From THE DEPARTMENT OF ONCOLOGY/PATHOLOGY
Karolinska Institutet, Stockholm, Sweden

VACCINATION AGAINST HER2/*NEU*-EXPRESSING CANCER USING CHIMERIC VIRUS-LIKE PARTICLES

Kalle Andreasson

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ABSTRACT

Thanks to the development of vaccines, children are these days protected against infectious diseases like polio, diphtheria and tetanus simply by receiving a few injections. Imagine a scenario where these injections would also result in protection against cancer. Vaccines against virus-induced cancer such as liver and cervical cancer are indeed already in use. Intense research is now focused on the generation of vaccines for protection also against cancer not induced by viruses. The overall aim of this thesis was to develop and determine the pre-clinical efficacy of a vaccine against tumours expressing the antigen Her2/*neu*.

Polyomaviruses are small non-enveloped viruses that are widespread in the population, and probably harmless as long as the immune system is normal. The viral capsid is composed of three structural proteins, the major structural protein VP1, and the two minor proteins VP2 and VP3. The mouse is host to two known polyomaviruses, murine polyomavirus (MPyV) and murine pneumotropic virus (MPtV), respectively.

It was known from before that VP1 of MPyV could self-assemble into viral capsids known as virus-like particles (VLPs), named so because of their morphological resemblance to natural viruses. In paper I, we showed that VP1 of MPtV could also self-assemble into VLPs, which were rapidly internalized by all tested cell types, including dendritic cells. However, the VLPs were not very efficient as vectors for gene therapy, possibly due to poor delivery of DNA into the nucleus of target cells.

The combination of efficient cellular uptake and poor nuclear delivery indicated that the VLPs could be more efficient as carriers of proteins for presentation to the immune system, since delivery into the cytoplasm would be sufficient in that case. We therefore attached Her2/*neu* to the inside of VLPs of both MPyV and MPtV and obtained so-called chimeric VLPs (cVLPs). In papers II and III, we showed that Her2/*neu*-cVLPs could protect against outgrowth of transplantable Her2/*neu*-expressing tumours in normal mice, as well as against spontaneously arising Her2/*neu*-positive carcinomas in transgenic mice. Her2/*neu*-cVLPs from MPtV also induced immunological memory and protected against tumour outgrowth in a therapeutic setting.

The purpose of paper IV was to determine the immune mechanisms responsible for tumour protection. We could demonstrate that Her2/*neu*-cVLPs induced Her2/*neu*-specific $CD8^+$ T cells, but did not induce anti-Her2/*neu* antibodies. However, we observed that mice were protected against tumour development also after depletion of either $CD8^+$, or $CD4^+$ T cells, but not after combined depletion of $CD4^+$ and $CD8^+$ T cells. This indicated that $CD4^+$ T cells could compensate for the absence of $CD8^+$ T cells and mediate tumour protection by a yet not fully clarified mechanism independent of both antibodies and $CD8^+$ T cells.

In conclusion, in this thesis it is shown that Her2/*neu*-cVLPs based on both MPyV and MPtV are efficient as prophylactic and therapeutic vaccines against Her2/*neu*-expressing tumours in mice, and that protection involves both $CD4^+$ and $CD8^+$ T cells.

LIST OF PUBLICATIONS

The thesis is based on the following papers, which in the text will be referred to by their roman numerals (I-IV):

I. Tegerstedt K, **Andreasson K**, Vlastos A, Hedlund KO, Dalianis T, 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.
Journal of General Virology, 2003, 84, 3443-3452

II. Tegerstedt K, Lindencrona JA, Curcio C, **Andreasson K**, Tullus C, Forni G, Dalianis T, Kiessling R, Ramqvist T;
A single vaccination with polyomavirus VP1/VP2Her2 virus-like particles prevents outgrowth of HER-2/neu-expressing tumours.
Cancer Research, 2005, 65, 5953-5957

III. **Andreasson K**, Tegerstedt K, Eriksson M, Curcio C, Cavallo F, Forni G, Dalianis T, Ramqvist T;
Murine pneumotropic virus chimeric Her2/neu virus-like particles as prophylactic and therapeutic vaccines against Her2/neu expressing tumours.
International Journal of Cancer, 2009, 124, 150-156

IV. **Andreasson K**, Eriksson M, Tegerstedt K, Ramqvist T, Dalianis T;
$CD4^+$ and $CD8^+$ T cells can act separately in tumour rejection after immunization with murine pneumotropic virus chimeric Her2/neu virus-like particles.
Manuscript

CONTENTS

LIST OF ABBREVIATIONS

ADCC	Antibody-Dependent Cellular Cytotoxicity
AIDS	Acquired ImmunoDeficiency Syndrome
APC	Antigen-Presenting Cell
β2m	β2-microglobulin
BCR	B Cell Receptor
BKV	BK Virus
BPV	Bovine PapillomaVirus
CAR	Coxsackie B virus and Adenovirus Receptor
CD	Cluster of Differentiation
CFA	Complete Freund's Adjuvant
CIN	Cervical Intraepithelial Neoplasia
CLR	C-type Lectin Receptor
CpG	Cytosine-phosphate-Guanosine
cr	conserved region
CTL	Cytotoxic T Lymphocyte
cVLP	chimeric Virus-Like Particle
DC	Dendritic Cell
DC-SIGN	Dendritic Cell-Specific ICAM-3 Grabbing Non-integrin
DNA	DeoxyriboNucleic Acid
EBV	Epstein-Barr Virus
E. coli	Escherichia coli
EGFP	Enhanced Green Fluorescent Protein
EGFR	Epidermal Growth Factor Receptor
ELISPOT	Enzyme-Linked ImmunoSPOT
ER	Endoplasmic Reticulum
FasL	Fas Ligand
Gal	Galactose
GalNac	N-acetyl-Galactosamine
GFP	Green Fluorescent Protein
GM	Ganglioside Monosialo
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
HaPyV	Hamster PolyomaVirus
HBsAg	Hepatitis B surface Antigen
HBV	Hepatitis B Virus
Her2	Human epidermal growth factor receptor 2
HIV	Human Immunodeficiency Virus
HPV	Human PapillomaVirus
i.d.	intradermal/ly
IFA	Incomplete Freund's Adjuvant
IFN	InterFeroN
IL	InterLeukin
i.m.	intramuscular/ly
JCV	JC Virus
kb	kilobase pairs

kDa	kiloDalton
KIPyV	KI PolyomaVirus
LAG-3	Lymphocyte Activation Gene-3
LCMV	Lymphocytic ChorioMeningitis Virus
LPS	LipoPolySaccharide
LT	Large T antigen
mAb	monoclonal Antibody
MAPK	Mitogen-Activated Protein Kinase
MCPyV	Merkel Cell PolyomaVirus
MGL	Macrophage Galactose-type Lectin
MHC	Major Histocompatibility Complex
MMTV	Mouse Mammary Tumour virus
MPtV	Murine Pneumotropic Virus
MPyV	Murine PolyomaVirus
MT	Middle T antigen
NeuAc	N-Acetyl Neuraminic acid
NK	Natural Killer
NLS	Nuclear Localization Signal
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
p.i.	post infection
PI3-kinase	Phosphatidyl-Inositol-3-Kinase
PML	Progressive Multifocal Leukoencephalopathy
PP2A	Protein Phosphatase 2A
PRR	Pattern Recognition Receptor
PTEN	Phosphatase and tensin homolog deleted on chromosome TEN
PTK	Protein Tyrosine Kinase
Rb	Retinoblastoma
SA12	Simian Agent 12
s.c.	subcutaneous/ly
ST	Small T antigen
SV40	Simian Virus 40
TCR	T Cell Receptor
Th	T helper/helper T
TLR	Toll-Like Receptor
TRAIL	TNF-Related Apoptosis Inducing Ligand
Treg	regulatory T cell
VLP	Virus-Like Particle
VP	Viral capsid Protein
WUPyV	WU PolyomaVirus

1 PREFACE

In December 2000, a 19-year-old student stepped into a laboratory at Karolinska Institutet. He had a bad hair day (as always), his blue jeans (bought by his mother) were full of holes and the T-shirt he was wearing (which he had received for free from a company) was completely washed out. Full of expectation, enthusiasm and energy he was eager to start working on a stimulating and exciting research project.

More than eight years have passed since that December day and it is about time I put my foot down and summarize these years. A lot has happened. Fortune and misfortune. Happiness as well as sorrow. My experience is that many not-in-research people tend to have a glamorous notion of scientists as people who do extremely exciting stuff the whole day like solving the riddle of cancer (which was by the way solved some twenty years ago). Most of the time research is indeed fun, exciting and intellectually stimulating but…without depreciating the significance of creativity, sound knowledge and not being afraid of questioning dogmas, I would claim that characteristics such as patience, stubbornness and a high working ability are at least as important. "Research" means in many cases hard highly repetitive routine work without the need for intellectual brilliance. Often it feels like the frontal bone itself is much more important than what is behind it. If you, dear reader, besides having a thick frontal bone think that overtime payment is overrated, see a fascination in unsocial working hours and cannot wait to look for lost orders, calibrate machines, make registries and clean plugholes (the janitor is busy as usual and cannot come before the next year…or was it the next decade?), maybe you should also consider a career as an experimental scientist.

During these years I have learnt that research is very competitive and that some people harass each other, falsify data and are involved in other kinds of academic misconduct in their striving for pride, glory and grant money. I have had the great privilege of working with several generous people, who have unselfishly involved me in their projects and for this I am forever grateful. Without these people's generosity my thesis would probably take a few more years to complete.

Since I am indeed a researcher, not a professional writer, I have allowed myself the slightly erratic pleasure of adding superfluous stories of doubtful value for the thesis (this preface is an excellent example), numerous odd personal thoughts and anecdotes as well as an overload of historical overviews. Some Latin, a tiny bit of Greek mythology, a cow and far too many footnotes have also slipped into the text.

Bonne lecture!

Solna on a sunny and snow-free spring day, 29th of March 2009

Kalle Andreasson

2 POLYOMAVIRUSES

2.1 SCIENTIFIC MUMBO JUMBO

To make the journey through this thesis as enjoyable as possible I think it is of importance to get into the scientific mood already at this very early stage. I would like to help you with that by introducing you to some of the most useful words in biomedical research, a little *dictionary of scientific words* if you like. The first word is *nonspecific*. If you get a result that you have not expected such as an extra band on a gel or an additional spot in an assay and you have no clue whatsoever what this extra band/spot represents, you can simply denote it as *nonspecific* and keep your fingers crossed that nobody will ask you about it. Another very useful word in the same category is *background*. If your negative controls give a strong response you can explain this by saying "there is a high background" without knowing, in accordance with nonspecific, what the background really consists of. The third and last word I would like to introduce you to is *artefact*[1]. If a result is odd you can, instead of being honest and admit that the result is odd, try to give the impression of being much cleverer than you really are by using the more academically correct word artefact. Artefact basically means "I don´t know at all what this is but I don´t want it to be in my experiment". Accordingly, artefact and nonspecific are to a large extent interchangeable. However, and this is the interesting point, in some cases nonspecific is not nonspecific at all but rather highly specific for something nobody has yet defined, a background is not always an annoying background but in fact consists of something extremely exciting that has not yet been explored and finally, in analogy with this, an artefact can actually be something very interesting just waiting to be studied. Many great discoveries have been made when scientists have looked into nonspecific results, mysterious backgrounds and peculiar artefacts. In other words, a scientist should have a prepared mind, be observant of unforeseen results and, last but not least, have a great deal of luck.

2.2 POLYOMAVIRUS PIONEERS

The American physician Ludwik Gross[2] had such a prepared mind and thanks to this he laid the foundations of the polyomavirus field. When inoculating extracts of leukemia into mice he noted that only very few mice developed the expected leukemia but instead a large proportion developed tumours in the cervical region, which were probably carcinomas arising in the salivary glands. Instead of muttering about artefact, background or nonspecific phenomenon he found this unexpected finding exciting and explored it further. Based on very simple but elegant experiments he could show that the agent causing leukemia was different from the one giving rise to salivary gland tumours and he called the two agents "Ak[3] tumor agent" and "Ak leukemic agent", re-

[1] *Artefact* originates from the Latin words *ars* meaning art and *facere/facesso* meaning do/make/create. The overall meaning of artefact (*artifact* in American English) is something that is artificial.
[2] His name is also spelled Ludwig Gross.
[3] *Ak* was the line of mouse used.

spectively, and published this in 1953 (1). A few years later Sarah Stewart, Bernice Eddy and co-workers showed that this "agent" was capable of inducing many different tumours, not only in mice (2, 3), but also in rats (4) and hamsters (5). Other scientists responded to Stewart's and Eddy's results by saying "they must have a hole in their filter or a hole in their heads" (6). Apparently both their filter and heads were in good shape since we now know that the results were true. Soon the sceptics had to eat humble pie and Peyton Rous was awarded the Nobel Prize in Physiology or Medicine in 1966 for his discovery that viruses can contribute to development of solid tumours (7, 8)[4]. The virus studied by Gross, Stewart and Eddy was given the name murine[5] polyomavirus (MPyV) (10), from the Greek words for *many tumours* and polyomavirus was later taken as the name of the whole virus family. A stickler for details could argue that the name is incorrect and misleading since most members of the polyomavirus family have nothing at all to do with tumour development, in particular not in their immunocompetent hosts. Nevertheless, without entering deeply into that discussion we can state that Gross, Stewart, Eddy and their colleagues had written the first chapters in the polyomavirus story. For the sake of completeness, it should be mentioned that some people consider Gross as the sole discoverer of MPyV, whereas other people state it was discovered independently by him and Stewart (11).

One major discovery in the polyomavirus field is often followed close in time by another one. The very same year as Gross (and Stewart?) published the discovery of MPyV, Lawrence Kilham and Helen W Murphy isolated yet another polyomavirus from mice (12). To be absolutely correct, the isolation of the virus was actually described in a publication the year before with Lawrence Kilham as sole author (13), but the publication from 1953 is by many considered as the first description of the virus. At this time discovered pathogens could be given a name based on the discoverer, and the virus was therefore named K-virus, which was later renamed Kilham virus (not to be mixed up with the parvovirus Kilham rat virus (14)) and the virus is nowadays known as murine pneumotropic virus (MPtV)[6] (15).

2.3 POLYOMAVIRUS – A SIMPLE CREATION

As we will see throughout this thesis, science is often complicated, tricky and abstract with different studies yielding contradictory results (this is at the same time in part the charm of science). However, one of the advantages of the polyomavirus field is that polyomaviruses have a very simple structure, both of the genome and of the virus particle. All polyomaviruses harbour a single copy of a circular double-stranded DNA molecule with a size of approximately 5 kb as schematically shown in figure 1. The genome is surrounded by a capsid but no envelope as depicted in figure 2. Polyomaviruses are in relation to most other viruses small with a diameter of around 45 nm. The

[4] Some people claim that the first tumour virus was not discovered by Rous but rather by Ellerman and Bang in 1908 (9) but that is a different story.
[5] *Murine* is derived from the Latin word *muris* meaning mouse or rat.
[6] *Pneumo* is the Greek word for air, based on the fact that the virus has a tropism for the endothelial cells in the lungs, see chapter 2.7.2.

genome is divided into three regions; a non-coding regulatory region (NCCR), an early and a late region[7], respectively (figure 1). Transcription occurs in both directions from opposite strands. The regulatory region contains an origin of replication as well as promoter and enhancer elements. The early region encodes two, or in some viruses three, regulatory proteins (see chapter 2.4), while the late region codes for three structural proteins and, in some cases, a fourth non-structural protein known as agnoprotein. The major structural protein, VP1 (45 kDa), is arranged in pentamers on an icosahedral lattice[8] (16, 17). To the inner side of each VP1 pentamer one of the two minor structural proteins, VP2 (35 kDa) and VP3 (23 kDa) is bound, implying in theory that each capsid is composed of 72 pentamers of VP1 and in total 72 monomers of VP2 and VP3 as shown in figure 2. The structural proteins will be described in more detail in chapter 2.5.

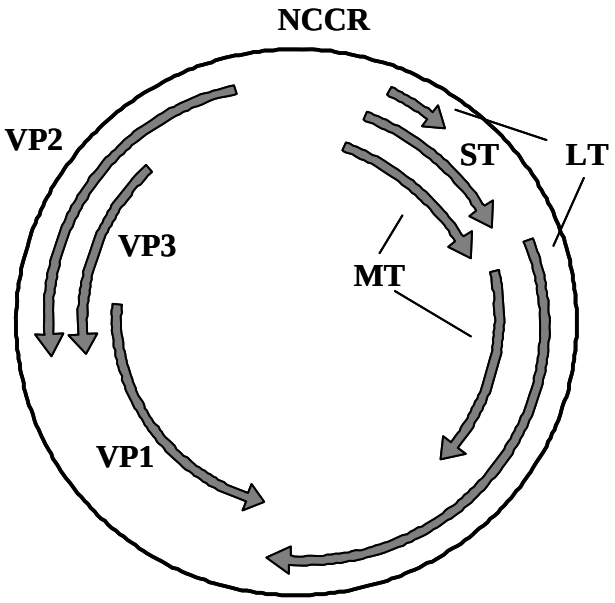


Figure 1. Structure of the genome of murine polyomavirus. Transcription occurs bidirectionally from the NCCR. Generally, polyomaviruses do not express middle T antigen. Some viruses express an additional protein, the agnoprotein, from the late region from an open reading frame located before the start codon of VP2. NCCR, non-coding control region; ST, small T antigen; MT, middle T antigen; LT, large T antigen

[7] *Early* and *late* refer to at what time points the regions are (or at least originally were thought to be) transcribed, the early region is transcribed before replication and the late region after replication.
[8] Most viral capsids are either *helical* (rod-shaped) or *icosahedral*. Icosahedral is derived from the Greek word *eikosaedron* ("twenty seats"), which is an almost spherical structure, originally having 20 sides.

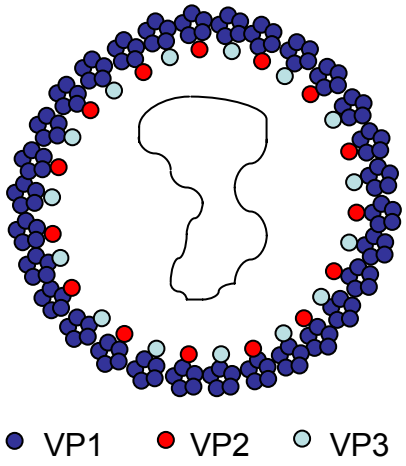


Figure 2. Schematic structure of a polyomavirus particle. A single copy of a circular double-stranded DNA molecule is surrounded by a capsid. The capsid is in turn composed of 72 pentamers of the major structural protein VP1 and to each pentamer either one VP2 or one VP3 molecule is bound.

2.4 PROTEINS ENCODED BY THE EARLY REGION

All polyomaviruses express the regulatory proteins small T[9] antigen (ST[10], 17 kDa[11]) and large T antigen (LT, 98 kDa) from the early region, while some members of the polyomavirus family express an additional regulatory protein denoted middle T antigen (MT, 52 kDa) (see table 1). The three mRNA molecules are generated through differential splicing from a common pre-mRNA (18). This results in identical N termini, while each T antigen has a unique C-terminal region (19). Some polyomaviruses like simian virus 40 (SV40) and JC virus (JCV) express additional early proteins created through alternative splicing (20, 21). In figure 3, many although not all of the domains present in the three tumour antigens are shown.

2.4.1 Large T antigen

LT is a multifunctional protein and as we will soon see it has a wide range of possible binding partners. The protein has a finger in regulation of both replication, transcription and transformation and studies in the middle of the 1970s showed that LT of SV40 was required for both the establishment and the maintenance of the transformed phenotype (22-25). The protein has been most extensively studied in SV40 and much of the description below is based on studies of this virus.

We will now have a look at the various domains in LT. If we start at the N terminus there is a highly conserved region denoted conserved region 1 (cr1), and mutations in this region make the virus unable to both transform cells[12] and make infectious virus particles (26). Then follows another highly conserved domain, the HPDKGG box, im-

[9] *T* refers to tumour.
[10] The nomenclature with regard to capital and small letters is not clear to me. I have decided to use the capital letter T for all proteins as well as capital letters for the abbreviations.
[11] The molecular weights differ to some extent between the various polyomaviruses.
[12] With regard to transformation *in vitro*, these LT mutants are defective if expressed from the normal viral promoters, but transforming activity is retained if LT is expressed from a "foreign" strong promoter.

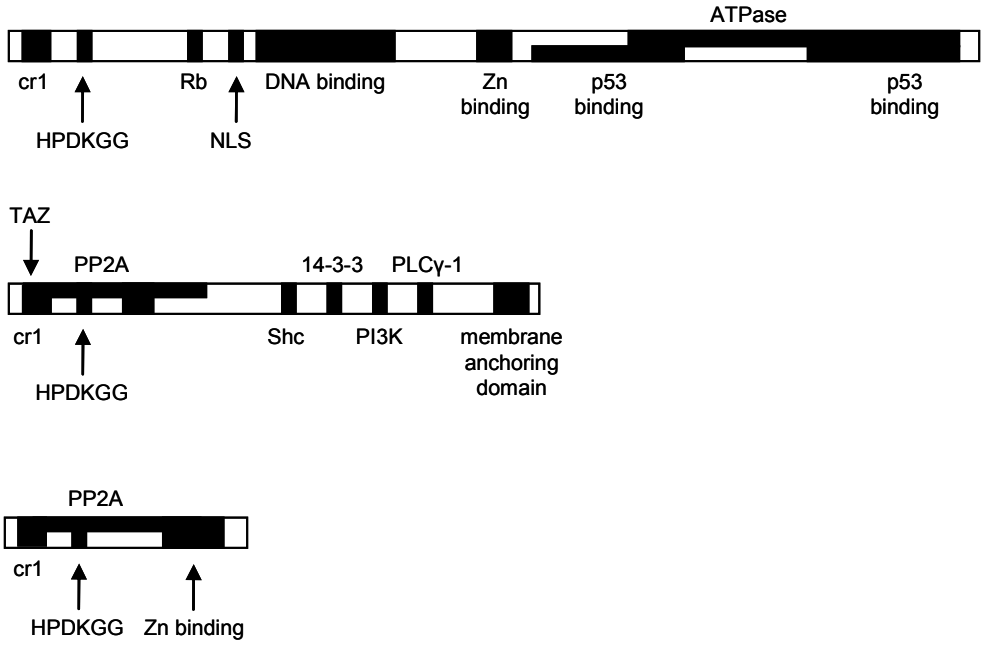


Figure 3. Schematic outline of the different domains in large T antigen (top), middle T antigen (middle) and small T antigen (bottom).

portant for replication (27). The next domain we encounter is the retinoblastoma protein (Rb) binding site (also called conserved region 2) harbouring the sequence LXCXE. It binds Rb and its cousins p107 and p130 (28-32). What is actually the rationale for a virus to bind Rb? The Rb protein is probably most well known as a tumour suppressor and inactivation of it increases the risk of cancer development. Could it be that the reason for Rb binding is that the virus has an inborn nastiness to give rise to cancer? I once asked George Klein why some viruses cause cancer, do they really gain anything from it? George immediately corrected me: "Viruses cannot CAUSE cancer, they only CONTRIBUTE to cancer development", i.e. a virus cannot cause cancer on its own, and other factors are needed as well. Anyhow, George then explained to me that if a virus-infected cell would develop into a malignant cell this would be a dead end for the virus, and the virus would definitely not gain anything from it. A malignant tumour will kill the individual unless treated and thereby the virus will die simultaneuosly, and this is not at all what the virus wants. So the question remains: Why does the virus bind and inactivate Rb? Viruses are unable to replicate on their own, and are dependent on the replication machinery of their host cells. This means that if a virus is going to replicate, the cell must replicate at the same time. Since a virus is highly egoistic and wants to produce progeny, it is a great advantage if it can push the host cell into the cell cycle in order for replication to occur. This is exactly what polyomaviruses do. The Rb protein keeps an eye especially on the transition of the cell from the quiescent phase G1 into the DNA synthesis phase S. Inactivation of Rb results in improper control of the G1-S checkpoint leading to progression of the cell into the S phase, and synthesis of the cellular as well as of the viral genome. However, there is a snag in this process, which we will come back to in a short while.

Before dealing with this snag we will return to the different domains in LT. LT is a nuclear protein and transportation into the nucleus is directed by a nuclear localization signal (NLS) located C terminal to the Rb binding domain. Thereafter we have a DNA binding region (reviewed in (33)) that we will have a little closer look at. In the NCCR there are multiple LT binding sites (34). LT binds to the origin of replication where it forms two hexamers (35, 36), unwinds the DNA molecule and initiates DNA replication (37). LT also coordinates replication with transcription of the viral genes by repressing transcription of the early region (38, 39) and inducing transcription of the late region, in order to produce the structural proteins and thereby render it possible for new virus particles to form (40, 41).

A zink finger region (42) follows the DNA binding domain and this region plays a role in oligomerization of the protein into hexamers and is thereby important for replication (43, 44). To fulfil all these different functions might seem as a sweaty work but this is not enough. As mentioned above, inactivation of Rb pushes the cell into the S phase. However, the tumour suppressor protein p53, also known as *the guardian of the genome*, will sense this perturbation of the cell cycle control and realize that this makes a threat to the cell and an enhanced risk of transformation. Therefore p53 will induce apoptosis[13], i.e. the cell be will sacrificed in order to save the individual from cancer. This appears to be a clever strategy by the cell, but…polyomaviruses are even cleverer since LT has a p53 binding domain and will bind to and inactivate p53 (45-47)[14]. In this way the transcriptional activity of p53 will be abrogated (48, 49), leading to avoidance of apoptosis and the cell will progress through the cell cycle and the final result will be that the virus will survive. As curios, p53 was discovered when people were studying LT of SV40 (46, 47).

