccinia virus Ankara) vaccines carrying *Mycobacterium tuberculosis* antigen 85A and IMX313. Enhanced CD4+ and CD8+ T cell responses to the *M. tuberculosis* antigen 85A were shown after DNA and MVA vaccination in mice and rhesus macaques (Spencer et al, 2012). Moreover, MVA85A-IMX313 vaccine was also evaluated in healthy BCG (Bacillus Calmette-Guérin) vaccinated adults. The vaccine has proved safe in humans but there was no significantly improved mycobacteria-specific cellular immune responses compared to MVA85A vaccination (Minhinnick et al, 2016) (INCT01879163). OVX313 protein has been also applied in malaria vaccines. Fusing *plasmodium falciparum* Pfs25 protein (Pfs25) to OVX313 in ChAd63 (chimpanzee adenovirus serotype 63) and MVA vectors resulted in increased antibody responses after vaccination (Li

et al, 2016b). A clinical trial with candidates ChAd63 Pfs25-IMX313 and MVA Pfs25-IMX313 was carried out in 2017 (NCT02532049), but the results have not been published until now. Our lab has developed HPV prophylactic monomeric vaccine PfTrx-L2 and heptameric vaccine PfTrx-L2 8mer-OVX313. The data indicated that antigen encompassing OVX313 scaffold presented a better performance regarding to anti-L2 antibody titers and cross-protection against different HPV types (Pouyanfard et al, 2018). Encouraged by this result, we continued to study if OVX313 can also aid CTL responses in both B-cell and T-cell epitopes comprised vaccines. We additionally incorporated a CTL epitope derived from ovalbumin or HPV16 E7 to construct monomeric antigen PADRE-PfTrx-L2-OVA and PADRE-PfTrx-L2-flank E7. As comparison, we also designed heptameric antigen PfTrx-L2 8mer-OVA-OVX313 and PfTrx-L2 8mer-flank E7-OVX313. It was apparently shown that antigens with OVX313 platform induced stronger CTL epitope-specific T cell responses. Moreover, we verified heptameric structure of PfTrx-L2 8mer-flank E7-OVX313 in SDS-PAGE (following shown). These may explain why PfTrx-L2 8mer-flank E7-OVX313 showed an effective therapeutic potential in mice tumor assays.

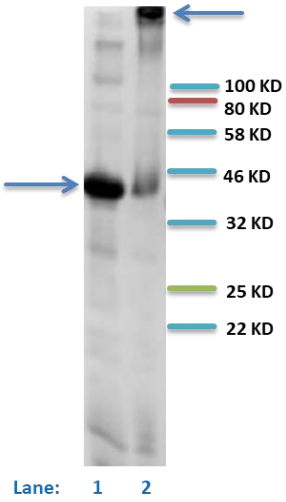


Figure 45. The monomeric or heptameric structure of PfTrx-L2 8mer-flank E7-OVX313. Lane 1: Sample was loaded under reducing conditions indicating a monomeric form of PfTrx-L2 8mer-flank E7-OVX313. **Lane 2:** Sample was under non-reducing conditions showing a heptameric form of the antigen.

5.3.2 The existence of T-helper epitopes in L2 8mer and OVX313

From the comparison of PfTrx-OVA-OVX313 and PfTrx-L2 8mer-OVA-OVX313, we saw that the addition of L2 8mer polytopes enhances OVA-specific T cell responses. Accordingly, we

were wondering if T-helper epitope exists in 8mer sequence. We designed 20mer-peptide set with 12 amino acids overlap covering the entire 8mer sequence in order to find out the T-helper epitope. Unfortunately, none of these 20mer-peptides showed a T cell response in IFN-γ ELISpot or intracellular cytokine staining. We supposed some reasons may be responsible for this. First, the quality of synthesized 20mer-peptides might be not good enough which may cause splenocytes uptake issues during *in vitro* stimulation. Another reason could be that IFN-γ ELISpot or intracellular cytokine staining assay is not suitable for the T-helper peptide screening in this case. The length with 20 amino acids might be too long for effective uptake or process by splenocytes especially in a limited time during the experiment.

Furthermore, a stronger T cell response was observed from the induction by the heptameric antigen compared to the monomeric one. This led us to explore the presence of T-helper epitope in OVX313. Likewise, we designed 20mer-peptide set with 12 amino acids overlap covering the entire OVX313 sequence. And we indeed observed a T cell response with peptide 'OVX313-I5' stimulation in IFN-γ ELISpot (section 4.2.4). However, this response could not be identified as being CD8+ or CD4+ T cell-specific by intracellular cytokine staining. Even though, we assumed that this is a T helper responses due to the long size (20 amino acids) of the stimulating peptide. But other assays need to be established in order to figure out T-helper epitopes in 8mer and to confirm the helper epitope 'I5' from OVX313.

