Novel approaches to an RSV vaccine: Papillomavirus-based delivery of a genetic vaccine and low-energy electron irradiation for the production of a killed vaccine

Dissertation
zur Erlangung des
Doktorgrades der Naturwissenschaften (Dr. rer. nat.)

der

Naturwissenschaftlichen Fakultät I – Biowissenschaften –
der Martin-Luther-Universität
Halle-Wittenberg,

vorgelegt von
Frau Lea Johanna Katrin Bayer (geb. Wierich)
geboren am 13. August 1987 in Stuttgart

öffentlich verteidigt am 28. November 2018

Gutachter:

Prof. Dr. Sven-Erik Behrens (Martin-Luther-Universität Halle-Wittenberg)
Dr. Thomas Grunwald (Fraunhofer Institut für Zelltherapie und Immunologie, Leipzig)
Prof. Dr. Frank Stubenrauch (Eberhard Karls Universität Tübingen)

CONTENT

I. List of Figures

Introduction

Methods

Results

DISCUSSION

APPENDIX

II. List of Tables

METHODS

RESULTS

III. List of Acronyms

AAV	Adeno-associated virus
Ad5	human adenovirus serotype 5
APC	Antigen presenting cell
AS	Adjuvant System
BPV1	bovine papillomavirus type 1
CcPV1	Caretta caretta papillomavirus type 1
CcrPV1	Crocuta crocuta papillomavirus type 1
CD	cluster of differentiation
CgPV1	Colobus guereza papillomavirus type 1
C_t	threshold cycle
DC	Dendritic cell
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
ERD	enhanced respiratory disease
F.Luc	Firefly luciferase
FBS	Fetal Bovine Serum
G.Luc	Gaussia luciferase
h	hour
HBSS	Hank's buffered salt solution
HE	hematoxylin-eosin
HPV	Human papilloma virus
HRP	Horseradish peroxidase
IFN	Interferon
IgG	Immunoglobulin G
IL	Interleukin
IRES	internal ribosome entry site
LEEI	Low-energy electron irradiation
MmPV1	Macaca mulatta papilloma virus type 1
MDA-5	melanoma differentiation-associated protein 5
MfPV6	Macaca fascicularis papilloma virus type 6
MfPV11	Macaca fascicularis papilloma virus type 11
MHC	Major histocompatibility complex
MPL	Monophosphoryl lipid A
MVA	modified vaccinia virus Ankara
ND10	Nuclear Domain 10
OPP	oriented polypropylene

PAMP	pathogen-associated molecular pattern
PcPV1	Puma concolor papillomavirus type 1
PEI	Polyethyleneimine
PlPV1	Procyon lotor papillomavirus type 1
PtPV1	Pan troglodytes papillomavirus type 1
RaPV1	Rousettus aegyptiacus papillomavirus type 1
RT	Room temperature
PRR	pattern-recognition-receptor
PsV	Pseudovirus / Pseudovirion
PV	Papilloma virus
RNA	Ribonucleic acid
TLR	Toll like receptor
VLP	Virus like particle
MEM	minimal essential medium

1 ABSTRACT

The presented thesis deals with two novel vaccine technologies, which were analyzed in view of developing a vaccine against the respiratory syncytial virus (RSV).

The first approach involved the use of non-human papilloma pseudovirions for the delivery of a DNA-vaccine. Ten different animal papilloma virus types were tested for their ability to form virus-like-particles and pseudovirus-particles. The latter was then used to encapsidate a reporter plasmid and deliver it into cells *in vitro*. The tested papilloma virus types showed great variability in their transduction efficiency. The two candidates that worked the most efficiently – MfPV11 and PcPV1 – were used for further analyses and *in vivo* studies. Both were able to transduce a reporter plasmid in mice after intramuscular injection, leading to the expression of the reporter protein. In the case of PcPV1, this expression was maintained over several weeks. In an immunization study, mice were vaccinated in a heterologous prime-boost regimen, intramuscularly and intranasally applying a vaccine plasmid coding for the RSV F-protein packaged in MfPV11 and PcPV1 pseudovirions. In comparison to non-vaccinated mice, immunized mice showed a significantly decreased viral load upon challenge with infectious RSV.

The second approach studied in this thesis dealt with the development and analysis of a novel technology to produce killed vaccines. RSV was irradiated with low-energy electrons, and the required dose for complete inactivation was determined as 20kGy. Analysis of the remaining antigenicity after low-energy electron irradiation (LEEI) revealed that at least 70% of viral proteins were conserved during the process. The LEEI-treated RSV material was used in vaccination studies and yielded a strong immune response and protection against RSV upon challenge with infectious virus. Although the use of different adjuvants did modify the immune response, it did not significantly influence the protective efficacy of the LEEI-RSV vaccine.

2 INTRODUCTION

A vaccine against the Respiratory Syncytial Virus (RSV) is urgently needed. To date, there is no vaccine against RSV available on the market. RSV is one of the leading causes of child mortality, especially in developing countries (Nair et al., 2010), but also presents a substantial issue in the elderly population (Falsey et al., 2005). In the hope that a two-pronged approach might increase the chances of developing a viable vaccine, two distinct research projects were conducted.

The first project aimed at developing a platform based on non-human papillomaviruses for viral delivery of genetic vaccines. Despite the many advantages of DNA-vaccines, there is currently no licensed genetic vaccine available for use in humans. The main issue lies in the application of genetic material, which is very inefficient when delivered by plain intramuscular injection. Human papillomavirus (HPV) has already been successfully used for the delivery of a vaccine plasmid (Graham et al., 2010). However, due to vaccinations against HPV, as well as natural infections, HPV is not a suitable vector for genetic vaccines. Therefore, ten different non-human papillomaviruses were tested for their ability to form pseudovirion particles, package a reporter- or vaccine plasmid inside and deliver the plasmid *in vitro* and *in vivo*.

Secondly, low-energy electron irradiation was tested as inactivation method for RSV with the goal of using the killed virus as vaccine. There is no vaccine against RSV available on the market yet, although the need is high. RSV is one of the leading causes of child mortality, especially in developing countries, but also presents a substantial issue in the elderly population Low-energy electron irradiation (LEEI) shows promise as a fast and gentle inactivation method for various pathogens (Fertey et al., 2016). RSV was subjected to LEEI, and tested as a vaccine in combination with a number of adjuvants for its ability to elicit an immune response and confer protection upon challenge in mice.

2.1 Vaccine immunology – an overview

The immune system of jawed vertebrates comprises two interlinked parts – the innate immune system and the adaptive (or acquired) immune system. The innate system detects invading pathogens through molecular-sensing mechanisms such as pattern recognition receptors (PRRs), and uses soluble effectors such as complement, and cellular players such as natural killer cells, monocytes, neutrophils, eosinophils, macrophages and dendritic cells (DCs). The role of the adaptive immune system is to form the second line of defense, which is antigen-specific and includes antibodies, cytokines and cytolytic molecules as effectors (Murphy, K., Travers, P., Walport, M., & Janeway, C., 2011).

Whenever a pathogen – both during an infection and in a vaccination scenario – enters the human body, it is first taken up by immature APCs such as macrophages or dendritic cells. Through PRRs expressed by the cells of the innate immune system, pathogen-associated molecular pattern (PAMPs) are recognized. There are four families of PRRs: toll-like receptors (TLR), nucleotide oligomerisation receptors (NLR), RIG-1 like receptors (RLR) and c-type lectin receptors (CLR). Signaling through these receptors leads to the expression of pro-inflammatory molecules, antimicrobial factors and chemotactic signals. This first response allows the invading pathogens to be damaged, and to recruit more supporting immune cells. In addition, APCs – DCs being the most specialized among them – process the antigens that they have taken up and carry them to the lymph node. This is where APCs bridge the innate and adaptive immune responses.

The DC's major histocompatibility complex (MHC) class II molecules present the antigen fragments on the cell's surface, allowing an interaction with the receptors of immature B- and T-cells in the lymph node. T-cells cannot recognize pathogens on their own, but need the presentation by APCs. A naïve T-cell expresses a unique antigen-specific receptor, but activation of the T-cell occurs only once the T-cell receptor binds to the antigen presented by an APC's MHC molecule. T-cells characterized by expressing the cluster of differentiation (CD)4 cell surface protein (CD4+ T-cells) are also referred to as helper T-cells (Th-cells) and primarily act by secreting cytokines. Several subgroups have been identified, characterized by the types of cytokines they secrete. Th1 and Th2 are the most intensely studied subgroups. CD4+ T-cells of the Th1 subgroup secrete mainly IFNγ, which restricts pathogen survival and spreading, and stimulates the differentiation of cytolytic cells. Th2 cells are primarily involved in the response to extracellular pathogens and parasites, and activate innate cells like eosinophils and mast cells by secreting cytokines. The activation of CD4+ T-cells is critical for the effective launch of the adaptive immune response. The antibody-producing B-cells and CD8-expressing cytolytic T-cells typically characterize the two effector cell populations.

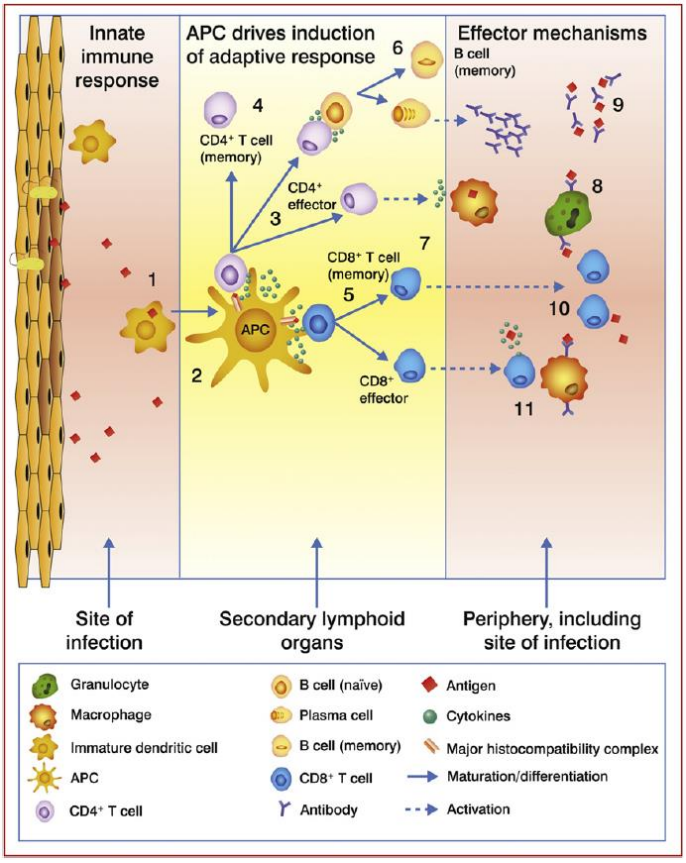


Figure 2.1-1 - The summarized human immune response

The innate immune response is initiated by immature innate immune cells upon entry of a pathogen (1) as they differentiate and travel to the lymph node (2). APCs activate T-cells (3-5) and naïve B-cells differentiate into plasma cells after activation by CD4+ T-cells. CD4+ T-cells further activate tissue-resident macrophages (7). Antibodies function as neutralizer of pathogens (9) and as enhancer of innate cells (8). Cytotoxic T-cells kill infected cells (10) and support the stimulation of phagocytes to eliminate intracellular pathogens (11).

(Leo et al., 2011)

CD8+ cells make use of the fact that MHC class I molecules are found on any nucleated cell, presenting fragments of intracellular proteins. CD8+ cells have the ability to detect pathogen-derived fragments presented on MHC I and to eliminate the infected cell, thereby interrupting the intracellular replication of the pathogen.

B-cells express a unique antigen receptor on their surface, which – in contrast to T lymphocytes – can bind directly to antigens without prior presentation by APCs. After binding of an antigen to the B-cell receptor, B-cells proliferate and differentiate into plasma cells, producing large amounts of antibodies, which are simply a soluble form of the B-cell receptor. Antibodies exert their function by binding directly to the surface of antigens, thereby blocking the infection of host cells, and furthermore by binding and therefore neutralizing toxins. Additionally, antigen binding can facilitate pathogen internalization by phagocytes ("opsonisation"), activate granulocytes and recruit effector molecules such as complement. Although a T-cell independent response is possible, optimal B-cell activation and production of high affinity antibodies is only achieved upon simultaneous activation of CD4+ T-cells (Murphy, K., Travers, P., Walport, M., & Janeway, C., 2011).

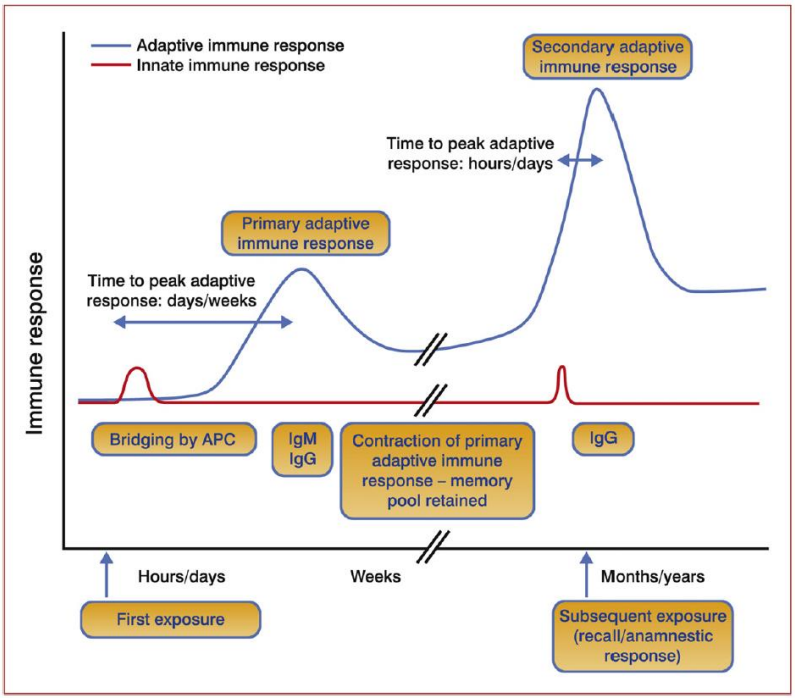


Figure 2.1-2 - Primary and memory immune response

Upon the first encounter with an antigen, e.g. in a prime vaccination, processing of innate immune system and presenting to cells of the adaptive immune system ("bridging by APCs") takes up days to weeks. The resulting memory cell population remains for long periods of time. Subsequent exposure to the antigen, e.g. during the booster vaccination, the innate immune system will briefly be activated, followed by a rapid and strong adaptive immune response.

IgM – immunoglobulin M, IgG, immunoglobulin G.

(Leo et al., 2011)

In the case of both B- and T-lymphocytes, some of these cells differentiate into effector cells and become antibody-producing plasma cells and cytokine-producing T-cells, respectively. A portion of the cells will become "memory cells", which have the ability to survive a long time. Upon secondary exposure to an antigen, the memory cells expand and differentiate rapidly, providing a strong immune response including high levels of specific antibodies. It is this immune memory on which all effective vaccines are based (Leo et al., 2011).

2.2 A brief history of vaccine technologies

Together with improved hygiene, vaccines have been the most effective measure against infectious diseases as they have drastically reduced the occurrence of many once common ailments. A true success story of vaccines, the WHO announced the eradication of smallpox in 1979 due to successful vaccination campaigns (Breman et al., 1980). Interestingly, smallpox is the virus, with which the development of vaccines began. The Chinese are reported to have been the first to inoculate humans with dried cowpox to prevent smallpox. Edward Jenner was then the first to conduct studies to show that inoculation with material from cowpox blisters elicits immunity against smallpox in 1796 (Riedel, 2005).

It then took about 100 years to develop the next vaccine, the rabies vaccine. Unlike Edward Jenner, who had no knowledge of the viral nature of smallpox, subsequent discoveries of the pathogen preceded the development of the respective vaccine in most cases (Bonanni and Santos, 2011). First vaccines were developed against diseases with high morbidity and mortality, such as smallpox, diphtheria and tetanus. Other important targets were “battlefield diseases” such as typhoid fever, plague and cholera. Today, the development of vaccines against pathogens causing morbidity and mortality is complemented by the addition of therapeutic vaccines.

When new vaccines are developed, the most direct approach is chosen first, which usually involves the whole pathogen. If safety, immunogenicity or practicability issues make this impossible, alternative strategies are employed, such as subunit and recombinant protein vaccines. The various vaccines and their technologies that have been developed over the last decades are summarized in Figure 2.2-1.

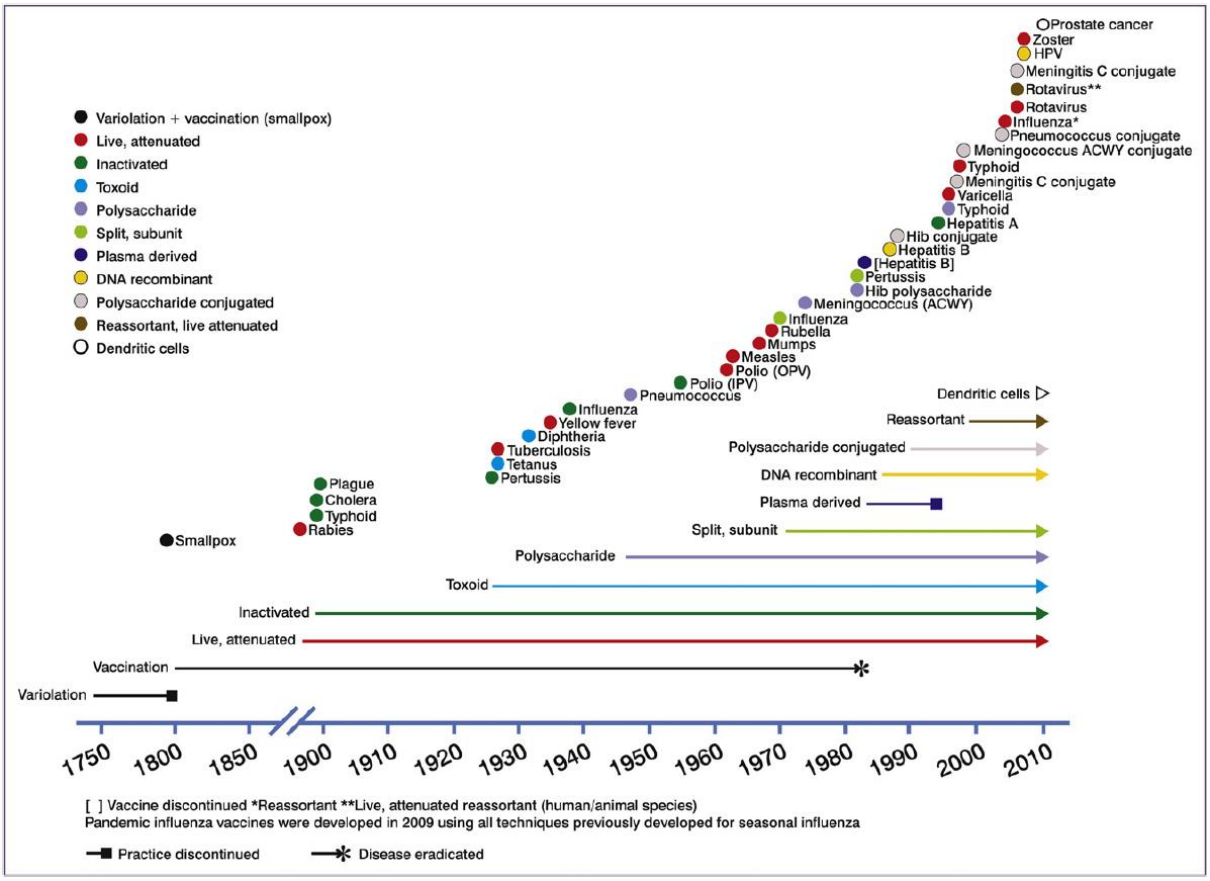


Figure 2.2-1 - Vaccines and technologies

Development of new vaccine technologies in the past centuries (Bonanni and Santos, 2011)

2.3 Inactivated vaccines

Typhoid, cholera and plague were the first pathogens against which inactivated vaccines were developed. In contrast to live attenuated vaccines, inactivated or killed vaccines do not present any residual risk of causing an infection or reverting back to virulent mutations of the vaccine strain. In inactivated vaccines, there is no selection of the most immunogenic antigen, as the whole pathogen is present in the vaccine preparation.

An old and still very common method for inactivation is the incubation with chemicals such as formaldehyde or β-propiolactone (Goldstein and Tauraso, 1970). The process of chemical inactivation using formaldehyde, however, leads to cross-linking of protein structures (FRAENKEL-CONRAT and OLCOTT, 1948) and therefore often negatively impacts the antigenicity of the resulting vaccine. Apart from the altering properties of the chemicals, the incubation usually takes up several days, and in some cases even weeks, to ensure inactivation. Following inactivation, the chemicals often have to be removed in labor-intensive downstream processes to avoid toxicity in the patient.

Various irradiation methods have been employed as alternatives to inactivate pathogens for vaccine production. One suitable method is gamma- or high-energy electron irradiation, which inactivates pathogens by damaging nucleic acids rather than protein structures (Seo, 2015). During gamma-irradiation, large amounts of X-rays and secondary radiation are emitted, necessitating extensive shielding. Successful inactivation can also be achieved using ultraviolet light, but substantial degradation of proteins is observed here (Delrue et al., 2009). Low-energy electron irradiation (LEEI) presents another irradiation method for pathogen inactivation, circumventing the use of any possibly structure-altering chemicals.

2.3.1 Low-energy electron irradiation (LEEI)

Low-energy electron irradiation has the ability to induce single and double strand breaks in DNA (Boudaïffa et al., 2000), while protein structures remain largely intact (Fertey et al., 2016). During LEEI, only low amounts of X-rays are emitted as a byproduct, which allows LEEI to be integrated into standard laboratories, including high biosafety level or GMP environments. Until now, LEEI has mainly been used for surface sterilization (Wetzel et al., 2010) due to its low penetration depth. In order for a liquid solution to be completely irradiated by LEEI, it has to be present as thin liquid film with a depth of less than 1mm. It has previously been shown that LEEI can be used for inactivation of both viruses and bacteria. Vaccination with LEEI-inactivated Influenza H3N8 induced an effective immune response and protection after challenge with H3N8 virus in mice (Fertey et al., 2016).

2.4 Adjuvants

Inactivated vaccines usually require repeated vaccination and the addition of an adjuvant for the vaccine to elicit a solid immune response (Garçon et al., 2011). Derived from the Latin word *adjuvare*, to help, an immunological adjuvant is defined by the National Cancer Institute as "a substance used to help boost the immune response to a vaccine so that less vaccine is needed" (NIH National Cancer Institute). Especially in neonates and the elderly, vaccines often do not work optimally. The immune systems of these populations are characterized by a reduced antigen-uptake and -presentation by APCs, as well as compromised responses by the adaptive immune system (Simon et al., 2015). Adjuvants are therefore especially important in vaccines targeting these populations to induce an immune response. In general, adjuvants are used to trigger the innate immune system in order to increase the recruitment of immune cells and the local cytokine response.

The 1920s witnessed a great advance in the vaccine research, when Alexander Glenny and Gaston Ramon discovered that addition of starch could substantially increase immune responses and antibody titers. Shortly thereafter, Glenny successfully tested aluminum salts for the precipitation of diphtheria toxoid for the immunization of rabbits (Glenny et al., 1931). To this day, aluminum salts remain the most commonly used adjuvants in vaccines and until recently represented the only licensed adjuvant (Kool et al., 2012). One of the main limitations of aluminum salts is their low effectivity in the induction of a Th1 type or cytotoxic T-cell response, as they exert a bias towards a Th2 response and antibody production (Didierlaurent et al., 2009). It has been shown that alum induces the secretion of IL-1β and IL-18, but conflicting reports exist whether the Nalp3 inflammasome is necessary for the adjuvant effect of alum (Eisenbarth et al., 2008; Franchi and Núñez, 2008). Although alum is the most frequently used adjuvant, the underlying mechanisms of its adjuvant properties are not yet entirely clear.

In 1997, the new adjuvant MF59 was approved in Europe. In the meantime, the MF59 adjuvanted influenza vaccine "Fluad" has been licensed worldwide in more than 20 countries (O'Hagan et al., 2012). MF59 is a squalene-based oil-in-water nano-emulsion (Banzhoff et al., 2009), which induces an inflammatory response by recruiting and activating antigen presenting cells. Due to an increased uptake of antigens by monoycytes and enhanced differentiation towards a mature phenotype, MF59 elicits a higher antibody and T-cell response in comparison to aluminum salts (Seubert et al., 2008). Besides MF59, further emulsion adjuvants are in development or in clinical trials. Montanide is a water-in-oil emulsion composed of mineral oil and mannide-mono-oleate as emulsifier. Although it has been shown to induce a strong immune response, Montanide is used mainly in immunotherapy because of mild to severe local reactions (Aucouturier et al., 2002).

QuilA, which is only licensed for veterinary vaccines due to toxicity and high local reactogenicity, is a saponin extracted from the bark of the *Quillaja saponaria* tree (McKee et al.,

2007). QuilA and its more tolerable derivative QS21 have been shown to stimulate a Th1 immune response by induction of IL-2 and IFN-γ, and to enhance the production of cytotoxic T-cells (Sun et al., 2009; Zhu and Tuo, 2016). There are two QS21-containing adjuvant mixtures, AS01 and AS02, which are currently being evaluated as part of different vaccine approaches in clinical trials (Garçon et al., 2007).

The development of novel adjuvants is not only motivated by the search for greater effectivity, but also to avoid unwanted side-effects, which can occur both locally and systemically. Swelling, local inflammation, pain, injection site necrosis, granulomas, lymphadenopathy, ulcers and the generation of sterile abscesses are examples of local reactions. Systemic side-effects include fever, nausea, eosinophilia, adjuvant arthritis, uveitis, anaphylaxis, allergy, organ specific toxicity and immunopathology. Often, a strong adjuvant effect correlates with enhanced toxicity. Although very potent, Freund's complete adjuvant, for example, is too toxic to be used in humans (Petrovsky and Aguilar, 2004). In 2011, Shoenfeld and Agmon-Levin coined the term "ASIA – Autoimmune/inflammatory Syndrome Induced by Adjuvants", a new syndrome, which describes a number of clinical conditions that are associated with the administration of adjuvants. One major criterion for ASIA is the exposure to an external stimulus, including vaccines, infections, adjuvants and silicone. Typical clinical manifestations are myalgia, myositis or muscle weakness, arthralgia and/or arthritis, chronic fatigue, neurological manifestations, cognitive impairment and pyrexia (Shoenfeld and Agmon-Levin, 2011). ASIA as a syndrome is however criticized for its ambiguous defining characteristics (Hawkes et al., 2015). The more detailed insights into the mechanisms of the innate immune system that are available now have inspired the development of new adjuvants. In a more rational approach, PRRs are addressed directly to induce a good innate immune response.

The poly-lysine stabilized, synthetic analog of double-stranded RNA Poly IC:LC leads to the activation of the innate immune system by binding to TLR3 (Matsumoto and Seya, 2008) and to the melanoma differentiation-associated protein 5 (MDA-5) (Andrejeva et al., 2004). As double-stranded RNA often presents a sign of virus infection, it induces the production of inflammatory cytokines and type I interferon (Akira et al., 2006; Gürtler and Bowie, 2013). Vaccination studies in rhesus macaques with Poly IC:LC showed its ability to induce Th1 and humoral immune responses (Stahl-Hennig et al., 2009). Poly IC:LC has been evaluated in clinical trials (Sabbatini et al., 2012), but is neither approved for veterinary nor human use. Another example of TLR-mediated adjuvants are synthetic oligodeoxynucleotides containing unmethylated CpG motifs, which are typically found in bacterial DNA. Binding of CpG DNA to TLR9 triggers an intracellular signal cascade, which leads to an NF-κB-mediated expression of inflammatory cytokines (Bode et al., 2011).

Newer approaches in the field of adjuvant development include combination adjuvants, which contain between two and three individual adjuvant components, as opposed to only one-

component adjuvants. Recently, a number of vaccines with new types of adjuvants have entered the market. One example is Adjuvant System (AS) 04, which is one of the newer generation adjuvants that is licensed for use in human vaccines. Here, aluminum salts are combined with the TLR4 agonist monophosphoryl lipid A (MPL). MPL causes APC maturation by TLR4 stimulation. Consequently, cytokine expression is induced, leading to the stimulation of T helper cells, which then produce a Th1 immune response. The aluminum salts were found to prolong the MPL-induced cytokine response (Didierlaurent et al., 2009). AS04 can therefore be seen as a successful modification of the traditional alum, shifting the induced immune response from Th2 to Th1. Another combination adjuvant platform (TriAdj) is comprised of a TLR agonist and a host defense peptide in a polyphosphazene carrier system. TriAdj has been tested in a number of different animals and has been shown to induce long-lasting humoral and cellular immunity against numerous infectious pathogens (Garg et al., 2017).

2.5 DNA vaccines

Vaccines based on nucleic acids have been gaining popularity due to the cheap production, easy adaptation and high room temperature stability of vaccine plasmids. Another main advantage is that the vaccinated individual itself expresses the encoded antigens, thereby ensuring the correct folding and post-translational modifications of the protein. Antigen-presentation via MHC I and MHC II triggers the activation of both cellular and humoral immune response (Grunwald and Ulbert, 2015; Liu, 2011).

The main hurdle with DNA vaccines lies in its application. When naked DNA is injected into the muscle, it is taken up by cells to a reasonable degree in rodents, leading to subsequent expression (Dupuis et al., 2000). In larger animals, especially non-human primates, however, additional stimuli are required to aid the uptake of the DNA.

Electroporation presents one of the most powerful tools to enhance DNA-uptake (Dey and Srivastava, 2011; Widera et al., 2000). In this method, needle electrodes are introduced into the skin or muscle after injection of the DNA, and an electric current is applied, making the cell membranes temporarily permeable and allowing the DNA to enter the cells (Grunwald and Ulbert, 2015).

Importantly, the antigen does not have to be produced by an APC. Crosspriming allows for the antigen to be transferred by cells such as muscle cells to professional APCs in order to generate an MHC class I-restricted cytolytic T lymphocyte response (Fu et al., 1997). Effective as it may be, electroporation relies on special equipment and is a painful and invasive procedure, requiring local anesthesia (Otten et al., 2004).

Several other developments have contributed to finding a solution to the problem of DNA-delivery. Physical methods include devices such as the pressure injector, gene gun (Fynan et al., 1993) or DNA tattooing (Pokorna et al., 2008). Chemical formulations such as polyethyleneimine (Pai Kasturi et al., 2006), block copolymers (Caputo et al., 2003) and cationic liposomes (Locher et al., 2003) are also used. While physical methods have the disadvantage of being highly dependent on specialized equipment, chemicals for DNA delivery are often cytotoxic (Kafil and Omidi, 2011).

2.6 Viral vectors

Viral vectors have been widely explored for their application as vaccines. The main considerations for the choice of virus are: 1) pre-existing immunity against the virus that is used as vector, 2) immunogenicity, 3) the size of foreign genomic insert that can be integrated into the viral genome, 4) replication competency of the vector, 5) chromosomal integration of the viral genome and 6) titer yields. While replicating vectors are usually considerably more immunogenic than non-replicating ones, replication-defective viruses are much safer. For gene therapy applications, viruses that integrate their genome into the human genome are an option. However, transient expression is favorable for vaccines, avoiding the risk of cancerous mutations (Ura et al., 2014).

A broad range of viruses has been used as viral vectors, including poxviruses, adenoviruses, alphaviruses, adeno-associated virus, vesicular stomatitis virus, measles virus, poliovirus and hepatitis B virus (Bråve et al., 2007). The most intensely used viral vectors, which have also been evaluated in clinical trials for different applications, are human adenovirus serotype 5 (Ad5), modified vaccinia virus Ankara (MVA) and adeno-associated virus (AAV) (Nieto and Salvetti, 2014; Ura et al., 2014). The use of AAVs is limited by their pre-existing immunity in humans, but several AAV serotypes have experimentally been explored for their use as vaccines (Nieto and Salvetti, 2014). For gene therapy, more than fifty candidates based on recombinant AAV are currently in different stages of clinical trials. "*Glybera*" is the only AAV gene therapy that is approved in Europe (Naso et al., 2017).

Viral vectors for DNA-delivery employ a slightly different technology than the viral vectors that incorporate a gene of interest: Instead of being an integrated part of the genome of the viral vector, the antigen-coding sequence is present on an episomal plasmid, while viral DNA is entirely absent. The idea here is that the virus functions simply as a delivery vehicle to introduce the plasmid into the cell. This approach allows the viral vector to become a very versatile tool, as only the plasmid needs to be exchanged for a new vaccine target, and the insertion step of the antigen sequence into the viral genome is omitted. This has previously been done with polyoma and papilloma viruses (Graham et al., 2010; Krauzewicz et al., 2000). Both are non-enveloped double-stranded DNA viruses with a circular genome, making them interesting candidates for the packaging and delivery of plasmid DNA.

2.6.1 Papillomaviruses as viral vectors

Papillomaviruses have several characteristics that make them promising candidates as DNA delivery vectors. They are quite stable, as they are not enveloped, and have the ability to package foreign DNA of at least 8kbp without specific packaging sequences (Buck et al., 2004; Xu et al., 2006). Some papillomaviruses infect mucosal tissue (Villiers et al., 2004), opening up

the possibility to use them for gene-delivery via the mucosa. Human papillomaviruses (HPV), especially type 16, have been used successfully as gene delivery vectors for transduction of various cell lines (Touze and Coursaget, 1998) and in vaccination studies targeting the mucosa (Graham et al., 2010) and the skin (Kines et al., 2015).

However, virus like particles (VLPs) of different papillomavirus types are increasingly being added to vaccines against HPV. Merck's "*Gardasil 9*" was approved by the FDA in December 2014 and contains VLPs of nine HPV types, 6, 11, 16, 18, 31, 33, 45, 52, and 58 (Merck, 2015). Consequently, these papillomavirus types cannot be used as gene carriers in *Gardasil 9* vaccinated individuals. Apart from vaccine-induced immunity, natural HPV infections occur frequently, rendering HPV a rather unreliable vector for DNA-vaccine delivery in the general population. Most research on papillomaviruses has been performed on human papillomavirus due to their cancerogenic potential, but a large number of animal papillomaviruses has also been described. 260 different papillomavirus types were identified by 2013, 112 of them non-human (Rector and van Ranst, 2013).

Naturally, papillomaviruses only replicate in the basal cells of the stratified epithelium (Schiller et al., 2010), which has made it impossible so far to cultivate this virus in cell culture. Therefore, methods have been developed to produce papilloma VLPs and pseudovirions (PsVs) in order to study this virus.

Although the nomenclature is sometimes not used uniformly in the literature, in this thesis the term "virus like particle" ("VLP") refers to the papilloma virus capsid structure consisting of protein L1 or of both L1 and L2. In contrast to VLPs, PsVs do contain genomic material – such as a reporter or vaccine plasmid – and can thus be used for the transfer of this genetic material.

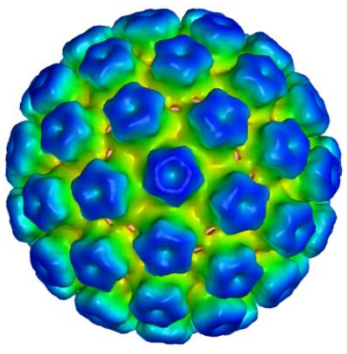


Figure 2.6-1 - HPV16 VLP

(Zhao et al., 2014)

The papillomavirus capsid consists of two proteins, major capsid L1 and minor capsid protein L2. L1 is arranged in pentamers, of which 72 are assembled on a T=7 icosahedral lattice to form the capsid, while L2 is found in substoichiometric amounts in the papilloma virion (Finnen et al., 2003). VLPs – as they are used for vaccines against HPV – are made up of only L1. L2 has been described to play a role in endosomal escape and is therefore necessary for infection (Roden et al., 2001; Richards et al., 2006). Additionally, the presence of L2 has been shown to increase the efficiency of DNA encapsidation (Zhao et al., 1998).

No specific receptor is known for the attachment of papillomaviruses to their target cells, but heparan sulfate proteoglycans (HSPGs) seem to be important primary attachment factors for epithelial cells (Giroglou et al., 2001). Interestingly, L1/L2 PsVs do not bind to or infect cultured primary keratinocytes (Day et al., 2008), and also have been found not to infect intact epithelial tissue *in vivo* (Roberts et al., 2007). The attachment to the cell induces conformational changes in the capsid protein L2, exposing a cleavage site for the endoprotease furin. After cleavage, L1 can bind to a receptor, which still remains to be identified (Figure 2.6-2). Uptake of the virion occurs by endocytosis.

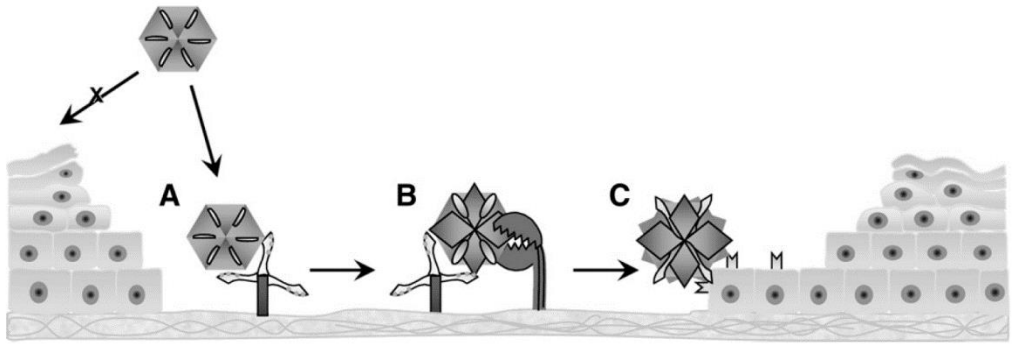


Figure 2.6-2 - Attachment of HPV virion

After disruption of the basal membrane, HPV virions bind to HSPGs (A). Binding induces a conformational change, allowing proprotein convertases like furin to cleave a site on L2 (B). After cleavage, a previously unexposed region of L1 binds to a secondary receptor (unknown) on the edge of the epithelial cells (C). (Schiller et al., 2010)

The precise mechanism of papillomavirus endocytosis is not yet entirely clear. While some studies suggest that endocytosis of HPV16 is mediated by clathrin (Day et al., 2003), caveolin (Laniosz et al., 2009) or dynamin-2 (Abban et al., 2008), others have found the virus entry to be independent of clathrin, caveolin, cholesterol and dynamin (Schelhaas et al., 2012). In addition, various human papillomavirus types were reported to use different endocytosis pathways (Bousarghin et al., 2003), making it even more difficult to make assumptions about the exact endocytotic entry pathway used by the non-human papillomaviruses studied in this thesis.

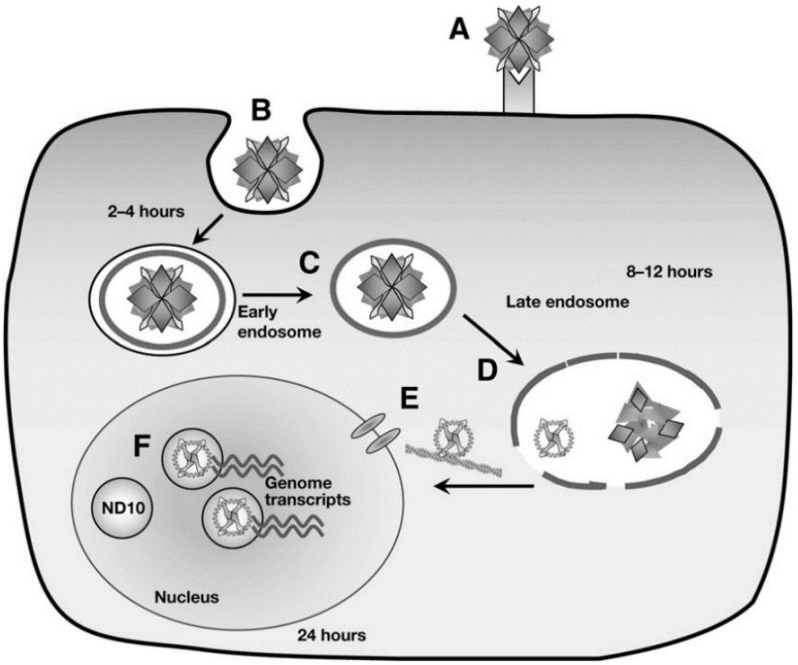


Figure 2.6-3 - Internalization and intracellular trafficking of HPV

The virus is transferred to a currently unknown receptor after binding to HSPGs and furin cleavage (A) and enters the cell using an endocytic pathway (B). Within 4h, the virus is found in the early endosome. The virus uncoats in the endosome within 12h, releasing the viral genome complexed with L2 (D). After 24h, the L2-genome complex will have trafficked through the cytoplasm, entering the nucleus (E). RNA transcription begins after the complex has co-localized with nuclear domain 10 (ND10) (F). (Schiller et al., 2010)

Like most DNA viruses, papillomaviruses traverse the cytoplasm in order to enter the nucleus and start replication (Figure 2.6-3). After expression of the capsid proteins, L2 moves into the nucleus and localizes to nuclear substructure nuclear domain 10 (ND10). L1 joins several hours later, after L2 has induced the reorganization of ND10 (Florin et al., 2002). The nuclear localization signal (NLS) of L1 overlaps with its DNA-binding domain, which is crucial for the interaction with the DNA that will be packaged inside the capsid (Schäfer et al., 2002).

In a natural infection, virus capsids mature in the superficial, dying keratinocytes, which provide an oxidizing environment due to the loss of mitochondrial oxidative phosphorylation (Doorbar et al., 2012). This allows the disulphide bonds between L1 proteins to accumulate, forming stable virions (Cardone et al., 2014; Finnen et al., 2003). Virion release is thought to be mediated by the abundant papillomavirus E4 protein, although this process has not been clearly unraveled (Doorbar et al., 2012). Papilloma VLPs or PsVs produced in cell culture do not leave the cell and have to be extracted by lysing the producer cells (Buck and Thompson, 2007).

2.7 Respiratory Syncytial Virus

The respiratory syncytial virus (RSV) belongs to the paramyxoviridae, family pneumoviridae, genus orthopneumovirus. Human RSV was first isolated from infected children in 1957. It was named after its characteristics to infect the respiratory tract and its ability to form large syncytia in cell culture (CHANOCK et al., 1957; CHANOCK and FINBERG, 1957).

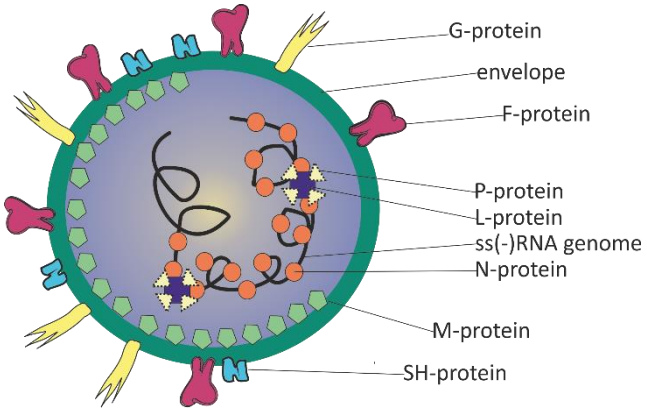


Figure 2.7-1 - RSV particle, schematic

RSV is an enveloped, non-segmented single-stranded RNA virus with a diameter of 150 to 250nm (Bächi and Howe, 1973). Its 11 proteins are encoded by 10 genes, two of which have overlapping open reading frames. The virus has two non-structural proteins (NS1 and NS2) and five structural proteins, the large protein (L), nucleocapsid protein (N), phosphoprotein (P), matrix protein (M) and the M2-1 protein. Three proteins are present in the viral envelope: the G glycoprotein, the fusion (F) glycoprotein and the small hydrophobic (SH) protein. The attachment to the host cell is mediated by the G protein, the F protein enables fusion and entry (Borchers et al., 2013).

Today, RSV belongs to the most important pathogens causing upper and lower respiratory tract infections in humans. Young children and infants are especially affected, and RSV is the primary cause of hospitalization in this group (Meissner, 2016), resulting in 59,600 to 118,200 deaths per year (Shi et al., 2017). Two further groups for which an RSV infection can have fatal consequences are the elderly (above the age of 65) and immune suppressed individuals (Falsey et al., 2005).

