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"Development of an OMV-based prophylactic vaccine against HPV: a Pan-HPV vaccine for cancer prevention"

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“Look at the stars,

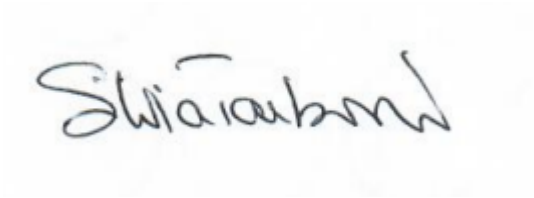
look how they shine for you”

Yellow - Coldplay

Declaration of authorship

I, Silvia Tamburini, confirm that this is my own work or work I have done together with other members of our group and the use of all material from other sources has been properly and fully acknowledged. Part of the work was done in collaboration with Prof. Martin Müller Lab – DKFZ – Heidelberg, Germany.

Trento, November 20th, 2023



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ABSTRACT

Human Papilloma Viruses (HPVs) are a large family of viruses with a capsid constituted by the L1 and L2 proteins, which bind to receptors of the basal epithelial cells, thus promoting virus entry. The majority of sexually active people become exposed to HPV, which is the most common cause of cervical cancer affecting more than 600.000 women every year. Moreover, every year more than 13.000 new cases of HPV-related cancers, including anal, penile and head and neck cancers, are diagnosed in men. Three vaccines are available based on the L1 capsid protein, which self-assembles and forms virus-like particles (VLPs) when expressed in yeast and insect cells. Although very effective, these vaccines are HPV type-restricted, and their costs limit broad vaccination campaigns, especially in low- and middle- income countries. Recently, vaccine candidates based on the conserved L2 epitope from serotypes 16, 18, 31, 33, 35, 6, 51 and 59 were shown to elicit broadly neutralizing anti-HPV antibodies, reaching a protection around 90% against all the HPV serotypes.

During my research activity, we have tested whether *E. coli* Outer Membrane Vesicles (OMVs) could be successfully decorated with L2 polytopes and whether the engineered OMVs could induce neutralizing antibodies. OMVs represent an attractive vaccine platform for their intrinsic adjuvanticity and their low production costs. We show that strings of L2 epitopes could be efficiently expressed on the surface of the OMVs and a polypeptide constituted by the L2 epitopes from serotypes 18, 33, 35 and 59 provided broad cross-protective activity against a large panel of HPV serotypes as judged by the *in vitro* pseudovirus neutralization assay.

In order to better characterize the vesicle and in perspective of future clinical studies of our HPV candidate vaccine, we also worked on the setting-up of a simple and reproducible production process at laboratory scale ready to be transferred at industrial level.

Moreover, we focused our attention on the strategy used for the engineering of the OMVs with the L2 epitopes and in particular on the carrier used for the delivery of the fusion construct in the surface of the vesicle. More in detail, since part of the carrier is a human cancer epitope, we tested whether a similar scaffold, with less homologies to the human gene could maintain the same properties in terms of: i) expression level of the fused epitopes in the OMVs, ii) localization on the surface of the vesicle and iii)

immunogenicity and efficiency to stimulate the immune system in order to produce anti-L2 antibodies.

Considering all the results described in this work combined with the points of strength of the OMV-based vaccine platform, as the simplicity of the production process, the yields of vaccine doses and the low cost/dose, our data provide a very promising prototype of universal anti-HPV vaccine.

Chapter 1

INTRODUCTION

1 .1 Human Papilloma Viruses

Human Papillomaviruses (HPVs) are a group of viruses included in Papillomaviridae family, which have been discovered in a wide range of vertebrates. Till now, 229 HPV serotypes have been classified[1], grouped in five different genera according to their tropism, either cutaneous or mucosal[2,3]. HPV mucosal serotypes can be also divided in “high-risk”, “probable high-risk” and “low-risk” serotypes, on the basis of their association to human tumors (see below). The “high-risk” serotypes include HPV16, 18, 31, 33, 35, 51, 52, 56, 58, 59, 68, 73 and 82[4]. Among the low-risk serotypes, the most frequent types are HPV6 and 11[4]. Moreover, in the recent years, the interest is focused also on cutaneous types which include HPV1, 2, 27 and 57 as the most frequent ones[5].

HPVs present a 50-55 nm non-enveloped icosahedral capsid with a small and circular double-stranded DNA genome of around 8 kb which encodes for eight different proteins (Figure 1A): six early (E) genes transcribed just after the infection and two late (L) genes necessary for the viral capsid formation[6].

The early genes are involved in impairing cell cycle control, viral DNA replication and immune system evasion[7]. Among them, E6 and E7 are two proteins involved in the carcinogenesis process due to their ability to bind p53 and Retinoblastoma (Rb) proteins, respectively[7,8]. E6 protein is mainly involved in the degradation of p53 leading

to the disruption of the apoptotic cycle and thus to the immortalization of the cells[9]. E7 protein is associated with the phosphorylation and the activation of the Rb protein[10], leading to a constitutive activation of E2F and all the downstream genes involved in the proliferation, inducing a transformation in already immortalized cells. Both actions are able to disrupt the activation of apoptotic pathway, promoting cell proliferation and leading to malignancy development[11].

The two late genes, L1 and L2 are the two structural proteins of the HPV capsid. They are designated major and minor, respectively, because the capsid contains more molecules of L1 than L2[6]. The L1 protein spontaneously forms pentamers, also known as capsomers, and 72 pentamers generate the skeletal of the HPV capsid[7,12]. The L1 protein was the one used for the classification of all the HPV serotypes into different branches of the phylogenetic tree. The L2, minor capsid protein, is able to occlude the center of each pentavalent capsomere, generating the final structure of the viral particle (Figure 1B). Its amino terminus contains cryptic epitopes that are broadly cross-neutralizing[6].

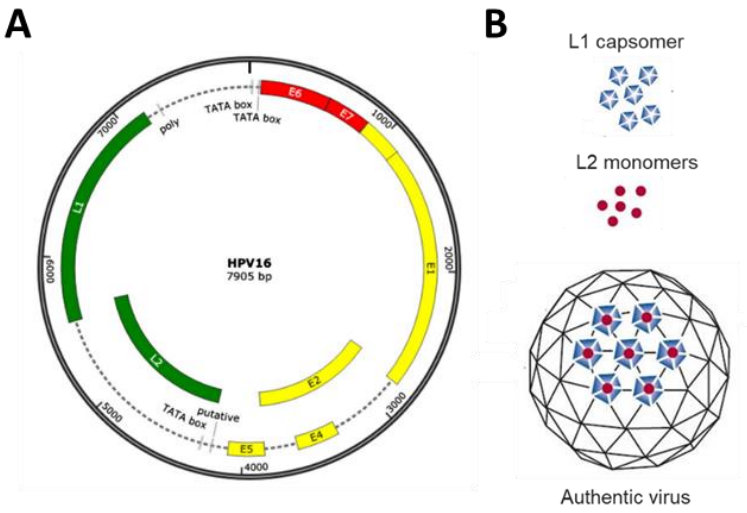


Figure 1 – HPV genome and structure. (A) The HPV16 genome is composed by seven different proteins, divided in early (E) and late (L) genes. Image from Ribeiro-Müller[13]. **(B)** The authentic virus is generated by the self-assembly of the L1 protein, generating 72 pentamers in the capsid structure, and a single copy of the L2 protein associated in the center of each pentamer. Adapted from Schiller & Müller[14].

1.1.1 Mechanism of HPV infection and HPV-mediated carcinogenesis

HPV accounts for the majority of sexual infections worldwide[6]. Almost all (85-90%) of sexually active women and men will acquire HPV at some point in their lives[15]. The viruses can infect the mucosa or the skin through tissue lesions[3] and reach the heparin sulfate proteoglycan (HSPG) receptors, a widely expressed and evolutionary conserved receptor allocated on the extracellular matrix (ECM) and on the surface of most cells, included basal keratinocytes[6,16]. The L1 protein binds the receptor, and the viral particles undergo a conformational change[8], allowing the exposure of the N-terminal region of the L2 protein[6,16], containing a highly conserved consensus recognition site for the furin protease. The cleavage of furin induces an additional conformational change of the virion, associated with the exposure of a secondary binding site on the L1 protein, necessary for the recognition with a specific cell receptor. At this point, the virus can be internalized in the basal layer of the mucosal cells (Figure 2).

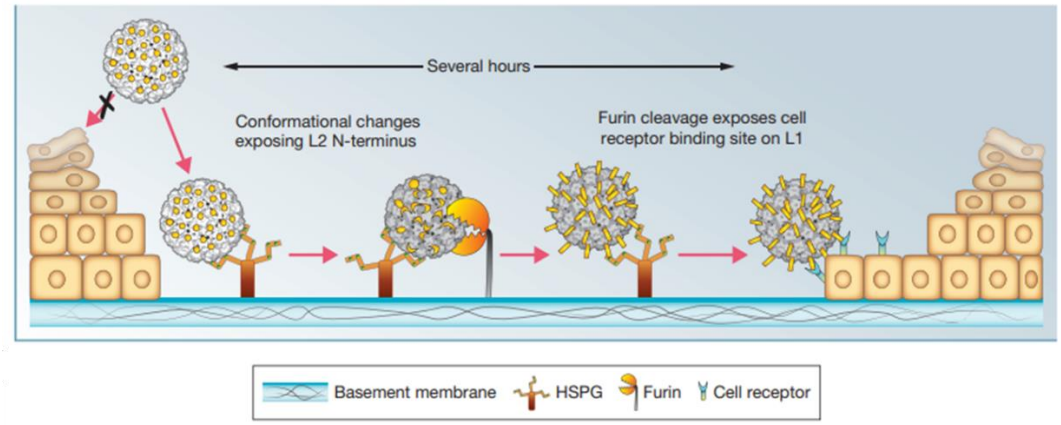


Figure 2 – HPV mechanism of infection. The virus reaching the basal membrane is able to bind the HSPG receptor. After a conformational change, furin protease cleaves the N-terminal region of the L2 protein, leading to an additional conformational change. The processed viral particle binds the specific receptor, allowing its entry in the epithelial cells. Image from Schiller, Lowy and Markowitz[6].

In many cases, the natural HPV infection is cleared by the immune system of the host, but in the 10% of the infected people, the virus persists, leading to the expression of the early genes[8]. The first hypothesis of the connection between HPV and cancer was postulated by Zur Hausen in 1977[17]. Some years later, in 1984, viral DNA was

identified in two different human cervical carcinomas[18]. This discovery allowed the scientific community to understand that the HPV genome can integrate into the human genome of 1% of infected people disrupting the open reading frame of different human genes and triggering cancer initiation[8]. Looking into the detail of the process, at the beginning of the localized infection, the number of viral genomes reach approximately 50–100 copies per cell in the basal epithelial cells[19]. This number of copies *per* dividing cells is maintained by the E1 and E2 proteins that bind to the viral origin of DNA replication and that are also fundamental for the initiation of replication. The expression of viral genes is minimal in the phases of plasmid or episomal DNA maintenance. In differentiated keratinocytes, the number of viral genomes *per* cells increases, reaching at least 1000 copies[20]. This leads to an increased expression of all the early genes of HPV genome, including those coding for E6 and E7 oncogenic proteins, which are involved in cellular proliferation, inhibition of apoptosis and genetic instability[21]. The L1 and L2 proteins are expressed in the superficial layers of the epithelium, and many thousands of viral genomes not integrated in human genome are encapsulated and infectious virus particles are shed[19], ready for a new cycle of infection (Figure 3).

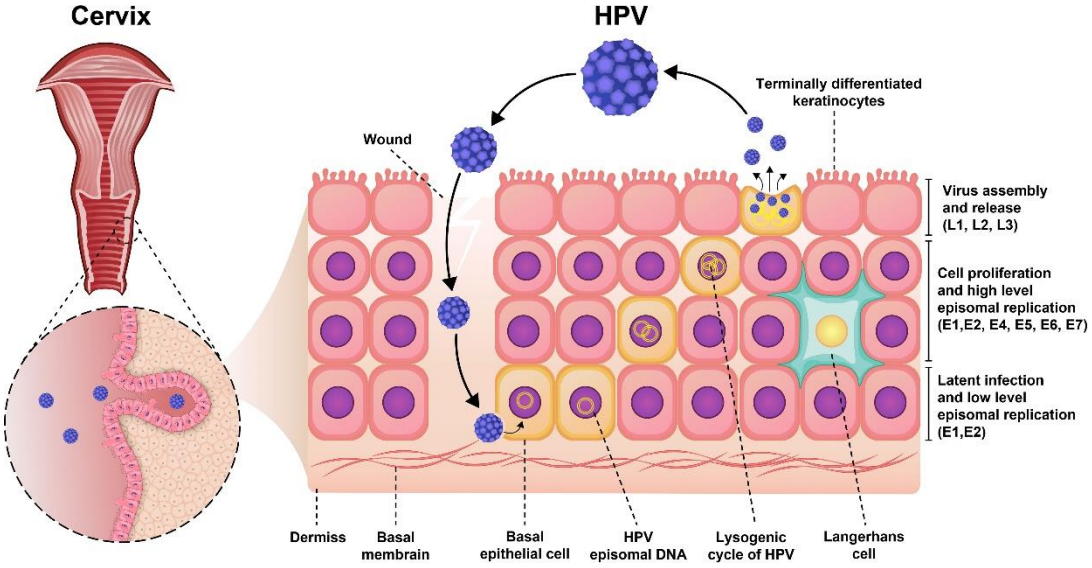


Figure 3 – Pathogenesis of HPV infection. Initially, the virus is latent inside the basal epithelial cells with a low proliferation rate. In the differentiated keratinocytes, the number of copies of viral genome *per* cells increases due to the high proliferation rate. Finally, the viruses are assembled with the expression of L1 and L2 capsid protein in the terminally differentiated cells and the particles are secreted from keratinocytes to repeat the infection cycle. Image from Yousefi[20].

During the infection process, the virus can start its lysogenic cycle, through which the viral genome is integrated into the human genome[19]. This process is induced also by the genomic instability generated by the high density of the viral DNA[21]. The integration of the genome, when occurred, is associated with the breakpoint in the E2 gene, resulting in the de-repression of the E6 and E7 viral oncogenes, leading to an extremely high expression of the two proteins they code for. The overexpression of E6 and E7 proteins give the cells a selective growth advantage and promote oncogenic progression, generating a positive feedback loop, thus stimulating more cells to integrate the viral genome and become transformant[21]. The entire process from infection to invasive cancer usually takes 1 to 3 decades[6]. It has been reported that approximately 10%–20% of individuals with a persistent cervical HPV infection showed a higher risk of cervical intraepithelial neoplasia (CIN) grade 2/3[2].

1.1.2 HPV-related cancers

Epidemiological studies showed that HPV, belonging to one of the serotypes 16, 18, 31, 33, 35, 51, 52, 56, 58, 59, 68, 73 and 82, is present in 99% of all cervical cancers, identifying the virus as the main etiological factor for cervical carcinoma[8] (Figure 4).

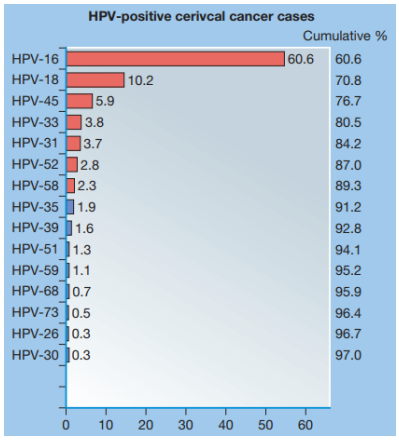


Figure 4 – Single and cumulative percentage of each oncogenic HPV serotype in the entity of invasive cervical cancer. Image from Plotkin's Vaccines[22].

Cervical cancer is characterized as the most common HPV-related malignancy[2]. This tumor type represents the 4th most common death cause among women worldwide[8], with 604.127 cervical cancer cases and more than 341.000 deaths globally as reported in 2020[23]. The virus was also detected in 91% of anal cancer, 63% of penile cancer, 75% of vaginal cancer and 69% of vulvar caricinomas[8,15] as presented by the Centers of Disease Control and Prevention of the USA. Moreover, recent studies also showed an increase in HPV-induced head and neck cancers, with particular interest in oropharyngeal cancer, notably squamous cell carcinomas arising from tonsils and base of tongue[24]. At this regard, Zhang and collaborators presented a projected association of HPV vaccination with oropharynx cancer incidence in the next decades[25]. In particular, the study was designed recruiting around 70.000 patients diagnosed with oropharynx cancer and treated with HPV vaccination between 1992 and 2017. They showed that under the same vaccination rate reached in our days, the diagnosis of this cancer will decrease till 2045 in younger adults, but not in older ones. This data suggest that continued efforts are needed to increase HPV vaccine uptake at younger ages among men and women to achieve population-level vaccine benefits[25].
Collectively, nowadays HPV-related tumors represent approximately 5% of all the cancers worldwide[26], with the most attributable cases reported in women and with half of the cases occurring before the age of 50 years[27].

Nowadays, thanks to the discovery of effective anti-HPV vaccines (See section 1.2.3) and to the extensive vaccination campaigns in many industrialized countries the incidence of HPV-associated tumors in women is declining, even though solid epidemiological data are still missing, considering the relatively recent introduction of the vaccination campaigns and the long elapse time between viral infection tumor diagnosis. However and not surprisingly, the number of cases is still very high in developing countries, in Sub-Saharan Africa, in Eastern European and in Latin America[8], where vaccination is still poorly utilized (Figure 5). Importantly, HPV-related anal and penile cancers have increased among men between 1962 and 2015, highlighting the need to include also men in global vaccination campaigns[28].

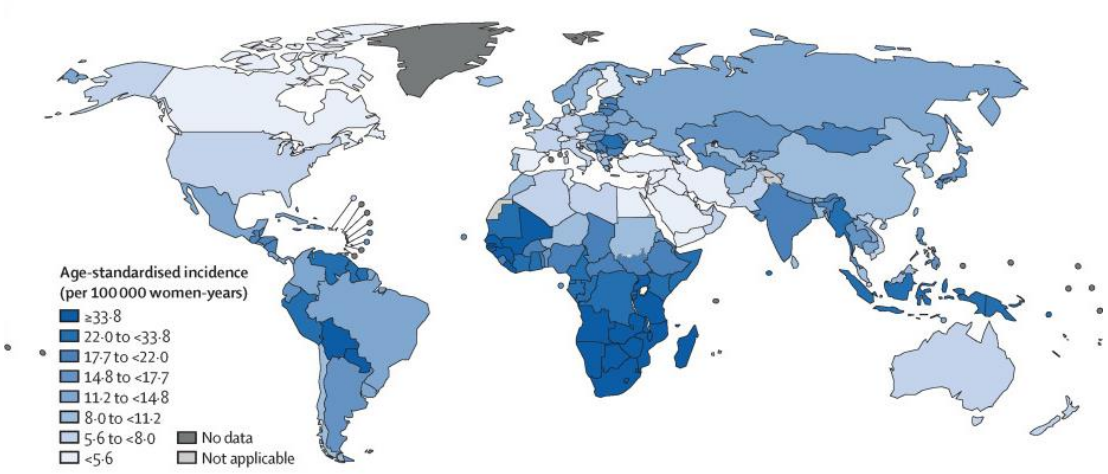


Figure 5 – Age-standardized incidence of cervical cancer by country in 2020. Image from Singh[23].

1.1.3 HPV-related genital warts

Even if not directly correlated with cancer, anogenital warts are the most common clinical manifestations of the HPV infections known worldwide[29]. Induced by low-risk HPV serotypes, anogenital wart worldwide incidence is estimated between 100 and 200 new cases per 100.000 individuals in the adult population[30,31]. HPV6 and HPV11 account for the majority of the anogenital warts[32], reaching approximately 90% of the reported cases[33]. Also, other HPV serotypes, including HPV42, 43 and 44, are associated with the development of these lesions in genital tracts, even at lower prevalence.

The social and economic impact of mucosal infections is of great concern for the society[29], but more importantly, these lesions are associated with an altered quality of life. For this reason, the HPV-related genital warts were a clear medical need, making necessary the introduction of the most frequent associated serotypes in the vaccine.

The quadrivalent Gardasil-4 vaccine and the upgraded Gardasil-9 vaccine (see Section 1.2.3) show high efficacy in preventing the onset of the lesions associated to the vaccine strains. However, the vaccine is not available worldwide due to the expensive production process and the difficulties in the delivery process (see Section 1.2.3), and the available vaccines are not effective in people already infected by HPVs[14].