Overlapping with the p53 binding domain is an ATPase domain (50, 51) and this domain makes the end of LT in many polyomaviruses, while some viruses like SV40, JCV, BK virus (BKV) and simian agent 12 (SA12) have a host range domain at their C terminus. The host range domain is required late in viral productive infection, probably in viral assembly (52, 53).

2.4.2 Middle T antigen

MT is as far as we know only expressed by MPyV and hamster polyomavirus (HaPyV) (54, 55). It should however be noted that the recently described human polyomavirus Merkel cell polyomavirus (56) has an open reading frame (ORF) with major similarities to the MT-encoding ORF in MPyV and HaPyV (my own observation and P. Moore (personal communication)) although expression of MT mRNA has so far not

[13] *Apoptosis* is Greek and means *falling off*, originally referring to the dropping off of petals/leaves from trees. Apoptosis is the process of programmed cell death used by the cell to commit suicide in the case of damage to the cell. The Nobel prize in Physiology or Medicine in 2002 was awarded to Sydney Brenner, Robert Horwitz and John Sulston for their "discoveries concering genetic regulation of programmed cell death" (8).

[14] The LT of MPyV and HaPyV lack the p53 binding domain but these viruses encode MT instead.

been detected for this virus (57). In the following only MT of MPyV will be discussed, since it has been better characterized than MT of HaPyV. MT is best known for its transforming capacity and is considered the major transforming protein. MT alone can transform established cells (58), while primary cells in addition require complementary proteins for transformation (59-61). Several mouse strains transgenic for MT have been created leading to spontaneous development of various tumours such as prostate carcinoma (62), breast cancer (63) and neuroblastoma (64). MT is attached to membranes of e.g. the endoplasmic reticulum (ER) (65, 66) and the plasma membrane (67, 68) through a stretch of hydrophobic amino acids at its C terminus (69, 70). Mutations that make MT cytoplasmic abrogates its transforming capacity (70). A key feature of MT is its ability to interact with proteins involved in cellular signalling and the function of MT has been compared to a constitutively active receptor tyrosine kinase. MT binds and inactivates protein phosphatase 2A (PP2A) (71, 72), which is a serine/threonine phosphatase that functions as a tumour suppressor (73). This interaction makes it possible for MT to bind protein tyrosine kinases (PTKs) of the Src[15] family (74-77). The PTKs subsequently phosphorylate MT on particular tyrosines (78, 79), and this in turn results in interaction between MT and other signalling molecules including PI3-kinase (80-82), PLCγ-1 (83) and Shc (84, 85). MT also interacts with several other proteins that I am not at all familiar with such as TAZ (86) and 14-3-3 proteins (87). However, MT is not only involved in transformation, but also in regulation of replication and transcription (88).

2.4.3 Small T antigen

Before leaving the early and carrying on to the late region we will have a quick look at ST. This protein is expressed by all polyomaviruses and as can be seen in figures 1 and 3, the N-terminal part is common to both MT and LT. Domains found in this part of the protein include the cr1 and the HPDKGG box. The ST unique C-terminal region is rich in cysteines that bind zinc ions, which is necessary for protein stability (89). The main function of ST seems to be to cooperate with and enhance the effects of LT and both proliferation and transformation by LT are facilitated by ST (reviewed in (90)). Expression of SV40 LT in mice results in fewer tumours compared to expression of both LT and ST (91, 92). The major cellular binding protein for ST is PP2A (71) and, in accordance with MT, this leads to PP2A inhibition (93, 94). Furthermore, ST increases expression from the early and late promoters (95).

2.5 PROTEINS ENCODED BY THE LATE REGION

As mentioned in chapter 2.3, the late region of all polyomaviruses encodes the three capsid proteins VP1, VP2 and VP3, while in some viruses this region in addition encodes a non-structural protein (agnoprotein). All three structural proteins are generated from a common pre-mRNA by alternative splicing. VP2 and VP3 are transcribed from the same ORF, while transcription of VP3 starts at an internal start codon meaning that

[15] The term Src is derived from Rous sarcoma virus, the same Nobel-prize awarded Rous as in chapter 2.2.

VP3 is identical to the C-terminal part of VP2 and this is clearly seen in figure 1. VP2 and VP3 are assumed to be completely hidden inside the capsid and thus not accessible to antibodies. This is an important feature when it comes to papers II-IV. Since the viral capsid encloses a DNA molecule it is likely that one or several of the structural proteins are able to bind DNA. Sure enough, a DNA binding domain has been identified in the N-terminal region of VP1 of MPyV (96, 97) and SV40 (98, 99), as well as in VP2/3 of SV40 (100), and there are good reasons to believe that at least one of the structural proteins of all polyomaviruses can bind DNA. The DNA binding domain does not only bind polyomavirus DNA but rather DNA in a sequence-independent manner, i.e. it can probably bind any sequence (97, 98). This is of paramount importance when it comes to the use of modified virus particles in gene therapy as in paper I.

A distinguished professor at our department, apparently with a good knowledge of geometry, has repeatedly pointed out that it should not be possible for a particle consisting of 72 identical subunits to form a spherical (icosahedral) structure. I have tried to elucidate this issue. An icosahedral capsid in which all subunits are identical and the interactions between all subunits are identical as well can only be composed of 60 subunits or less. In a capsid composed of more than 60 identical subunits, the units must interact with each other in a quasiequivalent (!) manner meaning that the interactions between the subunits are not the same throughout the capsid (101). Hypothesizing instead that not all subunits are identical, then it should be possible for an icosahedral capsid to form regardless of the number of subunits. It was initially thought that the polyomavirus capsid was made up of 420 subunits, 12 pentamers and 60 hexamers of VP1 (16). However, when the crystal structure of MPyV was revealed, it was obvious that all subunits were in fact pentamers (17). Today we know that the polyomavirus capsid is composed of 12 five-coordinated (pentavalent) and 60 six-coordinated (hexavalents) subunits. In simple words, 12 of the pentamers bind five other pentamers, while 60 of the pentamers interact with six pentamers and, abracadabra, an icosahedral polyomavirus capsid can be created (102). Now this issue has hopefully been solved once and for all.

We will return to the structural proteins in relation to the formation of virus-like particles (VLPs) in chapter 7. As mentioned above, in the 5' proximal part of the late region of some polyomaviruses, there is a gene named the agnogene (103) (table 1). The gene product, the agnoprotein, is small (8 kDa), mainly found in the cytosol (104, 105), and has multiple effects which are somewhat different between the various members of the polyomavirus family. In SV40, the protein seems to interact with VP1 during the late stages of development of new virus particles. SV40 agnogene-negative mutants are viable, but have a smaller plaque size than wild-type virus and produce virus particles more slowly (106), as well as have defective viral maturation (107, 108). In JCV, the agnoprotein suppresses both transcription and replication (109). A mutant deficient in agnoprotein expression is viable, but replicates less efficiently than the

wild-type virus (110). In BKV, phosphorylation of the agnoprotein is essential for normal virus production (111).

2.6 MURINE POLYOMAVIRUS

As mentioned in chapter 2.2, MPyV was discovered in the beginning of the 1950s (1, 11), and found to induce tumours in a multitude of tissues in various animal species (2, 4, 5). Later it was also shown to transform cells *in vitro* (112). For the purpose of gene and immune therapy using modified virus particles, which is the aim this thesis work, some aspects of the natural virus are of particular importance. One is which cell types the virus can enter. In some cases you only want the modified virus to enter some cells, whereas in other cases a more promiscuous uptake is desired. In this chapter we will therefore put much focus on the cell tropism of MPyV and also (try to) clarify the receptor. Furthermore, we will in brief discuss the mechanism(s) of uptake and the intracellular trafficking, since this is also of importance. Although it is not really relevant for the understanding of the thesis, I have included a description of the nature of natural MPyV infection and also a few words about tumour development, in order to provide some general information of the virus.

2.6.1 Natural infection

To my knowledge the route of spread of MPyV between mice has not been established, but the virus has been detected in both urine and samples taken from the mouth (113). To draw any general conclusions about the nature of a natural virus infection is somewhat difficult since it is depending on the mouse strain, the age and the immune status of the mice, the route of inoculation, as well as the strain of the virus[16]. If MPyV is present in a mouse colony[17], newborn mice are protected against virus infection thanks to the presence of maternal antibodies (114). However, newborn mice lacking maternal antibodies are susceptible and a persistent infection[18] will be established although the incidence of virus persistence is highly mouse strain dependent (115, 116). Nevertheless, in adult normal mice virus persistence does not occur regardless of the mouse strain and virus infection is cleared within approximately 1-6 months (117, 118). The organs that get infected are again dependent on the mouse strain, and in general the virus is found in a wide range of organs. According to the classification of Wirth from 1992 the organs can be divided into two groups (class I and II). In class I organs (mammary gland, skin and bone), viral replication is high in newborn and moderate in adult infected mice, while in class II organs (kidney, liver, lung), viral replication is high after infection of newborn and low after infection of adult mice (119). It should also be

[16] Two completely different kinds of strains are discussed here. *Virus strains* of MPyV are usually divided into large plaque strains such as A2, A3 and PTA and small plaque strains like RA. In addition, we have *mouse strains* such as BALB/c and C57Bl/6.
[17] Mice used in research are generally negative for MPyV.
[18] Virus infection is here defined as detection of viral DNA by PCR, *in situ* hybridization or Southern blotting. The definition of *persistent MPyV infection* differs between studies, some define it as presence of virus >5 weeks post infection while others define it as presence up to several months after infection.

noted that the sites of persistence are to some extent dependent on the route of virus inoculation (120, 121).

2.6.2 Tumour development

Although it was shown very early in the history of MPyV that the virus could induce tumours in various animals, tumour development was a relatively rare event. Subsequently, a line of evidence suggested that this was due to a potent immunologic control of viral infection and tumour induction. Today we know that polyomavirus does not give rise to tumours in immunocompetent mice, such as normal adult mice, but only in immunocompromised mice such as newborn mice (given that they lack protective maternal antibodies) and adult mice with certain immunodeficiences (115, 122-125). In analogy with the observation that various strains of mice have different susceptibility to polyomavirus infection, the incidence of tumours is also mouse strain dependent (119, 124).

It should though be noted that all wild-type laboratory strains of MPyV can transform established cells *in vitro* (126). Since the virus can infect a multitude of different cell types it is maybe not so surprising that tumours can arise in many different tissues/organs as well. The most common sites for tumour development are bone, mammary and salivary glands, hair follicles, skin, thymus and kidneys (123-126) but, once again, the tumour tissue profile is dependent on the strain of both the mouse and the virus (124, 126). Since tumours do not develop in immunocompetent mice, one might wonder how tumours could be so common in the pioneering studies by Gross, Stewart, Eddy and colleagues. Did they really use immunodeficient mice? Yes, they did! In references (1-3, 11) the inoculated mice were less than 1 day old. According to Ludwik Gross attempts to transmit leukemia between mice had failed until he learnt in 1951 that coxsackie virus had to be inoculated into mice less than two days old in order to induce paralysis. He therefore realized that the key to success with MPyV was to use newborn rather than adult mice (127). In addition, the polyomavirus pioneers were lucky in the sense that they used mice of the C3H strain, which is a strain extremely susceptible to polyomavirus-induced tumour development (124).

To make things even more complicated, not all virus strains are able to induce tumours in the mouse. The strains PTA and A2 induce tumours at a high frequency with short latency (also called *high tumour strains*) while the strains A3 and RA induce few or no tumours at all and the tumours that arise do so with a latency of 7 months to a year (*low tumour strains*) (126). I assumed that this divergence was related to differences in one or several of the early proteins, but this is not the case. Instead it is due to a single amino acid substitution in VP1 (128), which is important for discrimination between different sialic acid linkages as we will come back to later (129, 130). Apart from

tumours, MPyV has been shown to induce other pathologies in mice including runting syndrome[19] (119, 131), myeloproliferative disease (132) and polyarteritis[20] (133).

2.6.3 The receptor finally comes out of the cold…maybe

Superficial knowledge is often regarded as "bad" knowledge. However, sometimes I almost regret that I try to really understand things properly. Life would be so much easier with superficial knowledge only. The receptor for MPyV is a superb example. If one want to be lazy, one could say that MPyV binds to sialic acid on target cells. As a lawyer would put it: This is the truth, but not the whole truth.

We must put the clock back more than 50 years to find the traces of the first tentative efforts to identify the MPyV receptor. Already in the end of the 1950s it was shown that MPyV could hemagglutinate erythrocytes from various species and that *Vibrio cholerae* receptor-destroying enzyme (RDE) prevented hemagglutination (134, 135). What I can understand RDE is the same as neuraminidase, which is a substance that destroys sialic acid, although I do not think the specificity of RDE was known at this time. Whether it was known at the time that polyomavirus hemagglutinates by binding to sialic acid is not clear to me either, but later the study from 1958 described above (135) was taken as the first demonstration that sialic acid is important for MPyV absorption to cells (136). Subsequently it was also shown that neuraminidase could prevent infection of mouse embryo cells (137). At this stage it might be appropriate to give a brief description of sialic acid and to be able to do this I had to remove the dust from my old book in biochemistry (138). Sialic acid is a carbohydrate (monosaccharide). An amine group (NH_2) is attached to one of the carbon atoms and to the amine group an acetyl group (CH_3-CO-) is linked as shown in figure 4. Sialic acid makes up the end of many glycoproteins, glycolipids and gangliosides and is present on virtually every cell type (139). The most common sialic acid in humans is N-acetyl neuraminic acid (NeuAc).

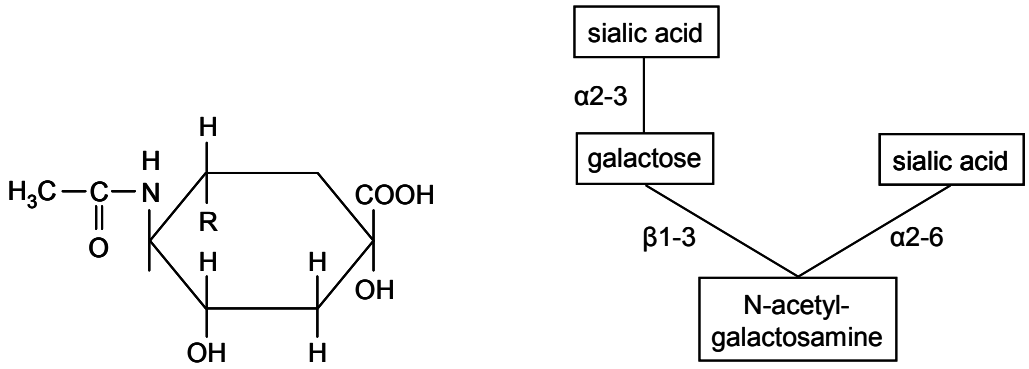


Figure 4. To the left, the structure of N-acetyl neuraminic acid, the most common sialic acid in humans, is shown. R denotes a 3-carbon chain with hydroxyl groups. To the right, the structure of NeuAc-α2,3-Gal-β1,3-(NeuAcα2,6)-GalNac is shown. NeuAc, N-acetyl Neuraminic acid; Gal, Galactose; GalNac, N-acetyl-Galactosamine.

[19] *Runting syndrome* is a kind of dwarfism seen in some animals like mice and pigs, usually accompanied with atrophy of the lymphoid organs.
[20] *Polyarteritis* means inflammation of the arteries.

Later reports indicated that MPyV did not bind to any sialic acid but only to specific molecules terminating in sialic acid. It was shown that if all sialic acid was removed from erythrocytes, binding by a large plaque strain could be restored by attaching sialic acid via an α2,3-bond,[21] but not an α2,6-bond (140), and that the sequence NeuAc-α2,3-Gal-β1,3-GalNAc[22] could serve as a specific cell surface receptor for this large plaque strain (136). As we have discussed before, various strains of MPyV differ in some respects. Could it also be that the various strains bound to different receptors? This issue was investigated by Cahan *et al* in 1983 (141), who showed that a large plaque strain bound to NeuAc-α2,3-Gal-β1,3-GalNAc, which is in line with the study above, while a small plaque strain could bind not only to this structure, but also to the branched disialyl[23] structure NeuAc-α2,3-Gal-β1,3-(NeuAcα2,6)-GalNac (figure 4) much stronger than the large plaque strain (141). Almost 20 years earlier it had been observed that the small plaque strain adsorbed to cells much better than the large plaque strain (142), and this could then be an explanation for that observation. In later studies these differences were found to be related to a single amino acid difference in VP1 as discussed above (130). In the middle of the 1990s several studies revealed the structure of MPyV in complex with sialyloligosaccharides (143-145). The question still remained whether MPyV bound to a unique receptor, or if it could bind to *any* molecule containing these sialic acid structures. Since MPyV was able to bind and infect a wide range of cell types it might seem likely that the virus could bind to multiple proteins/lipids, but the possibility still existed that the virus attached to *one* specific molecule present on many cell types.

When reading scientific literature you sometimes come across extremely ambitious studies. The one by Bauer and colleagues from 1999 (129) is an excellent example. They tested the hypothesis that MPyV binds to a unique molecule by incubating cells with supernatants from about 2000 (!) different hybridomas. The idea was that one or more antibodies would be able to prevent binding of MPyV to target cells. After testing these 2000 supernatants they could conclude that the results were negative, i.e. there was not a single hybridoma that was able to prevent infection. This suggested the likelihood of multiple receptor proteins/lipids (129). However, as often in research things would soon be turned upside-down. A study published in 2003 (146) showed that the gangliosides GD1a and GT1b *could* function as receptors for both small and large plaque strains. Gangliosides are glycolipids composed of a ceramide molecule[24] linked to a carbohydrate, often sialic acid. The structure of GD1a is schematically depicted in figure 5. They concluded that probably VP1 bound to the left branch, while the other sialic acid was highly unlikely to engage in contacts with VP1 (146). It should however

[21] This is a description of the bond between two carbon atoms. The carbons can be in either position α or β and the numbers (2 and 3 in this case) denote which carbon atoms in the molecule that make up the bond.
[22] This term might seem complicated but have a look at figure 5 and you will probably understand (Gal, galactose; GalNac, N-Acetylgalactosamine).
[23] *Disialyl* means that the molecule contains *two* sialic acid molecules.
[24] *Ceramide* is a lipid composed of sphingosine (a kind of alcohol) and a fatty acid.

be noted that the authors wrote that ”MPyV may utilize different receptors and follow different pathways of internalization depending in part on the target host cell”. Interestingly, the same group later showed that addition of GD1a to GD1a negative cells did not influence the overall level of virus binding but mediated the internalization and transit of virus to the ER (147). The group could also show that murine cells resistant to MPyV infection in fact bound MPyV but failed to allow entry, and the addition of GD1a restored infectibility (148).

Let us go back to the study by Bauer *et al* (129). From Cahan's study from 1983 it was known that a small plaque strain had a broader binding specificity than a large plaque strain (141). However, at the same time the small plaque strain was much less pathogenic than its large plaque counterpart as discussed in chapter 2.6.2[25]. Therefore Bauer and colleagues suggested the existence of pseudoreceptors *in vivo*, i.e. branched disialyl receptors that could bind MPyV, but would not allow viral entry, and thus such binding would not result in clinical disease (in line with study (148) above). They set up experiments to test this hypothesis and concluded that this was probably the case (129).

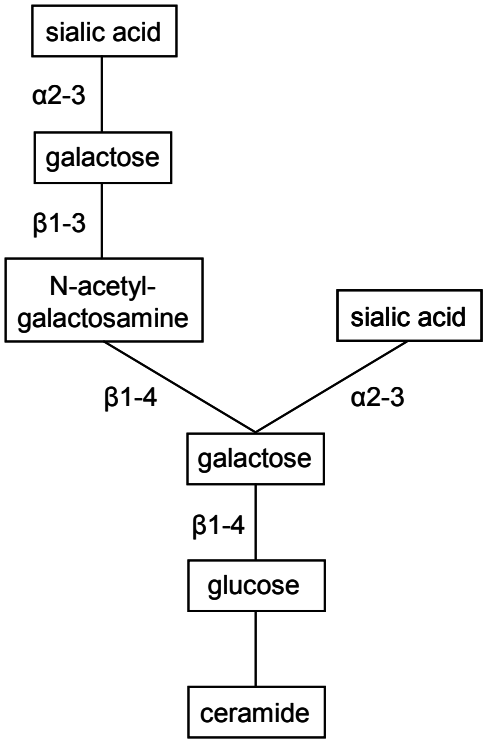


Figure 5. The structure of GD1a is shown. GT1b has an additional sialic acid attached via an α2-8 bond to the sialic acid molecule present in the right branch.

To conclude this chapter, some people today say more or less for sure that GD1a and GT1b *are* the receptors for MPyV (149). However, as the group that identified GD1a and GT1b as receptors for MPyV pointed out, MPyV may bind to different molecules on different target cells (146-148). The story will for sure continue.

[25] The literature is somewhat confusing. In the article by Bauer *et al* (129) it was claimed that small plaque strains were less pathogenic than large plaque strains. However, in the study by Dawe *et al* (126) it was in fact shown that both A3 (large plaque strain) and RA (small plaque strain) were *low tumour strains*.

2.6.4 Cellular uptake and intracellular trafficking

Finally we will discuss the fate of the virus within the cells. A crucial issue is how efficiently the DNA is delivered into the nucleus, since in gene therapy the DNA must reach the nucleus for the genes to be transcribed. Already in 1966 infection with MPyV was studied by electron microscopy and virus particles were initially observed within invaginations of the cell membrane. After a few hours the virus particles were seen in the cytoplasm and somewhat later between the two nuclear membranes[26]. It was proposed that the virus entered the cells through the process of pinocytosis[27] by these invaginations and that uncoating occurred between the nuclear membranes (150). However, another study suggested that the virus reached the nucleus intact (151). It was also shown that at 4°C the virus was able to attach to the cell membrane, but penetrate and enter only at 37°C. This is a phenomenon that we have utilized in paper I in our studies of the cellular uptake of MPtV. Two forms of membrane penetration were seen. Virus particles containing DNA entered in pinocytic vesicles, which appeared to migrate towards the outer nuclear membrane and intact virus particles were seen in the nucleus. The second form of penetration was one in which virus particles with transparent cores, i.e. without DNA, were observed to enter the cytoplasm in aggregates surrounded by membrane-structures looking like phagocytic[28] vesicles. These particles did not enter the nucleus but rather seemed to fuse with lysosomes for degradation. It was suggested that there was a nuclear transport recognition factor present on natural virus particles, while capsids must lack this factor and hence are penetrated in phagocytotic vesicles and destroyed (152-155). Later it was shown that the endocytic vesicles, which are made up of plasma membrane, were incorporated into the nuclear membrane (156). More than ten years later, in 2000, the characteristics of the vesicles were analyzed. It was now shown that neither clathrin-coated vesicles (JCV is taken up in such vesicles (157)) nor caveolae[29] were required for polyomavirus infectivity (159). The notion that viruses and "empty" particles used different ways of uptake was also challenged this year since a study now showed that VLPs composed of MPyV VP1 used the same mechanism of uptake as viruses. However, no intact viral particles were seen in the nucleus, which was suggested to be due to rapid uncoating (160). In accordance with these results, another study showed that there was no difference in adsorption, internalization and intracellular movements between natural MPyV virus particles, empty VP1 VLPs (lacking DNA) and full VP1 VLPs (containing DNA). In contrast to the results above, the virus particles and VLPs colocalized with caveolin. Cholesterol was important for the endocytosis indicating that MPyV entered cells through cholesterol-rich

[26] The *nuclear envelope* surrounding the nucleus has a rather complex structure and is made up of two nuclear membranes, inner and outer, in addition to several other parts.

[27] *Pinocytosis* is a form of endocytosis where extracellular fluid and small molecules are taken up non-specifically, i.e. without binding to receptors, in small vesicles.

[28] In contrast to pinocytosis, *phagocytosis* is endocytosis of large particles in a specific manner, i.e. it is receptor-dependent.

