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# Studies on polyomavirus virus-like particles

-as vaccines and vectors for immune and gene therapy

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## ABSTRACT

Virus-like particles (VLPs) are similar to natural virus particles except that they lack viral genes. They have a similar cellular uptake to the natural virus from which they are derived but are non-infectious and can therefore not reproduce themselves. VLPs have been used as a model to understand viral entry, infection and cell tropism, but have also been shown to be useful in other areas e.g. as vaccines against viral infection, as well as carriers for molecules in immune and gene therapy.

This thesis is based on VLPs from two related viruses, murine polyomavirus (MPyV) and murine pneumotropic virus (MPtV) and the aim has been to investigate their possible use as viral vaccines and as vectors in gene and immune therapy.

Both viruses consist of only a few genes and a protein capsid surrounding them. MPyV was discovered when it was shown to induce tumors in mice, thereof the name, “poly oma”, Greek for many tumors. It is easy to grow in cell culture, and because of its oncogenic potential and its small size it has been an important research tool in molecular biology. Studies on MPyV have led to many discoveries in understanding cellular events like DNA replication, cell growth regulation, and genes involved in tumor development. The MPtV on the other hand, is non-oncogenic, is difficult to grow and has not been well studied. We have been successful in producing VLPs from both these viruses, and used them as immunogens and as carriers for tumor antigens. They should both be suitable for therapeutic use in humans, since they are of non-human origin, and humans have no pre-existing immunity against them.

In the first paper, the aim was to optimize MPyV-vaccination by examining the importance of the route of administration and the VLP structure. All, even immune deficient, mice were protected against subsequent MPyV infection by changing from intraperitoneal to subcutaneous VLP vaccination. Furthermore, VLPs were more efficient, than the more linear GST-VP1 in viral protection and in the induction of an antibody response. The conclusion from this study was that, the route of administration, and the antigenic structure are important.

This antibody response is of value for MPyV vaccination, but more of an obstacle when using MPyV-VLPs as antigen carriers, where the antibodies may abolish the effect of a similar second treatment. Therefore the aim of the second study was to study VLPs from MPtV, as a possible complement to e.g. MPyV-VLPs. MPtV-VLPs were successfully produced; they entered all cell types tested and did not cross-react with MPyV-VLPs, which make them suitable as complement to MPyV-VLPs in prime-boost therapy.

In the third paper MPyV-VLPs carrying the oncoprotein Her2, Her2<sub>1-683</sub>PyVLPs, were successfully produced and were used to vaccinate against Her2-expressing tumors. Vaccination with Her2<sub>1-683</sub>PyVLPs efficiently protected mice from tumor outgrowth of the transplantable Her2 positive tumor D2F2/E2, as well as against spontaneous mammary carcinoma outgrowth in BALBneuT mice, transgenic for rat Her2.

In the fourth study, Her2<sub>1-683</sub>PyVLP vaccination was compared to vaccination with Her2<sub>1-683</sub>PyVLPs loaded on dendritic cells (DCs), with regard to efficiency and to anti-VLP response. Vaccination with Her2<sub>1-683</sub>PyVLP loaded DCs was more efficient in protecting mice against outgrowth of D2F2/E2 tumor where a lower Her2<sub>1-683</sub>PyVLP dose was sufficient for full protection, compared to vaccination with Her2<sub>1-683</sub>PyVLPs alone. Furthermore; vaccination with Her2<sub>1-683</sub>PyVLP loaded DCs resulted in lower anti-VLP titers.

In conclusion, VLPs derived from mouse polyomaviruses can be used to vaccinate against a subsequent polyoma infection. Moreover, they can be used as carriers for molecules in immune and gene therapy. We show that VLPs have substantial potential for use in cancer immune therapy, and that MPyV-VLPs and MPtV-VLPs, due to lack of cross-reactivity, should be complementary and suitable for prime-boost therapy.

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## LIST OF ABBREVIATIONS

a.a.	Amino acid
APC	Antigen-presenting cell
BKV	BK virus
Bp	Base pair
BTV	Bluetongue virus
CD	Cluster of differentiation molecule
CsCl	Cesium chloride
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DNA	Deoxyribonucleic acid
EC	Extra cellular
ELISA	Enzyme linked immunosorbent assay
GST-VP1	Glutathione-S-transferase-VP1
GM-CSF	Granulocyte macrophage-colony stimulating factor
HA	Hemagglutination
HAI	Hemagglutination inhibition
HPV	Human papillomavirus
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
i.p.	Intraperitoneal
JCV	JC virus
Kb	Kilo base pair
LT	Large tumor antigen
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
MLV	Murine leukemia virus
MT	Middle tumor antigen
MPtV	Murine pneumotropic virus
MPyV	Murine polyomavirus

PBS	Phosphate buffered saline
PML	Progressive multifocal leukoencephalopathy
PCR	Polymerase chain reaction
p.i.	Post infection
pRb	Retinoblastoma protein
RNA	Ribonucleic acid
s.c.	Subcutaneous
SCID	Severe combined immunodeficiency
ST	Small tumor antigen
SV 40	Simian virus 40
TAA	Tumor associated antigen
TCR	T-cell receptor
TM	Trans membrane
VLP	Virus-like particle
VP	Viral protein

## LIST OF ORIGINAL PAPERS

- 1. Vlastos A, Andreasson K, Tegerstedt K, Hollanderova D, Heidari S, Forstova J, Ramqvist T, Dalianis T.** VP1 pseudocapsids, but not a glutathione-S-transferase VP1 fusion protein, prevent polyomavirus infection in a T-cell immune deficient experimental mouse model. *J Med Virol.* 2003 Jun;70(2):293-300
- 2. Tegerstedt K, Andreasson K, Vlastos A, Hedlund KO, Dalianis T and Ramqvist T.** Murine pneumotropic virus VP1 virus-like particles (VLPs) bind to several cell types independent of sialic acid residues and do not serologically cross react with murine polyomavirus VP1 VLPs. *J Gen Virol.* 2003 Dec;84(Pt 12):3443-52
- 3. Tegerstedt K, Lindencrona JA, Curcio C, Andreasson K, Tullus C, Forni G, Dalianis T, Kiessling R and Ramqvist T.** A single vaccination with polyomavirus VP1/VP2Her2 virus-like particles prevents outgrowth of HER-2/neu-expressing tumors. *Cancer Res.* 2005 Jul 1;65(13):5953-7
- 4. Tegerstedt K, Franzén A, Ramqvist T and Dalianis T.** Dendritic cells loaded with polyomavirus VP1/VP2Her2 virus-like particles (VLPs) efficiently prevent outgrowth of a Her2/neu expressing tumor without inducing high anti-VLP serum titers. *Manuscript*

## **General aim**

The general aim of this thesis was to use virus-like particles (VLPs) derived from the murine polyomavirus (MPyV) and the murine pneumotropic virus (MPtV) as vaccines and as vectors in gene and immune therapy.

## **General introduction**

Viruses are small, intracellular organisms that consist of genetic material (RNA or DNA) and a protein shell surrounding it, the capsid. In some viruses a lipid membrane, an envelope, further encloses the capsid. Viruses are dependent on their host for survival and reproduction. In general, viruses enter the host and undergo massive reproduction at the infection site. Normally the immune system of the host manages to remove the virus within a few weeks, but some viruses can hide latently in the host cells for a long time and become reactivated much later, e.g. members of the herpes virus family[1].

Some viruses are associated with tumor development, for example members of the polyoma- and papilloma virus families. These viruses produce proteins that interact with tumor suppressor proteins, e.g. p53 and pRb, and this interaction may lead to uncontrolled cell growth. This is of benefit for the virus though it then can spread more readily; however, worse for the host since it can lead to tumor development. One of the best-known correlations between virus and tumor development is the one in cervix cancer, where human papillomavirus (HPV) is responsible for around 93 % of the cases [2].

Virus-like particles (VLPs) from different viruses are similar to authentic virus particles, with the difference that they lack viral genes and therefore are not able to reproduce themselves in the host. However, VLPs mimic the native virus and are potent inducers of the immune response with massive antibody production, and can therefore be used as vaccines against virus infection. There are ongoing clinical trials where HPV-VLPs are showing protection against HPV infection and these HPV-VLP vaccinations may therefore also protect against cancer [3, 4].

In addition, VLPs can be used in other areas. Because they are empty and lack DNA they can act as carriers for foreign DNA in gene therapy. Moreover, the efficiency by which they stimulate cellular and antibody responses have made them candidates as carriers for antigens for other diseases as well. One example is as carriers for a tumor antigen in cancer immunotherapy. However, in immune and gene therapy the substantial antibody response obtained after injection may limit the effect of a subsequent similar treatment. Therefore, the use of several different vectors or administration ways would be of benefit.

This thesis is based on studies on VLPs derived from the murine polyomavirus (MPyV) and the murine pneumotropic virus (MPtV). We have used MPyV-VLPs as vaccines against MPyV infection. We have also produced VLPs from MPtV in order to investigate if they can be used to complement MPyV-VLPs in immune and gene therapy. Finally, to investigate the possible use of VLPs in immunotherapy, we have constructed MPyV-VLPs carrying the tumor antigen Her2 and used them to vaccinate against Her2-expressing tumors.

# INTRODUCTION

## Viruses

Viruses are disease-causing organisms that are totally dependent on their host for survival and reproduction since they only can multiply inside cells. The first viruses were discovered in the end of the 19<sup>th</sup> century when the Russian botanist Iwanowski showed that extract from a diseased plant could transmit the disease to healthy plants even after passage through fine filters. By then a virus (from the Latin for poison) was referred to as “soluble living germ” and in 1898 Loeffler and Frosch showed that these agents also were responsible for foot-and-mouth disease in cattle, for historical review on viruses see Cann 2001 [1].

Viruses are the smallest known organisms and it was not until the 1930s that it was possible to separate viruses from their host cell material. After studies in bacterial viruses, e.g. bacteriophages, in the 1940s, it was confirmed that viruses consist of genetic material (RNA or DNA) and a protein shell, the capsid, which surrounds it [5]. The capsid helps to transport the virus and it also protects the virus from degradation when it is outside cells. Many viruses also have a lipid envelope surrounding the capsid. Each virus type can only infect a limited range of hosts and some viruses can only infect one species. Viruses are also normally tissue specific, for example some common cold viruses only infect cells of the upper respiratory tract.