RSV exists as a single serotype with two major antigenic subgroups, A and B. Usually both subtypes circulate with one of them predominating (Gilca et al., 2006). It is generally assumed that every child will have been infected with RSV by the age of 2 years (Glezen et al., 1986). Symptoms of an infection range from mild lower respiratory tract disease or middle ear inflammation to severe and potentially life-threatening lower respiratory tract infection including bronchiolitis, pneumonia and croup. Reinfections occur commonly throughout life, but in adults and older children symptoms are often not present or affect only the upper respiratory tract (Hall et al., 2001). The only licensed treatment against RSV is the prophylactic

administration of the humanized monoclonal IgG1 antibody *Palivizumab*, which is directed against the F-protein (Hu and Robinson, 2010). Although *Palivizumab* has been shown to be effective in preventing RSV-associated disease, its use is limited to high risk children due to the great costs and the necessity of repeated administration (Homaira et al., 2014).

Infection with RSV leads to the production of antibodies, but protective immunity is not conferred (Esposito et al., 2016). This is partially due to the mechanisms the virus has developed to evade the host immune system. Both the G and F protein induce neutralizing antibodies, but the intense glycosylation of G has been shown to hinder antibody recognition. Additionally, G is produced in a truncated, secreted form and can act as a decoy for neutralizing antibodies. NS1 and NS2 have the ability to interfere with the production of type I interferons (IFNα and IFNβ), thereby impeding the innate immune response of virus infected cells (van Drunen Littel-van den Hurk, Sylvia and Watkiss, 2012).

2.7.1 Vaccine candidates against RSV

Although more than five decades of research have been invested, there is still no licensed vaccine against RSV on the market. The first vaccine candidate, based on formalin-inactivated RSV, was tested in a clinical trial in 1966 with devastating consequences. Not only did the vaccine fail to induce protection, instead it caused an enhanced onset of the disease upon natural infection, leading to a hospitalization rate of 80% and even two fatalities (Kim et al., 1969). Post-mortem analyses revealed a predominance of mononuclear cells and neutrophils in the bronchial and bronchiolar epithelium (Prince et al., 2001).

Research that explains the exact mechanism behind the phenomenon of vaccine-induced enhanced respiratory disease (ERD) is still ongoing. One hypothesis attributes ERD to inadequately designed antibodies due to the deformation of the virus by formaldehyde, leading to the production of poorly neutralizing antibodies (Moghaddam et al., 2006). Further studies have shown vaccination with FI-RSV to trigger an inappropriately Th2-biased immune response causing lung eosinophilia (Openshaw et al., 2001; Polack et al., 2002).

Numerous novel vaccine approaches are currently being developed and evaluated (summarized in Figure 2.7-2). Among them we find the classical live-attenuated cold-passaged mutants, recombinant RS-virus with deletions of one or several virus proteins, vectored vaccines, and virus protein subunit and peptide vaccines. As vaccine-induced ERD is a much feared side effect of any RSV vaccine, a variety of adjuvants are included and examined for their ability to boost immunogenicity and in particular to avoid the induction of a Th2-skewed immune response. These adjuvants include monophosphoryl lipid A (Kamphuis et al., 2012), CpG oligodeoxynucleotides (Garlapati et al., 2012) and other PRR ligands (Shafique et al., 2012; Zeng et al., 2012).

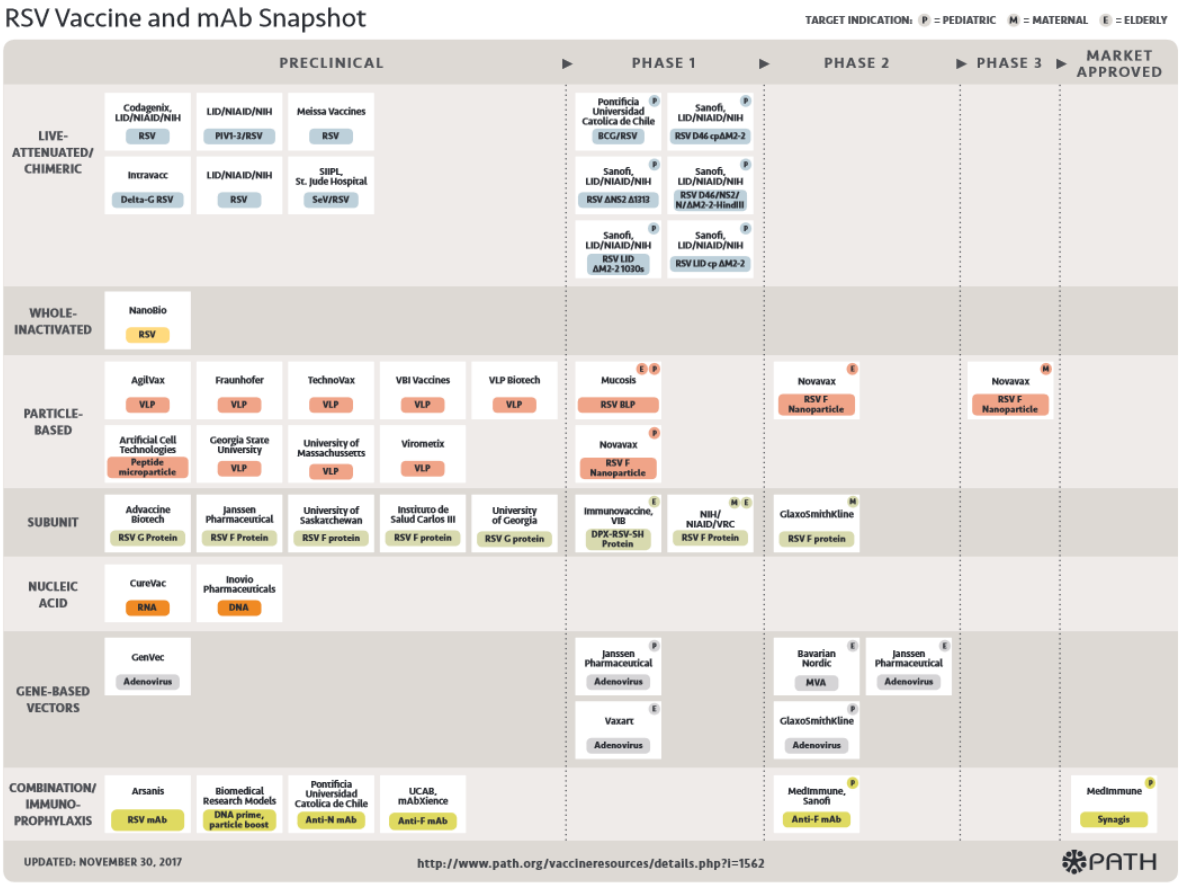


Figure 2.7-2 - RSV vaccines in development

Overview over RSV vaccines that are currently being developed in different stages (path, 2017).

Several RSV vaccine candidates have moved to clinical phases, but only the RSV-F nanoparticle vaccine for maternal immunization by Novavax is in phase 3 (ClinicalTrials.gov, 2018). In this trial, third trimester pregnant women are vaccinated with alum-adjuvanted F glycoprotein, with the goal of transplacental antibody transfer to provide immunity for the infants (Rezaee et al., 2017).

2.8 Motivation and goal

In this thesis, two different methods for the development of novel vaccines against RSV were explored. Both approaches have the potential to be expanded as general vaccine platforms.

Part I deals with the establishment of a viral vector platform based on non-human papilloma pseudovirions (PsVs). The goal was to identify non-human papillomavirus candidates that could be produced as transducing PsVs *in vitro* by using the published DNA-sequences of the viral capsid proteins. Ultimately, this would allow us to develop the PsVs such that they could be applied as universal vectors, packaging a vaccine plasmid for a genetic vaccination inside. In the case of RSV, genetic vaccination using a codon-optimized sequence of RSV-F (Ternette et al., 2007) has been shown to be highly effective in non-human primates (Grunwald et al., 2014). DNA-based vaccines have great potential due to their simple modification and cheap and fast production. Yet a method for safe and effective delivery – potentially both intramuscularly and via the mucosal route – is still lacking. In this thesis, selected non-human papilloma PsVs were tested *in vitro* and *in vivo* for their ability to transfer a reporter plasmid. Finally, two non-human papilloma PsVs were applied *in vivo* for the delivery of a vaccine plasmid in an immunization study against RSV.

Part II of this thesis seeks to evaluate a new pathogen-inactivation method for the production of killed vaccines. Low-energy electron irradiation (LEEI) has already been shown to effectively inactivate a number of pathogens, including viruses, bacteria and parasites (Fertey et al., 2016). As LEEI is a fast inactivation method without the use of any chemicals, it has great potential to be employed as new high-throughput system for killed-vaccine production.
One goal was to analyze the quality of LEEI-inactivated RSV in terms of viral protein conservation. Further, the LEEI-RSV vaccine preparation was tested in mice, with subsequent analysis of the elicited humoral response and virus load after challenge.

3 MATERIALS AND METHODS

Materials

3.1 Reagents

Carbenicillin	Carl Roth
Coelenterazin	p.j.k
DNA 100bp ladder	NEB
DNA 1kb ladder	NEB
DULBECCO's Mod. Eagle Medium (DMEM)	Gibco
Fetal Bovine Serum (BSA)	Thermo Scientific
Hank's Balanced Salt Solution (HBSS)	Thermo Scientific
Hygromycin	SantaCruz
Isofluran CP	CP-Pharma
Minimal Essential Medium (MEM)	Gibco
OptiPrep	Progen
Percoll	GE Healthcare
Phosphate-buffered saline (PBS), pH 7.4	Thermo Fisher Scientific
Pierce ECL	Thermo Scientific
Polyethylenimine (PEI)	Sigma-Aldrich
Protein ladder ProSieve QuadColor	Lonza
RotiBlock	Carl Roth
SYBR green	Thermo Fisher Scientific
TMB ELISA Substrate	BioLegend
Trypsin/EDTA	Thermo Scientific
Tween-20	Carl Roth
XenoLight D-Luciferin - K^+ Salt Bioluminescent Substrate	Perkin Elmer

Futher standard chemicals were purchased from Sigma-Aldrich and Carl Roth.

3.1.1 Kits

Bright-Glo™ Luciferase Assay System	Promega
EndoFree Plasmid Maxi Kit	Qiagen
E.Z.N.A. Plasmid Mini Kit	Omega Biotek
NucleoBond Plasmid PC500 Maxiprep Kit	Macherey-Nagel

Pierce™ BCA Protein Assay Kit	Thermo Fisher Scientific
QIAamp MinElute Virus Spin Kit	Qiagen
QIAamp Viral RNA Mini-Kit	Qiagen
QuantiNova SYBR Green PCR Kit	Qiagen
QuantitectTM Probe RT-PCR-Kit	Qiagen
TOPO® TA Cloning® Kit for Sequencing	Thermo Fisher Scientific

3.1.2 Enzymes

DNase I	NEB
Benzonase Nuclease	Santa Cruz
Plasmid Safe Nuclease	Biozym
DNA Ligation Kit, Version 2.1	Takara
Restriction Enzymes	NEB

3.1.3 Consumables

Amersham Protran nitrocellulose blotting membrane 0.2µm		GE Healthcare
Cell culture flasks (25cm^2, 75cm^2, 175cm^2)		Greiner BioOne
Cell culture plates		Greiner BioOne
Cell scraper		Greiner BioOne
Falcon tubes (50ml and 15ml)		VWR
GentleMACS C-tubes		Miltenyi Biotec
Microcentrifuge tubes (1.5ml, 2ml)		Greiner BioOne
Nunc Immuno 96-microwell plates	MaxiSorp PolySorp	Fisher Scientific
Pipette tips		Greiner BioOne
Polyallomer ultracentrifugation tubes (17ml and 36ml)		Beranek
Sterile filter pipette tips		Greiner BioOne
Sterile filters		TPP
Sterile serological pipettes		Greiner BioOne
Ultra-Clear tubes, 13x51 mm, thin-walled (5ml)		Beranek

3.1.4 Primers

Name	Sequence	Target
GFP fwd	5' ATC CTG GTC GAG CTG GAC GG 3'	EGPF
GFP rev	5' GAC GTA GCC TTC GGG CAT GG 3'	
G.Luc fwd	5‘ TGT TTG CCC TGA TCT GCA TCG CTG 3‘	gaussia
G.Luc rev	5‘ TGG CCC TGG ATC TTG CTG GC 3‘	luciferase
IRES fwd	5’ GCT TGC GTG GTT GAA AGC GA 3’	IRES
IRES rev	5’ TCC GAG GTT GGG ATT AGC CG 3’	
Fsyn fwd	5’ GAG GAG GTG CTG GCT TAC GT 3’	Fsyn
Fsyn rev	5’ GGC TGT TCA TGG TGT CGC AG 3’	
MfPV11 L1 fwd	5’ GAT CAC CCT GAC CAC CGA AG 3’	MfPV11 L1
MfPV11 L1 rev	5’ CTC GCT CTG CAC GAA TCT GT 3’	
MfPV11 L2 fwd	5’ CGG CTG ATC ACC TTC GAC AA 3’	MfPV11 L2
MfPV11 L2 rev	5’ CCC ACT CTG CTA TAC CGC AC 3’	
PcPV1 L1 fwd	5’ CCA CCC ATT CTT CGA CAT CT 3’	PcPV1 L1
PcPV1 L1 rev	5’ TTT CCA CGT CGC TGT ATC TG 3’	
PcPV1 L2 fwd	5’ AGC TAC CCC GAA GAG TTG GT 3’	PcPV1 L2
PcPV1 L2 rev	5’ GTC TTG TTA GGG TCG GTG GG 3’	
RSA-1	5’ AGA TCA ACT TCT GTC ATC CAG CAA 3’	RSV N-protein
RSA-2	5’ GCA CAT CAT AAT TAG GAG TAT CAAT 3’	
K-s	5’ GAC CAT CTA GCG ACC TCC AC 3’	Primers for
K-random-s	5’ GAC CAT CTA GCG ACC TCC ACM NNM NM 3’	PAN-PCR

Primers were ordered from Thermo Fisher Scientific and Biomers.net.

3.1.5 Antibodies

Primary antibodies (monoclonal)		
MD2H11	Papillomavirus L1	Martin Müller, DKFZ
18F12	RSV F-protein	Virology Dept., Bochum
3C4	RSV P-protein	Virology Dept., Bochum

Secondary antibodies (polyclonal)		
Peroxidase AffiniPure Sheep Anti-Mouse IgG (H+L)	Anti mouse IgG	Jackson Immuno (Dianova)
Peroxidase-AffiniPure Donkey Anti-Human IgG	Anti human IgG	Jackson Immuno (Dianova)
HRP Rat Anti-Mouse IgG1	Anti mouse IgG1	BD Bioscience
HRP Rat Anti-Mouse IgG2a	Anti mouse IgG2a	BD Bioscience

3.1.6 Expression plasmids

Name	Expressed protein 1	Expressed protein 2	Plasmid map
pcD3.1+ MfPV11 L1-IRES-L2	MfPV11 L1	MfPV11 L2	Figure 7.1-1
pcD3.1+ PcPV1 L1-IRES-L2	PcPV1 L1	PcPV1 L2	Figure 7.1-2
pcD3.1+ MfPV11 L1-IRES-L2 (Stuffer)	MfPV11 L1	MfPV11 L2	Figure 7.1-4
pcD3.1+ PcPV1 L1-IRES-L2 (Stuffer)	PcPV1 L1	PcPV1 L2	Figure 7.1-3
pCMV-G.Luc	gaussia luciferase	-	Figure 7.1-5
pEGFP	GFP	-	Figure 7.1-6
pcLuc 13	firefly luciferase	-	Figure 7.1-7
pVAX-Fsyn	RSV-F	-	Figure 7.1-8
pVAX-Fsyn plus PV insert	RSV-F	-	Figure 7.1-9
pcD3.1+ MfPV11 L1-IRES-G.Luc	MfPV11 L1	G.Luc	Figure 7.1-11
pcD3.1+ G.Luc-IRES-MfPV11 L2	G.Luc	MfPV11 L2	Figure 7.1-12
pcD3.1+ PcPV1 L1-IRES-G.Luc	PcPV1 L1	G.Luc	
pcD3.1+ G.Luc-IRES-PcPV1 L2	G.Luc	PcPV1 L2	
pcD3.1+ MfPV11 L1-IRES-Fsyn	MfPV11 L1	RSV-F	
pcD3.1+ Fsyn-IRES-MfPV11 L2	RSV-F	MfPV11 L2	
pcD3.1+ PcPV1 L1-IRES-Fsyn	PcPV1 L1	RSV-F	Figure 7.1-13
pcD3.1+ Fsyn-IRES-PcPV1 L2	RSV-F	PcPV1 L2	Figure 7.1-14

3.1.7 Adjuvants

Alhydrogel	Invivogen
AddaVax	Invivogen
QuilA	Invivogen
Poly IC:LC	Oncovir

3.1.8 Cell lines

HEp2	Human epithelial cells from larynx carcinoma	Virology Department, Bochum
HEK293TT	Human embryo kidney cells	Produced by Chris Buck by stable transfection of HEK293T cells with a plasmid carrying a sequence for SV40 large T antigen; kind gift from Martin Müller, DKFZ Heidelberg

3.1.9 Virus

RSV/A long	Laboratory isolate of the Respiratory Syncytial Virus, subgroup A, strain long	ATCC (VR-26)
rgRSV	GFP-expressing Respiratory Syncytial Virus	M. Peeples

3.2 Devices

Low-energy electron irradiation device	
EB Lab-200 (ebeam)	COMET
Incubator	
Hera Cell 240	Thermo Scientific
Clean Bench	
HERA Safe	Heraeus
Cell counting chamber	
Neubauer chamber	Marienfeld
Centrifuges	
Centrifuge 5424R	Eppendorf
Heraeus Multifuge 3SR+	Thermo Scientific

Chemiluminescence Imager	
Advanced Fluorescence Imager	Intas Science Imaging
ELISA Plate reader	
Tecan's infinite plate reader	Tecan
ELISA Plate washer	
Hydroflex	Tecan
Fridges and freezers	
Fridge MedLine -20°C	Liebherr
Deep freezer VIP Series -86	Sanyo
Deep freezer Forma -86C ULT Freezer	Thermo Scientific
Homogenizer	
gentleMACS	Miltenyi Biotec
Luminometer	
Centro XS³ LB 960 Microplate Luminometer	Berthold
Microscope	
Light microscope DMIL	Leica
Fluorescence microscope	Leica
PCR cycler	
Light Cycler	Roche
Applied Biosystems	ABI
Photometer	
Nanodrop ND-1000 Spectrophotometer	Peqlab (VWR)
Rotors	
Surespin 630	Thermo Scientific
Sw55ti	Beckman Coulter
Shaker	
Benchtop incubator shaker New Brunswick Innova 40	Eppendorf
Thermo cycler	
Biometra Tprofessional	Analytik Jena
Vortex	
VV3	VWR
Waterbath	VWR
Weigh scale	
Quintix2102-1S	Sartorius

Methods

3.3 Cell Culture

3.3.1 Cultivating HEK293TT and HEp2 cells

Cells were maintained in an incubator at 37°C, 5% CO_2 and 90% humidity in T75 cell culture flasks with 12ml cell culture medium. For HEK293TT cells, 400µg/ml Hygromycin were added for selection to maintain stable transfection with a plasmid containing a sequencing coding for the SV40 large T antigen. To split or seed cells, old media was removed, cells were washed with PBS and detached by incubation with trypsin/EDTA. Cells were counted using a Neubauer chamber and seeded in the desired amount.

Standard cell culture medium

DMEM with 4.5g/l glucose, 110mg/l sodium pyruvate and GlutaMAX
10% FBS
100U/ml penicillin and 100µg/ml streptomycin (pen/strep)

3.3.2 PEI-Transfection

Polyethylenimine (PEI) was used to transfect cells that were seeded on the previous day. $6x10^6$ cells were seeded in a T75 flask. For transfection, a total of 38µg DNA was mixed with 1ml of DMEM supplemented with penecillin/streptomycin. 50µl of 1mg/ml PEI was added, mixed and incubated at RT for 10min. Cell culture media on the cells was changed to 10ml DMEM + 1% FBS + pen/strep. After addition of the transfection mix, cells were incubated over night at 37°C, 5% CO_2. Approximately 44 hours after transfection, cells were harvested.

3.3.3 Harvest of VLPs and PsVs

Cells were harvested by scraping with a cell scraper. The collected the cell-medium suspension was centrifuged at 200g for 10min at 4°C. The cell culture supernatant was discarded, the cell pellet was resuspended in 1ml PBS with an additional 9.5mM $MgCl_2$ (PBS-Mg) and subsequently transferred into a siliconized 1.5ml microcentrifuge tube. After centrifugation at 200g for 10min at 4°C, the supernatant was discarded and the volume of the cell pellet was determined by side-by-side comparison with tubes containing defined volumes of liquid. The cell pellet was then resuspended in 1.4 volumes of PBS-Mg. 10% Triton-X 100 ($1/20^{th}$ of the volume), 1M ammonium sulfate, pH 9 ($1/40^{th}$ of the volume) and 1M HEPES ($1/100^{th}$ of the volume) were added, leading to final concentrations of 0.5% Triton-X 100, 25mM ammonium sulfate and 10mM HEPES. To digest free DNA and plasmids, 0.5µl Plasmid Safe endonuclease

and 0.5µl Benzonase nuclease were added to each tube. The PsV or VLP preparations were incubated at 37°C for 24h.

For the second part of the VLPs/PsV harvest, tubes were briefly put on ice and subsequently centrifuged at 5000g for 15min at 4°C. The VLP/PsV containing supernatant was collected and kept on ice. The pellet was resuspended in 100µl PBS (PcPV1) or 100µl PBS/0.8M NaCl (all other PV types), centrifuged as above and the supernatant was added to the previously collected supernatant. This clarified lysate was stored at -80°C or immediately purified by ultracentrifugation. The remaining pellet was discarded.

3.3.3.1 Purification by Ultracentrifugation

Papilloma VLPs and PsVs were purified by OptiPrep density gradient ultracentrifugation. OptiPrep, which is a solution of 60% iodixanol, was diluted using PBS (for PcPV1) or PBS/0.8M NaCl (for all other PVs) to 39%, 33% and 27%. Gradients were cast in 5ml sw55ti ultracentrifuge tubes by underlayering, such that 39% is the bottom layer, followed by 33% and finally 27% on the top. The clarified lysate was layered on top and ultracentrifuged using a Beckmann sw55ti rotor at 50,000g for 3½ hours at 16°C.

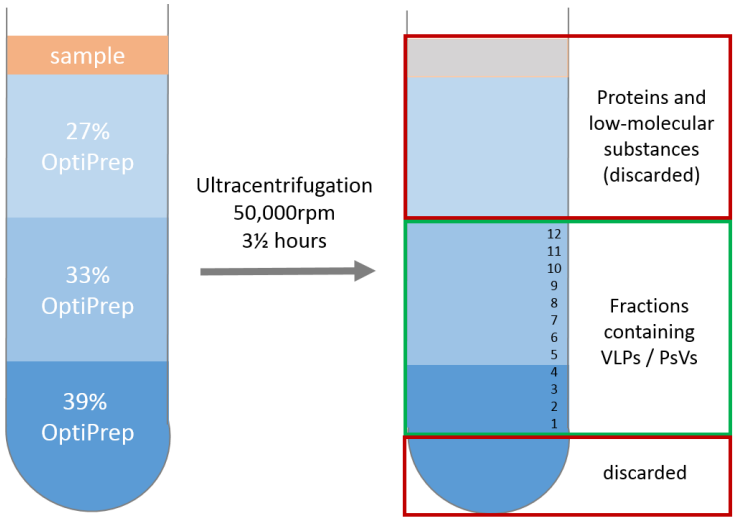


Figure 3.3-1 - OptiPrep gradient and fractions after ultracentrifugation

After the centrifugation, the most upper layer and 27% OptiPrep layer were removed. From here, 12 fraction were collected, containing 250µl each.

Alternatively, ultracentrifugation with Percoll was used to purify HPV16 and MfPV11 VLPs or PsVs. The clarified supernatant was layered on top of 4.5ml Percoll that has been diluted to 58.3% with PBS+0.8M NaCl in 5ml tubes. The centrifugation was carried out using an sw55ti rotor at 30,000 rpm at 16°C for one hour. Under these centrifugation conditions, Percoll forms a pellet. The liquid was removed from the tube, leaving only about 500µl above the pellet. The fraction right above the Percoll pellet contains the VLPs or PsVs, and was collected.

3.3.4 Transduction with papilloma PsVs

Transduction experiments with papilloma PsVs were conducted in 24- or 96-well plates. HEK293TT cells were seeded 24h before transduction at 8000 cells per well in 195µl standard cell culture media in 96-well plates or 50,000 cells per well in 24-well plates with 50µl cell culture media.

The PsV suspension was directly mixed into the cell culture medium. In experiments where gaussia luciferase (G.Luc) was used as reporter, 20µl of the cell culture supernatant were collected and stored at -20°C until G.Luc assay was performed. This allows to check for free G.Luc present in the PsV suspension.

In ι-carrageenan involving experiments, ι-carrageenan was dissolved in PBS, filter-sterilized and added to the cell culture medium immediately before addition of PsVs.

3.3.5 RSV propagation and titration

For propagation of RSV/A2 and rgRSV, HEp2 cells were inoculated with an MOI of approx. 0.1 in DMEM containing pen/strep. Virus containing medium was removed 3 hours after infection and replaced by DMEM with 1% FBS and pen/strep. The infected cells were incubated at 37°C for 2-3 days until formation of syncytia became visible. Cells and media were harvested using a cell scraper and centrifuged at 2,000rpm for 10min at 4°C. The supernatant was filtered through a 0.45µm filter and either immediately stored at -80°C or further purified by ultracentrifugation.

3.3.5.1 Purification of RSV by ultracentrifugation

5ml of 20% sucrose in PBS were filled in SureSpin™ 630 36ml-tubes or 2ml of 20% sucrose was filled in SureSpin™ 630 17ml-tubes. RSV-containing media was layered on top of the sucrose cushion and ultracentrifugation was performed at 10,600rpm for 3h at 4°C. After centrifugation, the cell culture supernatant and sucrose were discarded and the pellet was resuspended in 200µl or 500µl of 10% sucrose in PBS. The purified virus was stored at -80°C.

3.3.5.2 Titration for virus titer determination

Virus titer was determined by serially diluting the virus in 10-fold steps in a 96-well plate before adding 10,000 HEp2 cells per well in 200µl DMEM with 1% FBS and pen/strep. After incubation at 37°C for 48h, virus infected cells were visualized by immunocytochemistry. For rgRSV, virus-infected cells were visualized by fluorescent microscope.

3.3.5.3 Immunocytochemistry

Media was removed, followed by a 10min fixation step with 80% ethanol and subsequent rehydration of the cells with PBS + 0.05% Tween20 (PBS-T). The primary antibody 3C4 (directed against RSV-P) was diluted 1:300 in PBS-T, added to each well and incubated at 37°C for 60min. After three wash steps with PBS-T, 1:500 diluted secondary antibody (sheep anti-mouse HRP) was added and incubated at 37°C for 60min. Three wash steps with PBS-T were followed by the addition of the AEC-substrate (phosphate-citrate buffer pH 5.0 with 0.2% AEC and 0.036% H_2O_2) and incubation at 37°C for 30min. Substrate was removed, A.dest was added and red RSV-plaques were counted in the highest virus dilution at which infected cells were still detectable.

AEC-substrate

10ml phosphate-citrate buffer, pH 5.0
200µl AEC (3-amino-9-ethylcarbazole); 1% in ethanol
10µl 37% H_2O_2

Phosphate-citrate-buffer pH 5

1.46g Na_2HPO_4
1.02g $C_6H_8O_7$ x H_2O
Ad 100ml H_2O

3.3.6 RSV virus neutralization assay

To quantify the amount of RSV-neutralizing antibodies in mouse sera after vaccination, sera were diluted 1:6 in Hank's buffered salt solution (HBSS) and incubated at 56°C for 30min to inactivate complement. Complement-inactivated sera were then serially diluted in a 1:2 manner in a 96-well plate and rgRSV was added at a titer of approx. 50 plaques per well. Virus and antibodies were allowed to interact during a 60min incubation at 37°C. Finally, 10,000 HEp2 cells in DMEM containing 1% FBS and pen/strep were added per well. Wells containing only HEp2 cells and rgRSV without addition of serum were used as controls. After an incubation period of 48h, GFP-positive cells were analyzed, determining the serum-dilution, at which 50% of rgRSV-plaques compared to controls were detectable. This serum dilution was considered the IC_{50}. Neutralization assays were always carried out in at least three independent experiments.

3.4 Molecular Biology

3.4.1 Cloning and plasmid preparation

DNA sequences for papillomavirus L1 and L2, as well as an IRES sequence were ordered from GeneArt after codon optimization.
Sequences were cloned into a pcDNA3.1+ backbone, transformed into DH5α *E.coli* and plated on agar plates containing 100µg/ml carbenicillin for selection. After over-night incubation at 37°C, individual clones were picked and grown over night in 5ml LB-medium with 100µg/ml carbenicillin while shaking at 37°C. 2ml of the bacteria-suspension was used for plasmid isolation by mini prep.

LB-medium

1 % (w/v) tryptone
0,5 % (w/v) yeast extract
171mM NaCl
pH 7.4

3.4.2 Restriction digest and agarose-gel

To verify the correct ligation of a plasmid, approx. 300ng of the plasmids was digested for 60min at 37°C using 0.5µl per restriction enzyme (NEB) and 2µl 10x CutSmart buffer in a total volume of 20µl. After addition of the loading buffer, samples were loaded onto a 1% agarose gel containing ethidium bromide and were run at 100V for one hour. The separated bands were visualized by UV light.

TAE buffer

40mM Tris-HCl, pH 7.0
1mM EDTA

1% agarose gel

0.5g agarose
50ml TAE buffer

3.4.3 Transformation

Chemically competent DH5α *E.coli* were transformed by adding 10pg - 100ng plasmid and subsequent incubation for 30min on ice. Bacteria were heat shocked at 42°C for 1min and then put on ice for 2min. After addition of 900µl SOC-media, bacteria were shaken for 60min at 37°C, 400rpm. Bacteria were plated on agar-plates containing antibiotics for selection.

SOC-medium

2 % (w/v) tryptone
0,5 % (w/v) yeast extract
10mM NaCl
2.5M KCl
10mM $MgCl_2$
10mM $MgSO_4$
20mM glucose
pH 7.4

3.4.4 DNA-isolation from papilloma pseudovirus

10µl of ultracentrifugation-purified papilloma PsV suspension was used for the isolation of packaged DNA. Prior to DNA extraction, a DNase I incubation step was performed to digest any free DNA that might still be present in the PsV suspension.

10µl PsV suspension
78µl A.dest
10µl 10x DNase I buffer (NEB)
2µl DNase I (NEB)

The reaction mix was incubated at 37°C for 60min, followed by heat-inactivation of the enzyme at 75°C for 30min.

The entire sample (100µl) was then used for the DNA-isolation using Qiagen Qiamp MinElute Virus Spin Kit following the manufacturer's instructions. For the elution step, 100µl of AVE buffer was used.

3.4.5 RNA isolation from mouse lungs

Lungs were explanted into 2ml ice cold PBS in sterile homogenizer M-tubes and homogenized using a gentleMACS device. Homogenates were spun down at 2,000g for 5min at 4°C and RNA was extracted from 140µl lung homogenate supernatant using the QIAamp-Viral-RNA-Mini Kit following the manufacturer's instructions.

3.4.6 Quantitative PCR

Quantitative PCR (qPCR) was used for the quantification of papilloma PsVs and reverse transcription quantitative PCR (RT-qPCR) was used for the quantification of RSV. In both cases, nucleic acids standards of known concentrations were included in each PCR plate. To create the standard curves, logarithms (base 10) of plasmid copy numbers were plotted against threshold cycle (C_t) values. The equation corresponding to the regression curve (least square fit) was used

to calculate the absolute copy numbers in the samples based on the measured C_t values (Larionov et al., 2005).

3.4.6.1 qPCR for quantification of PsVs

Quantification of PsVs based on the encapsidated plasmids was done by quantitative PCR (qPCR) using the Qiagen QuantiNova SYBR-green qPCR Kit. As template, 5µl of the DNA isolated from PsVs was used (3.4.4).

Standard curves with known amounts of plasmids were included to allow for absolute quantification. Plasmids were diluted to be present at 30, 300, $3x10^3$, $3x10^4$ and $3x10^5$ per 5µl. Standard curves were measured in duplicates, while samples were measured in triplicates. Melting curve analysis was performed to ensure the specificity of the qPCR products.

Table 3.4-1 - qPCR reaction mix (QuantiNova SYBR Green)

Component	one reaction
RNase-free water	1.6µl
2x QuantiNova SYBR Green PCR mastermix	10µl
QN Rox reference dye	2µl
Primer (forward + reverse), 10µM	1.4µl
DNA-template	5µl
Total reaction volume	20µl

qPCR measurements were performed using an ABI 7900HT cycler.

Table 3.4-2 - Real time cycler conditions (ABI 7900HT)

Step	Duration	Temperature	
Activation of polymerase	2min	95°C	
Denaturation	5sec	95°C	35 cycles
Annealing and elongation	20sec	60°C	

3.4.6.2 RT-qPCR for quantification of viral RNA

Reverse transcription quantitative PCR (RT-qPCR) was used for the quantification of RSV-RNA in mouse lungs. After RNA-isolation from RSV-infected mice (3.4.5), RNA was diluted to a concentration of 45ng per 5µl. *In vitro* transcribed RSV RNA was diluted to $5x10^6$ to 50 copies per reaction (kindly provided by Vladimir Temchura and Klaus Überla, University of Bochum) and used as standard to allow absolute quantification.

SYBR green was pre-diluted 1:500 in DMSO stored at -20°C. The pre-diluted SYBR green was again diluted 1:20 in RNAse-free water to yield the 20x solution that was then used to prepare the RT-qPCR reaction mix (QuantitectTM Probe RT-PCR-Kit, Qiagen).

Table 3.4-3 - RT-qPCR reaction mix

Component	one reaction
RNase-free water	3µl
2x QuantiTect Probe RT-PCR Master Mix	10 µl
SYBR green (20x)	1 µl
QuantiTect RT Mix	0.2 µl
Primers RSA-1 (forward) and RSA-2 (reverse), 10µM	0.8µl
RNA-sample	5µl
Total reaction volume	20µl

Reverse transcription followed by quantitative PCR measurements were performed using a Roche Light Cycler 480.

Table 3.4-4 - Real-time cycler conditions (Light Cycler 480)

Step	Duration	Temperature	
Reverse transcription	30min	50°C	
Deactivation of reverse transcriptase and activation of polymerase	15min	95°C	
Denaturation	15sec	94°C	40 cycles
Annealing and elongation	60sec	60°C	

3.4.7 PAN-PCR

Particle-associated nucleic acid- (PAN-)PCR is a random PCR that allows sequence-independent amplification of nucleic acids, which is achieved by using primers with a degenerated 3' end. The randomly amplified PCR products are then cloned into a plasmid and sequenced (Stang et al., 2005).

For PAN-PCR, DNA was used that was extracted from purified PsVs (3.4.4).

During the primer extension step, random primers are annealed and extended. 1µl of DNA was mixed with 19µl annealing buffer and random-K-s primers (at a final concentration of 5pmol) and incubated at 22°C over night. The reaction mix was then heated to 65°C for 15min to dissolve secondary structures. Primer extension was performed by adding 1U T4 polymerase and 5µl

extension buffer and incubation at 12°C for 20min. T4 polymerase was inactivated by heating to 75°C for 10min.

Subsequently, 5µl of the primer extension product was PCR-amplified by adding 10µl PCR amplification mix and running under the following cycling conditions:

Table 3.4-5 - PAN-PCR cycling conditions

Step	Duration	Temperature	
Activation of polymerase	5min	95°C	
Denaturation	30sec	95°C	40 cycles
Annealing and elongation	60sec	72°C	
Final elongation	15min	72°C	

Annealing buffer

50mM Tris-HCl (pH 7.8)
10mM $MgCl_2$
10mM DTT
10mM ATP

Extension buffer

240mM NaCl
12mM MgCl2
0.4mM (each) dNTPs

PCR amplification mix

0.4µM primer K-s
0.2µM (each) dNTPs
6.6mM Tris-HCl (pH 9.0)
1mM $MgCl_2$
33.4mM KCl
0.75U Taq polymerase

The resulting amplicons was cloned into a TOPO TA plasmid. TOPO cloning allows cloning of DNA-fragments into a plasmid in a restriction site- and ligase-independent manner. Taq polymerase adds a single deoxyadenosine (A) to the 3'-end of the PCR product, which is used for TOPO cloning. The TOPO TA plasmid (*Figure 7.1-15*) is linearized and carries single 3' thymidine (T) overhangs as well as topoisomerase covalently bound to the plasmid.

Vaccinia virus topoisomerase I binds to double stranded DNA and cleaves the phosphodiester backbone of one strand after 5'-CCCTT (Shuman, 1991). The formation of a covalent bond between a tyrosyl residue of topoisomerase I and the 3' phosphate of the cleaved strand conserves the energy of the cleaved phosphodiester bond. The 5' hydroxyl of the original cleaved strand can attack the phospho-tyrosyl bond between the enzyme and the DNA, which reverses the reaction and releases the topoisomerase (Shuman, 1994). This reaction is used to clone PCR products that were amplified by Taq polymerase into a plasmid.
TOPO-TA cloning was performed following the manufacturer's instructions (Invitrogen).

The resulting plasmids were transformed into Dh5α *E.coli*, plated on an agar plate containing 100µg/ml carbenicillin for selection and incubated over night at 37°C. Clones were picked and grown in 5ml LB-medium with 100µg/ml carbenicillin over night at 37°C. After plasmid isolation, plasmids were digested with restriction enzymes *NotI* and *SpeI*, whose restriction sites flank the PCR product in the plasmid, and visualized on an agarose gel. Plasmids were sent to Eurofins Genomics for sequencing, using the M13 forward priming site.

3.5 Biochemical and Analytical Methods

3.5.1 BCA-Assay

Bicinchoninic acid (BCA) assay was used to measure protein concentrations in PsV or VLP preparations. 20µl of the sample or standard was pipetted into a 96-well plate. As standard, bovine serum albumin (BSA) was diluted to concentrations ranging from 5µg/ml to 500µg/ml using either 33% OptiPrep or 58.3% Percoll in PBS/0.8M NaCl to match the medium the PsVs or VLPs are in. 190µl of the working reagent (50 parts of BCA reagent A and 1 part of BCA reagent B, Pierce BCA Protein Assay Kit) were added to each well. After incubation at 37°C for 60min, absorbance at 562nm was measured and concentrations were calculated using the standard curves.

3.5.2 SDS-PAGE and Western Blot Analysis

SDS-polyacrylamide gels were cast as described in Table 3.5-1. Loading buffer was added to the samples before incubation at 98°C for 5min. Samples were loaded and gels were run at 80V until samples had passed the stacking gel. Voltage was then increased to 150V and gels were run until the dye from reached the bottom of the gel.

Table 3.5-1 - SDS-PAGE

	4x running gel (12%)	4x stacking gel (4%)
30% acrylamide solution	24ml	2.68ml
3M Tris-HCl, pH 8.8	7.5ml	-
0.5M Tris-HCl, pH 6.8	-	5ml
10% (w/v) SDS solution	600µl	200µl
A.dest	27.88ml	12.2ml
10% (w/v) APS	340µl	114µl
TEMED	50µl	14µl

After separation by SDS-PAGE, proteins were transferred onto a nitrocellulose blotting membrane by tank-blotting. The blotting membrane was briefly equilibrated in transfer buffer before placing it bubble-free on top of the gel, sandwiching both between Whatman filter paper. The transfer was performed at 100V for 2 hours with cooling, allowing migration of the proteins from the gel (at the anode) to the membrane (at the cathode).

The membrane was blocked for one hour at RT with blocking buffer before over night incubation with MD2H11 as primary antibody diluted 1:1,000 in blocking buffer at 4°C.
MD2H11 is a mouse monoclonal antibody that was raised against a conserved sequence in human papillomavirus L1 protein.

The membrane was washed three times with PBS containing 0.1% Tween (PBS-T) for 5min each. The HRP-conjugated secondary antibody (sheep anti-mouse) was diluted 1:5,000 in blocking buffer and incubated with the membrane at room temperature for 1.5 hours. After washing three times with PBS-T for 5min each, blots were developed using Pierce Enhanced Chemiluminescence (ECL) Substrate. Visualization was done using the Intas Advanced Fluorescence Imager.

10x SDS-PAGE running buffer

30.3g Tris
144g glycine
Ad 1l A.dest

6x Loading buffer

3.75ml 1M Tris-HCl, pH 6.8
0.75ml of A.dest
1.5g SDS
1.2ml 0.1% bromophenol blue
6ml 100% glycerol
3ml 14.3M β-mercaptoethanol (100% stock)

1x SDS-PAGE running buffer

100ml 10x SDS-PAGE running buffer
10ml 10% SDS
890ml A.dest

Transfer Buffer

80ml 10x SDS-PAGE running buffer
200ml methanol
720ml A.dest

Blocking Buffer

5% non-fat dry milk
0.1% Tween-20
in PBS

3.5.3 Luciferase-Assay

72h after transduction with PsVs carrying pCMV-G.Luc as reporter plasmid, 20µl of cell culture supernatant was transferred into black luminometer plates. The substrate was prepared by 1:1,000 dilution of 2mM coelenterazine in luciferase assay buffer. Injection of 100µl substrate and measurement of relative light units for 1sec after 1sec delay was performed for each individual well using a Centro XS³ luminometer.

Firefly luciferase assay was performed 72h after transduction of cells with PsVs carring pcLuc13 as reporter plasmid. Cells were lysed for 2min by adding Promega Bright-Glo reagent. Relative light units were measured within 5min after addition of the reagent using a Centro XS³ luminometer.

Luciferase assay buffer

1.1M NaCl
220mM K_2HPO_4/KH_2PO_4
0.44mg/ml BSA
1.3mM NaN_3
pH 5

3.5.4 ELISA

3.5.4.1 ELISA for analysis of viral proteins

Antigen recognition on RSV samples with and without irradiation or formaldehyde-treatment was determined by direct enzyme-linked immunosorbent assay (ELISA). NUNC maxisorp 96-well plates were coated with dilutions of RSV in coating buffer in a total volume of 100µl and incubated overnight at 4°C. Wells were blocked with 5% non-fat dry milk in PBS for one hour at RT. For detection of RSV F-protein, monoclonal antibody 18F12 was 1:200 diluted in 2% non-fat dry milk in PBS and 100µl were added to each well. Total virus protein was analyzed by using polyclonal serum from an RSV-infected individual as primary antibody in a 1:5,000 dilution. After a 2h-incubation at RT, plates were washed three times with PBS-T, and 1:1,000 diluted sheep anti-mouse HRP secondary antibody or 1:5,000 diluted donkey anti-human HRP secondary antibody was added. The plate was incubated for one hour at RT and then washed with PBS-T three times.

For color development, TMB-ELISA substrate was used. 10-30 minutes after adding the substrate, the reaction was stopped by adding 50µl 1M H_2SO_4 and absorbance at 450nm was determined with a reference wavelength of 520nm.

To calculate the viral protein conservation, the absorbance for untreated RSV "stock" was considered as 100%. Using this as reference, absorbance of treated samples are expressed in relative terms.

3.5.4.2 ELISA for analysis of antibodies in mouse sera

ELISA was used to analyze antibodies in mouse serum for the total amount of binding IgG antibodies, avidity and ratio of IgG1 to IgG2a binding antibodies after vaccination.

In all cases, $5x10^5$ pfu of heat inactivated (56°C, 30min), purified RSV in 100µl coating buffer were coated onto black NUNC polysorp 96-well plates and incubated for 24h at 4°C.

IgG and avidity experiments

Wells were blocked with 5% skim milk in PBS-T for 1h at RT. Mouse sera were diluted 1:2,000 in 2% skim milk in PBS-T, and 100µl were added per well in duplicates. This was done on two plates in parallel. After incubation for 2 hours at RT, wells were washed three times. One of the plates was washed additionally with 7M urea for 5min. Next, sheep anti-mouse HRP secondary antibody was diluted 1:1,000 in 2% skim milk in PBS-T, 100µl were added per well and plates were incubated at RT for one hour. Plates were washed three times with PBS-T, substrate was added and relative light units were measured (see below, "detection with luminol").