[29] *Caveolae* are invaginations of the plasma membrane, which are rich in lipids such as cholesterol and sphingolipids, and they are a form of lipid rafts. It has been a matter of debate whether they have a role in endocytosis or not but it has been shown that at least SV40 is taken up in caveolae (158). *Caveolin* is the classical marker of caveolae. Intracellular trafficking of SV40 occurs in an organelle termed *caveosome*.

lipid rafts, which would fit nicely with uptake in caveolae. No intact virus particles or even VP1 could be seen in the nucleus, and most VP1 accumulated around the outer nuclear membrane. In order to try to match these results with the earlier results showing that intact virus particles entered the nucleus, the authors had different suggestions. It was proposed that only a few entire virus particles penetrated the nuclear envelope, while most particles were disassembled and subsequently degraded in the cytoplasm. Another alternative was that the VP1 molecules of virus particles entering the nucleus were degraded more quickly than those remaining in the cytoplasm (161). The same group later showed that at approximately 3 h post infection (p.i.) the VP1 molecules seemed to be localized in the ER and at 6 h p.i. most of them were translocated from the ER into the cytosol. Again, only a minor fraction of the VP1 molecules entered the nucleus, and the rest remained spread around the nucleus. Of great interest for our studies, only a minor fraction of the genomes was transported into the nucleus (162). We have earlier discussed the interesting study from 2004 by Gilbert and Benjamin (147) showing the dependence on particular gangliosides for binding and uptake of MPyV. In this study they also showed that MPyV was transported to the ER and that the pathway was dependent on cholesterol and caveolae (159). They here suggested that MPyV trafficked via the caveosome to the ER and further to the nucleus and that disassembly probably occurred in the ER (147). In 2006, Liebl *et al* showed that the endocytic vesicles fused with endosomes. Although MPyV had been shown to localize in caveolae, they here showed that MPyV can be internalized by cells lacking such structures (163). Hence, summarizing the results from several studies it is possible that MPyV can be internalized both via a caveolae-dependent and a caveolae-independent pathway. Liebl and colleagues suggested the following trafficking pathway for MPyV: The virus is internalized through lipid-raft domains, often but not always, in caveolin-positive vesicles. These vesicles then fuse with early endosomes[30] and the virus is transported to the ER. Probably the virus can also enter the ER directly from vesicles, i.e. without going through endosomes. The virus is at least partially degraded in the ER and the destabilized particle is then translocated out of the ER into the cytosol where it encounters a low calcium environment. This low concentration of calcium likely contributes to further destablization of the virus particle. Exposure of the NLS in the viral capsid proteins that likely remain bound to the viral genome then directs transport of the genome across the nuclear pores (163). Later the same group has shown a similar way of uptake for VLPs based on MPyV (164, 165).

[30] *Endosomes* are divided into *early* and *late* endosomes. The material contained within an early endosome can either be recycled to the plasma membrane or further transported to a late endosome. The late endosome has a lower pH value and can mature into a lysosome.

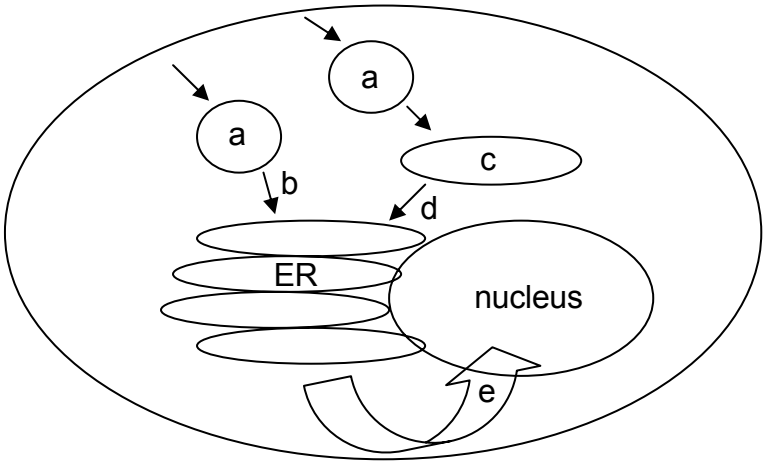


Figure 6. Cellular uptake of murine polyomavirus as suggested in (163) (simplified). The virus is taken up in endocytic vesicles (a), which can either enter the ER directly (b) or fuse with early endosomes (c), which in turn enter the ER (d). Uncoating, at least partial, occurs in the ER and the destabilized virus is broken down further in the cytosol. Some DNA molecules enter the nucleus bound to viral capsid proteins (e).

2.7 MURINE PNEUMOTROPIC VIRUS

Mice are host to two known polyomaviruses, MPyV and MPtV. Since these viruses share natural host one might assume they are biologically similar but in fact, they are not. Phylogenetically, MPtV is more closely related to polyomaviruses from birds, geese and cattle than to MPyV (166). Two major and important differences compared to MPyV are the lack of MT (167) and the probable lack of tumorgenicity *in vivo* (168, 169), although MPtV is capable of transforming cells *in vitro* (170). Being non-tumorigenic, it has not at all attracted as much attention as MPyV, and therefore the knowledge of MPtV is far more limited. Two studies have shown that MPtV hemagglutinates erythrocytes, although in one of these studies a large number of passages of the virus were required and only low titres of activity were observed (171, 172).

2.7.1 Structure and genomic organization

As we have discussed before, MPtV was discovered in the beginning of the 1950s (12, 13), but at that time is was unknown what kind of virus it was. Studies by electron microscopy in the beginning of the 1960s showed that the virus was morphologically similar and identical in size to SV40 and MPyV. These studies made it clear that MPtV most likely belonged to the papovavirus family (169, 173, 174) and later it was placed in the polyoma subfamily (now polyomavirus family) (175, 176). The genome was partially characterized in 1979 (177) and the complete genome sequenced in 1991, and it was then confirmed that it was a typical member of the polyomavirus family (167). When the late genes were sequenced in our laboratory in the beginning of the 2000s, several differences compared to the published sequence (177)[31] were found and we

[31] GenBank accession number NC_001505.

therefore resequenced the complete genome[32] (unpublished). It should also be noted that the genome of MPtV extracted from infected mice often differs in the NCCR from the "*in vitro* sequence" and in many cases a large "*in vivo*-insert" is present in this region (15).

2.7.2 Natural infection and cell tropism

Already in the very first publication of MPtV it was observed that mice developed respiratory distress when inoculated as newborns and died soon thereafter. At autopsy many were found to have consolidations in their lungs as a sign of pulmonary infection[33] (13). Subsequently it was shown that this was due to the inability of these very young mice to mount an antibody response, while adult mice lacking T cells did not develop any symptoms (178, 179). Regardless of at what age the mice were infected, the virus produced a systemic infection although the infection became more limited as the animals matured (180). While newborn mice developed severe respiratory symptoms, in older animals the infection was in fact clinically unapparent and in mice inoculated after 8 days of age no deaths occurred (12, 13, 178, 180, 181). When studying the infection in the lungs more closely, it was found that it was not a classical pulmonary infection, but rather the viral infection was restricted to the endothelial cells (182)[34].

The main site of primary MPtV infection is not completely clear to me. Several studies have shown that during the acute phase of the infection, viral replication takes place mainly in endothelial cells in the lungs, liver and spleen (180, 181, 183, 184), and probably also in the intestine (181, 184). However, one study showed that the earliest site of replication was actually the endothelial cells of the capillaries of the submucosa of the jejunum[35] and it was suggested that the virus was spread via the oral route with secondary spread to the lungs and the liver (183). In line with this it can also be mentioned that the virus can be recovered from stool (185), but the route of spread has to the best of my knowledge not been established. After about two months virus can be detected in epithelial cells lining the renal tubules and after approximately six months these cells represent the major site of viral persistence (181). Immunosuppression at this time results in viral reactivation (181, 186).

Not much is known about the receptor for MPtV and no permissive cell type for growth *in vitro* has been identified. While MPyV has a very wide cell tropism, the tropism of MPtV seems to be much more restricted as outlined above. A logical question is what this restricted tropism is due to. Can the virus particles only attach to a very limited number of cell types, i.e. is the receptor only present on some cells, or is the tropism

[32] Plasmid pKV19 kindly obtained from professor Göran Magnusson, Uppsala University, Sweden.
[33] This pulmonary infection is sometimes referred to as *pnemonia* and sometimes as *pneumonitis*, which are somewhat different kinds of infections in the lung, especially from a pathologist's perspective. To make things simple, in this thesis I will refer to this infection as *pulmonary infection*.
[34] One study has shown that macrophages of the lungs were also infected but maybe this was due to engulfment of infected cells (174).
[35] *Jejunum* is a part of the small intestine.

due to the fact that the virus is able to *replicate* only in a few types of cells? When a region of the NCCR of MPtV was replaced with a part of the transcriptional enhancer of MPyV, replication occurred in transfected mouse fibroblasts and increased expression of both the early and late genes was seen. In the same study it was shown that MPtV DNA could replicate in these cells when LT was expressed from a co-transfected plasmid under control of a strong promoter (187). As mentioned above, mutations in the NCCR are often found *in vivo* and many such mutations increase DNA replication (15). One of the purposes of paper I in this thesis was to determine some characteristics of the MPtV receptor.

2.8 POLYOMAVIRUSES IN HUMANS

It might seem a little bit beyond the scope of this thesis to discuss polyomaviruses in humans, but so much has happened in this field in recent years that I almost feel I have to devote a short chapter to it. As I have pointed out earlier, in the polyomavirus field several major discoveries have a tendency to coincide. So were the first two polyomaviruses, MPyV and MPtV, described almost at the same time (1, 11-13). Nearly two decades later, in 1971, two research groups in different parts of the world independently of each other identified the first two *human* polyomaviruses, JCV and BKV[36], respectively, and these discoveries were published in the very same issue of Lancet (188, 189).

2.8.1 BK and JC virus

According to current knowledge both BKV and JCV are harmless as long as we are healthy and have a normal immune system. This is good luck since most adults have latent life-long infections with both viruses (190). However, in the case of immunosuppression the viruses can be reactivated and cause severe diseases. With regard to BKV, the most well known diseases are hemorrhagic cystitis, a troublesome, very painful and bloody urinary tract infection (reviewed in (191)) following bone marrow transplantation (192, 193), and polyomavirus-associated nephropathy after kidney transplantation (194). Reactivation of JCV is associated primarily with progressive multifocal leukoencephalopathy (PML) (189, 195), a fatal demyelinating disease of the brain, mainly afflicting AIDS patients (reviewed in (196)). The interest in this disease has re-emerged recently, since the monoclonal antibody (mAb) natalizumab, used for the treatment of multiple sclerosis, can result in JCV reactivation and subsequent PML development (197, 198). It has also been proposed that BKV and JCV as well as SV40 can contribute to cancer development in humans, but convincing data to prove this association are still lacking (199-201).

2.8.1.1 Receptors

Studies on the receptors for polyomaviruses are often somewhat tricky to interpret, since in most cases attachment to only a limited number of cell types has been ana-

[36] The letters refer to the initials of the index patients.

lyzed. As we have discussed above for MPyV (chapter 2.6.3), it is possible that polyomaviruses bind to different receptors on different cell types.

With regard to BKV, it was shown already in 1990 that the virus could attach to gangliosides on the surface of target cells, and that the receptor was at least partially sensitive to neuraminidase (202). A more recent study has demonstrated that BKV interacts with the gangliosides GT1b and GD1b (203). α2,3-linked (204) and possibly also α2,8-linked sialic acid is essential for binding (203).

JCV attaches to molecules terminating in α2,6-linked (205-207) and maybe also α2,3-linked sialic acid (208). On the basis of the dependence on sialic acid, it is not surprising that the receptor is neuraminidase sensitive (206). Interestingly, Elphick and colleagues (205) showed that the serotonin receptor 5HT2A could act as a receptor for JCV[37], and antidepressive drugs (selective serotonin reuptake inhibitors) are indeed used for the treatment of PML (A. Näsman (personal communication)). As for BKV, gangliosides may also be involved in attachment to cells (207).

Studies in the end of the 1980s and beginning of 1990s showed that SV40 could attach to MHC class I on the surface of cells (209-211). The receptor was shown to be resistant to neuraminidase and trypsin (210), but sensitive to papain[38] (210). More recent studies have demonstrated that the ganglioside GM1 can also serve as a receptor (146, 148).

2.8.2 The renaissance

In the spring of 2007 a polyomavirus was identified by Allander and collaborators at Karolinska Institutet in a nasopharyngeal aspirate, and this polyomavirus was named KI polyomavirus (KIPyV) (166). Viral particles from this virus have not yet been observed by microscopy, nobody has managed to culture the virus and it remains to be proven that humans are indeed the natural host. However, a number of studies have shown the presence of this virus in various parts of the world like Europe (212, 213), Australia (214, 215), Asia (216, 217) and the US (218), and KIPyV is therefore considered as the third human polyomavirus. Accordingly, it took as long as 36 years from the discovery of the two first human polyomaviruses until the third human polyomavirus was found. I anticipated that it would take very long before another polyomavirus in humans would be identified, if it would ever happen. I was silly to neglect the fact that one major discovery in the polyomavirus field is usually followed within short by another one. It did not take more than one single month until a fourth polyomavirus in humans was described, WU polyomavirus (WUPyV) by Gaynor and colleagues at

[37] In this study, JCV initially interacted with α2,6-linked sialic acid and thereafter with 5HT2A, and the authors concluded that both components might be essential.

[38] Papain is a cysteine protease that cleaves peptide bonds of basic amino acids (lysine, arginine, histidine) as well as leucine and glycine. By contrast, trypsin is a serine protease that cleaves peptide bonds of lysine and arginine.

Washington University (219). Also this virus has been confirmed to be present in individuals throughout the world (214, 216, 217). This is not the end because one year ago a fifth polyomavirus in humans, Merkel cell polyomavirus (MCPyV), was identified by Feng *et al* at University of Pittsburgh in Merkel cell carcinoma (56), a rare malignancy of the skin (reviewed in (220)). Although not proven for sure, much speaks in favour of the fact that this virus is actually involved in the development of this cancer (56, 57, 221-223). If this would turn out to be the case, MCPyV would be the first human polyomavirus to be shown to be tumorigenic. All today known polyomaviruses are listed in table 1.

Table 1. Polyomaviruses known in the spring of 2009.

Virus	Year of discovery	Host[h]	Middle T	Agnoprotein[i]
Murine polyomavirus	1953 (1, 11)	Mouse	X (224)	
Murine pneumotropic virus	1953 (12, 13)	Mouse		
SV40	1960 (225)	Monkey (Rhesus macaque)		X (226, 227)
SA12[a]	1963 (228)	Monkey (Chacma baboon)		X (229)
Rabbit polyomavirus[b]	1964 (230)	Rabbit		
Hamster polyomavirus[c]	1968 (231, 232)	Hamster	X (54, 55)	
BK virus	1971 (188)	Human		X (233-235)
JC virus	1971 (189)	Human		X (105, 109, 236)
Bovine polyomavirus[d]	1974 (237)	Cattle		X (238)
Lymphotropic papovavirus[e]	1979 (239)	Monkey (African green monkey)		
Avian polyomavirus[f]	1981(240, 241)	Bird		X (242)
Rat polyomavirus	1984 (243)	Rat		
Baboon polyomavirus type 2[g]	1989 (244)	Baboon		
Cynomolgus polyomavirus	1999 (245)	Monkey (Cynomolgus)		
Goose hemorrhagic polyomavirus	2000 (246)	Goose		X (247)
Chimpanzee polyomavirus	2005 (248)	Chimpanzee		
Crow polyomavirus	2006 (249)	Bird (Crow)		X (249)
Finch polyomavirus	2006 (249)	Bird (Finch)		X (249)
KI polyomavirus	2007 (166)	Human		
WU polyomavirus	2007 (219)	Human		
Squirrel monkey polyomavirus	2008 (250)	Monkey (Squirrel monkey)		X (250)
MC polyomavirus	2008 (56)	Human		

[a] Also known as Baboon polyomavirus type 1.

[b] Also known as Rabbit Kidney Vacuolating Virus.

[c] In early publications referred to as hamster papovavirus but is nowadays called hamster polyomavirus.

[d] In 1974 a virus was isolated from the stump-tailed macaque (237), which was in 1976 identified as a member of the polyomavirus family and named stump-tailed macaque virus (251). The genome was sequenced in 1990 and it was then realized that it was a bovine rather than a simian virus and the virus was renamed bovine polyomavirus (238). I have denoted 1974 as the year of the discovery of the virus.

[e] Also known as African Green Monkey polyomavirus.

[f] Earlier known as Budgerigar Fledgling Disease Virus.

[g] Also known as Polyomavirus papionis-2 (according to this classification SA12/Baboon polyomavirus type 1 should be named Polyomavirus papionis-1).

[h] For some viruses the natural host is not known for sure, especially with regard to the polyomaviruses discovered in recent years.

[i] For some viruses such as SV40, BKV and JCV, expression of the agnoprotein has been confirmed experimentally (105, 109, 226, 227, 233). For other viruses like crow and finch polyomavirus as well as squirrel monkey polyomavirus a putative ORF for the agnogene (or a gene denoted ORF-X) has been found (249, 250), i.e. it is unknown whether the ORF in these viruses in fact encodes a protein.

3 MISCELLANEOUS VIRUSES

Polyomaviruses are morphologically similar to several other groups of viruses. For the understanding of this thesis, some polyomavirus-related viruses will be briefly described in this chapter.

3.1 PAPILLOMAVIRUS

Until recently papilloma- and polyomaviruses made up a group of viruses together known as the *papovavirus group* (pa, papilloma; po, polyoma; va, vacuolating agent (SV40)) (252), but nowadays they are separated into the polyoma- and papillomavirus families. More than 100 types of human papillomavirus (HPV) are known today (253) and several of them are carcinogenic in humans. HPV is particularly associated with cervical cancer (reviewed in (254)[39]). Similar to polyomaviruses, papillomaviruses are non-enveloped icosahedral viruses (255) with a circular double-stranded DNA genome divided into an early, a late and a non-coding control region (256). The papillomavirus capsid is very similar to its polyomavirus counterpart and consists of 72 pentamers of the major structural protein L1[40] (102, 257) and up to 72 copies of the minor structural protein L2 (258). The size of the virus is slightly larger than for polyomavirus (55 nm compared to 45 nm) (255). The L1 protein can self-assemble into VLPs (see chapter 7.4.2.3), either alone or together with L2, which has been shown for multiple HPV types (259-261). Papillomaviruses express up to eight regulatory proteins from the early region (E1-E8). Most extensively studied of these are E6, which binds to p53 (262) and promotes its degradation (263) and E7, which forms a complex with Rb (264) resulting in cell cyle progression.

3.2 PARVOVIRUS

Parvoviruses are found in many species, but only the human parvovirus[41] B19[42] and porcine parvovirus will be discussed in this thesis. Parvovirus is a non-enveloped icosahedral DNA virus with a size of only about 25 nm (265), carrying a single- rather than double-stranded genome that is linear rather than circular (266). The human parvovirus B19 (267) is the causative agent of the fifth disease[43] (268). Parvovirus encodes a major capsid protein (VP2, 58 kDa (269)) as well as a minor capsid protein (VP1, 84 kDa (269))[44] and the capsid is composed of 60 monomers of VP2/VP1 (269, 270). The sequence of VP1 is identical to VP2 except for the presence of an additional stretch of

[39] This review is written by Harald zur Hausen, who was awarded the Nobel Prize in Physiology or Medicine in 2008 “for his discovery of human papilloma viruses causing cervical cancer" (8).
[40] *L* refers to late, i.e. expressed late in the viral life cycle (after replication).
[41] *Parvus* is the Latin word for small and parvovirus is one of the smallest viruses known today (cf. the Swedish word *parvel* meaning little boy).
[42] The virus was originally discovered in a serum sample with number 19 in panel B and this is why it is called B19.
[43] *The fifth disease* is also known as *erythema infectiosum*, a disease of childhood characterized by fever and red skin rashes.
[44] Some parvoviruses encode additional structural proteins.

amino acids at its N terminus, the so called VP1 unique region (269, 271). The protein VP1 is mainly located inside an outer "shell" of VP2 (cf. VP1-VP2/VP3 in a polyoma-virus capsid) but a portion of the unique region is external to the capsid (272, 273). To make things extra complex, in the case of parvovirus the major capsid protein is called VP2 rather than VP1, although VP1 is in fact larger in size than VP2. The names originate from the fact that VP2 is more abundant in the capsid (>95% VP2, <5% VP1) (269). VP2 can self-assemble into VLPs, either on its own or together with VP1 (see chapter 7.4.2.2) (274, 275).

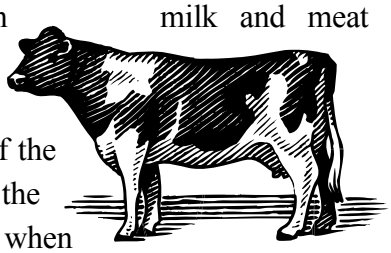
3.3 HEPATITIS B VIRUS

Hepatitis B virus (HBV) can cause acute as well as chronic infection of the liver and in the long run contribute to development of liver cancer. It is an enveloped virus with a size of 42 nm (276) and into the envelope is the surface antigen HBsAg incorporated. In HBV infected individuals, HBsAg particles self-assemble into 22 nm particles (277), which are present in serum together with 42 nm native virus particles (277). Such 22-nm particles, sometimes referred to as VLPs, can also be obtained after production of HBsAg in cells *in vitro* (278). The particles are effective in inducing humoral as well as cellular immune responses and the most commonly used vaccine today against hepatitis B virus is made up of such particles (279-283).

4 AN IMMUNOLOGICAL EXCURSION

4.1 IT ALL STARTED WITH A COW

The cow is one of my favourite animals. In my opinion it is very beautiful and much cleverer than most people believe. It supplies us with both milk and meat and is not fastidious at all, just provide it with some water and something to eat and it will be happy. In addition, we are indebted to the cow for the emergence of the whole field of immunology. Many people claim that the science of immunology began on 14th of May 1796 when Edward Jenner inoculated a young boy with extracts from blisters caused by cowpox, the bovine analogue of smallpox, from a woman who had in turn caught cowpox from a cow. In that way the young boy got protected against smallpox. Jenner named the procedure *vaccination* after the Latin word *vacca*, meaning cow.



In this chapter some components of the immune system will be described. The purpose is not to be complete, not even close to, but rather to give an overview of immunology with some extra emphasis on those parts that are of particular relevance for this thesis. I apologize for the infrequent citing of articles in general and the infrequent citing of original articles in particular in this chapter. Instead lots of review articles have been cited but to be honest, original articles are in many cases really tricky to find. When writing this chapter I was assisted by the textbook "Immunobiology, the immune system in health and disease" (284) as well as several excellent theses in the immunotherapy field written in recent years by my fellow PhD student colleagues.

4.2 INNATE IMMUNITY

I think it is amazing that we do not contract more infections than we actually do. Many people go to work despite a cold and every morning the metro is terribly packed with people coughing and sniffling at each other. Accordingly, we are constantly being exposed to viruses, bacteria and other microorganisms but, luckily enough, if these nasty guys manage to penetrate the physical barriers of the body like the skin and mucosal membranes, almost all of them will be destroyed within hours by the part of the immune system known as the *innate immune system*. Key players of innate immunity are cells such as granulocytes (neutrophils, eosinophils, basophils), mast cells, natural killer (NK) cells, macrophages and dendritic cells (DCs). The innate immune system responds quickly and there is no need for prior exposure to the invading organism.

How does the innate immune system recognize pathogens and especially, how does it distinguish *self* from *non-self*? Most pathogens carry molecules, which are highly conserved through evolution. In contrast to the *adaptive immune system* (chapter 4.3), which can recognize an almost incalculable number of antigens, the innate immune system can recognize only a limited number of molecules but these molecules are in-

stead present on many different pathogens. The receptors on the cells that bind these conserved molecules are referred to as pattern recognition receptors (PRRs), of which the most well known are Toll-Like Receptors (TLRs). The TLRs bind to molecules including lipopolysaccharide (LPS) found in the wall of many bacteria (285), double-stranded RNA present in certain viruses (286) and unmethylated CpG motifs in bacterial DNA (287). Most cells of the innate immune system have a phagocytosing capacity and following binding to PRRs, the pathogens will be engulfed by the cells, broken down into small pieces and presented to other cells of the immune system as will be discussed in further detail below.

Between the innate and adaptive immune system there is no big wall or deep ditch but rather an intense interplay and, as we will soon be aware of, without activation of innate immunity there will be no activation of adaptive immunity either.

4.2.1 Dendritic cells

Dendritic cells were discovered by Ralph Steinman and Zanvil Cohn at Rockefeller University in New York in the beginning of the 1970s (288-291). Steinman considered naming the cells after his wife but they were instead named dendritic cells due to the presence of dendritic processes[45] (292). The DCs are antigen-presenting cells (APCs), meaning that they present antigens to other cells of the immune system. Other APCs are e.g. B cells (see chapter 4.3.2) and macrophages, but DCs are regarded as the most powerful. In case a pathogen would manage to sneak into the body it will hopefully be caught by a DC in the peripheral tissues and be bound to one of its PRRs. The DC will then engulf the pathogen, mature and start to migrate to a lymph node.

On the surface of DCs, Major Histocompatibility Complex (MHC) class I and class II molecules are found. MHC class I is present on all cells with a nucleus, whereas only APCs generally express MHC class II. A pathogen that has been engulfed by an APC will be broken down into short peptides (10-15 amino acids) within the cell and these fragments will be presented on MHC class II, and thereby be exposed to surrounding $CD4^+$ T cells. While MHC class II presents peptides from particles taken up from the extracellular space, MHC class I presents peptides derived from proteins produced by the cell itself. Some copies of all proteins synthesized by a cell will be cleaved into short peptides (8-10 amino acids (293, 294)), which will be presented on MHC class I making recognition by $CD8^+$ T cells possible. MHC class I consists of an α-chain and a β2-microglobulin (β2m) chain (295), which we will return to when discussing knockout mice in paper IV. In contrast to the classical dogma, we know today that also peptides from proteins engulfed by APCs can be presented on MHC class I, and more about this in chapter 4.4. Presentation on MHC class I and class II molecules enables activation of different subsets of T cells, which we will return to later.

[45] *Dendrom* is Greek and means treelike.