Isolated virus particles are by definition unable to reproduce without assistance, since they lack enzymes and all other equipment necessary for making their own proteins. However, when they enter cells of a host, normally via the respiratory, gastrointestinal or genitourinary tract, they take control over the cellular machinery and undergo massive reproduction at the infection site. This virus multiplication is often lethal to the infected cell, which then lyses and releases numerous new virus particles that in turn can infect new cells. Many of the symptoms of a viral infection are due to this tissue damage, but also due to activation of the immune system, e.g. inflammation responses [6].

In general, the immune system manages to clear out the virus within some weeks. However, some viruses do not kill the cell; instead they are able to integrate their genes into the host genome and can stay more or less dormant in the host, and induce a persistent virus infection. Some such viruses may carry genes that regulate cell growth and can potentially transform normal cells to tumor cells; hence they are called tumor viruses [7].

## **Viruses and Cancer**

Shortly after the first viruses were discovered, some of these tiny agents were found to induce tumors in animals. The first tumor viruses were discovered in fowls by Ellermann *et al.*, in 1908 [8] and by Rous in 1911 [9]. Furthermore, in the 1930s Rous *et al.*, infected rabbits with cottontail rabbit papillomavirus and found that the rabbits developed tumors [10]. In 1951 Gross found the first murine tumor virus, the murine leukemia virus (MLV) [11]. Regarding human tumor viruses, it was not until 1976, that Zur Hausen could demonstrate that human papillomavirus (HPV) causes cervical cancer [12]. Today a number of tumor viruses have been identified and these include members of the retrovirus, papovavirus, adenovirus and herpesvirus groups [7].

About 15 % of all cancer cases are estimated to be caused by viruses. The strongest correlation is the one between HPV and cervical cancer where the virus has been estimated to be responsible for 93 % of the cases [2]. Moreover, liver cancer is linked to Hepatitis B and C virus infection [13]. Epstein-Barr virus (Burkitt's lymphoma), human herpes virus (Kaposi's sarcoma) and human T-lymphotropic retrovirus (leukemia) also represent identified human tumor viruses [7].

The link between viruses and different cancers has been studied in cell culture and animal models. A number of viral genes have been identified directly involved in triggering cancerous characteristics in cells. Many of these genes, viral oncogenes,

are not unique for tumor viruses; versions of these genes are also found in normal cells, where they are called proto-oncogenes. Proto-oncogenes code for proteins that regulate cell growth, and mutations in these genes can lead to uncontrolled cell growth and tumor development. However, tumor viruses do not necessarily need to carry their own oncogenes: they have evolved to interact with the host cell's proto-oncogenes; thereby increasing the rate of cell division in order to enhance their own reproduction [7].

## **Polyomaviruses**

Today at least 13 different polyomaviruses have been described in vertebrates, for a review see [14]. These viruses have been isolated in humans, monkeys, baboons, cattle, rodents, birds and geese. This thesis will however concentrate on the two murine polyomaviruses, the murine polyomavirus (MPyV) and the murine pneumotropic virus (MPTV), which both will be described in more detail later.

### *Human polyomaviruses*

More than 80 % of the human population is infected with the human polyomavirus JC virus (JCV) [15]. Infection occurs during early childhood and normally the virus remains latent throughout life. However, in immunosuppressed individuals it can be reactivated and can cause a lethal disease of the central nervous system, progressive multifocal leukoencephalopathy (PML) [16]. JCV was discovered in one such patient in 1971 [17]. Prior to the AIDS epidemic, PML was an extremely rare disease, but there are reports that between 5-8 % of HIV infected patients will develop PML [18]. JCV is also able to infect and transform primary cell cultures and JCV infection in laboratory animals can lead to tumor development. JCV infection has also been associated to human cancer but so far not consequentially and convincingly [19].

The second human polyomavirus, BK-virus (BKV) was first discovered in a renal patient with hemorrhagic cystitis [20]. After primary infection, BKV remains latent

in the kidneys but can be reactivated under certain immune deficiency conditions, for example in transplantation patients. BKV is able transform cells in culture and has been shown to be onogenic in mice, and has therefore been implicated in human tumors [21].

### *Simian Virus 40*

Simian virus 40 (SV40) is a virus naturally infecting rhesus macaques, but that has been implicated in human diseases, for review see Shah 2004 [22]. SV40 infection has been shown to transform cells in culture and to cause tumors in laboratory animals. The human poliovirus vaccine, which had been cultivated in renal cell lines from rhesus macaques, was later shown to be contaminated by SV40. This vaccine had been given to 100 million people in Europe and USA between 1955 and 1963. Therefore, the possibility that SV40 may cause human disease, particularly cancer, has been under debate since then. For example, SV40 has been described to be present in mesotheliomas in humans [23], but fortunately so far a major role of this virus in human tumors has not been established.

### **Murine polyomavirus**

The murine polyomavirus (MPyV) was discovered independently by Gross and Stewart in the early 1950s [11, 24]. This virus was able to induce many kinds of tumors in several tissues and thereof the name “poly oma” from the Greek words “many tumors” [25, 26].

MPyV was demonstrated to be easy to grow in cell culture, and because of its small genome, it was ideal for studies in molecular biology. The genome of MPyV was completely sequenced by Soeda already in the end of the 1970s [27, 28]. MPyV is a non-enveloped, double stranded DNA virus with a genome of 5.3 Kb, for review see [14]. Its genome is divided into an early, a late, and a regulatory region, see figure 1. The regulatory region contains the origin of replication and the enhancer. The early region of the genome comprises the genes coding for the proteins



[30]. It has been demonstrated that after separation and purification of the VP1 protein from the rest of the virus, the VP1 molecules are capable to self-assemble into empty virus-like particles (VLPs) [29]. Later, it was shown that it was possible to further increase the VLP production by expressing the VP1-gene in a eukaryotic baculovirus insect cell system, where the VP1-proteins self-assemble and form empty virus-like particles (VLPs) [31].



Figure 2. Structure of the polyomavirus capsid. Adopted from Cann 2001 [1].

### **Murine pneumotropic virus**

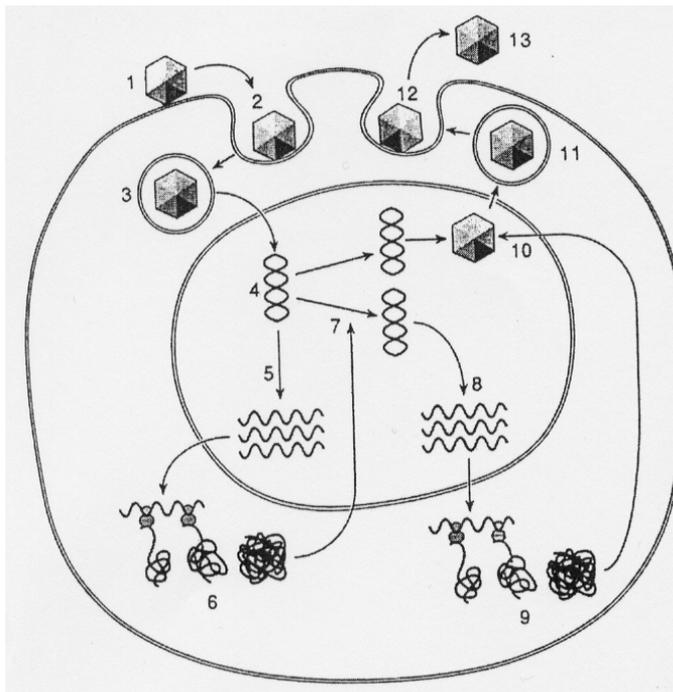
Murine pneumotropic virus (MPtV), also known as K-virus or Kilham polyomavirus, was the second murine polyomavirus to be identified. It was isolated in 1953 by Kilham and Murphy [32]. In contrast to MPyV it is not a tumor virus [33], it causes a lethal pneumonia in newborn mice, while in older animals it induces a non-apparent persistent infection [34]. In primary infection, MPtV infects the lung, liver and spleen. It has been shown to replicate in vascular endothelial cells, but later during persistence it is mainly found in the kidney [35].

The genome of MPtV consists of circular double stranded DNA of approximately 5 Kb. Its genome codes for three capsid proteins (VP1-VP3), and for small T and large T. However, in contrast to MPyV but similar to SV40, it lacks middle T [36]. The amino acid sequence of MPtV shows a close homology of 44 % with SV40, as compared to 36 % with MPyV. Nevertheless, the major capsid protein VP1 of MPtV has a 45 % homology to VP1 of MPyV [36].

MPtV has been less studied than MPyV, perhaps mostly since MPtV is non-oncogenic, but also since the virus is difficult to grow in cell culture [33]. MPtV has been suggested to only infect endothelial cells, whereas MPyV infects a wide range of cell types [37]. Still, the viral receptor is yet to be determined for MPtV. Moreover, prior to this thesis, attempts to produce VLPs by expression of the MPtV-VP1 gene had not been made.

### **Viral entry**

A schematic view of virus cell entry is presented in figure 3. The entry of animal viruses into their host cells starts with binding of the particles to receptors, consisting of lipids, proteins, or carbohydrates, on the cell surface. Often, the binding of the virus is followed by receptor-mediated endocytosis of the virus into the cytosol [38]. Tsai *et al* 2003 recently demonstrated that the receptor for MPyV consists of specific gangliosides [39] and it enters the cell via clathrin-independent, cholesterol-dependent endocytosis [40]. Mannova *et al* 2003 showed that VLPs enters cells in the same way as natural MPyV particles. They enter the cell in smooth monopinocytic vesicles that co-localize with the endoplasmic reticulum and accumulate on membranes around the cell nucleus. They also showed that after infections with MPyV, only a few virus particles delivered their DNA into the nucleus, and that most of the viral particles were degraded in the cytosol without ever entering the nucleus [41].



1. Attachment to the cell surface
2. Receptor-mediated endocytosis
3. Transport in smooth monopinocytotic vesicles
4. Early transcription
5. Translation of T-antigens
6. DNA replication
7. Late transcription
8. Translation of capsid proteins
9. Production of capsid proteins
10. Virus assembly
11. Virus export
12. Release of virus
13. Free virus

Figure 3. Viral cell entry and reproduction. Adopted from Cole *et al* 2001 [14].