5.4 Influence of immunization intervals

As we showed in section 4.3.1, T cell responses induced by a prime with PfTrx-L2 8mer-flank E7-OVX313 cannot be improved after a boost at a long immunization interval. This was also observed in the boost with a chimeric L1 VLP containing E6 or E7 polypeptides (Greenstone et al, 1998; Peng et al, 1998). Interestingly, we observed that T cell responses were significantly enhanced with the first boost at a short vaccination interval. But with the second boost, the responses reduced dramatically. It seems that the antibody induced by a previous immunization can interfere with T cell responses induced by the current vaccination. We speculated that the preexisting antibody can capture the new coming antigen then following an antibody mediated opsonization, which finally results in an invalid

boost. But immunization at short interval can overcome this due to insufficient time for antibody production, which can be definitely utilized in a T cell responses-desired assay. However, if we pursue both B cell and T cell responses induced by our combine vaccine, an appropriate time point for boost needs to be studied.

5.5 Optimize the vaccine with different strategies

5.5.1 The importance of linkers in the vaccine

In section 4.1.4, we showed that the E7-specifc T cell responses were significantly improved when we extended epitope $E7_{47-57}$ (RAHVYNIVTF) to $E7_{44-62}$ (QAEPDRAHVYNIVTFCCKCD). We suppose that this enhanced T cell response is attributed to the flanking sequences around CTL epitopes which facilitates proteasomal processing thus promoting the epitope presentation. Actually, several investigators have claimed that the design of linkers or flanking sequences between each epitope in their vaccines leads to an increased T cell responses (Bartkowiak et al, 2015; de Oliveira et al, 2015; Levy et al, 2007; Velders et al, 2001; Yamada et al, 2013). This is especially important if the vaccine harbors multi-epitopes. Remarkably, the linker "AAY (Ala-Ala-Tyr)" was widely employed in different vaccines. It was reported that the spacer residues AAY are preferred by proteasome for cleavage resulting in the correct epitope generation (Holzhutter et al, 1999; Nussbaum et al, 1998).

For example, one group (de Oliveira et al, 2015) developed a recombinant multi-epitope protein containing a string of immunogenic T cell epitopes of HPV16 E6 and E7 with AAY as spacers. An increased E6 and E7-specific immune responses were generated, which protected C57BL/6 mice from TC-1 tumor challenge. The group of Martin Kast designed a DNA vaccine consisting of CD8+ T cell, CD4+ T cell and B cell epitopes derived from HPV16 (Velders et al, 2001). The induced immune responses showed 100% protection for the mice against an otherwise lethal tumor challenge. Moreover, the separation of epitopes by AAY was proved very crucial for the therapeutic potential of the vaccine. A DNA multi-epitope vaccine containing HLA-restricted epitopes of HPV16 was evaluated in HLA-A*0201 transgenic mice. This vaccine elicited a T-cell response against multiple HPV16 epitopes, as well as significantly reduced tumor burden in mice (Eiben et al, 2002). We also tried to

express PADRE-Trx-(E7 trimers) with AAY as a linker between each E7 epitope. However, this protein cannot be expressed successfully in *E. coli*.

5.5.2 The design of multi-epitope vaccines

A multi-function vaccine is normally composed of three units: Adjuvant, CD8+ and CD4+ T cells epitopes. Except for spacer AAY, linkers such as HEYGAEALERAG, EAAAK and GPGPG were also applied to join different sections together (Nezafat et al, 2014). It has been claimed that AAY or HEYGAEALERAG motifs is employed between CTL epitopes. Helper epitopes are linked together via GPGPG. Adjuvant and CTL epitopes are conjugated together by EAAAK linker. Consequently, a designed vaccine embraces adjuvant, CD8+ T cell epitopes and CD4+ T cells epitopes in a string from N-terminal to C-terminal (Li et al, 2016a; Nezafat et al, 2014). This multi-epitope vaccine is able to gather a large number of helper epitopes and immunogenic CTL epitopes resulting in an improved therapeutic potential.

Thinking along this line, we would like to extend T cell epitopes with above mentioned linkers in our vaccine. For instance, we can graft the epitope PADRE in OVX313-antigens to enhance T-helper responses. And the usage of CTL epitope is not only restricted in HPV16 E7 protein. E6 and E2 are also expressed in the infected cells throughout life cycle. It was observed in both preclinical and clinical studies that the elimination of HPV-positive lesions is related to a specific immune response against E6 and E2 (de Jong et al, 2002b; de Jong et al, 2004; Farhat et al, 2009; Jacobelli et al, 2012; Selvakumar et al, 1995). Therefore, the E2 and E6 proteins present excellent therapeutic targets. It will be interesting to evaluate the therapeutic capacity of our vaccine with the expanded epitopes.