The total binding IgG is expressed as the measured relative light units. The relative avidity was calculated using the following equation:

$$\text{relative avidity [\%]} = \frac{\text{RLU with 7M urea wash}}{\text{RLU without urea wash}} \text{x100}$$

IgG1/IgG2a experiments

Wells were blocked with 5% skim milk diluted in PBS-T. Mouse sera were diluted 1:2,000 in 2% skim milk in PBS-T and 100µl were added per well in duplicates. This was done on two plates in parallel. After incubation for 2 hours at RT, the wells were washed three times. Next, secondary antibodies were diluted 1:1,000 in 2% skim milk in PBS-T. One plate was incubated with rat anti-mouse IgG1, the other plate with rat anti-mouse IgG2a for one hour at RT. Plates were washed three times with PBS-T, substrate was added and relative light units were measured (see below, "detection with luminol").

The ratio of IgG2a to IgGa binding antibodies is expressed as:

$$\text{IgG2a/IgG1} = \frac{\mathbf{RLU\ (IgG2a)}}{\mathbf{RLU\ (IgG1)}}$$

Detection with luminol

After the final wash step, a Berthold Centro XS^3 luminometer was used to inject 100µl of 1:10 in PBS diluted enhanced chemiluminescent substrate (ECL) into each well. After a delay of 1.5sec, relative light units were counted for 1sec.

Coating buffer	PBS-T	7M Urea
35 mM Na_2HCO_3 15 mM Na_2CO_3 pH 9.6	PBS 0.05% Tween-20	21.02g urea Ad 50ml PBS-T

3.5.5 Pulmonary histopathology

After challenge, at the end of a vaccination study, lungs were explanted and the right lobes were immersed in 4% neutral buffered formaldehyde for fixation. After incubation at 4°C for 48h, the lungs were embedded in paraffin, sectioned (5-7µm) and stained with hematoxylin and eosin (HE). Sections were analyzed for signs of inflammation and type2-hyperplasia by Prof. Dr. Robert Klopfleisch, FU Berlin. Scores are explained in Table 3.5-2.

Table 3.5-2 - Scores for signs of inflammation and type2-hyperplasia in HE-stained lung-sections

Score	Inflammation	Type-2 hyperplasia
0	healthy	healthy
1	few areas in which a mainly perivascular, lymphocytic and partially interstitial infiltration of low intensity can be observed	few areas that are affected by hyperplasia of type 2-pneumocytes of low intensity
2	moderate amounts of areas in which a mainly perivascular, lymphocytic and partially interstitial infiltration of low to moderate intensity can be observed	moderate amounts of areas that are affected by hyperplasia of type 2-pneumocytes of moderate intensity
3	high amounts of areas in which a mainly perivascular, lymphocytic and partially interstitial infiltration of high intensity can be observed	high amounts of areas that are affected by hyperplasia of type 2-pneumocytes of moderate intensity

3.6 Virus inactivation

3.6.1 Low-energy electron irradiation (LEEI)

Samples were prepared for irradiation by applying 70µl or 230µl RSV-solution into the center of a sterile 100mm petridish (Primaria™, Corning). Covering the solution with a round oriented polypropylene (OPP)-foil resulted in a liquid film of approx. 100µm thickness. Petridishes were irradiated with a 200keV electron beam (EB Lab-200 device, COMET AG, Switzerland). The final dose is calculated from the applied current and the speed, with which the sample holder moves. As the administered dose decreases with penetration depth, calculations were performed such that the indicated dose would have reached the bottom of the sample. Consequently, the top of the sample is irradiated with a slightly higher dose, while the 50-60µm thick OPP foil on top receives the highest dose.

Table 3.6-1 - EB Lab-200 settings

Target dose [kGy]	speed	Current [mA]	Calculated dose [kGy]
5	18m/min	1,11	6
10	18m/min	2,221	12
15	18m/min	3,331	18
20	18m/min	4,44	24

3.6.2 Formaldehyde inactivation

Formaldehyde inactivation was performed following the protocol for “Lot 100” (Kim et al., 1969). RSV containing cell culture supernatant was incubated with a final concentration of 0.025% (v/v) formaldehyde for 96 hours at 37°C. The virus was pelleted by centrifugation using a SureSpin 630 (17ml) rotor at 50,000g for 1 hour at 4°C. The supernatant was removed, leaving about 500µl of medium in the tube. 12ml minimal essential medium (MEM) and 58.56g $Al(OH)_3xH_2O$ were added, yielding a final concentration of 4mg/ml aluminum hydroxide. After incubation for 30min at RT, the precipitated virus was centrifuged at 1,000g for 30min at 4°C. The pellet (approx. 500µl) was resuspended in 1.5ml MEM and stored at 4°C.

3.6.3 Test for titer reduction and inactivation

After irradiation with different doses, approx. one third of the virus solution was used for titration as described in section 3.3.5.2 and visualized by immunocytochemistry (3.3.5.3).

Inactivated RSV that was intended to be used in a vaccine preparation was tested for successful inactivation by using at least 5% of the virus-solution. After five days, immunocytochemisty staining was performed to visualize any infected cells. To confirm complete inactivation, supernatants were passaged once to fresh HEp2 cells and immunocytochemistry staining was repeated after another five days.

3.6.4 LEEI-RSV vaccine preparation

In the scope of this thesis, two vaccination studies with LEEI-RSV were performed.

Vaccination study I with LEEI-RSV and FI-RSV

RSV-containing cell culture supernatant was inactivated either by LEEI with 20kGy (3.6.1) or with formalin (FI) following the "Lot 100" protocol (3.6.2), using the same virus stock for both inactivation methods. To complete the vaccine preparation, LEEI-RSV was mixed in a 1:1 ratio with Alhydrogel. FI-RSV was diluted 1:10 with PBS, as the precipitation step leads to a 5x concentration of the virus.

Vaccination study II with LEEI-RSV plus adjuvants

Ultracentrifuge purified RSV was irradiated with 20kGy for inactivation. For the vaccine preparation, inactivated virus was diluted 1:10 with 10% sucrose in PBS and mixed in a 1:1 ratio with Alhydrogel, QuilA, AddaVax and Poly IC:LC, respectively, as adjuvant. Concentrations of the adjuvants in the vaccine preparation are summarized in Table 3.6-2.

Table 3.6-2 - Adjuvants and their concentrations in vaccine preparation

Adjuvant	Final concentration of adjuvant in the vaccine preparation
Alhydrogel	5mg/ml
QuilA	150ng/ml
AddaVax	Not specified
Poly IC:LC	22.2ng/ml

3.7 In vivo experiments

Seven weeks old female BALB/c mice were obtained from Charles River (Sulzfeld, Germany). Mice were kept in isolated ventilated cages with unlimited access to water and standard rodent chow.

All animal experiments were carried out in accordance with the EU Directive 2010/63/EU for animal experiments and were approved by local authorities (TVV 07/15; DD24-5131/331/9 and TVV49/15; DD24-51-31/331/52).

3.7.1 Applications of vaccines and PsVs

Vaccinations were administered following a prime-boost regimen (Figure 3.7-1).

For the inactivated-RSV vaccination studies, 50µl of vaccine preparation were applied intramuscularly in each hind leg after isoflurane anesthesia.

In the vaccination study using papilloma PsVs, vaccine preparation was applied either intramuscularly (50µl per hind leg) or intranasally (50µl).

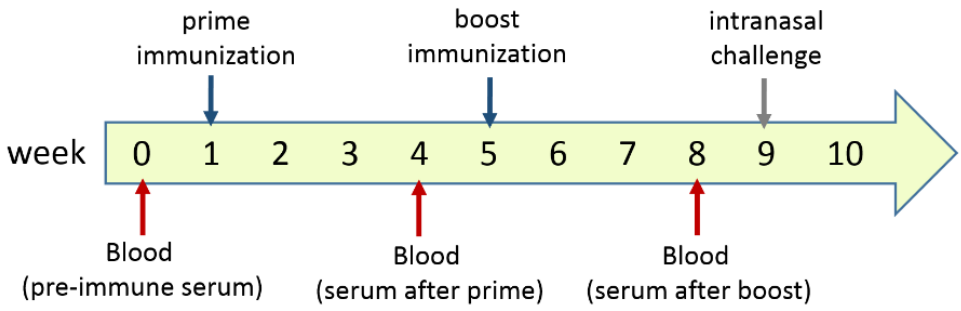


Figure 3.7-1 - Immunization schedule

Papilloma-PsVs with F.Luc reporter plasmid were administered intramuscularly (50µl) into one hind leg after inhalative anesthesia.

3.7.2 Blood collection and challenge

One week before the first immunization and three weeks after each immunization, mice were anesthetized with isoflurane and blood was collected from the retrobulbar plexus. Blood was allowed to coagulate before centrifugation at 8,000g for 10min. The serum was collected and stored at -20°C until analysis.

Four weeks after the last immunization, 10^6 pfu of infectious RSV/A long were prepared in a volume of 30µl and administered to each mouse intranasally after short inhalative anesthesia.

3.7.3 Bioluminescence imaging

For bioluminescence imaging, mice were anesthetized with isoflurane and 200µl of sterile 15mg/ml d-luciferin in PBS per mouse was administered intraperitoneally. The bioluminescence image was acquired (1min exposure, medium binning and f/1) using an IVIS SPEKTRUM (Xenogen, Perkin Elmer) 20min after injection of the substrate.

3.8 Statistical analysis

Statistical analyses were performed using GraphPad Prism6. Data were analyzed by t-test or by one- or two-way analysis of variance (ANOVA) and a suitable post test, as indicated. Differences were regarded as significant at $p<0.05$.

A p-value of 0.05 translates to a 5% chance of observing a difference as large as the detected difference even if the population means are in fact identical (GraphPad Statistics Guide).

Statistically significant differences are indicated in the graphs as follows:

* = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$, **** = $p<0.0001$, ns = not significant.

4 RESULTS

4.1 Part I – Non-human papilloma pseudovirions for gene delivery

Despite the many advantageous properties of genetic vaccines, no DNA vaccine has found its way into a licensed product as of now. One of the main reasons is the hurdle to deliver the DNA efficiently into the vaccinee's cells. This delivery problem can be overcome, for example, by using viral vectors. Human papilloma pseudovirions have been used repeatedly and successfully for the transfer of DNA *in vitro* and *in vivo* (Graham et al., 2010; Touze and Coursaget, 1998). As vector immunity due to previous exposure to HPV by infections or vaccinations limits the use of human papilloma pseudovirions, non-human papilloma viruses were explored in this thesis.

Part I (4.1) of this thesis describes the production and analysis of ten types of non-human papilloma pseudovirions for gene delivery. The main focus of the *in vitro* experiments was placed on the identification of papilloma virus types that can be produced reliably and that yield good transduction rates (4.1.3).

Further, another set of experiments sought to elucidate some of the DNA-packaging behavior of the animal papilloma virus types. For the purpose of using the virus particles as DNA-vaccine delivery vectors, it is desirable to create non-replicating PsVs that do not package the plasmids coding for capsid proteins or chromosomal DNA, but instead contain only the vaccine or reporter plasmid (4.1.4).

Finally, two non-human papilloma virus candidates were tested *in vivo*. First experiments involved the intramuscular delivery of a firefly luciferase reporter plasmid by PsVs and analysis by bioluminescent imaging (4.1.5). Lastly, mice were vaccinated in a heterologous prime-boost regimen with non-human papilloma PsVs, delivering a plasmid coding for RSV F-protein (4.1.6).

4.1.1 Generating expression plasmids for papilloma virus capsid proteins L1 and L2

DNA-sequences for the L1 and L2 capsid proteins of 10 different non-human papilloma virus types were identified in the NIH genetic sequence database GenBank (Table 4.1-1). Five of the ten animal papilloma viruses were chosen as they are classified as α-papillomaviruses, which have been shown to be efficient transducers in the case of the human papilloma viruses. Further papilloma viruses were chosen randomly to cover a range of different natural virus host species. The selected sequences were codon-optimized for expression in human cells by "*GeneOptimizer"* and ordered from *GeneArt*.

Table 4.1-1 - Non-human papillomaviruses selected for this thesis

Papilloma virus	Classification	Abbreviation	GenBank-Nr.
Caretta caretta papillomavirus type 1	Dyozeta 1	CcPV1	NC_011530
Colobus guereza papillomavirus type 1	Alpha 14	CgPV1	GU014532.1
Common chimpanzee papillomavirus type 1	Alpha 10	PtPV1	AF020905.1
Crocuta crocuta papillomavirus type 1	Lambda	CcrPV1	NC_018575
Macaca fascicularis papillomavirus type 6, isolate Mac39	Alpha 12	MfPV6	EF558840.1
Macaca fascicularis papillomavirus type 11, isolate Mac1637	Alpha 12	MfPV11	GQ227670.1
Procyon lotor papillomavirus type 1	Lambda 4	PlPV1	NC_007150
Puma concolor papillomavirus type 1	Lambda 1	PcPV1	AY904723
Rhesus papillomavirus type 1b, isolate Mac170	Alpha 12	MmPV1	EF591300.1
Rousettus aegyptiacus papillomavirus type 1	Psi 1	RaPV1	NC_008298.1

For each papillomavirus, the L1 and L2 sequences were cloned into the multiple cloning site of pcDNA3.1+ expression vector, downstream of the CMV promoter. L1 and L2 are connected by an internal ribosome entry site (IRES) sequence (Dirks et al., 1993) (see Figure 7.1-1 and Figure 7.1-2).

4.1.2 VLP formation of ten different non-human papilloma types

In order to check the expression of the capsid proteins and the assembly thereof into papillomavirus capsids, HEK293TT cells were transfected (PEI-transfection, 3.3.2) with each of the ten packaging plasmids. VLPs were harvested (3.3.3) and purified by OptiPrep density gradient ultracentrifugation (3.3.3.1).

4.1.2.1 Detection of VLPs after ultracentrifugation by western blot analysis

Fractions 2-9 of OptiPrep ultracentrifuged VLPs were separated by SDS-PAGE and blotted onto a nitrocellulose membrane. Papillomavirus L1 protein was visualized using MD2H11 as primary antibody, which was originally produced to bind to a conserved sequence in HPV L1. As no antibodies against the papillomavirus proteins that are used in this thesis are commercially available, MD2H11 was the only option to try for direct detection of L1. Interestingly, it was

possible to detect eight out of ten non-human papilloma L1 proteins, which have a molecular weight of approx. 60kDa (Figure 4.1-1). OptiPrep density gradient ultracentrifugation allows to separate VLPs or PsVs from single proteins as well as low-molecular substances, which are discarded by removal of the upmost part of the centrifuged sample. The purified VLPs – represented by the most intense bands in the blot – are usually found between fractions 4 and 6, which matches previous findings from human PVs (Buck et al., 2004). Deviations can likely be explained by the fact that fractioning is done by pipetting by hand, leading to slight variations in the fractions where most VLPs are found.

The SDS-PAGEs were run covering a range from 10 to 300kDa. Other than the bands shown in Figure 4.1-1, no further bands were detected on the blots.

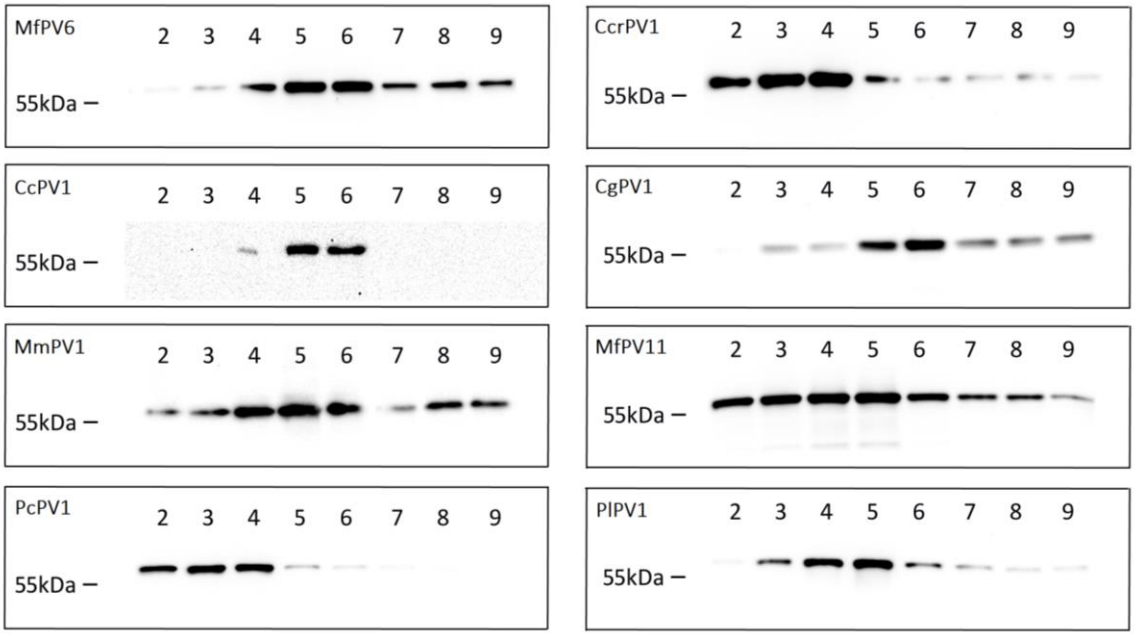


Figure 4.1-1 - Western blot analysis of VLP fractions after OptiPrep density-gradient ultracentrifugation

Non-human papilloma PsVs were purified by density gradient ultracentrifugation. The collected fractions (indicated by numbers above) were separated by SDS-PAGE, blotted onto a nitrocellulose membrane and probed for L1. Western blot analyses were performed in at least two independent experiments; shown is one representative experiment each.

Non-human papilloma virus types MfPV6, CcrPV1, CcPV1, CgPV1, MmPV1, MfPV11, PcPV1 and PlPV1 can therefore be produced as VLPs. Ultracentrifugation purified VLP preparations of RaPV1 and PtPV1 were additionally run on an SDS-PAGE and silver stained (not shown), as L1 could not be detected by western blot analysis using MD2H11. Since there was no detection of bands at 60kDa – corresponding to L1 – by silver staining, there were likely no VLPs present in the samples.

4.1.2.2 Electron Microscopy of non-human papilloma VLPs

In order to confirm that the produced particles are indeed papilloma VLPs, OptiPrep ultracentrifugation purified HPV16, PcPV1 and MfPV11 VLPs were visualized by transmission electron microscopy (TEM) after contrasting with phosphotungstic acid (Figure 4.1-2). The capsids present themselves similar to what has been described before by others (Cerqueira et al., 2017; Touze and Coursaget, 1998; Zhao et al., 2014) with a spherical shape and a diameter of approx. 60nm. We observed that contrasting with uranyl acetate led to capsids that are slightly smaller, with a diameter of approx. 40nm (not shown). Human papillomavirus capsids have been reported to have a size of approx. 50nm. Slight variations in the size of the capsids are likely due to the chosen method for preparation for electron microscopy. Although TEM is no method for quantification, the larger amount of HPV16 VLPs compared to PcPV1 and MfPV1 VLPs in the preparations is apparent, as all three batches were produced in parallel under the same conditions. The observation that HPV16 PsV preparations lead to higher transduction rates than PcPV1 and MfPV11 PsVs, as will be presented in section 4.1.3.1, can therefore possibly be explained by a higher PsV yield.

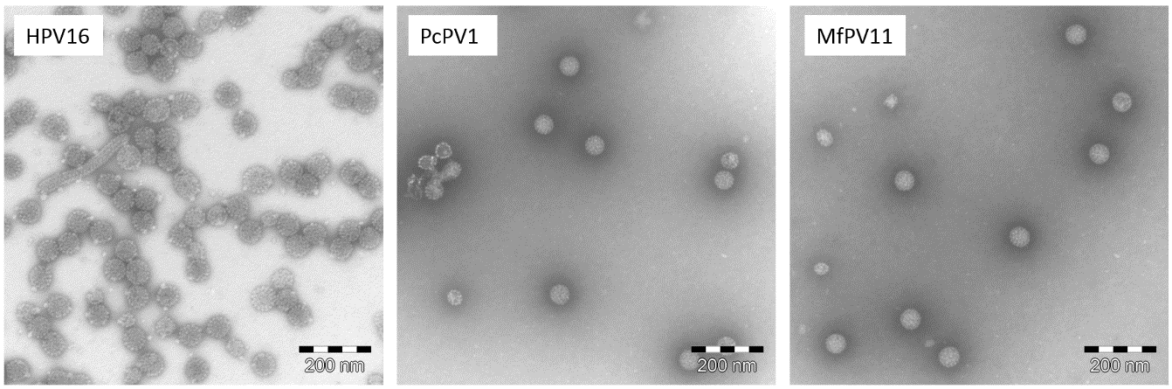


Figure 4.1-2 - Transmission electron microscopy of HPV16, PcPV1 and MfPV11 VLPs

Purified VLPs were fixed with formaldehyde for 24h at room temperature, contrasted with phosphotungstic acid and analyzed by transmission electron microscopy. The scale bare represents a length of 200nm.

4.1.3 Transduction of HEK293TT cells with non-human papilloma pseudovirus

All ten non-human papilloma viruses were produced as pseudoviruses, containing pCMV-G.Luc (Figure 7.1-5) or pEGFP (Figure 7.1-6) as reporter plasmid. For the PsV preparations, HEK293TT cells were cotransfected with a packaging plasmid containing the sequences for the capsid proteins L1 and L2 (Figure 7.1-1 and Figure 7.1-2), and the reporter plasmid. The PsVs were extracted from the producer cells and purified by density gradient ultracentrifugation (3.3.3). The resulting PsVs were added to HEK293TT cells and the transduction rates were analyzed measuring luciferase expression using luciferase assay or by quantifying GFP-expressing cells.

4.1.3.1 Comparison of transduction with 10 different non-human papilloma PsVs

For the comparison of the transduction rates of all ten non-human papilloma PsVs, gaussia luciferase was used as reporter. All PsVs were produced applying the harvest protocol using PBS/0.8M NaCl and OptiPrep ultracentrifgation for purification (3.3.3). For transduction, 10µl PsVs of fractions 3-8 were added to 50,000 HEK293TT cells (3.3.4). Transduction with these fractions of each preparation typically produce a pattern as shown in Figure 4.1-3, B, in which the highest transduction is achieved with fractions 4-6. Figure 4.1-3, A shows the relative light units (RLU) measured 72h after transduction from which the RLU measured in cell culture supernatant at time point 0h was subtracted. 0h measurements were subtracted to control for G.Luc background, which usually lay between 100 and 10,000 RLU. For each preparation, only the one fraction with the highest transduction rate was taken into account.

All PsVs yielded at least low levels of transduction that, however, differed greatly from type to type of the papilloma viruses. It is also worth noting that titers (as measured by luciferase assay after transduction of HEK293TT cells) showed high variability from preparation to preparation. While the efficiency of transfection for PsV production does influence the PsV yield, it does not explain all observed differences between PsV preparations.

Remarkably, all tested animal papillomaviruses yielded transduction efficiencies that were considerably lower than HPV16. PcPV1 and MfPV11, which showed an approx. 10-fold lower transduction than HPV16, proved to be the papilloma virus types, whose PsVs led to the highest transduction rates and are the most reliable to produce. These two papilloma viruses were hence analyzed in further experiments.

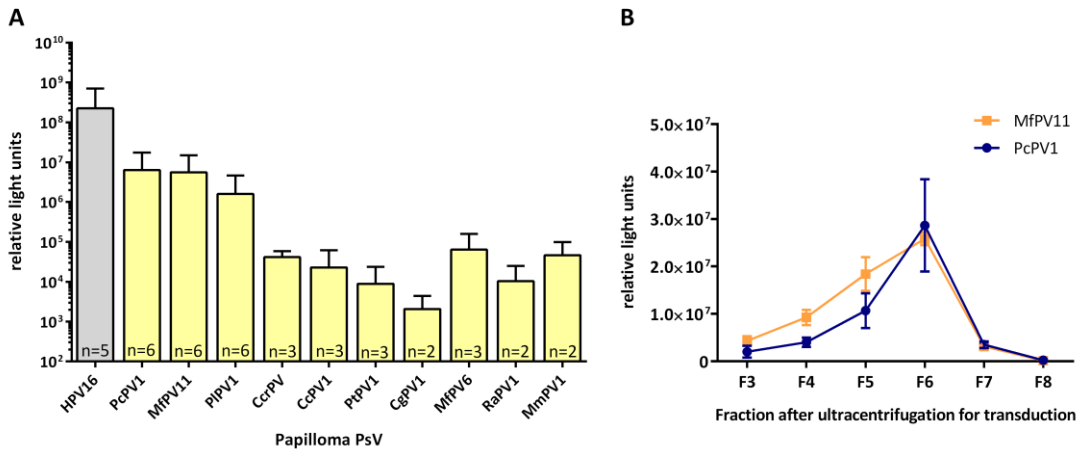


Figure 4.1-3 - Non-human papilloma PsVs with G.Luc reporter for transduction of HEK293TT cells

Fractions 3 to 8 of ultracentrifugation-purified non-human papilloma PsVs were used to transduce HEK293TT cells. 72h later, cell culture supernatant was used for luciferase assay and relative light units (RLU) from time point 0h were subtracted from measurements at time point 72h.

(A) Mean values and standard deviations of RLU measured after transductions with at least two independent PsV preparations are shown. For each preparation, only the fraction yielding the highest transduction was taken into account.

(B) RLU measured after transduction with fractions 3-8 of PcPV1 and MfPV11 PsVs. Shown are mean values and standard deviations of triplicate measurements of one representative experiment.

Apart from HEK293TT cells, further cell lines – HeLa-T, HEp2, Vero, NIH-3T3 and A549 cells – were used for transduction experiments with MfPV11 and PcPV1 PsVs containing pCMV-G.Luc as reporter plasmid. Equal amounts of cells were transduced with identical volumes of each papilloma PsV preparation. Only one PsV preparation was used for this experiment to allow a direct comparison of the transduction rates of different cell lines, which were measured by luciferase assay 72h after transduction. While HeLa-T, HEp2 and Vero cells showed expression of G.Luc after transduction (Figure 4.1-4), no expression of the reporter protein was observed for NIH-3T3 and A549 cells (not shown). As HEK293TT cells showed the highest susceptibility to the tested PsVs, further *in vitro* experiments were conducted with this cell line.

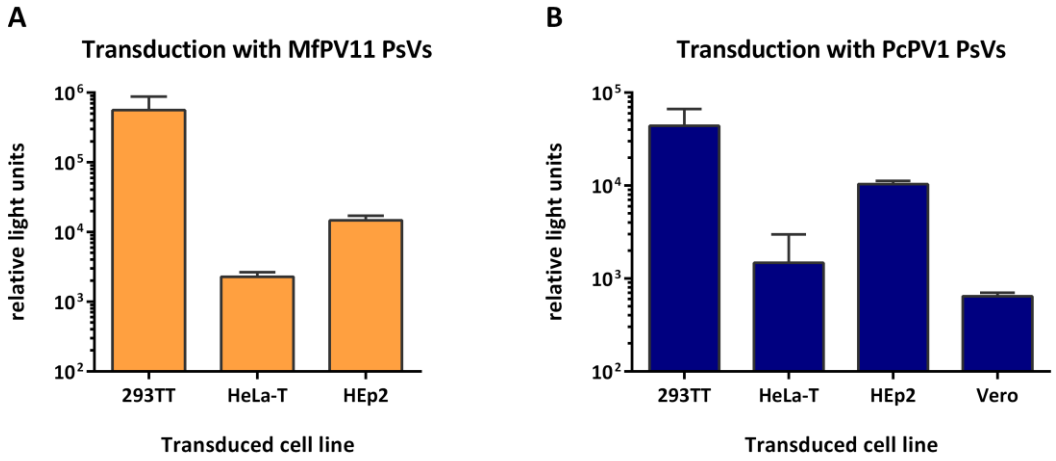


Figure 4.1-4 - Transduction of various cell lines with MfPV11 and PcPV1 PsVs

Different cell lines were transduced with MfPV11 (A) and PcPV1 (B) PsVs carrying pCMV-G.Luc as reporter plasmid. Expression of gaussia luciferase was measured by luciferase assay 72h after transduction, background measurements are subtracted. Shown is one representative experiment.

4.1.3.2 Optimization and adaptations of the protocol for PsV production

In direct comparison, where PsV preparations were produced in parallel, omitting the additional 0.8M NaCl in PBS yielded PcPV1 PsVs with significantly higher transduction rates in two independent experiments. Although in later experiment this unusually high transduction rate could not be reproduced, PBS without additional 0.8M NaCl was used for the production of PcPV1 PsVs (Figure 4.1-5, A).

Ultracentrifugation purification of PsVs with Percoll is much faster and easier than with OptiPrep, as the centrifugation with Percoll only takes 1 hour and leads to one easily identifiable fraction containing the PsVs. While purification of PcPV11 PsVs with Percoll led to barely any transduction-capable PsVs, it worked well for MfPV11 PsVs. The resulting purified MfPV11 PsVs showed no significant difference in their transduction rate compared to PsVs purified by OptiPrep ultracentrifugation (Figure 4.1-5, B). Therefore, for future experiments MfPV11 PsVs were purified using Percoll ultracentrifugation.

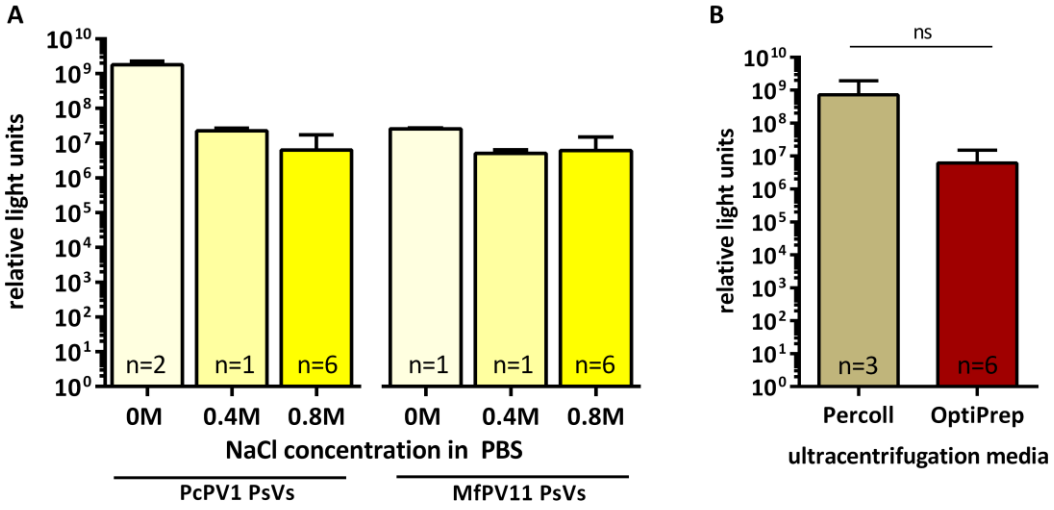


Figure 4.1-5 - Optimizing the PsV production

Purified PsVs (with G.Luc reporter plasmid) were used for transduction of HEK293TT cells. 72h later, transduction efficiency was measured by luciferase assay. Differences between the two groups were statistically analyzed by Mann-Whitney test.

(A) PBS with different concentrations of additional NaCl were used during the PsV-harvest and for the dilution of OptiPrep ultracentrifugation medium. Experiments were repeated as indicated. Shown are the mean values and standard deviations of the independent experiments, as indicated. In case of n=1, mean values of the triplicate measurements of the experiment are shown.

(B) For the purification of Mf11PV PsVs, ultracentrifugation with Percoll and OptiPrep medium was performed. Shown are mean values and standard deviations of the measured transductions.

4.1.3.3 Titer determination with GFP-reporter and qPCR

Using gaussia luciferase as reporter to analyze the transduction rate is very useful for many applications, but it does not allow titer determination as measured by individual transduced cells. Therefore, PcPV1 and MfPV11 PsVs carrying a pEGFP reporter plasmid were produced and used to transduce HEK293TT cells. The titer was determined by counting GFP-positive cells 72h after transduction, thereby quantifying functional, i.e. transducing, PsVs (Figure 4.1-6, A). PcPV1 PsVs yielded titers of approx. 6.5×10^3 GFP-transducing-units/ml and MfPV11 PsVs of approx. 1.9×10^3 GFP-transducing-units/ml.

Quantifying the reporter plasmid packaged inside the PsVs provides insight into the total amount of PsVs that could potentially transduce. Prior to DNA-extraction and quantification by qPCR, samples were treated with DNAse I to ensure the degradation of any DNA that might still be present outside of the PsV capsids (3.4.4). In each qPCR run, standard curves of each plasmid that was analyzed were included to allow absolute quantification.

Since quantification by qPCR does not differentiate between malformed and therefore dysfunctional and intact PsVs, the titer determined with this method will always be higher than the titer calculated from transduction experiments. Figure 4.1-6, B shows the drastic difference between PsVs from which the reporter plasmid (pEGFP) was isolated and transducing PsVs, which is approx. 1,000-fold.

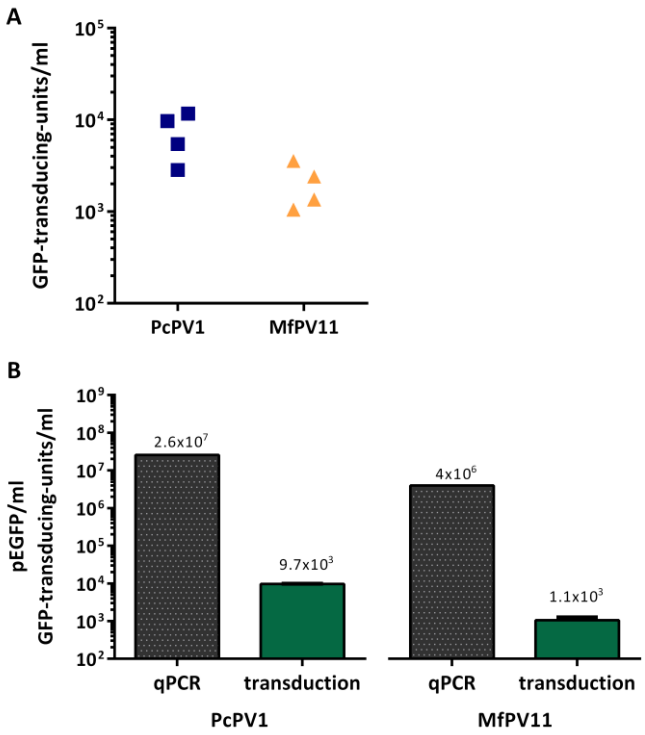


Figure 4.1-6 - Titer determination with qPCR and via GFP-reporter

Titers of PcPV1 and MfPV1 PsVs were analyzed with pEGFP reporter plasmid.

(A) GFP-transducing units/ml were measured by transduction of HEK293TT cells and counting of GFP-positive cells 72h after transduction. Each data point represents one independent PsV preparation.

(B) Additionally, the pEGFP reporter plasmid was quantified by qPCR and the pEGPF/ml concentration was calculated assuming that each PsV contains one plasmid. Shown is one representative PsV preparation for each type of papilloma virus.

4.1.3.4 Effect of ι-carrageenan on transduction with PcPV1 and MfPV11 PsVs

Carrageenan had previously been reported to be a potent inhibitor of HPV infection (Buck et al., 2006b) and was therefore analyzed for its ability to prevent PcPV1 and MfPV11 PsV-mediated transduction. While transduction with MfPV11 PsVs was reliably inhibited when ι-carrageenan was added to the cell culture medium right before addition of the PsVs, this was – unexpectedly – not the case for PcPV1 PsVs. In contrast, ι-carrageenan significantly enhanced transduction, as measured by expression of the luciferase reporter (Figure 4.1-7, A). To exclude

the possibility that luciferase expression rather than the transduction rate is increased by ι-carrageenan, the experiment was repeated using GFP as reporter and GFP-positive cells were quantified 72h after transduction. Here again, addition of ι-carrageenan led to complete inhibition of MfPV11 PsV-mediated transduction while PcPV1-mediated transduction was significantly enhanced (Figure 4.1-7, B).

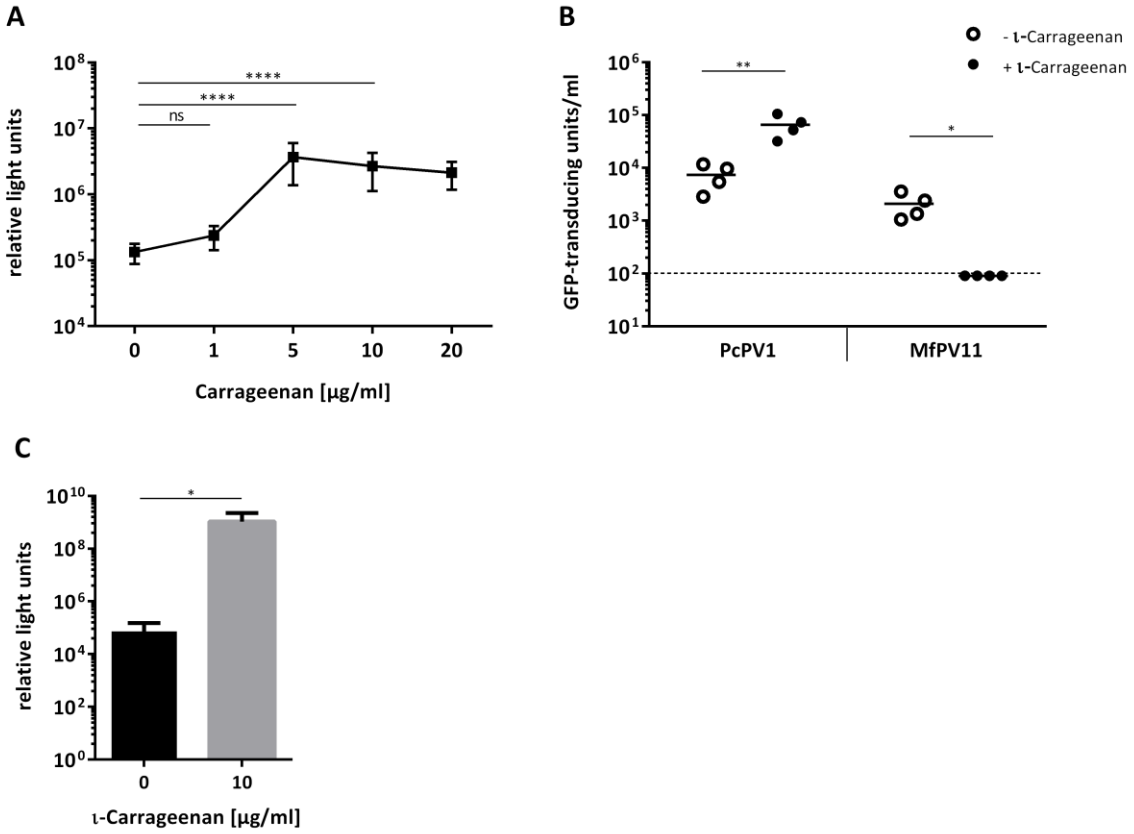


Figure 4.1-7 - Effect of ι-carrageenan on transduction with PcPV1, MfPV11 and MmPV1

(A) HEK293TT transduced with PcPV1 PsVs in the presence of increasing concentrations of ι-carrageenan. Transduction efficiency was measured by luciferase assay 72h after transduction. Shown are mean values and standard deviations of three independent experiments with different PsV preparations. Statistical analysis was performed by two-way ANOVA and Tukey's multiple comparison test.

(B) PcPV1 and MfPV11 PsVs with GFP-reporter were used to transduce HEK293TT cells with and without addition of 10µg/ml ι-carrageenan. GFP-positive cells were counted 72h after transduction and GFP-transducing units per ml were calculated. Each data point represents one experiment with one PsV preparation. Unpaired, two-sided T-test was used for statistical analysis.

(C) HEK293TT cells were transduced with MmPV1 PsVs carrying pCMV-G.Luc as reporter plasmid with or without addition of 10µg/ml ι-carrageenan. Luciferase assay was performed 72h after transduction. Shown are mean values and standard deviations of transduction experiments, performed in triplicates, with two different PsV preparations. Unpaired, two-sided T-test was used for statistical analysis.

While it has been described previously that ι-carrageenan does not have any effect on the transduction of some HPV types (Mistry et al., 2008), PcPV1 is the first papilloma virus, for which an ι-carrageenan-mediated enhancement of transduction has been observed. A similar effect was also found for MmPV1 PsVs (Figure 4.1-7, C), PlPV1 and CcrPV1, while transduction with MfPV6 PsVs was inhibited (data not shown).

4.1.3.5 Effect of the presence of empty VLPs on transduction

During each PsV production, a number of different particles are generated. Besides the PsVs, which contain the reporter- or vaccine plasmid, empty VLPs form, as well as deformed particles. Density gradient ultracentrifugation is thought to be able to separate these different particles from one another, especially PsVs from VLPs. To analyze the importance of purifying PsVs after the harvest and removing empty VLPs from the preparation, HEK293TT cells were transduced with PcPV1 and MfPV11 PsVs in the presence of the respective empty VLPs. The concentration of the VLPs was determined by BCA assay, calculating the amount of capsids based on the amount of protein measured (Buck et al., 2006a).

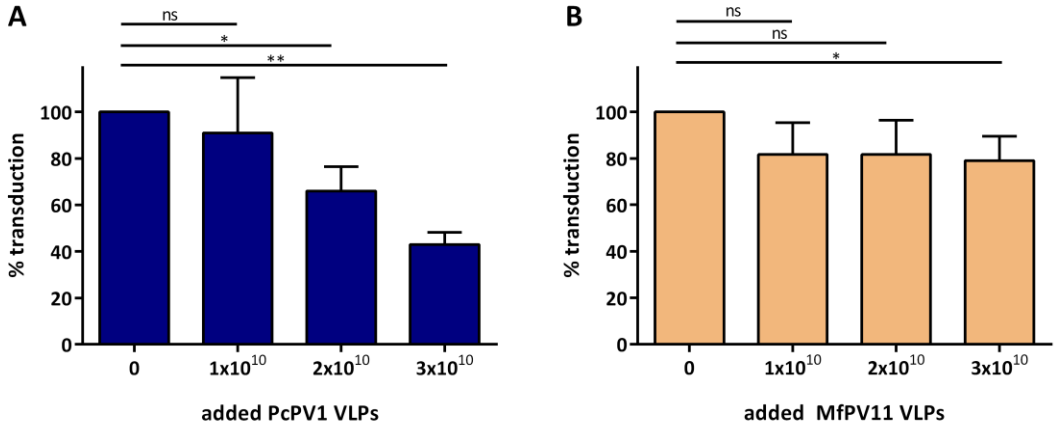


Figure 4.1-8 - Transduction with PsVs in the presence of VLPs

HEK293TT cells were transduced with PcPV1 (A) and MfPV11 (B) PsVs containing pCMV-G.Luc as reporter plasmid in the presence of empty VLPs of the same papilloma virus type. 72h later, transduction efficiency was measured by luciferase assay. Experiments were repeated with three independent PsV preparations. Shown are mean values and standard deviations. Statistical analysis was performed using two-way ANOVA and Tukey's multiple comparison test.

Per transduction $1x10^{10}$, $2x10^{10}$, $3x10^{10}$ and for controls no empty VLPs were added during the transduction with PsVs. Analysis of the resulting transduction efficiency, measured by luciferase assay, revealed that the addition of VLPs had a dose-dependent, negative effect on the amount of expressed luciferase. This effect was only pronounced for PcPV1, leading to the

assumption that transduction is reduced due to competition with the VLPs for cell binding and/or virus entry. In line with these findings, purification by Percoll ultracentrifugation did not yield PcPV1 PsVs with satisfying transduction efficiency in comparison to OptiPrep ultracentrifugation purified PcPV1 PsVs (described in section 4.1.3.2). During Percoll ultracentrifugation, empty VLPs are not separated from the PsVs, likely leading to the observed reduced transduction efficiency of PcPV1 PsVs. Consequently, this observation has implications for the purification step of PsV preparations, which should ensure the removal of empty VLPs especially from PcPV1 PsV samples.

4.1.4 Packaging of DNA inside PcPV1 and MfPV11 papilloma pseudovirus

For the production of PsVs, the producer cells have to be co-transfected both with plasmid containing the sequences coding for the capsid proteins and with the actual reporter- or vaccine plasmid. When PsVs are designed to deliver a plasmid for gene therapy or vaccination, it is important to ensure the absence of replication competence of the pseudoviral vector in the target cell. Therefore, only the desired plasmids should be packaged inside the particles, while the packaging plasmids containing the capsid protein sequences should be absent in the PsVs. Ensuring the specific packaging of plasmids is a challenge, as no packaging signal is known for papillomavirus genome packaging (Buck et al., 2004; Xu et al., 2006). For bovine papilloma virus 1 (BPV1) it has been reported that the efficiency of plasmid packaging will drastically decrease with increasing size of the plasmid. PsVs with reporter plasmids larger than 10kb did not show any measureable transduction (Buck et al., 2004), indicating that packaging specificity can be achieved by size adjustments of the plasmids used for PsV production.