Most of the polyomavirus have a sialic acid component in their receptors, for example MPyV [42], BK-virus [43], JC-virus [44, 45], and lymphotropic polyomavirus [46]. The receptors are therefore sensitive to the enzyme neuroaminidase and treatment of cells with neuroaminidase inhibits viral entry of these viruses. However, in contrast to all polyomaviruses described above, SV40 is the only known polyomavirus with a neuroaminidase resistant receptor [47] and MHC class I has been reported to be one receptor for SV40 [48]. As mentioned above, the receptor for MPtV is not known.

### Virus-like particles

Virus-like particles (VLPs) are similar to natural virus particles except that they lack viral genes. They are non-infectious, but have similar morphology and cell tropism as the natural virus, from which they are derived. They also show comparable cellular uptake and intracellular trafficking as the natural virus [49].

VLPs have been used as a model to understand viral entry, infection and cell tropism, and have in addition been shown to be useful in other areas as well; as vaccine against viral infection, as well as vectors for immune and gene therapy.

### *History*

When Crawford *et al* 1962 were purifying MPyV that had been produced in mouse cells, by centrifuging on a CsCl-gradient; they found two bands with different densities. They were able to separate the two bands and the band with the lower density was shown to consist of empty capsids without any genes [50].

When Michel *et al* [51] and Winicour [52] in the late 1960s, infected mouse kidney cells in culture with MPyV, they found that the cells were not only producing virions with MPyV DNA, but also produced particles carrying host DNA, and they called these latter particles pseudovirus. They also realized that these particles could be of potential value in gene therapy, since they could carry genes from one animal cell to another [51, 53].

Viral capsid proteins can be expressed in bacteria and eukaryotic cells, where they self-assemble into virus-like particles (VLPs), also known as pseudocapsids. Already in 1978 the MPyV major capsid protein VP1, chromatographically separated and purified, was shown to form pentamers that appeared as native virus pentomers [29]. In 1986 Salunke *et al* were able to express the VP1 gene in bacteria and after purification, VP1 molecules associated into pentamers, that subsequently self-assembled into capsid-like structures of the same size and shape as native virus particles [54]. They therefore concluded that the minor capsid proteins VP2 and VP3 were not required for capsid formation. Montross *et al* 1991 were later producing VLPs by expressing the VP1 gene in insect cells by baculovirus expression of VP1 [55].

VLPs that lack viral DNA and RNA have been produced from different viruses, e.g. HPV [56], hepatitis B [57], HIV [58, 59] and bluetongue [60]. VLPs can effectively vaccinate against the analogous virus. In addition, VLPs can also be used as carriers for other molecules, for example foreign DNA in gene therapy or

proteins in immunotherapy. The different use of VLPs will be discussed in more detail later.

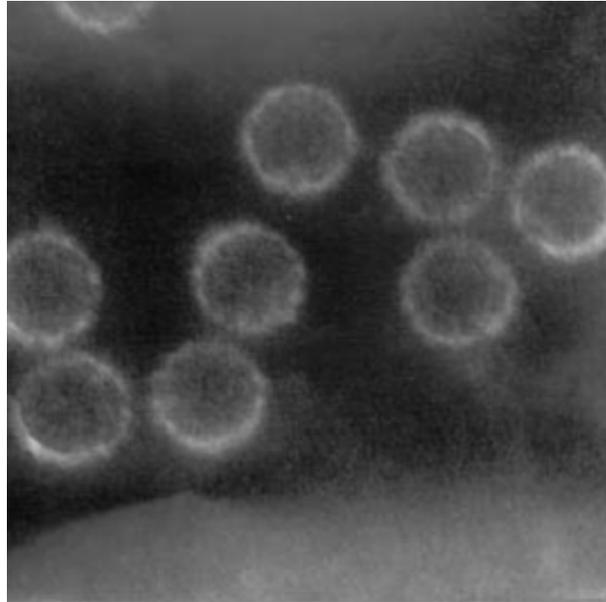


Figure 4. Purified VLPs demonstrated with electron microscopy [61].

### **Introduction to the immune system**

The immune system is a defense system that protects all animals from dying of infections from e.g. viruses, bacteria, parasites or fungi. The immune system should respond to foreign and it is important that it can separate self from non-self. Occasionally the immune system responds to harmless and non-infectious antigens, resulting in allergy. Sometimes, it may also react against self-antigens, leading to development of autoimmune diseases.

The immune system can be divided into an innate (non-specific) and an adaptive (specific) defense. The innate response is defined as a fast response that represents a first line of defense against a foreign pathogen. It includes anatomic (skin), physiological (temperature, pH and chemical), phagocytic (cells) and inflammatory

barriers. The adaptive response is slower, but can selectively eliminate a specific antigen. The specific response also induces immunologic memory, leading to immunity against a certain pathogen. The innate and adaptive responses cooperate, where the innate response keeps an infection under control long enough for an adaptive response to be generated.

### *The major histocompatibility complex (MHC)*

The major histocompatibility complex (MHC), also known as transplantation antigens, consists of molecules on the cell surface and they present self or non-self peptides to the immune system. This will help the immune system to determine, which cells that should live and which cells that should be eliminated. The MHC molecules are divided into two classes I and II. MHC class I molecules are expressed on nearly all cells and generally present intracellular particles, e.g. viruses, to the immune system, see figure 5. MHC class II molecules are mainly expressed on antigen presenting cells (APCs) and mainly present extracellular particles, e.g. bacterial toxins, to the immune system.

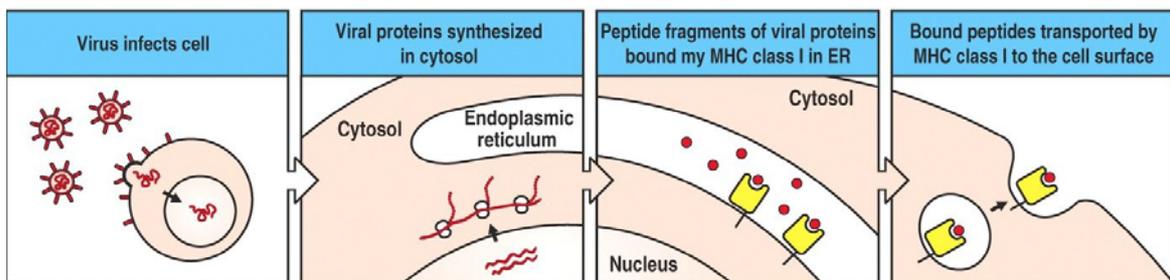


Figure 5. Cells present viral derived antigen on their MHC class I molecules. Adopted from Howard 2005 [62].

### *T lymphocytes*

T lymphocytes (T cells) arise from the bone marrow and mature in the thymus, for historical review on lymphocytes see Gowans 1996 [63]. Each T cell expresses a unique antigen-binding molecule, a T cell receptor (TCR) that recognizes antigen bound to the MHC molecule. In the thymus T cells undergo selection, and if the

TCR on a specific T cell recognizes a self-antigen, that particular T cell will be deleted.

### *T helper cells and cytotoxic T cells*

T cells are roughly divided into T helper cells (Th) that express CD4 molecules and cytotoxic T cells (CTL) that express CD8 molecules. When a CD4<sup>+</sup> T cell has recognized an antigen on a MHC II molecule, it matures into either a Th1 or a Th2 cell. Th1 cells are mainly involved in cellular immunity and are defined by the cytokines (signal molecules) they produce, e.g. IFN- $\gamma$ , IL-2 and TNF- $\beta$ . Th2 cells are most important in the humoral (antibody) response and produce the cytokines IL-4, IL-5 and IL-10. When a naïve CTL encounter an antigen it will mature into an effector CTL and expand in number. Those mature effector CTLs will then kill cells expressing that antigen, see figure 7.

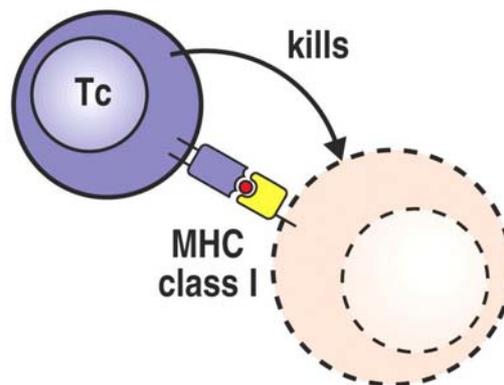


Figure 6. Cytotoxic T cells recognize antigen presented by MHC class I molecules.  
Adopted from Howard 2005 [62].

### *Regulatory T cells*

A subpopulation of CD4<sup>+</sup> T cells express CD25 and are called regulatory T cells. Regulatory T cells were first shown to be immunosuppressive by Sakaguchi *et al.* 1995 [64], where experiments in mice revealed that depletion of these cells induced several different autoimmune diseases. Subsequent depletion studies in mice have shown that regulatory T cells also inhibit anti-tumor immunity, since depletion improved tumor-specific immunity [65].

### *B lymphocytes*

B lymphocytes (B cells) arise and mature in the bone marrow. Each B cell expresses a unique antigen-binding receptor, a membrane-bound antibody, on its surface. When a B cell encounter an antigen that matches its receptor the B cell gets activated and starts to divide and differentiate into either a plasma cell or a memory B cell. Plasma cells produce large quantities of secreting antibodies that attack pathogens. Memory B cells keep their antibodies membrane bound, but they are long lived and are ready to get activated if the same pathogen will intrude the host once more [62].

### *Dendritic cells*

Dendritic cells (DCs) derive from the bone marrow and are considered to be the most potent APCs. DCs are important for linking the innate and adaptive responses and they play a major role in antiviral responses. Immature DCs rest in the peripheral tissues and blood where they take up antigens and process them, and then they migrate to lymphoid tissues where they present antigens on their MHC molecules to T cells.

DCs encounter viral antigens either by direct infection or indirectly through the infection of other cells. When infected directly, DCs present viral antigens on their MHC class I antigens. However, there are viruses that have evolved not to infect DCs in order to escape the immune defense, but then DCs are able to take up viral antigen by engulfing dying virus-infected cells, and will subsequently present viral peptides on their MHC class II molecules.