4.2.2 NK cells

NK cells were discovered by R. Kiessling, E. Klein, and H. Wigzell at Karolinska Institutet and by R. Herberman's group at the NIH in the US in the middle of the 1970s (296-299). This discovery is an excellent illustration of scientists who did not accept the concept of *background* in their assays. Instead they started to explore this background and found that it in fact consisted of cells with *natural cytotoxicity*, i.e. the cells could spontaneously kill tumour cells without prior sensitization. In 1981, Klas Kärre postulated in his thesis that NK cells recognize cells with low or absent expression of MHC class I (300), and this postulation has since been proven correct although incomplete. Further studies have shown that NK cells have two types of receptors, activating and inhibitory, and the function of NK cells depends on a balance between them. The inhibitory receptors bind to MHC class I molecules protecting these cells from NK cell-mediated lysis. However, it has been shown recently that some NK cells do not express inhibitory receptors specific for MHC molecules but still they do not attack cells of the body (301). If expression of MHC class I molecules would be decreased, which is common in malignant as well as virus-infected cells, inhibition will be lost and the target cells will be killed if the appropriate ligands for the activating receptors are present.

NK cells can kill target cells through two different mechanisms. The first one involves the use of perforin/granzyme. Within NK cells there are granules containing perforin and various granzymes and these are released onto target cells upon activation. Perforin creates pores in the plasma membrane and the granzymes were earlier thought to flow through these pores and induce apoptosis in the target cells (reviewed in (302)). Interestingly, it has been shown that at low levels of perforin, the formed pores are too small for the granzymes to pass through. Other mechanisms of uptake of granzyme have therefore been suggested such as receptor-mediated endocytosis (303). The other mechanism of killing is through binding of Fas Ligand (FasL) on NK cells to Fas on target cells and this also induces apoptosis. Other molecules such as TRAIL have the same effect but are probably of inferior importance (reviewed in (302)). NK cells can also secrete cytokines, of which the best known is IFNγ, which stimulates phagocytic cells as well as cytotoxic T lymhocytes (CTLs) (304). NK cells are also involved in antibody-dependent cellular cytotoxicity (ADCC) as briefly described in chapter 4.3.2.1.1.

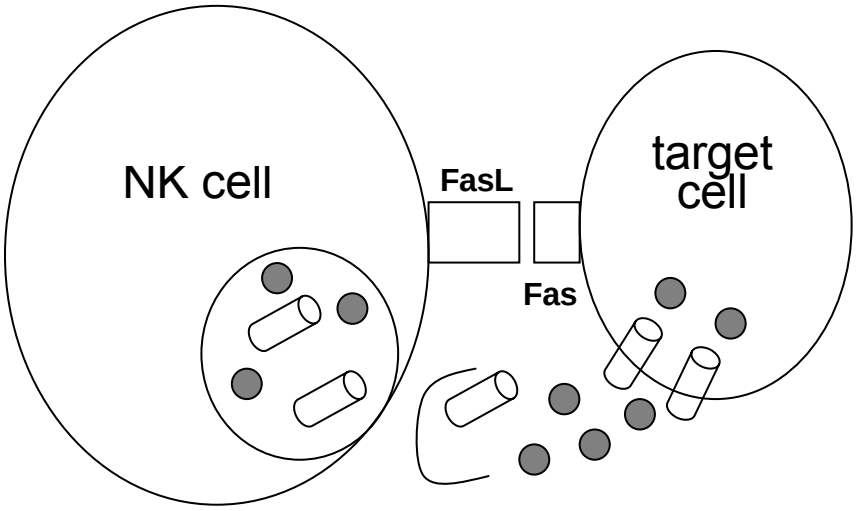


Figure 7. The mechanisms of NK cell-mediated killing are schematically shown. Killing occurs either through direct binding between e.g. FasL on the NK cell and Fas on the target cell, or through release of perforins (cylinders) and granzymes (gray dots). In both instances apoptosis is induced in the target cell.

4.3 ADAPTIVE IMMUNITY

4.3.1 T cells

Even if the innate immune system would collect its whole strength, there are some invaders that it cannot master and in that case it is the responsibility of the *adaptive immune system* to take care of the invading pathogen. In comparison with innate immunity, adaptive immunity is characterized by responding slowly and being extremely specific. To be more precise, each cell carries a receptor that only binds to one single antigen and as a consequence the number of cells that can bind and respond to any given antigen is very low. Another distinctive feature is immunological memory. The cells of the adaptive immune system, the T and B lymphocytes/cells, respond slowly the first time they encounter an antigen. However, if they come across the same antigen again, the second response will be more rapid and vigorous due to the presence of memory cells, a result of the previous antigen encounter. In this way, the cells of the adaptive immune system are said to *adapt* to pathogens.

4.3.1.1 $CD8^+$ cytotoxic T cells and $CD4^+$ helper T cells

$CD8^+$ T cells, also referred to as $CD8^+$ CTLs, kill target cells such as virus-infected cells and cancer cells. These T cells constitute the cellular arm of the immune response. Each CTL is specific for a particular antigen, namely a specific peptide bound to an MHC class I molecule on the surface of target cells. Killing occurs in the same way as for NK cells, i.e. through perforin/granzymes or receptor binding, but in addition they can also secrete cytokines that can have a direct cytotoxic effect. In order to be able to kill, the CTLs must be activated either by $CD4^+$ helper T (Th) cells or directly by APCs through a process known as cross-priming (see chapter 4.4).

With regard to $CD4^+$ T cells, we often equate them with Th cells. Today we know that this is not correct since several other types of $CD4^+$ T cells have been identified including regulatory T cells (305) (see chapter 4.5.2.1), $CD4^+$ CTLs (see chapter 4.3.1.4) and Th17 cells[46] (306, 307). $CD4^+$ Th cells activate other cells and are divided into Th1 and Th2 cells (308). Classically, Th1 cells activate ("prime") $CD8^+$ CTLs and produce "Th1 cytokines" such as IFNγ and IL-2, whereas Th2 cells produce "Th2 cytokines" like IL-3, IL-10 and TNFα and activate B cells (activation of B cells is further described in chapter 4.3.2).

4.3.1.2 Activation of helper T cells

Activation of Th cells does not occur in the peripheral tissues, i.e. at the site of infection, but rather in lymphoid tissues such as the lymph nodes. As we discussed in chapter 4.2.1, APCs and most notably DCs, engulf pathogens in the peripheral tissues, become activated and thereafter migrate to a regional lymph node where they encounter naïve $CD4^+$ T cells. Naïve $CD4^+$ T cells constantly pass through peripheral lymphoid organs, where they get trapped if they meet the correct peptide:MHC class II complex on the surface of an activated APC, i.e. a peptide that is specific for the T cell receptor (TCR) on the surface of the T cell. Activated APCs express high levels of molecules such as CD80 (B7.1), CD86 (B7.2) and MHC class I and II. The TCR binds to the peptide:MHC class II complex (309) and CD28 on the T cell interacts with CD80/86 of the APC. Besides binding to a peptide:MHC complex and CD80/86 of the APC, the T cell also needs stimulation by the cytokines produced by the APC, in order to become activated. The interaction between a DC and a $CD4^+$ T cell is schematically shown in figure 8.

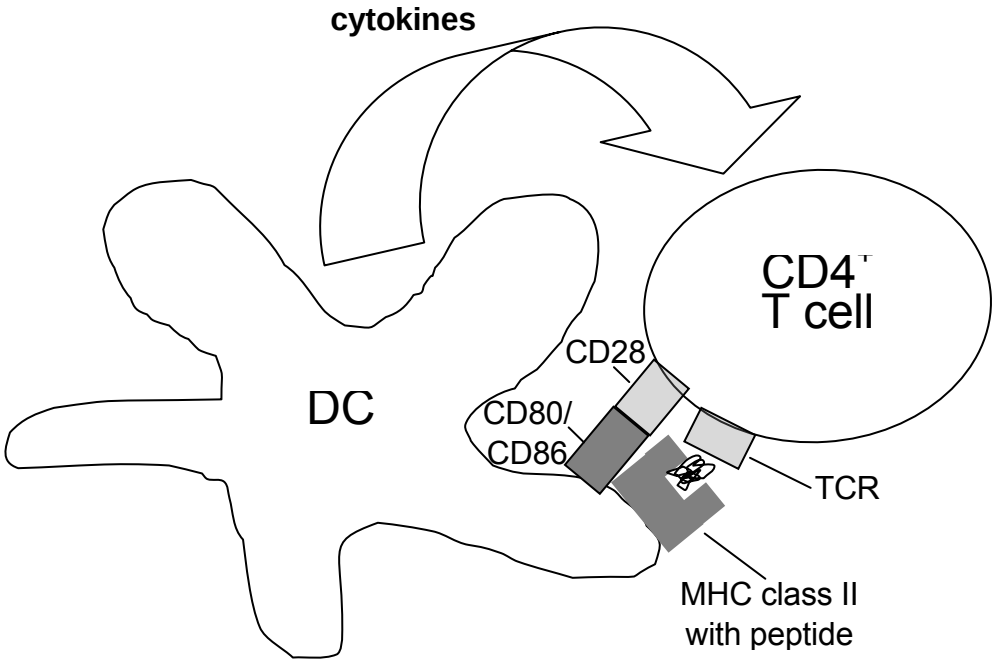


Figure 8. Activation of a $CD4^+$ Th cell by a dendritic cell.

[46] Th17 cells produce large amounts of IL-17.

4.3.1.3 Immunodominance

Since only short peptides are presented on MHC class I and II, the chopping of every single protein will result in a large number of peptides. This will in turn lead to activation of a multitude of T lymphocytes with various specificities, since each T lymphocyte will only respond to one specific peptide. However, T cells do not respond with the same efficacy to all peptides. The focusing of the immune system towards one or just a few peptides (epitopes[47]) is known as *immunodominance* (reviewed in (310)) and such peptides/epitopes are referred to as immunodominant epitopes. Immunodominance occurs for both $CD4^+$ and $CD8^+$ epitopes but mainly $CD8^+$ epitopes will be discussed here. In 2005, Gallo *et al* (311) identified in an enormous effort the immunodominant $CD8^+$ T cell epitopes of the Her2/*neu* protein on BALB/c background ($H\text{-}2^d$). They immunized mice with an adenovirus encoding the human Her2/*neu* protein and determined the frequency of IFNγ secreting cells by ELISPOT analysis. More than three hundred peptides spanning the complete human Her2/*neu* sequence were tested. Strong reactivity was found against a few epitopes and the strongest one was located in the extracellular domain (p63-71, TYLPTNASL) and was identified as a $CD8^+$ T cell immunodominant epitope. They also studied the rat homologue of Her2/*neu*[48]. Again they immunized with an adenovirus expressing the human Her2/*neu* gene but tested reactivity against the rat peptides corresponding to the immunodominant human peptides. Also a response to the rat homologue of the human p63-71 peptide (p66-74, TYVPANASL[49]) was seen in both BALB/c and the rat Her2/*neu* transgenic BALB-neuT mice (see chapter 6.5 for description of these mice). However, the response to the rat peptide was much weaker compared to the human counterpart as a consequence of the assumed tolerance to rat but not human Her2/*neu*. The p63-71 (human) and p66-74 (rat) peptides are therefore those that we have mainly used for ELISPOT analysis in papers III and IV. The two immunodominant peptides will in the following be referred to as $Her2_{63\text{-}71}$ (human peptide) and $neu_{66\text{-}74}$ (rat peptide), respectively.

4.3.1.4 $CD4^+$ cytotoxic T cells

4.3.1.4.1 Human $CD4^{\pm}$ cytotoxic T cells

As briefly mentioned above there are not only $CD8^+$ CTLs but also $CD4^+$ T cells with cytotoxic capacity. Since $CD4^+$ CTLs will be discussed in paper IV they will be quite extensively described here. First we will focus our attention on $CD4^+$ CTLs that are MHC class II-restricted and later deal with MHC class I-restricted $CD4^+$ CTLs. Several reports in the 1980s and 1990s showed the presence of MHC class II-restricted $CD4^+$ CTLs (312-315). If scientists who perform experiments *in vivo* want to criticize scientists who perform *in vitro* experiments, they can say that "the result is an *in vitro* artefact". Exactly this happened in this case primarily because long-term *in vitro* culture was needed to identify these cells. However, more recent reports have shown that *in vitro* stimulation is not required and that these cells can execute their cytotoxic effect in

[47] *Epitope* is also known as *antigen determinant*.
[48] 89% amino acid identity between human and rat Her2/*neu*.
[49] The human and rat peptides differ at two positions (underlined).

a peptide-specific and MHC class II-restricted manner. Usually the $CD4^+$ CTLs have been specific for viruses, most notably cytomegalovirus (316-319), but also other viruses such as BKV (320), varicella-zoster virus (321), hepatitis virus (322), herpes simplex virus (323), EBV (324) and HIV (325, 326). Most studies have demonstrated that the lytic activity is mainly dependent on granule exocytosis (perforin/granzyme) (316, 320, 322-331), although at least one report has implied a major role for death receptor-mediated killing (332). Interestingly, it has been shown that $CD4^+$ T cells with granules containing granzyme B and perforin are found at low frequencies in the circulation of most healthy individuals (325), and are increased in frequency in people with rheumatoid diseases (330, 331).

An interesting issue is what function these cells have and the impression I get from the literature is that nobody knows for sure. One possibility could be to kill virus-infected cells that present viral antigens on MHC class II as a kind of negative feedback (333).

Let us now switch to $CD4^+$, or even $CD4^-CD8^-$, CTLs that are MHC class I-restricted. A couple of studies from the 1990s reported the presence of such cells (334-339), but also these studies were criticized because of potential contamination with $CD8^+$ T cells, and the fact that nobody could show the antigenic specificity. However, in 1999, an MHC class I-restricted $CD4^+$ T cell clone was isolated from a melanoma patient. It recognized melanoma cell lines expressing MHC class I and the antigen was also identified (tyrosinase epitope). The cells were weakly cytolytic and the avidity was much lower than for most $CD8^+$ T cell clones (340). Earlier this year a $CD4^-8^-$ TCR^+ T cell clone from a melanoma patient was characterized. It recognized an MHC class I-restricted gp100-peptide and showed cytotoxicity against target cells in a perforin/granzyme-dependent manner (341).

4.3.1.4.2 Murine $CD4^\pm$ cytotoxic T cells

Several studies have shown that after virus infection (adenovirus, herpes simplex virus, LCVM, Sendai virus, poliovirus), virus-specific $CD4^+$ MHC class I- or II-restricted CTLs can be induced (342-346). The $CD4^+$ T cells do not necessarily function as direct effectors on target cells. Rather, it is possible that they provide help for other cells, e.g. secrete cytokines that increase expression of MHC class II on virus-infected target cells, rendering presentation of viral peptides on MHC class II possible. Studies have also demonstrated that following anti-tumour vaccination, $CD4^+$ CTLs, rather than $CD8^+$ CTLs, mediate the anti-tumour response, and especially so when $CD8^+$ CTLs are absent. However, this does not necessarily occur through direct killing of target cells either, but rather through indirect mechanisms, e.g. stimulation of monocytes and macrophages (347-350). It has also been shown that $CD4^+$ CTLs can kill APCs in an MHC class II-restricted manner (351, 352), which could then indicate a regulatory role in line with what has been suggested for human $CD4^+$ CTLs. I get the impression that much less is known about these cells in mice than in humans.

4.3.2 B cells

B cells make up the humoral[50] arm of the immune system. In accordance with T cells, each B cell is unique for a single antigen. The antibodies produced by the B cells circulate in the blood, in the lympatic vessels and are also found in tissues. Upon antigen encounter, one end of the antibody binds to the antigen and the other end binds to the B cell receptor (BCR) on a B cell, or to some other cell with an antibody receptor. The antibody/antigen complex will be endocytosed by the B cell, broken down into peptides and these peptides will be presented on MHC class II. In order to get activated the B cell must also meet an activated Th cell, i.e. a Th cell that has been activated by an APC as described in chapter 4.3.1.2. The activated Th cell binds through its TCR to the MHC class II:peptide complex on the B cell. Co-stimulation is received through contact between CD40 on the B cell and CD40 Ligand on the T cell (353, 354) as well as through cytokine stimulation, most important is IL-4 (355). This B cell activation can now result in somatic hypermutation (a process to improve the affinity of the antibodies) (reviewed in (356)), differentiation into plasma cells that produce large amounts of antibodies, or differentiation into memory cells (reviewed in (357)).

4.3.2.1.1 Antibodies

A few words should be said about antibodies. There are five classical classes of antibodies (IgM, IgG, IgE, IgD, IgA). Antibodies can exert their effects in several ways as follows:

- Antibody/antigen complexes are endocytosed by B cells and thereby destroyed.
- Antibodies bind to (coat) antigens in a process referred to as opsonization leading to ADCC, performed by e.g. NK cells, macrophages, DCs and neutrophils, which recognize opsonized antigens and kill the cells presenting them.
- Antibodies bind to antigens and thereby make the antigen non-functional. An example of this is antibody binding to the tumour antigen Her2/*neu*, which is discussed further in chapter 5.5. However, it should be noted that most tumour antigens are not exposed on the cell surface, making antibody binding impossible.
- Antibodies "attract" a group of proteins known as complements, which results in destruction of the antigen.

4.4 CROSS-PRESENTATION

Following antigen engulfment by an APC, fragments from the destroyed antigen will be presented on MHC class II according to the classical dogma of antigen presentation. This will in turn activate $CD4^+$ Th cells and finally activation of $CD8^+$ CTLs will occur. However, we have already touched upon the fact that $CD8^+$ T cells can be activated directly by APCs without the involvement of $CD4^+$ T cells. In fact, it is known that peptides from an engulfed antigen can be presented not only on MHC class II but also on class I in a process known as cross-presentation. My initial purpose was to give a

[50] Body fluids were once known as *humors* and since antibodies can be transferred from one individual to another by blood, this part of the immune system is referred to as *humoral immunity*.

very brief description of this phenomenon since it is not of major importance for the thesis. However, when writing this chapter I encountered a paradox, which aroused my interest and therefore I looked into it in more detail. This chapter is therefore much longer but hopefully also somewhat more interesting and exciting than planned.

Cross-presentation can result in either priming (cross-priming) or tolerization (cross-tolerance, see chapter 4.5) of $CD8^+$ T cells (reviewed in (358)). The dogma of presentation of exogenously derived antigens on MHC class II only was challenged by Michael J Bevan at the Salk Institute in the middle of the 1970s. Is this really correct? Already in the 1970s? The concept of presentation of peptides on MHC class I and class II had not yet evolved at that time, although the Nobel prize-awarding discoveries on MHC restriction "concerning the specificity of the cell mediated immune defence" by Rolf Zinkernagel and Peter Doherty (8) had been made in the years before (359-362). However, it would take another decade before it was shown that short peptides (363, 364) were presented on MHC class I (365, 366) and II (367, 368), respectively (although the fact that infectious agents made a complex with transplantation antigens was proposed already in 1959 (369) but this is, as we often say, a different story). Accordingly, Bevan could hardly have discovered presentation of exogenous antigens on MHC class I. Is there something fishy about this or could it be that Bevan was before his time? In other words: what did Bevan actually describe?

Bevan first showed that if a mouse ($H\text{-}2^d$) was immunized with allogeneic cells which carried the same H-2, splenocytes from the immunized mouse were only able to kill targets *in vitro* with the same H-2 as the allogeneic mouse. H-2 incompatibility ($H\text{-}2^b$), on the other hand, resulted in no lysis, i.e. killing was MHC restricted and most likely directed against minor histocompatibility antigens (370). Bevan then also observed something puzzling. When an $H\text{-}2^{b/d}$ mouse was immunized with allogeneic cells from an $H\text{-}2^b$ mouse, killing occurred not only of $H\text{-}2^b$ cells as in the previous study but also of $H\text{-}2^d$ cells. However, this required five days of *in vitro* culture of effector cells with the cells used for immunization ("*in vitro* secondary boost killing" [51]) (371, 372). MHC restriction was suddenly lost! I have made an attempt to illustrate these two experiments in figure 9. Later Bevan tried to elucidate the cellular basis of cross-priming and in the discussion section in that paper he hypothesized the following:

"At some stage the $H\text{-}2^b$ cells will be broken down; the minor histocompatibility gene products may then become dissociated from the cell membrane, taken up by macrophages in the $H\text{-}2^{d/b}$ mouse and re-presented to the immune system. Now the $H\text{-}2^b$ derived foreign minor histocompatibility gene products exist in a cell membrane which bears $H\text{-}2^b$ *and* $H\text{-}2^d$. Presented in this way, they may stimulate anti-$H\text{-}2^b$ and anti-$H\text{-}2^d$ CTL." (373)

[51] Today we refer to this as *in vitro* restimulation

Today we know, exactly as Bevan proposed, that the minor histocompatibility antigens were presented not only on H-2^b, but also on H-2^d, i.e. cross-reactivity between different H-2 haplotypes occurred and thereby the name *cross-priming*. In accordance, in those days cross-priming had nothing at all to do with presentation on MHC class I and II, respectively. It would take another decade before Bevan's observations could be confirmed (374, 375).

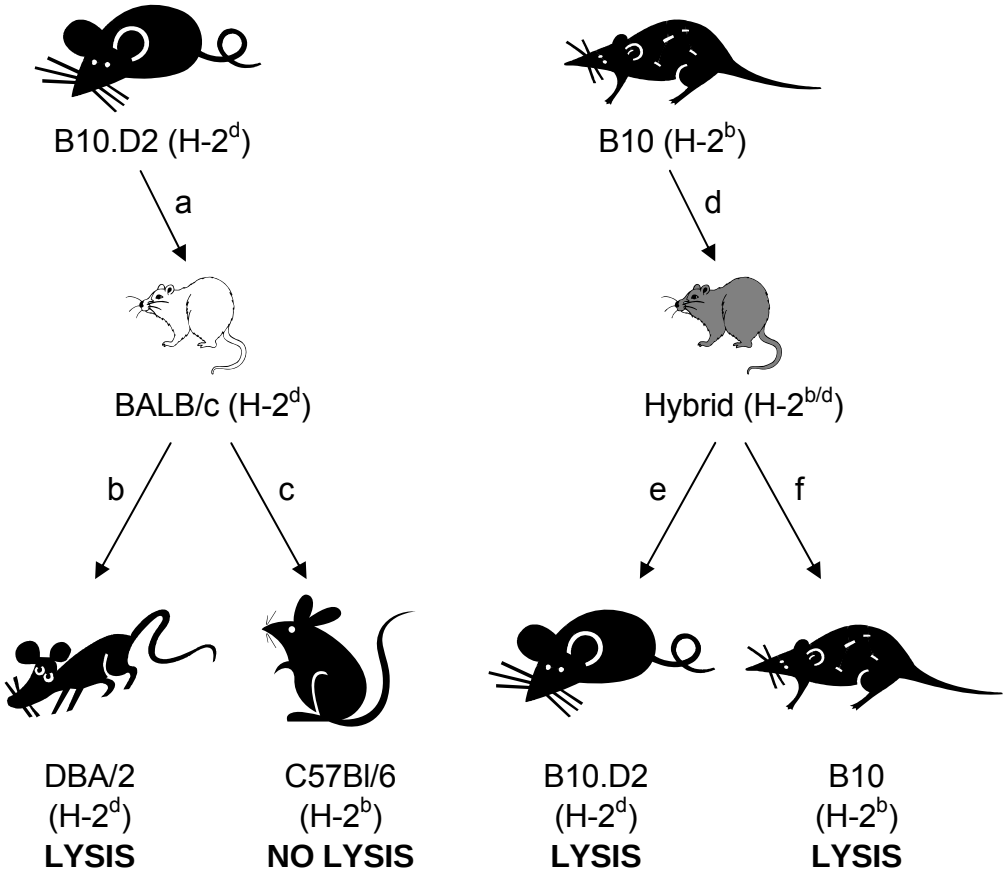


Figure 9. Left: A BALB/c mouse (H-2^d) was immunized with splenocytes from a B10.D2 mouse (H-2^d) (a). Cytotoxicity was shown against target cells with the same but not with different H-2 antigens, e.g. splenocytes from a DBA/2 mouse (H-2^d) were lysed (b), while splenocytes from a C57Bl/6 mouse (H-2^b) were not lysed (c).
Right: A hybrid mouse (H-$2^{b/d}$) was immunized with splenocytes from a B10 mouse (H-2^b) (d). Following *in vitro* culture of effector cells with the cells that were used for immunization, cytotoxicity was shown against target cells both carrying the same and different H-2 antigen. This means that splenocytes from both a B10.D2 mouse (H-2^d) (e) and a B10 mouse (H-2^b) were lysed (f).

The first cell type that was described as having the capability of cross-presenting antigens was to my knowledge the macrophage (376). Cross-presentation can also be achieved by DCs (377), B cells (378), neutrophils (379) and, very interestingly, possibly also endothelial cells (380).

When I studied basic immunology not so many years ago I was taught that $CD8^+$ T cells required activation by $CD4^+$ T cells in order to become effector cells and exert their cytotoxic function. However, now we have learnt that $CD4^+$ Th1 T cells are not

required for a $CD8^+$ CTL response to occur. A logical question then becomes: What is actually the function of $CD4^+$ Th1 cells? Are they needless and in fact created by mistake by nature? If I have understood it correctly, cross-presentation is particularly efficient in some conditions, e.g. cancer, while in other conditions such as during certain viral infections and in immune rejection, Th1 $CD4^+$ T cells are a prerequisite for proper activation of $CD8^+$ T cells. In these cases, $CD4^+$ T cells are assumed to induce upregulation of e.g. CD80/86 on APCs, through binding of the CD40 Ligand to CD40 on APCs and in this way promote the activation of $CD8^+$ T cells. $CD4^+$ Th1 cells also activate macrophages, produce cytokines like IL-2, which in turn induces proliferation of $CD8^+$ T cells, and IFNγ, which increases expression of MHC class I on target cells indirectly promoting the effector functions of $CD8^+$ T cells.