1.2 Prophylactic virus-associated cancer vaccines

The extraordinary impact on human health of vaccination against infectious diseases and the realization that the immune system plays a key role in continuously recognizing and eliminating transformed cells with tumorigenic potential have stimulated an intense research activity to develop vaccines that could prevent tumors. The rationale behind this strategy stems from the notion that tumors differ from normal tissues by virtue of the fact that they express different profiles of proteins, either in a qualitative or a quantitative term, or both. Therefore, the knowledge of which alterations are associated with specific tumors should allow the design of vaccines, which, upon administration to healthy individuals, would elicit immune responses capable of preventing future tumor development[34]. Although theoretically feasible, the development of prophylactic cancer vaccines turned out to be much more complicated than originally thought. The main reasons are (i) the existence of a very limited number of “public” mutations (mutations that are conserved in a large panel of patients carrying the same tumor type), (ii) the paucity of tumor-specific antigens (most of the tumor antigens are tumor-associated, meaning that they are expressed also in normal tissues though at lower concentrations), and (iii) the unacceptably long time of Phase 3 studies needed to demonstrate vaccine efficacy.

All this said, there are two extremely effective prophylactic cancer vaccines against hepatocellular carcinoma and cervical cancers and, not surprisingly, both of them were developed thanks to the fact that such cancers derived from viral infections (HBV and HPV, respectively[35,36]). The HBV vaccine was discovered by William J. Rutter in the 80s of the last century, and nowadays is broadly utilized (in 2019, the HBV vaccine reached a coverage of 85% worldwide[37]). The HPV vaccines became commercially available in 2007 and have the potential to annually prevent >500.000 cervical, anogenital, and oropharyngeal cancers worldwide[38].

1.2.1 Preclinical development of HPV vaccines

As it is the case for many infections caused by intracellular pathogens, HPV infections can be prevented in the presence of antibodies, which neutralize viral entry in the host cells. The role of neutralizing antibodies in HPV infections was first suggested in the late 1980s. Different works demonstrated that cattle can be protected against BPV infection by humoral responses elicited by immunization with either a recombinant

BPV-1 DNA vaccine expressing the major capsid protein[39] or *E. coli*-derived L1 protein[40]. Moreover, few years later, the role of neutralizing antibodies was confirmed in canine oral PV (COPV)-induced lesions[41]. Beagle dogs immunized with COPV L1 Virus-like particles (VLPs), purified from insect cells transfected with recombinant baculovirus, were completely protected from papilloma formation. This data was confirmed also by other experiments performed in rabbits. These animals are frequently infected by the cottontail rabbit papilloma virus (CRPV), which can cause cutaneous lesions. Rabbit immunization with VLPs expressing either L1 alone or L1 and L2 capsid proteins induces high neutralizing antibody titers[42]. Moreover, passive transfer of sera from rabbits immunized with CRPV L1-L2 VLPs fully protected animals against papilloma virus warts, while the ones which received pre-immune sera developed HPV-infection[42]. Finally, the effective role of the neutralizing antibodies was confirmed in mice which were protected from the mucosal challenge with pseudovirion particles after passive transfer of the sera from animals immunized with VLPs carrying L1 and L2 from the human serotypes[43,44].

Overall, these data confirm that the neutralizing antibodies are necessary and sufficient to confer protection against papilloma virus infections[6].

1.2.2 Mechanisms of protection of HPV-specific antibodies

The vaccine studies in the animal models clearly indicate that systemic immunization confers protection at mucosal sites. An interesting question is how serum antibodies can reach the mucosa at concentrations sufficiently high to prevent virus infection. Transudation from the vasculature was originally thought to be the main mechanism, even though it is known that the concentrations of IgGs released from the bloodstream to the mucosal sites are one-two orders of magnitude lower than the serum IgG concentration[45]. It was subsequently demonstrated that the many micro-lesions normally occurring on the genital mucosa represent the main source of mucosal antibodies. Exudation-dependent antibody release explains why mucosal antibody titers were not affected by the menstrual cycle[6] .

The understanding of the mechanisms of IgG deposition on the genital tract convinced vaccine developers that the intramuscular immunization with HPV vaccines could be highly effective at inducing protective titers of both serum and mucosal antibodies[46].

As far as the nature of neutralizing antibodies is concerned, most of the anti-L1 antibodies bind the L1 region which interact with HSPG receptors at the basal membrane of the epithelium (Figure 6A). Moreover, other neutralizing IgGs bind the viral particle before its interaction with cellular receptor expressed on the surface of keratinocytes[22] (Figure 6B).

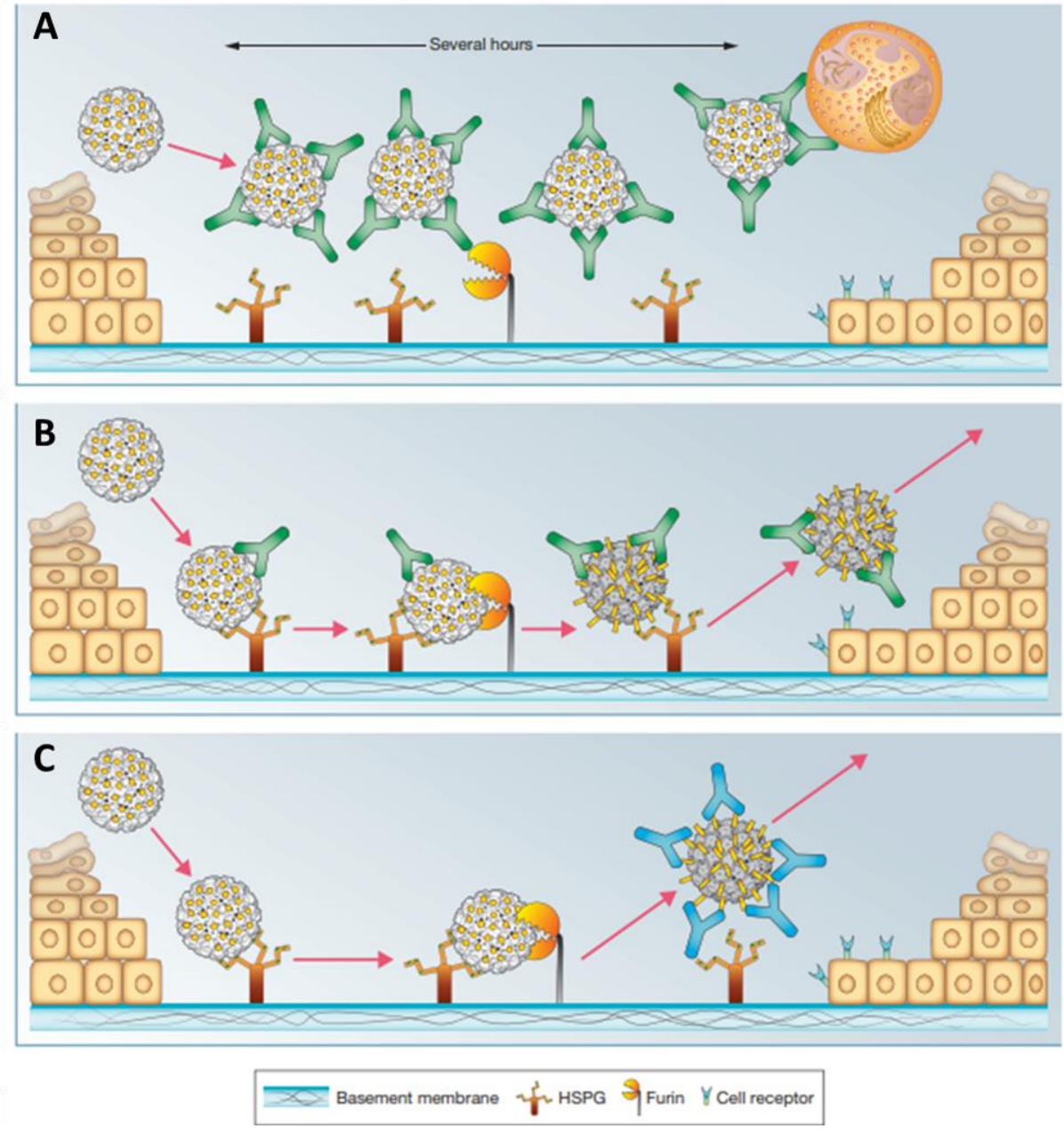


Figure 6 – Role of antibodies in HPV infection. **(A)** High level or **(B)** low levels of anti-L1 antibodies (green) can bind the virus at any moment when the particle is in solution, avoiding the binding with the **(A)** HSPG receptor or **(B)** keratinocytes receptor. **(C)** Anti-L2 antibodies (blue) can bind the viral particle only after the furin cleavage at the N-terminal of the L2 protein, impeding the binding of the viral particle with the keratocytes receptor. Adapted from Plotkin's Vaccines[22].

As previously described, another protein is present in the capsid, the minor capsid protein L2, which can also be the target of neutralizing antibodies[43,47]. N-terminal region of the L2 protein becomes exposed on the capsid surface as a consequence of L1 binding to HSPG receptors. Once exposed, this region of L2 is cleaved by furin, leading to the exposure of the cryptic neutralizing epitope, recognized by the anti-L2 antibodies[14,48,49] (Figure 6C), blocking the transfer of the particle from basement membrane HSPGs to the keratinocyte surface receptors. Also, antibodies preventing the protease from cleaving L2 would be highly effective in neutralizing virus entry in the cells, impeding the second conformational change and the binding with the cell specific receptor. However, till now no data have been reported demonstrating the presence of anti-L2 neutralizing antibodies in HPV-infected patients[44].

1.2.3 L1-based vaccines against HPV

Nowadays, three different prophylactic vaccines against HPV (Cervarix, Gardasil-4 and Gardasil-9) are available on the market. These vaccines are based on the L1 protein and take advantage of the fact that, when expressed in the cytoplasm of yeast and insect cells, L1 self-assembles to give rise virus-like particles (VLPs) morphologically and antigenically highly similar to the native virion[50] . Moreover, VLPs do not contain viral DNA and therefore they are non-infectious[51]. Immunologically, VLPs elicit durable responses even higher than the ones expected for a protein subunit vaccine[38].

Importantly, even though these vaccines are now classified as prophylactic cancer vaccines, their development was possible thanks to the fact that HPV-associated cancers are consistently preceded by mucosal lesions (CIN2 and CIN3), which occur soon after infections and several years before invasive carcinomas. Indeed, the vaccines are registered for preventing CIN2/CIN3 lesions and solid epidemiological data on the incidence of vaccination on cancer reduction will be available in the years to come[22].

In 2006 Merck licensed Gardasil-4, a quadrivalent vaccine composed by the L1 capsid protein of HPV16, 18, 6 and 11 formulated with aluminum salt and manufactured purifying L1-based VLP from recombinant yeast cells. This vaccine was associated with high efficacy (98-100%) in protection for $CIN2^{+}$ caused by HPV16 and HPV18 in

women DNA-negative for HPV vaccine serotypes and in HPV-naïve women[22]. Moreover, Gardasil-4 is effective also for genital warts caused by HPV 6 and HPV 11[52]. Almost simultaneously (2007), GSK introduced on the market the bivalent Cervarix vaccine. Cervarix is composed of VLPs produced in insect cells and carrying the L1 proteins from HPV16 and 18. Differently from the Merck' vaccine, Cervarix uses AS04 as adjuvant, a monophosphoryl lipid A-based adjuvant, which stimulates cell-mediated immunity[6,43]. In Phase III trials, Cervarix turned out to be very effective in protecting against CIN2$^+$ lesions, reaching 98% efficacy in women DNA-negative for vaccine serotypes and in HPV-naïve women[22], while there was no protection against genital warts induced by HPV6 and HPV11[52]. The rationale for using ASO4 as adjuvant was that, as it is the case for many viral infections, a Th1-skewed immune response should be important to optimally neutralizing HPV. The advantage of using ASO4 instead of Alum remains to be fully demonstrated. The data so far available indicate that Cervarix provides solid protection against CIN2$^+$ lesions for up to 9.4 years as opposed to the 7 years with Gardasil-4.

Seven years later, Merck launched an optimized version of Gardasil, Gardasil-9[22,53], which includes the VLPs from HPV31, 33, 45, 52 and 58[6,14]. This formulation extended the coverage against other "high risk" serotypes[54] (Figure 7).

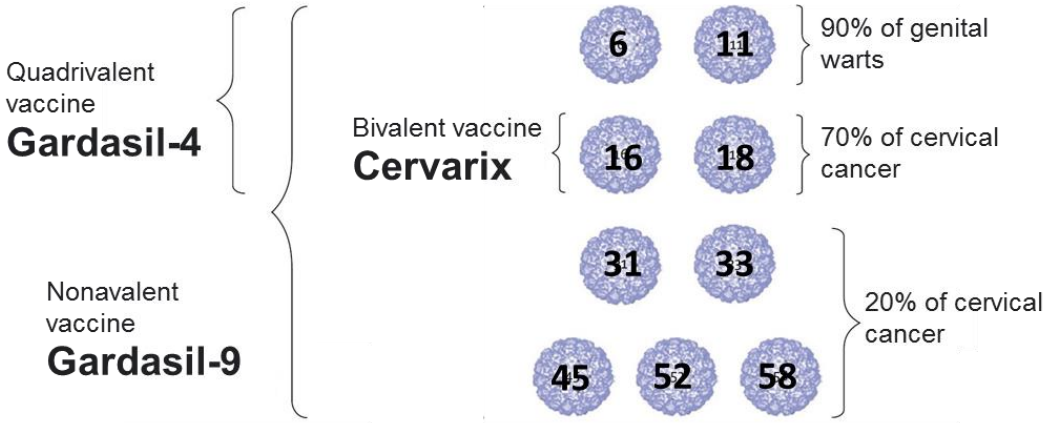


Figure 7 – Schematic representation of the three available vaccines on the market. Bivalent Cervarix constituted by VLPs of HPV16 and HPV18. Quadrivalent Gardasil-4 included also VLPs of HPV6 and HPV11, while nonavalent Gardasil-9 is implemented with VLPs of HPV31, 33, 45, 52, 58. Adapted from Schiller and Müller[14].

Regardless the vaccine used, the normal vaccination schedule consists in the administration of 3 doses over a period of 6-8 months[51], even though now different

studies are investigating the protective efficacy of one- and two-dose vaccination schedules[22]. Should the current promising results be confirmed, the introduction of shorter vaccination schedules could facilitate the introduction of vaccination campaigns (currently covering 12% of the population worldwide) even in the low-income countries where the incidence rate of cervical cancer is the highest[53].

Importantly, as anticipated, we have to consider that the manufacturing process of these vaccines is expensive. VLPs are purified from recombinant yeast or insect cells and the purification involves the use of chromatographic steps after dissociation into pentamers and subsequent reassembly into VLPs[6]. In addition, a cold chain is required for the stability of the product[14], making the vaccine distribution to low- and middle-income countries often impractical. It was estimated that the final cost of a single dose of the Gardasil-4 vaccine is around 30 times compared to the cost of a single dose of the measles vaccine[55].

Different and innovative vaccine platforms that allows high yields of vaccine at low costs are now being tested. Such platforms include the use of *E. coli* as factory for VLPs. For instance, Xiamen Innovax Biotech (Xiamen, China) produce VLPs in *E. coli* using proprietary L1 mutants and phase 3 clinical trials are in progress[56]. L1 monomeric protein has also been produced in recombinant *E. coli* strains, and the purified protein has been shown to induce neutralizing antibody titers in mice[57].

The main drawback of the L1-based vaccine is the low cross-protection due to the limited conservation of the neutralizing epitopes among the different serotypes. For this reason, the scientific community is focusing on the development of new vaccine prototypes based on more conserved regions of the virus, such as the conserved epitopes present in the minor capsid protein.

1.2.4 Candidate L2-based vaccines against HPV

The L2, the minor HPV capsid protein, is present with up to one copy per L1 capsomere, and a maximum of 72 copies per virion[6]. L2, a 500 amino acid protein whose structure has been fully characterized, is required for infection since it facilitates the encapsulation of the viral genome inside the HPV capsid and the exposure of the L1 receptor binding region[43]. No natural neutralizing anti-L2 humoral responses have been demonstrated till now, and immunization with L1 plus L2 VLPs does not induce such responses either. However, it has been discovered that regions at the N-terminus

of the protein, which become exposed upon viral binding to HPSG receptor (see above), contain a cryptic neutralizing epitope able to induce broad protection against different HPV types[58–60] and across species[61] (Figure 8).

This is an interesting property since, in contrast to the conformation-dependent L1 epitopes, immunizations with linear and conserved regions of the minor capsid protein L2 elicit broadly neutralizing responses[62]. Different studies were conducted in order to express the full-length L2 protein or part of the protein in multiple ways, included decoration of adenovirus[63], adeno-associated virus[64], bacteriophages[65] or bacteria or finally the use of linearized L2 concatemers or scaffolded in specific structure[58]. L2 vaccines have shown protection in mouse, rabbit, and calf challenge models, and protection can be passively transferred with immune sera[66].

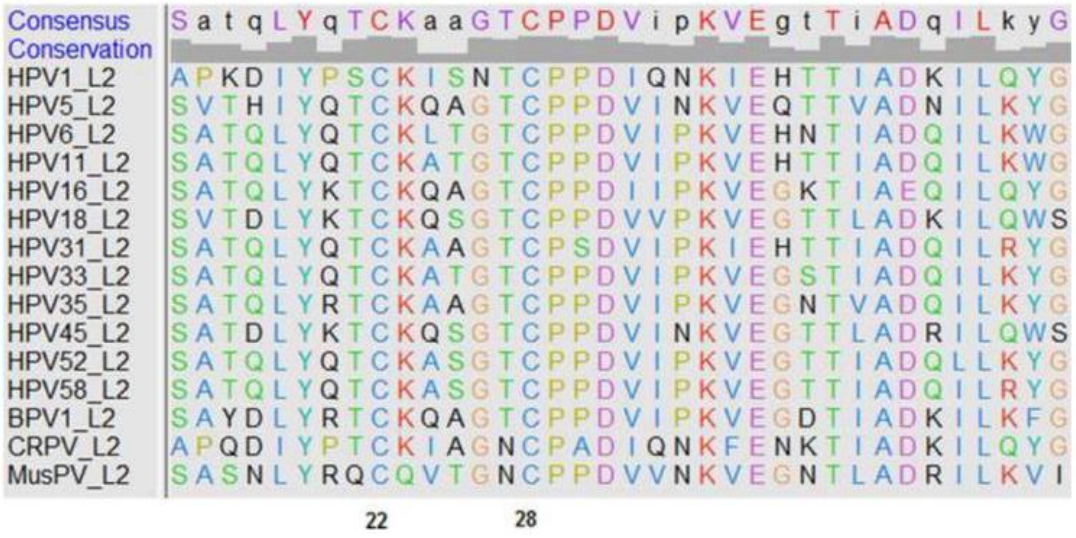


Figure 8 – Sequence alignment of the N-terminal part (aa 14-53) of the L2 protein of different Papillomavirus types (Human, Bovine, Cottontail Rabbit and Murine). Image from Wang[67]**.**

To identify the most immunogenic region of the L2 protein, Jagu and co-workers generated recombinant *E. coli* strains expressing multimeric L2 constructs coding for different amino acid regions (17-36 x 22, 11-88 x 5 and 11-200 x3) of a variety of HPV serotypes. This study allowed to conclude that the first 88 residues at the N-terminus of the protein contain important neutralizing epitopes[68]. In particular, the region comprising amino acids 20 to 38 ($L2_{20-38}$) has been identified as the most immunogenic one[58], not only for the induction of neutralizing antibodies, but also with regard to cross-protection[61,69,70]. This major cross-neutralizing epitope ($L2_{20-38}$) contains two cysteine residues (Cys 22 and Cys 28) highly conserved in the majority of known PVs. These

cysteine residues are buried, and disulfide bonded in mature HPV virions, and it has been suggested that the regulation of this disulfide bond may be critical for HPV infectivity in human cells[71]. Campos and coworkers showed that the HPV particles, which present mutations in these two specific amino acids (single or double), completely lose infection capability[71]. Not surprisingly, the role of these two cysteines is crucial also for L2-based vaccine efficacy: constructs that were fully reduced just before administration were less effective than the oxidized antigens at eliciting neutralizing responses, especially with regard to cross-protection[72]. Moreover, L2-neutralizing mAbs preferentially bind the oxidized L2 epitope[72].