In addition, DCs have a unique ability to present viral antigens via an MHC class I exogenous pathway, recognized as cross-presentation. The exact mechanism of cross-presentation is not known, but it is receiving growing interest since it has been shown to be necessary for a number of CD8<sup>+</sup> mediated responses, for example for different viruses [66].

In innate immunity, viral components (proteins, DNA or RNA) bind to Toll-like receptors (TLR) on DCs leading to an intracellular signaling cascade that triggers the DCs to mature. DCs are also crucial in adaptive immunity, where they activate both B and T cells.

DCs determine the outcome of an immune response by the cytokines they are producing and by their degree of maturation. Besides the antigen-receptor interaction between a T cell and a DC, the T cell need a second signal from the DC, which is delivered by co-stimulatory surface molecules, see figure 8. The absence of a co-stimulatory signal can instead of T cell activation lead to T cell tolerance, anergy, to an antigen [67].

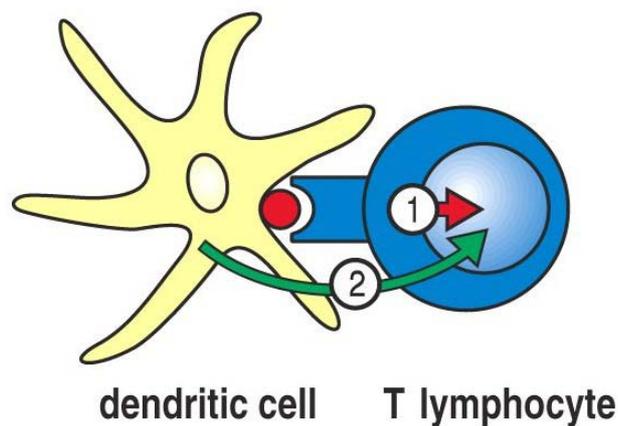


Figure 7. Antigen-receptor signal (1) and co-stimulation signal (2) from dendritic cell to T cell. Adopted from Howard 2005 [62].

## Vaccination

The first vaccines were used to prevent viral infections. Today there are also vaccines preventing bacterial infections, for example against pertussis [68] and against cholera and typhoid fever [69]. There are also attempts to develop vaccines against cancer; this will be described in more detail later.

The Chinese suffered from a smallpox outbreak around 1000 BC and it was realized already then, that survivors were protected against a subsequent infection. Therefore, already during this time period, it was common to take secretion from survivors, and scratch it onto the forearm of uninfected persons. This method, “variolation”, was shown to be effective in preventing disease and was practiced for centuries. However, it was also a hazardous method, since some of the individuals treated this way actually developed smallpox, and it was consequently a need for a safer alternative [1].

In 1796 Edward Jenner managed to vaccinate a boy against smallpox by inoculating him with cowpox (vaccinia). He named this successful procedure “Vaccination” and it was spread worldwide during the 19<sup>th</sup> century. Humans are not a natural host for vaccinia, and therefore develop only a very limited infection. This infection is however, sufficient to stimulate an immune response that is cross-reactive with smallpox and thereby confers protection.

Louis Pasteur produced an artificial virus vaccine, in contrast to Jenner’s live virus vaccine, and in 1885 he inoculated a child with a vaccine that had been developed by drying the spinal cord of rabies-infected rabbits. Since then a number of vaccines have been developed against many different viruses and these vaccines have without doubts saved millions of lives. Today vaccines are commonly divided into four basic types; live virus vaccines, inactivated vaccines, DNA vaccines and subunit vaccines [1].

#### *Live virus vaccines*

Vaccines against viral infections are often based on attenuated (weakened) live viruses. The advantage of these vaccines is that they are good immunogens and induce long-lived immunity. In 1980 the WHO announced the global eradication of smallpox, the first virus disease ever to be eliminated, thanks to vaccination with Vaccinia virus. Today, attenuated live vaccines are in use against polio, measles, mumps, rubella and varicella [70]. However, there is no general mechanism by which different viruses can be safely attenuated and still be effective. The reversion of an attenuated vaccine strain to a more virulent state can lead to development of

disease in vaccinated individuals, a phenomenon that has been observed in pregnant women and in immunosuppressed people [71].

### *Inactivated vaccines*

Inactivated, or “killed” virus vaccines are based on viruses that are chemically inactivated so that they cannot replicate. The purpose is to lose infectivity of the virus without the loss of immunogenicity. It is a delicate balance and generally these vaccines are not as effective as attenuated live vaccines, often because they fail to stimulate cell-mediated immunity. Still they are desirable since they can be effective and they bear little risk of vaccine-associated disease and thus are considered to be safer.

### *DNA vaccines*

DNA vaccines are one of the newest types of vaccines and are attractive due to its simplicity and would therefore be easily manufactured. DNA vaccines consist only of a DNA molecule, encoding the genes of interest, e.g. virus or tumor antigen, that will be expressed *in vivo* and hopefully the protein produced will prime a protective immune response. DNA vaccines have shown promising results in animal models but has so far not been effective alone in human clinical trials, for review see Donnelly *et al* 2005 [72].

### *Subunit vaccines*

Recombinant subunit vaccines consist of only one or a few components of the virus, e.g. a single viral protein, and have served as a safe alternative to live virus vaccines. Unfortunately, most of these vaccines have not been shown to be effective alone, and generally need the addition of adjuvant to induce a protective immune response. An adjuvant is a helper molecule that can increase the immune response to an antigen, for review see Pashine *et al* 2005 [73]. For human vaccines there are for safety reasons only a few adjuvants allowed, for example aluminum salts and oil in water emulsions. For animal models however, there are several

different adjuvants that are used to potentiate the efficacy of a vaccine, and many of these are of a microbial basis.

### *Virus-like particles as vaccines against viral infection*

VLPs are subunit vaccines that have been shown to efficiently protect against viral infections without the help of an adjuvant, for example against infections by HIV, and hepatitis B and C [74-76]. In these cases the VLPs have induced antibody, CD4 and CTL responses.

A human VLP vaccine against Hepatitis B, produced in yeast, which today is given on a routine basis to children in the USA, is so far the most successful VLP vaccine, and was also the first one to be commercialized [77]. These VLPs however do not mimic the natural virus, since the VLP lacks an envelope in contrast to the virus.

Papillomavirus and polyomaviruses are simpler in structure, and VLPs based on these viruses are more similar to natural virus particles. HPV-VLPs are the most studied so far; they are formed by expression of the major capsid protein L1 in yeast or insect cells [78-80]. HPV-VLPs have been shown to be safe in phase I clinical trials, and none of the vaccinated women were infected with HPV after HPV-VLP vaccination compared to 3.8 % in the control group [81]. Moreover, both GlaxoSmithKline [3] and Merck [4] have run phase II trials with HPV-VLP vaccines. Both vaccines seem safe and effective in preventing HPV infections and perhaps also cervical cancer and are currently running in phase III clinical trials.

The Bluetongue virus (BTV) is a virus that causes disease in sheep and cattle resulting in economic losses. BTV-VLPs have been produced by expression of the four major capsid proteins, VP2, VP3, VP5 and VP7, in the same baculovirus [82]. The resulting BTV-VLPs were used to vaccinate BTV susceptible sheep, which later were challenged with BTV and all vaccinated animals were protected from bluetongue disease [83].

Human polyomaviruses can cause disease in immunosuppressed patients. To be able to vaccinate against these viruses, especially in individuals with an immature immune system, or in immunosuppressed individuals, could be of value. In this thesis, we have used the murine polyomavirus (MPyV) mouse model in order to investigate the possibility to vaccinate against MPyV infection with MPyV-VLPs, both in normal and immunosuppressed mice.

## **Gene therapy**

Many diseases are genetic diseases, and these are diseases that are caused by a missing or defective gene, for example cancer. Genes code for all proteins in the body and if a gene is defective or deleted the corresponding protein cannot be produced or it will be produced in a non-functional state. The concept of gene therapy is to correct this defect by adding the gene of interest into cells in order to re-establish health. There have been a number of strategies in order to get the gene expressed in cells.

### *Viral vectors*

Since viruses are created to carry and deliver their genes into cells they were early of interest for the purpose of bringing foreign genes into cells, e.g. in viral vectors. When using viral vectors in gene therapy some of the viral genes have been exchanged for other genes and when the virus enters cells the inserted genes of interest will be expressed.

Retroviral vectors are the most well studied viral vectors for gene therapy. Retroviruses are able to enter a variety of different cells and can integrate the genes of interest into the host genome and genes introduced this way are expressed for a long period of time. This strategy has been shown to be very effective, but has some major side effects, for example, the genes will be randomly integrated in the genome and this might disrupt the expression of other important genes. There have

also been reports that outbreaks of the wild type virus have occurred. In 2000, 9 of 10 children, with a severe immune deficiency, SCID-X1, that normally leads to death before the age of 3 years, were successfully treated with stem cells infected with a retrovirus [84]. Unfortunately, three years later two of the children developed leukemia, since the retrovirus had been integrated in a position, where it lead to the expression of an oncogene [85]. Since then the use of retroviral vector has been under further detailed investigation, and intensive risk evaluation, before allowing any further use in humans.

Adenoviruses have also been used as viral vectors. They can introduce large genes that are expressed efficiently in cells. This expression may however be short-lived and many humans also have antibody immunity against adenoviruses, and these antibodies may neutralize the efficiency of some of these vectors [86]. Newer developed helper-dependent adenoviral vectors have shown reduced immunogenicity, but unfortunately also reduced transduction efficiency [87].

#### *Non-viral vectors*

Since the use of viral vectors has in some cases been shown to be hazardous, a number of non-viral vectors are being developed. For example, lipids, polymers, and different proteins have been used as DNA carriers. The main advantages of using non-viral vectors are non-expensive large-scale production, lack of immunogenicity and high degree of safety. However, they have so far not been as effective as viral vectors and have also shown limited *in vivo* stability [88].

#### *Virus-like particles in gene therapy*

Since Michel *et al* 1967 [51] and Winicour *et al* 1968 [53] demonstrated that VLPs could carry DNA, VLPs based on several different viruses have been explored for their ability to be used as vectors for gene therapy.

The use of VLPs as vectors for gene transfer has several advantages over the use of adeno- and retroviruses. Most importantly VLPs do not contain any viral genes,

which make the system safer, since retained viral genes have a potential danger of activating viral oncogenes.