Based on these findings for BPV1, using a packaging plasmid coding for papilloma virus capsid proteins L1 and L2 of approx. 9100bp – which is about 1kb longer than the papilloma virus genome – was expected to prevent the packaging of this plasmid. To analyze the packaged plasmids, purified PsVs were treated with DNAse to eliminate any free non-packaged DNA, and DNA was extracted from the PsVs using Qiagen Qiamp MinElute Virus Spin Kit (3.4.4). Surprisingly, in all PsV preparations tested by qPCR, the packaging plasmids (Figure 7.1-1 and Figure 7.1-2) were found in amounts at least as high as the small reporter plasmid pCMV-G.Luc of 5764bp (Figure 7.1-5), if not even higher. To increase the size of the packaging plasmid, a stuffer element was cloned into the packaging plasmid, leading to a size of approx. 10,200bp (Figure 7.1-3 and Figure 7.1-4). Still, this modification was not able to prevent or at least reduce the packaging of the packaging plasmid in the PsVs (Figure 4.1-9). To rule out off-target amplification by qPCR, it was confirmed experimentally that the primers used for detection of the packaging plasmids do not bind to the genomic DNA of the producer cell line HEK293TT (not shown).

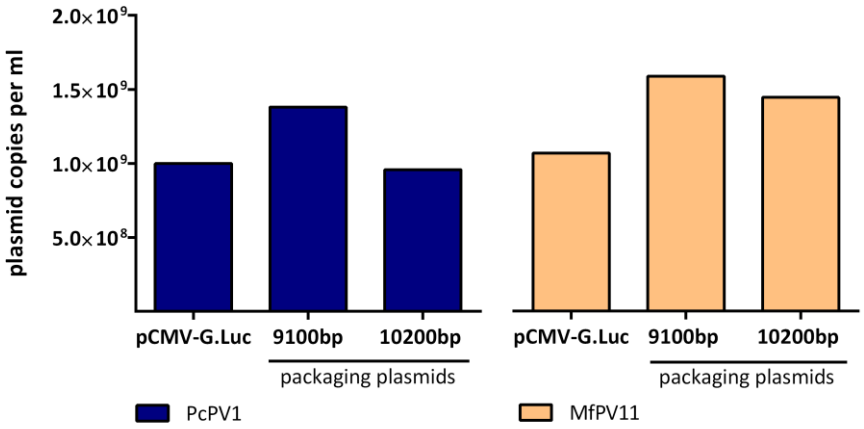


Figure 4.1-9 - Packaging plasmids of different sizes

Packaging plasmids of different sizes (9,100bp and 10,200bp) and pCMV-G.Luc as reporter plasmid were used for transfection to produce PsVs. DNA was extracted and plasmids in each PsV samples were quantified by qPCR. Shown are the copy numbers per ml of each plasmid in one PcPV1 and one MfPV11 sample. Shown is one representative experiment for PcPV1 and MfPV11 PsVs.

The observation that the packaging plasmid was found in the PsVs so efficiently despite its large size led to the question whether the explanation for this phenomenon could lie in the sequence of the plasmid. Unlike the reporter- or vaccine plasmid, the packaging plasmid contains papilloma virus sequences that might have a positive effect on the packaging. In order to test this hypothesis, PcPV1 and MfPV11 PsVs were produced using pVAX-Fsyn – a plasmid coding for RSV F-protein (Figure 7.1-8) – as vaccine plasmid to be packaged. Papilloma virus L1 and L2 sequences, respectively, were cloned additionally into pVAX-Fsyn upstream of the promoter such that they were present only as potential packaging sequence without being expressed (Figure 4.1-10).

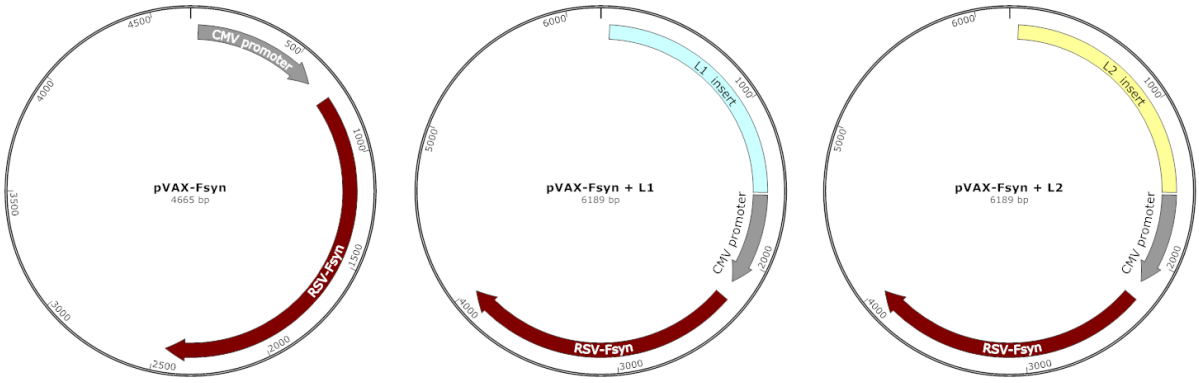


Figure 4.1-10 - pVAX-Fsyn plasmid and pVAX-Fsyn with additional papilloma virus L1 or L2 sequences

Subsequently, three different PsV preparations were produced for both PcPV1 and MfPV11 (see Table 4.1-2).

Table 4.1-2 - Plasmid combinations used for PsV production

		Plasmids for PsV production	
		Packaging plasmid	Vaccine plasmid
Figure 4.1-11, A PcPV1 PsVs	pVAX-Fsyn	pcD3.1+ PcPV1 L1-IRES-L2 (Figure 7.1-2)	pVAX-Fsyn
	pVAX-Fsyn (+Pc L1)		pVAX-Fsyn +PcPV1 L1
	pVAX-Fsyn (+Pc L2)		pVAX-Fsyn +PcPV1 L2
Figure 4.1-11, B MfPV11 PsVs	pVAX-Fsyn	pcD3.1+ MfPV11 L1-IRES-L2 (Figure 7.1-1)	pVAX-Fsyn
	pVAX-Fsyn (+Mf11 L1)		pVAX-Fsyn +MfPV11 L1
	pVAX-Fsyn (+M11 L2)		pVAX-Fsyn +MPV11 L2

While the same packaging plasmids were used in all three cases, the vaccine plasmids differed in that either the original plasmid pVAX-Fsyn or pVAX-Fsyn with the additional L1 sequence or pVAX-Fsyn with the additional L2 sequence was used. After DNA-extraction from the PsVs, plasmids were quantified by qPCR-analysis using primers binding to the IRES to quantify the packaging plasmid, and primers binding in the Fsyn sequence to quantify the vaccine plasmids. The analysis revealed that the addition of the L1 or L2 sequence did not enhance the packaging of the pVAX-Fsyn plasmid to bring it up to the level of the packaging plasmid, which was still preferably packaged (Figure 4.1-11).

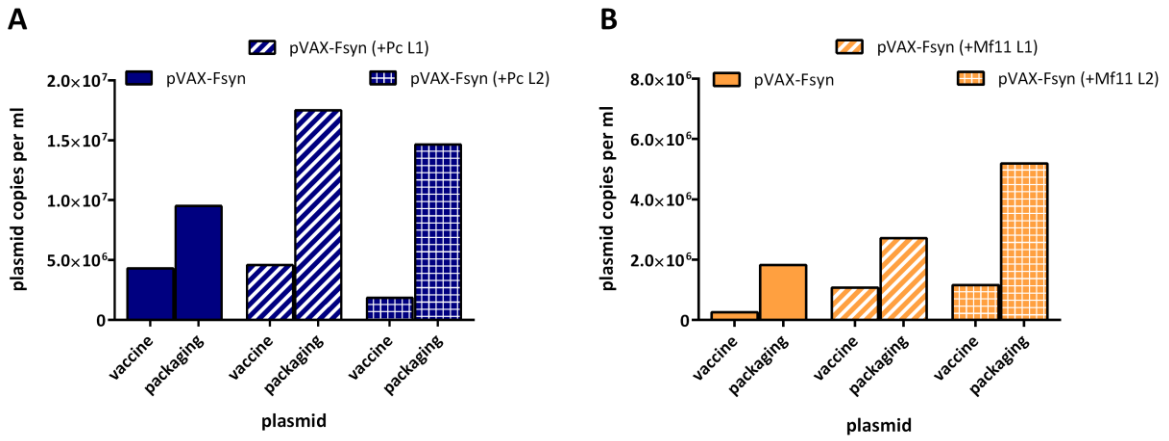


Figure 4.1-11 - Effect of adding L1 and L2 as potential packaging sequence

Three preparations of PsVs (both for PcPV1 (A) and MfPV11 (B)) were produced by transfection with the packaging plasmid and different variations of the pVAX-Fsyn plasmid, which is the vaccine plasmid that is meant to be packaged. Besides the original pVAX-Fsyn plasmid, two variations of the plasmid were used, containing the sequence for L1 and L2 respectively. Shown are plasmid copies per ml as determined by qPCR after DNA extraction from the PsVs. "Vaccine" refers to plasmid pVAX-Fsyn, with or without modification; "packaging" refers so plasmid pcD3.1+ L1-IRES-L2. PcPV1 (A), MfPV11 (B). Shown is one experiment for PcPV1 and MfPV11 PsVs.

4.1.4.1 PAN-PCR and sequencing of DNA extracted from PsVs

As it remained difficult to derive any rules explaining the packaging behavior of PcPV1 and MfPV11 PsVs, "Particle-associated-nucleic acid PCR" (PAN-PCR, 3.4.7) was chosen for a sequence-independent analysis of nucleic acids that are packaged inside the PsVs. This method uses degenerated primers to bind to random DNA sequences in the first step, thereby allowing the unspecific amplification of any DNA sequences present in the sample.

The goal of this experiment was to check for the presence of other DNA sequences, such as genomic DNA from the producer cells that may also have been packaged inside the PsVs.

For this experiment, PsVs carrying pVAX-Fsyn (described above, section 4.1.4) were used. The DNA was extracted from the PsVs (3.4.4) and amplified by PAN-PCR (3.4.7). These amplified sequences were cloned into a TOPO-TA plasmid (Figure 7.1-15), which was transformed into E.Coli. The resulting plasmids were restriction digested to excise the sample sequences. The small bands (between 100 and 1,000bp) in the agarose gel (Figure 4.1-12) represent the amplified sequences that were cloned into the TOPO-TA plasmids and were subsequently excised by restriction digest.

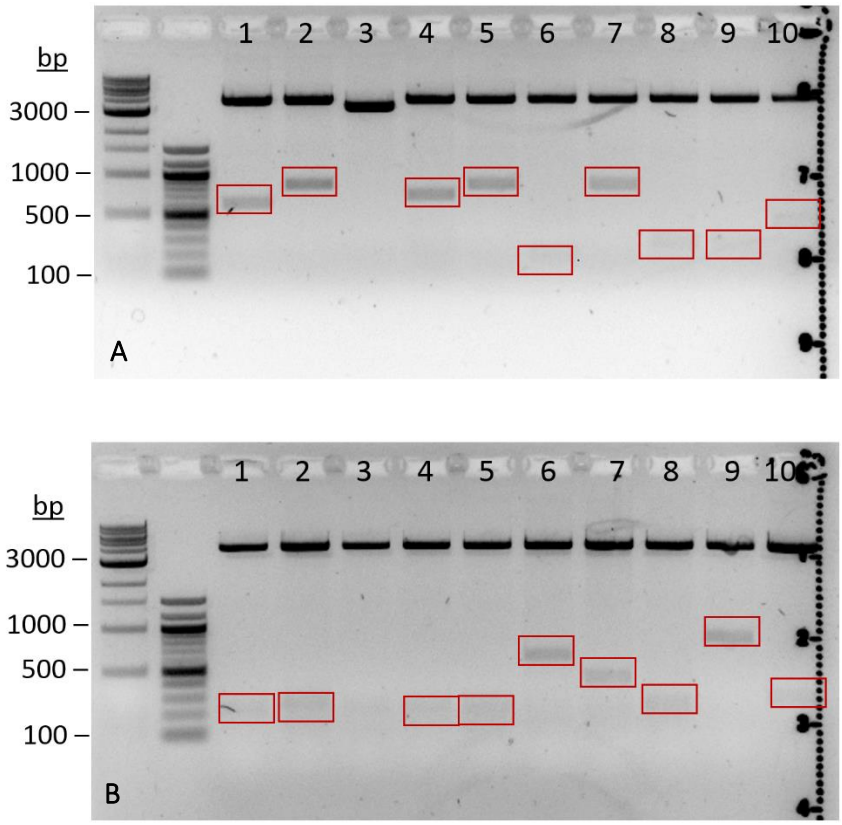


Figure 4.1-12 - PAN-PCR products cloned into TOPO-TA plasmid

Sequences amplified by PAN-PCR were cloned into TOPO-TA plasmid and excised by restriction digest. The resulting DNA-fragments were separated by agarose gel electrophoresis and visualized with UV light. PcPV1 (A), MfPV11 (B).

For DNA extracted from PcPV1 and MfPV11 PsVs (3.4.4), six of the submitted plasmids each yielded sequencing results. In the case of Mf11PV1, all six sequences were identified to stem from the packaging plasmid pcD3.1+MfPV11 L1-IRES-L2. For PcPV1, five of the sequences were identified to originate from the packaging plasmid pcD3.1+PcPV1 L1-IRES-L2, while one sequence was found to originate from *Homo sapiens* chromosome 3.

In a second round, purified PcPV1 and MfPV11 PsVs carrying pCMV-G.Luc were used for DNA-extraction, PAN-PCR, TOPO-TA cloning and subsequent sequencing the same way as described above. Ten plasmids each were submitted for sequencing. For PcPV1 PsVs, seven plasmids yielded sequencing results that were all identified by NCBI Nucleotide BLAST[1] to originate from human chromosomal DNA. Six plasmids containing DNA sequences extracted from MfPV11 PsVs returned sequencing results, which were again all identified to originate from human chromosomal DNA.

Identifying the packaging plasmids (pcD3.1+ PcPV1 L1-IRES-L2 and pcD3.1+ MfPV11 L1-IRES-L2) in the DNA extracted from the PsVs confirms the findings of the qPCR (section 4.1.4;

[1] https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Nucleotides

Figure 4.1-9) that the packaging plasmids are indeed encapsidated by the PsVs despite their size, which exceeds the length of the PV genome. Further, we find that the encapsidation is not limited to plasmid DNA, but also genomic DNA is found inside the PsV particles. This is in line with previous reports stating that papilloma PsVs do package genomic DNA to some degree (Cerqueira et al., 2017).

The applied method using PAN-PCR, however, does not allow to come to quantitative conclusions concerning the amount of plasmid DNA in comparison to chromosomal DNA that is encapsidated by papilloma PsVs.

4.1.4.2 Design of new “combination” plasmids

To circumvent the problem that the packaging plasmids are packaged inside the PsVs to such a large extent, new “combination” plasmids were designed. Instead of keeping L1 and L2 sequences on one plasmid and having a separate vaccine or reporter plasmid, packaging and vaccine/reporter sequences were now combined:

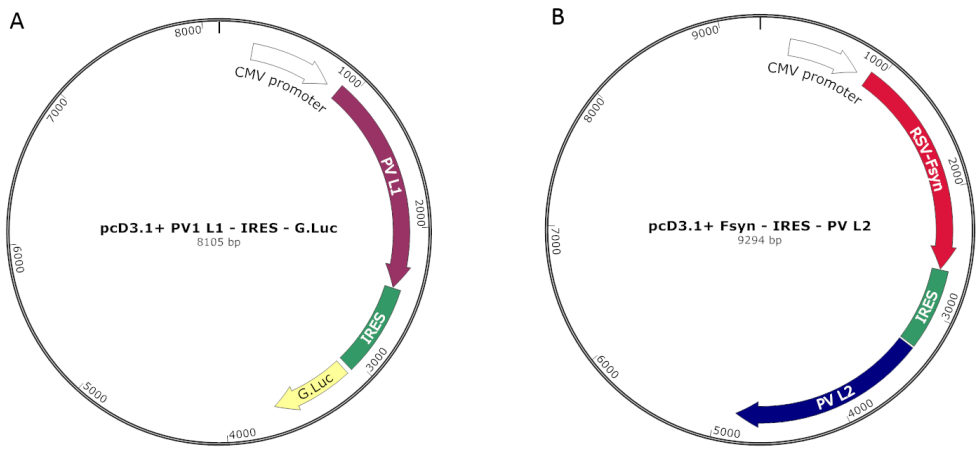


Figure 4.1-13 - New "combined" plasmids

(A) Plasmid containing the sequence for papilloma capsid protein L1 and gaussia luciferase (G.Luc)

(B) Plasmid containing the vaccine sequence coding for the RSV fusion protein F, and the sequence for papilloma capsid protein L2.

For detailed plasmid maps see appendix (Figure 7.1-11, Figure 7.1-12, Figure 7.1-13 and Figure 7.1-14).

The rationale behind this design is threefold:
First, it considers the possibility that the packaging might occur in a cotranslational manner. Secondly, safety issues are addressed as the separation of the L1 and L2 sequences onto two different plasmids reduces the chance of the production of new PsVs in the vaccinee. Finally, every transduced cell will now express the vaccine or reporter protein.

To check the transduction efficiency of the new PsVs with the “combined” plasmid constructs, PcPV1 and MfPV11 PsVs were produced by transfecting HEK293TT with L1-IRES-G.Luc and G.Luc-IRES-L2 containing plasmids. The resulting PsVs were used to transduce HEK293TT cells and G.Luc expression was measured by luciferase assay. In comparison to PsVs that were produced using L1-IRES-L2 plasmids plus pCMV-G.Luc as separate reporter plasmid, PsVs carrying the combined plasmids did not show statistically significantly different transduction rates, measured as G.Luc expression (Figure 4.1-14, A).

qPCR analysis of the packaged plasmids revealed that the distribution of L1-IRES-G.Luc and G.Luc-IRES-L2 was about 1:1 with 49% L1-IRES-G.Luc and 51% G.Luc-IRES-L2 for PcPV1, and 56% L1-IRES-G.Luc and 44% G.Luc-IRES-L2 for MfPV11 (Figure 4.1-14, B).

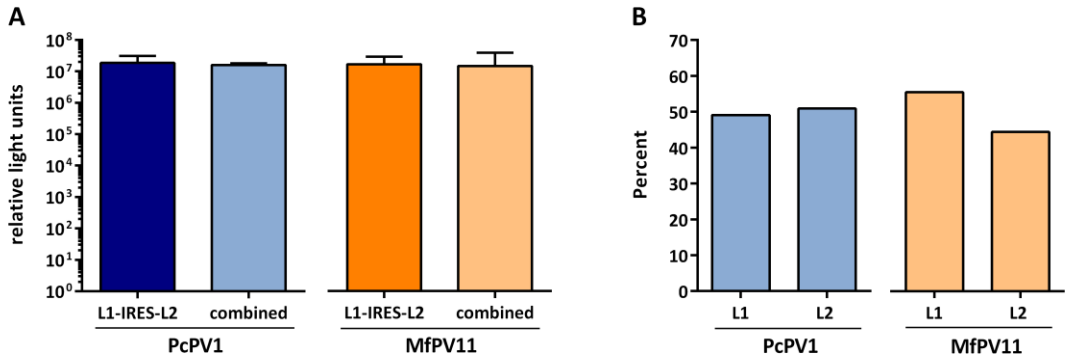


Figure 4.1-14 - Transduction and qPCR analysis with PsVs produced with "combination" plasmids

(A) RLU measured by luciferase assay after transduction of HEK293TT with PsVs, produced either with “L1-IRES-L2” plasmids and additional pCMV-G.Luc or with “combined” plasmids (L1-IRES-G.Luc and G.Luc-IRES-L2). Shown are mean values and standard deviations of transduction experiments of at least two independent PsV preparations each.

(B) qPCR analysis of plasmids extracted from one PsV preparation each that was produced with “combined” plasmids containing both G.Luc and L1 or L2 respectively. Shown is one experiment.

The above described “combined” plasmids were later used for the production of PsVs for a vaccination against RSV, which will be described in section 4.1.6.

4.1.5 PcPV1 and MfPV11 pseudovirus with Firefly Luciferase Reporter in vivo

In order to test whether papilloma PsV-mediated transduction also worked *in vivo*, PcPV1 and MfPV11 PsVs carrying a reporter plasmid coding for firefly luciferase (pcLuc13, for plasmid map see Figure 7.1-7) were produced. For this first proof-of-principle, PsVs were produced using the packaging plasmids coding for L1 and L2, and the additional reporter plasmid pcLuc13. Previous experiments with ultracentrifugation-purified PsVs with pcLuc13 as reporter had yielded only low F.Luc expression *in vivo* in the case of PcPV1 PsVs, while no F.Luc expression was observed after application of MfPV11 PsVs *in vivo* (data not shown). In the experiment described here, we decided to use PsVs without purification (3.3.3). Due to the extensive nuclease treatment, residual plasmid DNA in the PsV preparation was not considered a potential problem.

The functionality of the PsVs was first assessed *in vitro* by transducing HEK293TT cells and measuring firefly luciferase (F.Luc) expression by F.Luc assay (3.5.3). Both batches of PsVs showed transduction at similar levels with $4.71x10^6$ RLU/ml for MfPV11 and $3.09x10^6$ RLU/ml PcPV1 (not shown). Female BALB/c mice were injected intramuscularly with 50µl of the respective PsV suspensions into the left hind leg. As the use of non-purified PsV preparations poses the risk of containing free F.Luc, mice were subjected to bioluminescence imaging about 3h after injection to check for the presence of F.Luc in the muscle. No F.Luc was detectable in any of the mice (Figure 4.1-15, “Day 0”). Mice were subsequently monitored by bioluminescence imaging in a weekly manner. Mice that had been injected with PcPV1 PsVs showed a marked F.Luc expression on day 7. In mice injected with MfPV11 PsVs, F.Luc was detectable for the first time on day 28, though quite weak and only visible in three of the five animals. PcPV1 PsVs with pcLuc13 reporter plasmid yielded a stable expression of F.Luc until day 98 after injection, which is when the last bioluminescent imaging was performed (not shown).

These results show that both PcPV1 and MfPV11 PsVs have the capability of transducing murine muscle cells, leading to long-lasting expression of a reporter protein. Although transduction rates of PcPV1 and MfPV11 PsV preparations were comparable *in vitro*, injection of PcPV1 PsVs led to much more F.Luc expression than MfPV11 PsVs. Quantitative analysis of the bioluminescence imaging data was not performed as this requires very exact and equal positioning of all animals on the stage of the bioluminescence imager, which was not possible with the available set-up.

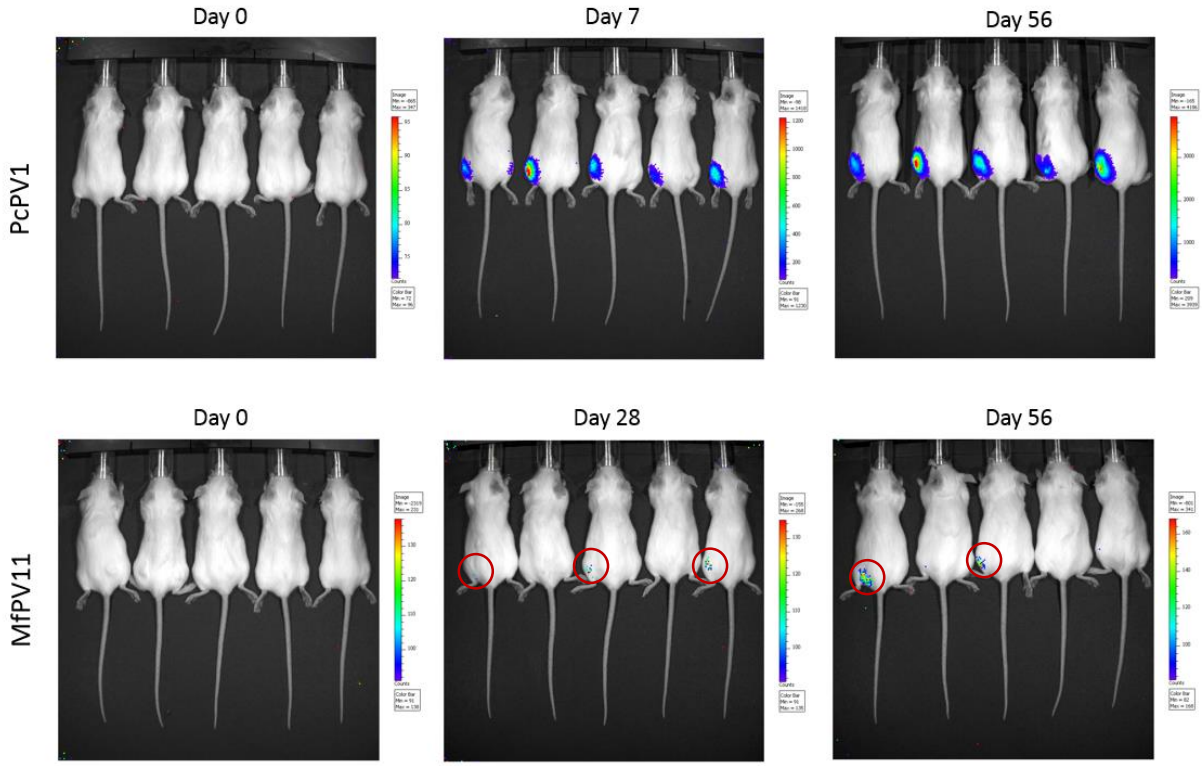


Figure 4.1-15 - Bioluminescence imaging

PcPV1 and MfPV11 PsVs with F.Luc reporter plasmid were injected intramuscularly in the left hind leg of BALB/c mice. Bioluminescent imaging was performed approx. 3h after injection (day 0) and subsequently in a weekly manner.

4.1.6 Genetic vaccination against RSV using PcPV1 and MfPV11 as vectors

Having shown that intramuscular transduction of a reporter plasmid is possible using PcPV1 and MfPV11 PsVs, PsVs were applied in a vaccination setting. PsVs were harvested (3.3.3) and purified by ultracentrifugation (3.3.3.1) using OptiPrep for PcPV1 PsVs and Percoll for MfPV11 PsVs. For the production of the vaccine PsVs, we used the combined plasmids, coding for L1 and G.Luc on one plasmid (Figure 7.1-11) and for RSV-Fsyn and L2 on the second plasmid (Figure 7.1-14). This set-up enables the performance of *in vitro* transduction experiments, measuring the expression of G.Luc as quality control and for identification of preparations and fractions after ultracentrifugation that show the highest transduction rates. Fsyn is a modified, codon-optimized version of the native RSV-F sequence that is expressed at much higher rates (Ternette et al., 2007).

Several PsV preparations were pooled to obtain enough material for vaccination. A sample of the two vaccination batches was tested to confirm that no free RSV-F protein was present (Figure 4.1-16, B).

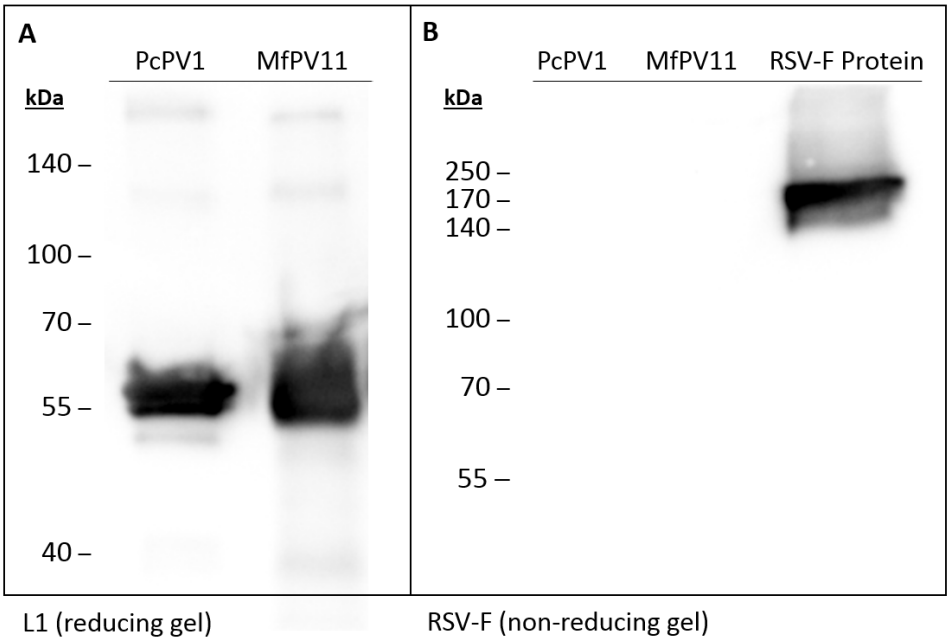


Figure 4.1-16 - Western Blot analysis of PsVs vaccine preparations

Proteins of PcPV1 and MfPV11 PsV preparations were separated by SDS-PAGE and blotted. (A) MD2H11 antibody was used to visualize the papilloma virus L1 protein. (B) 18F12 antibody was used for the detection of any free RSV-F protein in the PsV preparations. RSV-F protein, obtained from RSV-F transfected cells, was loaded as positive control in the very right lane.

In a vaccination setting using viral vectors, vector immunity has to be considered a potential problem. It is feasible that the use of a certain papillomavirus type for the prime vaccination would elicit an immune response against the vector itself. Using the same vector for

the boost may then lead to the vector being eliminated by the immune system before it can transduce any cells. In order to circumvent vector immunity in the scope of this immunization study, mice were vaccinated in a heterologous prime-boost regimen, using one PV type for the first immunization and the other type for the second immunization. Additionally, two different routes of administration – intramuscularly and intranasally – were used for the boost.
Besides checking the transduction efficiency of the vaccine-PsVs *in vitro* using G.Luc as reporter, qPCR analysis was performed to calculate theoretical titers based on the Fsyn-containing plasmid. Concentrations were calculated to be $1.32x10^9$/ml for MfPV11 PsVs and $2.27x10^9$/ml for PcPV1 PsVs. 100µl of the PcPV1 PsV preparation therefore contains 2.3ng of pcD3.1+Fsyn-IRES-L2 plasmid (Figure 7.1-14). Control groups were vaccinated intramuscularly with 2.3ng pcDFsyn plasmid (Figure 7.1-10) to be able to identify effects of PsV-mediated transduction in comparison to the administration of naked DNA. The vaccination groups, each containing six female BALB/c mice, are summarized below in Table 4.1-3.

Table 4.1-3 - Vaccination groups

Vaccination group	Prime	Boost
P-m / M-m	PcPV1 intramuscular (2x 50µl)	MfPV11 intramuscular (2x 50µl)
P-m / M-n	PcPV1 intramuscular (2x 50µl)	MfPV11 intranasal (50µl)
M-m / P-m	MfPV11 intramuscular (2x 50µl)	PcPV1 intramuscular (2x 50µl)
M-m / P-n	MfPV11 intramuscular (2x 50µl)	PcPV1 intranasal (50µl)
pcDFsyn	2,3ng pcDFsyn plasmid (50µl)	2,3ng pcDFsyn plasmid (50µl)
Non-vaccinated	-	-

The serum that was collected three weeks after prime and three weeks after boost, was analyzed for the presence of RSV-F binding antibodies as well as RSV-neutralizing antibodies. No binding antibodies were detectable in the sera after prime, but after boost, binding antibodies were present in the serum of some animals (Figure 4.1-17, A). Neutralizing antibodies were not detectable in the serum after prime or boost (data not shown).

Unexpectedly and suddenly, approx. three weeks after the intranasal boost immunization in group "P-m / M-n", within three days, all of the animals died or had to be euthanized due to their condition. Pathological examination (*GVG Diagnostics*, Leipzig) did not reveal a clear reason for the peracute death of the mice. However, the animals showed elevated signs of inflammation in their lungs, leading to the assumption that the cause might indeed lie in the intranasal application of the PsV suspension. It remains unclear why these symptoms occurred as late as three weeks after application.

Finally, mice of all remaining groups were challenged intranasally with 10^6 pfu RSV four weeks after the last immunization. Five days post challenge, RNA was extracted from the mouse lungs (3.4.5) and viral RNA was quantified by RT-qPCR (3.4.6.2). Mice, which had received a PsV-based vaccine by two intramuscular injections (P-m/M-m and M-m/P-m), showed a significant reduction of the viral load. Vaccination with PsVs by intramuscular and intranasal application (M-m/P-n), as well as pcDFsyn as naked DNA did not yield a significant reduction of the viral load (Figure 4.1-17, B).

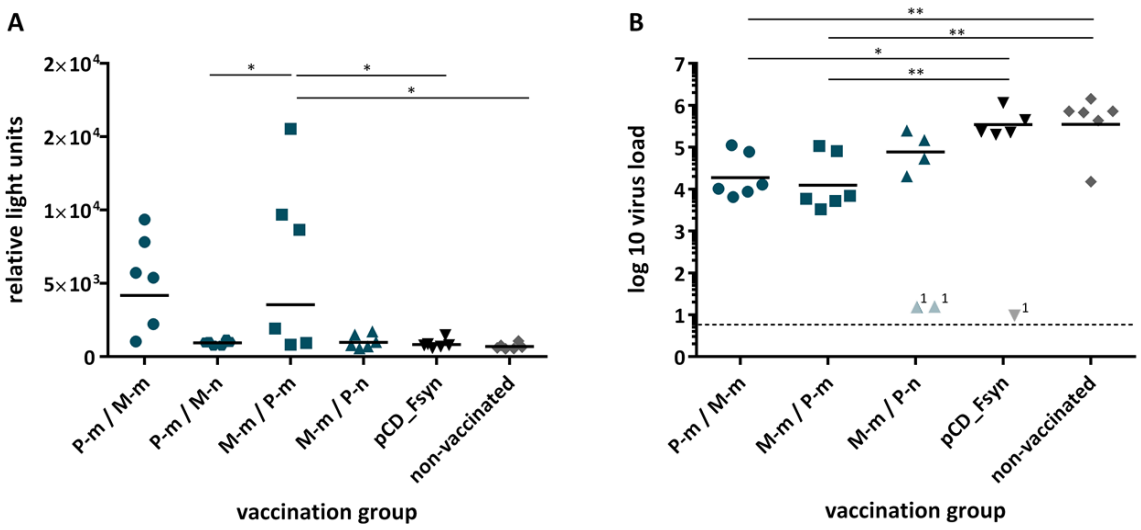


Figure 4.1-17 - Binding antibodies in serum after boost and viral load after challenge

Serum of vaccinated mice after boost was tested for RSV-binding antibodies by coating an ELISA-plate with purified RSV and probing with mouse sera (A). Mice were challenged with RSV/A2 four weeks after the last immunization. RNA was extracted from lungs five days later and used for RT-qPCR quantification of virus load.
[1] Indicated data points were not taken into account for the statistical analysis (B). Statistical analysis was performed by one-way ANOVA and Tukey's multiple comparison test.

Calculations of the viral load reductions were performed using the geometric mean value of each group and are summarized in Table 4.1-4. The indicated data points ([1]) were not taken into account for the statistical analysis and for the calculation of the viral load reduction. As an almost complete protection was not expected with this type of vaccination due to the low amount of DNA administered, it is more likely that the infection with RSV during the challenge did not work optimally in these three animals. Therefore, the resulting data points were excluded from further analyses.

Immunization using intramuscular injection of MfPV11 PsVs and subsequent intramuscular application of PcPV1 PsVs yielded the highest reduction of the viral load (29-fold). Only the groups, which were vaccinated twice intramuscularly, showed a significant reduction of the viral load compared to the non-vaccinated animals. This is in correspondence with the

measured binding antibody titers, which were only detectable in groups vaccinated exclusively by intramuscular injection.

Table 4.1-4 - Viral load reduction

Group	Fold viral load reduction (compared to non-vaccinated control)
P-m / M-m	19.2
M-m / P-m	28.6
M-m / P-n	4.9
pcDFsyn	1.1

4.1.6.1 Lung histopathology

In the process of the development of an RSV vaccine, it is important to rule out the possibility of inducing enhanced respiratory disease (ERD) upon infection after immunization. Therefore, after challenge, lung sections were HE-stained and analyzed for signs of inflammation and type 2 hyperplasia (3.5.5).

Differences between the groups were not very pronounced and mostly not statistically significant. Mice vaccinated with the M-m/P-n regimen did, however, show significantly lower signs of both inflammation and type 2 hyperplasia in comparison to the M-m/P-m group (Figure 4.1-18). It is worth noting that the mice of the M-m/P-m group showed the highest lung histopathology scores, and also had the highest (29-fold) reduction in viral load after challenge (Table 4.1-4). However, a correlation between the degree of inflammation or type 2 hyperplasia and protection was not found.

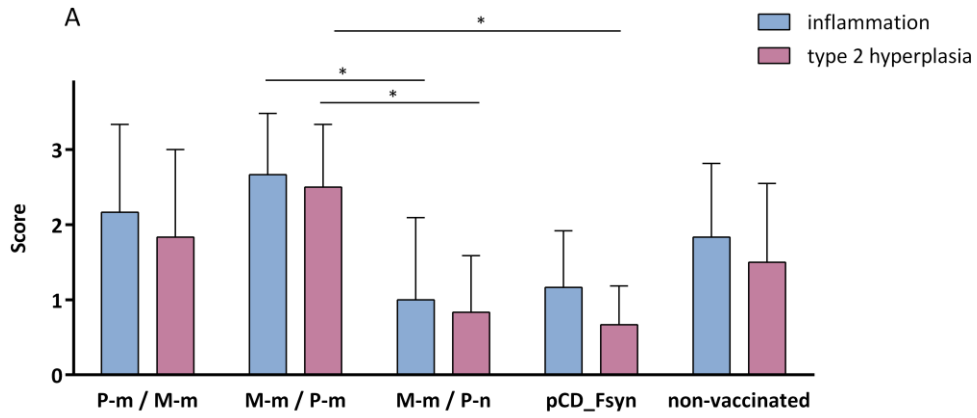


Figure 4.1-18 - Lung histopathology after immunization and challenge

Formaldehyde-fixed, HE-stained lung sections were analyzed for signs of inflammation and type 2 hyperplasia. Scores range from 0-3, as described in Table 3.5-2. Shown are mean values of all 6 mice per group and standard deviation. Statistical analyses were performed by two-way ANOVA and Tukey's multiple comparison test.

4.1.7 Summary and conclusion Part I

The analysis of ten animal papilloma virus types revealed that it was possible for most of them to use the sequences for the capsid proteins L1 and L2 to produce VLPs and even PsVs with at least basal transduction rates *in vitro*. Two papilloma PsVs which transduced markedly better than the remaining eight – MfPV11 and PcPV1 – were analyzed in more detail. The packaging of a reporter plasmid inside the PsVs was confirmed by qPCR, which also showed that the packaging plasmid, coding for the papilloma virus L1 and L2, was found inside the capsids to a large degree. For this reason, new plasmids were constructed, coding for either L1 or L2 plus the actual vaccine sequence of the RSV-F protein. MfPV11 and PcPV1 PsVs carrying these plasmids were used to vaccinate mice. The elicited immune response after two intramuscular immunizations allowed a significant reduction of the viral titer when the animals were challenged with infectious RSV. Additionally, intramuscular injection of MfPV11 and PcPV1 PsVs carrying a plasmid coding for firefly luciferase (F.Luc) led to the expression of F.Luc, which lasted several weeks in the case of PcPV1 PsVs.

The surprising finding that the *in vitro* transduction of a number of animal papilloma PsVs – such as PcPV1 – can be enhanced by the addition of ι-carrageenan, could be a way to increase the transduction also *in vivo*.

In summary, pseudovirions based on non-human papilloma viruses are an attractive vehicle to deliver DNA, and should be considered for further development in the fields of vaccines or gene therapy.

4.2 Part II – vaccination with RSV inactivated by low-energy-electron-irradiation

Inactivation methods for the viral vaccine production currently include mainly chemical inactivation by β-propriolactone or formaldehyde. Due to long incubation periods and subsequent purification steps, these are rather time consuming methods. In addition, the propensity of chemicals to alter protein structures could yield less effective vaccines. Especially in the case of RSV, chemically inactivated vaccines preparations are not considered anymore due to the devastating consequences of a formaldehyde-RSV vaccine trial in the 1960s.

Part II of this thesis deals with an alternative method for pathogen inactivation in the scope of vaccine production. Low-energy electron irradiation (LEEI) was tested for its ability to inactivate RSV (4.2.2), and conservation of viral antigens upon irradiation was analyzed (4.2.3).

The LEEI-inactivated virus material was then used in two mouse-vaccination studies to test its immunogenic potential and its ability to confer protection upon infection with RSV. The first study (4.2.4) focuses on a comparison of LEEI-RSV with formaldehyde-inactivated (FI-) RSV as vaccine. In the following second study (4.2.5), the LEEI-RSV vaccine preparation was refined by adding a purification step and by testing various adjuvants. The alum-based adjuvant Alhydrogel and MF59-equivalent AddaVax were used since these adjuvants are approved for use in humans. In addition, QuilA – approved for veterinary use – was tested. Poly IC:LC was considered to be an especially suitable adjuvant in a RNA-virus vaccine due to its dsRNA nature and was therefore included in the experimental design.

4.2.1 LEEI-inactivated Influenza A (H3N8) virus

Prior to the experiments with RSV, which will be described in detail in the following sections, pilot experiments were performed with Influenza A (H3N8). Irradiation dose curves revealed that an irradiation with 30kGy reproducibly inactivates Influenza A (H3N8) (not shown). LEEI-treated H3N8 was compared to H3N8, which was inactivated with 0.05% formaldehyde. The incubation with formaldehyde was 16 hours at 4°C (FA short) or 7 days at 37°C (FA long), as inactivation times in the vaccine manufacturing setting are usually several days or weeks at 37°C.

The analysis of the antigenicity was performed by coating the H3N8 virus particles onto an ELISA plate and probing with serum from an H3N8 infected pig. Figure 4.2-1, A shows the conservation of viral proteins relative to untreated virus, which served as positive control. The 7-day incubation with formaldehyde (FA long) resulted in significantly reduced viral surface proteins (around 40%), which are recognized by the polyclonal serum antibodies. In contrast, both LEEI and FA short treatment of the virus allowed an approx. 90% conservation of viral antigens. The observation that inactivation by LEEI performed significantly better than the long formaldehyde treatment leads to the conclusion that LEEI is a much more gentle inactivation method than what is currently used for the vaccine production, resulting in a higher amount of antigens in the vaccine preparation.

In order to test whether LEEI-treated Influenza A (H3N8) is suitable as a vaccine, mice were immunized intramuscularly with alum-adjuvanted LEEI-H3N8 in a prime-boost regimen (Figure 3.7-1). Another group was immunized with alum-adjuvanted H3N8, inactivated by a 16 hour formaldehyde treatment at 4°C. The control group was injected with alum-adjuvanted mock-infected cell culture supernatant. Each group contained six female BALB/c mice. Finally, the mice were challenged intranasally with a dose of $2x10^5$ infectious H3N8 particles per animal. Three days later, the viral load in each mouse's lung was determined by quantitative real-time PCR (Figure 4.2-1, B).

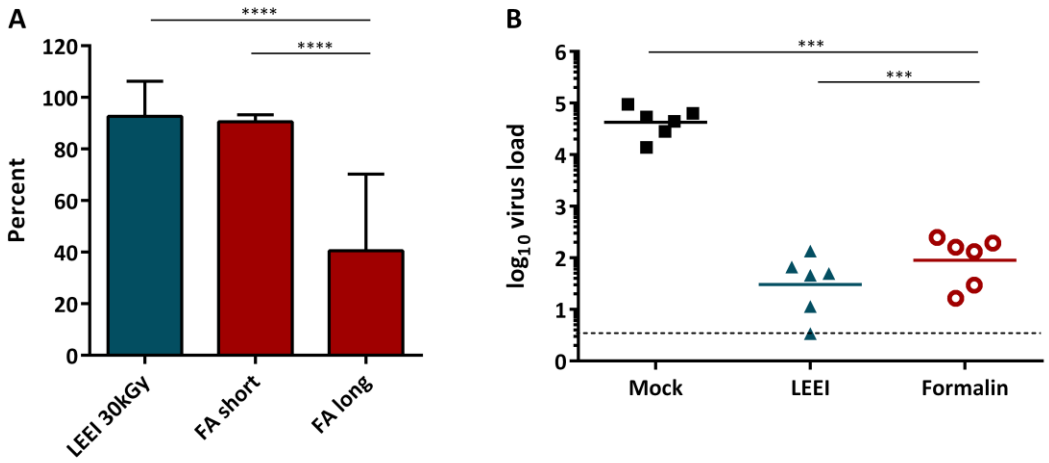


Figure 4.2-1 - LEEI-inactivated Influenza H3N8

(from Fertey et al., 2016, adapted)

(A) Analysis of the antigen structures after inactivation. Influenza A (H3N8) was inactivated by 30kGy LEEI-treatment, by incubation with 0.05% formaldehyde (FA) for 16h at 4°C ("short") or for 7 days at 37°C ("long"), or left untreated. Error bars indicate the standard deviation. Statistical analysis was performed by one-way ANOVA and Tukey's multiple comparison test. Graph provided by Dr. Jasmin Fertey.