Different studies showed that mouse immunization with vaccines constituted by the L2 epitope from a single HPV serotype[68,73,62] or by strings of the L2 epitope derived from multiple serotypes[44] elicited robust neutralizing antibody responses. In particular, the use of these concatenated multiple L2 fusion proteins was proposed as a Pan-HPV vaccine due to its high cross-neutralization capability[68]. L2 vaccines were shown to protect against cervicovaginal challenge by heterologous types in rabbit cutaneous and oral models and in mouse cutaneous and cervicovaginal challenge models[49,74,75].

However, L2-based vaccines presented some limitations. Firstly, the titers obtained with these candidate L2 vaccines are lower than the ones obtained with L1 vaccines[44,68]. Although the neutralization titers elicited by the L2 vaccines are above the threshold necessary and sufficient to protect against HPV infections (as judged by the neutralizing antibody titers induced by natural infection[22]), it could be difficult to introduce new vaccines which do not respect the “non-inferiority” rule. Second, it is not yet known the longevity of the protection induced by the L2-based vaccines. Animal studies have shown that the L2-based vaccines are still protective after one year from the immunization, as judged by the pseudovirions of HPV16 challenge[76] but additional studies are needed.

Third, the production and purification process of the vaccines based on recombinant proteins is time- and cost-consuming and the cold chain is expected to be required to present stability during distribution and storage.

Altogether, these limitations point to the need of developing new approaches to optimize the L2-based vaccines.

1.3 Bacterial outer membrane vesicles

Outer membrane vesicles (OMVs) are closed, spheroid particles of 50-300 nm in diameter which are produced by pathogenic and non-pathogenic Gram-negative bacteria, through a “budding” out process from the bacterial outer membrane during their growth[77] (Figure 9).

The process of OMV production performed by Gram-negative bacteria can be altered by multiple external factors like pH, temperature, oxidative environment, nutrient availability and different stress conditions[78,79].

OMVs are composed by outer membrane and periplasmic components including lipopolysaccharides (LPS), lipoproteins and periplasmic prioteins[80,81].

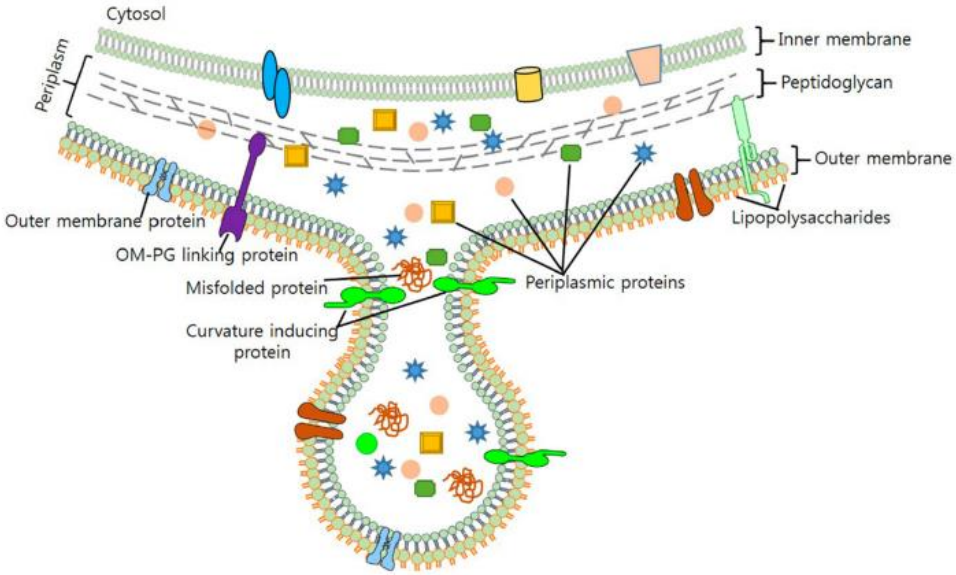


Figure 9 – Biogenesis of OMV production in Gram-negative bacteria. Periplasmic proteins and peptidoglycan are entrapped in the lumen of the vesicle, while outer membrane proteins are allocated in the OMV membrane. Image from Caruana et al.[82]

Bacteria exploit the vesicles for multiple biological functions, including inter and intra species cross talk, biofilm formation, defense against host immune response and toxin delivery to host cells[80,83,84]. Vesiculation is also used by bacterium as a way of constitutively expelling substances such as toxic molecules or misfolded proteins[84].

In the last years, OMVs become increasingly attracting for a novel vaccine platform for three main characteristics.

First, OMVs carry several bacterial elements, such as LPS, lipoproteins and peptidoglycan, classified as pathogen-associated-molecular patterns (PAMPs) which

allow the stimulation of the immune system[85]. Moreover, OMVs can elicit potent Th1-skewed immune response[86–88], a type of immunity which is required to fight intracellular pathogens and cancer. OMVs are non-replicative particles thus avoiding the risk of vaccine-associated infections[89].

Second, the OMV protein content can be easily manipulated by genetic manipulation of the OMV-producing bacteria. In fact, OMVs can be engineered with heterologous antigens either expressed in the lumen of the vesicle or exposed from the surface[90–92]. This feature was demonstrated for the first time by Kesty and Kuehn who showed the possibility to incorporate heterologous proteins in the OMV lumen[92]. Following this observation, an increasing number of heterologous proteins have been successfully delivered to OMVs using a variety of strategies[91,93]. In our laboratory, different bacterial antigens were delivered to the lumen or exposed on the surface of *E. coli* vesicles by fusing their coding sequences to a leader peptide for secretion[87].

Third, the simplicity and the low costs of production processes make OMVs ideal vaccines for the low- and middle- income countries.

The unique characteristics and proprieties of the OMV-based vaccine have been already exploited for the development of vaccines for human use. The MenB vaccine composed by *Neisseria meningitidis* OMVs is licensed by GSK (Bexsero)[94] and other candidate formulations based on OMVs are now in clinics[95,96].

1.3.1 *E. coli* strains releasing "proteome minimized" OMVs

We have recently developed an innovative and unique vaccine platform based on OMVs released by non-pathogenic *Escherichia coli (E. coli)* strains. Non-pathogenic *E. coli* represents an attractive organism as a factory for OMV-based vaccines. Unfortunately, the wild-type strains present some limitations: i) they produce low amount of OMVs, ii) they release vesicles rich of endogenous proteins and iii) the derived OMVs carry wild-type, highly reactogenic LPS.

With our platform we have overcome these limitations. In particular, through a CRSPR/Cas9 Synthetic Biology approach, we have created a panel of genetically modified *E. coli* strains deprived of a large set of endogenous proteins. In particular, the workhorse "proteome minimized" strain, called *E. coli* BL21(DE3)Δ60[97] (Figure 10), releases OMVs ($OMVs_{\Delta 60}$) deprived of 60 endogenous proteins.

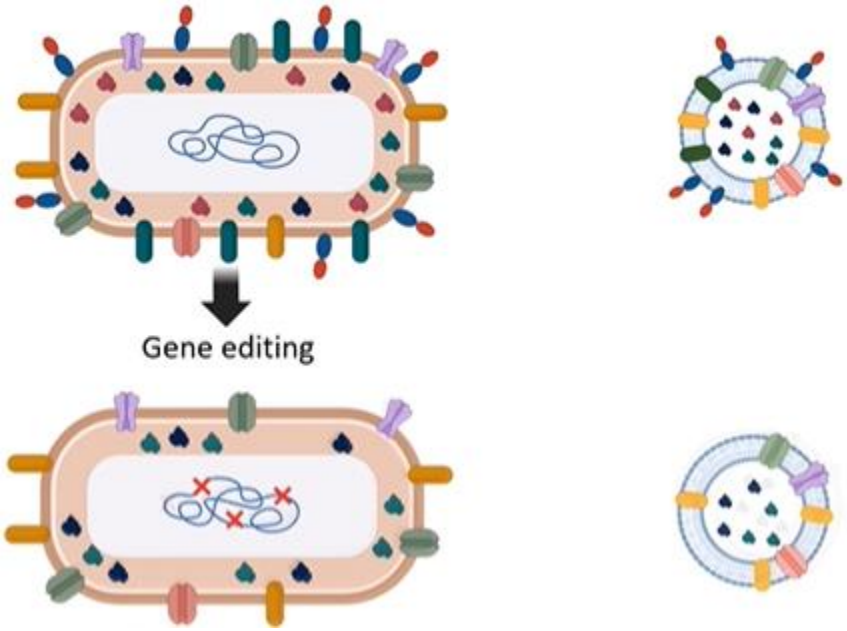


Figure 10 – Construction of *E. coli* BL21(DE3)Δ60 releasing proteome minimized OMVs. Genes encoding proteins present in wild type *E. coli* BL21(DE3) (upper) have been sequentially inactivated, generating *E. coli* BL21(DE3)Δ60 (lower) which releases OMVs carrying a reduced number of proteins. Image from website www.biomvis.com.

This strain has been genetically reprogrammed to acquire characteristics that make it an ideal factory in terms of immunogenicity and safety for human vaccine application. Our *E. coli* BL21(DE3)Δ60 presents many advantages. Firstly, our strain presents ipervesiculating capabilities. Under laboratory conditions, the OMV production yield can exceed 50 mg/L. Moreover, the elimination of dispensable endogenous proteins has substantially increased the loading capacity of the vesicles with respect to recombinant proteins[97], which can represent up to 30% of the total OMV proteins. This guarantees a high immunogenicity even with minute quantities (<1 μg) of OMVs *per* vaccine dose[97]. Finally, we also worked on the reactogenicity and safety of our vaccine and in particular, acting on the LPS pathway[97], we obtained OMVs with a “detoxified” LPS. Such modified LipiLPS carries a pentacylated lipid A, featuring excellent adjuvanticity proprieties, but highly reduced reactogenicity.

In parallel, our laboratory developed a panel of strategies for rapidly and effectively decorating the OMVs with heterologous antigens, including bacterial antigens, viral antigens or cancer antigens[98].

1.3.2 OMV engineering

Different strategies have been developed in our laboratories to express heterologous antigens in the lumen or in the membrane of the vesicle[99,100]. As reported by Fantappiè et al.[101], the fusion of a heterologous protein with the leader peptide of a lipoprotein was identified as a valid strategy to translocate it into the membrane of the vesicle[99]. Alternatively, proteins/protein domains are fused to the C-terminus of selected carrier proteins, thus allowing their compartmentalization in the lumen or in the membrane of the vesicle[98,100,102].

Lipidated antigens not only can accumulate in the vesicular compartment at concentrations as high as 20-30% of total OMV proteins[101,103] but they are also often exposed on the OMV surface. This is an unexpected result considering that essentially all natural *E. coli* lipoproteins (approximately 100 as predicted by genome analysis) face the periplasmic compartment, anchored to either the inner or the outer membrane[101]. For example, the lipoprotein factor H binding protein (fHbp) of *N. meningitidis* which is transported on the cell surface of *E. coli* and protrudes out of the membrane vesicle[101]. We also showed that fHbp can be exploited as a carrier protein. Fantappiè et al. described a highly expressed and surface-exposed fusion construct formed by the N-terminal domain of fHbp (fHbp-DomA) with the repeated epitope "variable number of tandem repeats region (VNTR)" of MUC1 protein[101]. MUC1 is a human tumor-specific antigen which is highly expressed in a wide range of tumor tissues, including head and neck cancers[104,105], and HPV^+ cervical carcinomas[106]. Interestingly, MUC1 elicits a strong antibody response, and, in addition, it carries MHC I and on MHC II restricted epitopes $CD8^+$ T cells and $CD4^+$ T cells, thus leading to both cellular and humoral immunity[107].

AIM OF THE THESIS

The overall objective of my PhD project is to demonstrate the feasibility of developing a broadly protective HPV vaccine based on OMVs engineered with selected strings of conserved L2 epitopes.

The project is organized in four main lines of activities:

1. Demonstration that single and multiple copies of the L2 epitope ($L2_{20-38}$) from different HPV serotypes can be efficiently expressed on the surface of the *E. coli* OMVs

2. Demonstration that OMVs engineered with the L2 epitopes can elicit high L2-specific antibody titers in mice

3. Demonstration that L2-specific antibodies can neutralize HPV infection using an *in vitro* pseudovirus infectivity assay

4. Optimization a production process for the OMV-based HPV vaccine, as a first step for the preparation of a Good Manufacturing Practice (GMP) vaccine formulation to be used in future clinical trials.

Chapter 2

MATERIALS AND METHODS

2.1 Bacterial strains and cultures

E. coli HK100 strain was used for cloning experiments using the polymerase incomplete primer extension (PIPE) method[108]. The newly generated plasmids of interest were transformed in *E. coli* BL21(DE3)Δ60 strain[97]. For each recombinant strain, a stock preparation (Master Seed) in Luria Bertani medium (LB) (Sigma-Aldrich, St. Louis, MO, USA) + 20% glycerol was prepared from an overnight (ON) culture and stored at -80°C. Bacteria were grown in LB and when required ampicillin or chloramphenicol was added to a final concentration of 100 μg/ml and 30 μg/ml, respectively.

2.2 Cloning of the L2 epitopes

Figure 2.1 shows the strategy used to clone the L2 epitopes fused at the N-terminal domain of Factor H binding protein from *N. meningitidis* (*Nm*fHbp). All the primers used for the PCRs were purchased from Metabion (Planegg, Germany) and they are summarized in Table I. Briefly, plasmid pET_Nm-fHbpDomA_MUC1[101], coding the MUC1 peptides fused to the C-terminus of the Domain A of fHbp (amino acids 1-120), was PCR-linearized with the primers pET_F and MUC3x_R (Figure 11A).

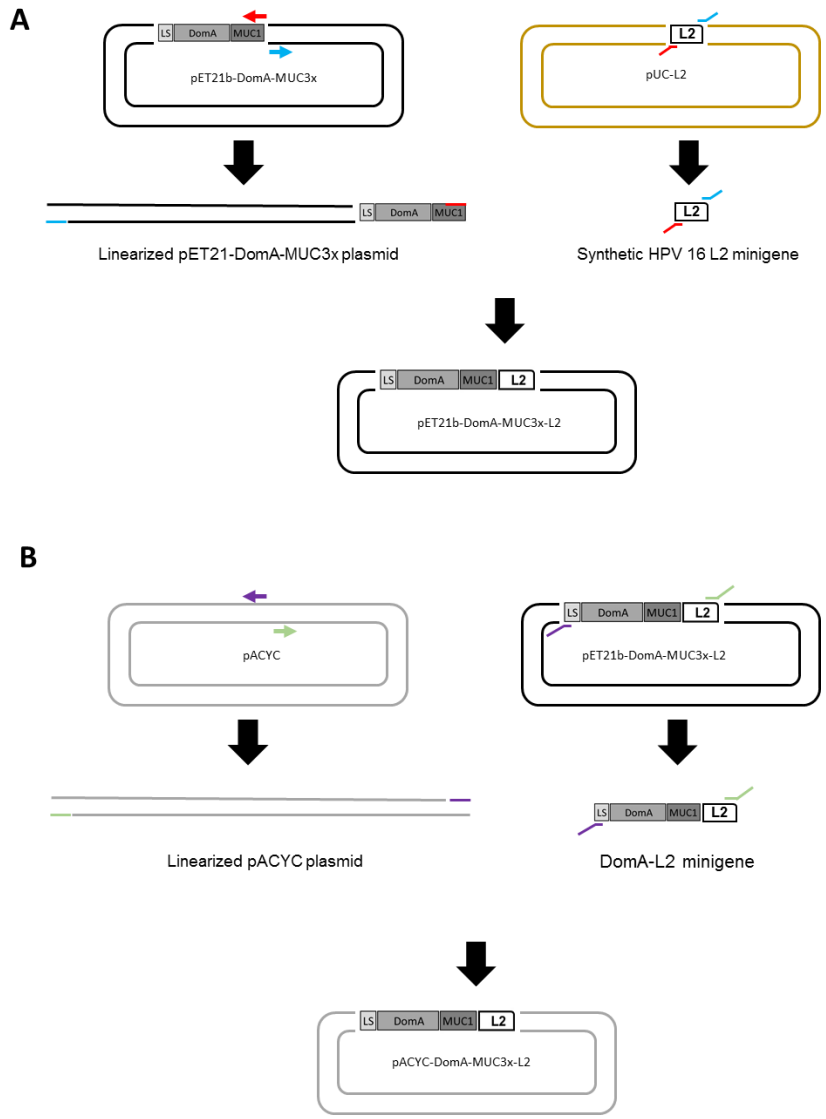


Figure 11 – Cloning strategy of HPV16 L2 fusion. (A) Cloning of fHbp-DomA-MUC3x-$L2_{16}$ gene in pET. pET_Nm-fHbpDomA_MUC1 was linearized with two divergent primers (see Table 1) at the C-terminus of the MUC1 epitope. In parallel the $L2_{16}$ coding sequence was amplified from pUC plasmid carrying the synthetic DNA encoding the $L2_{16}$ epitope. Finally, the linearized pET-fHbp-DomA-MUC3x and the amplified $L2_{16}$ minigene were combined to generate plasmid pET-fHbp-DomA-MUC3x-$L2_{16}$. **(B)** Cloning of fHbp-DomA-MUC3x-$L2_{16}$ gene in pACYC. The low copy number plasmid pACYC was linearized with primers annealing downstream from the T7 promoter. In parallel the fHbp-DomA-MUC3x-L216 gene was amplified from pET-fHbp-DomA-MUC3x-$L2_{16}$. The fHbp-DomA-MUC3x-$L2_{16}$ fragment and the linearized pACYC were mixed together to generate pACYC-DomA-MUC3x-$L2_{16}$. The cloning strategy just described was exactly the same used also to generate all the other L2 fusions described in the text.

The synthetic genes coding for the L2 epitopes (GeneArt, ThermoFisher, Waltham, MA, USA) were inserted into the linearized plasmid using the PIPE method. In particular, each synthetic gene was amplified using the primers listed in Table I, carrying overhangs complementary to the termini of the linearized pET_Nm-

fHbpDomA_MUC1. Four plasmids were generated named pET-fHbp-DomA-MUC3x-$L2_{16}$, pET-fHbp-DomA-MUC3x-4merA, pET-fHbp-DomA-MUC3x-4merB and pET-fHbp-DomA-MUC3x-8merAB.

The fusion constructs fHbp-DomA-$L2_{16}$, fHbp-DomA-4merB and fHbp-DomA-8merAB were finally transferred into plasmid pACYC[109]. To this aim, pACYC was linearized using the two primers PACYC_F and PACYC_R (Figure 11B) and the fusion constructs were amplified from the pET plasmid using the primers listed in Table I.

The amplified fragments and the PCR-linearized plasmid, mixed together in *E. coli* HK100 strain, generated the different plasmids. Positive clones were identified by colony PCR using GoTaq® Green Master Mix (Promega Corporation, Fitchburg, WI, USA) and gene specific primers (Table I) and then were subjected to sequence analysis (Eurofins Genomics, Ebersberg, Germany) using primers T7-P and T7-T or pACYC-up1 and PACYC-down1 (Table I) to verify the correctness of the cloning.

This procedure generates the three plasmids: pACYC-fHbp-DomA-MUC3x-$L2_{16}$, pACYC-fHbp-DomA-MUC3x-4merB and pACYC-fHbp-DomA-MUC3x-8merAB. Finally, the plasmids were used to transform *E. coli* BL21(DE3)Δ60 obtaining the following recombinant strains: *E. coli* BL21(DE3)Δ60(pACYC-fHbp-DomA-MUC3x-$L2_{16}$), *E. coli* BL21(DE3)Δ60(pET-fHbp-DomA-MUC3x-4merA), *E. coli* BL21(DE3)Δ60(pACYC-fHbp-DomA-MUC3x-4merB) and *E. coli* BL21(DE3)Δ60(pACYC-fHbp-DomA-MUC3x-8merAB).