Attempts have for example been made to use VLPs from HPV for gene therapy [89], as well as VLPs from different polyomaviruses, e. g. SV40 [30], JCV [90] and BKV [91]. We and others have focused on VLPs from MPyV and we have been able to show that MPyV-VLPs can introduce foreign DNA into cells *in vitro* and *in vivo* [92-94]. MPyV-VLPs would be suitable as vectors in humans since humans have no pre-existing immunity against MPyV, which is an advantage compared to human polyomaviruses and adenovirus systems [90]. Furthermore, MPyV has a broad tissue distribution and receptors for MPyV are expressed on most mouse and human cells, which extend the potential of MPyV-VLPs as vectors.

However, when using MPyV-VLPs *in vivo*, they give rise to anti-VLP antibodies and this is useful for vaccinating against MPyV infection [95]. On the other hand, this antibody response is inappropriate for gene therapy, since it can reduce or abolish the effect of a second or third similar inoculation. In order to avoid the hinder with neutralizing antibodies, and increase the efficiency of gene therapy by prime-boost gene therapy, another vector that does not cross react with MPyV-VLPs would be of value. In this thesis, another murine polyomavirus, the murine pneumotropic virus (MPtV) [36], was studied for the purpose of producing MPtV-VLPs, to use as a complement to MPyV-VLPs.

## **Tumor immunology**

According to the immunosurveillance hypothesis [96], the immune system surveys the body for detection of tumor cells. This hypothesis was supported by the findings that mice treated with chemicals developed tumors that could be immunogenic [97]. However, the immunosurveillance hypothesis has received a lot of criticism since not all tumors are immunogenic and tumors do escape immune detection and do develop even in immunocompetent individuals [98, 99].

Various experiments in mice have led to the discovery that it is possible to elicit a protective immune response against some types of tumors. It was shown that irradiation and chemical drugs can induce tumors in mice and that these tumors can be transplanted between mice from the same strain [100, 101]. It was also shown that, if the tumor was transplanted into a mouse with different MHC molecules than that of the tumor, the tumor was attacked and destroyed by the immune system [99]. Moreover, it was shown that if mice were injected with irradiated tumor cells, which are unable to grow in mice, and later challenged with viable cells from the same tumor, the mice were able to reject the tumor [102]. In contrast, if the same mice were challenged with viable cells from a different tumor, there was no protection and the mice died from their tumor.

### *Tumor antigens*

The anti-tumor protection has later been demonstrated to be T cell dependent, since it could not be demonstrated in T cell deficient mice. From these results it was suggested that tumor cells express foreign antigens, so called tumor associated antigens (TAAs), which could induce specific T cell responses, for review see [103]. These antigens are presented to T cells by MHC molecules on the surface of tumor cells, and they are targeted because they are not expressed on normal cells, and thus not recognized as self. Tumor antigens can be divided into different categories. First, there are tumor antigens that are strictly tumor specific; they are results of mutations in oncogenes or tumor suppressor genes leading to mutated proteins. Viral oncoproteins can also be viewed as strictly tumor specific. There are also germ cell antigens, and these are antigens normally only expressed by male germ cells, that do not express MHC molecules, and if these antigens are presented on MHC molecules they will be viewed as foreign for T cells. Moreover, tumor antigens can be differentiation antigens, genes normally expressed only in certain type of tissue, for example melanin in melanoma cells. Finally, there are self-antigens that are strongly overexpressed, due to amplification or mutation, and this high level of a self- protein can also be recognized by T cells.

In many of these cases, the immune system has the ability to elicit an anti-tumor response against the tumor antigen. For example, antibodies against the tumor antigen [104] and tumor specific T cells can be found in the peripheral blood of patients with different tumors. Infiltrating CTLs have also been found in different tumors [105]. However, these immune responses seem not to be strong enough to kill the tumor cells and the concept of immune therapy is to potentate these responses in different ways.

### *Different strategies for immunotherapy*

The choice of antigen is of great importance for the development of a cancer vaccine, and choosing an antigen, which is not truly tumor specific might be hazardous because the risk of inducing autoimmune diseases. Melanoma is perhaps the most studied cancer for immunotherapy, and perhaps also the one with most promising clinical response [106]. A number of melanoma antigens have been identified and since these antigens are expressed specifically in melanocytes, they are considered as rather safe targets. However, since immunotherapy directed against melanoma even kills normal cells expressing melanin, a side effect can be symptoms of the autoimmune disease vitiligo [107].

There are several different strategies used for immunotherapy. Cytokines have been used as a non-specific stimulator of the immune system. Interleukin-2 (IL-2) is essential not only in the activation of the adaptive response to antigens by T cells, but also in triggering the innate immunity and this was the first cytokine to be used for immunotherapy. The pioneer studies of Rosenberg *et al* 1994, with high doses of IL-2 in several hundred cancer patients, showed an anti-tumor effect in approximately 20 % of the patients. This treatment, however, was associated with a considerable systemic toxicity [108]. Today in immunotherapy, cytokines, in particular IL-2, IFN, GM-CSF and IL-12, are mostly used in lower doses as adjuvants to improve antigen presentation and T cell expansion [109].

Monoclonal antibodies (mAbs) can target specific tumor antigens and can rather easily be produced in massive amounts [110]. In 1997, the humanized anti-CD20

antibody rituximab (Rituxan), became the first mAb approved by the US Food and Drug Administration (FDA) for use in human cancer and it has demonstrated impressive clinical responses against B-cell lymphomas [111]. Moreover, in combination with chemotherapy, an anti-epidermal growth factor antibody, cetuximab (Erbix), and an anti-vascular endothelial growth factor antibody, bevacizumab (Avastin), both prolong survival in patients with colorectal cancer [112, 113]. The humanized Her2-specific mAb trastuzumab (Herceptin, Roche) will be described in more detail below.

Nevertheless, most immunotherapeutic strategies aim at stimulating specific T cell responses and Rosenberg 2004 argues that there are some basic requirements for immune therapy to be effective against cancer. First of all there have to be tumor-specific T cells in the host. Furthermore, these T cells have to be in sufficient number and capable of reaching the tumor site. Finally, they have to have effector mechanisms to kill the tumor cells [114].

An attractive way of achieving this goal is by using adoptive T cell transfer. In adoptive T cell transfer, tumor-specific T cells are isolated from a tumor-bearing patient, expanded *in vitro* and then re-infused to the patient. This strategy has showed promising results in melanoma patients [115]. It has further been shown that depletion of lymphocytes before the transferred T cells further enhances the effect in melanoma patients [116], the mechanism might probably be due to elimination of regulatory T cells [117].

### *Dendritic cells in immunotherapy*

Dendritic cells (DCs) are known as the most potent antigen presenting cells with a unique T cell stimulatory potential, and they have therefore been of special interest in immunotherapy, for review see Figdor *et al* 2004 [118]. Most tumor antigens are poor immunogens because they are self antigens, against which it can be difficult to break tolerance. DCs can supply tumor antigens with necessary co-stimulatory ligands, and migrate to secondary lymphoid organs, where the DCs efficiently can present the tumor antigens to T cells.

DC-based vaccination strategies have attracted attention because of promising results of applying tumor antigen-loaded murine DCs in several tumor models. DCs can be obtained from peripheral blood, loaded or transfected *in vitro* with tumor antigen based on protein, RNA or DNA and these DCs are then re-infused *in vivo*. Clinical trials indicate that DC vaccines are safe, with minimal side effects [119]. It has been shown that mature DCs are more potent than immature DCs in inducing potent immune responses. The maturation degree of the DCs is of importance and it has been shown that non-activated DCs can induce tolerance instead of immunity [120]. Although early clinical trials have indicated that DC vaccines can induce anti-tumor immune responses in some cancer patients, much is still to be learned regarding antigen loading, maturation, route of administration and migration of DC-based vaccines [118].

#### *Virus-like particles in immunotherapy*

The notion that VLPs so efficiently induce immune responses have made them candidates as carriers for antigens for other diseases as well, for example for tumor antigens in cancer immunotherapy. Their excellent efficiency is probably due to the ability of VLPs to be taken up by antigen-presenting cells, and this way prime CTL responses [121].

Minor capsid proteins, e.g. VP2 and L2, are known to bind specifically to the inside of major capsid proteins, e.g. VP1 and L1. Therefore any molecule fused to either VP2 or L2, would potentially be efficiently introduced into cells by the VLPs [122]. For example in cancer immunotherapy, it would be of value to introduce a tumor antigen with the help of VLPs and this way elicits an anti-tumor immune response.

Greenstone *et al* 1998 have produced HPV-VLPs consisting of the major capsid protein L1 plus the E7 oncoprotein fused to the L2 minor capsid protein [123]. They show that vaccination with these VLPs protected mice from an E7-expressing tumor challenge. This protection was also demonstrated in MHC class II-deficient

mice, but not in  $\beta_2$ -microglobulin knockout mice, suggesting that protection was mediated by MHC class I-restricted CTLs [123].

Moreover, Brinkman *et al* 2005, show that they are able to treat OVA-expressing melanoma-bearing mice with MPyV-VLPs carrying an ovalbumin epitope, OVA<sub>257-264</sub>, and this way induce tumor remission [124]. The OVA epitope is a self-antigen, which serves as an excellent model antigen against which it is easy to detect CTL responses. Brinkman *et al* 2005 were also successfully able to correlate the anti-tumor immunity to OVA<sub>257-264</sub> specific CTLs. These experiments showed that it is possible to break T cell tolerance against a differentially expressed self-antigen using VLPs.

In this thesis, we have used the Her2 tumor antigen as a model antigen and produced MPyV-VLPs carrying a part of this oncoprotein and investigated their possible use as vaccine against Her2-expressing tumors.

## **Her2**

The proto-oncogene Her2 (also known as ErbB2 and neu) is one of the four receptor members in the epidermal growth factor (EGF) receptor family. When ligands bind to these receptors, they dimerize and this dimerization initiates a complex signaling cascade that activates cell proliferation. Her2 receptors do not bind ligands themselves but form heterodimers with other Her receptors. The Her signaling network is mostly active during embryonic development and the expression in normal tissue is very low [125]. However, the Her2 gene is amplified in many different malignancies leading to overexpression of the Her2 oncoprotein. This Her2 overexpression, which occurs in e.g. 25 % of breast cancers, is associated with aggressive tumors and poor prognosis [126]. Moreover, Her2 is also implicated as a possible marker of resistance to tamoxifen [127].