4.5 TOLERANCE

As we have discussed earlier, the immune system should react to *foreign* and ignore *self*. In other words, it should be *tolerant* to self. Since the TCRs and BCRs are generated randomly it would be unlikely that all TCRs/BCRs react to foreign. Lymphocytes with receptors specific for self should also develop and this is exactly what happens, an enormous number of self-reactive lymphocytes are created. Autoimmune diseases are caused by such self-reactive cells, but only a minority of the population develops autoimmune pathology, implying that most self-reactive cells must be removed or inactivated in some way. The Australian scientist Sir Frank Macfarlane Burnet thought about this issue. Not only the whole field of immunology, but also the theory of tolerance started with a cow, or at least some calves. In 1945 it was shown that most twin calves were born with a mixture of erythrocytes, meaning that the calves must have exchanged erythrocytes and precursors thereof during foetal life. The cells were assumed to be capable of becoming established in the hemapoietic system of the "new" host (381). Since this mixture often retained throughout life, the calves must have adapted and developed tolerance to the foreign erythrocytes since the erythrocytes otherwise should have been destroyed. Later it was shown that dizygotic twin calves could accept skin grafts from each other, but not from unrelated calves (382, 383). With these studies in his mind, Burnet predicted that a mechanism must exist making it possible for the immune cells to distinguish between self and non-self/foreign during their maturation. As we discussed in the chapter about MPyV infection (2.6.1 and 2.6.2), the immune system is not mature at birth, in fact it develops over time. Burnet suggested that the capacity of recognizing self cannot be an inherited property, and instead must be gradually acquired. Moreover, he assumed that immunity develops as a result of contact with self-substances, whereby the immune system learns to recognize and remember self. He put forward the *theory of acquired immunological tolerance* (384). This theory was proposed by Burnet, but it was Peter Medawar and co-workers, who proved the prediction experimentally. They inoculated foreign tissues into mouse embryos and after birth, when the immune system was mature, the mice received grafts. The mice accepted not only self-tissue, but also foreign tissue as long as it originated from the same mouse strain as the foreign tissue introduced during foetal

life. Accordingly, the inoculation of foreign tissue into the foetus resulted in immunological unresponsiveness, *tolerance*, to tissues from that strain (385). Sir Frank Macfarlane Burnet and Peter Medawar were jointly awarded the Nobel Prize in Physiology or Medicine in 1960 (8). However, at this time it was not known how these processes were governed.

4.5.1 Central tolerance

Today the mechanisms responsible for tolerance have at least partially been delineated. Tolerance is divided into *central tolerance*, that occurs in the thymus for T cells and in the bone marrow for B cells, and *peripheral tolerance* that takes place in peripheral tissues. To start with, we will have a look at how central tolerance develops in the case of T cells. In the thymus, APCs will present all proteins synthesized by thymic cells to the developing T cells. The T cells that bind weakly to MHC molecules will be selected and continue to survive (positive selection), while T cells that do not bind at all to MHC will die since they are non-functional (386, 387). The surviving T cells will then undergo negative selection in order to get rid of self-reactive T cells and it is negative selection that mediates central tolerance (reviewed in (388)). What I can understand, four outcomes of negative selection are known. The most important is probably clonal deletion. This occurs when there is strong binding of a T cell to a self-peptide/MHC complex and the cell will die by apoptosis (389). However, later it has been shown that if a T cell binds strongly to a self-peptide it does not undergo apoptosis immediately, but rather it has the possibility to rearrange its TCR. Such a rearrangement, known as *receptor editing*, will hopefully result in replacement of the self-reactive receptor with a new receptor without autoreactivity (reviewed in (390)). Other fates of negative selection are anergy, which has been more extensively studied with regard to peripheral tolerance (see below) and a process referred to as clonal diversion meaning that high-affinity thymocytes are skewed into regulatory T cells (reviewed in (388)).

Let us now switch to B cells. The processes by which B cells undergo central tolerance in the bone marrow are very similar to those for T cells in the thymus. In the case of strong binding to a self-antigen, they can rearrange their receptors (receptor editing) (reviewed in (390)) or, if this would fail, they instead undergo clonal deletion (391). If a soluble self-antigen is weakly rather than strongly cross-linked to the BCR, the B cell will survive and enter a state of permanent unresponsiveness (anergy). Such cells will leave the thymus but will be unable to respond to antigens in the periphery (392). B cells that bind self-antigens with low affinity and/or without cross-linking become ignorant. These cells will also leave the thymus but have a low affinity for self-antigens and even if they would encounter their antigen in the periphery the interaction between the antibody and the antigen would not be strong enough to result in activation.

4.5.2 Peripheral tolerance

Despite central tolerance, many self-reactive B and T cells escape from the thymus and the bone marrow into the periphery (393, 394). This is at least partly due to the fact that

not all self-antigens are expressed in the thymus/bone marrow, and in fact many proteins have specialized functions and are produced only by cells of certain tissues. When lymphocytes, both of the B and T cell subset, encounter self-antigens in the periphery either of three processes can occur: deletion, anergy or ignorance. Similar to central tolerance, deletion is the consequence if there is strong binding to an antigen (395, 396). Anergy was initially assumed to occur as a consequence of absence of co-stimulatory activity (397), but later work has implicated signalling through "incorrect" receptors rather than lack of co-stimulation (398, 399). Ignorant cells develop either because the antigen is sequestered in a site not easily accessible to the blood/lymph-borne immune system (400), or because the amount of antigen does not reach the threshold required to trigger a response (396).

4.5.2.1 Regulatory T cells

Peripheral tolerance is also maintained by regulatory T cells (Tregs) (305), which suppress effector T cells (401). This cell type has really come into fashion in recent years. Tregs are somewhat tricky to define. Immunologists like to define cells based on marker phenotype and Tregs are sometimes a little bit sloppily defined as $CD4^+CD25^+$ cells[52]. Other commonly used markers are CTLA-4, CD127, GITR and the transcription factor Foxp3, however, Tregs can lack some of these markers. It has also been proposed by several groups that Tregs should not be defined on the basis of marker phenotype, but rather by their immunosuppressive function (402-404), while a third category of scientists claim that a mixture of marker phenotype and function should be applied. Tregs are normally divided into *naturally occurring*, which mature in the thymus, and those which are induced from naïve T cells in the periphery (reviewed in (405)), referred to as *adaptive Tregs*. Depletion of Tregs in mice improves tumour immunity (406-409). Moreover, cancer patients frequently have an increased number of Tregs, which could potentially promote tumour progression (410-412).

[52] *CD25* is the α-chain of the receptor for IL-2.

5 HER2/*NEU*

5.1 DISCOVERY AND INITIAL CHARACTERIZATION

For about ten years, a mAb has been used in the clinic for the treatment of Her2/*neu*-expressing cancer, and we will come back to this soon. The translational momentum of this research is of course important, but as we will see in this chapter, the initial discoveries that laid the basis of the development of this antibody were actually made by studies on chickens and rats. The discovery and initial characterization of Her2/*ne*u are described rather carefully below and although perhaps not of absolute relevance for the thesis, I think this story is an interesting and fascinating piece of scientific history, and is also an excellent example of how technically fairly simple and at the same time ingenious experiments can result in major discoveries and publications in high-impact journals.

In 1974, Schubert and colleagues (413) injected a carcinogenic substance (nitrosoethylurca) into pregnant rats resulting in central nervous system tumours in the offspring. From these tumours, cell lines were established and when analyzing these lines some of them were classified on the basis of their morphology as neuroblastoma cell lines[53]. In the end of the 1970s and the beginning of 1980s, proto-oncogene research was really in fashion. A classical experiment to study cellular oncogenes was to extract DNA from malignant cells and use it to transfect non-malignant ones, usually mouse fibroblasts such as NIH/3T3, and look for foci of transformed cells[54]. Bob Weinberg's group at MIT carried out such an experiment and could show that DNA from one of the neuroblastoma cell lines established by Schubert *et al* (413) indeed induced transformation (415). Since it was known that only 0.1% of the transfected DNA was integrated into the genome of each transfected cell, the transformation was most likely caused by one single gene (416), and efforts started to identify this gene. Mice were injected with these tumour cells and the sera from the mice precipitated a phosphoprotein of a molecular mass of 185 kDa[55] from the neuroblastoma cells (417).

In 1983, a new avian erythroblastosis virus, named AEV-H, was established, which caused erythroblastosis[56] and sarcoma in chicken (418). The avian erythroblastosis viruses that already existed at that time were known to encode two oncogenes, erbA and erbB (419, 420), where erbB had a molecular weight of around 68 kDa and was located in the cell membrane (421). However, AEV-H turned out to express erbB only (422). Soon it was shown that the erbB gene was similar to a large region of the

[53] Neuroblastomas are not, at least not in humans, found in the central nervous system but rather in the sympathetic nervous system.
[54] Known as focus-forming assay (414).
[55] 185 kDa is today the established molecular weight of Her2/*neu* and the protein is often referred to as p185neu.
[56] *Erythroblastosis* is a kind of leukemia characterized by a large number of immature erythrocytes in peripheral blood.

receptor for human epidermal growth factor (Her). The authors proposed that "AEV-H may have acquired the cellular gene sequences[57] of a truncated epidermal growth factor receptor (EGFR) (or closely related protein), which lacked the external EGF-binding domain, but retained the transmembrane domain and a domain involved in stimulating cell proliferation" (423). The fact that the gene was assumed to encode a truncated version could also explain the finding that the protein product had a size of only 68 kDa as compared to 185 kDa for p185.

The 185 kDa protein expressed by neuroblastoma cell lines and the EGFR were both phosphorylated cell surface glycoproteins with similar molecular weights (424, 425). Based on this information, Weinberg's group speculated that there might be a relationship also between erbB and *neu*[58] and could show that the sequences of the two genes were highly similar (426). Accordingly, if erbB and *neu* were related, and erbB was related to the EGFR, there should naturally also be a relationship between the EGFR and *neu*. As a partial confirmation of this, Weinberg and colleagues showed that these two proteins were serologically related but they were not the same protein since they coexisted in rat cells (426).

5.2 HER2 AND *NEU* BECOME HER2/*NEU*

In 1985, lots of things happened almost at the same time. Semba and colleagues searched the cellular genome for genes related to the viral erbB gene (v-erbB) and found two genes. They named one of them c-erbB-1 and it was suggested to be the same as the EGFR gene. The other one, similar but not identical, was named c-erbB-2 and had a sequence similar to the *neu* gene. They also showed that the cellular gene was amplified about 30 times in a human adenocarcinoma of the salivary gland (427). Very interestingly, while Semba and colleagues performed their study at a lab in Tokyo, Coussens *et al* (428) investigated exactly the same thing in a completely different part of the world, at Genentech in San Francisco. They also looked for the cellular homologue of the v-erbB gene and mapped it to chromosome 17q21 and found that the gene encoded a protein of 1255 amino acids. Due to its similarity with the human EGFR, they named the gene HER2 for Human Epidermal growth factor Receptor 2. Accordingly, both groups found the same gene but gave it different names, c-erbB-2 and HER2, respectively. Three months earlier the Coussens group had mapped the *neu* gene to the same location in the human genome (429), while it was known from before that the EGFR gene is found on chromosome 7 (430) and accordingly the two genes were definitely distinct genes. Concurrently, King *et al* (431) identified a DNA sequence in a human breast cancer sample with a sequence similar to the gene of the EGFR and v-erbB and this gene was amplified 5-10 times.

[57] In 1989 Michael Bishop and Harold Warmus were awarded the Nobel Prize in Physiology or Medicine for the discovery that the oncogenes present in retroviruses were not true viral genes, but were normal cellular genes, which the viruses had acquired from the host cells during replication (8).
[58] This is most likely the first time the term *neu* is used and is the same as the p185 protein above.

The following year also c-erbB-2 was mapped to human chromosome 17q21[59] (432) meaning that c-erbB-2, HER2 and *neu* had all been mapped to the same region of the human genome! In a gastric carcinoma cell line the c-erbB-2 gene was shown to be amplified about 30-fold (432). Weinberg's group once again performed a focus forming assay, and this time they also transfected cells with the normal *neu* gene and those cells did not form foci. A comparison between the two versions of the gene revealed a single amino acid difference at position 664 in the transmembrane domain. This difference resulted in a substitution of valine for glutamic acid (414), and as we will come back to in chapter 6.5 this is relevant with regard to mice transgenic for the *neu* gene. In 1986 it was shown that the protein product of *neu* had tyrosine kinase activity and that EGF was not a ligand (433). It was not difficult to conclude that HER2, *neu* and c-erbB-2 were in fact the same gene. Still today various names are used and in addition to those above, the term Her2/*neu* is common. *neu* normally refers to the rat or mouse gene.

5.3 STRUCTURE, SIGNALLING AND FUNCTION

There are four known members of the human epidermal growth factor receptor family (Her1-4). They are all tyrosine kinase receptors with an extracellular, a single membrane-spanning (transmembrane) and an intracellular tyrosine-kinase domain as shown in figure 10. The extracelluar part is in turn composed of four domains. Multiple ligands have been identified for all members except Her2, which is still considered as an orphan receptor (434). Ligands for Her1 include EGF, TGF-α, epiregulin and amphiregulin while neuregulin 1 and 2 bind to Her3 and Her4 (435). Dimerization occurs upon ligand binding, activating the receptor leading to autophosphorylation of tyrosine residues in the intracellular domain. Receptor overexpression can also induce dimerization (436) and homo- as well as heterodimers can form. Although Her2 lacks ligands, it

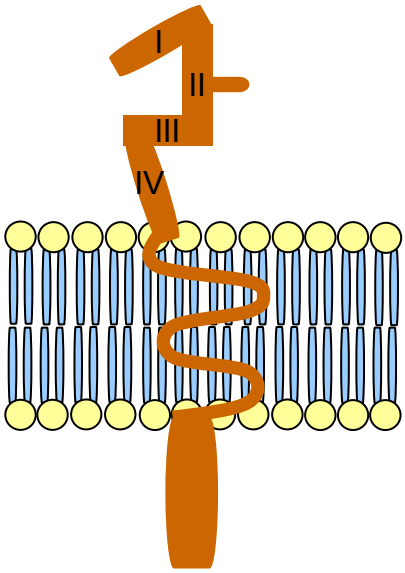


Figure 10. The structure of a Her2/*neu* monomer. The four domains of the extracellular region are indicated.

[59] In rat the protein is composed of 1259 amino acids and the gene is located on chromosome 10.

can heterodimerize with all other receptors and Her2 is actually the favoured dimerization partner for the other members (437-439), and signalling via a heterodimer including Her2 is more potent and long-lasting compared to dimers lacking Her2 (440). The phosphorylated tyrosines in turn act as binding sites for intracellular signalling molecules, and a cascade of intracellular signalling is initiated. The two main pathways downstream of Her2 are the RAS/MAPK and the P13K/Akt pathways, which stimulate proliferation and inhibit cell death. Normally PTEN (441) opposes the activation of the P13K/Akt pathway (442). During the embryonic period Her2 is important for normal heart development (443), and absence of Her2 in adult mice results in enlargement of the heart (444). This might be the reason for the cardiotoxicity of trastuzumab seen in some patients, especially those receiving concurrent treatment with anthracyclins (see chapter 5.5.1 below) (445).

5.4 ROLE IN CANCER

The first study to show Her2/*neu* gene amplification in human cancer (breast cancer) was published in 1985 (431). Then in 1987, Her2/*neu* gene amplification (2-20-fold) was reported in 30% of breast tumours and this was also the first study to show that Her2/*neu* amplification was an independent predictor of time to relapse and overall survival (446). Soon after, correlation with metastatic disease was shown (447). Now we know that Her2/*neu* is amplified mainly in adenocarcinomas e.g. breast, ovarian (448), pancreatic (449), gastric (450), kidney (450), colorectal (451) and non-small cell lung cancer (452), but also in cancers of other histological origin such as osteosarcoma (453) and malignant melanoma (454). Her2/*neu* has been most extensively studied in breast cancer and around 20-25% of all breast cancers have Her2/*neu*-overexpression (reviewed in (455)). Her2/*neu* positivity is associated with aggressive behaviour, poor differentiation, high grade, high proliferation rate and an increased rate of recurrence, particularly in patients with lymp node metastases (456-458). In most patients with Her2/*neu*-overexpressing cancer mutations are not found in the gene (448, 459), although mutations both in the transmembrane and intracellular domains have been described (460, 461), but the significance of such mutations is unknown.

5.5 CURRENT THERAPIES AGAINST HER2/*NEU*-EXPRESSING CANCER

5.5.1 Trastuzumab

In 1989, a murine antibody against Her2/*neu* (mAb4D5) was produced (462) and a humanized version of the antibody was developed a few years later (463). This antibody is nowadays known as trastuzumab (Herceptin), which binds to domain IV in the extracellular domain (see figure 10) (464).

In 1998, Herceptin received FDA approval for use in women with Her2/*neu*-overexpressing metastatic breast cancer both as a single agent and in combination with paclitaxel after a study had showed promising results (465). Since then, several studies have shown that chemotherapy in combination with Herceptin, is more efficient than chemotherapy alone in women with Her2/*neu*-overexpressing metastatic breast cancer

(445, 466). In 2005, several large studies showed that Herceptin also had positive effect in patients with early-stage breast cancer either as monotherapy or in combination with chemotherapy (467, 468). In Sweden today, Herceptin is approved for treatment of both metastatic Her2/*neu*-overexpressing breast cancer, as well as Her2/*neu*-overexpressing early-stage breast cancer (469). It is used both as a single agent and in combination with chemotherapy and anti-hormonal treatment (469).

The mechanism(s) of action of trastuzumab is still a matter of debate. The antibody has several putative mechanisms of action but their relative importance is difficult to determine *in vivo*. Her2/*neu*-overexpressing tumours in mice lacking activating antibody receptors showed an impaired response to trastuzumab, indicating a role for immune cells in trastuzumab-mediated killing (470). Binding of trastuzumab to the surface of cancer cells leads to killing via ADCC (see chapter 4.3.2.1.1), and one study showed that patients responding to trastuzumab had a more pronounced tumour infiltration of leukocytes compared to non-responders (471). Furthermore, trastuzumab induces receptor endocytosis leading to Her2/*neu* downregulation and inhibition of intracellular signalling (462, 472), and it also stabilizes and activates PTEN (473), which is an inhibitor of the P13K/Akt pathway as mentioned above. Whether the antibody can block receptor dimerization is to me not clear since studies are contradictory. What I understand the domain that trastuzumab binds to (IV) is not involved in dimerization of the receptor (464, 474).

5.5.2 Lapatinib

In recent years several inhibitors of tyrosine kinase receptors have reached the clinic, among them are erlotinib (Tarceva) for the treatment of non-small cell lung cancer, and sunitinib (Sutent) for the treatment of kidney cancer and gastrointestinal stromal cell tumours (469). It is therefore not very surprising that inhibitors of Her2/*neu* have also been developed. Lapatinib (Tykerb/Tyverb) inhibits autophosphorylation of both Her1 and Her2 (475, 476). Importantly, cells resistant to trastuzumab were not cross-resistant to lapatinib and, additionally, the two drugs seemed to have synergistic effects *in vitro* (475, 477). At least two phase III trials have been performed and both showed better effect with chemotherapy in combination with lapatinib compared to chemotherapy alone in Her2/*neu*-overexpressing metastatic breast cancer (478, 479). In Sweden, lapatinib is approved in combination with capecitabin for use in patients with metastatic or advanced Her2/*neu*-overexpresssing breast cancer, who have progressed despite treatment with trastuzumab (469).

5.6 WHY DEVELOP ADDITIONAL ANTI-HER2/*NEU* THERAPIES?

Considering the fact that a drug directed against Her2/*neu* is already on the market, what is the purpose of developing new anti-Her2/*neu* therapies? Are we simply inventing the wheel again or is there any rationale for our scientific efforts? I would claim that there are several reasons as follows:

- **Resistance**

Not all patients with Her2/*neu*-overexpressing breast cancer respond to trastuzumab (primary resistance), and many of those who initially respond develop resistance over time (secondary resistance) (480, 481). Several mechanisms of resistance are possible. Her2/*neu* can lack the extracellular domain and the receptor is then denoted p95Her2 (482), and this of course makes it impossible for trastuzumab to bind, but these tumours seem to be sensitive to lapatinib (483). If the Her2/*neu* signalling pathay is non-functional, the cell can use compensatory pathways such as the pathways for insulin growth factor-1 and fibroblast growth factor (484).

It is much easier to develop resistance in the case of monotherapy compared to combination therapy and this knowledge is used in cancer treatment when cytostatic drugs with different mechanisms of action are used in conjunction. Trastuzumab binds to a particular region of Her2/*neu* and so is the case for lapatinib. If we instead would apply active vaccination in the form of a Her2/*neu* cancer vaccine, a multitude of antibodies specific for various parts of the extracellular domain would be raised and this would potentially decrease the risk of resistance. It should also be noted that expression of Her2/*neu* is usually retained after resistance to trastuzumab has developed.

- **Improved efficacy**

Trastuzumab is considered as a very efficient therapy for breast cancer. However, if we scrutinize the effect of trastuzumab in e.g. patients with metastatic breast cancer, the addition of trastuzumab to chemotherapy prolongs survival with *a couple of months* (445, 466). Accordingly, there is a need for better treatments.

- **Long-lasting effect**

Both trastuzumab and lapatinib are passively transferred molecules, i.e. there is no endogenous production in the patient of these drugs, and the drugs will therefore slowly disappear from the body and repeated administrations are required. In the case of lapatinib the half-life is around 24 hours and the drug should be taken on a daily basis. With regard to trastuzumab, the half-life is actually fairly long (28.5 days) and it is sufficient to administer it once a week or even once every third week. However, when it comes to active immunization such as administrations of Her2/*neu*-VLPs (see the results and discussion chapter), the patient's immune system will be activated, immune cells will start to proliferate and memory cells will be induced. Hopefully, this will result in a more long-lived response compared to trastuzumab and lapatinib and repeated administrations will not be required.

6 TUMOUR IMMUNOTHERAPY

6.1 TO COMBAT OR NOT TO COMBAT CANCER

Have you ever reflected upon the fact why we actually have an immune system? Probably not because for most people the answer is self-evident. Even a fourteen-year-old person would consider such a question trivial and reply that the immune system protects us against infections. If a professor of tumour immunology would be asked the same question, he or she would claim that the immune system also makes up a defence against cancer. The fourteen-year-old person's answer is definitely correct, while the importance of the immune system with regard to protection against cancer is still under debate[60].

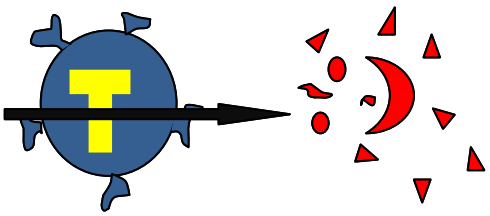


Figure 11. Is killing of cancer cells (red) by the immune system (in this case represented by a T cell (blue)) truth or myth? Reality or utopia? Cartoon by Helena Tufvesson. Reproduced with kind permission.

It is more or less a tradition that a thesis about cancer immunotherapy has a chapter about the history of the field, about immunosurveillance and about immunoediting, but since there has already been so much of history in this thesis I have decided to skip the theories of Paul Ehrlich (485), Sir Frank Macfarlane Burnet and Lewis Thomas (486-488) *et al*. To cut a very long story short, we know today that in most cancer patients the immune system reacts against the malignant cells. However, as we will soon be faced with, the immune response is generally very weak.

This chapter is arranged as follows. First there is a description of how the immune system can recognize tumour cells and this is then followed by some examples of approaches used to strengthen the immune system against cancer cells. In order to keep the second section within reasonable bounds, I have focused almost exclusively on studies involving Her2/*neu*. Some areas have been completely excluded, like adoptive transfer of T cells, not because they are less important but rather because they are not of direct relevance for this thesis.

6.2 HOW DOES THE IMMUNE SYSTEM RECOGNIZE CANCER CELLS?

The fact that the immune system reacts against malignant cells creates paradox. According to the concept of tolerance, the immune system should not raise a response

[60] For the sake of completeness, it should be noted that there is no doubt about the fact that the immune system is important with regard to protection against *viral-induced* cancer. However, for cancer not caused by viruses the role of the immune system is uncertain.

against cancer cells since the cancer cells are not foreign. However, malignant cells are different from normal healthy cells, and if we simplify a little, the immune system will *consider* them as foreign and may therefore raise an immune response. Cancer cells can differ from normal cells in several different ways as exemplified below:

- If a virus has contributed to the development of the cancer, e.g. HBV in liver cancer and HPV in cervical cancer, virus-encoded proteins are produced by the cancer cells.
- Cancer cells carry mutated genes and when they are expressed, the protein products can look different compared to the wild-type proteins, e.g. mutated p53.
- Some proteins normally only expressed during embryonic development can be re-expressed in malignant cells, e.g. the carcinoembryonic antigen.
- Proteins normally expressed only in immune-privileged sites[61] are sometimes expressed in malignant cells, e.g. the MAGE antigens normally expressed in the testis.
- Overexpression of a non-mutated protein, e.g. Her2/*neu*. Her2/*neu* is not only expressed by malignant but also by normal cells, but then at a very low level. Today we assume that this low expression is below the level of detection of the immune system (ignorance), and an immune response is only evoked as a consequence of overexpression. With regard to Her2/*neu* it should also be pointed out that it is present in the cell membrane and exposed on the surface. This implies that a Her2/*neu*-overexpressing cancer cell will differ in two ways from a normal cell, both by the presentation of Her2/*neu* peptides on MHC class I, and by the presence of the native Her2/*neu* protein on the cell surface. Accordingly, a Her2/*neu*-positive tumour cell can be recognized by the immune system both through binding of $CD8^+$ CTLs to MHC class I:peptide complexes and through direct binding of antibodies to Her2/*neu*.