2.3 Generation of MUC1 mutants

In order to fuse different epitopes at the C-terminus of the Domain A of fHbp, pET_Nm-fHbpDomA_MUC1[101] was linearized with the two primers, pET_F and fhDa_R. Synthetic DNA fragment expressing MUC_{mutB}3x in triplicate were purchased by GeneArt, (ThermoFisher, Waltham, MA, USA). The construct was amplified with the corresponding primers described in Table I. Through PIPE method, the fragments present overhangs both at the right and at the left flank, necessary for the annealing with the PCR-linearized plasmid. The generated plasmid, named pET-fHbp-DomA-MUC_{mutB}3x, was mixed with chemically competent *E. coli* HK100 strain, followed by chemical shock. Positive clones were identified by colony PCR using GoTaq® Green Master Mix (Promega Corporation, Fitchburg, WI, USA) and gene specific primers (Table I) and then were subjected to sequence analysis (Eurofins Genomics,

Ebersberg, Germany) using primers T7-P and T7-T (Table I) to verify the correctness of the cloning. Sequenced plasmid were inserted in our “proteome minimized” *E. coli* BL21(DE3)Δ60 strain obtaining *E. coli* BL21(DE3)Δ60(pET-fHbp-DomA-MUC$_{mutB}$3x).
In order to generate the plasmid pET-fHbp-DomA-MUC$_{P>A}$3x, we used the already linearized plasmid free at the C-termiuns of the Domain A of fHbp. MUC$_{P>A}$3x fragment gene was purchased by GeneArt, (ThermoFisher, Waltham, MA, USA) and amplified with the two primers, MUCP>A3x_F and MUCP>A3x_R. The amplified fragment and the PCR-linearized plasmid, mixed together in *E. coli* HK100 strain, generated the plasmid named pET-fHbp-DomA-MUC$_{P>A}$3x. Also in this case, positive clones were selected by colony PCR screening and then were sent for sequence analysis (Eurofins Genomics, Ebersberg, Germany). Corrected plasmid sequences were used to transform *E. coli* BL21(DE3)Δ60, generating the *E. coli* BL21(DE3)Δ60(pET-fHbp-DomA-MUC$_{P>A}$3x).
In order to test the feasibility of the mutated MUC1 linker as carrier for L2 epitope, the pET-fHbp-DomA-MUC$_{mutB}$3x was linearized using the couple of primers: pET_F and MUCmutB3x_R, just after the linker MUCmutB. At this point, the L2 of HPV16 fragment gene was amplified with the L2-MUCmutB3x_F and the and the reverse on the C-terminus of L2 epitope with the overhang specific for the pET plasmid (L2_R_pET). The mixing of the two elements in *E. coli* HK100 strain allows the generation of the plasmid pET-fHbp-DomA-MUC$_{mutB}$3x-L2, which was selected and sequenced as previously described. The plasmid was then inserted in *E. coli* BL21(DE3)Δ60, generating the *E. coli* BL21(DE3)Δ60(pET-fHbp-DomA-MUC$_{mutB}$3x-L2) strain.

2.4 OMV preparation, purification and analysis of the protein content

Recombinant strains were grown in 600 ml LB at 30°C under shaking at 200 rpm. At OD_{600} = 0.8, the recombinant antigen expression was induced adding Isopropyl β-D-thiogalactoside (IPTG) at a final concentration of 0.1 mM. Normally, after 2-3 h, depending on the growth curve, the bacterial biomass was separated from the supernatant through a centrifugation of the bacterial culture at 6000x g for 30 min. The supernatant was collected and filtered through a 0.22 μm pore size filter (Fisher Scientific part of ThermoFisher, Waltham, MA, USA) followed by the addition of 1 U/ml of benzonase (Sigma-Aldrich, St. Louis, MO, USA). For small-volume cultures, the

supernatant was concentrated with a 100 kDa ultrafiltration membrane (Sigma-Aldrich, St. Louis, MO, USA) and the OMVs were collected by ultracentrifugation (200.000 x g for 2 h). For large-volume cultures, the OMVs were purified from the supernatant trough an ÄKTA flux Tangential Flow Filtration (TFF) (GE Healthcare, Chicago, IL, USA) using an 500 kDa Hollow Fibre cartridge UFP-500-C-3MA (GE Healthcare, Chicago, IL, USA), dialyzed against 1.5 L of sterile PBS and concentrated until a final volume of about 15 ml. Purified OMVs were filtered through a 0.22 μm pore size filter (Fisher Scientific part of ThermoFisher, Waltham, MA, USA). The protein content was determined using DC Protein Assay (Bio-Rad, Hercules, CA, USA), which consists in a colorimetric assay which allows the measurement of protein concentration following detergent solubilization. The quality of the OMVs was analyzed by SDS-PAGE loading 20 μg of total OMV proteins on a NuPAGE 4-12% Bis-Tris gel (Invitrogen, Waltham, MA, USA) which was finally stained with Coomassie Blue (Giotto, Sesto Fiorentino, Italy). The expression of each antigen was analyzed performing a densitometric analysis by Image Studio Lite software (LI-COR Biosciences, NE, USA).

2.5 Dynamic Light Scattering

Size distribution profile of OMVs was determined by Dynamic Light Scattering (DLS) based on laser diffraction method using Zetasizer Nano-ZS90 (Malvern, UK). The OMV diameter of the batch preparation diluted at a final concentration of 0.5 mg/ml in PBS was determined by measuring the 90° side scatter size at 25°C. Three measurements (between 15 to 20 experimental runs for each measurement) were averaged to determine the vesicle size.

2.6 Confocal microscopy analysis of recombinant *E. coli* strains

The surface localization of the L2 epitopes in recombinant strains was analyzed by confocal microscopy using anti-HPV16-L2 monoclonal antibodies K18 designed against the $L2_{19-37}$ epitope of HPV16[58]. Briefly, the *E. coli* BL21(DE3)Δ60(pACYC-fHbp-DomA-MUC3x-$L2_{16}$), *E. coli* BL21(DE3)Δ60(pET-fHbp-DomA-MUC3x-4merA) and *E. coli* BL21(DE3)Δ60(pACYC-fHbp-DomA-MUC3x-8merAB) recombinant strains were grown in LB at 30°C. At OD_{600} = 0.5, 0.1 mM IPTG was added to the cultures and bacteria were grown for two additional hours. Three ml of each culture were harvested

by centrifugation at 11.000x g for 2 min at 4°C and resuspended in 4% para-formaldehyde solution in PBS, incubated 15 min at room temperature (RT), and then centrifuged at 7000x g for 2 min. Bacteria were washed three times with 1 ml PBS, suspended in 1 ml of blocking buffer (PBS containing 1% BSA), and incubated 20 min at RT. Primary monoclonal antibodies against L2 of HPV 16[58] were diluted in PBS containing 1% BSA at a final concentration of 5 μg/ml and incubated 1 h at RT. After two washes with PBS, bacteria were incubated for 20 min at RT with a solution containing the secondary Alexa Fluor® 488-labelled goat anti-mouse antibody (Molecular Probes, Eugene, USA) diluted 1:400 and the DAPI (ThermoFisher, Waltham, MA, USA) at the final concentration of 1:2500. Labeled bacteria were washed twice with PBS and placed on the slides (BioSigma, Cona (VE), Italy) with the ProLong Gold anti-fade reagent (Thermo Scientific part of ThermoFisher, Waltham, MA, USA). Confocal microscopy analysis was performed with a SP5 microscope (Leica, Wetzlar, Germany) and images were obtained using Leica LASAF4.0 software (Leica, Wetzlar, Germany).

2.7 Analysis of surface localization of L2 epitopes by Proteinase K Assay

Newly prepared OMV protein samples (2 μg) were treated with 100 μg/ml of Proteinase K (Applichem, Monza, Italy) in the presence or absence of 1% SDS. After incubation for 30 min at 37°C, the peptidase inhibitor phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich, St. Louis, MO, USA) was added at a final concentration of 3 mM. The OMV protein samples (0.05 to 0.5 μg) were separated by SDS-PAGE and the integrity of the fusions were analyzed by Western Blot. Briefly, after separation on SDS-polyacrylamide gels, the proteins were transferred to PVDF membranes (Invitrogen, Waltham, MA, USA). The membranes were then incubated 1 h at RT in 10% skimmed milk (Sigma-Aldrich, St. Louis, MO, USA), 0.05% Tween in PBS under mild agitation. Subsequently, the membranes were incubated for 1 h at RT in a PBS solution containing 1% skimmed milk, 0.05% Tween and 0.5 μg/ml of an anti-HPV16-L2 monoclonal antibody K18[58] or 0.6 μg/ml of anti-fHbp antibody produced in our laboratories. After three washing steps in PBS containing 0.05% Tween, the peroxidase-conjugated anti-mouse immunoglobulins (Sigma-Aldrich, St. Louis, MO, USA), for the membrane incubated with the anti-L2 antibody or peroxidase-conjugated

anti-rabbit immunoglobulins (Sigma-Aldrich, St. Louis, MO, USA), for the membrane incubated with anti-fHbp antibody was added. The secondary antibody was diluted 1:4000 in PBS containing 1% skimmed milk and 0.05% Tween and incubated on the filters for 1 h. The membranes were washed three times with PBS and then the immunoreactive signals were detected with the ImageQuant LAS4000 (GE, Chicago, IL, USA) using the SuperSignal West Pico chemiluminescent substrate (Thermo Scientific part of ThermoFisher, Waltham, MA, USA).

2.8 Negative staining electron microscopy analysis

A volume of 5 μl of OMVs diluted at 80 ng/μl in PBS were loaded onto a copper 200-squaremesh grid of carbon/formvar rendered hydrophilic by glow discharge using a Q150R S (Quorum, Laughton, UK). The excess solution was blotted off after 30 s using Whatman filter Paper No.1. The grids were negatively stained with NanoW (Nanoprobes, Yaphank, NY, USA) for 30 s, then blotted using Whatman filter Paper No.1 and finally let air dry. Micrographs were acquired using a G2 Spirit Transmission Electron Microscope (Tecnai, Hillsboro, OR, USA) equipped with a CCD 2kx4k camera at a final magnification of 120000x.

2.9 Interleukin 6 (IL-6) reactogenicity assay

For the IL-6 assay, THP-1 human leukemic monocyte cells were cultured in RPMI (Sigma-Aldrich, St. Louis, MO, USA) supplement with 10% FBS. For differentiation of the monocytes into macrophages 10 ng/mL of phorbol 12-myristate 13-acetate (PMA) were added into the culture medium and maintained for 48 h. Then, after medium replacement with complete RPMI, cells were maintained in culture for additional 24 h. Different amounts (diluted in a final volume of 100 μL RPMI) of “Empty” OMVs, 4merB-OMVs or commercially available Bexsero vaccine (based on the OMV concentration per dose of vaccine) were added to 1.5×10^5 differentiated macrophages. The OMVs were diluted starting from an initial concentration of 1.000 ng/ml to a final concentration of 0.01 ng/ml with 10-fold serial dilutions. Plates were incubated for 15 h at 37°C. The amount of IL-6 released in supernatants was measured by Human IL-6 Uncoated ELISA™ Kit (Thermo Fisher Scientific, Waltham, MA USA) following the manufacturer’s protocol. Briefly, Corning Costar ELISA plates were coated with anti-

human IL-6 antibody (100 μl/well) by overnight incubation at 4°C. The day after, the blocking solution was added to each well (Thermo Fisher Scientific, Waltham, MA USA) and subsequently 100 μl/well of cell supernatants were transferred to the plates and incubated 2 h at RT. Finally, Biotin-conjugated anti-IL-6 human antibodies, Streptavidin-HRP and tetramethylbenzidine (TMB) substrates were added to each well according to the manufacturer's instructions and the plates were read at 450 nm using a Varioskan apparatus (ThermoFisher, Waltham, MA, USA). The results were analyzed comparing the value to the standard curve obtained with different concentrations of purified human IL-6 (2 to 200 pg/ml).

2.10 Animal experiments

Four groups of CD1 female mice (6-8 weeks/old) (4 or 5 mice per group) were purchased by Charles Rivers (Charles River Laboratories Italia, Lodi, Italy) and were i.p. immunized (200 μl/mouse) with 10 μg/dose of $L2_{16}$-OMVs, 4merA-OMVs, 4merB-OMVs or 8merAB-OMVs, respectively, formulated with 2 mg/ml Alum (InvivoGen, Toulouse, France). The vaccination with 8merAB-OMVs was also repeated in another group of four mice using 25 μg/dose of OMVs formulated again with 2 mg/ml Alum (InvivoGen, Toulouse, France). For each experiment, three immunizations were performed at two-week intervals. One week after the last immunization, mice were sacrificed, and immune sera were collected from each mouse (Figure 12).
Moreover, another round of immunization was performed with the $MUC_{mutB}L2_{16}$-OMVs (10 μg/dose) formulated with 2 mg/ml Alum (InvivoGen, Toulouse, France) in a group of four CD1 mice with the same scheme of immunization.

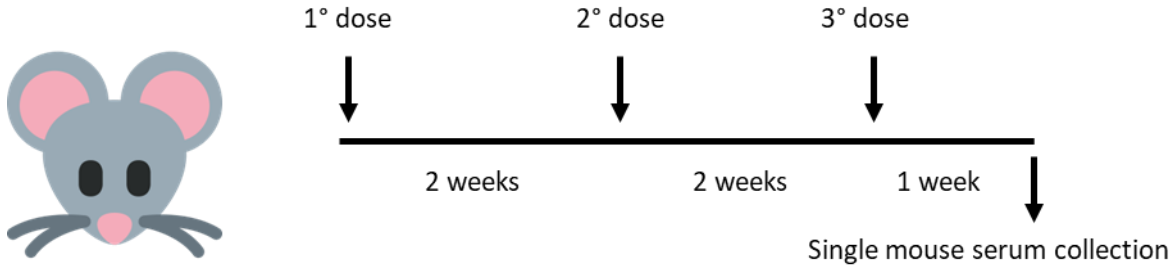


Figure 12 – Schematic schedule of immunization performed in CD1 mice. Immunization was performed with 10 μg/dose or 25 μg/dose of the OMVs as described in the text.

2.11 Ethics statement

The animal study was reviewed and approved by the Animal Ethical Committee of the University of Trento, by the Animal Ethical Committee of the Toscana Life Sciences and by the Italian Ministry of Health.

Mice were monitored twice per day to evaluate early signs of pain and distress, such as respiration rate, posture, and loss of weight (more than 20%) according to humane endpoints. Animals showing such conditions were anesthetized and subsequently sacrificed in accordance with experimental protocols.

2.12 Enzyme Linked ImmunoSorbent Assay (ELISA)

Sera from immunized mice were collected and analyzed with Enzyme Linked ImmunoSorbent Assay (ELISA). Covalink 96-well plates (ThermoFisher, Waltham, MA, USA) were coated with 100 μl/well of 100 mM sodium carbonate solution containing 5 μg/ml of each synthetic peptide corresponding to L2 from HPV 16, 31, 51, 6, 18, 33, 35 and 59 (Genescript, Piscataway, NJ, USA) (Table II). After an ON incubation at 4°C, the plates were blocked with 1% BSA in PBS for 1 h at 37°C. Mice sera were 3-fold serially diluted starting from 1:100 to 1:218700 in a solution containing 0.1% BSA in PBS and incubated for 1 h at 37°C. After three washes with 200 μl/well of 0.05% Tween in PBS, goat anti-mouse IgG conjugated with alkaline phosphatase (Sigma-Aldrich, St. Louis, MO, USA) was added at a 1:2000 dilution. After 45 min at 37°C and three washes with 0.05% Tween in PBS the substrate p-nitrophenyl phosphate (pNPP, Sigma-Aldrich, St. Louis, MO, USA) containing 100 mM Glycine, 1 mM $ZnCl_2$, 1 mM $MgCl_2$ (100 μl/well) was added and the plates were read at 405 nm wavelength using the Varioskan apparatus (ThermoFisher, Waltham, MA, USA).

2.13 Eukaryotic cell cultures

THP-1 human leukemic monocyte cells were cultured in RPMI (Sigma-Aldrich, St. Louis, MO, USA) containing 10% FBS (Sigma-Aldrich, St. Louis, MO, USA) and 5% glutamine (Sigma-Aldrich, St. Louis, MO, USA).

Human cervix epithelial cell line derived from HPV18 positive cervical carcinoma from Henrietta Lacks and stably expressing one copy of the SV40 large T- Antigen, named HeLaT-K4 cells were cultivated in Dublecco Modified Eagle Medium (DMEM) (Sigma-

Aldrich, St. Louis, USA) 1000 mg glucose, added with 10% FBS, 1% glutamine, 1% Pen/Strep (Sigma-Aldrich, St. Louis, MO, USA).
All the cells were cultured at 37°C, 5% CO_2 and 95% humidity.

2.14 Pseudovirions preparations

Different types of pseudovirions (PsVs) were prepared by cotransfection of the human fibroblast cell line 293TT with plasmids carrying humanized HPV L1 and L2 coding sequences plus a reporter plasmid expressing Gaussia luciferase, and purification was performed by iodixanol gradient ultracentrifugation according to a previously described protocol[110].

2.15 Neutralization assays

Sera were collected from immunized mice, and they were diluted in a 96-well tissue culture plate (Corning, New York, NY, USA) from 1:50 to 1:12.150 with a three-fold titration. Following the dilution, the sera were incubated with pseudovirus for 20 min, after which HelaT-K4 cells, having a concentration of 3.3×10^5 cell/ml, were introduced to the mixture. The plates were incubated under 5% CO_2, 37°C for 48 h. Afterwards, 10 μl of the supernatant from each well was extracted and placed in a 96-well white plate, and 100 μl/well of the substrate for Gaussia luciferase (PJK, Kleinblittersdorf, Germany) was added. Finally, the luminescence was measured after 15 min upon addition of the substrate. By comparing with the luminescence of the infection lane, where only the medium was added instead of the sera, the neutralizing antibody titers of each serum can be analyzed. EC_{50} is defined as the titer of serum that could neutralize half of the pseudovirus.

Table I – Primers used for synthetic gene cloning strategies.

Name	Sequence	Use
pET_F	CATCACCATCACCATCACGATTACA	Linearization plasmid pET
MUC3X_R	ATGCGCCGGCGGCGC	Linearization plasmid pET
L2_F_pET	GCGCCGCCGGCGCATGGCGGCCCGAAAACC	Cloning L2, 4merA and 8merAB
L2_R_pET	GATGGTGATGGTGATGTTATGGACCACCGCCTTCCAC	Cloning L2
4merA_R_pET	GATGGTGATGGTGATGTTATGGCCCGCCATGCTCCA	Cloning 4merA
4merB_F	GCGCCGCCGGCGCATGGTGGGCCGAAAACGTGT	Cloning 4merB
4merB_R_pET	GATGGTGATGGTGATGTTACGGACCACCCCCTTCGAC	Cloning 4merB and 8merAB
PACYC_F	AGCCAGGATCCGAATTCGAGC	Linearization plasmid pACYC
PACYC_R	GGTATATCTCCTTATTAAAGTTAAAC	Linearization plasmid pACYC
fHDa_F_pACYC	ATAAGGAGATATACCGTGAATCGAACTGCC	Cloning entire constructs (fHDa-MUC3x + epitopes) in pACYC
L2_R_pACYC	ATTCGGATCCTGGCTTTATGGACCACCGCCTTCCAC	Cloning L2
4merB_R_pACYC	ATTCGGATCCTGGCTTTACGGACCACCCCCTTC	Cloning 4merB and 8merAB
T7-P	TAATACGACTCACTATAGGG	Screening and sequencing in pET
T7-T	GCTAGTTATTGCTCAGCGG	Screening and sequencing in pET
PACYC-up1	GGATCTCGACGCTCTCCCT	Screening and sequencing in pACYC
PACYC-down1	GATTATGCGGCCGTGTACAA	Screening and sequencing in pACYC
fhDa_R	TTGTTTGTATACTTGGAACTC	Sequencing in pACYC
MUCmutB_F	CAAGTATACAAACAAGGCGCGAGCACCGTG	Cloning MUCmutB
MUCmutB3x_R	GATGGTGATGGTGATGTTAATGCAACGGTGGCAC	Cloning MUCmutB
MUCP>A3x_F	CAAGTATACAAACAAGGCGTGACCAGCGCG	Cloning MUCP>A3x
MUCP>A3X_R	GATGGTGATGGTGATGTTAATGGGCCGCTGCGGC	Cloning MUCP>A3x

L2-MUCmutB3x-F	GTGCCACCGTTGCATGGCGGCCCGAAAACC	Cloning L2 for the new linker MUCmutB

Table II – Peptides used for ELISA coating plate.