Due to its selective overexpression in tumor tissue, Her2 is considered an important therapeutic target in immunotherapy against cancer. This notion is further supported

by the fact that Her2-specific antibodies [104] and T-cells [128] have been reported to occur naturally in patients with Her2-positive tumors, indicating that these tumor can be immunogenic.

Several types of therapies, including monoclonal antibodies, small molecules, and vaccines, have been developed to interfere with the Her2 signaling in tumors. For example, treatment with the humanized Her2-specific monoclonal antibody trastuzumab (Herceptin, Roche) is now regularly used in patients with Her2-expressing breast tumors. This antibody reduces the Her2 expression from the tumor cells and thereby inhibits cell proliferation [129]. In a large study, Piccart-Gebhart *et al* 2005 showed profit in disease-free survival after treatment with trastuzumab in the Her2 positive breast cancer patients [130]. In addition, trastuzumab in combination with chemotherapy improved disease-free survival with 12 % compared to breast cancer patients only receiving chemotherapy [131].

Her2 derived vaccines based on e.g. plasmid DNA, proteins or peptides have also been developed in order to induce rejection of Her2 positive tumors. When tested in animal models and in clinical trials, these vaccines have clearly shown that it is possible to break tolerance to Her2 and generate effective T and B cell responses leading to tumor resistance, for review see Kiessling *et al* 2002 [132].

For example, it has been demonstrated that an intramuscular injection of Her2 DNA co-administered with DNA expressing GM-CSF induced complete rejection of a lethal challenge with the Her2-expressing tumor D2F2/E2 in BALB/c mice [133, 134]. In these studies they demonstrate protecting anti-tumor immunity against Her2 even in the absence antibodies.

#### *BALBneuT mice*

Many of the studies on Her2 derived vaccines are done in transplantable tumor models in mice. One problem with the transplantable models is that it is difficult to show that the effect would also prevent endogenous malignancies. Transgenic mice that spontaneously develop tumors would therefore be a more natural system in where to estimate tumor vaccine efficiency. Already in 1988 Muller *et al* presented

the first mouse model transgenic for rat Her2 [135]. In 1998, Boggio *et al* presented another mouse model transgenic for rat Her2, the more aggressive BALBneuT [136]. BALBneuT mice express Her2 from week 3 of age and already at week 10 all 10 mammary gland display in situ carcinomas [137]. The carcinomas become invasive between the 10th and the 20th week and metastasize after the 35th week [138].

Different vaccination strategies with Her2 derived vaccines based on DNA, whole cells, and proteins have been tested in these mice. For example, vaccination with the extracellular (EC) and the transmembrane (TM) domain of rat Her2 DNA [139, 140] have shown to be effective against tumor development in these mice. The importance of the combined administration of the Her2 antigen, and a nonspecific cytokine, IL-12, has been noted in several studies [141-143]. However, in these cases the vaccinations had to be repeated six to eight times, starting from week six in order to completely inhibit tumor outgrowth. Both anti-Her2 antibodies and specific CTL have been induced in this model and implicated as important for protection [144].

In this thesis, we have produced MPyV-VLPs carrying a part of the Her 2 oncoprotein and investigated their ability to vaccinate against Her2-expressing tumors, both in BALBneuT mice and against Her2-expressing D2F2/E2 tumor cells.

## VLP-PRODUCTION

In this thesis, VLPs have been produced by baculovirus expression in insect cells. The genes of interest were cloned into the baculovirus transfer vectors pAcDB3, (to the left), which were used for production of four different VLPs, as illustrated schematically as cross sections to the right.

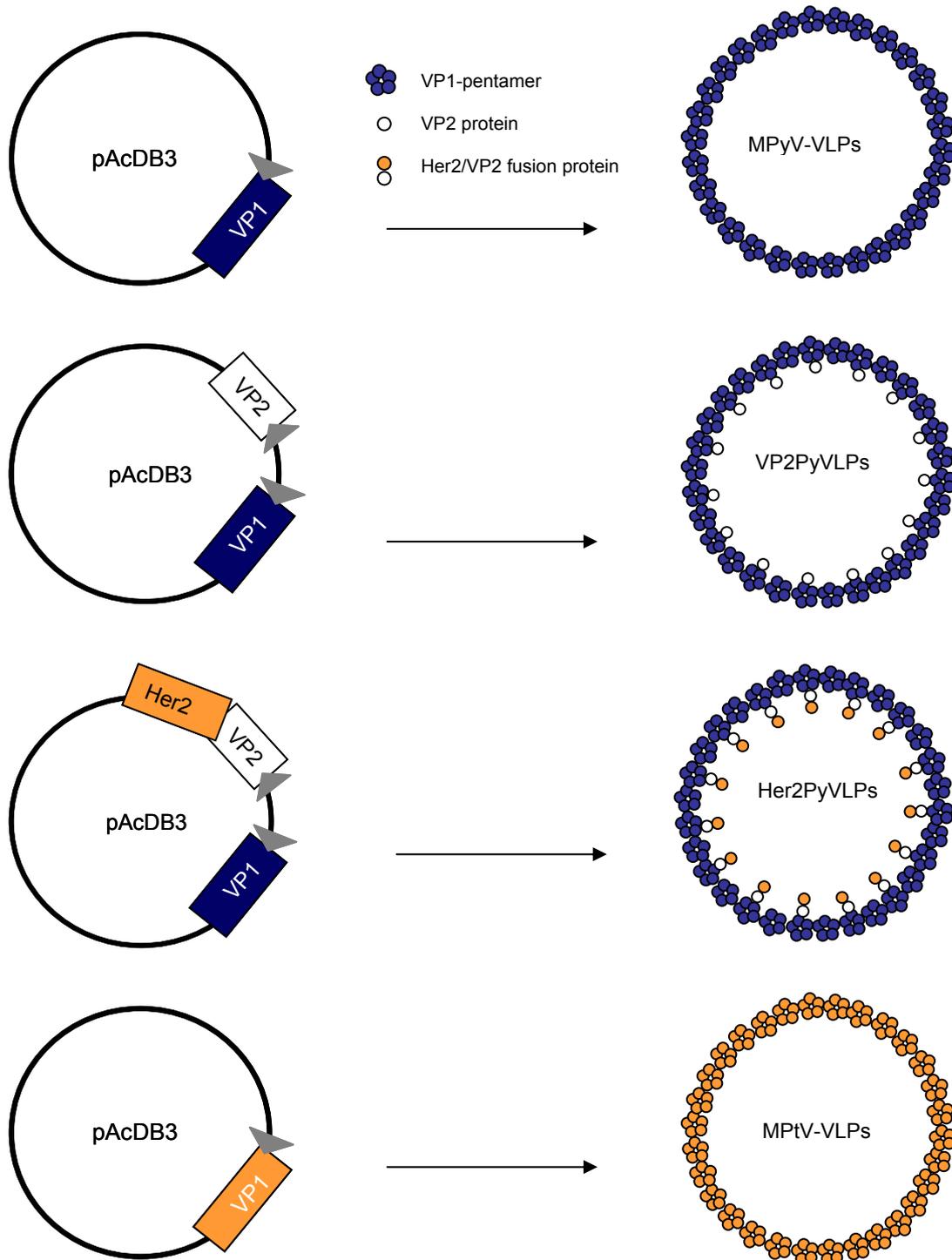


Figure 8. Plasmids used for VLP-production illustrated to the left and purified VLPs as cross-section to the right.

Insect cells were co-transfected with the plasmid of interest and baculovirus DNA. This way the genes of interest and the baculovirus DNA recombine in the nucleus, resulting in a baculovirus carrying genes for the capsid proteins, that can infect other insect cells. The genes are expressed and the resulting VP1 molecules self-assemble into VLPs. For production of Her2<sub>1-683</sub>PyVLPs and VP2PyVLPs, the fusion genes are also expressed, and the resulting fusion protein binds to VP1 and ends up on the inside of the VLP. After three days of culture the cells were lysed and the VLPs are purified on a CsCl-gradient.

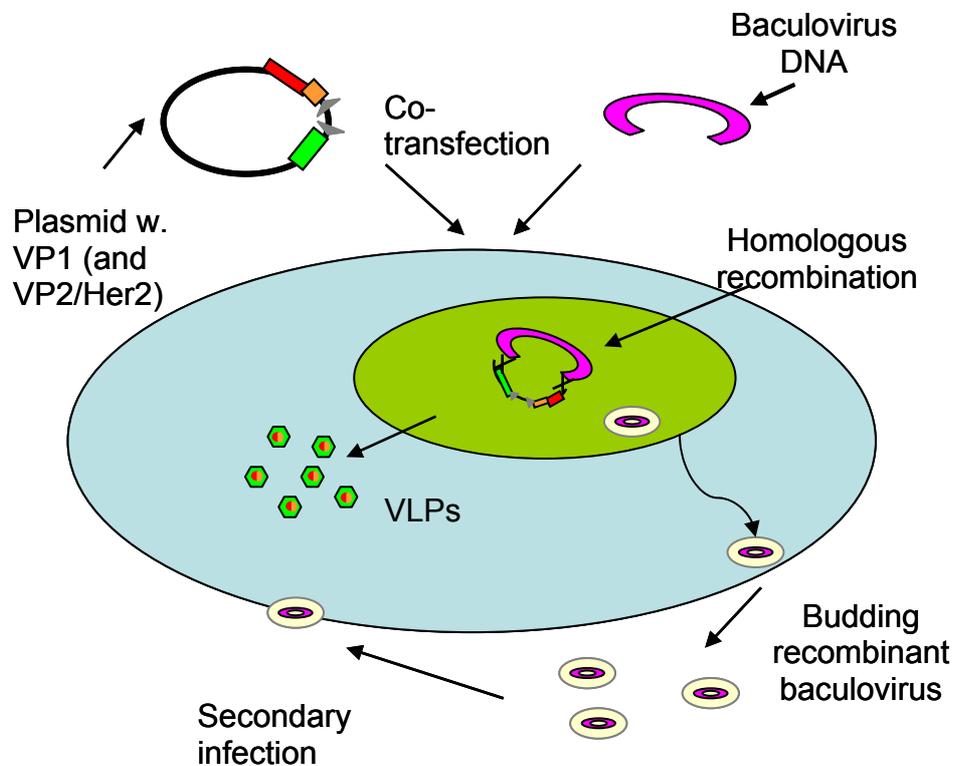


Figure 9. VLPs production in insect cells.