Malignant cells can also be recognized by NK cells due to low or absent expression of MHC class I. In addition, as we will see in chapter 6.3, cancer cells often have a changed glycosylation pattern of their proteins, which is recognized by DCs and probably also antibodies.

This sounds good but…why do we sometimes get cancer? We can also put it this way: How come that the immune system does not always kill the malignant cells? The answer to this question is complex and there are several underlying reasons for the failure of the immune system to protect us from cancer. One important reason is that the immune response induced by malignant cells is normally very weak. Another reason is that the tumour escapes from the immune system in various ways, however this will not be further discussed in this thesis. In our bodies both T cells and antibodies directed against self-antigens are circulating, but they are low in number and of low

[61] Sites that the cells of the immune system are unable to reach, e.g. the testis since it is "hidden" behind the tight blood-testis-barrier.

avidity (489, 490). The purpose of cancer vaccination is to stimulate and activate these types of cells and antibodies, as well as induce cells and antibodies with higher avidity. This might seem simple. It is simple, in theory, but slightly more difficult when it comes to real life.

6.3 HOW DO DENDRITIC CELLS RECOGNIZE CANCER CELLS?

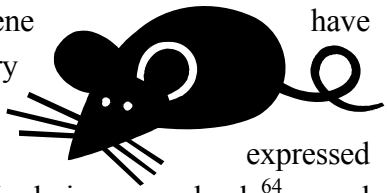
From the above section we can conclude that malignant cells differ from normal cells in their pattern of protein expression, and due to this they are recognized by the immune system. $CD8^+$ CTLs need several signals in order to be activated and subsequently kill targets. Apart from recognizing target cells through binding to an MHC class I:peptide complex, CTLs also need co-stimulation. Tumour cells can only provide the presentation of peptides on MHC class I and, unless the tumours cells are APCs themselves[62], they lack co-stimulatory capacity. As discussed in chapter 4.5, MHC class I presentation without co-stimulation will result in tolerance in the form of anergy and this is probably quite a common phenomenon in cancer patients. Co-stimulation can be obtained either from a DC through cross-presentation, or from a $CD4^+$ Th cell. In the latter case, the $CD4^+$ Th cell must be activated by a DC anyway. Accordingly, activated DCs seem indispensable. In chapter 4.2 we discussed that DCs get activated by binding to conserved molecules like LPS, dsRNA and CpG present in pathogens but how do DCs then recognize tumour cells, which do not carry such molecules? Dendritic cells can bind and engulf dying cells, primarily those undergoing apoptosis but also necrosis, via receptors such as $\alpha_v\beta_5$ integrins and DEC-205 and thereafter cross-present the antigens (491, 492). It is now clear that DCs can also interact with living cells including malignant ones, and it was in fact shown already in 1986 that allogeneic lymphocytes were phagocytosed by DCs (493). One mechanism of such recognition involves binding of antibodies and engulfment through ADCC. Apart from antibody recognition, DCs can acquire antigens from living cells for cross-presentation by a process termed "nibbling" (494), and it seems like this is at least partly mediated by scavenger receptors (495). With regard to PRRs, the kind of PRRs known as TLRs (see chapter 4.2) mainly recognize invading pathogens and from what I can understand they are not directly involved in tumour cell recognition. However, there is another group of PRRs on DCs called C-type lectin receptors (CLRs) including the DC-SIGN and MGL receptors. The CLRs recognize carbohydrates and malignant cells have often changes in protein glycosylation as briefly mentioned above (reviewed in (496)). Two well studied examples are carcinoembryonic antigen (reviewed in (497)) and MUC1 (reviewed in (498)). It has been demonstrated that DCs recognize modified glycosylations on these two proteins through DC-SIGN and MGL respectively, while DCs do not interact with the same unmodified proteins on normal cells (499, 500).

[62] A B cell lymphoma is an excellent example where the cells are malignant cells and APCs at the same time.

6.4 HER2/*NEU* AS A TUMOUR ANTIGEN

Her2/*neu* is a very attractive antigen in tumour immunotherapy for several reasons. First of all, it is selectively overexpressed by malignant cells, while its low expression by normal cells results in a low risk of autoimmune responses. Secondly, it is exposed on the cell surface making it accessible for both antibodies and T cells. Thirdly, the malignant phenotype is dependent on expression of Her2/*neu* reducing the risk of antigen-negative variants, although the tumour cells can overcome the dependence on Her2/*neu* as discussed in chapter 5.6. An important issue is tolerance. In order for tumour vaccination[63] to work, a prerequisite is the existence of CTLs and/or antibodies. Fortunately, it has been shown repeatedly that antibodies (501-504) as well as T cells (501, 505, 506) specific for Her2/*neu* are found in patients with Her2/*neu*-overexpressing cancer.

6.5 MOUSE MODELS OF HER2/*NEU*-EXPRESSING CANCER



Several lines of mice transgenic for the rat Her2/*neu* gene have been created (507-510). Generally the mouse mammary tumour virus (MMTV) promoter is used to direct gene expression to the breast. However, this promoter is expressed not only in breast tissue, but also in the parotid and Harderian glands[64], and the epididymis. The first Her2/*neu* transgenic mouse strain was created by Muller and colleagues in 1988 (508). These mice are on FVB/N background (H-2^q) and are transgenic for the activated rat Her2/*neu* gene, i.e. there is a mutation at position 664 as described in chapter 5.2 (414, 511). This mutation results in a bond between the Her2/*neu* molecule and a neighbouring Her2/*neu* molecule or other receptor molecules in the EGFR family. Such dimerization leads to constitutive receptor activation and transformation (511). At 8 weeks of age these mice display multiple foci of hyperplasia in their mammary glands and normally all mice have developed mammary tumours by day 90-100. This strain will in the following be referred to as FVB/*neu*-T[65]. Since no mutation in the human gene had been described at this time, it was assumed that a mouse carrying unmutated rat Her2/*neu* would be more similar to the human situation and such a mouse strain was created a few years later (507). Also these mice are on FVB/N background but in contrast to the strain above, these mice develop tumours at much higher age and normally begin to appear at 4 months of age. In the best-characterized line (N202), 50% of mice have developed tumours by the age of 200 days. These transgenic mice will in the following be referred to as *neu*-N mice. With regard to tolerance to Her2/*neu*, in these mice Her2/*neu* is expressed in the thymus in adult but

[63] Tumour vaccination in this case refers to active vaccination/immunotherapy. For e.g. adoptive therapy of T cells no pre-existing antibodies/T cells are needed.

[64] The *Harderian glands*, also known as the Harder's lacrimal glands, were described in the late 17th hundred by the Swiss physician Johann Harder. They are located in the orbit (behind the eye) and the secretions (including lots of red porphyrin) lubricate the eye. Humans have a rudimentary gland. In rodents sickness, stress, poor diet etc result in overproduction of porphyrin, which can form a red crust below the eye and this is one of the signs of an unhappy laboratory mouse.

[65] No consensus seems to exist with regard to naming of the Her2/*neu* transgenic mice on FVB/N background.

not in young mice. In order to obtain tumour outgrowth of a transplantable tumour, a dose at least 100-fold lower can be used in *neu*-N compared to the parental FVB/N mice, which has been interpreted as a proof of tolerance to rat Her2/*neu* (512).

The transgenic mice used in this thesis were created by Lucchini *et al* in 1992 (509). These mice carry the activated rat Her2/*neu* gene under the MMTV promoter. The mice (on CD-1 background) were mated with BALB/c (H-2^d) mice and further backcrossed with BALB/c mice by Guido Forni's and Federica Cavallo's group at the University of Turin, Italy, to obtain transgenic mice on a H-2^d background (513). This strain is nowadays referred to as BALB-neuT mice. They are heterozygous for Her2/*neu* since the homozygotic mice die *in utero*. In these mice, hyperplasia is seen at 3 weeks of age and at around 8 weeks carcinoma *in situ* is present. Invasiveness is initiated between 10 and 20 weeks and the tumours metastasize after the 35th week (513, 514). The Her2/*neu* gene is expressed in the thymus and induces deletion of T cells recognizing dominant epitopes with high affinity (515). The BALB-neuT mice are therefore usually considered tolerant to rat Her2/*neu* but not to human Her2/*neu*. It has also been shown that Tregs expand as a consequence of tumour progression starting at about 7 weeks of age (407).

The strain created by Bouchard and colleagues is also on BALB/c background and carries the activated rat Her2/*neu* gene under the MMTV promoter. Mammary tumours appear stochastically between 5 and 10 months of age (510). These mice will be referred to as MMTV*neu*T mice.

In 2003, a strain on C57Bl/6 background expressing the unmutated human Her2/*neu* gene was constructed (hereafter referred to as Her2 Tg mice). These mice do not develop tumours spontaneously but are tolerant to human Her2/*neu* (516).

6.6 ADJUVANTS – THE IMMUNOLOGISTS' DIRTY LITTLE SECRET

An adjuvant is a compound that is co-delivered with a vaccine in order to augment the induced immune response. Many vaccines are composed of live or attenuated viruses or bacteria and these vaccines are normally very potent also in the absence of adjuvant. However, vaccines composed of e.g. recombinant proteins are weaker inducers of immunity and the effect of such vaccines is often dramatically improved by an adjuvant. In addition to making the immune response stronger, an adjuvant can break tolerance to self and skew the immune response towards a Th1 or a Th2 response.

6.6.1 CpG

Already in 1984, it was shown that bacterial DNA could induce anti-tumor immunity (517) and it was later demonstrated that particular CpG structures were responsible for the effect (518). In 2000, the receptor for CpG was identified as TLR 9 (287). A CpG sequence consists of an unmethylated CpG dinucleotide flanked by certain nucleotides. We have utilized CpG 1826 and the sequence of this molecule is shown in figure 12.

The bonds between the nucleotides are phosphorothioate-modified in order to increase resistance to nuclease degradation (519). CpG has been shown to be well-tolerated in humans (520, 521). It has a range of functions including activation of B cells (518), monocytes, NK cells and various subsets of DCs (522, 523). It both stimulates Th1 and Th2 immunity (524). Several studies in mice have shown that CpG can mediate tumour protection in an NK cell-dependent manner (525-528). Of interest for this thesis is the fact that CpG can improve tumour protection but simultaneously weaken CTL responses (529, 530). As mentioned above, these sequences are present also in natural DNA, but more common in bacteria than in vertebrates, and this partly explains the relatively high immunogenicity of DNA vaccines since such vaccines are normally composed of a plasmid produced in bacteria (518, 531).

TCCATGACGTTCCTGACGTT

Figure 12. Sequence of CpG 1826. CpG motifs underlined.

6.6.2 Freund's adjuvant

Not surprisingly, the classical adjuvant known as Freund's adjuvant was developed by a person called Freund, Jules Freund to be more precise (532). It is a water-in-oil emulsion and increases the efficacy of a vaccine by providing continuous release of antigens. When killed Mycobacterium tuberculosis bacteria are included it is known as Complete Freund's Adjuvant (CFA), and without bacteria it is referred to as Incomplete Freund's Adjuvant (IFA). Freund's adjuvant stimulates both humoral- and cell-mediated immune responses (533, 534), but due to side effects the use of at least CFA is limited today (reviewed in (535)). The adjuvant used in paper I contains, in addition to mycobacteria, paraffin oil and the emulsifying agent mannide monooleate[66] (536).

6.6.3 Cytokines

A small selection of cytokines and other immunostimulatory molecules will be presented here, since they will be mentioned later in this chapter. *Granulocyte macrophage colony-stimulating factor* (GM-CSF) is a cytokine with multiple functions. In immunotherapy its most well known effect is probably recruitment of DCs as well as stimulation of growth and differentiation of these cells. It is commonly used as an adjuvant in cancer immunotherapy including clinical trials. *Interleukin-12* (IL-12), also known as NK cell stimulatory factor, is a cytokine that stimulates a Th1-skewed response (537, 538), upregulates MHC class I and II and activates NK cells. IL-12 also promotes expression of LAG-3 (Lymphocyte Activation Gene-3/CD223) on activated $CD4^+$ and $CD8^+$ T cells as well as NK cells. LAG-3 is thought to stimulate presentation of peptides on MHC class I by DCs (539).

[66] Four immunizations were performed in paper I, the first with CFA and the other three with IFA.

6.7 IMMUNOTHERAPY OF HER2/*NEU*-EXPRESSING CANCER

This chapter is a review of studies on various immunotherapy approaches to combat Her2/*neu*-expressing cancer with emphasis on active vaccination. Some readers would probably consider this part of the thesis as dull, heavy and boring with lots of data lined up in a long row. Others would instead find this chapter very informative and comprehensive, at least those who have a profound interest in Her2/*neu* vaccines. In case you would not like to read the complete chapter, I have summarized the chapter in section 6.7.5.

6.7.1 Administration of antibodies and cytokines

As discussed in chapter 5.5.1, many studies have shown the effect of anti-Her2/*neu* antibodies on Her2/*neu*-expressing cells both *in vitro* and *in vivo*. An example using transgenic mice (MMTV*neu*T) is the study by Katsumata *et al* (540), who showed that repeated injections starting at 6 weeks of age delayed tumour onset and completely protected 50% of mice against tumour outgrowth. In BALB-neuT mice, repeated administrations of IL-12 beginning at 2 weeks of age caused delayed tumour onset but protected only a minor fraction against outgrowth of spontaneous tumours. Depletion of $CD8^+$ T cells did not have any impact. In *neu*-N mice, the same treatment completely protected half of the mice against tumour outgrowth (513, 541). Combining IL-12 with tamoxifen from week 20 in these mice resulted in 70% protection, while either treatment alone only delayed tumour onset marginally (542). As noted above (chapter 6.5), in BALB-neuT mice Tregs expand during tumour progression. Repeated injections of an antibody against these cells starting at 6 weeks of age resulted in both a cellular and a humoral response to rat Her2/*neu* accompanied with a delay in tumour onset (407). Furthermore, injection of CpG next to the mammary carcinomas into these mice lead to infiltration into the tumour tissue of a multitude of cell types including macrophages, plasma cells, B cells and both $CD4^+$ and $CD8^+$ T cells. This caused a delay in tumour onset and in some mice complete tumour protection. Very interestingly, in this study CpG administration also increased the lytic activity of NK cells and depletion of NK cells abrogated tumour inhibition (526).

6.7.2 Whole-cell vaccines

Cefai and colleagues (543) immunized FVB/*neu*-T mice with allogeneic fibroblasts transfected with the rat Her2/*neu* gene, which protected 80% of mice against tumour challenge, and this effect was improved further by boosting. Based on additional experiments they concluded that the response was mainly mediated by $CD8^+$ T cells. Reilly *et al* (512) immunized *neu*-N mice with a whole-cell vaccine expressing rat Her2/*neu* in combination with GM-CSF and observed induction of both antibodies and CTLs. This caused a delay in outgrowth of a transplantable tumour in *neu*-N mice, while parental FVB/N mice, not tolerant to rat Her2/*neu*, were completely protected. Although there was a marked difference concerning the induced immune response between the parental and transgenic mice, the induction of an immune response in *neu*-N mice proved that tolerance to Her2/*neu* could be broken. Depletion of $CD4^+$ T

cells prior to vaccination completely abrogated the effect, while depletion of $CD8^+$ T cells had a less dramatic effect and NK cell depletion did not affect at all. The following year the same group investigated the possibility of improving the immunization effect by combining the vaccine with chemotherapeutic agents (544). A combination of either paclitaxel or cyclophosphamide and vaccine was better in both parental FVB/N and transgenic *neu*-N mice than either modality alone, whereas addition of doxorubicin and cisplatin had no effect. When trying to analyze the immune mechanisms they made the interesting observation that the addition of cytostatic agents resulted in an increased number of T cells, secreting primarily IFNγ indicative of a Th1 response. In another study (545), the same investigators showed that the effect of the whole-cell vaccine expressing rat Her2/*neu* and GM-CSF could be improved further with mAbs against Her2/*neu* and especially if two different antibodies against various epitopes were used. Depletion of either $CD4^+$ or $CD8^+$ T cells after vaccination abrogated the effect, i.e. the response was T-cell dependent. BALB-neuT mice were vaccinated repeatedly with allogeneic rat Her2/*neu* positive tumour cells starting at 6 weeks of age in combination with administration of IL-12, since repeated administrations of IL-12 had recently shown to increase tumour latency as described above (513). This approach protected almost 90% of mice against tumour outgrowth, which was much better than treatment with either cells or IL-12 alone, and the effect was shown to be dependent on IFNγ (546). When crossing BALB-neuT mice with different strains of knockout mice, protection was lost in mice lacking IFNγ, and in B-cell knockout mice that did not develop an antibody response, while protection was maintained in B-cell knockout mice that still raised antibodies (547). In addition to GM-CSF, a whole-cell vaccine expressing either IL-2, IL-12, IL-15 or IFNγ was constructed, and the most efficient protection of BALB-neuT mice was conferred by the IL-12 expressing cells. The effect was lost in BALB-neuT mice crossed with mice lacking IFNγ and markedly reduced when crossed with mice lacking B cells. The protection afforded by the whole-cell vaccine expressing IL-12 was equal to that provided by the systemic administration of IL-12 in combination with cells. However, cells expressing IL-12 have the advantage of giving much lower circulating concentrations of IL-12, and therefore fewer potential side effects and less systemic toxicity (548).

6.7.3 Peptides and proteins

It has been suggested that T cells develop tolerance to the immunodominant epitopes of self proteins but ignore the subdominant ones, and that tolerance can be circumvented by immunizing with peptides but not with the complete protein (549, 550). Based on this assumption, Disis *et al* (551) immunized rats with groups of peptides from the intra- or extracellular domains of rat Her2/*neu*. In both cases $CD4^+$ T cell immunity as well as antibodies were observed, while rats immunized with full-length protein did not raise any such response. Esserman and colleagues (552) immunized with the extracellular domain of rat Her2/*neu* together with CFA, which induced a cellular as well as a humoral response and reduced tumour incidence in FVB/*neu*-T mice. In a study by Dakappagari and collaborators (553) potential B-cell epitopes of the extracellular

domain of the human Her2/*neu* protein were fused to a Th epitope of a measles virus protein and these fusion proteins induced high titres of antibodies in rabbits. In *neu*-N mice, repeated immunizations starting at 4 weeks of age completely protected more than 80% of mice against tumour development. Manjili *et al* (554) linked heat-shock protein 110 to the intracellular domain of human Her2/*neu* and this induced both a cellular and humoral response in mice and in *neu*-N mice this construct induced production of IFNγ and IL-4, while the extracellular domain alone did not. Repeated immunizations delayed tumour onset. In this model depletion of $CD4^+$ T cells had no effect while depletion of $CD8^+$ T cells impaired, although not completely abrogated protection (555).

6.7.4 Gene therapy

The purpose of gene therapy can be to introduce a single gene into a patient with a monozygotic disorder (cystic fibrosis is an example), introduce a foreign gene into a mouse and thereby create a transgenic mouse strain, or delete a gene from a mouse resulting in a knock-out mouse. When it comes to tumour immunotherapy, gene therapy normally means introduction of one or several genes in order to obtain endogenous production of a tumour antigen such as Her2/*neu*.

6.7.4.1 Transfer of naked DNA

Let us start very simple. If naked DNA such as a plasmid is added to cells the DNA will be taken up non-specifically and the gene/s encoded by the transferred DNA will hopefully be expressed. Usually the demonstration by Wolff *et al* in 1990 (556) that DNA can be taken up and expressed following intramuscular (i.m.) injection is taken as the first example of this although it had been shown six years earlier that following intrahepatic injection naked hepatitis DNA yielded expression of hepatitis genes in the liver (557). Anyhow, the study by Wolff and colleagues was spectacular since it was previously assumed that DNA had to be formulated with particles in order to be introduced into cells, i.e. at that time it was considered as "impossible" for naked DNA to be taken up (558). As a matter of curiosity, this study is an excellent example of when the control became the experiment. Naked DNA was in fact used as a negative control (DNA formed with chemicals made up the real samples) but worked better than the samples! Accordingly, gene therapy might seem pretty simple. However, a major drawback of using naked DNA is the low efficacy since gene expression normally occurs in only a minor fraction of cells and for a limited period of time. This could be due to several factors such as destruction of DNA before reaching the cells, poor uptake into cells and few DNA molecules reaching the nucleus. It is not so difficult to imagine that the uptake is promiscuous and it is not possible to target the DNA to specific cell types. Advantages of naked DNA are low toxicity, simple and relatively inexpensive production, no pre-existing immunity, low risk of integration into the cellular genome and induction of both cellular and humoral responses. In addition, as mentioned in chapter 6.6.1, bacterial DNA contains immunostimulatory CpG motifs. A DNA vaccine is most commonly made up of a plasmid produced in bacteria where the gene(s) of interest is

placed under control of a strong promoter and in some cases the gene is codon optimized[67] in order to obtain as high gene expression as possible.

6.7.4.1.1 Improving transfer of naked DNA

Various strategies have been attempted to improve the efficacy of transfer of naked DNA and a few of them, which are relevant for the understanding of this thesis, will be briefly discussed here. One is by the way association with VLPs and this topic will be further discussed in chapter 7.4.1. *Gene gun* means that the DNA is bound to small particles, often gold particles, which are then "shot" into the cells with high speed ("particle bombardment"), leading to penetration of the plasma membrane. *Liposomes* are small lipid particles, which are positively charged and thereby can make a complex with the negatively charged DNA and this has been shown to improve uptake. *Electroporation* means that following injection of DNA, an electrical current is applied over the site of injection and this is thought to create pores in the plasma membrane (559), making it easier for DNA to enter cells. Electroporation results in enhanced gene expression levels (560), leading to improved humoral as well as cellular responses to the expressed protein (561).

6.7.4.1.2 Naked DNA encoding Her2/*neu*

6.7.4.1.2.1 Studies in wild-type and Her2 Tg mice

Already in 1996 an interesting study was performed by Concetti *et al* (562). Immunization with DNA encoding the rat Her2/*neu* protein induced antibodies in BALB/c mice, but not in rats. However, a plasmid containing the human gene resulted in an antibody response in rats. These results together indicated that the heterologous, but not the homologous Her2/*neu* gene, induced antibodies. A danger of using the wild-type Her2/*neu* gene is that it could potentially function as a normal gene in the transfected cells and give rise to cellular transformation, and this would be especially hazardous in case the gene would be integrated. To circumvent this risk, Wei and colleagues (563) mutated amino acid 753 in the intracellular domain of the human Her2/*neu* protein to alanine instead of glycine. This substitution eliminates autophosphorylation. BALB/c mice immunized i.m. three times with the full-length wild-type gene were completely protected against challenge with D2F2/E2 (human Her2/*neu* positive), while the full-length mutated gene yielded only partial protection. They also created constructs lacking the ER localization signal in order to direct the protein into the MHC class I processing pathway, but this strategy failed and no protection at all was seen. However, despite this failure they could later show that a combination of a plasmid encoding a protein lacking the ER localization signal and a plasmid encoding either GM-CSF or IL-2 gave the same protection against a transplantable tumour as a plasmid encoding the full-length wild-type protein. The full-length constructs induced antibodies, while

[67] *Codon optimization* means that the codons encoding the various amino acids are changed to better match the tRNA molecules present in mammalian cells, rather than the tRNAs present in bacteria. However, it is now known that also other factors increase the expression of the gene since the amount of mRNA is enhanced as well.

no humoral responses were induced with plasmids encoding the gene without the ER localization signal, thus proving that anti-tumour immunity can occur in the absence of antibodies. As a further proof of the role of T cells, depletion of $CD8^+$ T cells impaired although not completely abrogated protection, while depletion of $CD4^+$ T cells did not have any effect at all (564). They also constructed a gene encoding the N-terminal 505 amino acids of the human Her2/*neu* protein encompassing most of the extracellular domain, and this construct yielded protection against D2F2/E2 outgrowth in 90% of the mice. Protection was improved to 100% when this construct was combined with the plasmid encoding the gene lacking the ER localization signal. This combination was shown to result in both a strong CTL and a potent antibody response (565). The same year (2001), Lachman and colleagues (566) could show that multiple immunizations, but not a single immunization, with a plasmid encoding the rat Her2/*neu* gene reduced tumour incidence of a transplantable tumour in BALB/c mice.