Name	Sequence
L2 HPV16	KTCKQAGTCPPDIIPKVEG
L2 HPV31	QTCKAAGTCPSDVIPKIEH
L2 HPV51	STCKAAGTCPPDVVNKVEG
L2 HPV6	QTCKLTGTCPPDVIPKVEH
L2 HPV18	KTCKQSGTCPPDVVPKVEG
L2 HPV33	QTCKATGTCPPDVIPKVEG
L2 HPV35	RTCKAAGTCPPDVIPKVEG
L2 HPV59	KTCKQAGTCPSDVINKVEG

Chapter 3

RESULTS

3.1 The L2 epitope of HPV16 is efficiently expressed on the surface of *E. coli* and accumulate in the OMVs

As previously mentioned, different strategies can be adopted to engineer the vesicle with heterologous antigens. In the case of the L2 epitope ($L2_{20-38}$)[58], it has to be considered that that the disulfide bond between Cys 22 and Cys 28 is essential to elicit functional antibodies[72]. Therefore, in selecting the optimal strategy for L2 expression in the OMVs, we focused our attention on the fHbp-DomA-MUC1 as a carrier. As demonstrated by Fantappiè[101] this protein fusion accumulates in the vesicular and protrudes out of the membrane. Therefore, when fused to the C-terminus of fHbp-DomA-MUC1, L2 is expected to find a proper oxidizing environment, which should allow the formation of the required disulfide bonds. Moreover, the presence of three copies of the MUC1 repeat should provide enough flexibility to the construct, thus reducing the risk of potential steric hindrance.

To test the feasibility of the approach, we first inserted the coding sequence of a single L2 epitope, the HPV16-L2 ($L2_{16}$), flanked by the Gly-Gly-Pro (GGP) spacer, in frame with the fHbp-DomA-MUC1 gene present in the plasmid pET_Nm-fHbpDomA_MUC1[101] (Figure 13).

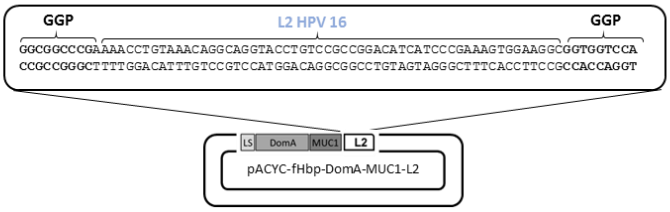


Figure 13 – Schematic representation of the cassette of expression of the L2 epitope of HPV16 fused in frame with the Domain A of fHbp gene and three copies of MUC1 epitope.

The entire construct fHbp-DomA-MUC3x-$L2_{16}$ was transferred into the low copy number pACYC plasmid. The recombinant plasmid pACYC-fHbp-DomA-MUC3x-$L2_{16}$ was then used to transform the *E. coli* BL21(DE3)Δ60 strain, generating *E. coli* BL21(DE3)Δ60(pACYC-fHbp-DomA-MUC3x-$L2_{16}$). The recombinant strain was grown at 30°C under shaking condition at 200 rpm. At OD_{600} = 0.8, the recombinant antigen expression was induced adding IPTG. The growth was monitored for 2-3 hr, depending on the growth curve and then the bacterial biomass was separated from the supernatant through a centrifugation of the culture. The OMVs were purified from the culture supernatant of the recombinant strain *E. coli* BL21(DE3)Δ60(pACYC-fHbp-DomA-MUC3x-$L2_{16}$) through an ultracentrifugation step. As shown in Figure 14A, the fHbpDomA-MUC3x-$L2_{16}$ fusion (hereinafter DomA-$L2_{16}$) was expressed in the OMVs with high efficiency, representing approximately the 24% of total OMV proteins as judged by densitometric scanning of the SDS-polyacrylamide gel. Moreover, the fusion was exposed on the surface of the OMVs, as judged by the proteinase K “shaving” assay. In this assay, purified OMVs were treated with proteinase K and subsequently the integrity of the fusion protein was analyzed by Western Blot, using a monoclonal antibody designed against the $L2_{19\text{-}37}$ epitope of HPV16[58]. As shown in Figure 14B, the band corresponding to DomA-$L2_{16}$ fusion protein in the OMV compartment almost completely disappeared after protease treatment. As a control, we can see that in presence of both Proteinase K and 1% SDS, which completely disintegrate the phospholipid bilayer, no signal is detected, while in presence of only 1% SDS the detected signal by the monoclonal antibody is comparable to the not treated OMVs.

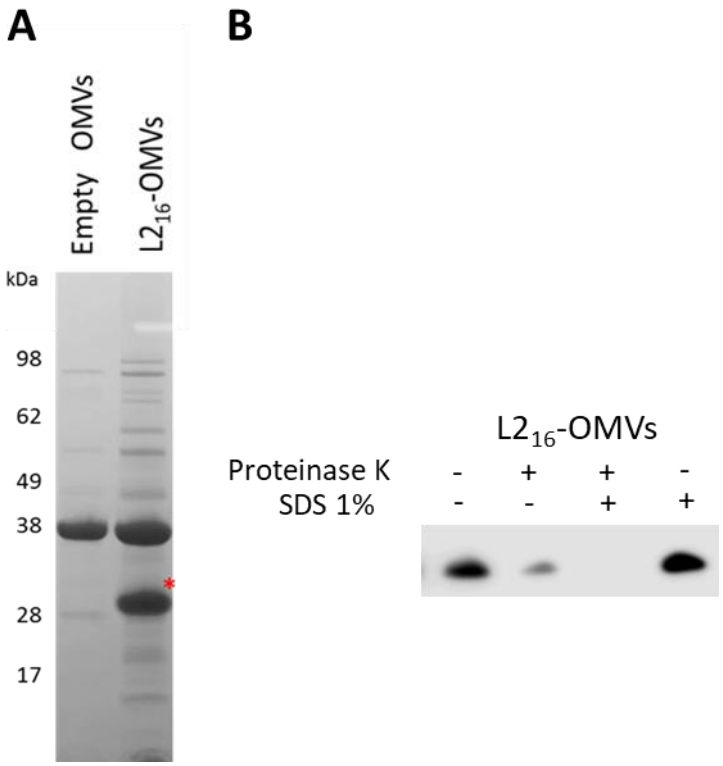


Figure 14 – Engineering of OMVs with L2$_{16}$ epitope. (A) Aliquots of 20 μg of OMVs purified from the culture supernatants of the engineered *E. coli* BL21(DE3)Δ60 strains were loaded on a polyacrylamide gel and stained with Coomassie Blue. As negative control the "Empty OMVs", purified from *E. coli* BL21(DE3)Δ60 strain, were loaded. Red asterisk (*) indicate the band of interest. **(B)** Assessment of L2 epitope localization on engineered OMVs. Purified OMVs (1 μg) were treated for 30 min with proteinase K in the presence (+) or absence (-) of SDS and the integrity of the fusion proteins was analyzed by Western Blot using an anti-L216 monoclonal antibody[58].

3.2 L2 multiepitopes are efficiently expressed on the surface of *E. coli* and accumulate in OMVs

Having demonstrated that the L2 epitope of HPV16 was efficiently transported to the OMV compartment, we next investigated whether strings of L2 epitopes from different HPV serotypes could similarly be incorporated in the vesicles. To properly select the L2 epitopes, the L2 sequences belonging to the most frequent "high-risks" and the "low-risk" serotypes were aligned (17 HPV serotypes in total). The alignment allowed to sub-group the 17 serotypes into 8 subgroups[111]. Next, we selected one specific L2 epitope from each subgroup (arrowed in Figure 15), the rational being any representative of each sub-group should elicit cross-reactive antibodies against the other serotypes belonging to the same sub-group. For instance, we expected that the L2 epitope of HPV18 can confer protection against HPV45, 39 and 68.

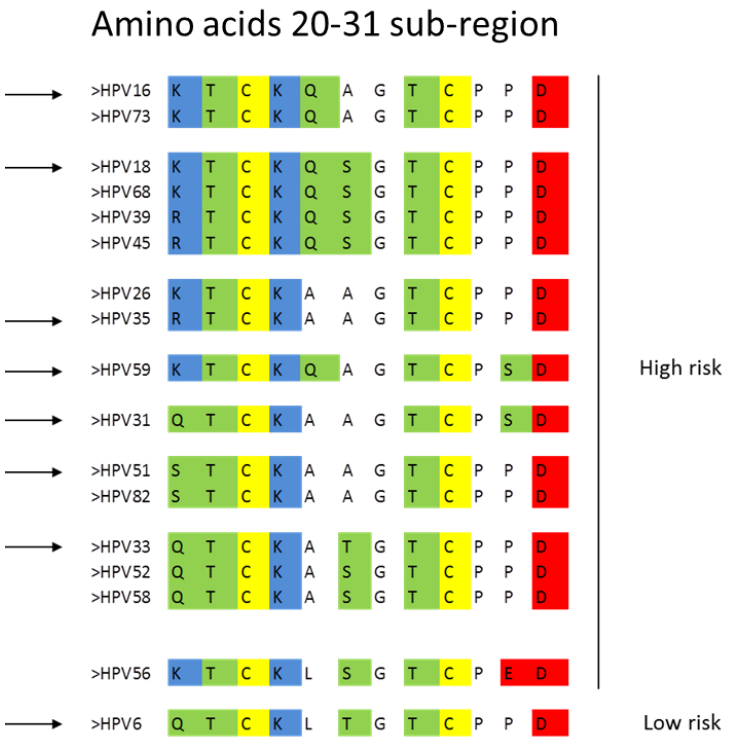


Figure 15 – Schematic alignment of the epitope comprising amino acids 20-31 of the L2 of 17 different HPV serotypes. Amino acids are colored as follow: *green*: polar amino acids; *blue*: basic amino acids; *white*: non-polar amino acids; *red*: acidic amino acids; *yellow*: cysteine residues conserved in the L2 proteins from all HPV types. Arrows indicates the serotypes selected for the generation of the strings of the L2 multiepitope. Modified from Spagnoli[111].

Three different DomA fusions were generated: DomA-4merA, carrying the L2 epitopes from HPV serotypes 16, 31, 51, 6; DomA-4merB, carrying the L2 epitopes from serotypes 18, 33, 35 and 59; and DomA-8merAB, in which 4merA and 4merB were fused together. In all three constructs each L2 epitopes were separated from the other by the GGP spacer. The three synthetic genes were fused to the Domain A of fHbp and the three copies of MUC1 epitope as previously described using the pipe technology. The three plasmids expressing the genes coding for the three fusions (Figure 16) were used to transform the "proteome minimized" *E. coli* BL21(DE3)Δ60 generating the three recombinant strains *E. coli* BL21(DE3)Δ60(pET-fHbp-DomA-4merA), *E. coli* BL21(DE3)Δ60(pACYC-fHbp-DomA-4merB) and *E. coli* BL21(DE3)Δ60(pACYC-fHbp-DomA-8merAB).

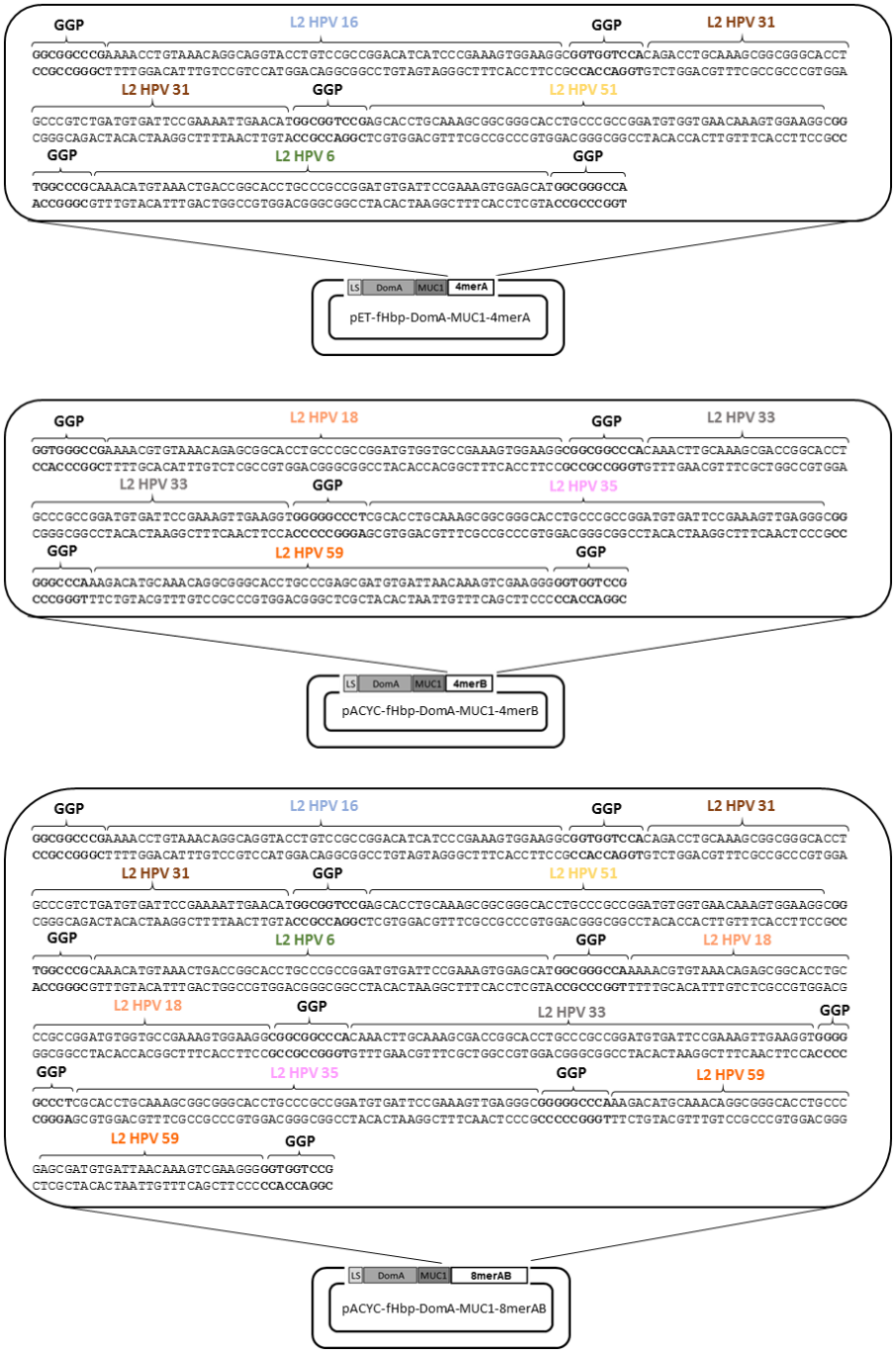


Figure 16 – Schematic representation of the cassette of expression of the L2 multiepitope fused in frame with the Domain A of fHbp and three copies of MUC1 epitope.

The recombinant strains were grown in LB medium at 30°C under shaking condition at 200 rpm. At OD_{600} = 0.8, the expression of the fusions was induced adding IPTG. After 2-3 h, the bacterial biomass was separated from the supernatant through a centrifugation of the bacterial culture and the OMVs, purified from the culture supernatants, were analyzed by SDS-PAGE. As shown in Figure 17A, similarly to

DomA-L2$_{16}$, all three fusions were efficiently incorporated in the OMVs, as judged by densitometric analysis (from 13% to 16% of total OMV proteins). Moreover, the fusion proteins, and in particular the strings of the L2 epitopes, were well exposed on the surface of the outer membrane as judged by the proteinase K "shaving" assay performed on engineered OMVs (Figure 17B).

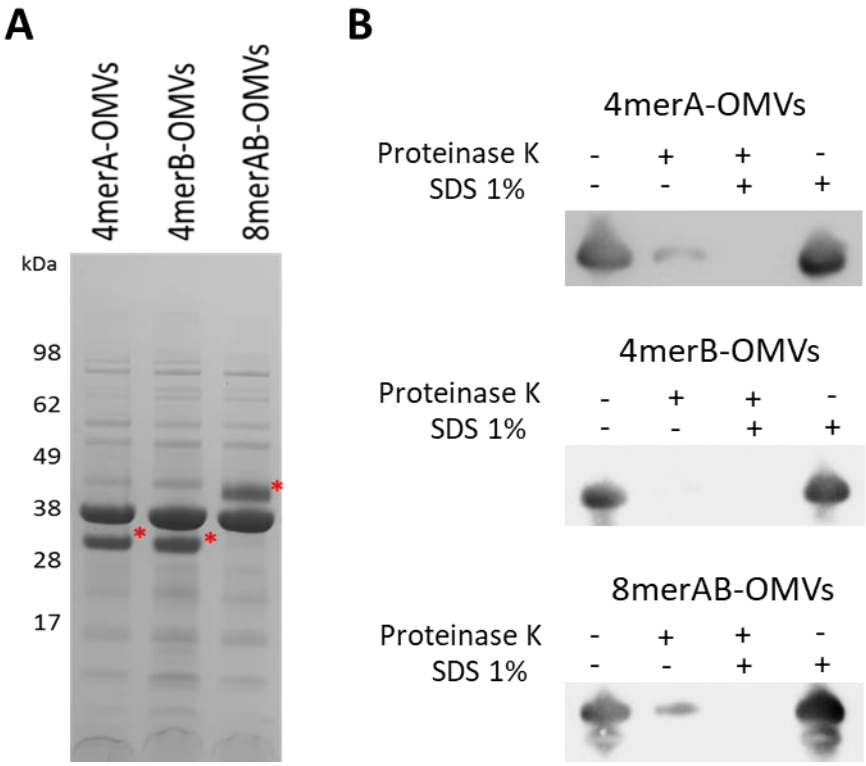


Figure 17 – Engineering of OMVs with a string of multiple L2 epitopes. (A) Aliquots of 20 μg of OMVs purified from the culture supernatants of the engineered *E. coli* BL21(DE3)Δ60 strains were loaded on a polyacrylamide gel and stained with Coomassie Blue. Red asterisk (*) indicate the band of interest. **(B)** Assessment of L2 epitope localization on engineered OMVs. Purified OMVs (1 μg) were treated for 30 min with proteinase K in the presence (+) or absence (-) of SDS and the integrity of the fusion proteins was analyzed by Western Blot using an anti-L216 monoclonal antibody (see Text for details).

The surface exposure of the epitope was also demonstrated by confocal microscopy of the engineered bacteria in non-permeabilized condition stained with the monoclonal antibody K18, specific for the L2 epitope of HPV16[58] (Figure 18A).

Finally, electron microscopy analysis showed that 4merA-OMVs, 4merB-OMVs and 8merAB-OMVs had similar size and morphology, which appeared indistinguishable from the OMVs of the recipient strain "Empty OMVs" (Figure 18B), suggesting no alteration due to the presence of the heterologous antigens in the vesicle.