## **SPECIFIC AIMS OF THE STUDY**

To improve MPyV-VLP vaccination against MPyV infection by changing the route of administration, and to examine the importance of the VLP structure for protection (Paper 1).

To study and characterize a new vector, MPtV-VLP, for its possible use as a complement to MPyV-VLP in gene and immune therapy (Paper 2).

To vaccinate against Her2 expressing tumors, using MPyV-VLPs carrying the oncoprotein Her2, Her2<sub>1-683</sub>PyVLPs, or dendritic cells loaded with Her2<sub>1-683</sub>PyVLPs (Paper 3 and 4).

# RESULTS AND DISCUSSION

## Paper 1

**VP1 pseudocapsids, but not a glutathione-S-transferase VP1 fusion protein, prevent polyomavirus infection in a T-cell immune deficient experimental mouse model.**

*The aim of this study was to investigate the importance of the route of administration and of the VLP structure for vaccination against MPyV infection.*

We have previously shown that intraperitoneal vaccinations with MPyV-VLPs protected half of the vaccinated mice, both normal and T cell deficient, against a subsequent MPyV challenge [95]. In this study, to improve these results and to hopefully get full protection against polyoma infection, we changed the route of vaccination from the intraperitoneal (i.p.) to the subcutaneous (s.c) route. Furthermore, to evaluate the importance of the VLP structure, we vaccinated mice with the glutathione-S-transferase-VP1 fusion protein (GST-VP1), which forms dimers and pentamers, but are unable to form VLPs. However, GST-fusion proteins are easier and less expensive to produce in large quantities and can still generate antibodies that protect against viral infections [145]. Finally, the degree of protection against MPyV infection was compared and correlated with the corresponding antibody response.

In the first set of experiments, T cell deficient  $CD4^{-/-}8^{-/-}$  mice were immunized four times s.c. with VLPs, and for comparison, normal mice were vaccinated with VLPs. One week after the last immunization all immunized mice and controls were challenged with MPyV. Three weeks later the mice were sacrificed, and MPyV DNA could be detected in non-immunized mice, but not in any of the immunized mice. Anti VLP-antibodies were obtained in sera from all immunized mice, but not in sera from non-immunized mice. However, the titers increased with the VLP dose.

In the second set of experiments, CD4<sup>-/-</sup>8<sup>-/-</sup> and normal mice were immunized s.c. with VLPs or GST-VP1 and later challenged with MPyV. As expected, MPyV DNA was found in all control mice, but not in any of the VLP vaccinated mice. Immunization with GST-VP1 protected all normal, but only 3/5 of the CD4<sup>-/-</sup>8<sup>-/-</sup> mice. Sera from both VLP and GST-VP1 immunized mice were compared for their antibody titers against VLPs, and against GST-VP1. Sera from normal and CD4<sup>-/-</sup>8<sup>-/-</sup> mice immunized with VLPs had approximately similar antibody titers against both VLPs and GST-VP1. Sera from GST-VP1 immunized normal mice showed high antibody titers against GST-VP1, while the response against VLPs was very low. In GST-VP1 immunized CD4<sup>-/-</sup>8<sup>-/-</sup> mice, no antibodies could be detected against either GST-VP1 or VLPs.

VLPs and GST-VP1 were also compared for their ability to hemagglutinate guinea pig erythrocytes and we observed that only VLPs induced hemagglutination. In concordance, hemagglutinating inhibiting antibodies were only obtained after immunization with VLP, and not after GST-VP1 immunization.

In summary, it was possible to improve vaccination against MPyV infection by changing the route of immunization. Hence, s.c. VLP vaccination, resulted in complete protection, in comparison to i.p. VLP vaccination, resulting in partial protection [95]. It is possible that the s.c. distribution of VLPs prevents its rapid degradation, and allows the antigen to be exposed to the immune system for a longer period.

Our data also suggest that prevention of MPyV infection is mainly due to the presence of antibodies directed against the viral capsid. This explanation is supported by serological assays, where anti-VLP antibodies are observed more readily in both CD4<sup>-/-</sup>8<sup>-/-</sup> and normal mice after VLP immunization, rather than in mice immunized with GST-VP1. We propose that this was due to that VLPs, because of their virus-like structure have a better ability compared to the more linear structure of GST-VP1, to induce antibody responses. Nevertheless, similar to the present study, in a normal immune context, immunization with GST-L1

has been shown to elicit a sufficient amount of antibodies to get protection against canine papilloma infection in dogs [146].

In conclusion, VLP s.c. immunization induces a protective response against primary MPyV infection in both T cell deficient and normal mice. GST-VP1 immunization however, only fully protects normal mice and the antibody response correlates to this results. The data suggest that it should be possible to vaccinate both healthy and T cell immune suppressed individuals against polyomavirus infections with VLPs. However, vaccination with GST-VP1 could still be an alternative in healthy individuals and it is easier and less expensive to produce in large scale.

## Paper 2

**Murine pneumotropic virus VP1 virus-like particles (VLPs) bind to several cell types independent of sialic acid residues and do not serologically cross react with murine polyomavirus VP1 VLPs.**

*The aim of this study was to produce VLPs based on the murine pneumotropic virus (MPtV) with the main goal to use them as a complement to MPyV-VLPs in repeated immune and gene therapy.*

Others and we have previously demonstrated that MPyV-VLPs can introduce foreign DNA into cells *in vivo* and *in vitro* and thus should be suitable as vectors in gene therapy. However, inoculation with MPyV-VLPs in mice gives rise to an antibody response that may reduce the effect of a second treatment. For this reason another vector that does not cross react serologically with MPyV-VLPs would be of value for prime boost gene therapy.

We therefore produced VLPs from VP1 of another murine polyomavirus, the murine pneumotropic virus (MPtV) as a possible complement to MPyV-VLPs. Since the MPtV is not a well studied virus, some of its general characteristics were examined. MPtV-VLPs were used to estimate the potential of MPtV to attach to different cells and to assess some characteristics of the MPtV receptor.

MPtV-VLPs were also tested for potential serological cross-reactivity with MPyV-VLPs, and for their DNA transduction efficiency.

MPtV-VLPs were successfully produced and their morphology was similar to that of other polyomaviruses. In contrast to most polyomaviruses, MPtV-VLPs did not haemagglutinate erythrocytes [47]. Demonstrated *in vitro* and *in vivo*, MPtV-VLPs did not cross react with MPyV-VLPs. The lack of cross-reactivity between the two VLPs could be due to that the induced antibody response is directed against surface epitopes, where VP1 of MPyV and MPtV only show a homology of 44 % [36] whereas regions with a higher homology are mainly situated within the non-exposed areas of the capsid. These results suggest that it should be possible to use MPtV-VLP and MPyV-VLP for prime-boost gene therapy.

MPtV-VLPs were then tested for their ability to bind to different cells with flow cytometry, and were demonstrated to bind to all cells tested, including the ones lacking MHC class I expression. Furthermore similar to SV40 [147] and MPyV [148] MPtV uptake was rapid.

Receptor qualities of MPtV-VLPs were tested using cells treated with neuraminidase, papain or trypsin, before incubation with VLPs. We could show that the binding of MPtV-VLPs was neuraminidase resistant, but trypsin and papain sensitive. These results suggest that the MPtV-VLP receptor consists of proteins. Furthermore, these results confirmed that MPtV binding is independent of sialic acids, in concordance with the fact that MPtV-VLPs do not hemagglutinate erythrocytes.

Hence, MPtV and SV40 show similarities in that their receptors are neuroaminidase resistant. However, in contrast to what has been demonstrated for SV40, where SV40 also binds to MHC class I and, MPtV-VLPs also bind to cells that lack MHC class I expression. These findings implicate that MHC class I is not an important receptor for MPtV. We could also show that the internalization of MPtV-VLPs was a very rapid process with a significant uptake

already after 5–10 min, which is similar to that of SV40 [147] and MPyV [148] and also indicating a specific mechanism of uptake.

Finally, we tested if MPtV-VLPs could transduce DNA *in vitro* and transfer target DNA into different cell tissues *in vivo*. The transduction efficiency of MPtV-VLPs was low in our present experiments. This might be due to that whereas the VLPs efficiently enter cells, only a small portion enters the nucleus [41].

In conclusion, MPtV-VLPs, and most likely also MPtV, can bind to several different cell types, and binding is neuraminidase resistant. Furthermore, MPtV-VLPs may have the potential to complement MPyV-VLPs in repeated gene and immune therapy, since the two VLPs do not cross-react. However, for both types of VLPs, the transduction efficiency using the present protocol was low and needs to be improved. Nevertheless, the VLPs could be more efficient as carriers of antigen for immunotherapy, where it is necessary for the VLPs to enter the cytosol, as investigated in paper 3 below.

### **Paper 3**

#### **A single vaccination with polyomavirus VP1/VP2Her2 virus-like particles prevents outgrowth of HER-2/neu-expressing tumors.**

*The aim of this study was to investigate the possibility to vaccinate against Her2-expressing tumors with MPyV-VLPs carrying the Her2 oncoprotein.*

Her2 is a proto-oncogene that is overexpressed in about 25 % of all breast cancer and this overexpression is correlated to a poor prognosis [126]. Her2 is also considered a good target for immunotherapy, because of its selective overexpression in tumor tissue. The fact that VLPs are so efficient in inducing immunity, suggests that they could be excellent carriers for the Her2 protein with the purpose to vaccinate against tumors.

MpYV-VLPs containing a fusion protein between VP2 and the extracellular (EC) and transmembrane (TM) domain of human Her2, Her2<sub>1-683</sub>PyVLPs, were produced and tested for their ability to vaccinate against Her2-expressing tumors in two different *in vivo* models. More specifically, they were used to vaccinate against a challenge with the transplantable Her2-expressing mammary carcinoma, D2F2/E2, and against spontaneous development of mammary carcinomas in transgenic BALB-neuT mice. BALBneuT mice are transgenic for the rat Her2 gene, and spontaneously develop mammary tumors by the age of 15 weeks.