(B) Virus load determination after challenge of vaccinated and unvaccinated mice by RT-qPCR. Statistical analysis was performed by one-way ANOVA and Tukey's multiple comparison test.

After vaccination, the number of viral genome copies in the lung was reduced 982-fold in LEEI-H3N8 vaccinated animals and 382-fold in Formalin-H3N8 vaccinated mice. All vaccinated mice showed therefore a significantly reduced viral load compared to unvaccinated mice, but the differences between the LEEI and the formalin groups are not statistically significant.

In summary, the presented data show that vaccination with LEEI-inactivated Influenza A (H3N8) results in an effective immune response and protection against infection.

Due to the promising results obtained with LEEI-inactivated Influenza A (H3N8), LEEI-experiments were conducted with RSV, as presented in the following sections.

4.2.2 Irradiation dose curve for RSV inactivation

In order to determine the required dose of LEEI for reliable inactivation of RSV, different RSV preparations were subjected to irradiation between 4 and 20kGy. Controls (0kGy) were prepared in the same manner, but left without irradiation. The virus containing, irradiated fluid was serially diluted, and HEp2 cells were used in an infectivity assay to determine the resulting virus titers. Virus infected cells were visualized by immunocytochemistry 48h later (3.3.5.3).

The virus titer was reduced in a dose dependent manner (Figure 4.2-2). Although the necessary dose for complete inactivation positively correlated with the virus titer, RSV was reproducibly inactivated by LEEI at 20kGy up to a titer of 10^9pfu/ml (Figure 4.2-2, B and data not shown).

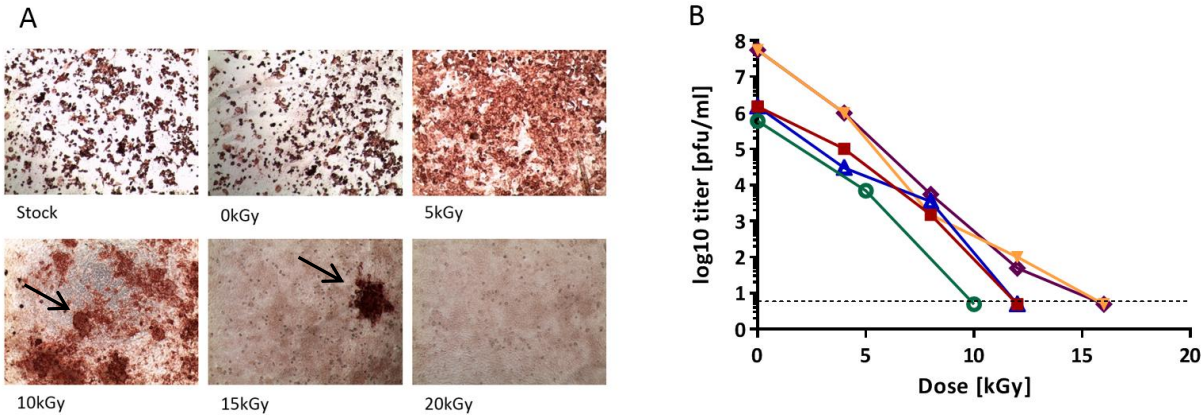


Figure 4.2-2 - LEEI-dose dependent reduction of RSV titer

RSV was irradiated with increasing doses of low-energy electrons and used for infection of HEp2 cells. Immunocytochemistry staining was used 48h later to visualize virus-infected cells (red plaques, see arrows; A). RSV preparations with titers ranging from $6x10^5$ to $6x10^7$ pfu/ml were subjected to increasing doses of LEEI. HEp2 cells were infected with serially diluted virus to determine the titer. One irradiation experiment of one preparation is represented by one curve in the graph; the dashed line indicates the limit of virus detection (B).

4.2.3 Conservation of RSV proteins after LEEI

In the process of virus inactivation for vaccine preparation, it is critical to ensure the preservation of the antigens that will be important for the induction of a specific and effective immune response. Therefore, RSV was irradiated with LEEI at doses between 4 and 20kGy and coated onto ELISA plates. Human polyclonal serum was used to probe for RSV antigens in general, while monoclonal anti RSV-F antibody was used to probe specifically for RSV F-protein, the most important antigen for a protective immune response. Non-treated stock was used as control and its ELISA-signal was set to 100%; all other values were calculated relative to the stock control.

Both RSV antigens in general (Figure 4.2-3, A) and RSV fusion protein F (Figure 4.2-3, B) showed a reduction in binding antibodies in an irradiation-dose dependent manner. After irradiation with 20kGy, about 70% of antibody-detectable RSV proteins and an average of 74% of RSV-F remained measureable compared to the non-treated stock. This degree of antigen conservation was reproducible in at least five independent experiments. In contrast, RSV inactivation by a 3-day incubation with 0.025% of formaldehyde at 37°C led to a highly variable RSV-F protein conservation measured by ELISA, ranging from 34% to 116% (Figure 4.2-3, C).

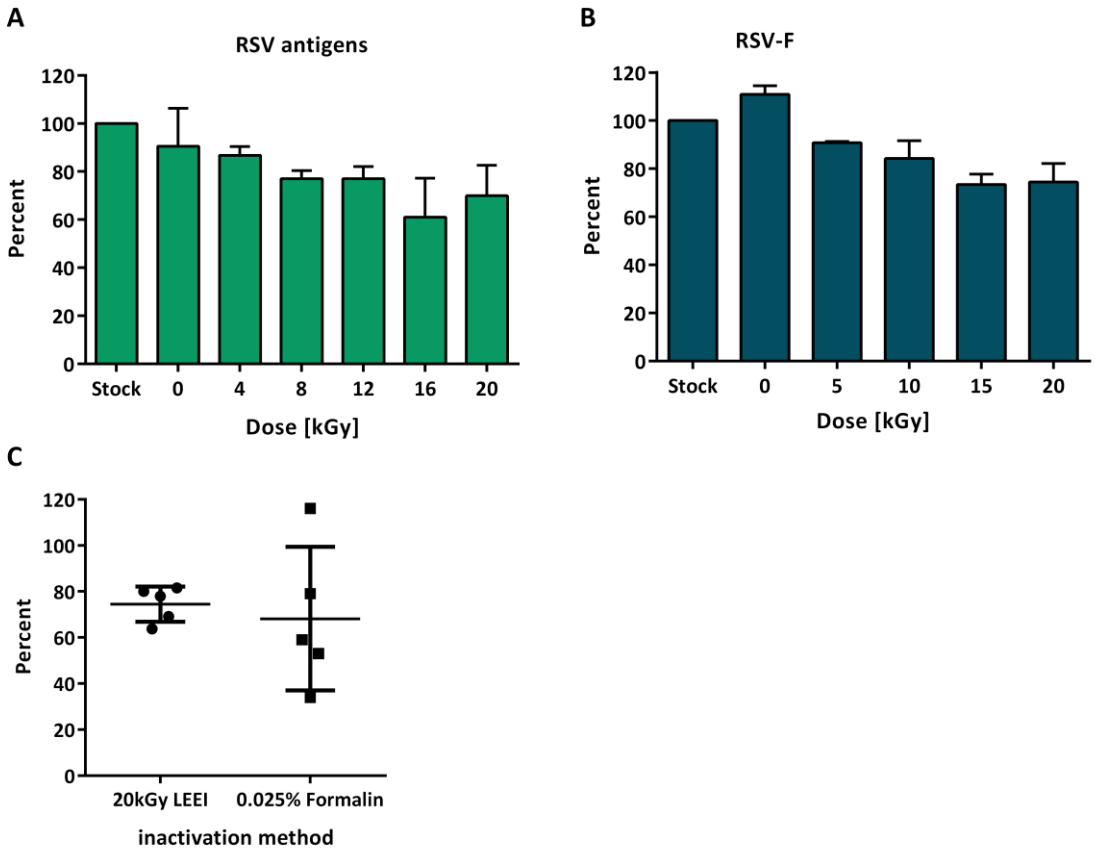


Figure 4.2-3 - Conservation of RSV proteins after inactivation

RSV was subjected to increasing doses of LEEI, or was left non-irradiated (0kGy). RSV material was coated on ELISA plates and probed with serum from an RSV-positive human (A) and with anti-RSV-F antibody (18F12) (B), respectively. Non-treated RSV stock was used as reference and measured absorbance was set to 100%. Medians and standard deviations of the relative signals of three or more independent experiments are shown. Similarly, conservation of RSV after inactivation with formalin and 20kGy, respectively, was measured using ELISA by coating virus material on ELISA plates and probing with anti-RSV-F antibody (18F12). Each data point represents one independent RSV preparation (C).

4.2.4 Vaccination study I: LEEI and formalin inactivated RSV

The first vaccination study sought to directly compare formaldehyde and LEEI as inactivation methods in terms of the resulting vaccine's abilities to elicit a protective immune response. RSV-containing cell culture supernatant was inactivated by formaldehyde following the protocol for "Lot100" ((Kim et al., 1969); 3.6.2). The same virus stock was used for LEEI inactivation with 20kGy and adjuvanted with Alhydrogel.

4.2.4.1 Immune response after vaccination with LEEI-RSV and FI-RSV

Mice were immunized intramuscularly following a homologous prime-boost regimen (3.7.1), using six female BALB/c mice per group. RSV specific antibodies were detectable by ELISA (3.5.4.2) in both vaccinated groups after prime, and an increase in binding antibodies was observed after boost (Figure 4.2-4, A). The avidity of the binding antibodies was measured in a modified ELISA (3.5.4.2). After prime, the average relative avidity of antibodies of the FI-RSV group was at 20%, mice from the LEEI-RSV group developed antibodies with an average relative avidity of 24%. In both groups, the avidity was increased by the boost, leading to an average relative avidity of 55% for the FI-RSV group and 66% for the LEEI-RSV group (Figure 4.2-4, B). Differences between both vaccinated groups are not statistically significant.

Titers of neutralizing antibodies are important measures for protectivity and were therefore determined by neutralizing antibody assays (3.3.6). Neutralizing antibodies were present in sera after prime and after boost of both vaccinated groups. The titers increased after boost to an average of 1:1086 in the LEEI group and 1:684 in the FI-RSV group. The differences in titers between both vaccinated groups are not statistically significant different (Figure 4.2-4, C).

Finally, the mice were challenged intranasally with 10^6 pfu of RSV four weeks after the last immunization to test the ability of the vaccines to protect from RSV infection. Three days post challenge, the viral load was quantified from the lungs of the infected mice. After isolation of the viral RNA, an 80bp sequence of the RSV N-gene was reverse transcribed and amplified by real time PCR. In comparison to control animals, which had received mock infected cell culture supernatant as vaccine, the viral load was reduced approx. 77-fold in mice vaccinated with LEEI-RSV and about 180-fold in mice vaccinated with FI-RSV (Figure 4.2-4, D). Again, differences between the two vaccinated groups are not statistically significant.

In summary, this immunization study shows that RSV inactivated by LEEI is suitable as vaccine, leading to a reduction in viral load upon challenge with RSV, which is comparable to FI-RSV. Aiming for a stronger and more protective immune response, the LEEI-RSV formulation was adjusted and different adjuvants were tested in a subsequent immunization study (4.2.5).

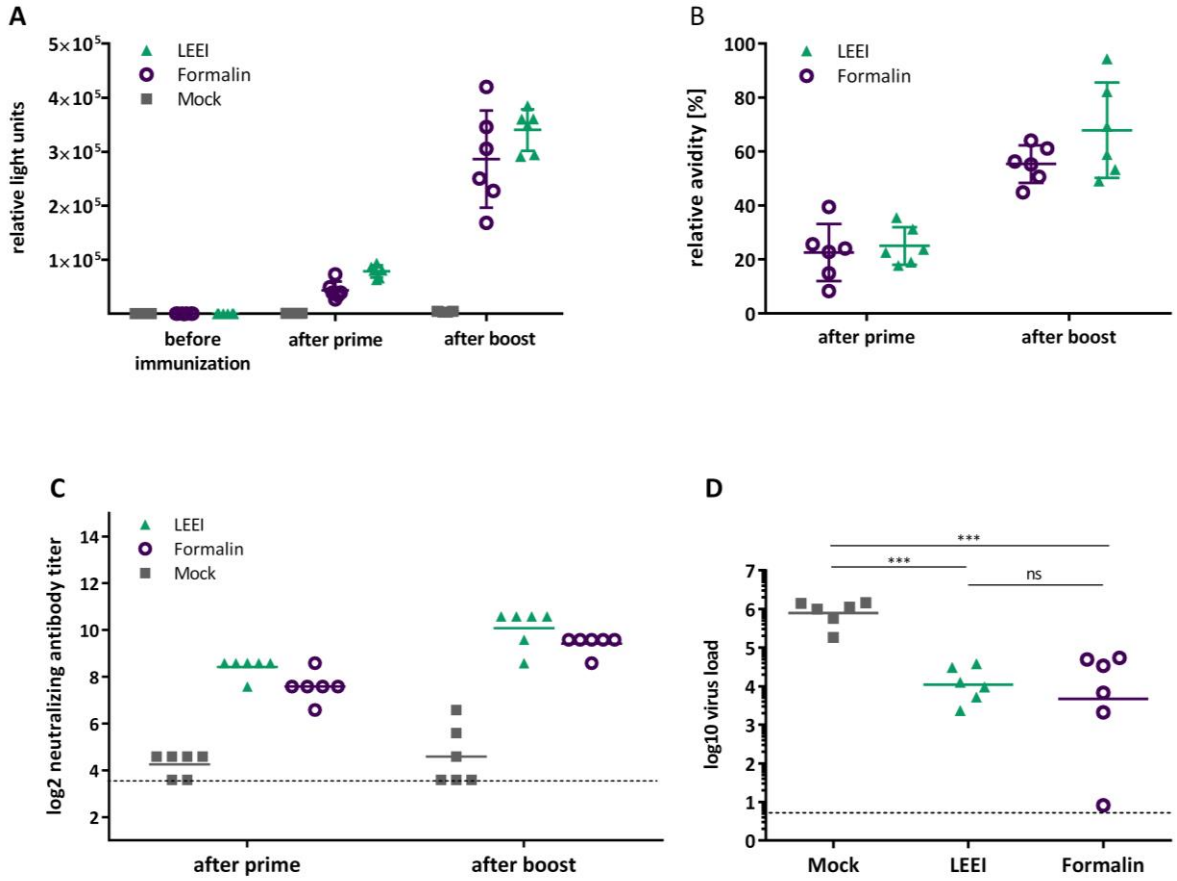


Figure 4.2-4 - Analysis of the immune response after vaccination with LEEI-RSV and FI-RSV

ELISA-plates were coated with purified RSV and probed with sera from immunized mice after prime and after boost to analyze total IgG binding antibodies (A). In order to calculate avidity, a 7M urea wash step was included after incubation with sera (B). Neutralizing antibodies were measured by serial dilution of sera and incubation with rgRSV before addition of HEp2 cells. GFP-positive plaque forming units were counted 48h later. Shown are reciprocal values of the serum dilutions, at which 50% of pfu were counted in comparison to controls without serum. ELISAs were performed in three independent experiments; shown is one representative experiment. Neutralization assays were performed in at least three independent experiments; shown is the geometric mean of the calculated neutralizing antibody titer.
Mice were challenged four weeks after the last immunization and RNA was extracted from lungs three days post infection. Virus load was determined in triplicates by RT-qPCR as copies per 45ng RNA. Statistical analysis was performed using one-way ANOVA and Tukey's multiple comparison test (D).

LEEI: group vaccinated with LEEI-inactivated RSV-containing cell culture supernatant; Formalin: group vaccinated with formalin-inactivated RSV-containing cell culture supernatant; Mock: group vaccinated with mock-infected cell culture supernatant. Alhydrogel was added as adjuvant in all groups.

4.2.5 Vaccination study II: LEEI-RSV and the effect of adjuvants

In an attempt to improve the LEEI-RSV vaccine, two parameters we changed compared to the preceding vaccine production (3.6.4). Firstly, RSV was purified from the virus-containing cell culture supernatant by ultracentrifugation with a 20% sucrose cushion. Secondly, for the final vaccine preparation different adjuvants were added. Apart from the alum-containing Alhydrogel, AddaVax, QuilA and Poly IC:LC were tested. 50µl of each adjuvant were added to 50µl of purified RSV, which had a titer of approx. 10^8 pfu/ml, respectively. Final concentrations of the adjuvants are summarized in Table 3.6-2.

Again, a homologous prime-boost regimen was used for the vaccination of six female BALB/c mice per group, while control mice were left unvaccinated. All mice were bled for the collection of sera prior to immunization, three weeks after prime and three weeks after boost.

4.2.5.1 Analysis of induced RSV-specific antibodies

Sera were used to determine RSV-binding antibodies as well as these antibodies' avidity and ratio of IgG1 to IgG2a isotypes elicited upon vaccination. Unlike unvaccinated mice, all immunized mice developed RSV-specific antibodies, the titer of which was significantly ($p<0.0001$) increased after the boost in all groups (Figure 4.2-5, A).

The avidity was measured in a modified ELISA including an additional urea wash step (3.5.4.2). In all vaccinated groups, the boost led to a significant increase in avidity ($p<0.0001$) – with the exception of Poly IC:LC, where the avidity was not increased after the boost. Poly IC:LC adjuvanted LEEI-RSV provoked not only significantly lower amounts of RSV-binding antibodies in general (Figure 4.2-5, A), but the antibodies' avidity was also significantly poorer than in groups vaccinated with LEEI-RSV plus Alhydrogel, QuilA or AddaVax (Figure 4.2-5, B). While antibodies of the Alhydrogel, QuilA and AddaVax groups showed avidities of 78%, 71% and 60%, respectively, antibodies of the Poly IC:LC group had a comparably low average avidity of 20% after boost.

The IgG1 and IgG2a specific ELISA (3.5.4.2) showed that all vaccinated groups developed RSV-binding antibodies of both Th2-associated subtype IgG1 and Th1-associated IgG2a. Antibodies elicited in mice vaccinated with LEEI-RSV + Alhydrogel stood out in comparison to all other vaccinated groups, as subclass IgG1 was present to a much higher extent, while less antibodies of subclass IgG2a were measurable, indicating a Th2 bias (Figure 4.2-5, C). This is consistent with previous findings that alum is an adjuvant triggering mainly Th2 immune responses (Brewer et al., 1996).

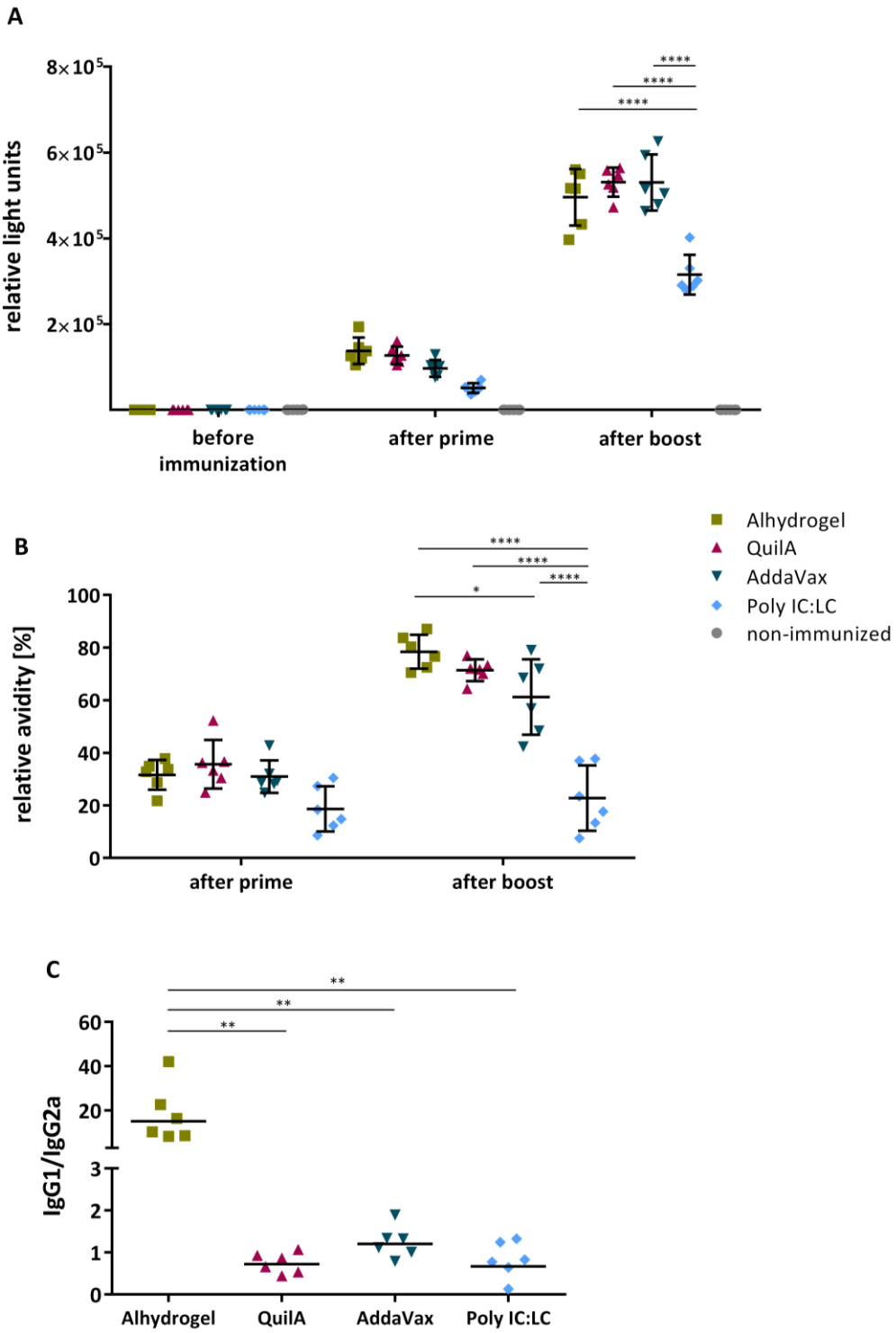


Figure 4.2-5 - Antibody response after vaccination with LEEI-RSV plus different adjuvants

ELISAs were performed to determine RSV binding IgG antibodies (A), avidity (B) and IgG1/IgG2a ratio (C) by coating RSV virions on ELISA plates and probing with sera from vaccinated mice. Avidity was determined by including a 7M urea wash step after incubation with sera. Shown is the relative avidity as percentage of bound antibodies after urea wash (B). Ratio of IgG1 to IgG2 antibodies was determined in serum after boost (C). Statistical analyses were performed using one-way ANOVA and

Tukey's multiple comparison test. ELISAs were repeated in three independent assays; shown is one representative experiment.

4.2.5.2 Neutralizing antibodies and viral load in mice's lungs after RSV-challenge

As main indicators of protection, neutralizing antibodies in the mice's sera were measured, as well as the lung viral load after challenge with RSV.

Prime immunization induced the production of neutralizing antibodies in all groups, with the exception of mice that had been vaccinated with Poly IC:LC adjuvanted LEEI-RSV (Figure 4.2-6, A). In the latter case, neutralizing antibody titers did not differ from non-immunized control animals in a statistically significant way. Boost immunization led to a strong increase in neutralizing antibody titers in all groups, but titers of the Poly IC:LC group were still significantly lower than of all other immunized groups (Figure 4.2-6, B).

Finally, the mice were challenged with infectious RSV four weeks after the last immunization. Five days later, mice were sacrificed and the viral RNA was extracted from their lungs (3.4.5). 45ng of RNA were used for quantitative RT-PCR (3.4.6.2), which contained an average viral copy number of 4.8×10^5 in the control group (Figure 4.2-6, C). All vaccinated groups showed a significant reduction in viral load ($p<0.0001$) with no statistically significant differences between the individual vaccinated groups. Mice vaccinated with LEEI-RSV adjuvanted with QuilA und AddaVax presented the strongest response, as the viral load was reduced approximately 10^4-fold. Since the limit of quantitation lies at 50 due to the standard curve, a viral load of 50 was conservatively assumed for all data points below the limit of quantitation for the calculations of the virus load reduction.

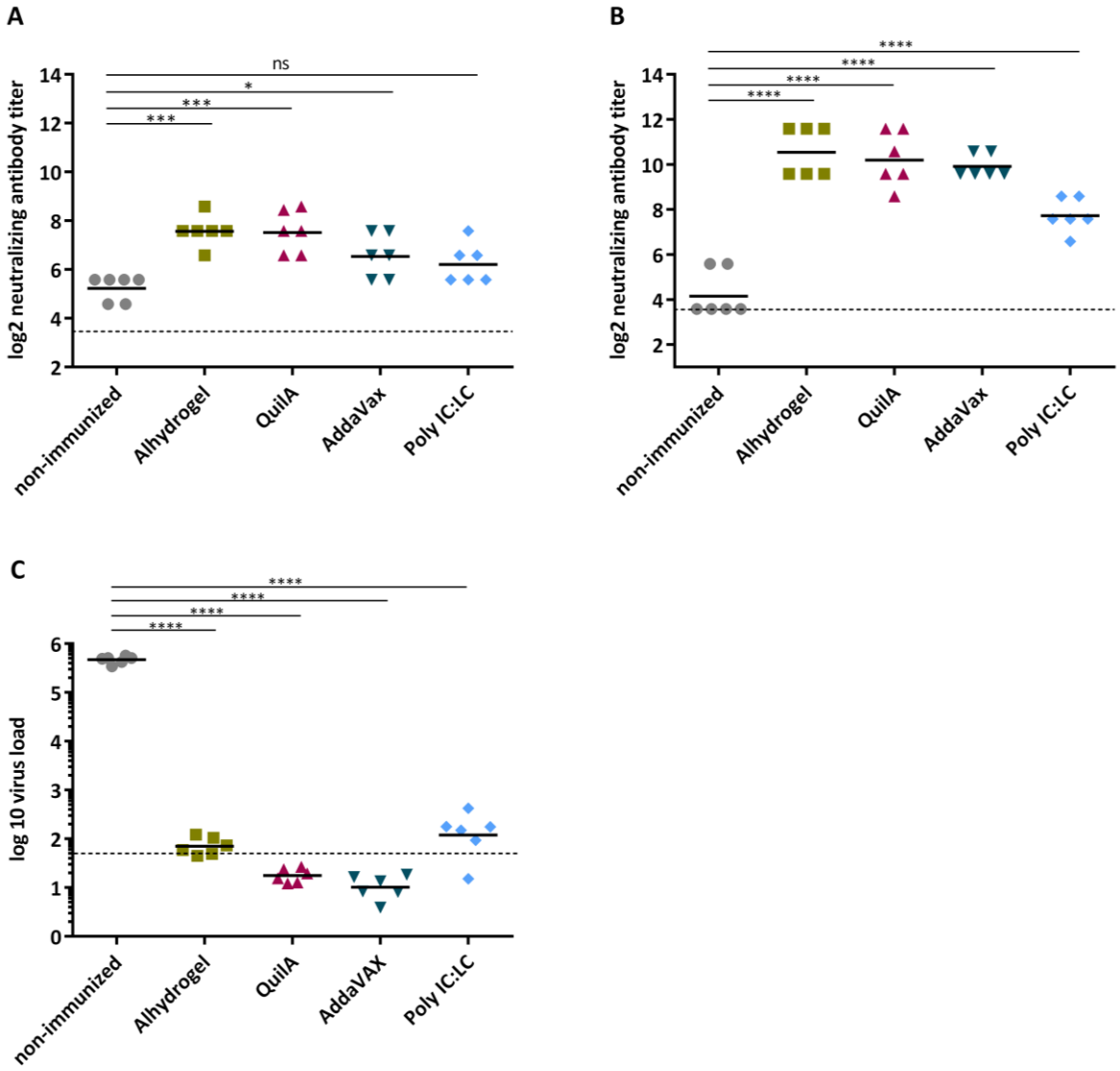
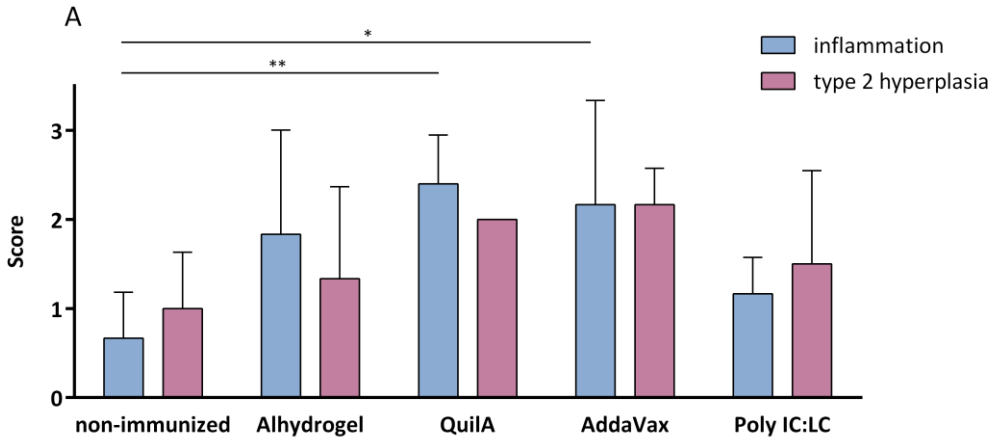


Figure 4.2-6 - Neutralizing antibodies and virus load in lungs after challenge

Neutralizing antibodies were measured in serum after prime (A) and boost (B) by serial dilution of sera and incubation with rgRSV before addition to HEp2 cells. GFP-positive plaque forming units were counted 48h later. Shown are reciprocal values of the serum dilutions at which 50% of pfu were counted in comparison to controls without serum. The dashed line indicates the limit of detection. Neutralization assays were performed in at least three independent experiments; shown is the geometric mean of the calculated neutralizing antibody titer for each mouse. One-way ANOVA and Bonferroni's multiple comparison test was used for statistical analysis. Mice were challenged four weeks after the last immunization and RNA was extracted from lungs three days post infection. Virus load was determined in triplicates by RT-qPCR as copies per 45ng RNA. Statistical analysis was performed using one-way ANOVA and Tukey's multiple comparison test (C).

4.2.5.3 Lung histopathology

Following the devastating 1960's FI-RSV vaccine trial, inactivated RSV vaccine candidates have come under particular scrutiny, especially with respect to their propensity to induce enhanced respiratory disease (ERD). Typical signs of ERD like weight loss or ruffled fur upon challenge (Knudson et al., 2015) were not noted. For a more detailed analysis, one lobe of each mouse lung was formaldehyde-fixed, sectioned, HE-stained and assessed histopathologically for signs of inflammation and type 2 hyperplasia (3.5.5). No significant difference between the individual groups regarding type 2 hyperplasia were observed, but QuilA and AddaVax groups did show enhanced lung inflammation in comparison to non-immunized mice (Figure 4.2-7, A). A correlation between protection and scores does not exist.



B

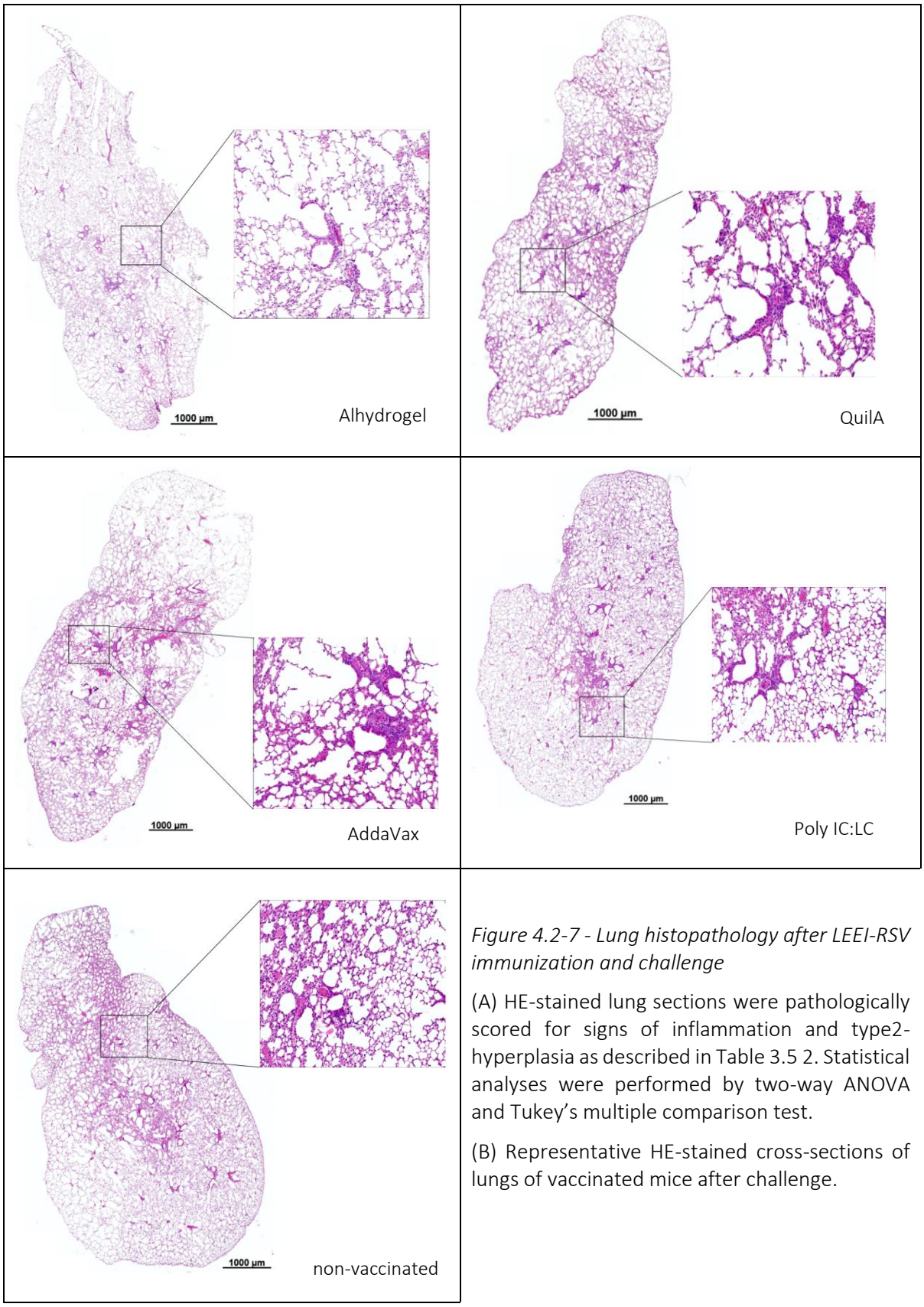


Figure 4.2-7 - Lung histopathology after LEEI-RSV immunization and challenge

(A) HE-stained lung sections were pathologically scored for signs of inflammation and type2-hyperplasia as described in Table 3.5 2. Statistical analyses were performed by two-way ANOVA and Tukey's multiple comparison test.

(B) Representative HE-stained cross-sections of lungs of vaccinated mice after challenge.

4.2.6 Summary and conclusion Part II

The presented data show that LEEI is a suitable method for the reliable inactivation of RSV, keeping viral antigens intact to a large degree. In contrast to chemical inactivation methods, LEEI is a very fast process, only taking seconds. This makes the LEEI-technology especially attractive for the vaccine production. Vaccination of mice with adjuvanted LEEI-RSV resulted in the production of RSV-specific binding and neutralizing antibodies, and conferred protection upon challenge with infectious RSV. The addition of various adjuvants showed that QuilA, AddaVax and Poly IC:LC steered the immune response in a Th1 direction, in contrast to Alhydrogel, which led to a Th2-biased response. Finally, there were no indications that vaccination with LEEI-RSV induces an enhanced respiratory disease upon infection with the virus, although mice that had received AddaVax or QuilA did show slightly elevated signs of lung inflammation.

In summary, LEEI-RSV is a promising procedure for the production of a killed RSV vaccine. Further studies should be performed to gain more insight into the functionality of the vaccine-induced immune response, including an analysis of the cellular immune response.

5 DISCUSSION

The experiments performed within the scope of this dissertation explored novel approaches to vaccine platform technologies that can be applied to a large number of pathogens.

The use of non-human papilloma pseudovirions combines the application of a DNA vaccine with a viral delivery system. To date, genetic vaccines have not been licensed for the use in humans, given that the DNA-delivery technologies are not yet efficient and workable. The existence of a large amount of gene therapy candidates based on recombinant adeno-associated viruses, shows that virus-mediated gene delivery is a technology that is currently actively being pursued.

The identification of suitable papilloma virus candidates through functional tests, analyzing the delivery of a target plasmid *in vitro* and especially *in vivo*, presented the main task. Further, several questions concerning packaging and transduction characteristics were addressed.

While viral delivery of DNA is a rather recent vaccine approach, killed vaccines have been in use for many years. Pathogen inactivation through low-energy electron irradiation (LEEI) is a novel technology that has several advantages over chemical inactivation. The presented studies sought to analyze the immunogenicity and protective efficacy of an LEEI-inactivated virus vaccine in combination with a number of adjuvants.

An RSV vaccine should induce sufficient immunity, protecting against lower respiratory tract infections and apnea, while sterilizing immunity is not necessary and possibly not even feasible (Modjarrad et al., 2016). RSV vaccine candidates in recent preclinical and clinical development include live-attenuated, inactivated whole virus, particle-based, subunit vaccines as well as nucleic acid and gene-based vector approaches (Grunwald et al., 2014; Karron et al., 2015; Kohlmann et al., 2009; McGinnes et al., 2011; Raghunandan et al., 2014; Ternette et al., 2007). Depending on the target group, certain approaches might be more efficient than others. There are four target populations that need to be considered for RSV vaccines: RSV naïve young infants, RSV naïve children (≥6 months of age), pregnant women, and the elderly (Anderson et al., 2013). Live-attenuated RSV vaccines, for instance, are not immunogenic in adults, but are considered for antigen-naïve infants as they are not associated with ERD (Graham, 2016; Higgins et al., 2016; Karron et al., 2013).

Although both approaches presented in this thesis – LEEI-RSV and papilloma PsVs-mediated genetic vaccination – are meant as generally applicable vaccine platform technologies, all vaccination studies were performed using RSV, against which no vaccine is currently available in spite of an urgent need.

5.1 Non-human papilloma pseudovirions for gene transfer in vitro and in vivo

Although genetic vaccines hold great potential, the difficulty to efficiently deliver nucleic acids to the vaccinee has hampered the step of turning genetic vaccines into licensed products. The results of this thesis show that non-human papilloma pseudovirions present a viable means for vector-mediated delivery of DNA-plasmids. The wide range of non-human papilloma virus (PV) types presents a great opportunity to develop a platform containing numerous non-human PV based vectors. L1 and L2 gene sequences are available for a large amount of PV genotypes, making the production of different types of PsVs feasible. At the same time, predicting the suitability of the more than one hundred virus types for this specific application poses a major challenge.

5.1.1 Identification of suitable non-human papilloma virus types

Papillomaviruses exhibit a strict species tropism (Rector et al., 2007; Zur Hausen, 2002), but these restrictions are thought to be caused by tissue-specific cellular enhancers rather than by specific membrane receptors (Müller et al., 1995; Roden et al., 1994; Steinberg et al., 1989). As PVs have been found to enter a wide range of cells (Müller et al., 1995), the tropism should not directly restrict the use of PsVs as gene delivery vehicle.

HPVs can be assigned to two different tropism groups. HPVs infecting the mucosa belong predominantly to the α-PVs, while those HPVs, which infect the skin, include β-PVs as well as further HPV genus (Mistry et al., 2008). Most research in the papilloma virus field has been done with HPV, especially type 16, due to its propensity to cause cervical cancer (Zur Hausen, 2009). Successful transduction with HPV16, as well as with many other α-PVs, has previously been shown *in vitro* and in mice (Cerqueira et al., 2017), suggesting that suitable candidates for gene delivery might be found in this genus. For this reason, five of the ten tested non-human PV types were chosen from the α-papillomaviruses, all of which normally infect non-human primates (CgPV1, PtPV1, MfPV6, MfPV11 and MmPV1, see Table 4.1-1). Interestingly, substantial differences became apparent, as MfPV11 was the only α-PV yielding reproducible, good transduction rates *in vitro* (Figure 4.1-3). PcPV1 PsVs showed comparable transduction rates *in vitro*, but the following *in vivo* experiments painted a different picture. Upon intramuscular injection, Mf11PV PsV-mediated transduction and subsequent reporter expression were weak and not even detectable in all mice of the group. PcPV1 PsVs – which belong to the genus of λ-PVs – on the other hand, sufficiently transduced the reporter plasmid to yield a strong and long lasting expression (Figure 4.1-15, B). These observations lead to the conclusion that *in vitro* transduction efficiency does not allow predictions of the *in vivo* applicability, nor is there a correlation between the PV genus and its *in vivo* (or at least intramuscular) transduction capacity. As slowly evolving viruses (Rector et al., 2007), the latter could be explained since PV

classification occurs exclusively on the genomic level, and does not include serology or mechanistic characteristics. PVs are considered to belong to the same genus if they share 60% - 70% nucleotide identity in L1 (Villiers et al., 2004). The observation that there is substantial variability across PV types in terms of their production of capsids and infectious virions has also been reported by others (Cerqueira and Schiller, 2017). It is therefore currently a matter of trial and error to identify PV types that are suitable as gene delivery vectors.

5.1.2 Transduction characteristics

The experiments included in this thesis did not aim to elucidate the exact entry mechanisms employed by the different animal papillomaviruses, yet dissimilarities in their infection characteristics were indeed observed. PcPV1 and MfPV11 differ in their infection characteristics, as witnessed when ι-carrageenan or empty VLPs were added upon transduction with the PsVs *in vitro*.

In line with previous findings describing ι-carrageenan as a potent inhibitor of papilloma virus infection by preventing virion-to-cell binding (Buck et al., 2006b), transduction with MfPV11 PsVs was obliterated when ι-carrageenan was added to the cell culture medium. In contrast, ι-carrageenan has never been reported to enhance transduction *in vitro*, as observed for PcPV1 PsVs. Studies on the HPV attachment mechanism show that the virus uses negatively charged heparan sulfate proteoglycans (HSPG) as primary attachment factors on epithelial cells (Schiller et al., 2010; Shafti-Keramat et al., 2003), with which the L1 protein interacts. These findings are in agreement with the observation that negatively charged sulfated polysaccharides like heparin or carrageenan (Figure 5.1-1) can block HPV infections (Buck et al., 2006b; Joyce et al., 1999).

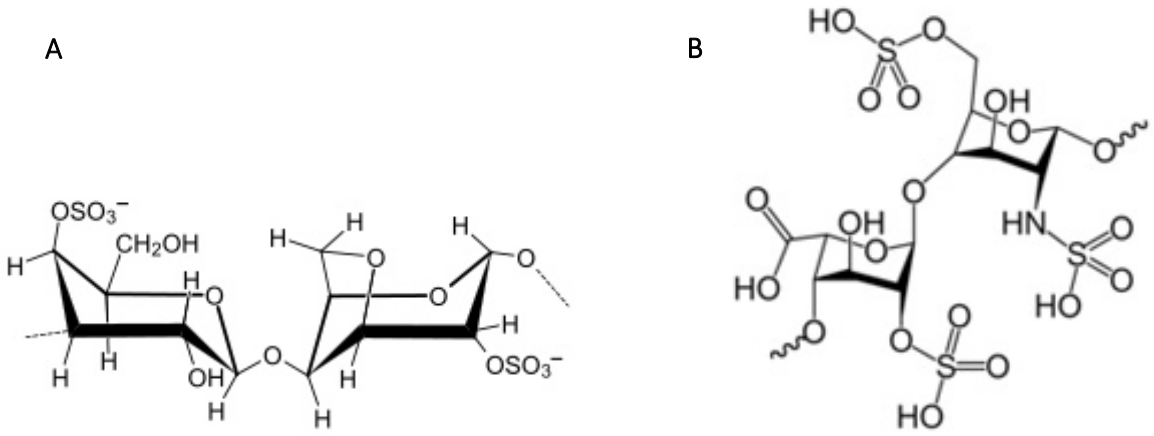


Figure 5.1-1 - ι-carrageenan and heparin

Chemical structures of ι-carrageenan[2] (A) and heparin[3] (B)

Studies analyzing the surface charge of different HPV-types found that the predicted isoelectric point (pI) of the L1 capsid protein correlates with the cutaneous or mucosal tropism. HPV16 (mucosal) has a positive surface charge at physiological pH, while HPV5 (cutaneous) has a negative surface charge at pH 7.4. Uptake of HPV5 was not inhibited by heparin, while uptake of HPV16 was abrogated by heparin (Mistry et al., 2008). These findings suggest that negatively charged large molecules like heparin or ι-carrageenan might interact with the positively charged L1 and compete with its HSPG interaction, thereby blocking the interaction between PsV and cell surface. While this hypothesis explains why heparin (or ι-carrageenan) does not have an effect on negatively charged PsVs (Mistry et al., 2008), it does not allow for an explanation as to why ι-carrageenan may also have enhancing effects on certain PsVs. Especially interesting is the finding that, in contradiction to all other α-PVs which have been reported to be inhibited by ι-carrageenan, transduction of HEK293TT cells with MmPV1 (an α-PV) PsVs was enhanced by the addition of ι-carrageenan *in vitro* (Figure 4.1-7, C). This observation leads to the postulation that the classification of the papilloma virus genus alone is not sufficient to deduce their infection characteristics and their susceptibility to ι-carrageenan-mediated transduction enhancement or inhibition.