In 2003, the Wei-Zen Wei group in Detroit constructed the Her2 Tg mice, which were on C57Bl/6 background (see chapter 6.5). Weak antibody responses were induced in these mice with a plasmid given i.m. carrying either the full-length human Her2/*neu* gene, or two plasmids encoding the extracelluar domain and GM-CSF, respectively. The combination of the two plasmids resulted in protection of 33% of the mice against tumour cell challenge (remember these mice do not develop tumours spontaneously) if five immunizations were given, implying that tolerance could be broken (516). Last year this group published a very exciting study with regard to the use of Her2/*neu* vaccines in patients (567). In BALB/c mice, i.m. immunization combined with electroporation with a plasmid carrying the extracellular and transmembrane domains of the rat Her2/*neu* gene and a plasmid encoding GM-CSF, completely protected against tumour outgrowth. Protection was obtained regardless of if the tumour cells were sensitive to anti-Her2/*neu* antibodies or a tyrosine kinase inhibitor. They also showed that $CD4^+$ and $CD8^+$ T cells were necessary for protection against drug-resistant but not against drug-sensitive tumours using this vaccine. Interestingly, when immunizing with a plasmid encoding GM-CSF in conjunction with a plasmid encoding only the extracellular domain of rat Her2/*neu*, a cellular but not a humoral response was induced and both drug-sensitive and drug-resistant tumour were rejected. Consequently, anti-Her2/*neu* antibodies were not necessary for rejection. In 2003, Curcio and colleagues (568) immunized BALB/c mice intradermally (i.d.) with DNA encoding the extracellular and transmembrane domains of rat Her2/*neu* using the gene gun technique. This resulted in a cellular as well as humoral immune response and complete protection against challenge with TUBO. They very carefully dissected the underlying immune mechanisms and showed that depletion of $CD4^+$ T cells during immunization completely abolished the effect while depletion of the same cells during the effector phase had no impact. Depletion of $CD8^+$ T cells during the effector phase impaired although not completely abrogated protection. Mice lacking either β2m, μIg, FcγRI/III, IFNγ or perforin were all protected while protection was lost in mice lacking both IFNγ and perforin as well as in mice lacking polyomorphonuclear cells (granulocytes). In a thera-

peutic setting, immunization was commenced at a tumour size of 2 mm and repeated vaccinations completely cured the mice. Interestingly, in contrast to the prophylactic setting, when treating already established tumours μIg- and FcγRI/III-knockout mice were not cured. These results indicate that different immune effector mechanisms are responsible for *preventing* tumour outgrowth and *eradicating* already established tumours. Lindencrona *et al* (569) obtained almost complete protection against D2F2/E2 challenge using co-immmunization with plasmids encoding human Her2/*neu* and GM-CSF, respectively. Protection was lost following depletion of either $CD4^+$ or $CD8^+$ T cells during the effector phase as well as lost in mice lacking IFNγ, while close to complete protection was observed in B-cell knockout mice. Recently the same group tested the efficacy of a plasmid expressing human Her2/*neu* in mice transgenic for the human MHC class I molecule A2 (570). I.d. injection followed by electroporation resulted in complete protection against outgrowth of a transplantable human Her2/*neu*-expressing tumour in a CTL-independent manner.

6.7.4.1.2.2 STUDIES IN BALB-NEUT MICE

In BALB-neuT mice a wide range of DNA immunization strategies have been explored. In 2000, Rovero *et al* (571) compared the efficacy of a plasmid encoding rat Her2/*neu* to protect against a transplantable tumour with its efficacy to inhibit spontaneous tumour outgrowth. First they showed that a plasmid encoding either the extracellular domain only or both the extracellular and transmembrane domains injected i.m. completely protected against TUBO challenge in normal BALB/c mice. Thereafter they showed that in BALB-neuT mice, DNA more efficiently protected against spontaneous tumour development than against challenge with TUBO and, additionally, that the mice protected against spontaneous outgrowth were still susceptible to TUBO. Four DNA immunizations starting at 6 weeks of age completely protected 57% of mice against spontaneous tumour development. In order to try to improve the effect of IL-12 administration (see chapters 6.7.1 and 6.7.2), IL-12 was given in combination with a plasmid encoding the extracellular and transmembrane domains of rat Her2/*neu*. Multiple immunizations starting at week 6 of age protected 58% of mice. Neither IL-12 nor DNA induced a CTL response while DNA with or without IL-12 raised antibodies to rat Her2/*neu* (572). LAG-3 co-administered with a plasmid given i.m. encoding the extracellular and transmembrane domains of rat Her2/*neu* was superior to DNA alone. Immunization at week 4 and 7 of age protected almost 70% of the mice against outgrowth (573). A study by Quaglino and co-workers (574) showed that the same plasmid given i.m. at weeks 10 and 12 delayed tumour onset but induced complete protection of only a minor fraction of the mice. The efficacy was improved by co-administration of rat Her2/*neu* positive tumour cells expressing IFNγ, but the effect was completely abolished in BALB-neuT mice lacking B cells. In another study (575), the same investigators showed that if the plasmid was electroporated instead of simply injected i.m., the effect was improved and repeated immunizations completely protected all mice. As in their previous study (575), the effect was completely lost in BALB-neuT mice lacking B cells and the same was shown for mice lacking IFNγ. On the basis of

this they concluded that protection was mediated by antibodies in combination with IFNγ secreting cells. As a next step they investigated if they could immunize even older mice, i.e. when the mammary carcinomas were more advanced. Electroporation at weeks 16 and 18 did not cause a delay in tumour onset but combined with IL-12 complete protection of more than 60% of the mice was seen and protection of all mice could be obtained with further boosting (576). Last year another group published a study where they had utilized a plasmid with a codon optimized version of a gene encoding the extracellular and transmembrane domains of rat Her2/*neu* and concurrently they supplied the drinking water with an agonist for TLR 7 (577). Electroporation of DNA at weeks 13 and 15 protected 80%, while only a minor effect was observed when the immunizations were performed at weeks 16 and 17. The TLR 7 agonist did not improve the cellular response, but increased the antibody titres. So far it had been shown that one single immunization of BALB-neuT mice had a very limited effect and multiple immunizations were required in order to obtain a potent anti-tumour response. The Forni/Cavallo group had demonstrated that electroporation improved the effect of DNA immunization compared to simple i.m. immunization as described above (575), and in a study published in 2008 they evaluated various electroporation protocols (578). Using *constant-current electroporation* they could protect all BALB/c mice against TUBO challenge with as little as 10 μg of a plasmid encoding the extracellular and transmembrane domains of rat Her2/*neu*. In BALB-neuT mice, immunization with 50 μg at 10 weeks of age followed by repeated immunizations every 10 weeks protected 8 out of 9 mice, while one single immunization at 15 weeks of age protected 50%. To my knowledge, this is the best result so far in the BALB-neuT model. This year the same group investigated how much of the Her2/*neu* gene/protein that was needed in order to obtain protection (579). They could show that in BALB/c mice the N-terminal 310 amino acids, i.e. about 50% of the extracellular domain, were dispensable for protection against TUBO. In BALB-neuT mice, a plasmid encoding amino acids 311-689 and lacking the first 310 amino acids resulted in 36% protection, which was at the same level as the protection afforded by a plasmid encoding the extracellular and transmembrane domains (amino acids 1-689). An interesting strategy was explored by Sloots and colleagues in 2008 (580). They constructed a plasmid encoding a fusion protein between the extracellular domain of human CTLA-4 and the N-terminal 222 amino acids of human Her2/*neu*. The idea was that such a fusion protein would be targeted specifically to DCs[68]. In BALB/c mice, this plasmid protected the majority of mice against a transplantable human Her2/*neu*-positive tumour, which was much better than a plasmid encoding the 222 amino acids of Her2/*neu* without CTLA-4. A plasmid containing the N-terminal region of the rat Her2/*neu* gene in place of the human gene completely protected against TUBO outgrowth in BALB/c mice, while the plasmid carrying the human gene had no effect at all. In BALB-neuT mice, i.m. immunization in combination with electroporation at 10 and 12 weeks of age with the plasmids containing rat Her2/*neu* in combination with CTLA-4 induced an antibody response but no CTL response. A delay in tumour outgrowth was observed, whereas the plasmid

[68] CTLA-4 binds to B7 molecules (CD80/86) on the surface of DCs.

carrying the human Her2/*neu* gene had no effect on tumour development, implying that there was no cross-reactivity between the human and rat protein.

Jacob *et al* (581) made a comparison of Her2/*neu* immunization in BALB-neuT and Her2 Tg mice respectively, and also studied cross-reactivity between human and rat Her2/*neu*. In all cases they immunized i.m. with a plasmid encoding the extracellular and transmembrane domains of the rat Her2/*neu* protein together with a plasmid encoding GM-CSF. They started off by immunizing normal BALB/c and C57Bl/6 mice and plasmids encoding either human or rat Her2/*neu* induced cross-reactive T cells, but only species-specific antibodies in both strains. In BALB/c mice, rat Her2/*neu* completely protected against a challenge with rat Her2/*neu*-expressing cells and partially against human Her2/*neu*-positive cells, while human Her2/*neu* protected against both human and rat Her2/*neu*-positive tumour cells. Due to the lack of cross-reactive antibodies the cross-reactivity must have been mediated by cross-reactive T cells. In Her2 Tg mice, no or only weak antibody responses were induced with either human or rat Her2/*neu* (tolerance), whereas T cells were induced following rat but not human Her2/*neu* immunization. In BALB-neuT mice, rat but not human Her2/*neu* induced antibodies and caused a delay in tumour onset. However, Her2/*neu* of both rat and human origin induced T cells in these mice, but depletion of $CD8^+$ T cells had no impact on tumour outgrowth indicating that the anti-tumour response was mediated by antibodies rather than T cells.

6.7.4.1.2.3 *Studies in neu*-N and FVB/*neu*-T mice

In *neu*-N mice, DNA encoding the full-length human Her2/*neu* protein and given i.m. protected 60% of mice against tumour outgrowth when repeated immunizations starting at week 12 were performed (582). Another study showed that DNA encoding full-length rat Her2/*neu* and given i.d. could delay tumour outgrowth of a transplantable tumour in these mice (583). The effect was improved by co-administration of plasmids encoding CD80 and CD86, but no mouse was ever protected against outgrowth. The FVB/*neu*-T strain has been more frequently used in tumour vaccination studies. In 1998, Amici *et al* (584) showed that three i.m. injection with a plasmid encoding the full-length rat Her2/*neu* protein starting at week 6 delayed tumour onset but only protected 20% of the mice completely. Two years later plasmids encoding either full-length rat Her2/*neu*, the extracellular domain only or the extracellular and transmembrane domains in combination were compared in FVB/*neu*-T mice (585). The plasmid encoding the extracellular and transmembrane domains conferred the best protection although there were no major differences. Co-injection of a plasmid encoding IL-12 improved the effect further but only a minor fraction of the mice were in fact completely protected despite repeated immunizations. A few years later the same group compared different routes of DNA inoculation and i.m. immunization, especially in combination with electroporation, was superior to both i.d. (without electroporation) and gene gun immunization (586).

6.7.4.2 Viral vectors

Another way of improving uptake of DNA is to make use of viruses since they have a natural propensity not only to break into cells but also to have their genes expressed. Several viruses have been tested and the first to be used in gene therapy was retrovirus (587, 588). Retroviruses only infect dividing cells and the viral genome integrates into the host chromosome. This results in long-term gene expression but the risk is integration into an unfavourable site. In this thesis retroviral vectors will not be discussed, and I will instead focus on a viral vector, which has been used extensively for vaccination against Her2/*neu*, and that is the one based on adenovirus.

6.7.4.2.1 Adenoviral vectors

The most commonly used viral vectors today in clinical trials are those based on adenoviruses (589). Adenovirus is a non-enveloped virus with a double-stranded linear DNA molecule. In contrast to retroviruses, they can infect both dividing and non-dividing cells. Their cell tropism is wide since the primary receptor, coxsackie-adenovirus receptor (CAR) (590), has a very broad tissue distribution. The DNA of adenovirus does not integrate into the cellular genome (591), which results in short-term gene expression and no risk of interaction with cellular genes. Gene therapy and different gene therapy vectors is definitely not my area of expertise, but from what I can understand vectors based on adenoviruses were in the beginning of the gene therapy era made replication-deficient by deleting certain genes. Now replication-competent adenoviruses are back in business. This might seem risky but note that adenoviruses are widely spread in the population, at least certain serotypes (592-594), generally without any high pathogenicity[69]. The high seroprevalence has however a major disadvantage, pre-existing immunity, which considerably reduces the effect of systemic inoculation. In the section below there are some examples of pre-clinical testing of adenoviral vectors encoding Her2/*neu* including strategies to overcome the broad tissue tropism and pre-existing immunity.

6.7.4.2.1.1 ADENOVIRUSES ENCODING HER2/*NEU*

An interesting feature of adenovirus that I came across when writing this thesis is the fact that the adenovirus E1A protein suppresses the Her2/*neu* promoter and induces apoptosis of Her2/*neu*-positive cells (595-597). Several studies have shown that infection of Her2/*neu*-expressing ovarian carcinoma and osteosarcoma cell lines with an adenovirus decreased growth rate, increased cell death and down-regulated Her2/*neu* expression as well as slowed down the growth rate of established tumours in mice (598-600). Clinical trials have been conducted in breast, ovarian and head-and-neck cancer patients using the E1A gene in complex with liposomes, which has resulted in E1A gene expression in most patients but down-regulation of Her2/*neu* expression was obtained only in a minority (601-603).

[69] Adenoviruses commonly cause infections in e.g. the eyes and respiratory tract but although troublesome, this can hardly be defined as "high pathogenicity".

In order to limit the cell tropism to Her2/*neu*-expressing cells, trastuzumab has been linked to the surface of adenovirus particles (604, 605). At the same time binding to CAR has been removed by deleting amino acids in the CAR binding region (605), or alternatively, the induction of neutralizing antibodies has been reduced via conjugation of PEG[70] to the viral surface (604). To circumvent the problem of pre-existing immunity, Peruzzi *et al* (606) used an adenovirus based on the chimpanzee serotype 3 expressing the extracellular and transmembrane domains of rat Her2/*neu*. In BALB-neuT mice, this adenovirus induced a cellular as well as a humoral response although slightly weaker than for a virus based on human adenovirus serotype 5. When studying the effect on spontaneous tumour development, two immunizations at weeks 8 and 9 protected 60% of mice against outgrowth, which was slightly lower than for the human adenovirus (80% protection).

Gallo and colleagues (311) showed that immunization with an adenovirus expressing human Her2/*neu* induced comparable titres of anti-Her2/*neu* antibodies in BALB/c and BALB-neuT mice, which cross-reacted with rat Her2/*neu*. However, no comparison with the titres induced by an adenovirus-expressing rat Her2/*neu* was made. An adenovirus expressing human Her2/*neu* induced much stronger cellular responses to human Her2/*neu* than electroporated DNA and in both cases a very weak cross-reactivity to the rat Her2/*neu* protein was seen. Immunization with DNA encoding rat Her2/*neu* induced a stronger cellular response to the rat protein both in BALB-neuT and BALB/c mice compared to DNA encoding human Her2/*neu*, although the response was weaker in BALB-neuT than BALB/c in line with tolerance of the former to rat Her2/*neu*. When comparing adenoviruses expressing human and rat Her2/*neu*, in both BALB/c and BALB-neuT mice the rat gene induced a more potent cellular response to the rat protein. Accordingly, with regard to cellular responses against rat Her2/*neu* following vaccination of BALB-neuT mice, adenovirus is definitely superior to DNA and rat Her2/*neu* is superior to human Her2/*neu*. Immunization of BALB-neuT mice with an adenovirus expressing human Her2/*neu* at 6 and 9 weeks of age delayed tumour outgrowth slightly but did not protect mice against tumour development.

In a subsequent study (607) they used a codon-optimized version of the rat Her2/*neu* gene and could once again show that in BALB-neuT mice adenovirus induced a stronger cellular response to the rat Her2/*neu* protein compared to electroporated DNA. In addition, they showed that adenovirus induced a stronger humoral response as well. Since adenoviruses induce neutralizing antibodies a heterologous prime-boost regimen including e.g. adenovirus for priming and DNA for boosting could be of value. Various combinations of adenovirus and DNA were tested, but addition of DNA to adenovirus did not improve the cellular or the humoral response. Immunization with a rat Her2/*neu* encoding adenovirus at weeks 8 and 11 almost completely protected mice against spontaneous tumour outgrowth, while DNA immunization had a weak effect only.

[70] *PEG* is short for *polyethylene glycol* and is frequently used to inhibit protein-protein interactions.

Immunization of BALB-neuT mice with adenovirus at weeks 10 and 12 delayed tumour outgrowth and completely protected 40%.

Park *et al* (608) showed that four immunizations starting between week 7 and 9 with an adenovirus expressing the extracellular and transmembrane domains of rat Her2/*neu* protected all BALB-neuT mice against tumour outgrowth. In BALB/c mice, one single immunization was sufficient to completely protect against a TUBO challenge. Depletion of $CD4^+$ T cells at the time of immunization abrogated the effect, while depletion of $CD8^+$ T cells had no effect in either BALB/c or BALB-neuT mice. Interestingly, simultaneous depletion of $CD4^+$ and $CD8^+$ T cells during the effector phase had no effect in BALB/c mice and so was the case for NK cells. BALB/c mice deficient in β2m were protected, but not mice lacking B cells. They could also show that mice lacking the antibody receptors FcγRI/III were protected meaning that the anti-tumour effect was not mediated by ADCC. Depletion of $CD4^+$ T cells 36 hours after immunization had no effect, i.e. those cells were only needed at the time of immunization. Three years later the same group examined the effect of this adenovirus on already established tumours (609). Complete regression was seen when immunization was performed up to 15 days after challenge with TUBO cells. In accordance with their previous study, $CD4^+$ and $CD8^+$ T cells had no effect during the effector phase, while $CD4^+$ T cells were required during the immunization phase. Complete regression was again seen in β2m- and FcγRI/III-knockout mice, but abrogated in mice lacking B cells.

Wang and co-workers (610) demonstrated that immunization of BALB/c mice with an adenovirus expressing full-length rat Her2/*neu* protected 90% of mice against a challenge with A2L2 tumour cells (rat Her2/*neu*-positive) when given before challenge, but no effect was obtained when given after challenge. They also tested a prime-boost immunization strategy. They could show that if DNA encoding rat Her2/*neu* was given two days after challenge followed by boosting with an adenovirus on days 9 and 16, no effect on the growth of the primary tumour was seen, but this approach still inhibited pulmonary metastases and prolonged survival.

6.7.5 Summary of preclinical testing of Her2/*neu* vaccines

6.7.5.1 Transplantable tumour models

Human and rat Her2/*neu* have 89% amino acid identity, while the corresponding figure for rat and mouse Her2/*neu* is 94%. Very interestingly, to the best of my knowledge not a single study has been performed in mice where the murine Her2/*neu* gene/protein has been used for vaccination, but rather the human or rat counterpart has always been utilized. Such studies are somewhat artificial since tolerance does not have to be broken. In a prophylactic setting, complete or close to complete protection against either human or rat Her2/*neu*-expressing tumours has been obtained with a range of vaccines and vaccination protocols (512, 563, 565, 567-571, 578, 581, 608, 610). In

some systems the anti-tumour effect has been antibody dependent (570, 581, 608), and in others antibody independent (567, 569, 581).

Of relevance for papers II and III in this thesis is cross-reactivity between human and rat Her2/*neu*. Sloots *et al* (580) showed that it was possible to obtain complete protection against TUBO outgrowth with a plasmid encoding a fusion protein between CTLA-4 and the N-terminal region of rat Her2/*neu*, while a plasmid containing human Her2/*neu* did not have any effect. The latter construct induced antibodies against human Her2/*neu*, which did not cross-react with rat Her2/*neu*, and the induced CTLs showed weak cross-reactivity with neu_{66-74}. In the study by Jacob and colleagues (581), a DNA vaccine expressing the extracellular and transmembrane domains of rat Her2/*neu* in combination with a plasmid expressing GM-CSF induced complete protection against rat and partial protection against human Her2/*neu*-expressing tumour cells. When a plasmid expressing human Her2/*neu* was used, complete protection against both human and rat Her2/*neu*-positive tumours was obtained. The plasmid encoding rat Her2/*neu* induced a T cell response to both neu_{66-74} and $Her2_{63-71}$. Although the response was much stronger to the former than the latter, this indicated that T cells to rat Her2/*neu* were cross-reactive with human Her2/*neu*. The same pattern was seen when mice were immunized with human Her2/*neu*, i.e. T cells strongly reactive with $Her2_{63-71}$ were detected, which cross-reacted with neu_{66-74}. In this study only species-specific antibodies were induced. Accordingly, despite the fact that both Sloots *et al* (580) and *Jacob et al* (581) used a plasmid for immunization, as well as the same mouse strain, the T cell response was very different with regard to cross-reactivity.

6.7.5.2 BALB-neuT mice

BALB-neuT mice represent a very aggressive model of spontaneous tumour development. To obtain protection against tumour outgrowth, repeated immunizations (311, 513, 541, 546, 571-577, 580, 606-608) as well as adjuvants (546, 572-574, 576, 577, 581) are generally required and in most cases the immunizations must be commenced at an early stage of tumour progression (311, 513, 541, 546, 571-573, 606, 608). In addition, usually only delayed onset of tumour outgrowth is seen, while complete tumour protection is a rare event (311, 513, 541, 571, 572, 574, 577, 579-581, 606, 607). However, no rule without exception and Curcio *et al* showed in 2008 (578) that one single i.m. injection of a rat Her2/*neu*-expressing plasmid followed by electroporation at 15 weeks of age completely protected as many as 50% of the mice.

In all studies where the immune mechanisms responsible for tumour inhibition have been studied, the anti-tumour response has been mediated by antibodies (547, 548, 572, 574, 575, 580, 581, 608). In some studies, in addition to antibodies, dependence on IFNγ has been observed (547, 548, 575), but what I can recall I have not come across one single study where the anti-tumour response has been convincingly shown to be mediated by CTLs. The reason for this overwhelming effect of antibodies is not clear to me. One possibility could be that it is easier to break B-cell tolerance than T-cell tole-

rance in these mice. It should also be noted that in most studies either DNA or an adenovirus has been used for immunization and in those cases endogenous production of the Her2/*neu* protein is obtained. The protein is then exposed on the cell surface resulting in an antibody response.

With regard to cross-reactivity between human and rat Her2/*neu* in this model, Sloots and colleagues (580) observed a delay in tumour outgrowth with a plasmid containing a fusion gene between CTLA-4 and the N-terminal region of rat Her2/*neu*, while the human gene had no effect at all. The rat gene induced antibodies but no CTLs against rat Her2/*neu*. Human Her2/*neu* did not induce either antibodies or CTLs cross-reactive with rat Her2/*neu*. Jacob *et al* (581) showed that a DNA vaccine expressing rat Her2/*neu* caused a delay in tumour onset, while a plasmid carrying the human gene had no effect. Here rat Her2/*neu* induced T cells reactive with *cells* expressing the rat Her2/*neu* protein, but not with neu_{66-74}, indicating deletion in the thymus of T cells recognizing this immunodominant epitope. These T cells did not cross-react with human Her2/*neu*. Immunization with the human gene induced T cells to $Her2_{63-71}$, which cross-reacted with rat Her2/*neu*. Exactly as in the transplantable tumour model, these two studies showed divergent results considering the specificity/cross-reactivity of the induced T cells, despite the use of a DNA vaccine in both studies. In 2005 Gallo and collaborators (311) could show that an adenovirus expressing human Her2/*neu* induced antibodies as well as T cells to human Her2/*neu*, which cross-reacted with the rat Her2/*neu*. However, a much stronger response to rat Her2/*neu* was seen with an adenovirus expressing the rat instead of the human protein. The human Her2/*neu*-expressing adenovirus induced a minor delay in tumour outgrowth but no side-by-side comparison with a rat Her2/*neu*-expressing adenovirus was made.

6.7.5.3 Other interesting observations

The addition of chemotherapy to a Her2/*neu* vaccine can in fact *increase* the T-cell response (544). Tumour vaccination can be effective against Her2/*neu*-expressing cells that are resistant to both chemotherapy and tyrosine kinase inhibitors (567). The immune mechanisms responsible for preventing tumour outgrowth (prophylactic setting) are not necessarily the same as those responsible for eradicating an already established tumour (therapeutic setting) (568).

6.7.6 Clinical trials with Her2/*neu* vaccines

The peptide $p369\text{-}377^{71}$ in the human Her2/*neu* protein was originally described by Fisk *et al* in 1995 (611) as an immunodominant peptide recognized by ovarian cancer-specific CTL lines. It is restricted to the human MHC class I molecule A2, which is expressed in roughly 50% of the population. This peptide has become the most studied Her2/*neu*-derived peptide both *in vitro* and *in vivo*. In 1998, it was shown that the peptide induced CTLs that recognized peptide-pulsed target cells but not tumour cells (612). In 2000, autologous DCs loaded with E75 induced CTLs in patients (613). The

following year potential Th epitopes, which also contained A2 binding motifs, increased the number of peptide-specific A2-specific T cells in most patients and these cells could lyse tumour cells (614). One study showed that the E75 peptide given together with GM-CSF induced short-lived CTL responses with low magnitude (615) and another investigation showed induction of CTLs that lysed tumour cells (616). In 2002, immunization with potential Th epitopes together with GM-CSF evoked T-cell immunity against peptides as well as the native Her2/*neu* protein. Epitope spreading[72] was seen in most patients and significantly correlated with protein-specific T-cell immunity (617). In another study, immunization with Th peptides and GM-CSF resulted in T-cell immunity in 25% of the patients and antibody responses in 50%, but none developed protein-specific immunity (618). In 2004, vaccination with pools of different peptides was attempted and 60% of the patients developed antibodies against at least one of the peptides and fewer to the native Her2/*neu* protein (619). The same year immunization with the intracellular domain in combination with GM-CSF yielded T-cell as well as antibody-immunity to this domain in most patients. The dose did not affect the magnitude of the response but the response developed more rapidly in patients receiving a high dose (620). Most clinical trials have been phase I trials and clinical responses have not been published. However, in 2005, E75 administered in combination with GM-CSF in breast cancer patients induced CTLs that lysed tumour cells and appeared to reduce the recurrence rate (621). Several phase I trials with plasmids or viruses encoding Her2/*neu* are ongoing or have been completed (622).