In the transplantation model, mice were vaccinated once with Her2<sub>1-683</sub>PyVLPs. Immunized and control mice were later challenged with a lethal dose of Her2-expressing D2F2/E2 cells and 87 % of the Her2<sub>1-683</sub>PyVLPs immunized mice rejected the tumor, whereas the control mice developed tumors.

Encouraged by these results, Her2<sub>1-683</sub>PyVLP immunization was attempted in the well-established Her2 transgenic BALBneuT mouse system. Groups of six BALBneuT mice were immunized at week 6 of age with Her2<sub>1-683</sub>PyVLPs. When mice, 6 weeks of age, were vaccinated with Her2<sub>1-683</sub>PyVLPs, 5 out of 6 mice were completely protected against tumor outgrowth, whereas all non-immunized mice developed tumors in each mammary gland.

Successful vaccination was thus observed in two *in vivo* models, despite the low amount of the Her2 protein in the VLPs, approximately 800 ng per vaccination. The high efficiency induced by the VLPs was most likely due to their ability to efficiently be taken up by antigen-presenting cells, and this way prime CTL responses [121]. The MpYV receptor is expressed at a high quantity on most cell types [42] and the VP2Her2 fusion protein is most likely protected from extra cellular breakdown. Moreover, as suggested by Rollman *et al* [149] the VLPs themselves may act as an adjuvant and induce a nonspecific stimulation of the immune response.

It has earlier been demonstrated that an intramuscular injection of Her2 DNA does not induce a complete rejection of a D2F2/E2 challenge in BALB/c mice.

However, complete protection can be achieved by the co-immunization of DNA expressing a GM-CSF [133, 134]. Anti-tumor responses have been demonstrated with different strategies in the BALB-neuT model as well [139], [143]. However, combined administration of the Her2 antigen and a nonspecific cytokine, IL-12, has been necessary and the vaccinations had to be repeated several times to completely inhibit tumor outgrowth [141, 142]. With Her2<sub>1-683</sub>PyVLP vaccination complete protection was obtained after only one single immunization. Nevertheless, our vaccine is based on human Her2 and the BALBneuT mice express rat Her2. The strong anti-tumor effect could be due to the high homology between the two proteins and a cross-protective response, but it cannot be excluded that the effect is so strong due to a xenogenic antigen. Nonetheless, it is not necessarily so, since we have a strong effect against D2F2/E2, that in fact expresses human Her2.

Her2-specific antibodies were however, not detected after Her2<sub>1-683</sub>PyVLP vaccination in either of the models, but a Her2-specific T cell response was demonstrated in an ELISPOT assay. In other studies, the immune response has been shown to either B cell or T cell depending on the vaccination strategy. In BALB/c mice immunized with Her2 DNA and challenged with Her2 expressing D2F2/E2 cells, the rejection was shown not to be antibody dependent [133]. Our results are in agreement with Pilon *et al* 2001, who found that protecting immunity could be induced against Her2 in the absence antibodies [134].

MPyV-VLPs are attractive antigen carriers for use in humans, since humans have no pre-existing immunity to MPyV-VLPs. Although MPyV-VLPs have not yet been used in humans, VLPs from several other viruses e.g. HPV [3, 150] HIV [151] and Norwalk virus [152] have been used in clinical trials and this mode of vaccination is now established as safe and often also efficient.

In conclusion, vaccination with Her2<sub>1-683</sub>PyVLPs efficiently protected mice from tumor outgrowth both in a transplantation model, as well as in a Her-2 transgenic mouse model. VLP based vaccination strategy is of special interest out of safety reasons, because of its avoidance of viral vectors and DNA. Thus Her2<sub>1-683</sub>PyVLPs should be possible to use also as a prophylactic vaccine in healthy

persons, especially those with a predisposition of developing Her2 expressing carcinomas.

## Paper 4

**Dendritic cells loaded with polyomavirus VP1/VP2Her2 virus-like particles (VLPs) efficiently prevent outgrowth of a Her2/*neu* expressing tumor without inducing high anti-VLP serum titers.**

*The aim of this study was to compare the vaccination effect of Her2<sub>1-683</sub>PyVLPs with Her2<sub>1-683</sub>PyVLP-loaded dendritic cells, and to reduce the anti-VP1 titers.*

As demonstrated above (Paper 3), one vaccination with Her2<sub>1-683</sub>PyVLPs protects against Her2-expressing tumors in two *in vivo* systems. However, when vaccinating with VLPs, a high amount of anti-VLP antibodies is obtained and this response may reduce the efficiency of a repeated vaccination with the same type of VLP. The aim of this study was to examine the vaccination potential of dendritic cells (DCs) loaded with Her2<sub>1-683</sub>PyVLPs, and also to measure the anti-VLP antibody response after this type of vaccination. An additional aim was also to examine if it was possible to optimize Her2<sub>1-683</sub>PyVLP vaccination using DCs. and therefore using a tenfold lower Her2<sub>1-683</sub>PyVLP dose, compared to the one in paper 3, was also tested.

Immunization with the high dose Her2<sub>1-683</sub>PyVLPs alone, as well as vaccination with DCs loaded with a high dose of Her2<sub>1-683</sub>PyVLPs, protected mice from tumor outgrowth, in agreement with the data from paper 3. With the lower dose however, a significant difference in tumor protection was seen between vaccination with DCs loaded with Her2<sub>1-683</sub>PyVLPs and Her2<sub>1-683</sub>PyVLPs alone. The former vaccination procedure protected all mice against tumor outgrowth while immunization with Her2<sub>1-683</sub>PyVLPs alone, gave only a 50% protection.

In addition, DC-vaccination with Her2<sub>1-683</sub>PyVLPs reduced the anti-VLP titers by six-fold compared to the response obtained by immunizing with Her2<sub>1-683</sub>PyVLPs alone. This is could be due to that VLPs are not fully exposed when

bound to DCs, and that the actual number of VLPs on the surface of DCs is reduced compared to when VLPs are used alone. The lower antibody titers may be of significance if several vaccinations are required for tumor rejection, since neutralizing antibodies may limit the effect of a boosting vaccination, as has been shown for HPV-VLPs [123]

VLPs have been shown to efficiently protect against viral infections and in these cases the VLPs have induced both CD4 and CTL responses [74-76]. Their efficiency is probably due to the ability of VLPs to be taken up by antigen-presenting cells, and this way prime CTL responses [121]. The tumor protection obtained with Her2<sub>1-683</sub>PyVLPs is most likely also cell-mediated. However, when DCs were incubated with Her2<sub>1-683</sub>PyVLPs, no upregulation of DC maturation markers on human or murine DCs could be demonstrated. This is in agreement with recently published results from Boura *et al.* 2005 [153] who were unable to detect upregulation of DC maturation markers. Furthermore, human polyomaviruses BK and JC failed to induce maturation of human DCs *in vitro* [154]. However, Her2<sub>1-683</sub>PyVLPs might still activate DCs *in vivo*, although it cannot be demonstrated *in vitro*. Boura *et al.* 2005 [153], suggest an alternative mechanism of cross-presentation by gap junction-mediated immunological coupling as a possible explanation for the induction of cellular immunity *in vivo*, as has recently been described by Neijssen *et al.* 2005 [155].

In conclusion, DC-vaccination with Her2<sub>1-683</sub>PyVLPs against tumor outgrowth was successful and it was even more efficient than vaccination with Her2<sub>1-683</sub>PyVLPs alone. A tenfold lower dose of Her2<sub>1-683</sub>PyVLPs could be used for full protection and moreover the serum titers of anti-VLP antibodies were reduced six fold compared to vaccination with Her2<sub>1-683</sub>PyVLP alone. The reduction of antibodies could also be of value if several vaccinations would be required for tumor rejection. It may also be possible that the combination of DC-vaccination and vaccination with Her2<sub>1-683</sub>PyVLPs alone could be efficient for prime boost therapy.

## CONCLUSIONS

Subcutaneous MPyV-VLP vaccination protects both normal and T cell deficient mice against MPyV infection. Furthermore, VLPs are more efficient than the more linear GST-VP1 in inducing antibody response and for protection (paper 1).

A new murine polyomavirus vector, MPtV-VLP, has been produced. It does not cross react with MPyV-VLPs and should therefore be useful as a complement in gene- and immune therapy (paper 2).

Her2<sub>1-683</sub>PyVLP immunization efficiently protects mice against outgrowth of Her2-expressing tumors. This protection is enhanced by loading the Her2<sub>1-683</sub>PyVLPs on DCs. The latter vaccination strategy also reduces anti-VLP titers (paper 3 & 4).

## FUTURE PERSPECTIVES

The main future challenges are now to improve the anti-tumor immune response generated by VLPs carrying the Her2 antigen, and to understand the mechanisms behind the immune response.

In this thesis, MPtV-VLPs have been characterized with the aim to use them for prime-boost immunotherapy. We now have preliminary data showing that vaccination with MPtV-VLPs carrying a fusion protein between MPtV-VP2 and Her2, Her2<sub>1-683</sub>PtVLPs, also give full protection against the D2F2/E2 tumor. The next step will be to examine if they, in combination with Her2<sub>1-683</sub>PyVLPs, in prime-boost immunotherapy can further increase anti-tumor immunity.

Her2<sub>1-683</sub>PyVLPs have been shown to be efficient for preventive vaccination against Her2-expressing tumors however; they would be even more attractive if they could also be used as therapeutic vaccines. The possibility to use Her2<sub>1-683</sub>PyVLP vaccination alone, or in prime-boost vaccination with Her2<sub>1-683</sub>PtVLPs, or with DCs loaded with Her2<sub>1-683</sub>PyVLPs as therapeutic vaccines will be examined.

The mechanism by which the VLPs induce the anti-tumor protection also needs further investigation. This can be done by comparing tumor protection after vaccination in different knock-out mice, affecting components of the immune system, or by depleting certain cell populations *in vivo* before vaccination.

Her2<sub>1-683</sub>PyVLPs are based on human Her2 and BALBneuT mice express rat Her2. In order to exclude that the strong anti-tumor response is due to a xenogenic effect, we have produced VLPs, from both MPyV and MPtV, carrying rat Her2. These will now be used to vaccinate BALBneuT mice.

To optimize VLP immunotherapy, we are now increasing the amount of Her2 antigen inside VLPs, by constructing VLPs with different parts of the Her2 protein, as well as with shorter VP2 fragments.

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