Nonetheless, virus charge may indeed play a critical role for the cell-virus-interface. Apart from papillomaviruses, it has been shown for certain adenoviruses that the net charge is important for the virus-cell receptor binding (Arnberg et al., 2002). In fact, HSPGs present central cell surface endocytosis receptors for a large variety of macromolecules and viruses. RSV (Cagno et al., 2014; Krusat and Streckert, 1997), Dengue-2 virus (Lin et al., 2002), varizella zosta virus (Zhu et al., 1995) and Hepatitis B virus (Schulze et al., 2007) have been shown to use HSPGs for

[2] https://biology.stackexchange.com/questions/1611/how-does-iota-carrageenan-achieve-an-antiviral-effect
[3] https://www.heparinscience.com/

cell binding, and infection can be inhibited by heparin. Further examples of viruses, whose *in vitro* infection can be repressed with heparin, are herpes simplex virus (HSV) (Nahmias and Kibrick, 1964) and HIV (Ito et al., 1987). HSV-2 infection in mice was shown to be inhibited by carrageenan (Zacharopoulos and Phillips, 1997). *In vitro* studies using HeLa cells demonstrated that carrageenan was active against HSV-1, HSV-2, Semliki Forest, vaccinia, encephalomyocarditis virus and swine fever viruses. No effect was observed against vesicular stomatitis virus, poliovirus, adenovirus and measles virus (González et al., 1987). Due to the broad *in vitro* activity of ι-carrageenan against respiratory viruses, especially rhinoviruses (Grassauer et al., 2008), it has even been tested as a nose spray to lessen cold symptoms and viral load. In several small clinical trials, subjects receiving ι-carrageenan via nose spray did indeed show a significantly lower viral load and slight reduction of cold symptoms (Eccles et al., 2010; Eccles et al., 2015; Koenighofer et al., 2014; Ludwig et al., 2013).

Interestingly, studies to investigate the mode of action of carrageenan-mediated inhibition of HSV-1 infection in HeLa cells found that it was not the attachment that was prevented (González et al., 1987). Instead, it is speculated that carrageenan and the virus particles are co-internalized in the endosome, and that the polysaccharide might prevent virus replication. These findings, however, do not agree with studies on papillomaviruses and carrageenan, which identify the binding process as the inhibited step (Buck et al., 2006b). As the experiments conducted in the scope of this thesis contain PsVs rather than replication competent virus, ι-carrageenan indeed seems to prevent the binding or entry of HPV16 and MfPV11 into the cells. Therefore, this polysaccharide apparently applies its anti-viral mechanism on different levels. In the case of PcPV1, it is likely the attachment/entry step that is enhanced by ι-carrageenan. Experiments using PcPV1 PsVs with GFP as reporter showed an increased number of transduced (i.e. GFP-positive) cells instead of a higher GFP expression per cell (Figure 4.1-7).

Other examples of viral infection enhancers belong to the group of cationic polymers, such as protamine sulfate and polybrene. The latter is commonly used to improve retroviral gene transfer *in vitro* by increasing the virus' adsorption to cell membranes (Davis et al., 2002). A similar mode of action has been reported for "*Semen-Derived Amyloid Fibrils*" (SEVI) (Münch et al., 2007), acting as a polycationic bridge between HIV and the negatively charged cell surface (Roan et al., 2009). However, this effect was only observable *in vitro*, and SEVI failed to enhance transmission in the mouse (van Dis et al., 2016).

One could speculate that a number of PVs (such as PcPV1) use a different cell entry mechanism than what has been described until now, which is enhanced or stabilized by ι-carrageenan. In line with this hypothesis, the addition of empty VLPs caused a significant reduction of the transduction for PcPV1, but not for MfPV1 PsVs (Figure 4.1-8). PcPV1 may, for instance, use cell surface receptors or structures that are less abundant than HSPGs, for which

PsVs and VLPs would then compete. The difference in cell entry could also lie in the secondary receptor (as of now elusive), to which the capsid binds after the initial interaction with HSPGs. Although controversial, α6-integrin has been suggested as a potential secondary receptor (Horvath et al., 2010). In the attempt to elucidate the cell entry mechanism of papillomaviruses (see Figure 2.6-2 and Figure 2.6-3), it is important to keep in mind that differences have been found between entry *in vitro* and *in vivo.* HPV entry *in vitro* has been shown to involve the interaction with a cell surface receptor, while the basement membrane has been identified as the primary binding site of the virus *in vivo* (Roberts et al., 2007).

5.1.3 DNA-packaging by non-human papilloma PsVs

Although packaging signals have to be present for most viruses to ensure selective encapsidation of the viral genome, no such sequences have been identified for papilloma viruses (Stauffer et al., 1998; Zhou et al., 1994). DNA-binding regions are found in both the L1 and the L2 protein, but this interaction is presumably based on ionic interactions with the DNA's backbone rather than on specific sequences (El Mehdaoui et al., 2000; Li et al., 1997; Zhou et al., 1994). Studies analyzing the plasmid packaging behavior of the bovine papilloma virus type 1 (BPV1) and HPV18 showed that the PsV transduction efficiency negatively correlates with the size of the reporter plasmid. This suggests a sequence-unrelated packaging, only requiring the plasmid size not to exceed the size of the PV genome, which is about 8kb (Buck et al., 2004; Stauffer et al., 1998). It has recently been proposed that papillomaviruses employ a size discrimination mechanism for packaging, in which the capsid undergoes multiple assembly/disassembly cycles in the nucleus, sampling different DNA molecules for packaging. As soon as episomal DNA of an appropriate size has been identified, it is packaged, yielding more stable capsids that do not undergo further assembly/disassembly cycles (Cerqueira et al., 2015).

In light of this data, packaging plasmids of the animal PVs to be analyzed were designed as bicistronic plasmids, coding for both L1 and L2 (Figure 7.1-1 and Figure 7.1-2), thereby exceeding the PV genome size of 8kb by more than 1kb. When these packaging plasmids were, surprisingly, found by qPCR to be packaged, the packaging plasmid was further enlarged by addition of a stuffer sequence, yielding plasmids of more than 10.2kb (Figure 7.1-3 and Figure 7.1-4). Unexpectedly, this did not prevent the plasmids from being packaged. The most likely explanation for these controversial findings seems to be that the qPCR amplifies plasmid DNA that actually sticks to the outside of the capsids rather than being packaged inside. In order to avoid this false positive amplification, the PsV preparation includes an overnight treatment with Benzonase nuclease before purification by ultracentrifugation. In addition, purified PsV preparations were subjected to DNAseI treatment before DNA extraction. A very similar protocol for quantification of HPV-encapsidated DNA has been published, even omitting the DNAseI step

(Çuburu et al., 2015). In this protocol, primers amplifying a 200bp product of the f1 ori are used, therefore detecting any f1 ori-containing plasmid. As the f1 ori is present on most plasmids, this method is not suitable to distinguish between different packaged plasmids. Concerning the experiments of this thesis, unspecific binding of the primers (3.1.4) used to amplify a product in the IRES-sequence of the packaging plasmids has been ruled out experimentally using HEK293TT genomic DNA as template, which showed no amplification. In addition, analysis by PAN-PCR supports the findings that the packaging plasmid does get packaged into the PsVs (4.1.4.1). Next generation sequencing of a large amount of samples would allow a quantitative analysis of the DNA that is encapsidated by papilloma PsVs.

Making use of the efficient incorporation of the packaging plasmid, "combination" bicistronic plasmids were designed, carrying either L1 or L2 and an additional gene of interest for expression. It is thinkable that the plasmid packaging may be coupled to the expression of the viral capsid proteins L1 and L2, leading to an increased encapsidation of plasmids from which L1 or L2 are expressed. This is a rather speculative hypothesis, as co-translational packaging has only been described for lenti- and retroviruses thus far. A comparison of the transduction with papilloma PsVs containing the packaging plasmid plus reporter plasmid versus PsVs containing the combined plasmids did not reveal any differences (Figure 4.1-14), leading to the conclusion that translation and packaging are probably separate events in papillomaviruses.

The "combined plasmid" system brings several safety advantages, however, as every plasmid-containing PsV now delivers the vaccine- or reporter sequence into the cell, ensuring that each successful transduction event leads to the expression of the vaccine or reporter protein. In addition, one PsV alone never contains the genetic information for both L1 and L2, which are required in combination to produce new transduction-competent PsVs. Therefore, the separation of L1 and L2 onto two different plasmids reduces the risk of further mobilization of the vaccine construct in the vaccinee.

In order to enhance the packaging efficiency of the vaccine plasmid and reduce the amount of empty VLPs that are generated as a byproduct, one could employ a systematic screening of the papillomavirus genome for a potential packaging sequence. By successively adding sequences of the papillomavirus genome in 1,000bp increments to the vaccine plasmid, it may be possible to identify putative packaging sequences.

5.1.4 Papilloma PsV mediated DNA delivery in vivo

First functional studies analyzing the PsV-mediated DNA delivery in mice were conducted using a Firefly Luciferase (F.Luc) reporter plasmid (pcLuc13, Figure 7.1-7). Intramuscular injection of PcPV1 and MfPV11 PsVs carrying pcLuc13 resulted in very different efficiencies of F.Luc expression. While F.Luc was clearly detectable in mice injected with PcPV1 PsVs on day 7,

it took 28 days to measure the expression of F.Luc in mice injected with Mf11PV. This late detection leads to the assumption that enough F.Luc has to accumulate until a detectable amount is present. However, it has been shown that there is only little intracellular accumulation of F.Luc (Naylor, 1999), which already has a rather short half-life of approx. 3h (Wu et al., 2001) due to its sensitivity to proteolysis (Pazzagli et al., 1992). Another explanation for the late detection of F.Luc could be the unusually slow entry process employed by papilloma viruses, which includes an extended halt on the cell surface (Horvath et al., 2010). This might lead to a drawn-out process of gradual entry of PsVs into the cells, resulting in a slow but steady increase of transduced cells, which then express F.Luc. In most previously published experiments using PVs as gene vectors, PsVs were applied to the mucosa. Therefore, very little is known about their behavior when injected intramuscularly. Kinetic analyses of F.Luc expression after intravaginal infection with different HPV types showed a peak in expression on day two after administration, and the disappearance of the luciferase signal within seven days (Cerqueira et al., 2017; Graham et al., 2010). Importantly, viral infectivity was found to be highly ineffective as long as the mucosal epithelium was still intact. Only after mechanical, hormonal or chemical disruption of the epithelium, infection was possible. These findings differ substantially from the results obtained in this thesis, where the luciferase signal remained detectable for several weeks, probably due to the intramuscular administration route. Others found a strong luciferase expression until day eight after intramuscular injection of replication-deficient adenovirus (Ad.Hu5) vector expressing firefly luciferase. The same group reported the induction of a CD8+ T-cell mediated immune response against F.Luc. Although not shown in the publication, as mice were sacrificed for spleen cell harvest, one would expect this immune response ultimately to lead to the clearance of all infected and therefore F.Luc-expressing cells (Limberis et al., 2009). Surprisingly, we continued to detect clear luciferase signals even three months after the injection of PcPV1 PsVs. HPV VLPs are able to enter dendritic cells (Horvath et al., 2010), which should therefore lead to cross-presentation and activation of CD8+ T-cells to kill infected, F.Luc-expressing cells. It is feasible, however, that the stimulus exerted by the non-human PV PsVs is not sufficient for a strong engagement of the innate immune response and subsequent adaptive immune reaction. This is in line with the finding that vaccination with PcPV1 and MfPV11 PsVs against RSV led to a rather weak immune response, causing a maximum 29-fold reduction of the viral load after challenge (Table 4.1-4). Considering the low amount of DNA delivered (2.3ng), however, it is remarkable that an immune response can be observed at all. The absence of detectable RSV-neutralizing antibodies and the low levels of RSV-binding antibodies (Figure 4.1-17, A) speak for a T-cell mediated response rather than an antibody-based protection. In order to generate stronger immune responses, it would probably be advisable to add adjuvants in future vaccination studies. Especially nucleic acid-based adjuvants such as Poly IC:LC or CpG DNA, which could possibly be delivered by PsVs, would be of interest.

Interestingly, only two subsequent intramuscular immunizations led to a measurable immune response as observed by the presence of RSV-binding antibodies and a reduction in viral load after the challenge. Heterologous immunization by intramuscular injection followed by intranasal application probably did not deliver sufficient amounts of antigen, leading to the assumption that the PsVs are not efficiently taken up by the lung tissue. Further studies using PsVs with reporters such as F.Luc may shed light on the uptake and expression efficiency in the lung after intransasal application.

An important difference between the PsV preparations used in the F.Luc study and the vaccination study is the ultracentrifugation purification step, which was omitted for the preparation of the pcLuc13-carrying PsVs. Analysis of non-purified PsVs in cell culture is difficult due to the presence of detergents and residual cell debris and proteins. This did not present any problems upon intramuscular injection, but for an intranasal application it would not have been acceptable. Thus, purification is indeed necessary for certain applications. Unfortunately, ultracentrifugation is associated with great titer losses, as has also been experienced by others who recovered only 3% of their HPV16 pseudovirus preparations (Pastrana et al., 2004). The virus' propensity to absorb nonspecifically to polypropylene (Volkin et al., 2002) could be one possible explanation for the observed purification problems. Despite intense efforts to find alternative methods for purification and maybe even for concentration, ultracentrifugation is – as of now – still the most efficient. Ultracentrifugation with Percoll is a viable and much faster alternative for the purification of MfPV11 capsids (Figure 4.1-5, B), but did not yield good results for PcPV1 PsVs. This could possibly be explained as Percoll centrifugation yields only one fraction containing both PsVs with plasmid and empty VLPs, while OptiPrep centrifugation allows for fractionation that separates PsVs from other particles. As observed in other experiments, transduction with PcPV1 PsVs was significantly reduced upon addition of empty VLPs (Figure 4.1-8). These observations imply that purification methods should be tested for each individual PV type.

Concerning the functionality of PsV-mediated gene-delivery, cross-reactivity between antibodies in HPV-vaccinated individuals and animal papilloma-PsVs needs to be ruled out. Several studies, including HPV vaccination trials, have found that neutralizing antibodies induced by HPV VLP vaccination are genotype-restricted, with the exception of very closely related HPV genotypes (Drolet et al., 2015; Schiller and Lowy, 2009). For this reason, more and more papilloma virus types are continuously being added to HPV vaccines, resulting in Merck's "Gardasil 9", a nonavalent vaccine containing VLPs of nine HPV types (Merck, 2015). Consequently, cross-reactive antibodies are not expected. In contrast to PsVs used for gene delivery *in vivo*, VLPs contained in the HPV vaccines are composed only of capsid protein L1. L2, however, which is part of the PsVs, has been reported to elicit antibodies that are more cross-reactive between HPV types (Pereira et al., 2009). As it has been shown that L2 is necessary for

DNA binding, endosome escape as well as DNA-to-nucleus trafficking (Horvath et al., 2010; Kämper et al., 2006; Pereira et al., 2009; Schiller et al., 2010), PsVs composed only of an L1 capsid would likely not be functional *in vivo*. It is therefore important to analyze to which degree PsV-based vaccinations elicit antibodies that are cross-reactive between different animal-PV genotypes.

5.2 Low-energy electron irradiation as a novel method to produce a killed-RSV vaccine

The development of a vaccine to prevent disease caused by an RSV-infection has been difficult ever since a formaldehyde-inactivated RSV vaccine induced enhanced respiratory disease (ERD) in a clinical trial (Higgins et al., 2016). This outcome has been attributed in part to the disruption of key epitopes by formaldehyde during the inactivation process (Murphy and Walsh, 1988; Prince et al., 1986; Widjaja et al., 2016). DNA vaccinations avoid this problem, as the antigen is produced by the cells of the vaccinee, thereby guaranteeing the correct formation of the protein.

Another approach to ensure the presence of important antigens on the surface of RSV in a vaccine preparation is the use of inactivation methods, which allow the conservation of proteins in their native structures. Results obtained in the course of this thesis show that low-energy electron irradiation (LEEI) presents a promising method to inactivate the virus while reproducibly keeping viral antigens intact to a large degree. In contrast, using formaldehyde for chemical inactivation of RSV showed high inter-preparation variance in the virus antigen conservation (Figure 4.2-3), which might explain why FI-RSV vaccine studies often differ in outcome (Acosta et al., 2015; Castilow et al., 2007; Delgado et al., 2009; Knudson et al., 2015). The reproducibility concerning both inactivation of RSV at 20kGy and virus antigen conservation make LEEI an attractive method for the production of killed RSV vaccines.

For killed vaccine preparations such as the LEEI-RSV vaccine, adjuvants are crucial components. T-cells require three stimulatory signals to become activated: the antigen presented by an APC, engagement of CD28 on the T-cell surface and lastly cytokines. The presence of an inflammatory environment, signaled by the presence of cytokines, is especially important as antigen presentation in a non-inflammatory setting leads to anergy. Killed viral vaccines often lack sufficient DAMPs to activate the innate immune response to a degree which provides strong activating signals. This role is then adopted by adjuvants. The finding that vaccination with RSV inactivation by UV irradiation can, similar to formaldehyde, lead to ERD unless TLR agonists are added (Delgado et al., 2009), highlights the significance of additional adjuvants for a more pronounced stimulation of the immune system. In the presented LEEI-RSV vaccination study, only adjuvanted vaccine preparations were used. A comparison between Alhydrogel, AddaVax, QuilA and Poly IC:LC showed that in all cases the viral load was reduced approx. 10^4-fold upon RSV-challenge. Analyses of the elicited antibodies, however, did reveal differences between the immunized groups. Poly IC:LC adjuvanted LEEI-RSV yielded the lowest antibody titers over all, including neutralizing antibodies, as well as the lowest avidity. Although Poly IC:LC was used at a concentration of 0.5mg/kg, which is well within the range of concentrations that are commonly used (between 0.1 and 1mg/kg; Wong et al., 2005; Stahl-Hennig et al., 2009), one could possibly have achieved a stronger effect at a higher

concentration. Studies show that the adjuvant effect of Poly IC:LC is dose-dependent (Stahl-Hennig et al., 2009). Especially interesting is the finding that despite the comparably small amount of antibodies with low avidity in mice vaccinated with LEEI-RSV plus Poly IC:LC, the animals still showed a strong reduction in viral load after the challenge.

This leads to further questions concerning the role of the elicited antibodies for the protection against RSV. Antibodies can confer protection not only through neutralization and high avidity, but they may also have effector functions such as antibody-dependent cell-mediated cytotoxicity or opsonophagocytic capacity (Pulendran and Ahmed, 2011). In some cases, antibody titers and neutralization can be poor predictors of protective immunity. A rather new approach termed “systems serology” analyzes the vaccine-induced humoral immunity using a large panel of parameters. The polyclonal antibodies elicited by vaccination are profiled by examining Fc-effector functions, antibody-dependent natural killer activities, as well as biophysical properties such as subclass identification (Ackerman et al., 2017). This approach was used to compare the outcome of the HIV-vaccine trials with VAX003 (non-protective) and RV144 (partially protective), and found that the specific antibody profiles can be used to predict protection (Chung et al., 2015). Similarly, profiles of antibodies elicited by RSV vaccination have been reported to show correlations between protected and non-protected individuals (Alter, 2017).

Using the example of the yellow fever vaccine, a highly effective and successful vaccine, antigen-specific CD8+ T-cell responses and neutralization were predicted using a “systems biology” approach, generating gene expression and cytokine profiles from the vaccinees’ sera (Querec et al. 2009). Using these parameters to predict protection early after vaccination can be especially of interest for diseases where an effective vaccine is lacking – such as RSV.

The good protection against RSV found in LEEI-RSV + PolyIC:LC vaccinated mice, despite the comparably lower antibody response, could be due to parameters that were not analyzed in this study. Especially the involvement of the cellular response should be elucidated. It is also possible that the induced immune response was still strong enough to protect from infection with the chosen RSV-titer.

Analysis of the elicited antibody isotypes exposed further dissimilarities in the immune responses, which were induced by the four tested adjuvants. In contrast to all other groups, Alhydrogel-adjuvanted LEEI-RSV rarely elicited any IgG2a antibodies, but large amounts of IgG1 antibodies, indicating a strong Th2-skewed immune response. In all other groups (AddaVax, QuilA and Poly IC:LC), we found similar IgG1/IgG2a ratios (between 1 and 2), showing almost equal amounts of IgG1 and IgG2a antibodies. Interestingly, the elicited antibody isotype does not seem to influence the protection from the RSV challenge, which is not statistically different among the four vaccination groups.

Recent detailed analyses of the RSV F-protein conformation have emphasized that the prefusion/postfusion conformation of the F-protein in a vaccine preparation may play an important role for the induced immune response.

During the intracellular virus maturation process, furin cleaves the type I fusion F-protein, producing two disulfide-like fragments, F1 and F2. As the virus enters a cell, F1 and F2 rearrange from a prefusion trimer conformation to a postfusion trimer conformation (Figure 5.2-1). McLellan et al. characterized the structure of the RSV-F glycoprotein and identified binding sites in prefusion-F, to which potent neutralizing antibodies bind (McLellan et al., 2013b). Studies comparing the vaccination with postfusion F-protein to prefusion F-protein found a better immune response and protective efficacy when prefusion-F was used for the immunization (Liang et al., 2015; McLellan et al., 2013a).

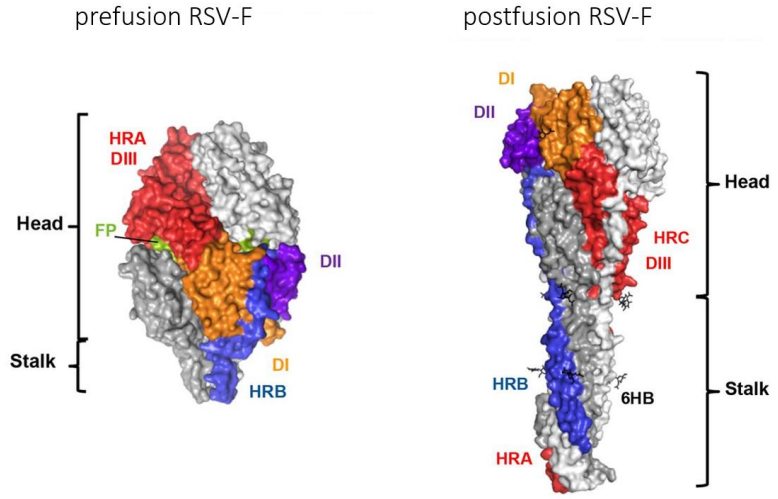


Figure 5.2-1 - Prefusion and postfusion conformation of RSV-F
(from Swanson et al., 2014, modified)

Another study using VLP-based RSV vaccines with either prefusion-F or postfusion-F or a combination of both conformations on the VLP surface found that the highest titers of neutralizing antibodies as well as an enhanced Th1-mediated immune response were induced when both conformations of F were present (Cimica et al., 2016). Interestingly, the prefusion-F conformation was found to be absent on the surface of formaldehyde-inactivated RSV, as the chemical transforms the prefusion-F dominant fresh RSV into the postfusion-F dominant FI-RSV (Killikelly et al., 2016). It would therefore be very interesting to analyze LEEI-inactivated RSV for the presence of pre- and postfusion-F on the viral surface. Natural infection with RSV elicits antibodies against a number of epitopes that are present on both the prefusion and postfusion conformation, all of which contribute to the overall protection (Widjaja et al., 2016).

We hypothesize that immunization with a genetic vaccine coding for RSV-F allows the expression of the prefusion conformation, as well as the conversion to the postfusion conformation. This would consequently permit the elicitation of antibodies against pre- and postfusion-F as well as possibly other intermediate conformations, leading to a broad spectrum of antibodies.

5.3 Vaccine safety and risk evaluation

While protective efficacy is an obvious goal when developing new vaccines, the issue of safety is paramount, especially given the history of RSV vaccine development. As all adult vaccine recipients will have been primed by live virus infections, enhanced respiratory disease (ERD) upon infection after vaccination is a concern only in RSV-naïve infants. At the same time, infants are the most important target group, on which many vaccines in development focus (Anderson et al., 2013). The RSV-naïve BALB/c mice, which were used for the vaccination studies in this thesis, therefore present a model for vaccine recipients who encounter RSV antigens for the first time by means of immunization. In none of the conducted vaccination studies – including mice that were immunized with FI-RSV – were signs of ERD observed, such as weight loss or ruffled fur (Knudson et al., 2015). The induction of ERD by FI-RSV in mice seems to be reproducible in some labs (Knudson et al., 2015; Moghaddam et al., 2006), while others fail to see this phenomenon (e.g. Li et al., 1998; Ternette et al., 2007), and it is not clear why this is the case. It is imaginable that the RSV preparations that are propagated and used over years in the various laboratories may acquire mutations, which alter certain characteristic of the virus.

Mice' lungs in the LEEI-RSV vaccination study II, as well as in the papilloma PsV vaccination study, were examined by a pathologist for a more detailed analysis of a potentially induced inflammation. QuilA and AddaVax groups (in LEEI-RSV vaccination study II) did show signs of inflammation that were significantly more pronounced than in non-vaccinated mice. However, not all features of a typical ERD were fulfilled, such as low neutralizing antibodies titers or low avidity (Delgado et al., 2009; Kim et al., 1969; Polack et al., 2002). Most importantly, while cases of ERD usually show a high viral load, all of the vaccinated mice presented significantly lower virus loads compared to non-immunized control mice. Analyzing the correlation between viral load and scores for inflammation and type 2 hyperplasia in the lung showed that neither high nor low scores correlated with a high RSV load in both vaccination studies.

Attention has been drawn especially to the naïve CD4+ T helper cell differentiation induced by RSV vaccines, as a strong Th2 response has been linked to ERD (Waris et al., 1996). It has been shown that depletion of IL-4 and IL-10 – two important Th2-associated interleukins – prevented the FI-RSV-induced enhanced pulmonary histopathology in mice (Connors et al., 1994). The differentiation into Th1 or Th2 cells is determined by the antigen that is presented by APCs, which subsequently secrete the corresponding cytokines (Murphy, K., Travers, P., Walport, M., & Janeway, C., 2011). Antibody isotypes reflect the CD4+ T-cell differentiation. A Th1 immune response is characterized by the presence of mainly IgG2a antibodies, while IgG1 antibodies dominate in a Th2 setting in the mouse. Isotype analysis of the antibodies elicited by LEEI-RSV underline the importance of a well-chosen adjuvant in a vaccine preparation. A balanced Th1/Th2 response was stimulated by all adjuvants (QuilA, AddaVax, Poly IC:LC) but Alhyodrogel. Non-vaccinated mice subjected to secondary RSV infections similarly produce both

IgG1 and IgG2a antibodies (Lindell et al., 2011). A more clearly Th1-skewed immune response would have been expected for Poly IC:LC, as double stranded RNA presents a strong signal for a viral infection. Mice vaccinated with Poly IC:LC-adjuvanted LEEI-RSV, however, showed a similar antibody isotype profile as mice immunized with AddaVax- or QuilA-adjuvanted LEEI-RSV. In contrast, Alhydrogel evidently triggered a distinct Th2-directed immune response. Previously it has been reported that vaccination with FI-RSV induces a Th2 bias, leading to the production of IL-4 and IL-5, which in turn cause a pronounced pulmonary eosinophilic response (Openshaw et al., 2001). Since "Lot100" was produced by formaldehyde inactivation, followed by precipitation of the virus with aluminum hydroxide (Kim et al., 1969), it can be speculated that not only deformed antigens, but also the choice of adjuvant could in part be responsible for the failure of this vaccine (Mullard, 2008). Although an unbalanced Th2 response is thought to cause an undesirable immune response to an RSV vaccine, some studies suggest that an unbalanced Th1 response might similarly pose a risk, leading to augmented pulmonary pathology (Alwan, 1994; Cannon, 1988).

Unrelated to vaccine-induced ERD, the most severe adverse effect observed in any of the vaccination studies were lung inflammations in mice immunized intranasally with MfPV11 in Percoll, which ultimately led to their demise. Percoll is described as "completely non-toxic" by the manufacturer, and a study, in which Percoll was injected into ovaries and uterine horns of rabbits, showed no inflammatory cell infiltration after four weeks (Arora et al., 1994). However, since Percoll apparently has not been applied intranasally before, it is feasible that this substance should not be administered to the lung. It would also be thinkable that the MfPV11 PsVs mediated the observed lung-inflammation. Further studies will have to evaluate the *in vivo* applicability of Percoll and MfPV11 PsVs separately, especially addressing the late reaction that was observed three weeks after administration.

One major concern with viral vector systems is the unspecific packaging of nucleic acids from the producer cell lines inside the viral particles. Especially in the absence of clear packaging signals, which are currently unknown for papilloma viruses, it is difficult to ensure with certainty the production of PsVs that contain nothing but the desired plasmid. Protocols to avoid the packaging of chromosomal DNA inside papilloma PsVs have been developed that omit the DNA-digestion step with Benzonase during the PsV-harvest from the producer cells (Buck and Thompson, 2007). That way, chromosomal DNA is believed to remain intact – and therefore too large – to be packaged into the PsV particles. An even safer way to address this issue is the production in cell-free systems, which is even thought to comply with good manufacturing practice (GMP) (Cerqueira et al., 2017).

5.4 Conclusion and outlook

More than fifty years of RSV vaccine research have not led to a licensed product, spurring increased efforts and novel approaches. RSV is an especially threatening pathogen for immunologically compromised populations, i.e. neonates and the elderly. For vaccine development, the challenge is to strike a balance between sufficient attenuation and immunogenicity.

One must keep in mind that all findings were premised on models: the RSV laboratory strain and mice as vaccination subjects. RSV clinical isolates have been shown to differ markedly from RSV/A2 with respect to viral growth kinetics, cytopathogenicity and pro-inflammatory responses induced upon infection of primary pediatric bronchial epithelial cells (Villenave et al., 2011). Conducting further vaccination studies with various RSV strains may paint a more complete picture of the necessary features to produce an efficient and protective vaccine.

Two very distinct approaches for vaccine development were analyzed in this dissertation. LEEI has proven to be a viable alternative to chemical inactivation of RSV and potentially many more pathogens, due to its fast and reproducible mode of action and antigen conservation. Mice vaccinated with adjuvanted LEEI-RSV showed a great reduction in the viral load after challenge, presenting the protection conveyed by the vaccine. The biggest challenge for the implementation of LEEI for the vaccine production at a larger scale is the low penetration depth of low-energy electrons, and the requirement to prepare the pathogen solution as a liquid film of less than 1mm. Devices to fulfill this prerequisite are currently being constructed at the *Fraunhofer Institute for Manufacturing Engineering and Automation*, Stuttgart.

The second approach for vaccine production addressed in this dissertation has several advantages over the classical killed vaccine. Using non-human papilloma PsVs to deliver a genetic vaccine avoids live virus handling and the check for inactivation, which is performed for a killed vaccine. Furthermore, antigens can be easily adapted by changing the DNA sequence. Since the antigen is expressed by the vaccinees themselves, correct folding and protein-modifications are always ensured. Out of a pool of ten different animal papillomaviruses, PcPV1 PsVs were found to be the most promising for *in vitro* and *in vivo* delivery. While PVs do have the potential to also infect the mucosa, each PV type has to be tested *in vivo* for its capability to enter the cells of particular tissues. A future expansion of this viral platform could be drastically accelerated if correlates between *in vitro* measurable characteristics and *in vivo* transduction efficiency were identified.

The only moderate reduction of virus load, as well as the small amount of RSV-specific antibodies after PV PsV-mediated delivery of an RSV-F encoding plasmid, can probably be attributed to the low PV PsV titers and subsequent low expression of RSV-F. Currently, attempts are being undertaken to produce PsVs in insect cells with the goal of yielding higher titers. Further plans to develop the next generation of PV PsVs include the use of inducible and

repressable promoters controlling the expression of capsid proteins and vaccine antigens. The goal is to limit L1 and L2 expression to the PsV production step *in vitro*, while the vaccine antigen will exclusively be expressed *in vivo* after application of the PsVs. This approach would also increase the vaccine's safety, as PsV replication in the vaccinee would not be possible.

In summary, the presented data show that LEEI has the potential to become a viable alternative to chemical inactivation for vaccine production and might even deliver a new RSV vaccine candidate. The various adjuvants applied in the LEEI-RSV experiment have shown that they have the ability to influence the direction of the immune response. It is therefore encouraging to see the portfolio of licensed adjuvants expand. A killed vaccine like LEEI-RSV would probably be suitable for the elderly and for maternal vaccination. While gene-based vectors may be suitable for all population groups, they appear especially appropriate in the pediatric setting (Anderson et al., 2013; Higgins et al., 2016). DNA-based vaccines are anticipated to minimize the risk of ERD in RSV-naïve children, associated with the MHC class II presentation pathway (Higgins et al., 2016).

The abundance of existing animal papillomaviruses presents a huge field for further exploration of this viral DNA delivery system. A combination of the LEEI-RSV vaccine with a PV PsV-mediated genetic vaccination could elicit an even broader immune response.

6 REFERENCES

Abban, C.Y., Bradbury, N.A., Meneses, P.I., 2008. HPV16 and BPV1 infection can be blocked by the dynamin inhibitor dynasore. American journal of therapeutics 15 (4), 304–311. 10.1097/MJT.0b013e3181754134.

Ackerman, M.E., Barouch, D.H., Alter, G., 2017. Systems serology for evaluation of HIV vaccine trials. Immunological reviews 275 (1), 262–270. 10.1111/imr.12503.

Acosta, P.L., Caballero, M.T., Polack, F.P., 2015. Brief History and Characterization of Enhanced Respiratory Syncytial Virus Disease. Clinical and vaccine immunology : CVI 23 (3), 189–195. 10.1128/CVI.00609-15.

Akira, S., Uematsu, S., Takeuchi, O., 2006. Pathogen recognition and innate immunity. Cell 124 (4), 783–801. 10.1016/j.cell.2006.02.015.

Alter, G., 2017. Dissecting correlates of vaccine mediated protection using systems serology, 20 September 2017, San Diego, CA, USA.

Alwan, W.H., 1994. Distinct types of lung disease caused by functional subsets of antiviral T cells. Journal of Experimental Medicine 179 (1), 81–89. 10.1084/jem.179.1.81.

Anderson, L.J., Dormitzer, P.R., Nokes, D.J., Rappuoli, R., Roca, A., Graham, B.S., 2013. Strategic priorities for respiratory syncytial virus (RSV) vaccine development. Vaccine 31 Suppl 2, B209-15. 10.1016/j.vaccine.2012.11.106.

Andrejeva, J., Childs, K.S., Young, D.F., Carlos, T.S., Stock, N., Goodbourn, S., Randall, R.E., 2004. The V proteins of paramyxoviruses bind the IFN-inducible RNA helicase, mda-5, and inhibit its activation of the IFN-beta promoter. Proceedings of the National Academy of Sciences of the United States of America 101 (49), 17264–17269. 10.1073/pnas.0407639101.

Arnberg, N., Kidd, A.H., Edlund, K., Nilsson, J., Pring-Akerblom, P., Wadell, G., 2002. Adenovirus type 37 binds to cell surface sialic acid through a charge-dependent interaction. Virology 302 (1), 33–43.

Arora, M., Carver-Ward, J.A., Jaroudi, K.A., Sieck, U.V., 1994. Is Percoll safe for in vivo use? Fertility and Sterility 61 (5), 979–981. 10.1016/S0015-0282(16)56718-1.

Aucouturier, J., Dupuis, L., Deville, S., Ascarateil, S., Ganne, V., 2002. Montanide ISA 720 and 51: a new generation of water in oil emulsions as adjuvants for human vaccines. Expert review of vaccines 1 (1), 111–118. 10.1586/14760584.1.1.111.

Bächi, T., Howe, C., 1973. Morphogenesis and Ultrastructure of Respiratory Syncytial Virus. Journal of virology 12 (5), 1173–1180.

Banzhoff, A., Gasparini, R., Laghi-Pasini, F., Staniscia, T., Durando, P., Montomoli, E., Capecchi, P.L., Capecchi, P., Di Giovanni, P., Sticchi, L., Gentile, C., Hilbert, A., Brauer, V., Tilman, S., Podda, A., 2009. MF59-adjuvanted H5N1 vaccine induces immunologic memory and heterotypic antibody responses in non-elderly and elderly adults. PloS one 4 (2), e4384. 10.1371/journal.pone.0004384.

Bode, C., Zhao, G., Steinhagen, F., Kinjo, T., Klinman, D.M., 2011. CpG DNA as a vaccine adjuvant. Expert review of vaccines 10 (4), 499–511. 10.1586/erv.10.174.

Bonanni, P., Santos, J.I., 2011. Vaccine evolution. Perspectives in Vaccinology 1 (1), 1–24. 10.1016/j.pervac.2011.05.001.

Borchers, A.T., Chang, C., Gershwin, M.E., Gershwin, L.J., 2013. Respiratory Syncytial Virus—A Comprehensive Review. Clinical Reviews in Allergy & Immunology 45 (3), 331–379. 10.1007/s12016-013-8368-9.

Boudaïffa, B., Cloutier, P., Hunting, D., Huels, M.A., Sanche, L., 2000. Resonant formation of DNA strand breaks by low-energy (3 to 20 eV) electrons. Science (New York, N.Y.) 287 (5458), 1658–1660.

Bousarghin, L., Touze, A., Sizaret, P.-Y., Coursaget, P., 2003. Human Papillomavirus Types 16, 31, and 58 Use Different Endocytosis Pathways To Enter Cells. Journal of virology 77 (6), 3846–3850. 10.1128/JVI.77.6.3846-3850.2003.

Bråve, A., Ljungberg, K., Wahren, B., Liu, M.A., 2007. Vaccine delivery methods using viral vectors. Molecular pharmaceutics 4 (1), 18–32. 10.1021/mp060098+.

Breman, J.G., Arita, I., Unit, S.E., World Health Organization, 1980. The confirmation and maintenance of smallpox eradication.

Brewer, J.M., Conacher, M., Satoskar, A., Bluethmann, H., Alexander, J., 1996. In interleukin-4-deficient mice, alum not only generates T helper 1 responses equivalent to freund's complete adjuvant, but continues to induce T helper 2 cytokine production. European journal of immunology 26 (9), 2062–2066. 10.1002/eji.1830260915.

Buck, C., Pastrana, D., Lowy, D., Schiller, J., 2006a. Generation of HPV Pseudovirions Using Transfection and Their Use in Neutralization Assays, in: Davy, C., Doorbar, J. (Eds.), Human Papillomaviruses, vol. 119. Humana Press, pp. 445–462.

Buck, C.B., Pastrana, D.V., Lowy, D.R., Schiller, J.T., 2004. Efficient Intracellular Assembly of Papillomaviral Vectors. Journal of virology 78 (2), 751–757. 10.1128/JVI.78.2.751-757.2004.

Buck, C.B., Thompson, C.D., 2007. Production of papillomavirus-based gene transfer vectors. Current protocols in cell biology Chapter 26, Unit 26.1. 10.1002/0471143030.cb2601s37.

Buck, C.B., Thompson, C.D., Roberts, J.N., Müller, M., Lowy, D.R., Schiller, J.T., 2006b. Carrageenan is a potent inhibitor of papillomavirus infection. PLoS pathogens 2 (7), e69. 10.1371/journal.ppat.0020069.

Cagno, V., Donalisio, M., Civra, A., Volante, M., Veccelli, E., Oreste, P., Rusnati, M., Lembo, D., 2014. Highly sulfated K5 Escherichia coli polysaccharide derivatives inhibit respiratory syncytial virus infectivity in cell lines and human tracheal-bronchial histocultures. Antimicrobial Agents and Chemotherapy 58 (8), 4782–4794. 10.1128/AAC.02594-14.

Cannon, M.J., 1988. Cytotoxic T cells clear virus but augment lung pathology in mice infected with respiratory syncytial virus. Journal of Experimental Medicine 168 (3), 1163–1168. 10.1084/jem.168.3.1163.

References

Caputo, A., Gavioli, R., Altavilla, G., Brocca-Cofano, E., Boarini, C., Betti, M., Castaldello, A., Lorenzini, F., Micheletti, F., Cafaro, A., Sparnacci, K., Laus, M., Tondelli, L., Ensoli, B., 2003. Immunization with low doses of HIV-1 tat DNA delivered by novel cationic block copolymers induces CTL responses against Tat. Vaccine 21 (11-12), 1103–1111. 10.1016/S0264-410X(02)00555-8.

Cardone, G., Moyer, A.L., Cheng, N., Thompson, C.D., Dvoretzky, I., Lowy, D.R., Schiller, J.T., Steven, A.C., Buck, C.B., Trus, B.L., 2014. Maturation of the human papillomavirus 16 capsid. mBio 5 (4), e01104-14. 10.1128/mBio.01104-14.

Castilow, E.M., Olson, M.R., Varga, S.M., 2007. Understanding respiratory syncytial virus (RSV) vaccine-enhanced disease. Immunologic Research 39 (1), 225–239. 10.1007/s12026-007-0071-6.

Cerqueira, C., Pang, Y.-Y.S., Day, P.M., Thompson, C.D., Buck, C.B., Lowy, D.R., Schiller, J.T., 2015. A Cell-Free Assembly System for Generating Infectious Human Papillomavirus 16 Capsids Implicates a Size Discrimination Mechanism for Preferential Viral Genome Packaging. Journal of virology 90 (2), 1096–1107. 10.1128/JVI.02497-15.

Cerqueira, C., Schiller, J.T., 2017. Papillomavirus assembly: An overview and perspectives. Virus research 231, 103–107. 10.1016/j.virusres.2016.11.010.

Cerqueira, C., Thompson, C.D., Day, P.M., Pang, Y.-Y.S., Lowy, D.R., Schiller, J.T., 2017. Efficient Production of Papillomavirus Gene Delivery Vectors in Defined In Vitro Reactions. Molecular therapy. Methods & clinical development 5, 165–179. 10.1016/j.omtm.2017.04.005.

CHANOCK, R., FINBERG, L., 1957. Recovery from infants with respiratory illness of a virus related to chimpanzee coryza agent (CCA). II. Epidemiologic aspects of infection in infants and young children. American journal of hygiene 66 (3), 291–300.

CHANOCK, R., ROIZMAN, B., MYERS, R., 1957. Recovery from infants with respiratory illness of a virus related to chimpanzee coryza agent (CCA). I. Isolation, properties and characterization. American journal of hygiene 66 (3), 281–290.

Chung, A.W., Kumar, M.P., Arnold, K.B., Yu, W.H., Schoen, M.K., Dunphy, L.J., Suscovich, T.J., Frahm, N., Linde, C., Mahan, A.E., Hoffner, M., Streeck, H., Ackerman, M.E., McElrath, M.J., Schuitemaker, H., Pau, M.G., Baden, L.R., Kim, J.H., Michael, N.L., Barouch, D.H., Lauffenburger, D.A., Alter, G., 2015. Dissecting Polyclonal Vaccine-Induced Humoral Immunity against HIV Using Systems Serology. Cell 163 (4), 988–998. 10.1016/j.cell.2015.10.027.

Cimica, V., Boigard, H., Bhatia, B., Fallon, J.T., Alimova, A., Gottlieb, P., Galarza, J.M., 2016. Novel Respiratory Syncytial Virus-Like Particle Vaccine Composed of the Postfusion and Prefusion Conformations of the F Glycoprotein. Clinical and vaccine immunology : CVI 23 (6), 451–459. 10.1128/CVI.00720-15.