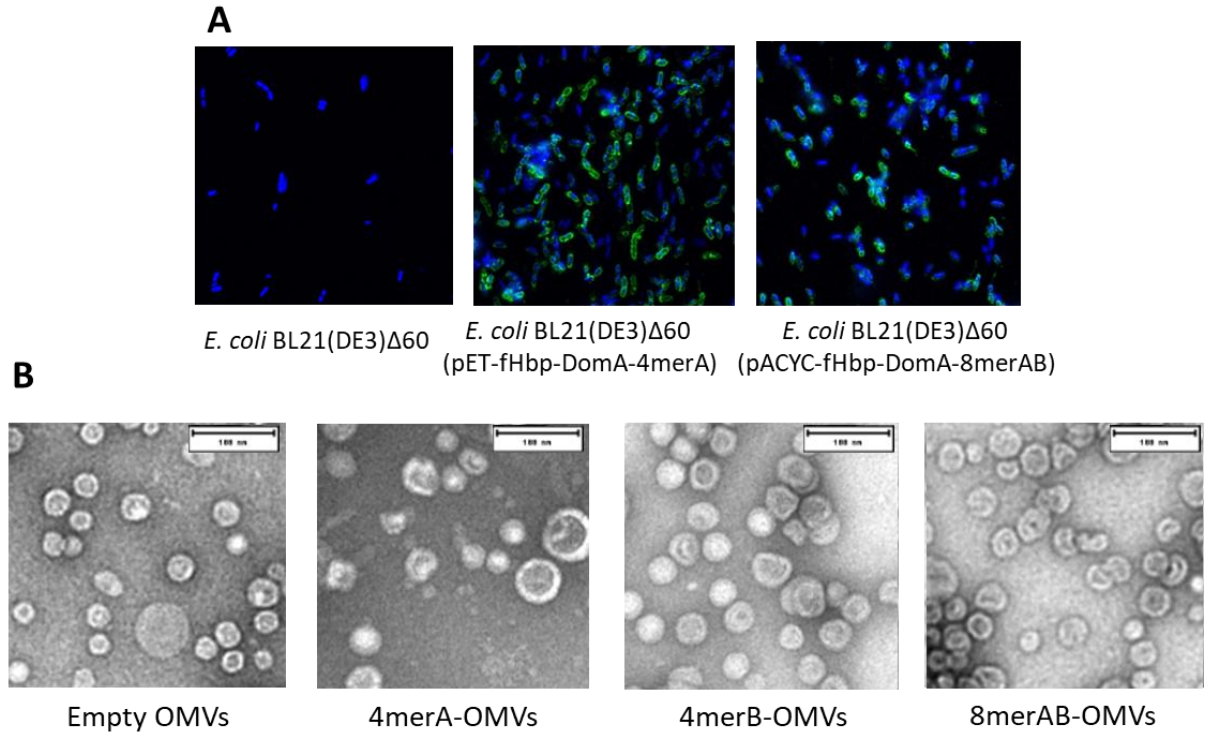


Figure 18 – Confocal microscopy of bacterial cells and electron microscopy of OMVs (A) Assessment of localization of L2 epitope by confocal microscopy. Induced bacterial cells of *E. coli* BL21(DE3)Δ60, *E. coli* BL21(DE3)Δ60(pET-fHbp-DomA-MUC3x-4merA) and *E. coli* BL21(DE3)Δ60(pACYC-fHbp-DomA-MUC3x-8merAB) were fixed with 2% formaldehyde and stained with a mAb specific for the L2 epitope of HPV16. The binding of the antibody was visualized with an anti-mouse AlexaFluor488 antibody (green) and the nucleus were stained with DAPI (blue). **(B)** Negative staining Electron Microscopy of OMVs. Five μl of OMVs purified from *E. coli* BL21(DE3)Δ60, *E. coli* BL21(DE3)Δ60(pET-fHbp-DomA-MUC3x-4merA), *E. coli* BL21(DE3)Δ60(pET-fHbp-DomA-MUC3x-4merB) and *E. coli* BL21(DE3)Δ60(pACYC-fHbp-DomA-MUC3x-8merAB) were negatively stained with NanoW for 30 s and micrographs were acquired using a G2 Spirit Transmission Electron Microscope at a final magnification of 120000x.

3.3 Immunization with L2-engineered OMVs elicits L2-specific IgG titers

Next, we investigated whether the OMVs engineered with the L2 epitope of HPV16 could induce L2-specific antibodies. In order to do so, a group of 5 CD1 mice were immunized three times at two-week intervals with 10 μg of $L2_{16}$-OMVs formulated with 2 mg/ml Alum. Seven days after the last immunization, sera were collected and used to measure the L2-specific total IgG titers by ELISA. The assay was carried out coating the plates with the synthetic peptide corresponding to the L2 epitope of HPV16 and comprising amino acid 20-38. As negative control, sera from mice immunized with

Empty OMVs derived from *E. coli* BL21(DE3)Δ60 strain were used. As shown in Figure 19, $L2_{16}$-OMVs elicited L2-specific antibodies. The titers were at least as good as the titers obtained immunizing mice with the recombinant thioredoxin-L2 fusion previously described (the serum was gently provided by Martin Müller lab (DKFZ, Heidelberg)).

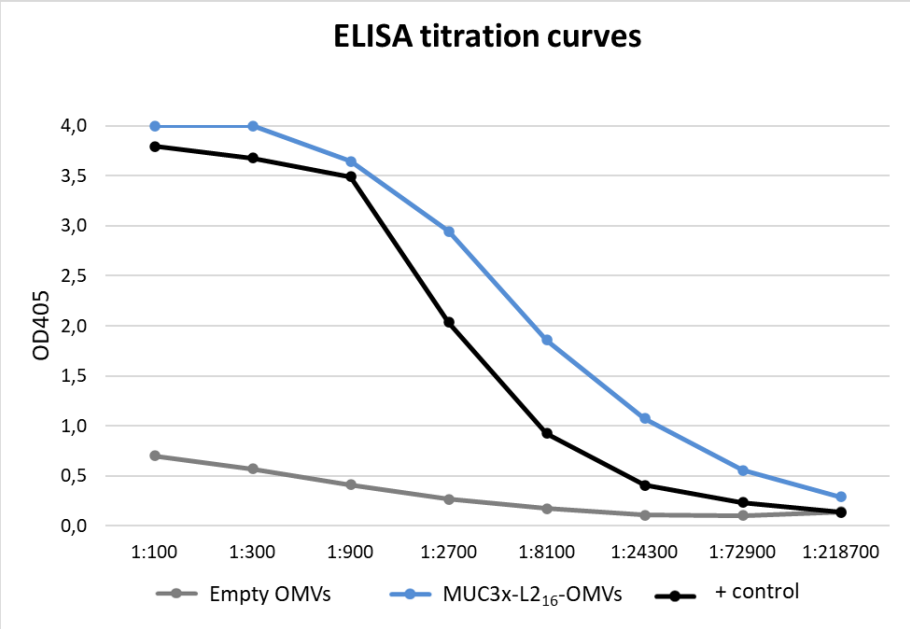


Figure 19 – Anti-L2 ELISA curves in sera from mice immunized with $L2_{16}$-OMVs. Group of 5 CD1 mice were immunized i.p. (three immunizations) with "Empty OMVs" (negative control) and $L2_{16}$-OMVs (10 μg/dose) and sera were collected and pooled 7 days after the last immunization. Total IgGs titers of the pooled sera against the L2 epitope of HPV16 were analyzed by ELISA using plates coated with the corresponding synthetic peptide (0.5 μg/well). As a positive control, a serum provided by Martin Müller lab (DKFZ, Heidelberg) was used.

Once demonstrated the capability of the $L2_{16}$-OMVs of inducing HPV16 L2-specific antibodies, we investigated whether the vesicles engineered with the strings of the L2 epitopes could maintain this feature. To this aim, three groups of five CD1 mice each were immunized three times, two weeks apart, with 10 μg of either 4merA-OMVs, or 4merB-OMVs or 8merAB-OMVs, formulated with 2 mg/ml Alum. Seven days after the last immunization, the serum from each mouse was collected and used to measure the L2-specific total IgG titers by ELISA. The assay was carried out coating the plates with each of the eight synthetic peptides corresponding to the selected L2 epitopes. As negative control, sera from mice immunized with Empty OMVs derived from *E. coli* BL21(DE3)Δ60 strain were used, and the value titers obtained from the assay were lower than the calculable limit (<100).

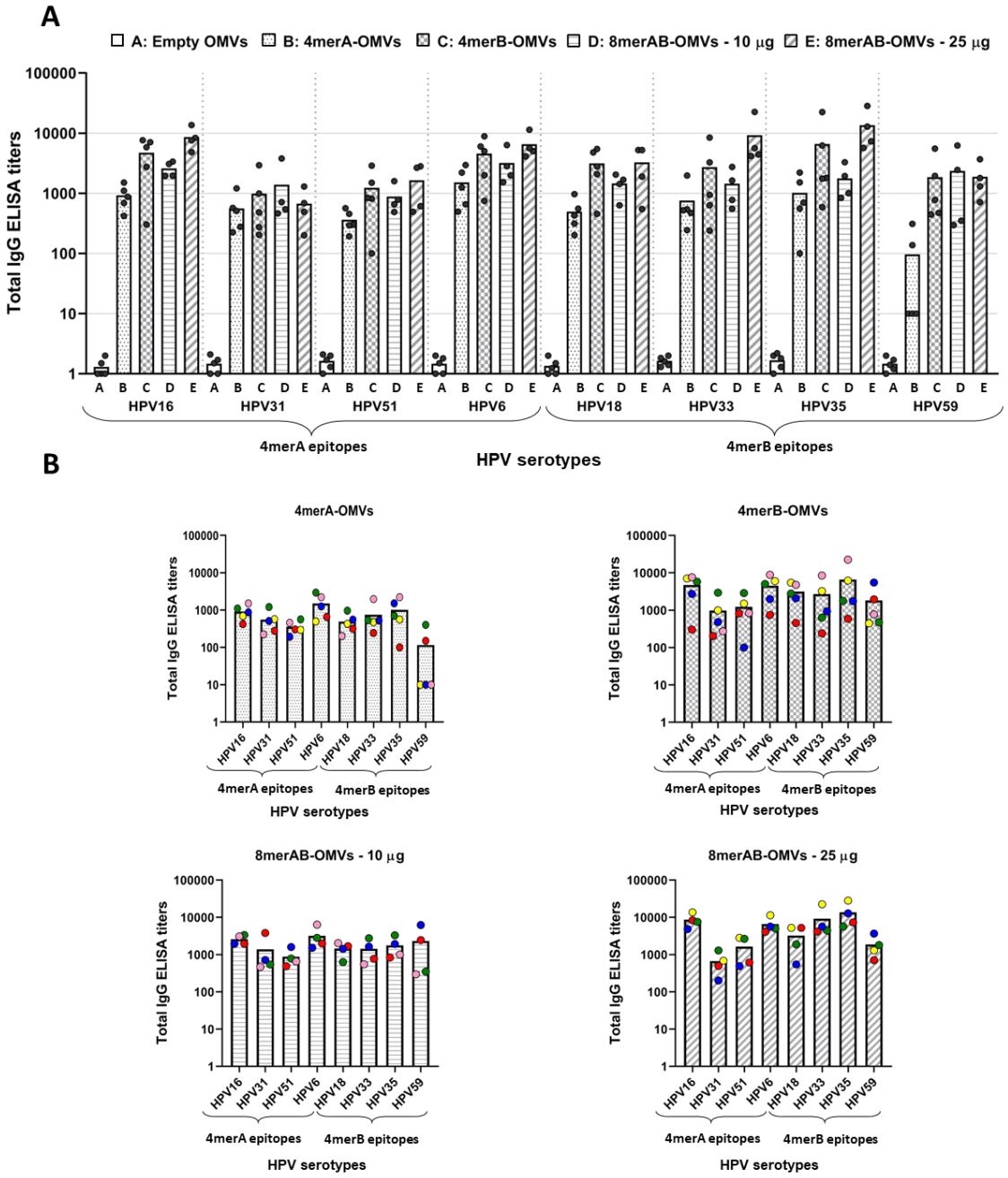


Figure 20 – Anti-L2 ELISA titers in sera from mice immunized with engineered OMVs with L2 multiepitope[112]. (**A**) Group of 4 or 5 CD1 mice were immunized i.p. (three immunizations) with "Empty OMVs" (negative control), 4merA-OMVs, 4merB-OMVs, 8merAB-OMVs (10 μg/dose) and 8merAB-OMVs (25 μg/dose) and sera were collected 7 days after the last immunization. Total IgGs titers of each mouse serum against the different L2 epitopes were measured by ELISA using plates coated with the corresponding synthetic peptides (0.5 μg/well). Cumulative representation of the titers elicited by sera from mice immunized with the different vaccine formulations, as reported in the graph legend, tested against all selected HPV serotypes. Each vaccinated mouse is represented as a grey circle and ELISA titers correspond to the serum dilution that gives an OD_{405} value = 1.5 expressed on a logarithmic scale. **(B)** Representation of the ELISA titers against the eight L2 epitopes grouped by vaccine formulation. Each mouse serum is represented by a circle with a different color code. Graphs were generated using GraphPad 8 software.

From the ELISA titers reported in Figure 20A, the following conclusions can be drawn. First, 4merA-OMVs and 4merB-OMVs immunizations elicited antibodies, which recognized their own L2 epitopes.

Second, the sera from mice immunized with 4merA-OMVs and with 4merB-OMVs also cross-reacted with the epitopes present in the other construct, with the exception for the L2 of serotype 59, which was poorly recognized by the anti-4merA-OMV sera. Cross-recognition was particularly effective in sera from mice immunized with 4merB-OMVs, which gave titers $\geq 1x10^3$ against all the eight synthetic peptides in most of the vaccinated mice.

Third, 8merAB-OMVs induced antibodies specific for all the tested L2 epitopes. However, when 10 μg were used, in particular, the titers against serotypes HPV16, 6, 51, 18, 33 and 35 appeared to be slightly lower than the titers obtained with 4merB-OMVs. This was probably due to the lower amount of each epitope present in 10 μg of 8merAB-OMVs with respect to its presence in 10 μg of the tetramer construct. Indeed, when the immunization was repeated using 25 μg of 8merAB-OMVs, the titers reached the highest levels against all eight L2 peptides, except for HPV31 and 59 in which the titers of the two immunization schedules are similar.

Forth, from the inspection of single mouse sera (Figure 20B), presented with a color-code, it appears that titer variability was not epitope-specific, meaning that a mouse that present high titer for a specific HPV serotype, tendentially present high titers against all the serotypes and *vice versa*.

Fifth, as expected, the immunization with the Empty OMVs does not provide any specific antibody titers against L2 epitopes.

In conclusion, OMVs carrying L2 epitopes were capable of eliciting high levels of anti-L2 antibodies. Moreover, the immunization with 10 μg of 4merB-OMVs was particularly effective: not only the formulation elicited antibodies cross-reacting with all eight L2 serotypes tested, but also it performed equally well with respect to the immunization with 25 μg of 8merAB-OMVs.

3.4 L2-specific IgGs elicited by OMV immunization neutralize HPV *in vitro*

We next asked the question whether the L2-specific antibodies induced by OMV immunization had functional activity. Since no reliable animal models are available to study human HPV infection, the *in vitro* L1 pseudovirus neutralization assay (L1-PBNA) was used[62]. According to this assay, pseudovirus, constituted by L1 and L2 proteins from selected HPV serotypes and by Gaussia luciferase-expressing genome, were used to infect HeLaT-K4 cells in the presence or absence of mouse sera, and the capacity of serum antibodies to inhibit pseudovirus infection was quantified following the luciferase activity in the cells. All the data were normalized to the negative control, which was obtained mixing the pseudoviruses and the cell in absence of the serum, where the infection is standardized as 100%.

Pseudoviruses corresponding to all eight HPV serotypes selected for the L2 polytope constructs were prepared and systematically tested in the *in vitro* neutralization assay using the sera from mice immunized with 4merA-OMVs, 4merB-OMVs and with the 10 μg/dose of 8merAB-OMVs. As negative control, sera from mice immunized with Empty OMVs derived from *E. coli* BL21(DE3)Δ60 strain were used, and the value titers obtained from the assay were lower than the calculable limit (<100).

As shown in Figure 21, the three vaccine formulations elicited neutralization titers against all serotypes tested. The two tetramers induced antibodies that not only neutralize the corresponding pseudoviruses, but also the pseudoviruses belonging to the four serotypes included in the other formulation, confirming the cross-neutralization capability of the L2 epitope. However, but in line with the ELISA titers, the neutralization titers elicited by the 4merA-OMVs construct were low against HPV59 serotype. Also, HPV51 pseudovirus appeared to be the most difficult pseudovirus to neutralize, regardless the formulation used. Nonetheless, Seitz et al[62] showed that even a 40% neutralization in the L1-PBNA correlated with a total protection in mice challenged with HPV51 after passive transfer of anti HPV51-L2 sera.

Paralleling the ELISA titers, the 4merB-OMVs outperformed both the 4merA-OMVs and the 8merAB-OMV-formulation given at 10 μg/dose. This could be appreciated by comparing the graphs shown in Figure 21B: the neutralization titers elicited by the 4merB-OMVs against each serotype were never lower, and in most of the cases higher, than the titers induced by the other two formulations.

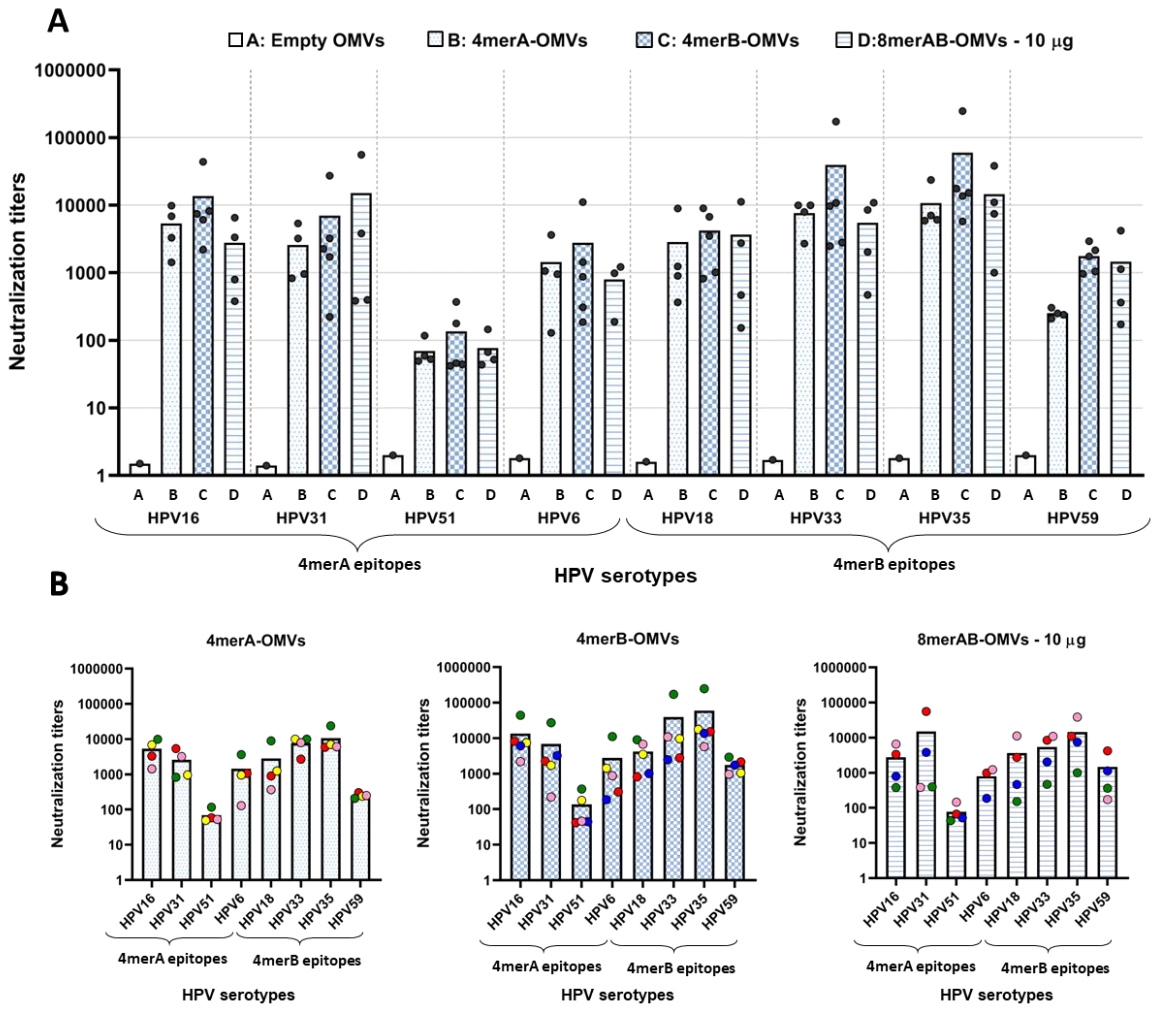


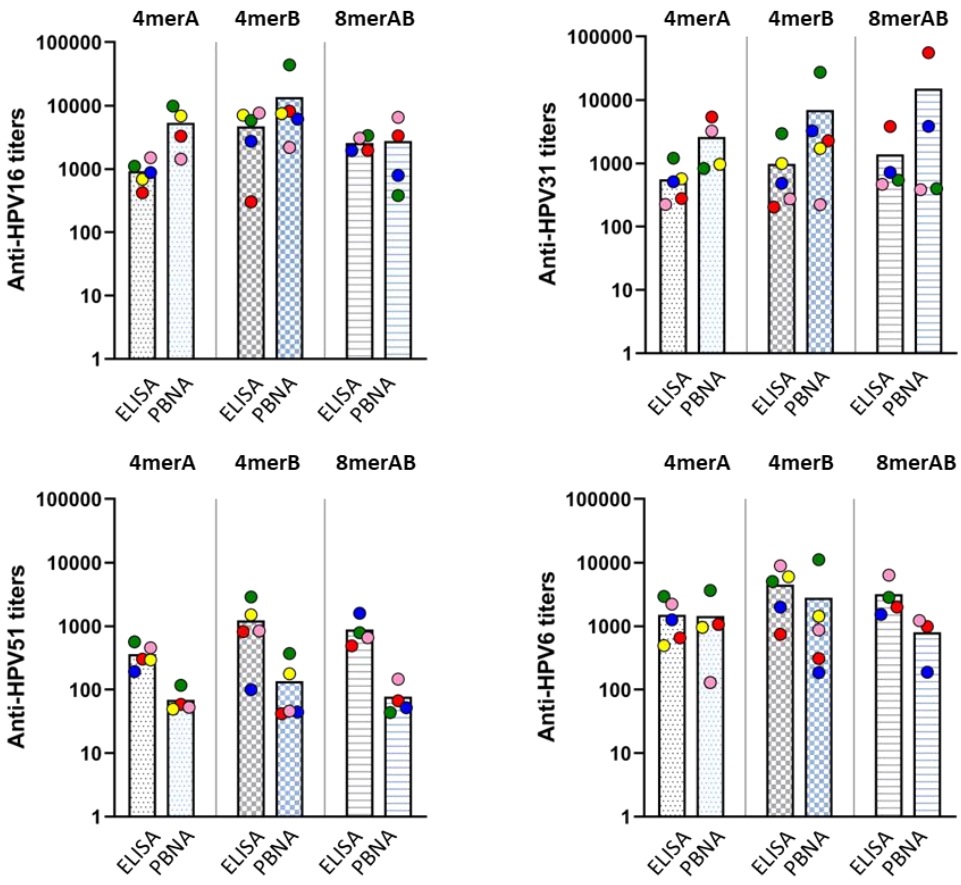
Figure 21 – Neutralization titers of OMV-based vaccines[112]**. (A)** Group of 4 or 5 CD1 mice were immunized i.p. with "Empty OMVs" (negative control), 4merA-OMVs, 4merB-OMVs or 8merAB-OMVs. Sera were collected 7 days after the last immunization and analyzed singularly by L1 pseudovirus-based neutralization assay (L1-PBNA) using eight different HPV pseudoviruses (see Text for details). Cumulative representation of the neutralization titers elicited by sera from mice immunized with the different vaccine formulations tested against all selected HPV serotypes. Each vaccinated mouse is represented as a grey circle apart from the negative control ("Empty" OMVs) in which the sera were pooled. In graph, the EC_{50} value is represented, defined as the titer of serum that could neutralize half of the pseudovirus. Values are expressed in a logarithmic scale. **(B)** Representation of the neutralization titers against the eight pseudoviruses grouped by vaccine formulation. Each mouse serum is represented by a different color code. Graphs were generated using GraphPad 8 software.