5.5.3 The fusion with organelle targeting signals

Another approach used to optimize the vaccine by improving antigen processing, MHC loading and epitope presentation is guiding proteins to a cellular compartment. For example, antigen of interest can be fused with ubiquitin to target it to the protein degradation pathway which promotes CTL precursor processing (Rodriguez et al, 1998). Other carrier proteins including calreticulin, FM4, heat shock proteins or herpesvirus glycoprotein D have shown efficacy in increasing CTL responses and antitumor immunity (de Oliveira et al, 2015). This gives us an inspiration to design our vaccine fused to a targeting sequence to expect a better antitumor activity.

5.6 Development of HPV prophylactic and therapeutic combined vaccine with a HLA-A2 restricted HPV16 E7 epitope

HPV16 E7 (11-19) was identified as HLA-A*0201 restricted epitope (Riemer et al, 2010). HPV prophylactic and therapeutic combined vaccine Trx-L2 8mer-flank $E7_{(49-57)}$-OVX313 showed a potent therapeutic potential in C57BL/6N mice. Based on this inspiring result, we have tried to develop a HPV vaccine that can induce humoral and cellular responses in a human genetic background. So we designed a vaccine Trx-L2 8mer-flank $E7_{(11-19)}$-OVX313 (flank $E7_{(11-19)}$ epitope is from 11-19 of E7 sequence with additional five amino acids afore and behind it.) and tested T cell responses in A2.DR1 transgenic mice which can express HLA-A2.1 and HLA-DR1 molecules (Pajot et al, 2004). Unfortunately, no E7-specific T cell responses were observed in IFN-γ ELISpot. There are two factors likely to limit the detection of anti-E7 T cell responses in A2.DR1 mice. One is that only one third of CD8+ T cells can be found in PBMCs (peripheral blood mononuclear cells) of A2.DR1 mice compared to wild type C57BL/6N mice. Besides, HLA-A2 restricted $E7_{(11-19)}$ epitope is thought to be not as immunogenic as $H2\text{-}D^b$ restricted $E7_{(49-57)}$ epitope (Kruse et al, 2018). These two reasons definitely can influence the intensity of T cell responses. Even though there is a T cell response induced by Trx-L2 8mer-flank $E7_{(11-19)}$-OVX313, but the level might be not enough for detection in IFN-γ ELISpot.

With the aim of enhancing the T cell responses against $E7_{(11-19)}$, we can add PADRE T-helper epitope in antigen Trx-L2 8mer-flank $E7_{(11-19)}$-OVX313. As known that the T helper pathway plays an indispensable role in cytotoxic T cell responses in most cases. PADRE may provide a positive influence on anti-E7 CD8+ T cell responses. Another strategy is to use HPV16 E7 protein instead of $E7_{(11-19)}$ epitope in the vaccine. The protein usually contains miscellaneous T-helper and cytotoxic T epitopes which could lead to an improved E7-specific T cell response. One potential issue in Trx-L2 8mer-HPV16 E7-OVX313 antigen is solubility which is strongly dependent on the protein size and structure. The insertion of E7 protein will definitely increase the size of the whole antigen. Moreover, several cysteines and zinc finger motifs contained in E7 make the purification of Trx-L2 8mer-HPV16 E7-OVX313 hard to predict. Another possible solution to achieve a better T cell response is synergistic use of PADRE-Trx-L2 8mer-flank $E7_{(11-19)}$-OVX313 and checkpoint inhibitors in A2.DR1 mice. As discussed above, the improved immune microenvironment by checkpoint blockades would promote CD8+ cytotoxic T cell responses.

Interestingly, 'OVX313-I5' which was identified in C57BL/6N mice as a T-helper epitope also showed a low but detectable T cell response in A2.DR1 mice. Besides, it was proved previously by our group that 'OVX313-I5' also induced T cell responses in BALB/c mice (Pouyanfard et al, 2018). It seems that 'OVX313-I5' peptide could work as a universal T-helper epitope in both mice and human MHC genetic background.

5.7 Concluding remarks

Our concept of developing vaccines went from simple to complex antigen design. We started to study the cytotoxic T cell responses against the OVA CTL epitope, later being replaced by the HPV16 E7 CTL epitope. The utilized scaffold for immunogens was from a monomeric structure to a multimerized platform. The function of the antigen was developed from therapeutic alone to a combined prophylactic and therapeutic efficacy. Ultimately, we obtained a heptameric HPV vaccine Trx-L2 8mer-flank $E7_{(49-57)}$-OVX313 which can induce HPV neutralizing antibodies, as well as perform a potent antitumor activity in C57BL /6N mice. But for vaccine development, a dilemma of deciding whether continue optimization or move forward with the current candidate always exists. Since our final aim is to translate the most promising antigen from animals to humans and performance of antigen Trx-L2 8mer-flank $E7_{(11-19)}$-OVX313 fell short of our expectations, more efforts need to be made on promoting T cell responses in a human genetic background.

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