ClinicalTrials.gov, 2018. A Study to Determine the Safety and Efficacy of the RSV F Vaccine to Protect Infants Via Maternal Immunization: NCT02624947. https://clinicaltrials.gov/ct2/show/NCT02624947. Accessed 25 April 2018.

Connors, M., Giese, N.A., Kulkarni, A.B., Firestone, C.Y., Morse, H.C., Murphy, B.R., 1994. Enhanced pulmonary histopathology induced by respiratory syncytial virus (RSV) challenge of formalin-inactivated RSV-immunized BALB/c mice is abrogated by depletion of interleukin-4 (IL-4) and IL-10. Journal of virology 68 (8), 5321–5325.

Çuburu, N., Cerio, R.J., Thompson, C.D., Day, P.M., 2015. Mouse model of cervicovaginal papillomavirus infection. Methods in molecular biology (Clifton, N.J.) 1249, 365–379. 10.1007/978-1-4939-2013-6_27.

Davis, H.E., Morgan, J.R., Yarmush, M.L., 2002. Polybrene increases retrovirus gene transfer efficiency by enhancing receptor-independent virus adsorption on target cell membranes. Biophysical Chemistry 97 (2-3), 159–172. 10.1016/S0301-4622(02)00057-1.

Day, P.M., Lowy, D.R., Schiller, J.T., 2003. Papillomaviruses infect cells via a clathrin-dependent pathway. Virology 307 (1), 1–11.

Day, P.M., Lowy, D.R., Schiller, J.T., 2008. Heparan sulfate-independent cell binding and infection with furin-precleaved papillomavirus capsids. Journal of virology 82 (24), 12565–12568. 10.1128/JVI.01631-08.

Delgado, M.F., Coviello, S., Monsalvo, A.C., Melendi, G.A., Hernandez, J.Z., Batalle, J.P., Diaz, L., Trento, A., Chang, H.-Y., Mitzner, W., Ravetch, J., Melero, J.A., Irusta, P.M., Polack, F.P., 2009. Lack of antibody affinity maturation due to poor Toll-like receptor stimulation leads to enhanced respiratory syncytial virus disease. Nature medicine 15 (1), 34–41. 10.1038/nm.1894.

Delrue, I., Delputte, P.L., Nauwynck, H.J., 2009. Assessing the functionality of viral entry-associated domains of porcine reproductive and respiratory syndrome virus during inactivation procedures, a potential tool to optimize inactivated vaccines. Veterinary research 40 (6), 62. 10.1051/vetres/2009047.

Dey, A.K., Srivastava, I.K., 2011. Novel adjuvants and delivery systems for enhancing immune responses induced by immunogens. Expert review of vaccines 10 (2), 227–251. 10.1586/erv.10.142.

Didierlaurent, A.M., Morel, S., Lockman, L., Giannini, S.L., Bisteau, M., Carlsen, H., Kielland, A., Vosters, O., Vanderheyde, N., Schiavetti, F., Larocque, D., van Mechelen, M., Garçon, N., 2009. AS04, an aluminum salt- and TLR4 agonist-based adjuvant system, induces a transient localized innate immune response leading to enhanced adaptive immunity. Journal of immunology (Baltimore, Md. : 1950) 183 (10), 6186–6197. 10.4049/jimmunol.0901474.

Dirks, W., Wirth, M., Hauser, H., 1993. Dicistronic transcription units for gene expression in mammalian cells. Gene 128 (2), 247–249. 10.1016/0378-1119(93)90569-O.

Doorbar, J., Quint, W., Banks, L., Bravo, I.G., Stoler, M., Broker, T.R., Stanley, M.A., 2012. The biology and life-cycle of human papillomaviruses. Vaccine 30 Suppl 5, F55-70. 10.1016/j.vaccine.2012.06.083.

Drolet, M., Bénard, É., Boily, M.-C., Ali, H., Baandrup, L., Bauer, H., Beddows, S., Brisson, J., Brotherton, J.M.L., Cummings, T., Donovan, B., Fairley, C.K., Flagg, E.W., Johnson, A.M., Kahn,

J.A., Kavanagh, K., Kjaer, S.K., Kliewer, E.V., Lemieux-Mellouki, P., Markowitz, L., Mboup, A., Mesher, D., Niccolai, L., Oliphant, J., Pollock, K.G., Soldan, K., Sonnenberg, P., Tabrizi, S.N., Tanton, C., Brisson, M., 2015. Population-level impact and herd effects following human papillomavirus vaccination programmes: A systematic review and meta-analysis. The Lancet Infectious Diseases 15 (5), 565–580. 10.1016/S1473-3099(14)71073-4.

Dupuis, M., Denis-Mize, K., Woo, C., Goldbeck, C., Selby, M.J., Chen, M., Otten, G.R., Ulmer, J.B., Donnelly, J.J., Ott, G., McDonald, D.M., 2000. Distribution of DNA Vaccines Determines Their Immunogenicity After Intramuscular Injection in Mice. The Journal of Immunology 165 (5), 2850–2858. 10.4049/jimmunol.165.5.2850.

Eccles, R., Meier, C., Jawad, M., Weinmüllner, R., Grassauer, A., Prieschl-Grassauer, E., 2010. Efficacy and safety of an antiviral Iota-Carrageenan nasal spray: a randomized, double-blind, placebo-controlled exploratory study in volunteers with early symptoms of the common cold. Respiratory research 11, 108. 10.1186/1465-9921-11-108.

Eccles, R., Winther, B., Johnston, S.L., Robinson, P., Trampisch, M., Koelsch, S., 2015. Efficacy and safety of iota-carrageenan nasal spray versus placebo in early treatment of the common cold in adults: the ICICC trial. Respiratory research 16, 121. 10.1186/s12931-015-0281-8.

Eisenbarth, S.C., Colegio, O.R., O'Connor, W., Sutterwala, F.S., Flavell, R.A., 2008. Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. Nature 453 (7198), 1122–1126. 10.1038/nature06939.

El Mehdaoui, S., Touze, A., Laurent, S., Sizaret, P.-Y., Rasschaert, D., Coursaget, P., 2000. Gene Transfer Using Recombinant Rabbit Hemorrhagic Disease Virus Capsids with Genetically Modified DNA Encapsidation Capacity by Addition of Packaging Sequences from the L1 or L2 Protein of Human Papillomavirus Type 16. Journal of virology 74 (22), 10332–10340. 10.1128/JVI.74.22.10332-10340.2000.

Esposito, S., Scarselli, E., Lelii, M., Scala, A., Vitelli, A., Capone, S., Fornili, M., Biganzoli, E., Orenti, A., Nicosia, A., Cortese, R., Principi, N., 2016. Antibody response to respiratory syncytial virus infection in children <18 months old. Human Vaccines & Immunotherapeutics 12 (7), 1700–1706. 10.1080/21645515.2016.1145847.

Falsey, A.R., Hennessey, P.A., Formica, M.A., Cox, C., Walsh, E.E., 2005. Respiratory syncytial virus infection in elderly and high-risk adults. The New England journal of medicine 352 (17), 1749–1759. 10.1056/NEJMoa043951.

Fertey, J., Bayer, L., Grunwald, T., Pohl, A., Beckmann, J., Gotzmann, G., Casado, J.P., Schonfelder, J., Rogner, F.-H., Wetzel, C., Thoma, M., Bailer, S.M., Hiller, E., Rupp, S., Ulbert, S., 2016. Pathogens Inactivated by Low-Energy-Electron Irradiation Maintain Antigenic Properties and Induce Protective Immune Responses. Viruses 8 (11). 10.3390/v8110319.

Finnen, R.L., Erickson, K.D., Chen, X.S., Garcea, R.L., 2003. Interactions between Papillomavirus L1 and L2 Capsid Proteins. Journal of virology 77 (8), 4818–4826. 10.1128/JVI.77.8.4818-4826.2003.

Florin, L., Sapp, C., Streeck, R.E., Sapp, M., 2002. Assembly and Translocation of Papillomavirus Capsid Proteins. Journal of virology 76 (19), 10009–10014. 10.1128/JVI.76.19.10009–10014.2002.

FRAENKEL-CONRAT, H., OLCOTT, H.S., 1948. The reaction of formaldehyde with proteins; cross-linking between amino and primary amide or guanidyl groups. Journal of the American Chemical Society 70 (8), 2673–2684.

Franchi, L., Núñez, G., 2008. The Nlrp3 inflammasome is critical for aluminium hydroxide-mediated IL-1beta secretion but dispensable for adjuvant activity. European journal of immunology 38 (8), 2085–2089. 10.1002/eji.200838549.

Fu, T.M., Ulmer, J.B., Caulfield, M.J., Deck, R.R., Friedman, A., Wang, S., Liu, X., Donnelly, J.J., Liu, M.A., 1997. Priming of cytotoxic T lymphocytes by DNA vaccines: Requirement for professional antigen presenting cells and evidence for antigen transfer from myocytes. Molecular medicine (Cambridge, Mass.) 3 (6), 362–371.

Fynan, E.F., Webster, R.G., Fuller, D.H., Haynes, J.R., Santoro, J.C., Robinson, H.L., 1993. DNA vaccines: protective immunizations by parenteral, mucosal, and gene-gun inoculations. Proceedings of the National Academy of Sciences 90 (24), 11478–11482.

Garçon, N., Chomez, P., van Mechelen, M., 2007. GlaxoSmithKline Adjuvant Systems in vaccines: concepts, achievements and perspectives. Expert review of vaccines 6 (5), 723–739. 10.1586/14760584.6.5.723.

Garçon, N., Leroux-Roels, G., Cheng, W.-F., 2011. Vaccine adjuvants. Perspectives in Vaccinology 1 (1), 89–113. 10.1016/j.pervac.2011.05.004.

Garg, R., Babiuk, L., van Drunen Littel-van den Hurk, Sylvia, Gerdts, V., 2017. A novel combination adjuvant platform for human and animal vaccines. Vaccine 35 (35 Pt A), 4486–4489. 10.1016/j.vaccine.2017.05.067.

Garlapati, S., Garg, R., Brownlie, R., Latimer, L., Simko, E., Hancock, R.E.W., Babiuk, L.A., Gerdts, V., Potter, A., van Drunen Littel-van den Hurk, S., 2012. Enhanced immune responses and protection by vaccination with respiratory syncytial virus fusion protein formulated with CpG oligodeoxynucleotide and innate defense regulator peptide in polyphosphazene microparticles. Vaccine 30 (35), 5206–5214. 10.1016/j.vaccine.2012.06.011.

Gilca, R., Serres, G. de, Tremblay, M., Vachon, M.-L., Leblanc, E., Bergeron, M.G., Dery, P., Boivin, G., 2006. Distribution and clinical impact of human respiratory syncytial virus genotypes in hospitalized children over 2 winter seasons. The Journal of infectious diseases 193 (1), 54–58. 10.1086/498526.

Giroglou, T., Florin, L., Schäfer, F., Streeck, R.E., Sapp, M., 2001. Human papillomavirus infection requires cell surface heparan sulfate. Journal of virology 75 (3), 1565–1570. 10.1128/JVI.75.3.1565-1570.2001.

Glenny, A.T., Buttle, G.A.H., Stevens, M.F., 1931. Rate of disappearance of diphtheria toxoid injected into rabbits and guinea - pigs: Toxoid precipitated with alum. J. Pathol. 34 (2), 267–275. 10.1002/path.1700340214.

Glezen, W.P., Taber, L.H., Frank, A.L., Kasel, J.A., 1986. Risk of primary infection and reinfection with respiratory syncytial virus. American journal of diseases of children (1960) 140 (6), 543–546.

Goldstein, M.A., Tauraso, N.M., 1970. Effect of formalin, beta-propiolactone, merthiolate, and ultraviolet light upon influenza virus infectivity chicken cell agglutination, hemagglutination, and antigenicity. Applied microbiology 19 (2), 290–294.

González, M.E., Alarcón, B., Carrasco, L., 1987. Polysaccharides as antiviral agents: Antiviral activity of carrageenan. Antimicrobial Agents and Chemotherapy 31 (9), 1388–1393.

Graham, B.S., 2016. Vaccines against respiratory syncytial virus: The time has finally come. Vaccine 34 (30), 3535–3541. 10.1016/j.vaccine.2016.04.083.

Graham, B.S., Kines, R.C., Corbett, K.S., Nicewonger, J., Johnson, T.R., Chen, M., LaVigne, D., Roberts, J.N., Cuburu, N., Schiller, J.T., Buck, C.B., 2010. Mucosal delivery of human papillomavirus pseudovirus-encapsidated plasmids improves the potency of DNA vaccination. Mucosal immunology 3 (5), 475–486. 10.1038/mi.2010.31.

GraphPad Statistics Guide. The most common misinterpretation of a P value. https://www.graphpad.com/guides/prism/7/statistics/index.htm?common_misinterpretation_of_a_p_value.htm. Accessed 13 February 2018.

Grassauer, A., Weinmuellner, R., Meier, C., Pretsch, A., Prieschl-Grassauer, E., Unger, H., 2008. Iota-Carrageenan is a potent inhibitor of rhinovirus infection. Virology journal 5, 107. 10.1186/1743-422X-5-107.

Grunwald, T., Tenbusch, M., Schulte, R., Raue, K., Wolf, H., Hannaman, D., Swart, R.L. de, Uberla, K., Stahl-Hennig, C., 2014. Novel vaccine regimen elicits strong airway immune responses and control of respiratory syncytial virus in nonhuman primates. Journal of virology 88 (8), 3997–4007. 10.1128/JVI.02736-13.

Grunwald, T., Ulbert, S., 2015. Improvement of DNA vaccination by adjuvants and sophisticated delivery devices: vaccine-platforms for the battle against infectious diseases. Clinical and experimental vaccine research 4 (1), 1–10. 10.7774/cevr.2015.4.1.1.

Gürtler, C., Bowie, A.G., 2013. Innate immune detection of microbial nucleic acids. Trends in microbiology 21 (8), 413–420. 10.1016/j.tim.2013.04.004.

Hall, C.B., Long, C.E., Schnabel, K.C., 2001. Respiratory syncytial virus infections in previously healthy working adults. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 33 (6), 792–796. 10.1086/322657.

Hawkes, D., Benhamu, J., Sidwell, T., Miles, R., Dunlop, R.A., 2015. Revisiting adverse reactions to vaccines: A critical appraisal of Autoimmune Syndrome Induced by Adjuvants (ASIA). Journal of autoimmunity 59, 77–84. 10.1016/j.jaut.2015.02.005.

Higgins, D., Trujillo, C., Keech, C., 2016. Advances in RSV vaccine research and development - A global agenda. Vaccine 34 (26), 2870–2875. 10.1016/j.vaccine.2016.03.109.

Homaira, N., Rawlinson, W., Snelling, T.L., Jaffe, A., 2014. Effectiveness of Palivizumab in Preventing RSV Hospitalization in High Risk Children: A Real-World Perspective. International journal of pediatrics 2014, 571609. 10.1155/2014/571609.

Horvath, C.A.J., Boulet, G.A.V., Renoux, V.M., Delvenne, P.O., Bogers, J.-P.J., 2010. Mechanisms of cell entry by human papillomaviruses: an overview. Virology journal 7, 11. 10.1186/1743-422X-7-11.

Hu, J., Robinson, J.L., 2010. Treatment of respiratory syncytial virus with palivizumab: a systematic review. World journal of pediatrics : WJP 6 (4), 296–300. 10.1007/s12519-010-0230-z.

Invitrogen. TOPO® TA Cloning® Kit for Sequencing: Five-minute cloning of Taq polymerase-amplified PCR products for sequencing. Catalog Number 450030, MAN0000109.

Ito, M., Baba, M., Sato, A., Pauwels, R., Clercq, E. de, Shigeta, S., 1987. Inhibitory effect of dextran sulfate and heparin on the replication of human immunodeficiency virus (HIV) in vitro. Antiviral Research 7 (6), 361–367.

Joyce, J.G., Tung, J.-S., Przysiecki, C.T., Cook, J.C., Lehman, E.D., Sands, J.A., Jansen, K.U., Keller, P.M., 1999. The L1 Major Capsid Protein of Human Papillomavirus Type 11 Recombinant Virus-like Particles Interacts with Heparin and Cell-surface Glycosaminoglycans on Human Keratinocytes. J. Biol. Chem. 274 (9), 5810–5822. 10.1074/jbc.274.9.5810.

Kafil, V., Omidi, Y., 2011. Cytotoxic impacts of linear and branched polyethylenimine nanostructures in a431 cells. BioImpacts : BI 1 (1), 23–30. 10.5681/bi.2011.004.

Kämper, N., Day, P.M., Nowak, T., Selinka, H.-C., Florin, L., Bolscher, J., Hilbig, L., Schiller, J.T., Sapp, M., 2006. A membrane-destabilizing peptide in capsid protein L2 is required for egress of papillomavirus genomes from endosomes. Journal of virology 80 (2), 759–768. 10.1128/JVI.80.2.759-768.2006.

Kamphuis, T., Meijerhof, T., Stegmann, T., Lederhofer, J., Wilschut, J., Haan, A. de, 2012. Immunogenicity and protective capacity of a virosomal respiratory syncytial virus vaccine adjuvanted with monophosphoryl lipid A in mice. PloS one 7 (5), e36812. 10.1371/journal.pone.0036812.

Karron, R.A., Buchholz, U.J., Collins, P.L., 2013. Live-attenuated respiratory syncytial virus vaccines. Current topics in microbiology and immunology 372, 259–284. 10.1007/978-3-642-38919-1_13.

Karron, R.A., Luongo, C., Thumar, B., Loehr, K.M., Englund, J.A., Collins, P.L., Buchholz, U.J., 2015. A gene deletion that up-regulates viral gene expression yields an attenuated RSV vaccine with improved antibody responses in children. Science translational medicine 7 (312), 312ra175. 10.1126/scitranslmed.aac8463.

Killikelly, A.M., Kanekiyo, M., Graham, B.S., 2016. Pre-fusion F is absent on the surface of formalin-inactivated respiratory syncytial virus. Scientific reports 6, 34108. 10.1038/srep34108.

Kim, H.W., Canchola, J.G., Brandt, C.D., Pyles, G., Chanock, R.M., Jensen, K., Parrott, R.H., 1969. Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. American journal of epidemiology 89 (4), 422–434.

Kines, R.C., Zarnitsyn, V., Johnson, T.R., Pang, Y.-Y.S., Corbett, K.S., Nicewonger, J.D., Gangopadhyay, A., Chen, M., Liu, J., Prausnitz, M.R., Schiller, J.T., Graham, B.S., Tregoning, J.S., 2015. Vaccination with Human Papillomavirus Pseudovirus-Encapsidated Plasmids Targeted to Skin Using Microneedles. PLoS ONE 10 (3), e0120797. 10.1371/journal.pone.0120797.

Knudson, C.J., Hartwig, S.M., Meyerholz, D.K., Varga, S.M., 2015. RSV vaccine-enhanced disease is orchestrated by the combined actions of distinct CD4 T cell subsets. PLoS pathogens 11 (3), e1004757. 10.1371/journal.ppat.1004757.

Koenighofer, M., Lion, T., Bodenteich, A., Prieschl-Grassauer, E., Grassauer, A., Unger, H., Mueller, C.A., Fazekas, T., 2014. Carrageenan nasal spray in virus confirmed common cold: individual patient data analysis of two randomized controlled trials. Multidisciplinary respiratory medicine 9 (1), 57. 10.1186/2049-6958-9-57.

Kohlmann, R., Schwannecke, S., Tippler, B., Ternette, N., Temchura, V.V., Tenbusch, M., Uberla, K., Grunwald, T., 2009. Protective efficacy and immunogenicity of an adenoviral vector vaccine encoding the codon-optimized F protein of respiratory syncytial virus. Journal of virology 83 (23), 12601–12610. 10.1128/JVI.01036-09.

Kool, M., Fierens, K., Lambrecht, B.N., 2012. Alum adjuvant: some of the tricks of the oldest adjuvant. Journal of medical microbiology 61 (Pt 7), 927–934. 10.1099/jmm.0.038943-0.

Krauzewicz, N., Cox, C., Soeda, E., Clark, B., Rayner, S., Griffin, B.E., 2000. Sustained ex vivo and in vivo transfer of a reporter gene using polyoma virus pseudocapsids. Gene therapy 7 (13), 1094–1102. 10.1038/sj.gt.3301219.

Krusat, T., Streckert, H.-J., 1997. Heparin-dependent attachment ofrespiratory syncytial virus (RSV) to host cells. Arch. Virol. 142 (6), 1247–1254. 10.1007/s007050050156.

Laniosz, V., Dabydeen, S.A., Havens, M.A., Meneses, P.I., 2009. Human papillomavirus type 16 infection of human keratinocytes requires clathrin and caveolin-1 and is brefeldin a sensitive. Journal of virology 83 (16), 8221–8232. 10.1128/JVI.00576-09.

Larionov, A., Krause, A., Miller, W., 2005. A standard curve based method for relative real time PCR data processing. BMC bioinformatics 6, 62. 10.1186/1471-2105-6-62.

Leo, O., Cunningham, A., Stern, P.L., 2011. Vaccine immunology. Perspectives in Vaccinology 1 (1), 25–59. 10.1016/j.pervac.2011.05.002.

Li, M., Cripe, T.P., Estes, P.A., Lyon, M.K., Rose, R.C., Garcea, R.L., 1997. Expression of the human papillomavirus type 11 L1 capsid protein in Escherichia coli: characterization of protein domains involved in DNA binding and capsid assembly. Journal of virology 71 (4), 2988–2995.

Li, X., Sambhara, S., Li, C.X., Ewasyshyn, M., Parrington, M., Caterini, J., James, O., Cates, G., Du, R.-P., Klein, M., 1998. Protection against Respiratory Syncytial Virus Infection by DNA Immunization. The Journal of Experimental Medicine 188 (4), 681–688.

Liang, B., Surman, S., Amaro-Carambot, E., Kabatova, B., Mackow, N., Lingemann, M., Yang, L., McLellan, J.S., Graham, B.S., Kwong, P.D., Schaap-Nutt, A., Collins, P.L., Munir, S., 2015. Enhanced Neutralizing Antibody Response Induced by Respiratory Syncytial Virus Prefusion F Protein Expressed by a Vaccine Candidate. Journal of virology 89 (18), 9499–9510. 10.1128/JVI.01373-15.

Limberis, M.P., Bell, C.L., Wilson, J.M., 2009. Identification of the murine firefly luciferase-specific CD8 T-cell epitopes. Gene therapy 16 (3), 441–447. 10.1038/gt.2008.177.

Lin, Y.-L., Lei, H.-Y., Lin, Y.-S., Yeh, T.-M., Chen, S.-H., Liu, H.-S., 2002. Heparin inhibits dengue-2 virus infection of five human liver cell lines. Antiviral Research 56 (1), 93–96. 10.1016/S0166-3542(02)00095-5.

Lindell, D.M., Morris, S.B., White, M.P., Kallal, L.E., Lundy, P.K., Hamouda, T., Baker, J.R., Lukacs, N.W., 2011. A novel inactivated intranasal respiratory syncytial virus vaccine promotes viral clearance without Th2 associated vaccine-enhanced disease. PloS one 6 (7), e21823. 10.1371/journal.pone.0021823.

Liu, M.A., 2011. DNA vaccines: an historical perspective and view to the future. Immunological reviews 239 (1), 62–84. 10.1111/j.1600-065X.2010.00980.x.

Locher, C.P., Putnam, D., Langer, R., Witt, S.A., Ashlock, B.M., Levy, J.A., 2003. Enhancement of a human immunodeficiency virus env DNA vaccine using a novel polycationic nanoparticle formulation. Immunology Letters 90 (2-3), 67–70. 10.1016/j.imlet.2003.02.001.

Ludwig, M., Enzenhofer, E., Schneider, S., Rauch, M., Bodenteich, A., Neumann, K., Prieschl-Grassauer, E., Grassauer, A., Lion, T., Mueller, C.A., 2013. Efficacy of a carrageenan nasal spray in patients with common cold: a randomized controlled trial. Respiratory research 14, 124. 10.1186/1465-9921-14-124.

Matsumoto, M., Seya, T., 2008. TLR3: interferon induction by double-stranded RNA including poly(I:C). Advanced drug delivery reviews 60 (7), 805–812. 10.1016/j.addr.2007.11.005.

McGinnes, L.W., Gravel, K.A., Finberg, R.W., Kurt-Jones, E.A., Massare, M.J., Smith, G., Schmidt, M.R., Morrison, T.G., 2011. Assembly and immunological properties of Newcastle disease virus-like particles containing the respiratory syncytial virus F and G proteins. Journal of virology 85 (1), 366–377. 10.1128/JVI.01861-10.

McKee, A.S., Munks, M.W., Marrack, P., 2007. How do adjuvants work? Important considerations for new generation adjuvants. Immunity 27 (5), 687–690. 10.1016/j.immuni.2007.11.003.

McLellan, J.S., Chen, M., Joyce, M.G., Sastry, M., Stewart-Jones, G.B.E., Yang, Y., Zhang, B., Chen, L., Srivatsan, S., Zheng, A., Zhou, T., Graepel, K.W., Kumar, A., Moin, S., Boyington, J.C., Chuang, G.-Y., Soto, C., Baxa, U., Bakker, A.Q., Spits, H., Beaumont, T., Zheng, Z., Xia, N., Ko, S.-Y., Todd, J.-P., Rao, S., Graham, B.S., Kwong, P.D., 2013a. Structure-based design of a fusion glycoprotein vaccine for respiratory syncytial virus. Science (New York, N.Y.) 342 (6158), 592–598. 10.1126/science.1243283.

McLellan, J.S., Chen, M., Leung, S., Graepel, K.W., Du, X., Yang, Y., Zhou, T., Baxa, U., Yasuda, E., Beaumont, T., Kumar, A., Modjarrad, K., Zheng, Z., Zhao, M., Xia, N., Kwong, P.D., Graham, B.S., 2013b. Structure of RSV fusion glycoprotein trimer bound to a prefusion-specific neutralizing antibody. Science (New York, N.Y.) 340 (6136), 1113–1117. 10.1126/science.1234914.

Meissner, H.C., 2016. Viral Bronchiolitis in Children. The New England journal of medicine 374 (1), 62–72. 10.1056/NEJMra1413456.

Merck, 2015. Merck's 9-Valent HPV Vaccine, GARDASIL®9, Recommended by CDC's Advisory Committee on Immunization Practices for Females Aged 9-26 and Males Aged 9-2. http://www.mrknewsroom.com/news-release/vaccine-news/mercks-9-valent-hpv-vaccine-gardasil9-recommended-cdcs-advisory-committee-.

Mistry, N., Wibom, C., Evander, M., 2008. Cutaneous and mucosal human papillomaviruses differ in net surface charge, potential impact on tropism. Virology journal 5, 118. 10.1186/1743-422X-5-118.

Modjarrad, K., Giersing, B., Kaslow, D.C., Smith, P.G., Moorthy, V.S., 2016. WHO consultation on Respiratory Syncytial Virus Vaccine Development Report from a World Health Organization Meeting held on 23-24 March 2015. Vaccine 34 (2), 190–197. 10.1016/j.vaccine.2015.05.093.

Moghaddam, A., Olszewska, W., Wang, B., Tregoning, J.S., Helson, R., Sattentau, Q.J., Openshaw, P.J.M., 2006. A potential molecular mechanism for hypersensitivity caused by formalin-inactivated vaccines. Nature medicine 12 (8), 905–907. 10.1038/nm1456.

Mullard, A., 2008. Vaccine failure explained. Nature 140, 543–546. 10.1038/news.2008.1302.

Müller, M., Gissmann, L., Cristiano, R.J., Sun, X.Y., Frazer, I.H., Jenson, A.B., Alonso, A., Zentgraf, H., Zhou, J., 1995. Papillomavirus capsid binding and uptake by cells from different tissues and species. Journal of virology 69 (2), 948–954.

Münch, J., Rücker, E., Ständker, L., Adermann, K., Goffinet, C., Schindler, M., Wildum, S., Chinnadurai, R., Rajan, D., Specht, A., Giménez-Gallego, G., Sánchez, P.C., Fowler, D.M., Koulov, A., Kelly, J.W., Mothes, W., Grivel, J.-C., Margolis, L., Keppler, O.T., Forssmann, W.-G., Kirchhoff, F., 2007. Semen-derived amyloid fibrils drastically enhance HIV infection. Cell 131 (6), 1059–1071. 10.1016/j.cell.2007.10.014.

Murphy, B.R., Walsh, E.E., 1988. Formalin-inactivated respiratory syncytial virus vaccine induces antibodies to the fusion glycoprotein that are deficient in fusion-inhibiting activity. Journal of clinical microbiology 26 (8), 1595–1597.

Murphy, K., Travers, P., Walport, M., & Janeway, C., 2011. Janeway's immunobiology, 8th ed., New York.

Nahmias, A.J., Kibrick, S., 1964. Inhibitory effect of heparin on herpes simplex virus. Journal of bacteriology 87 (5), 1060–1066.

Nair, H., Nokes, D.J., Gessner, B.D., Dherani, M., Madhi, S.A., Singleton, R.J., O'Brien, K.L., Roca, A., Wright, P.F., Bruce, N., Chandran, A., Theodoratou, E., Sutanto, A., Sedyaningsih, E.R., Ngama, M., Munywoki, P.K., Kartasasmita, C., Simões, E.A.F., Rudan, I., Weber, M.W., Campbell, H.,

2010. Global burden of acute lower respiratory infections due to respiratory syncytial virus in young children: A systematic review and meta-analysis. The Lancet 375 (9725), 1545–1555. 10.1016/S0140-6736(10)60206-1.

Naso, M.F., Tomkowicz, B., Perry, W.L., Strohl, W.R., 2017. Adeno-Associated Virus (AAV) as a Vector for Gene Therapy. BioDrugs : clinical immunotherapeutics, biopharmaceuticals and gene therapy 31 (4), 317–334. 10.1007/s40259-017-0234-5.

Naylor, L.H., 1999. Reporter gene technology: the future looks bright. Biochemical pharmacology 58 (5), 749–757.

Nieto, K., Salvetti, A., 2014. AAV Vectors Vaccines Against Infectious Diseases. Frontiers in immunology 5, 5. 10.3389/fimmu.2014.00005.

NIH National Cancer Institute. NCI Dictionary of Cancer Terms: immunological adjuvant. https://www.cancer.gov/publications/dictionaries/cancer-terms?cdrid=43987. Accessed accessed on 3 January 2018.

O'Hagan, D.T., Ott, G.S., Gregorio, E. de, Seubert, A., 2012. The mechanism of action of MF59 - an innately attractive adjuvant formulation. Vaccine 30 (29), 4341–4348. 10.1016/j.vaccine.2011.09.061.

Openshaw, P.J., Culley, F.J., Olszewska, W., 2001. Immunopathogenesis of vaccine-enhanced RSV disease. Vaccine 20 Suppl 1, S27-31.

Otten, G., Schaefer, M., Doe, B., Liu, H., Srivastava, I., Zur Megede, J., O'Hagan, D., Donnelly, J., Widera, G., Rabussay, D., Lewis, M.G., Barnett, S., Ulmer, J.B., 2004. Enhancement of DNA vaccine potency in rhesus macaques by electroporation. Vaccine 22 (19), 2489–2493. 10.1016/j.vaccine.2003.11.073.

Pai Kasturi, S., Qin, H., Thomson, K.S., El-Bereir, S., Cha, S.-C., Neelapu, S., Kwak, L.W., Roy, K., 2006. Prophylactic anti-tumor effects in a B cell lymphoma model with DNA vaccines delivered on polyethylenimine (PEI) functionalized PLGA microparticles. Journal of controlled release : official journal of the Controlled Release Society 113 (3), 261–270. 10.1016/j.jconrel.2006.04.006.

Pastrana, D.V., Buck, C.B., Pang, Y.-Y.S., Thompson, C.D., Castle, P.E., FitzGerald, P.C., Krüger Kjaer, S., Lowy, D.R., Schiller, J.T., 2004. Reactivity of human sera in a sensitive, high-throughput pseudovirus-based papillomavirus neutralization assay for HPV16 and HPV18. Virology 321 (2), 205–216. 10.1016/j.virol.2003.12.027.

path, 2017. RSV Vaccine and mAb Snapshot. https://www.path.org/publications/files/CVIA_RSV_snapshot_fs.pdf. Accessed 25 April 2018.

Pazzagli, M., Devine, J.H., Peterson, D.O., Baldwin, T.O., 1992. Use of bacterial and firefly luciferases as reporter genes in DEAE-dextran-mediated transfection of mammalian cells. Analytical Biochemistry (204), 315–323.

Pereira, R., Hitzeroth, I.I., Rybicki, E.P., 2009. Insights into the role and function of L2, the minor capsid protein of papillomaviruses. Archives of virology 154 (2), 187–197. 10.1007/s00705-009-0310-3.

Petrovsky, N., Aguilar, J.C., 2004. Vaccine adjuvants: current state and future trends. Immunology and cell biology 82 (5), 488–496. 10.1111/j.0818-9641.2004.01272.x.

Pokorna, D., Rubio, I., Müller, M., 2008. DNA-vaccination via tattooing induces stronger humoral and cellular immune responses than intramuscular delivery supported by molecular adjuvants. Genetic vaccines and therapy 6, 4. 10.1186/1479-0556-6-4.

Polack, F.P., Teng, M.N., L.Collins, P., Prince, G.A., Exner, M., Regele, H., Lirman, D.D., Rabold, R., Hoffman, S.J., Karp, C.L., Kleeberger, S.R., Wills-Karp, M., Karron, R.A., 2002. A Role for Immune Complexes in Enhanced Respiratory Syncytial Virus Disease. J Exp Med 196 (6), 859–865. 10.1084/jem.20020781.

Prince, G.A., Curtis, S.J., Yim, K.C., Porter, D.D., 2001. Vaccine-enhanced respiratory syncytial virus disease in cotton rats following immunization with Lot 100 or a newly prepared reference vaccine. The Journal of general virology 82 (Pt 12), 2881–2888. 10.1099/0022-1317-82-12-2881.

Prince, G.A., Jenson, A.B., Hemming, V.G., Murphy, B.R., Walsh, E.E., Horswood, R.L., Chanock, R.M., 1986. Enhancement of respiratory syncytial virus pulmonary pathology in cotton rats by prior intramuscular inoculation of formalin-inactiva ted virus. Journal of virology 57 (3), 721–728.

Pulendran, B., Ahmed, R., 2011. Immunological mechanisms of vaccination. Nat Immunol 131 (6), 509–517. 10.1038/ni.2039.

Raghunandan, R., Lu, H., Zhou, B., Xabier, M.G., Massare, M.J., Flyer, D.C., Fries, L.F., Smith, G.E., Glenn, G.M., 2014. An insect cell derived respiratory syncytial virus (RSV) F nanoparticle vaccine induces antigenic site II antibodies and protects against RSV challenge in cotton rats by active and passive immunization. Vaccine 32 (48), 6485–6492. 10.1016/j.vaccine.2014.09.030.

Rector, A., Lemey, P., Tachezy, R., Mostmans, S., Ghim, S.-J., van Doorslaer, K., Roelke, M., Bush, M., Montali, R.J., Joslin, J., Burk, R.D., Jenson, A.B., Sundberg, J.P., Shapiro, B., van Ranst, M., 2007. Ancient papillomavirus-host co-speciation in Felidae. Genome biology 8 (4), R57. 10.1186/gb-2007-8-4-r57.

Rector, A., van Ranst, M., 2013. Animal papillomaviruses. Virology 445 (1-2), 213–223. 10.1016/j.virol.2013.05.007.

Rezaee, F., Linfield, D.T., Harford, T.J., Piedimonte, G., 2017. Ongoing developments in RSV prophylaxis: a clinician's analysis. Current opinion in virology 24, 70–78. 10.1016/j.coviro.2017.03.015.

Richards, R.M., Lowy, D.R., Schiller, J.T., Day, P.M., 2006. Cleavage of the papillomavirus minor capsid protein, L2, at a furin consensus site is necessary for infection. Proceedings of the National Academy of Sciences of the United States of America 103 (5), 1522–1527. 10.1073/pnas.0508815103.

Riedel, S., 2005. Edward Jenner and the history of smallpox and vaccination. Proceedings (Baylor University. Medical Center) 18 (1), 21–25.

Roan, N.R., Münch, J., Arhel, N., Mothes, W., Neidleman, J., Kobayashi, A., Smith-McCune, K., Kirchhoff, F., Greene, W.C., 2009. The cationic properties of SEVI underlie its ability to enhance human immunodeficiency virus infection. Journal of virology 83 (1), 73–80. 10.1128/JVI.01366-08.

Roberts, J.N., Buck, C.B., Thompson, C.D., Kines, R., Bernardo, M., Choyke, P.L., Lowy, D.R., Schiller, J.T., 2007. Genital transmission of HPV in a mouse model is potentiated by nonoxynol-9 and inhibited by carrageenan. Nature medicine 13 (7), 857–861. 10.1038/nm1598.

Roden, R.B., Day, P.M., Bronzo, B.K., Yutzy, W.H., Yang, Y., Lowy, D.R., Schiller, J.T., 2001. Positively charged termini of the L2 minor capsid protein are necessary for papillomavirus infection. Journal of virology 75 (21), 10493–10497. 10.1128/JVI.75.21.10493–10497.2001.

Roden, R.B., Kirnbauer, R., Jenson, A.B., Lowy, D.R., Schiller, J.T., 1994. Interaction of papillomaviruses with the cell surface. Journal of virology 68 (11), 7260–7266.

Sabbatini, P., Tsuji, T., Ferran, L., Ritter, E., Sedrak, C., Tuballes, K., Jungbluth, A.A., Ritter, G., Aghajanian, C., Bell-McGuinn, K., Hensley, M.L., Konner, J., Tew, W., Spriggs, D.R., Hoffman, E.W., Venhaus, R., Pan, L., Salazar, A.M., Diefenbach, C.M., Old, L.J., Gnjatic, S., 2012. Phase I trial of overlapping long peptides from a tumor self-antigen and poly-ICLC shows rapid induction of integrated immune response in ovarian cancer patients. Clinical cancer research : an official journal of the American Association for Cancer Research 18 (23), 6497–6508. 10.1158/1078-0432.CCR-12-2189.

Schäfer, F., Florin, L., Sapp, M., 2002. DNA binding of L1 is required for human papillomavirus morphogenesis in vivo. Virology 295 (1), 172–181. 10.1006/viro.2002.1361.

Schelhaas, M., Shah, B., Holzer, M., Blattmann, P., Kühling, L., Day, P.M., Schiller, J.T., Helenius, A., 2012. Entry of human papillomavirus type 16 by actin-dependent, clathrin- and lipid raft-independent endocytosis. PLoS pathogens 8 (4), e1002657. 10.1371/journal.ppat.1002657.

Schiller, J.T., Day, P.M., Kines, R.C., 2010. Current understanding of the mechanism of HPV infection. Gynecologic Oncology 118 (1), S12-S17. 10.1016/j.ygyno.2010.04.004.

Schiller, J.T., Lowy, D.R., 2009. Immunogenicity testing in human papillomavirus virus-like-particle vaccine trials. The Journal of infectious diseases 200 (2), 166–171. 10.1086/599988.

Schulze, A., Gripon, P., Urban, S., 2007. Hepatitis B virus infection initiates with a large surface protein-dependent binding to heparan sulfate proteoglycans. Hepatology (Baltimore, Md.) 46 (6), 1759–1768. 10.1002/hep.21896.

Seo, H.S., 2015. Application of radiation technology in vaccines development. Clinical and experimental vaccine research 4 (2), 145–158. 10.7774/cevr.2015.4.2.145.

Seubert, A., Monaci, E., Pizza, M., O'Hagan, D.T., Wack, A., 2008. The adjuvants aluminum hydroxide and MF59 induce monocyte and granulocyte chemoattractants and enhance monocyte differentiation toward dendritic cells. Journal of immunology (Baltimore, Md. : 1950) 180 (8), 5402–5412.

Shafique, M., Wilschut, J., Haan, A. de, 2012. Induction of mucosal and systemic immunity against respiratory syncytial virus by inactivated virus supplemented with TLR9 and NOD2 ligands. Vaccine 30 (3), 597–606. 10.1016/j.vaccine.2011.11.054.

Shafti-Keramat, S., Handisurya, A., Kriehuber, E., Meneguzzi, G., Slupetzky, K., Kirnbauer, R., 2003. Different heparan sulfate proteoglycans serve as cellular receptors for human papillomaviruses. Journal of virology 77 (24), 13125–13135.

Shi, T., McAllister, D.A., O'Brien, K.L., Simoes, E.A.F., Madhi, S.A., Gessner, B.D., Polack, F.P., Balsells, E., Acacio, S., Aguayo, C., Alassani, I., Ali, A., Antonio, M., Awasthi, S., Awori, J.O., Azziz-Baumgartner, E., Baggett, H.C., Baillie, V.L., Balmaseda, A., Barahona, A., Basnet, S., Bassat, Q., Basualdo, W., Bigogo, G., Bont, L., Breiman, R.F., Brooks, W.A., Broor, S., Bruce, N., Bruden, D., Buchy, P., Campbell, S., Carosone-Link, P., Chadha, M., Chipeta, J., Chou, M., Clara, W., Cohen, C., Cuellar, E. de, Dang, D.-A., Dash-yandag, B., Deloria-Knoll, M., Dherani, M., Eap, T., Ebruke, B.E., Echavarria, M., de Freitas Lázaro Emediato, Carla Cecília, Fasce, R.A., Feikin, D.R., Feng, L., Gentile, A., Gordon, A., Goswami, D., Goyet, S., Groome, M., Halasa, N., Hirve, S., Homaira, N., Howie, S.R.C., Jara, J., Jroundi, I., Kartasasmita, C.B., Khuri-Bulos, N., Kotloff, K.L., Krishnan, A., Libster, R., Lopez, O., Lucero, M.G., Lucion, F., Lupisan, S.P., Marcone, D.N., McCracken, J.P., Mejia, M., Moisi, J.C., Montgomery, J.M., Moore, D.P., Moraleda, C., Moyes, J., Munywoki, P., Mutyara, K., Nicol, M.P., Nokes, D.J., Nymadawa, P., da Costa Oliveira, Maria Tereza, Oshitani, H., Pandey, N., Paranhos-Baccalà, G., Phillips, L.N., Picot, V.S., Rahman, M., Rakoto-Andrianarivelo, M., Rasmussen, Z.A., Rath, B.A., Robinson, A., Romero, C., Russomando, G., Salimi, V., Sawatwong, P., Scheltema, N., Schweiger, B., Scott, J.A.G., Seidenberg, P., Shen, K., Singleton, R., Sotomayor, V., Strand, T.A., Sutanto, A., Sylla, M., Tapia, M.D., Thamthitiwat, S., Thomas, E.D., Tokarz, R., Turner, C., Venter, M., Waicharoen, S., Wang, J., Watthanaworawit, W., Yoshida, L.-M., Yu, H., Zar, H.J., Campbell, H., Nair, H., 2017. Global, regional, and national disease burden estimates of acute lower respiratory infections due to respiratory syncytial virus in young children in 2015: A systematic review and modelling study. The Lancet 390 (10098), 946–958. 10.1016/S0140-6736(17)30938-8.

Shoenfeld, Y., Agmon-Levin, N., 2011. 'ASIA' - autoimmune/inflammatory syndrome induced by adjuvants. Journal of autoimmunity 36 (1), 4–8. 10.1016/j.jaut.2010.07.003.

Shuman, S., 1991. Recombination mediated by vaccinia virus DNA topoisomerase I in Escherichia coli is sequence specific. Proceedings of the National Academy of Sciences of the United States of America 88 (22), 10104–10108.

Shuman, S., 1994. Novel approach to molecular cloning and polynucleotide synthesis using vaccinia DNA topoisomerase. Journal of Biological Chemistry 269 (51), 32678–32684.

Simon, A.K., Hollander, G.A., McMichael, A., 2015. Evolution of the immune system in humans from infancy to old age. Proceedings. Biological sciences 282 (1821), 20143085. 10.1098/rspb.2014.3085.

Stahl-Hennig, C., Eisenblätter, M., Jasny, E., Rzehak, T., Tenner-Racz, K., Trumpfheller, C., Salazar, A.M., Uberla, K., Nieto, K., Kleinschmidt, J., Schulte, R., Gissmann, L., Müller, M., Sacher, A., Racz, P., Steinman, R.M., Uguccioni, M., Ignatius, R., 2009. Synthetic double-stranded RNAs are adjuvants for the induction of T helper 1 and humoral immune responses to human papillomavirus in rhesus macaques. PLoS pathogens 5 (4), e1000373. 10.1371/journal.ppat.1000373.