Finally, the availability of both ELISA and neutralization titers from each mouse serum allows to establish whether there was a correlation between the titers of the epitope-binding antibodies and functional (neutralizing) antibody titers. In general, but not always, looking at the color-coded sera in Figure 22, this appeared to be the case. Those sera with the highest ELISA titers against a specific serotype often performed

well in the neutralization assay, and *vice versa.* For example, for HPV31, all the mice immunized with the 8merAB-OMVs present a similar trend comparing titers obtained with ELISA and PBNA: the mice represented with the same color move similarly in both assays.

We did not measure the neutralization titers induced by the 25 μg 8merAB-OMVs formulation for time reasons. However, based on the above consideration, we predict that the titers should approach those observed with the 4merB-OMVs formulation and in some case, they could reach even higher values.

All the neutralization data were obtained in collaboration with the Tumorvirus-specific Vaccination Strategies laboratories (DKFZ, Heidelberg, Germany), the laboratory where I spend one year for my Master Thesis.



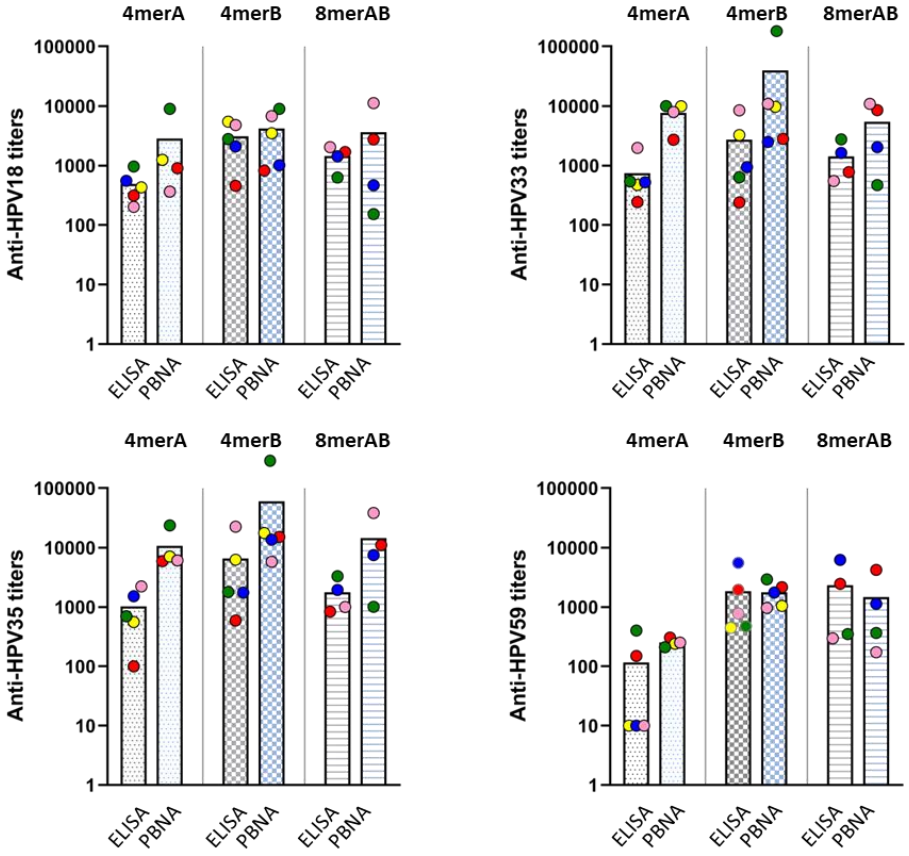


Figure 22 – ELISA and neutralization (PBNA) titers induced by OMV-based vaccines[112]**.** ELISA (Grey) and PBNA (Light blue) neutralization titers of the sera from mice immunized with 4merA-OMVs, 4merB-OMVs and 8merAB-OMVs (same sera described in Figures 2 and 3). Each graph groups the ELISA and neutralization titers against one of the selected HPV serotypes, elicited by the three vaccine formulations. To follow the correlation between the ELISA and the neutralization titers induced by each vaccine formulation, to each mouse serum has been assigned a circle with a different color code. ELISA titers correspond to the serum dilution that gives an OD_{405} value = 1.5 and the PBNA EC50 value calculated as the titer of serum that could neutralize half of the pseudovirus. Both titers are expressed in logarithmic scale. Depending upon serum availability, four or five sera from each group were analyzed. Graphs were made with GraphPad 8 software.

3.5 Set-up of a laboratory scale production process of 4merB-OMVs

From the ELISA and neutralization data reported above, it appears that the 4merB-OMVs is an attractive HPV vaccine candidate since, similarly to 8merAB-OMVs, it elicits broadly protective neutralizing antibodies against the majority of oncogenic high-risk HPV serotypes, as tested by PBNA. Moreover, OMV quantities as low as 10 μg/dose were sufficient to induce good antibody titers. The use of low quantities of OMVs is an important aspect in consideration of their high adjuvanticity which might lead to reactogenicity issues. The anti-Meningococcus B human Bexsero vaccine

contains 25 μg/dose of OMVs combined with 3 mg/ml of Alum and we expect that the use of equal, or possibly lower, doses of both adjuvants (OMVs and Alum) should ease the regulatory authorization path of novel OMV-based vaccines.

Therefore, in view of future clinical studies of our 4merB-OMVs vaccine, we investigated the possibility of setting-up a reproducible production process (Figure 23).

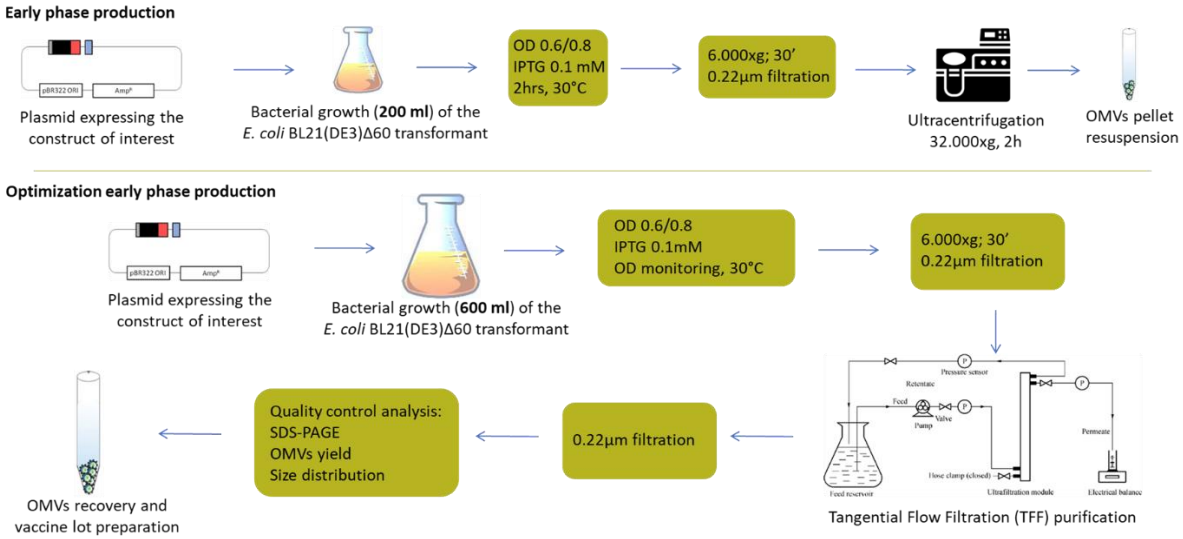


Figure 23 – Schematic workflow with the comparison between early phase production and the optimization of the process for OMV-vaccine lot preparation.

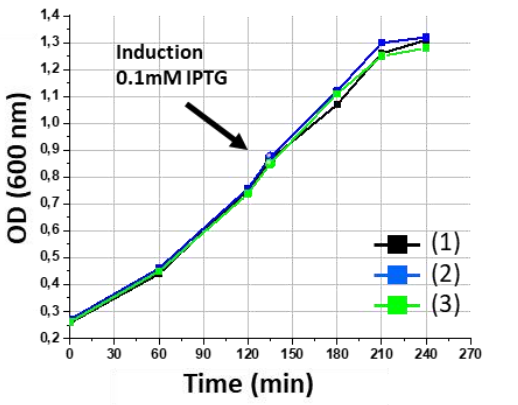
Three independent cultures of 100 ml of LB were inoculated with 0.1 ml of *E. coli* BL21(DE3)Δ60(pACYC-fHbp-DomA-4merB) working seeds (stored at -80°C in glycerol) previously prepared taking the cultural bacteria during their exponential growth. The cultures were incubated at 30°C for 15 hours under agitation at 200 rpm. Subsequently, 60 ml of each culture (approx. OD_{600} = 2.6) were added to 540 ml of LB in presence of the correct antibiotic and the three independent cultures were grown at 30°C under agitation. At OD_{600} = 0.8, 0.1 mM IPTG was added to induce the expression of fHbp-DomA-4merB fusion protein, and the growth was continued at 30°C for two additional hours, monitoring the optical density over the time. Next, the supernatants of the cultures were collected by centrifugation and left at 4°C for 18 hours in the presence of 1 U/ml of Benzonase. Finally, the OMVs were purified from the supernatant by Tangential Flow Filtration and filtered with a 0.22 μm PVDF filter.

In order to analyze the robustness of the OMVs preparation process, three independent cultures were carried out. As reported in Figure 24A, growth curves were highly reproducible. After OMV purification from the culture supernatants, OMVs were

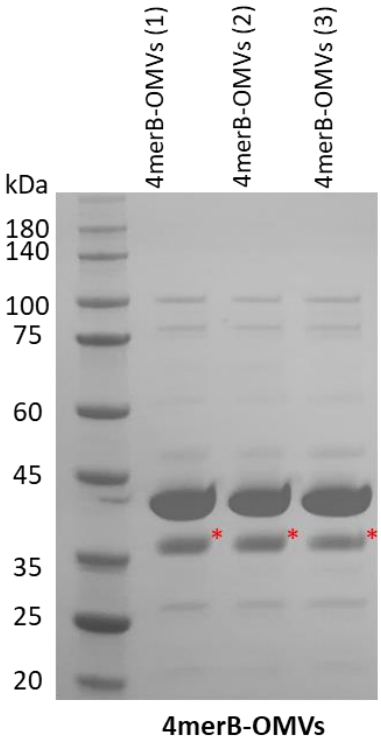
quantified by DC assay and 20 μg were analyzed by SDS-PAGE. As shown by Figure 24B, the three batches of OMVs were comparable in terms of protein profile and the fusion protein accumulated in the vesicular compartment at similar quantities. OMV yield was also reproducible, corresponding to 11.4 ± 1.05 mg/L (Figure 24C). Moreover, the vesicles were analyzed by Dynamic Light Scattering, which allows the measurement of the size distribution (Figure 24D). The mean of the OMV diameter of the three preparations was 40.2 nm with a standard deviation of 3.4. The 4merB-OMVs were compared to the OMVs derived from the recipient *E. coli* BL21(DE3)Δ60 strain.

Finally, the 4merB-OMV vaccine was tested in the IL-6 release assay, the validated assay used to follow reactogenicity and lot consistency of the Bexsero vaccine[113]. Increasing quantities of 4merB-OMVs were added to differentiated THP-1 cells and the IL-6 released in the supernatant was compared to the amount of IL-6 released by different quantities of commercially available Bexsero vaccine. As shown in Figure 24E, the 4merB-OMVs and Bexsero released similar quantities of IL-6, indicating that our 4merB-OMVs are in compliance with IL-6 levels measured for vaccine lots used in humans[95,114].

A

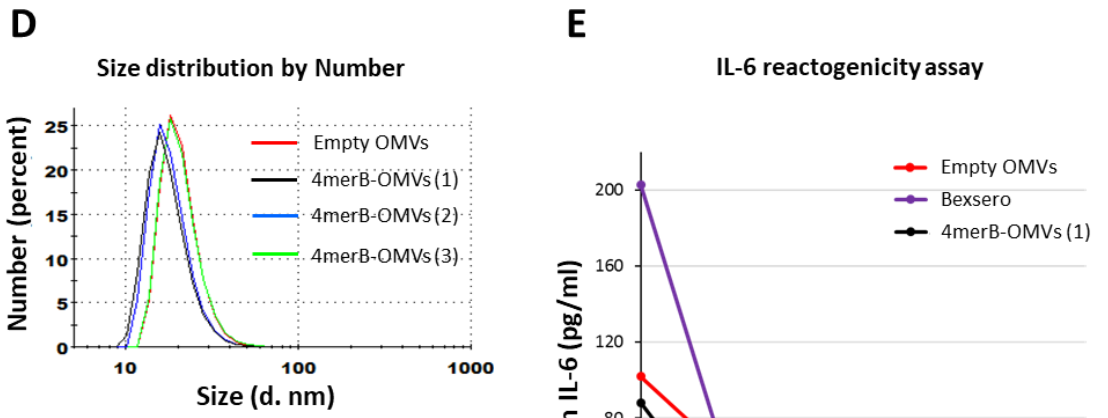


B



C

OMVs production	Yield (mg/L)
4merB-OMVs (1)	11,31
4merB-OMVs (2)	12,45
4merB-OMVs (3)	10,5



Sample	Size (nm)
Empty OMVs	37.90
4merB-OMVs (1)	43.62
4merB-OMVs (2)	39.29
4merB-OMVs (3)	37.65

Figure 24 – Reproducibility of the 4merB-OMVs laboratory scale production process[112]. **(A)** *E. coli* BL21(DE3)Δ60(pACYC-fHbp-DomA-MUC3x-4merB) strain was grown in LB in triplicate (growth curves 1, 2 and 3) starting from three different overnight cultures and the growth was monitored at 30-minute intervals. When the cultures reached an OD_{600} value of 0.8, the expression of the DomA-L2 fusions was induced by addition of 0.1 mM of IPTG. Three different OMVs batches were purified from the bacterial culture supernatants trough TFF. **(B)** Aliquots of 20 μg of OMVs purified from each culture were loaded on an SDS-PAGE polyacrylamide gel and stained with Coomassie Blue. The red asterisk indicates the band corresponding to DomA-4merB fusion. **(C)** Amount of 4merB-OMVs (expressed as mg/L of total proteins) recovered from each culture. **(D)** Size distribution profile of the three 4merB-OMVs preparations (Black, Blue and Green curves) determined by DLS using NanoZS90. The size of the 4merB-OMVs was compared with the purified "Empty" OMVs derived from recipient *E. coli* BL21(DE3)Δ60 (Red curve). The table reports the means of the vesicle diameter (nm) of each 4merB-OMV batch and of "Empty OMVs"**.** **(E)** IL-6 release assay. THP-1 human leukemic monocyte cells (1.5×10^5 cells in 100 μl/well) differentiated to macrophages with PMA were incubated with different amounts of either Empty OMVs or 4mer-OMVs or OMVs present in the Bexsero vaccine in a final volume of 200 μl/well. The IL-6 released in the supernatant was measured in duplicate by ELISA.

All together these data confirm the reproducibility of the 4merB-OMVs laboratory scale production process and show the low-reactogenicity profile of the "proteome minimized" strain, paving the way to the development of a pilot scale production process to run future toxicity studies in animals and Phase I studies in human volunteers.

3.6 Substitution of the MUC1 hinge region

MUC1 in the fusion proteins has a double role. First, it acts as a flexible hinge, which keeps the L2 epitopes sufficiently separated from the carrier protein (fHbp-DomA), thus favoring the recognition of L2 by B cell receptor. Second, as thoroughly explained in the discussion section, MUC1 is a tumor-specific epitope expressed in a large fraction of cancers, including those associated with HPV infection[115–117], and several clinical studies are being carried out aiming at demonstrating the ability of MUC1 vaccination to prevent cancer. Therefore, the combination of L2 epitopes and MUC1 is expected to have a synergistic effect in the protection against HPV-associated tumors.

Having said that, we tested the possibility to use a different hinge featuring low homology with human sequences but still capable of providing the necessary flexibility to the fusion proteins.

From the inspection of the amino acid composition of MUC1 repeat, three main features emerge: 1) the presence of the five proline residues which provide a certain rigidity to the repeat; 2) two closely located charged amino acids (aspartic acid and arginine); 3) 13 short chain polar or partially hydrophobic amino acids. On the basis of these features and guiding by the idea to preserve chemical motif of the repeated, we design a MUC1 mutant (MUC_{mutB}) in which the aspartic acid and the arginine were converted into glutamic acid and lysine and the polar/short chain amino acids were swapped from one position to another in order to maintain the same charge of the epitope. The proline residues were kept unchanged. The sequence of MUC_{mutB} is shown in Figure 25A. Moreover, in order to test whether the Prolines have a role in the conformational rigidity of our fusion protein, a construct in which all the Prolines were substituted with Alanines ($MUC_{P>A}$) was generated (Figure 25A).

The mutant repeats were fused in three copies to the C-terminus of fHbpDomA and the expression of the fusion protein in the OMVs was analyzed by SDS-PAGE. As shown in Figure 25B, the two proteins were expressed at a level similar to the one observed with wild type MUC1. Similarly to fHbp-DomA-MUC3x, fHbp-DomA-MUC_{mutB}3x was also at least partially exposed on the surface on the vesicle, as judged by the proteinase K shaving assay (Figure 25C). On the contrary, the band corresponding to fHbp-DomA-$MUC_{P>A}$3x protein is persistently visible after addition of Proteinase K with the same entity of the not treated sample, suggesting that the

Prolines are necessary for the exposure of the epitope outside from the membrane (Figure 25C).