Stang, A., Korn, K., Wildner, O., Uberla, K., 2005. Characterization of virus isolates by particle-associated nucleic acid PCR. Journal of clinical microbiology 43 (2), 716–720. 10.1128/JCM.43.2.716-720.2005.

Stauffer, Y., Raj, K., Masternak, K., Beard, P., 1998. Infectious human papillomavirus type 18 pseudovirions. Journal of molecular biology 283 (3), 529–536. 10.1006/jmbi.1998.2113.

Steinberg, B.M., Auborn, K.J., Brandsma, J.L., Taichman, L.B., 1989. Tissue site-specific enhancer function of the upstream regulatory region of human papillomavirus type 11 in cultured keratinocytes. Journal of virology 63 (2), 957–960.

Sun, H.-X., Xie, Y., Ye, Y.-P., 2009. Advances in saponin-based adjuvants. Vaccine 27 (12), 1787–1796. 10.1016/j.vaccine.2009.01.091.

Swanson, K.A., Balabanis, K., Xie, Y., Aggarwal, Y., Palomo, C., Mas, V., Metrick, C., Yang, H., Shaw, C.A., Melero, J.A., Dormitzer, P.R., Carfi, A., 2014. A monomeric uncleaved respiratory syncytial virus F antigen retains prefusion-specific neutralizing epitopes. Journal of virology 88 (20), 11802–11810. 10.1128/JVI.01225-14.

Ternette, N., Tippler, B., Uberla, K., Grunwald, T., 2007. Immunogenicity and efficacy of codon optimized DNA vaccines encoding the F-protein of respiratory syncytial virus. Vaccine 25 (41), 7271–7279. 10.1016/j.vaccine.2007.07.025.

Touze, A., Coursaget, P., 1998. In vitro gene transfer using human papillomavirus-like particles. Nucleic Acids Research 26 (5), 1317–1323.

Ura, T., Okuda, K., Shimada, M., 2014. Developments in Viral Vector-Based Vaccines. Vaccines 2 (3), 624–641. 10.3390/vaccines2030624.

van Dis, E.S., Moore, T.C., Lavender, K.J., Messer, R.J., Keppler, O.T., Verheyen, J., Dittmer, U., Hasenkrug, K.J., 2016. No SEVI-mediated enhancement of rectal HIV-1 transmission of HIV-1 in two humanized mouse cohorts. Virology 488, 88–95. 10.1016/j.virol.2015.11.005.

van Drunen Littel-van den Hurk, Sylvia, Watkiss, E.R., 2012. Pathogenesis of respiratory syncytial virus. Current opinion in virology 2 (3), 300–305. 10.1016/j.coviro.2012.01.008.

Villenave, R., O'Donoghue, D., Thavagnanam, S., Touzelet, O., Skibinski, G., Heaney, L.G., McKaigue, J.P., Coyle, P.V., Shields, M.D., Power, U.F., 2011. Differential cytopathogenesis of respiratory syncytial virus prototypic and clinical isolates in primary pediatric bronchial epithelial cells. Virology journal 8, 43. 10.1186/1743-422X-8-43.

Villiers, E.-M. de, Fauquet, C., Broker, T.R., Bernard, H.-U., Zur Hausen, H., 2004. Classification of papillomaviruses. Virology 324 (1), 17–27. 10.1016/j.virol.2004.03.033.

Volkin, D.B., Shi, L., Sanyal, G., 2002. Stabilized human papillomavirus formulations. Google Patents. https://www.google.de/patents/US6358744.

Waris, M.E., Tsou, C., Erdman, D.D., Zaki, S.R., Anderson, L.J., 1996. Respiratory synctial virus infection in BALB/c mice previously immunized with formalin-inactivated virus induces enhanced pulmonary inflammatory response with a predominant Th2-like cytokine pattern. Journal of virology 70 (5), 2852–2860.

Wetzel, C., Schönfelder, J., Schwarz, W., Funk, R., 2010. Surface modification of polyurethane and silicone for therapeutic medical technics by means of electron beam. Surface and Coatings Technology 205 (5), 1618–1623. 10.1016/j.surfcoat.2010.07.103.

Widera, G., Austin, M., Rabussay, D., Goldbeck, C., Barnett, S.W., Chen, M., Leung, L., Otten, G.R., Thudium, K., Selby, M.J., Ulmer, J.B., 2000. Increased DNA Vaccine Delivery and Immunogenicity by Electroporation In Vivo. The Journal of Immunology 164 (9), 4635–4640. 10.4049/jimmunol.164.9.4635.

Widjaja, I., Wicht, O., Luytjes, W., Leenhouts, K., Rottier, P.J.M., van Kuppeveld, Frank J M, Haijema, B.J., de Haan, Cornelis A M, 2016. Characterization of Epitope-Specific Anti-Respiratory Syncytial Virus (Anti-RSV) Antibody Responses after Natural Infection and after Vaccination with Formalin-Inactivated RSV. Journal of virology 90 (13), 5965–5977. 10.1128/JVI.00235-16.

Wu, J.C., Sundaresan, G., Iyer, M., Gambhir, S.S., 2001. Noninvasive optical imaging of firefly luciferase reporter gene expression in skeletal muscles of living mice. Molecular therapy : the journal of the American Society of Gene Therapy 4 (4), 297–306. 10.1006/mthe.2001.0460.

Xu, Y.-F., Zhang, Y.-Q., Xu, X.-M., Song, G.-X., 2006. Papillomavirus virus-like particles as vehicles for the delivery of epitopes or genes. Archives of virology 151 (11), 2133–2148. 10.1007/s00705-006-0798-8.

Zacharopoulos, V.R., Phillips, D.M., 1997. Vaginal formulations of carrageenan protect mice from herpes simplex virus infection. Clinical and Diagnostic Laboratory Immunology 4 (4), 465–468.

Zeng, R., Cui, Y., Hai, Y., Liu, Y., 2012. Pattern recognition receptors for respiratory syncytial virus infection and design of vaccines. Virus research 167 (2), 138–145. 10.1016/j.virusres.2012.06.003.

Zhao, K.N., Sun, X.Y., Frazer, I.H., Zhou, J., 1998. DNA packaging by L1 and L2 capsid proteins of bovine papillomavirus type 1. Virology 243 (2), 482–491. 10.1006/viro.1998.9091.

Zhao, Q., Potter, C.S., Carragher, B., Lander, G., Sworen, J., Towne, V., Abraham, D., Duncan, P., Washabaugh, M.W., Sitrin, R.D., 2014. Characterization of virus-like particles in GARDASIL® by cryo transmission electron microscopy. Human Vaccines & Immunotherapeutics 10 (3), 734–739. 10.4161/hv.27316.

Zhou, J., Sun, X.Y., Louis, K., Frazer, I.H., 1994. Interaction of human papillomavirus (HPV) type 16 capsid proteins with HPV DNA requires an intact L2 N-terminal sequence. Journal of virology 68 (2), 619–625.

Zhu, D., Tuo, W., 2016. QS-21: A Potent Vaccine Adjuvant. Natural products chemistry & research 3 (4). 10.4172/2329-6836.1000e113.

Zhu, Z., Gershon, M.D., Ambron, R., Gabel, C., Gershon, A.A., 1995. Infection of cells by varicella zoster virus: Inhibition of viral entry by mannose 6-phosphate and heparin. Proceedings of the National Academy of Sciences of the United States of America 92 (8), 3546–3550. 10.1073/pnas.92.8.3546.

Zur Hausen, H., 2002. Papillomaviruses and cancer: From basic studies to clinical application. Nature reviews. Cancer 2 (5), 342–350. 10.1038/nrc798.

Zur Hausen, H., 2009. Papillomaviruses in the causation of human cancers - a brief historical account. Virology 384 (2), 260–265. 10.1016/j.virol.2008.11.046.

7 APPENDIX

7.1 Plasmid maps

7.1.1 Packaging plasmids

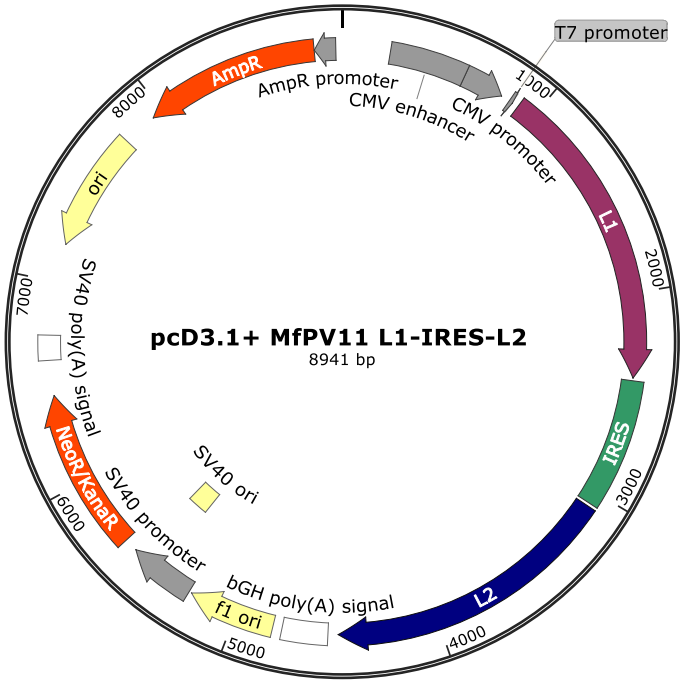


Figure 7.1-1 - pcD3.1+ MfPV11 L1-IRES-L2

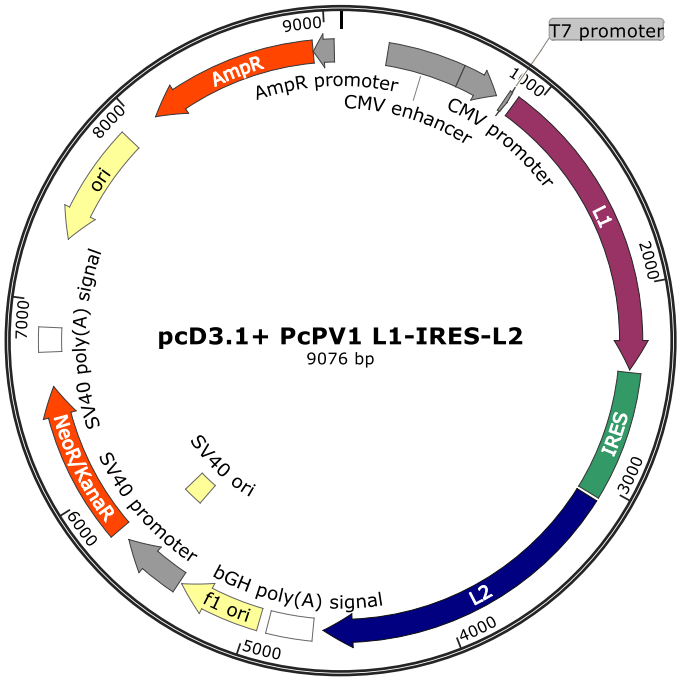


Figure 7.1-2 - pcD3.1+ PcPV1 L1-IRES-L2

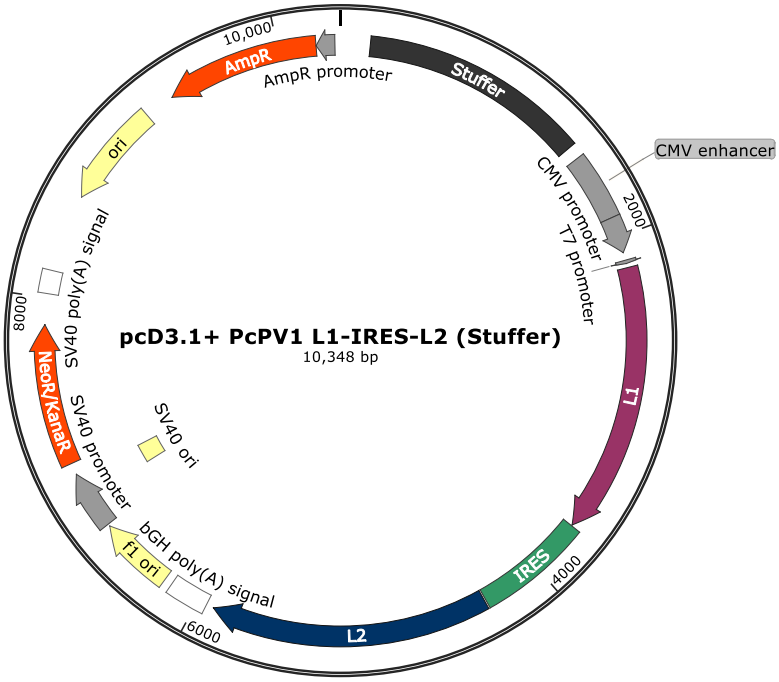


Figure 7.1-3 - pcD3.1+ PcPV1 L1-IRES-L2 (Stuffer)

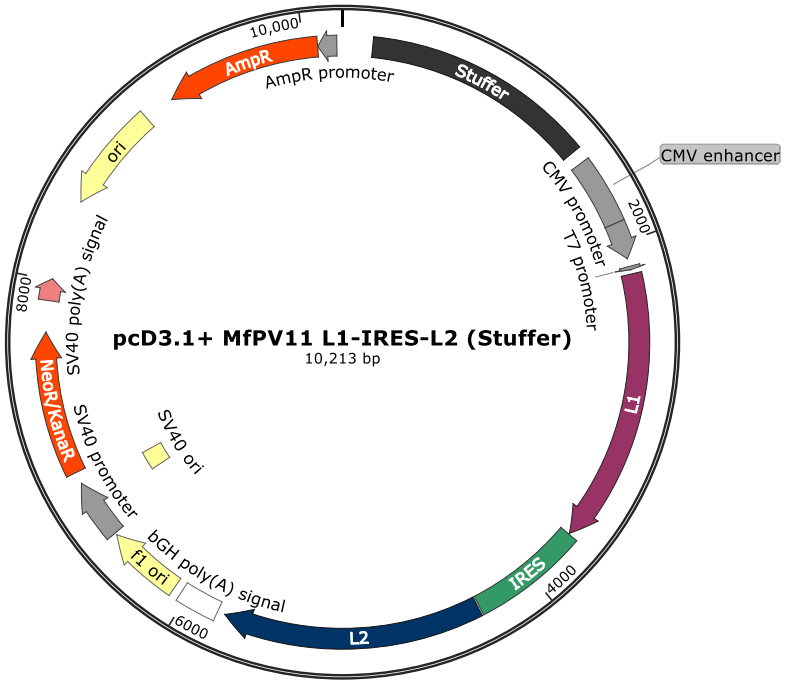


Figure 7.1-4 - pcD3.1+ MfPV11 L1-IRES-L2 (Stuffer)

7.1.2 Reporter plasmids

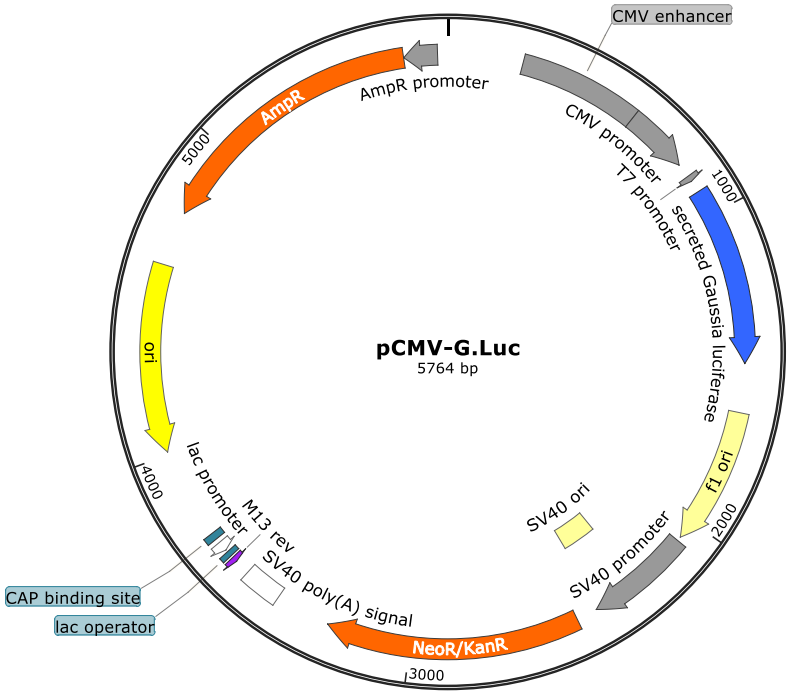


Figure 7.1-5 - pCMV-G.Luc

Expression plasmid with gaussia luciferase reporter

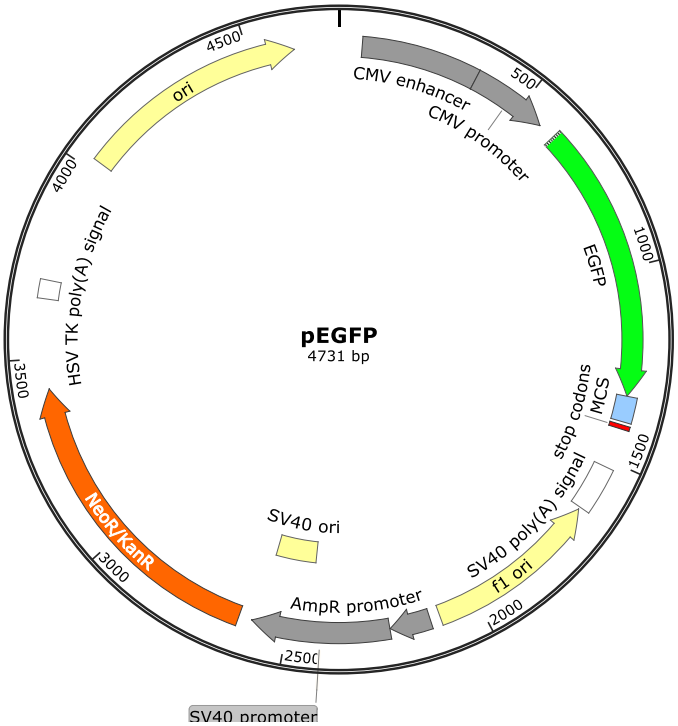


Figure 7.1-6 - pEGFP

Expression plasmid with GFP reporter

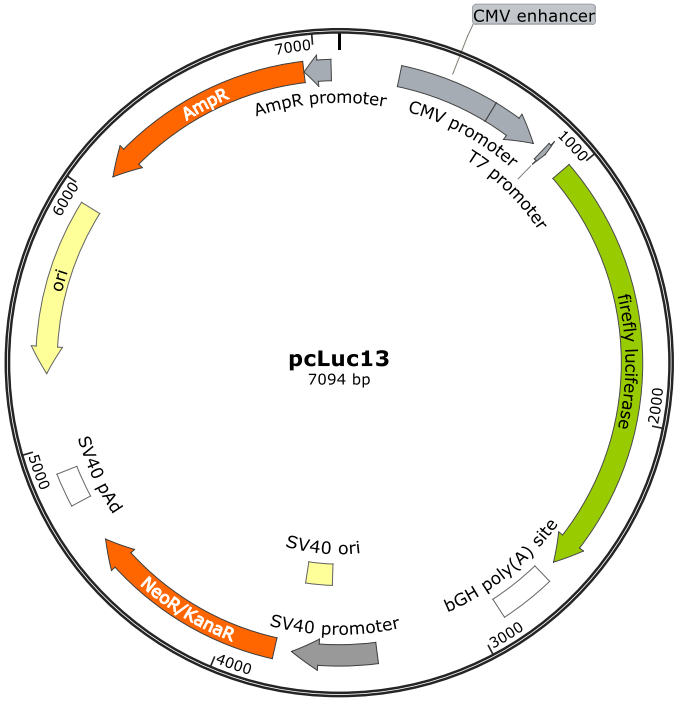


Figure 7.1-7 - pcLuc13

Expression plasmid with GFP reporter

7.1.3 RSV-F vaccine plasmids

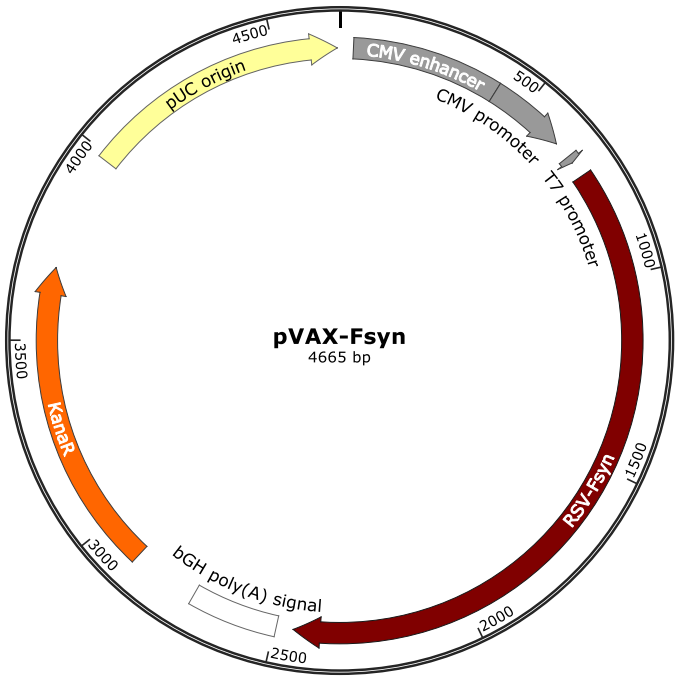


Figure 7.1-8 - pVAX-Fsyn

Vaccine expression plasmid coding for RSV-F

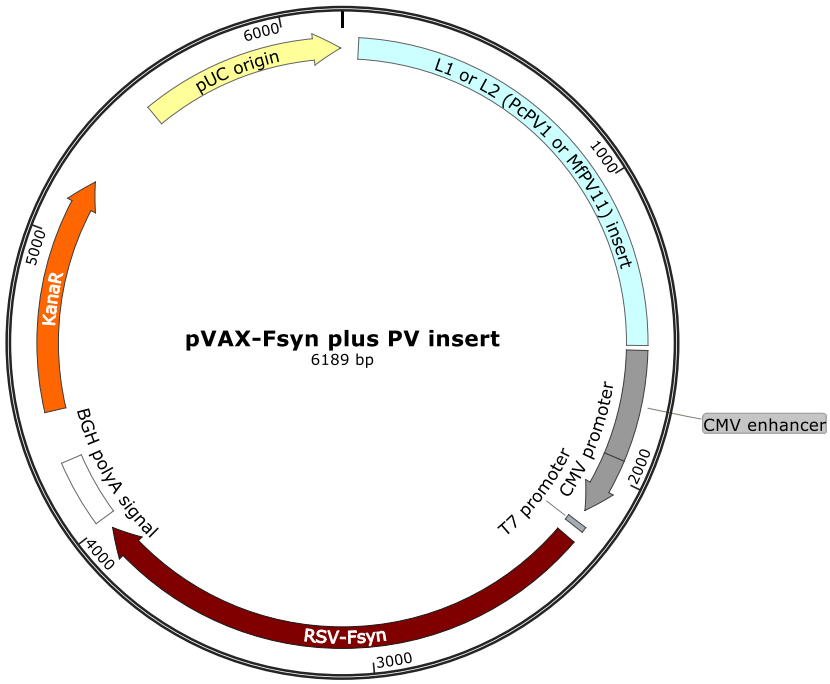


Figure 7.1-9 - pVAX-Fsyn (PV insert)

Vaccine expression plasmid coding for RSV-F. As additional insert, PcPV L1, PcPVL2, MfPV11 L2 or MfPV11 L2, respectively, have been cloned into the vector.

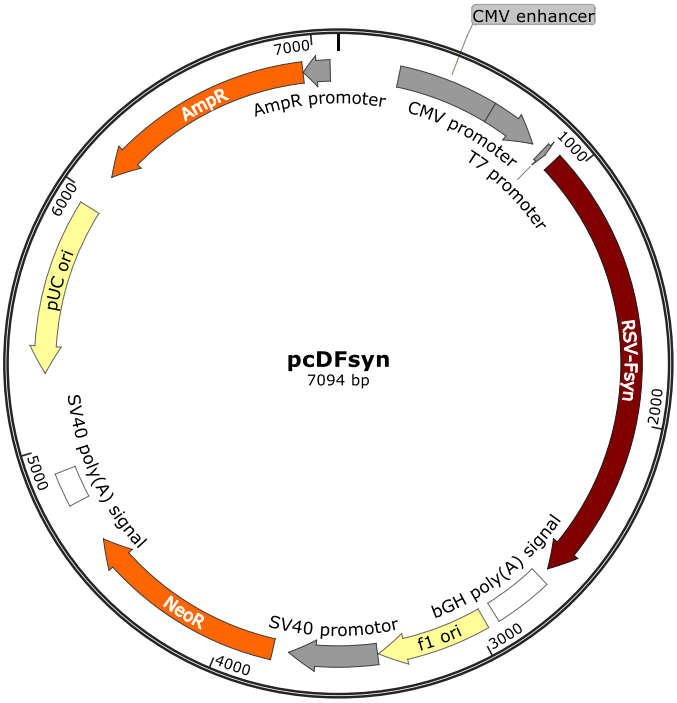


Figure 7.1-10 - pCDFsyn

Expression plasmid of RSV-Fsyn in pcDNA3 backbone.

7.1.4 Combination plasmids

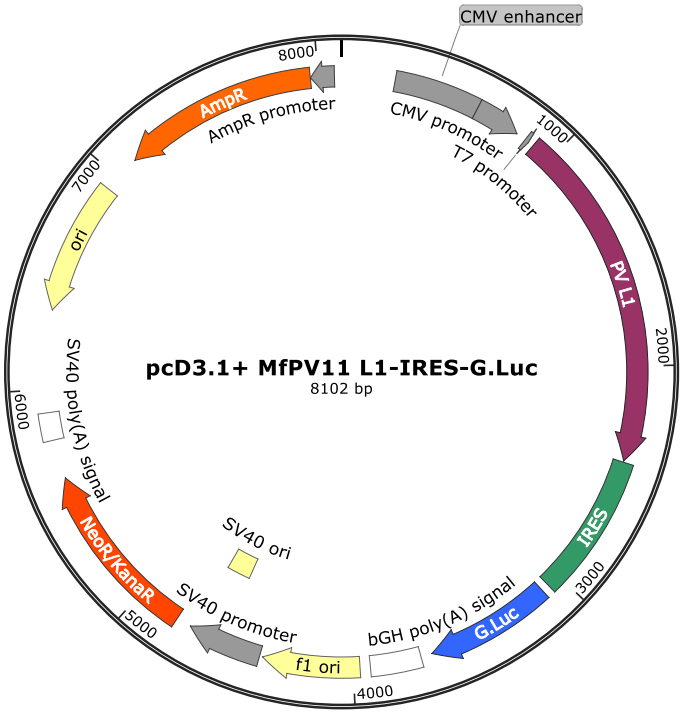


Figure 7.1-11 - pcD3.1+ MfPV11 L1-IRES-G.Luc

Expression plasmid coding for both papillomavirus L1 and G.Luc.

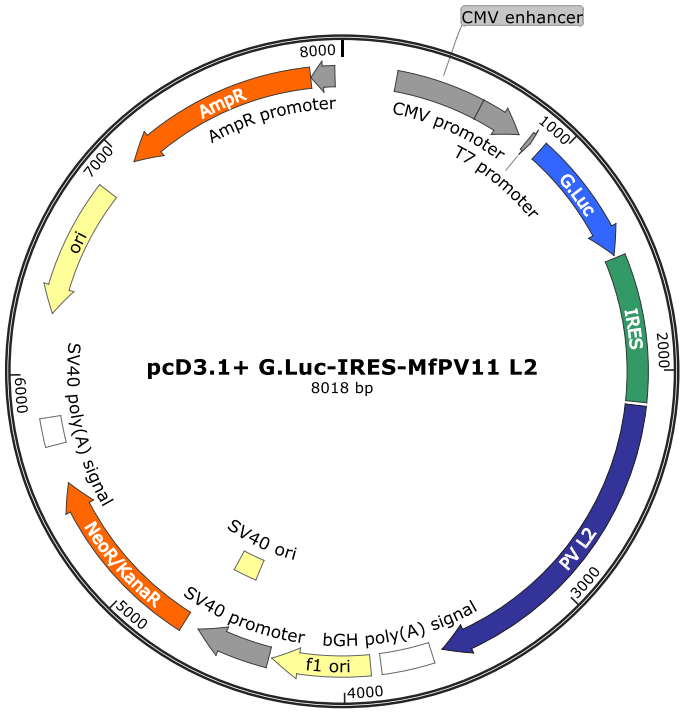


Figure 7.1-12- pcD3.1+ G.Luc-IRES-MfPV11 L2

Expression plasmid coding for both papillomavirus L2 and G.Luc.

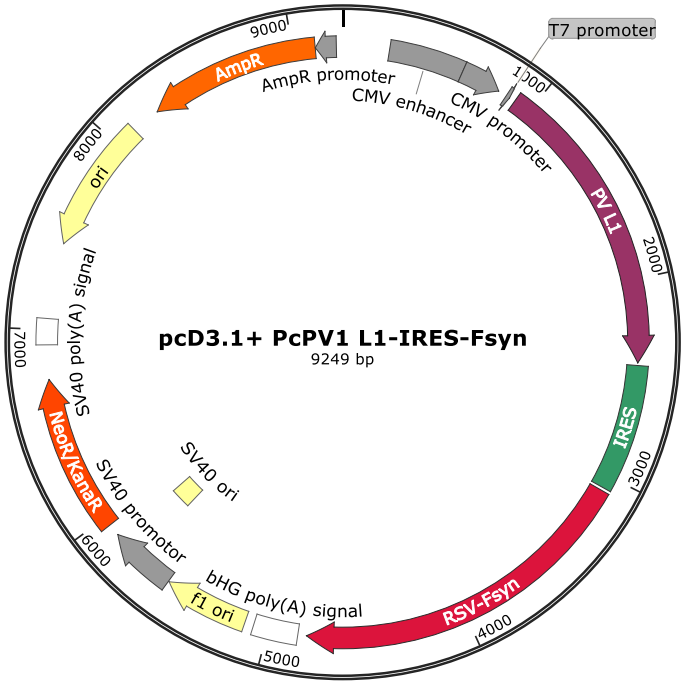


Figure 7.1-13 - pcD3.1+ PcPV L1-IRES-Fsyn

Expression plasmid coding for both papillomavirus L1 and RSV-Fsyn.

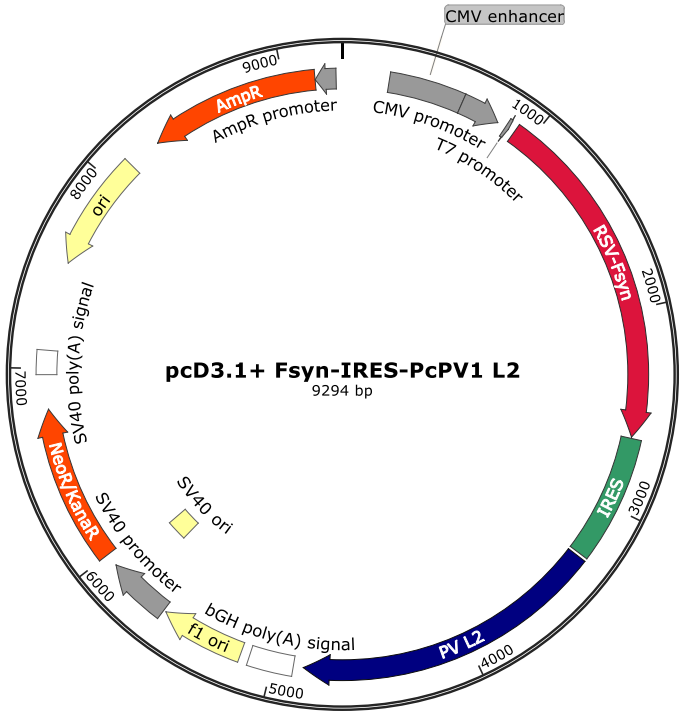


Figure 7.1-14 - pcD3.1+ Fsyn-IRES-PcPV L2

Expression plasmid coding for both papillomavirus L2 and RSV-Fsyn.

7.1.5 Commercial plasmids

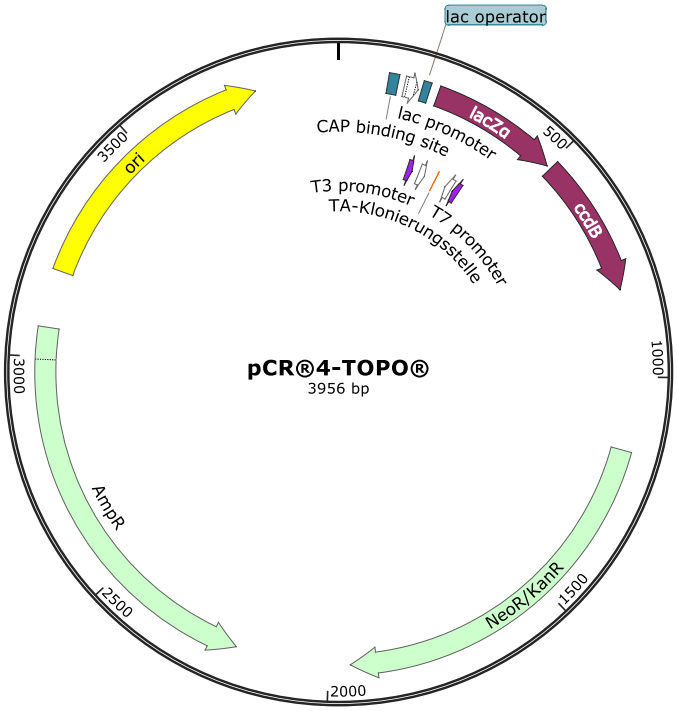


Figure 7.1-15 - pCR4-TOPO

ACKNOWLEDGEMENT

Mein größtes Dankeschön geht an Dr. Thomas Grunwald für die Möglichkeit, unter seiner Supervision mit vielen Freiräumen so spannende Fragestellungen zu bearbeiten. Es hat mir große Freude bereitet, nicht nur die experimentellen Abläufe, sondern auch den Aufbau des Labors aktiv mitgestalten zu können. Dafür, dass du immer ein offenes Ohr für mich hattest und dass wir alle Ergebnisse gemeinsam diskutiert haben, bin ich sehr dankbar. Auch die Möglichkeit, regelmäßig an Konferenzen teilzunehmen, hat mich wissenschaftlich sehr vorangebracht.

Prof. Dr. Sven-Erik Behrens danke ich ganz herzlich dafür, dass er die Betreuung dieser Dissertation als mein Doktorvater übernommen hat, und dass wir in regelmäßigen Abschnitten den Fortschritt dieser Arbeit gemeinsam besprechen konnten.

Vielen Dank an Prof. Dr. Frank Stubenrauch für seine Gutachtertätigkeit.

Bei Dr. Gerd Hause bedanke ich mich für die schönen elektronenmikroskopischen Aufnahmen der Papilloma VLPs am TEM.

Der gesamten AG Grunwald sowie der AG Wagner gilt mein großer Dank für die tolle Zusammenarbeit im Labor und für die Unterstützung bei der Umsetzung der Experimente. Wenke Fröhlich, Isabell Schulz, Jana Holm – vielen Dank für eure wertvolle Hilfe bei den vielen Neutralisationsassays, ELISAs, Zellkultur, etc. Leila Issmail danke ich für ihren konstanten Einsatz, große Hilfsbereitschaft und immer die Bereitschaft, Viruspräps zu teilen. Dankeschön an Jessica Gümpel für ihre tolle Mitarbeit im Papillomvirus-Projekt.

Der AG Ulbert bin ich dankbar dafür, dass wir die Laborräumlichkeiten bei so einem schönen Arbeitsklima teilen konnten. Vielen Dank an Dr. Jasmin Fertey und Steffen Jakob, dass ihr immer hilfreiche Ansprechpartner für Fragen methodischer Natur für mich wart. Bei Dr. Sebastian Ulbert möchte ich mich bedanken, dass ich die Gelegenheit zur Mitarbeit im spannenden ELVIRA-Projekt hatte und damit auch die Arbeit in einem institutsübergreifenden, interdisziplinären Projekt kennen lernen durfte.

Den Tierpflegern danke ich herzlich dafür, dass sie immer gut für das Wohl der Mäuse gesorgt haben. Ganz besonderer Dank an Jacqueline Krause dafür, dass auf sie einfach immer Verlass ist.

Nadine Didwischus und Nadja Lindner – danke, dass ihr so großartige Bürokollegen seid. Es war für mich immer wertvoll, bei einem so konzentrierten und produktiven Arbeitsklima am Schreibtisch sitzen zu können, und bei der ein oder anderen Tasse Kaffee gemeinsam die Gedanken zu sortieren.

Außerhalb der Institutsräumlichkeiten möchte ich ganz besonders meiner Familie für Ihren Rückhalt danken und für Ihre konstante Unterstützung bei all meinen Wegen und Umwegen.

Last but not least: Chris, you know why you’re in here. For essentially contributing to my mental health and being my best advisor. (And for simply being you.)

DECLARATION OF AUTHORSHIP

EIDESSTATTLICHE ERKLÄRUNG

Hiermit erkläre ich an Eides statt, dass ich mich bisher mit dieser Arbeit weder an der Martin-Luther-Universität Halle-Wittenberg noch an einer anderen Einrichtung um die Erlangung eines akademischen Grades beworben habe.
Ich versichere weiterhin, dass die vorliegende Arbeit selbstständig und nur unter Benutzung der angegebenen Quellen und Hilfsmittel erstellt wurde. Die den genutzten Werken wörtlich oder inhaltlich entnommenen Stellen sind als solche gekennzeichnet.

Leipzig, 30. April 2018

_____

Lea Bayer

CURRICULUM VITAE AND LIST OF PUBLICATIONS

Personal Information

Name	Lea Johanna Katrin (Wierich) Bayer
Date and place of birth	13 August 1987, Stuttgart, Germany
Nationality	Germany

Education

Fraunhofer IZI, Leipzig / Martin-Luther University Halle 05/2014 – 04/2018	Doctoral thesis Preclinical Validation, Dr. Thomas Grunwald *"Novel approaches to an RSV vaccine: Papillomavirus-based delivery of a genetic vaccine and low-energy electron irradiation for the production of a killed vaccine"*
University of Lübeck 10/2010 – 02/2013	Master of Science „Molecular Life Science" Final grade 1.5 („very good")
University of Lübeck 10/2007 – 10/2010	Bachelor of Science „Molecular Life Science" Final grade 1.8 („good")
Meadow Lake, Canada 08/2004 – 07/2005	Exchange year at Carpenter High School
Gymnasium Glinde 1998 - 2007	Abitur Final grade: 1.5

Practical Experience

Tulane University New Orleans, USA 04/2013 – 03/2014	Research scholar at the Applied Stem Cell Lab *„Role of the CCR5-receptor in LTR activation of HIV-infected cells"*
Universität Münster / OxProtect GmbH 05/2012 – 02/2013	Master Thesis *"Studies on the FDP-lysine formation on the surface of platelets and methods for detection of misfolded proteins"* (grade: 1.0 - "very good")
Fraunhofer EMB, Lübeck 10/2010 – 07/2011	Student research assistant

Nanyang Technological University, Singapur 08/2011 -11/2011	Internship at School of Biological Sciences *"Respiratory Syncytial Virus G-Protein: Role of the N-Terminus and Interaction with Caveolin-1"*
Centro de Investigación I-Mar, Puerto Montt, Chile 12/2011 – 02/2012	Internship *„Set-up of a cell culture laboratory and establishment of cultivation of cells obtained from fish organs"*
Fraunhofer EMB, Lübeck/ University of Lübeck 04/2010 – 07/2010	Bachelor Thesis *"Establishment of the cultivation and electrophysiological measurement of porcine heart slices"* (grade: 1.0 - "very good")

Leipzig, 30. April 2018

_____

Lea Bayer

List of Publications

Parts of this thesis were published in scientific journals:

Lea Bayer, Jessica Gümpel, Gerd Hause, Martin Müller, Thomas Grunwald (2018): Non-human papillomaviruses for gene delivery in vitro and in vivo. In: PLOS ONE.
DOI: 10.1371/journal.pone.0198996.

Lea Bayer, Jasmin Fertey, Sebastian Ulbert, Thomas Grunwald (2018): Immunization with an adjuvanted low-energy electron irradiation inactivated respiratory syncytial virus vaccine shows immunoprotective activity in mice. In: Vaccine. DOI: 10.1016/j.vaccine.2018.02.014.

Jasmin Fertey, Lea Bayer, Thomas Grunwald, Alexandra Pohl, Jana Beckmann, Gaby Gotzmann, Javier Portillo Casado, Jessy Schönfelder, Frank-Holm Rögner, Christiane Wetzel, Martin Thoma, Susanne M. Bailer, Ekkehard Hiller, Steffen Rupp, Sebastian Ulbert (2016): Pathogens Inactivated by Low-Energy-Electron Irradiation Maintain Antigenic Properties and Induce Protective Immune Responses. In: Viruses 8 (11). DOI: 10.3390/v8110319.

Conferences – oral presentations

Lea Bayer, Jessica Gümpel, Martin Müller, Thomas Grunwald. Non-human papillomaviruses for gene delivery in vitro and in vivo. 28th Annual Meeting of the Society for Virology from 14-17 March 2018 in Würzburg, Germany

Sarah Wilmschen, Sabrina Schneider, Lea Bayer, Thomas Grunwald, Dorothee von Laer, Janine Kimpel. VSV-GP as vaccine vector for RSV. 28th Annual Meeting of the Society for Virology from 14-17 March 2018 in Würzburg, Germany

Jasmin Fertey, Lea Bayer, Ekkehard Hiller, Susanne Bailer, Steffen Rupp, Alexandra Pohl, Christiane Wetzel, Thomas Grunwald, Sebastian Ulbert. Inactivating viruses with low-energy electron irradiation for vaccine production. 15th workshop of the study group “Immunobiology of Viral Infections” of the Society of Virology in Tauberbischofsheim, September 28-30, 2016

Conferences – poster presentations

Leila Issmail, Lea Bayer, Thomas Grunwald. Immunization with DNA vaccines encoding different variants of human respiratory syncytial virus glycoproteins. 28th Annual Meeting of the Society for Virology from 14-17 March 2018 in Würzburg, Germany

Lea Bayer, Jasmin Fertey, Ekkehard Hiller, Susanne Bailer, Steffen Rupp, Alexandra Pohl, Christiane Wetzel, Sebastian Ulbert, Thomas Grunwald. Inactivation of Influenza A Virus and RSV via Low-Energy Electron Irradiation Provides a Versatile Method for Efficacious Vaccine Production. 11th Vaccine Congress, 17-20 September 2017, San Diego, CA, USA

Lea Bayer, Jasmin Fertey, Ekkehard Hiller, Susanne Bailer, Steffen Rupp, Alexandra Pohl, Christiane Wetzel, Sebastian Ulbert, Thomas Grunwald. Inactivation of Influenza A Virus and RSV via Low-Energy Electron Irradiation Provides a Versatile Method for Efficacious Vaccine Production. 27th Annual Meeting of the Society for Virology from 22-25 March 2017 in Marburg, Germany

Lea Bayer, Jasmin Fertey, Sebastian Ulbert, Thomas Grunwald T. Immunization of low energy electron irradiation inactivated respiratory syncytial virus vaccine shows immunoprotective activity. ReSViNET; RSV VACCINES FOR THE WORLD MEETING 2017, 29 November - 1 December 2017, Malaga, Spain

Lea Bayer, Nadja Lindner, Jasmin Fertey, Anna Leichsenring, Eckehard Hiller, Susanne Bailer, Steffen Rupp, Alexandra Pohl, Christiane Wetzel, Franziska Lange, Sebastian Ulbert, Thomas Grunwald. Vaccine and Drug Testing by Viral Infection Models in Mice. Fraunhofer Life Science Symposium, 8-9 September 2017, Leipzig, Germany

Lea Bayer, Nadja Lindner, Jasmin Fertey, Anna Leichsenring, Eckehard Hiller, Susanne Bailer, Steffen Rupp, Alexandra Pohl, Christiane Wetzel, Sebastian Ulbert, Franziska Lange, Thomas Grunwald. Vaccine and drug testing by viral infection models in mice. World Preclinical Congress Europe, 15-17 November 2016, Lissabon, Portugal

Patents

The gene transfer technology based on non-human papilloma PsVs (section 4.1) is patented under Deutsche Patentanmeldung Nr. 10 2018 220 276.9