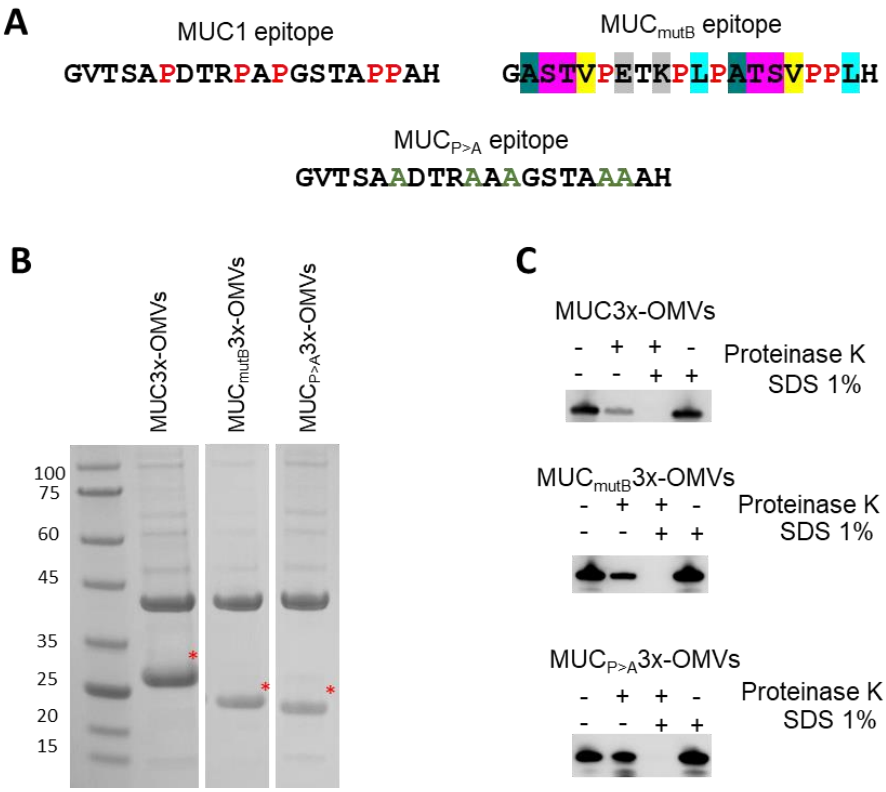


Figure 25 – Analysis of MUC1 mutant construct. **(A)** Sequences in single copy of MUC1 epitope, wild-type, MUC_{mutB} and $MUC_{P>A}$ sequence. **(B)** Aliquots of 20 μg of OMVs expressing the three fusion proteins of MUC1 wild-type and mutated form in triplicate were loaded on an SDS-PAGE polyacrylamide gel and stained with Coomassie Blue. **(C)** Assessment of MUC1 epitope localization on engineered OMVs. Purified OMVs (5 μg) were treated for 30 min with proteinase K in the presence (+) or absence (-) of SDS and the integrity of the fusion proteins was analyzed by Western Blot using an anti-fHpb antibody.

Next, we fused the L2 epitope of HPV16 to fHbp-DomA-MUC_{mutB} and we analyzed the expression and surface localization of the fusion protein in the OMVs. As shown in Figure 26A and B, the fusion protein behaved in a manner very similar to the original, non-mutated construct.

Finally, 10 μg of MUC_{mutB}3x-$L2_{16}$-OMVs were prepared and mixed with Alum for the immunization of four CD1 mice. Seven days after the third immunization, sera were collected and analyzed by ELISA. As shown in figure 26C, L2-specific antibodies were induced. A further investigation will be required for the analysis of the functional activity of the antibodies.

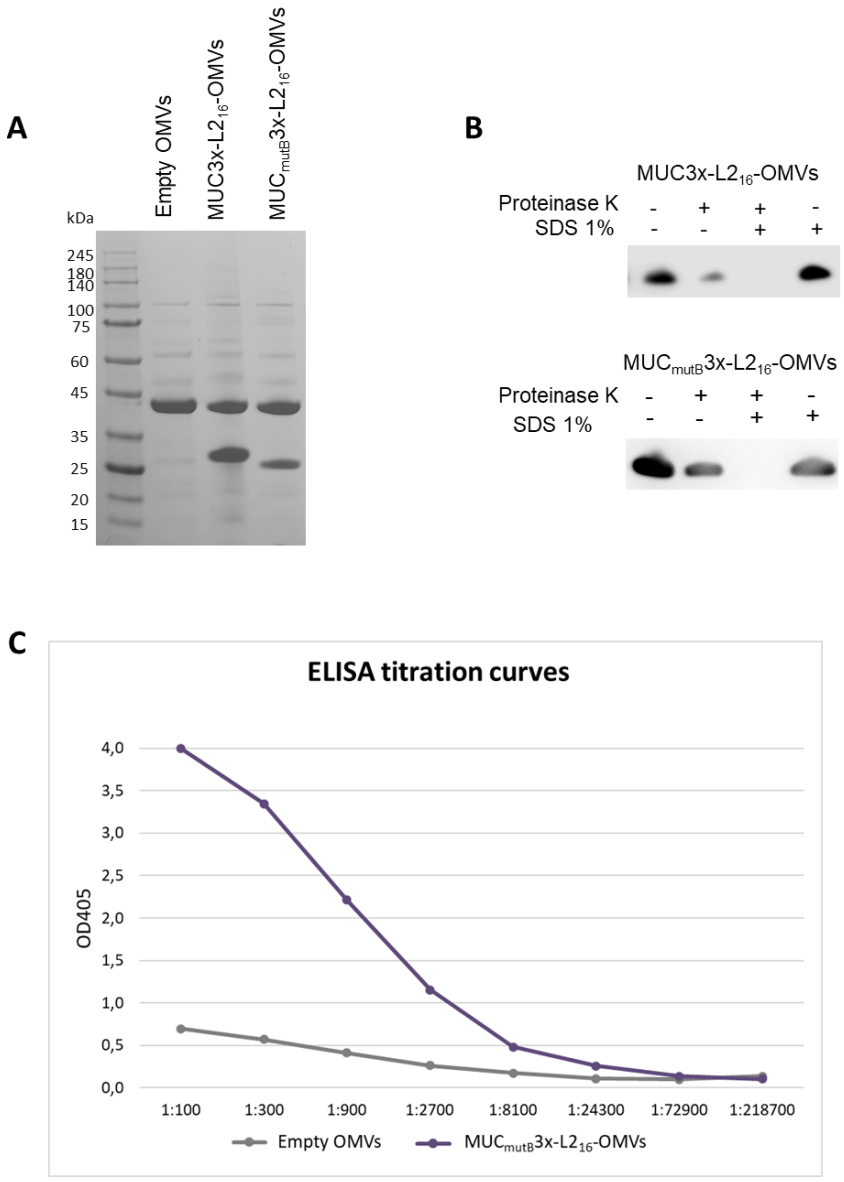


Figure 26 – Mutated from of MUC1 as carrier for HPV epitope (A) Aliquots of 20 μg of OMVs expressing the three construct MUC3x-L2$_{16}$ and MUC$_{mutB}$3x-L2$_{16}$ were loaded on an SDS-PAGE polyacrylamide gel and stained with Coomassie Blue. **(B)** Assessment of MUC1 epitope localization on engineered OMVs. Purified OMVs (1 μg) were treated for 30 min with proteinase K in the presence (+) or absence (-) of SDS and the integrity of the fusion proteins was analyzed by Western Blot using an anti-L216 monoclonal antibody (see Text for details). **(C)** ELISA curve of the pooled sera of CD1 mice immunized with MUC$_{mutB}$3x-L2$_{16}$-OMVs (10 μg/dose). Total IgGs titers of pool sera against the L2 epitope of HPV16 were analyzed by ELISA using plates coated with the corresponding peptide (0.5 μg/well).

All together these results suggest that the Prolines have a key role in favoring the exposure of this specific epitopes outside from the membrane. In this way, we obtained a specific and efficacious stimulation of the immune system, allowing to consider the use the of these carriers, the wildtype (MUC3x) or the mutated form ($MUC_{mutB}3x$), for future applications.

Chapter 4

DISCUSSION AND FUTURE PERSPECTIVES

Current HPV vaccines, based on the major L1 capsid protein, represent extremely powerful strategies for preventing HPV infections. However, vaccine costs and serotype specificity are major hurdles, which limit the introduction of global vaccination campaigns that would drastically reduce the HPV-related pathologies, cancer above all. It has been estimated that in vaccinated women, a reduction of 85-90% of HPV-16/18-related high-grade dysplasia was observed[118]. For this reason, the development of an HPV vaccine which can be produced with lower costs, in order to include low- and middle- income country population in the vaccination campaign, is an actual medical need.

4.1 Our candidate 4merB-OMVs vaccine induces high neutralizing antibody titers

In this work we have presented a new formulation that has the potential to overcome the limitations of the L1-based vaccines, which are the coverage restricted to the HPV vaccine serotypes and the high production and distribution costs. Taking advantage of previous works showing that the L2 HPV protein carries a conserved neutralizing B cell epitope[58], and that a string of L2 epitopes selected among eight relevant HPV serotypes provides broad cross-protection[44,62], we tested whether *E. coli* OMVs could

be decorated with L2 repeats and whether such OMVs could elicit anti-HPV functional antibodies. Our data show that, indeed, the expression of the L2 repeats fused to the N-terminal domain of neisserial fHbp was highly efficient: the fusion proteins represent approximately 13%-16% of total OMV proteins. Moreover and importantly, the immunization of mice with the engineered OMVs elicited high titers of anti-L2 antibodies, which could efficiently neutralize the *in vitro* infection induced by a panel of eight HPV serotypes, as judged by the L1 pseudovirus-based neutralization assay.

Interestingly, previous work predicted that vaccines containing the L2 epitopes from the eight selected serotypes (16, 31, 51, 6, 18, 33, 35, and 59) protect against more than 90% of all circulating oncogenic HPV and genital wart associated strains[111], including the serotypes which are the target of the current nonavalent Gardasil vaccine[44]. Our neutralization data show that the expression of the 18-33-35-59 tetramer in OMVs (4merB) was necessary and sufficient to neutralize with high efficiency not only the homologous pseudovirus but also the 16, 31, 51, 6 pseudoviruses. Although the vaccine neutralization capacity has not been tested yet against other serotypes, this result might suggest that the L2 epitopes are presented on the surface of the OMVs in a configuration that allow the elicitation of antibodies with a cross-protective activity broader than what originally predicted. This was confirmed by Pouyanfard et al.[44] in which sixteen HPV serotypes were tested against the sera from mice immunized with the eight HPV serotypes included in our vaccine. For the majority of them, the protection is provided also even though the specific L2 epitope is not included in the formulation.

A relevant question is whether the neutralizing activity induced by our L2-expressing OMVs would be sufficient to protect HPV infection in humans and how the neutralization titers compare with the titers induced by the L1-based vaccines. To start addressing this question it would be necessary to run head-to-head comparative studies immunizing mice with commercially available vaccines. However, the performance of our OMV-based vaccine with respect to the L1 vaccines can be inferred from the published neutralization data measured using the pseudovirus assay. For instance, Romanowski et al.[119] reported that in humans the HPV16 neutralizing antibody titers induced by the HPV-16/18 AS04-adjuvanted vaccine and measured 7 months after the third vaccination dose were in the range of 2×10^4. Should our OMV vaccine be in humans as immunogenic as in mice, the neutralization titers of the L2-based vaccine and the L1-based vaccine would not be too dissimilar.

Moreover, considering the relatively long period of time during which the L2 epitope remains exposed on the virus surface to allow the furin cleavage[120] the role of anti-L2 antibodies might be crucial to prevent HPV infections.

4.2 Potential applications of L2-OMV vaccine against HPV

This said, it has to be pointed out that it is currently not known the threshold of anti-L2 functional antibody titers necessary and sufficient to provide protection against HPV infection. Also, it is not known whether to be protective anti-L1 and anti-L2 antibodies have to reach the same functional antibody threshold. These pieces of information can ultimately only be collected in future clinical trials.
Clinical trials using L2-based vaccines are considered particularly complicated in view of the availability of effective anti-L1-based vaccines. However, it is important to remind that the human population is seronegative with respect to the L2 N-terminal epitope since neither natural infection nor vaccination elicit antibodies against this particular L2 epitope[44]. Therefore, it is possible to envisage studies whereby volunteers vaccinated with a L1 vaccine receive our OMVs (4merB) vaccine whose immunogenicity can be followed comparing anti-L1 and anti-L2 antibody titers with respect to control volunteers. Subsequently, OMVs (4merB) vaccine efficacy can be established following the incidence of infection/disease caused by non-L1 vaccine strains.

A second possible clinical application of our OMV vaccine would be its combination with one of the existing L1 vaccines. We believe that such combination would be extremely attractive for three main reasons. First, the anti-L1 and anti-L2 antibodies are expected to synergize, thus providing extremely high neutralization titers. Second, the neutralizing antibody titers should be further enhanced by the potent adjuvant contribution of the OMVs. Third, considering the broad protective activity of the string of L2 epitopes, the vaccine combination is likely to become a universal PAN-HPV vaccine.

The undisputable advantages of the OMV-based vaccines are the simplicity of the production process, which could be easily set-up in any local production facility at extremely low costs. Indeed, our experiments show that, at least under laboratory conditions, the production process of our OMVs (4merB) vaccine is robust and reliable. Data provided are in line with the data published by Zanella et al[97] in which the recipient

strain was analyzed. OMV distribution size and OMV-induced reactogenicity were compared to the proteome-minimized strain, obtaining optimal results. With regard to the OMV-production yield, the amount of OMVs obtained with laboratory flasks for our 4merB-OMV vaccine was promising, considering also that it can be improved using a fermentation unit. In fact, considering that using a 2 L fermentation unit we routinely obtain, with our "proteome minimized" strain[97], 20-50 mg of OMVs/L of culture and assuming a vaccination schedule of three injections of 10 to 20 μg of OMVs/dose, more than to 10^4 three-dose vaccines could be produced from a small 100 L fermenter unit.

4.3 The advantage of the MUC1 linker for a combinatorial effect against different cancer types

To deliver the strings of the L2 epitopes to the surface of the OMVs we used the 120 amino acids long N-terminal domain of the neisserial fHbp carrying three copies of the 20 amino acid MUC1 epitope at its C-terminus. The motivation to use this fusion is threefold. First, we previously demonstrated that such fusion is exposed on the surface of the outer membrane and accumulates in the vesicular compartment with remarkable efficiency, the fusion representing more than 20% of total OMV proteins[101]. Second, the MUC1 repeat is expected to provide flexibility to the L2 polyepitope, thus facilitating the elicitation of proper humoral responses. Third, MUC1 is one of the most characterized and interesting tumor-specific epitopes[121]. The epitope, named VNTR and having the sequence GVTSAPDTRPAPGSTAPPAH, is found repeated 20 to 150 times in the extracellular domain of the transmembrane glycoprotein Mucin1[115]. Mucin 1 protein plays an essential role in forming protective mucous barriers on epithelial surfaces and it behaves differently in healthy and tumoral tissue. In normal epithelia, VNTR is highly glycosylated in Serine and Threonine whereas in most carcinomas, such as those of breast, ovary, colon, pancreas, lung, head and neck and in premalignant lesions, MUC1 becomes over-expressed and hypoglycosylated, thus making the non-glycosylated VNTR epitope immunogenic and tumor-specific. The differentiated nature of MUC1 epitope between normal and cancerous tissues has been the rational to design vaccines based on the non-glycosylated MUC1 epitope, which are currently in clinical trials. Interestingly, as reported in *clinicaltrial.gov*, over 400 clinical trials have been conducted using MUC1 epitope in the prophylactic and in

the therapeutic settings and excellent safety data with promising efficacy have been reported[122].

On the basis of the above consideration, we believe that the presence of MUC1 in our formulation brings two potential advantages. First, in addition to anti-L2 antibodies, the vaccine is expected to induce anti-MUC1 antibodies. Therefore, the 4merB-OMVs vaccine candidate could represent a valid alternative to the prophylactic peptide-based MUC1 vaccines, which are currently being tested in clinical trials and which could potentially prevent the large number of MUC1-positive epithelial adenocarcinomas, such as lung, liver, pancreatic, breast, ovarian and head and neck cancers[106]. Second, since MUC1-specific $CD8^+$ T cells have been shown to kill MUC1 expressing cancer cells and have been proposed to play a role in cancer therapy[107,123], it is tempting to speculate that our OMV-based vaccine could have a potential therapeutic application. Knowing that L1 and L2 protein-based vaccines does not provide any therapeutic effect due to their selective expression on terminal differentiated keratinocytes[125], different clinical trials designed for the treatment of HPV-related cancers with peptides expressing early proteins E6 and E7 or dendritic cells pulsed with HPV16/18-E7 oncoprotein are ongoing[124]. Our proposed OMV-based vaccine could synergize such vaccines and potentially be effective against head and neck MUC1-positive tumors irrespectively to the presence or absence of HPV infection. Should this therapeutic effect be demonstrated, the 4merB-OMV vaccine would offer an additional advantage over the current HPV vaccines which are not effective at eliminating pre-existing infections[125].

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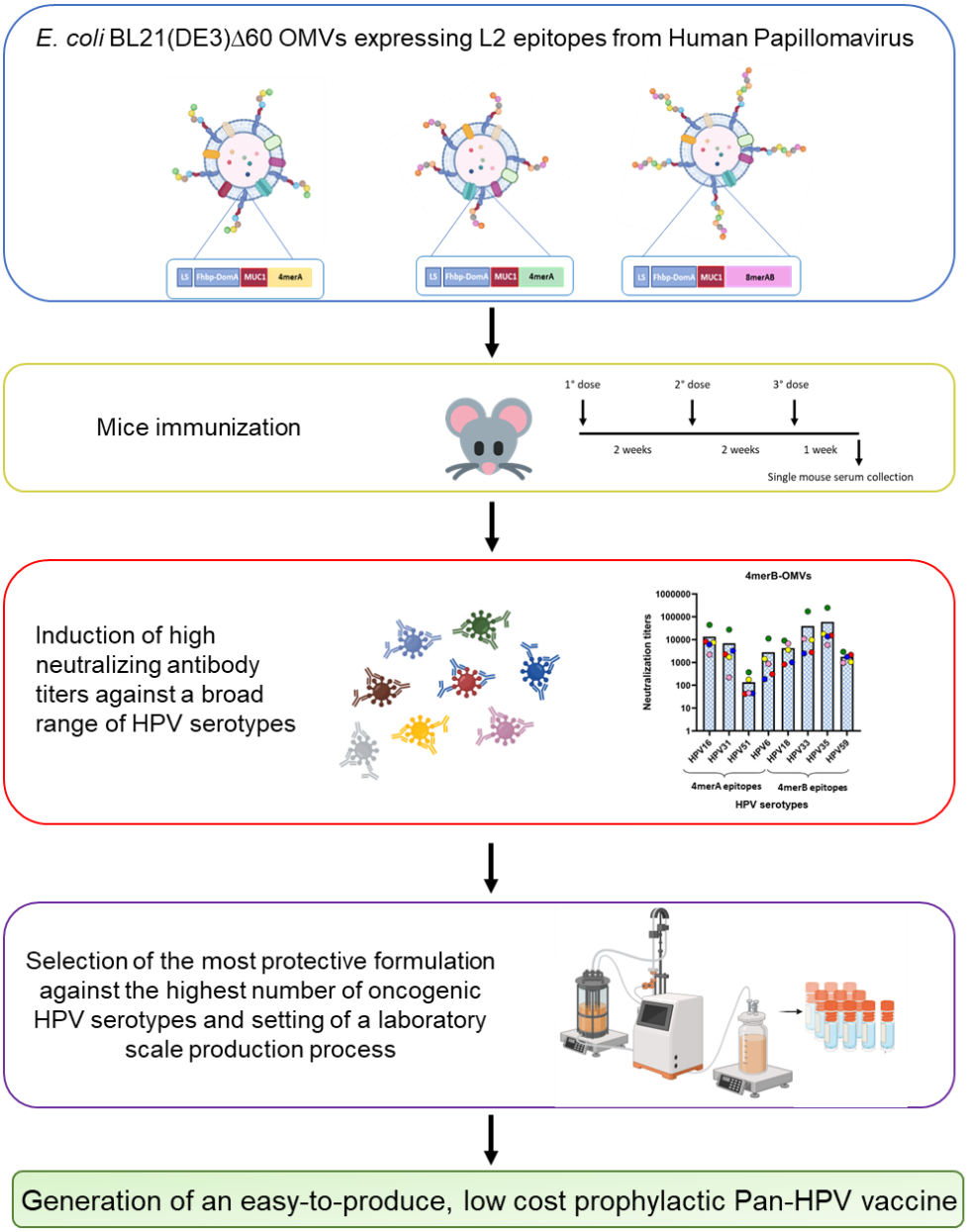
GRAPHICAL ABSTRACT



PUBLICATIONS

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