[71] This peptide is commonly referred to as E75.

[72] *Epitope spreading* means that an immune response is evoked against peptides not included in the vaccine.

7 VIRUS-LIKE PARTICLES

7.1 A VIRUS LOOKALIKE

According to a PubMed search the term *virus-like particles* was first used 60 years ago when particles looking like viruses were seen in papillomas of the skin (623). Today we normally use the term for a recombinant particle that looks like a virus, i.e. has a morphology that is very similar or even identical to the natural viral particle, but lacks the viral genome. In figure 13, a schematic cross-section of a polyomavirus particle is shown to the left where the circular genome is enclosed by the capsid composed of VP1, VP2 and VP3. To the right is a virus-like particle and as one can see it is identical except for the absence of the viral DNA molecule, i.e. when watching it from the outside it looks exactly as a viral particle. To be absolutely correct, this is not completely true, at least not for papillomavirus since it has been shown that parts of L2 stick out on the outside of the capsid (624), but whether the same is the case for polyomavirus is not clear to me.

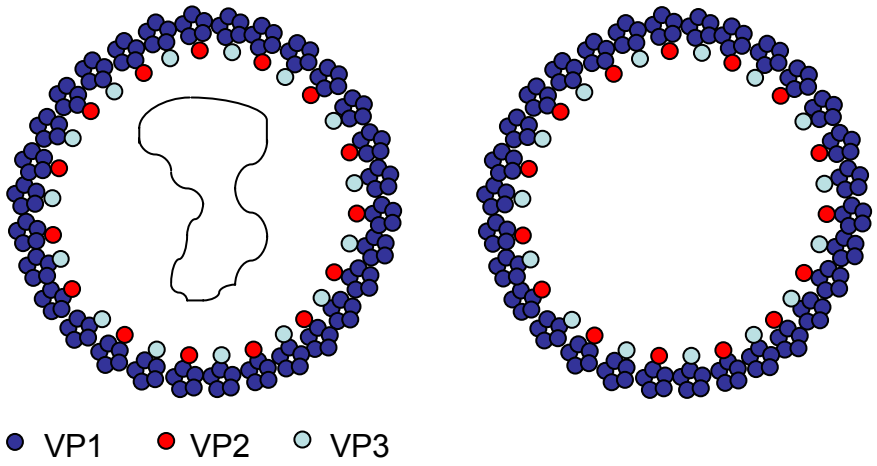


Figure 13. Schematic overview of the structure of a polyomavirus particle (left). A double-stranded DNA molecule is surrounded by a capsid composed of pentamers of VP1 and monomers of VP2 and VP3, respectively. To the right is a virus-like particle made up of VP1, VP2 and VP3.

A polyomavirus VLP can either be composed of all three capsid proteins (625) as in the figure above, VP1 only, VP1 and VP2 or VP1 and VP3. The formation of VLPs has been shown for many polyomaviruses such as MPyV (626), SV40 (627, 628), JCV (629), BKV (630), LPV (631), HaPyV (632), goose hemorrhagic polyomavirus (633) and avian polyomavirus (634). As mentioned in chapter 3, several other viruses can also assemble into VLPs, among them are human papillomavirus (259-261), parvovirus (274, 275) and hepatitis B virus[73] (278).

[73] As mentioned in chapter 3, VLPs based on hepatitis B virus do not resemble the native virus but are sometimes called VLPs anyhow.

7.2 THE MAGIC SELF-ASSEMBLY OF VIRUS-LIKE PARTICLES

Let us again put the clock back and see how it all started. In the end of the 1970s, Brady and Consigli (635) showed that it was possible to denaturate MPyV particles into VP1, VP2 and VP3 and thereafter renaturate the VP1 monomers into pentamers. A few years later, Bob Garcea's group in Boston published an article in Cell describing the self-assembly of MPyV VP1 into viral capsids (636). Garcea and his colleagues produced MPyV VP1 in bacteria (E. coli) with the purpose of obtaining crystals for structural studies. They did not obtain any crystals but instead observed something very exciting. First of all the VP1 monomers spontaneously formed pentamers in the bacteria and no monomers at all were seen. Additionally, to their surprise, at high ionic strength[74] these pentamers automatically assembled into structures looking like normal viral capsids, what we today refer to as VLPs. Accordingly, they could show that neither VP2/VP3 nor posttranslational modifications[75] were required for capsid assembly (636). Figure 14 shows schematically the self-assembly of polyomavirus VLPs. Five years later, the same group could show that in eukaryotic cells (Sf9)[76], expression of VP1 resulted in self-assembly into VLPs, which was in contrast to E. coli where only pentamers were formed. Formation of VLPs from various viruses was later shown to occur also in yeast (638).

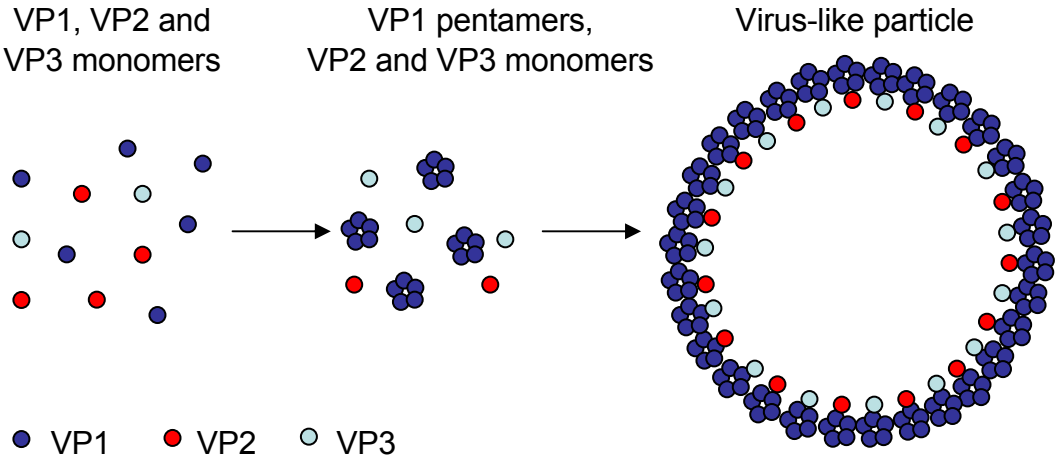


Figure 14. The process of self-assembly of a virus-like particle from monomers of VP1, VP2 and VP3.

7.3 EFFECTS ON DENDRITIC CELLS

In 2001, Lenz *et al* showed that VLPs from HVP16 as well as bovine papillomavirus (BPV) induced phenotypic maturation of DCs from mice (upregulation of MHC I and II, CD40, CD80 and CD86), while the same was not seen with VLPs from BKV and

[74] *Ionic strength* is a function of the concentration and charge of all ions in a solution. In the publication by Salunke *et al* (636) mainly high concentations of NaCl (1 M) were used, and in the case of NaCl ionic strength is equal to the ion concentration.

[75] Prokaryotic cells such as bacteria are (almost) unable to carry out post-translational modifications in contrast to eukaryotic cells. MPyV VP1 is normally modified by acetylation as well as phosphorylation (637).

[76] Sf is an insect (*Spodoptera frugiperda*) and cells from this insect are commonly used for protein production.

JCV (639). One concern is that they studied the effect of VLPs from *human* and *bovine* viruses on *murine* cells. However, a few years later it was shown that VLPs based on MPyV did not induce upregulation of maturation markers on either human or on murine DCs, but did induce production of IL-12 (164). Contrary to these results, two studies have shown that VLPs from MPyV induce maturation of human (640) and murine DCs (641), respectively, and it has also been demonstrated twice that HaPyV VLPs induce maturation of human DCs (640, 642). A study from our group showed that VLPs from MPyV did not induce upregulation of maturation markers on human or murine DCs, although increased production of IL-12 was observed (643).

7.4 THE VERSATILE USE OF VIRUS-LIKE PARTICLES

Virus-like particles can be used for several purposes, although few of the applications have reached the clinic so far. Most well-known are the VLP vaccines against infection with HPV for the prevention of cervical cancer (644, 645). These vaccines have turned out to be very efficient and starting 2010 there will be general vaccination of girls in Sweden. The concept is easy. If we simplify a little, since a virus particle of HPV and an HPV-VLP look identical from the outside, the immune system is unable to distinguish between these two and will respond to the VLP (almost) in the same way as it will respond to the natural virus. Virus-like particles can also be used as carriers of DNA or proteins and these topics are further described below.

7.4.1 Gene therapy using polyomavirus virus-like particles

As mentioned in chapter 6.7.4.1 the efficacy of uptake of naked DNA into cells is low and different vehicles are being tested to increase the uptake. Since viruses efficiently deliver DNA into cells, it was assumed that VLPs could do the same. Virus-like particles based on different viruses have therefore been exploited as gene therapy vectors, but only VLPs based on various polyomaviruses will be described in this chapter.

With regard to packaging of DNA into VLPs, several different methods have been used. Most common is the one denoted *osmotic shock* (630, 646-650). It is known that infection with MPyV yields two different forms of particles, empty particles lacking DNA, and full particles containing DNA (651). In 1975, it was shown that if polyomavirus DNA was mixed with such particles, DNA formed complexes with empty particles but not with full particles. The authors suggested that the empty capsids contained some site with affinity for DNA, and that this site was attached to or was a part of the inner surface of the capsid (652). Today we now that this suggestion was completely correct. However, the attached DNA was not protected against the action of DNase (652). This strategy was further developed by Barr *et al* (653), who lowered the ionic strength by adding H_2O and in that way obtained DNA/capsid complexes where the DNA was protected against DNase. It is assumed that the lower ionic strength leads to formation of pores in the capsid facilitating uptake of DNA, however I do not think this has ever been proven. Another method of DNA packaging is disassembly/reassembly. The VP1 protein binds calcium (654), and calcium is important

for stability of the capsid (655-657). If calcium is removed, e.g. by adding EGTA, and disulfide bridges are broken simultaneuosly, e.g. by addition of DTT, the capsid will disassemble into pentamers (658). These pentamers can thereafter be reassembled by addition of calcium and removal of EGTA (658). To pack DNA using this procedure, the capsid is first disassembled into pentamers, thereafter DNA is added and finally the pentamers are reassembled into a capsid and it was assumed that the DNA would be enclosed by the capsid in this way. Other packaging methods include mixing of intact capsids with DNA at room temperature (630, 659, 660), and sonication of the capsids prior to mixing with DNA (661). A relevant question is which method is the most efficient, and also if the DNA is really packaged within the capsid, and we will come back to these issues soon.

Gene transfer using VLPs from polyomavirus was first shown by Forstová and colleagues in 1995 (646). They showed that VLPs[77] based on MPyV VP1 could package DNA and transfer it into cells *in vitro* more efficiently than both calcium phosphate and lipofectin. When treating the VLP/DNA complexes with DNase, only 5-30% of the DNA was protected but on the other hand DNA associated with VP1 pentamers was not protected at all. Later the same group showed that these VLP/DNA complexes were able to transfer DNA also into cells *in vivo* (mice) and expression persisted for at least seven weeks, and this was superior to naked DNA (650). In 1999, it was shown that VP1 from JCV self-assembled into VLPs in E. coli (in contrast to the study from Garcea's group where only pentamers were formed in E. coli (636)) and could transfer DNA into cells *in vitro* (649). Now scientists started to study the association between VLPs and DNA in more detail. What happened actually during the osmotic shock procedure? Were the entire DNA molecules really packaged within the VLPs? Stokrová and colleagues (662) studied the VLP/DNA complexes by electron microscopy and found that in most preparations *circular* DNA was not at all associated with VLPs! Rather it was a mixture of VLPs and DNA without any physical connection between them. However, the authors speculated that this could be due to disruption of the complexes during preparation for electron microscopy, i.e. an electron microscopy artefact. For *linearized* DNA, the situation was somewhat different. Most of the DNA interacted with the VLPs, but the VLPs were mainly located at both ends of the DNA chain forming something that the authors called a "skipping-rope"-like arrangement, i.e. only a minor fraction was actually enclosed by the VLPs. Interestingly, despite this low degree of encapsidation, up to 2.5 kb of DNA was protected against the action of DNase. In line with these results, another study showed that a large proportion of the DNA was not packaged within the VLPs but was in solution and it appeared that the majority of the VLP/DNA complexes formed large, loosely associated structures as observed by electron microscopy (648). During the years as a PhD student I have several times been asked how the VLP/DNA complexes are internalized by cells. Are they taken up exactly in the same way as natural polyomaviruses or is there a different way of uptake? We have not studied this issue ourselves, but it was investigated in this

study and something exciting was observed. When cells were treated with neuraminidase to destroy sialic acid (see chapter 2.6.3), the expression of the gene present in the VLP/DNA complexes was lost while, interestingly, the VLPs were still taken up by the cells. The authors speculated that there were two routes of uptake of VLP/DNA complexes, one that was sialic acid dependent and resulted in gene expression, and one that occurred independent of sialic acid but did not result in expression of the gene (648). The same group showed in another study the same year (660) that DNA could be transferred also into cells *ex vivo* (cultures of rabbit corneas) more efficiently than naked DNA, as well as in *in vivo* both into immunodeficient (nude) and immunocompetent mice, and also this was better than DNA alone. Our group showed simultaneously that gene transfer into cells *in vivo* was more efficient with VLP/DNA complexes than with naked DNA and that this was more efficient in immunodeficient (both T- and B-cell deficient) mice than in normal mice (661). The same year (2000) it was again shown that VLPs (formed *in vitro* after expression in E. coli) transferred DNA into cells *in vitro* better than naked DNA (647). Touzé *et al* (630) showed that VLPs based on BKV VP1 could transfer DNA into cells *in vitro*. Gene transfer using linear DNA was always better than that observed with circular DNA. They also performed another interesting experiment, where they compared the different DNA packaging methods. Surprisingly, they showed that simple mixing of DNA and VLPs was the most efficient method for a linearized plasmid with regard to both gene transfer and protection against the action of DNase. Another study showed that VLP/DNA complexes were not superior to naked DNA *in vitro* although it was superior *in vivo* (mice) (659). Recently, it was shown that also VP1 from HaPyV can form VLPs spontaneously both in insect cells (Drosophila) and E. coli and can transfer DNA into cells *in vitro* (663).

7.4.2 Chimeric virus-like particles

For the purpose of variation, I think this is a perfect time to switch to Greek mythology for a short while. You might know that in Greek mythology there is a monstrous creature that is made up of elements from different animal species. The body is derived from a lion, the tail terminates in the head of a snake and on the back of the creature, there is the head of a goat. This monster is called *chimera*. In accordance with this, a *chimeric virus-like particle* (cVLP) is a mixture of proteins from various sources, i.e. a VLP to which an unrelated protein has been linked. There is no strict definition of a cVLP but it can be a VLP with several different modifications as follows:

- A protein or a part of a protein is fused to the major capsid protein and thereby either exposed on the surface or located within the VLP.
- A protein or a part of a protein is fused to one of the minor capsid proteins and in that way located ("hidden") within the VLP.
- A protein is bound to the outer surface of an already assembled VLP.

[77] At this time the VLPs were referred to as *pseudocapsids*.

A few words should be said about the difference between protein insertions into the major compared to one of the minor capsid proteins. For cVLP from HPV it has been shown that it is not possible to insert more than 60 amino acids into L1 without disrupting the integrity of the VLP (664). However, many more amino acids can be fused to the L2 protein and still intact particles can be obtained, but the disadvantage is that the *number* of inserted peptides/proteins is lower. If the protein would be inserted into L1 it means that as many as 360 copies would be present in each cVLP (corresponding to the number of L1 molecules in a VLP), whereas maximum 72 copies would be found in each cVLP if the protein would be inserted into L2 (corresponding to the possible number of L2 proteins in each VLP). For polyomaviruses this issue has to the best of my knowledge never been studied but one could assume that the situation is similar.

Below is an odyssey of the field of cVLPs. I have decided to include not only cVLPs based on polyomavirus, but also on papillomavirus, parvovirus and hepatitis B virus since studies on cVLPs from these viruses often have walked hand in hand. One could argue that I am unfair to e.g. the HIV field since VLPs/cVLPs based on HIV have been completely omitted but for space purposes and the fact that HIV VLPs/cVLPs are of minor importance for cVLPs based on polyomavirus they will not be discussed.

7.4.2.1 The early years

We must go back at least to the middle of the 1980s to find the first steps in the field of cVLPs, although this was long before the designation chimeric virus-like particle had been introduced. Rather these particles were referred to as *hybrid virus-like particles* or *hybrid pseudo-particles*. In 1986, it was shown that an epitope from poliovirus could be inserted into HBsAg and that these particles assembled into 22 nm spherical particles (665) as described in chapter 3.3. When inoculated into mice, these particles induced neutralizing antibodies to poliovirus. However, it would take almost another decade before the field received an impetus. Several studies from the middle of the 1990s made use of the same approach but used different parvoviruses in place of hepatitis B. Peptides from various viruses were inserted either into VP1 or VP2 and following inoculation into mice, a T and/or B cell response was induced to the inserted peptides (666-668). Later it was also shown that such cVLPs carrying a $CD8^+$ T cell epitope from LCMV induced a strong and long-lasting CTL response in mice, which protected against a lethal challenge with LCMV (669). Soon papovavirus scientists realized that it would be of value to investigate if also cVLPs from papillomavirus could work in the same way. In 1997, Müller and colleagues (664) deleted the C-terminal part of HPV16 L1 and inserted 60 amino acids of the E7 protein instead, and showed that this L1-E7 protein could assemble into cVLPs, which could enter cells *in vitro*. One of the main reasons for constructing cVLPs based on HPV was that L1 VLPs can prevent primary HPV infection, but have no therapeutic effect. By constructing a cVLP one could possibly kill two birds with one stone, since such a vaccine would be capable not only of preventing primary infection but also of treating an already established infection.

7.4.2.2 Chimeric parvovirus virus-like particles

Some interesting data has accumulated over the years from studies on cVLPs from various parvoviruses. In 1998, it was shown that parvovirus cVLPs with peptides inserted into VP2 were in fact presented on both MHC class I and II (670). Another finding of importance for the work presented in this thesis was the one by Sedlik *et al* (671), who analyzed if priming with "empty" parvovirus VLPs, i.e. VLPs without insertion of a foreign peptide/protein, would affect subsequent boosting with cVLPs. They could show that if boosting was performed 15 days after priming with "empty" VLPs, cytotoxicity was lost completely, while if boosting was done one month or more after VLP priming, as strong cytotoxic response was seen as if no priming with "empty" VLPs had been performed. The same group later showed that *in vivo*, DCs were the only APCs capable of efficiently processing cVLPs based on porcine parvovirus and *in vitro* DCs incubated together with cVLPs presented the foreign epitope to MHC class I-restricted T cells, i.e. cross-presentation occurred. They also analyzed DCs in more detail and could show that the cVLPs induced DC maturation and that several subtypes of DCs were involved in uptake and presentation (672).

7.4.2.3 Chimeric papillomavirus virus-like particles

In the papillomavirus field, cVLPs based on both human and bovine papillomavirus 1 (BPV-1) have been investigated. In 1998, Peng *et al* (673) showed that cVLPs composed of L1 of BPV-1 fused to either an E7 or an HIV gp160 epitope induced cytotoxicity, and in the case of the E7 peptide also protection against outgrowth of E7-expressing tumour cells. The same year John Schiller's group at NCI/NIH could protect mice against outgrowth of E7-expressing tumour cells by vaccinating with a cVLP made up of HPV16 L1 and a fusion protein between L2 and the full-length E7 protein (674). Protection was seen also in MHC class II-deficient mice, as well as in mice depleted of NK cells, but not in β2m or perforin knockout mice. They concluded that the anti-tumour response was mediated by MHC class-I restricted CTLs and that cross-priming occured. This means that it had now been shown both for cVLP based on parvo- and papillomavirus that they could be cross-presented. In line with these results, the following year Schäfer and colleagues (675) showed that cVLPs made up of a fusion protein between C-terminally truncated HPV16 L1 and an E7 epitope induced cytotoxicity as well as tumour protection in mice. In continuation, Nieland and colleagues (676) demonstrated that similar cVLPs carrying a different peptide protected against outgrowth of the P815 mastocytoma cell line, while immunization with the naked peptide in combination with CFA induced tolerance. Liu *et al* (677) developed the concept further and inserted five epitopes after one another in BPV-1 L1 (one CTL epitope of HPV16 E7, three HIV CTL epitopes, and one HPV16 E7 B-cell epitope) and induced cytotoxicity against all CTL epitopes and antibodies against the B-cell epitope. One concern with the use of VLPs/cVLPs is the presence of pre-existing antibodies in humans already infected with the virus as well as the high antibody titres induced by VLPs/cVLPs. This issue has already been touched upon by Sedlik *et al* as described above (671), and was also analyzed by Da Silva and colleagues (678). Mice were

inoculated with HPV16 VLPs lacking foreign antigen or injected with hyperimmune serum[78] and thereafter immunized with cVLPs carrying E7. Mice with pre-existing antibodies were not protected from outgrowth of an E7-expressing tumour. Later they tried to bypass this inhibition in different ways, and showed that this was not possible using the mucosal route for immunization, however *heterologous boosting*, i.e. inoculation with cVLPs from HPV of a different species, resulted in both an improved T-cell response and better tumour protection (679).

In 2002, it was shown that both antibody and T-cell responses against HIV-1 gp120 peptides were induced with a cVLP based on L1 of BPV-1 (680), and the same year Wakabayashi and co-workers published a very interesting study (681). They compared cVLPs composed of a fusion protein between L1 and an E7 peptide with cVLPs made up of L1 and a fusion protein between L2 and the same E7 peptide. As mentioned above, the number of E7 peptides should be much higher in the former cVLP and this cVLP was also more efficient. However, the yield obtained from the insect cells was considerably higher for the L1-L2/E7-cVLP than for the L1/E7-cVLP. Later it was shown that cVLPs carrying an HIV-1 gp41 peptide induced antibodies that neutralized HIV-1 *in vitro* (682). Last year another strategy was presented. Chimeric VLPs composed of HPV16 L1 and a fusion protein between L2 and the adjuvant mLTK63[79] induced higher antibody titres and stronger proliferation of splenocytes compared to the corresponding VLPs without the adjuvant (683). Chimeric papillomavirus virus-like particles used for the purpose of breaking B-cell tolerance have also been investigated and this is described in chapter 7.4.2.5.

7.4.2.4 Chimeric polyomavirus virus-like particles

What about cVLPs from polyomaviruses then? Most of this work has been performed using cVLPs from HaPyV. The first study I have found is from the year 2000. An epitope from hepatitis B virus was inserted into VP1 of HaPyV in order to expose it on the surface and antibodies against the epitope were induced in mice (684). The same group has since published a couple of papers where they have shown the same thing for insertions of the nucleocapsid protein from Puumala hantavirus (685), peptides from carcinoembryonic antigen (686), as well as MUC1 (642). In 2004, a study with high relevance for the work presented in this thesis was published. It had earlier been shown that the 45 C-terminal amino acids of VP2 were sufficient in order to obtain stable binding to the inner surface of the VP1 pentamer (687). Abbing and colleagues (688) now made use of this information and attached green fluorescent protein (GFP) and methotrexate, respectively, to the 47 C-terminal amino acids of MPyV VP2 and could show encapsidation of both these substances into cVLPs. Importantly, although maybe not very surprisingly, they showed that GFP and methotrexate were internalized into cells *in vitro*. A schematic figure of a cVLP based on polyomavirus is shown in figure 15.

[78] Serum containing high titres of antibodies against the L1 protein.
[79] *mLTK63* is a modification of the E. coli heat-labile enterotoxin.

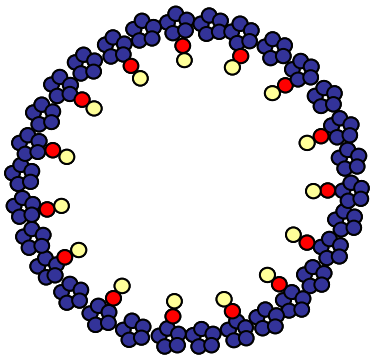


Figure 15. Schematic figure of a cVLP based on polyomavirus where the foreign protein is "hidden" within the cVLP. A foreign protein (yellow) is fused to VP2 (red). VP1 in blue.

7.4.2.5 Breaking B-cell tolerance

Chimeric virus-like particles can also be used to break B-cell tolerance and most of this work has been done in John Schiller's lab at the NCI/NIH. Their work was in turn founded on a study published in Science in 1993 (689). In this study, it was suggested that the arrangement of an antigen affects B-cell responses to the antigen. This suggestion was based on very simple but clever experiments, which showed that it is much easier to break B-cell tolerance in mice if the antigen is densely packed and repetitively organized compared to if the antigen is present in a less organized way (689).

The first study on this topic was published in 1999 (690). A peptide of 16 amino acids of CCR5[80] was inserted into an immunodominant site of L1 from BPV-1. As a result, 360 copies of the CCR5 peptide were exposed on the surface of the cVLPs in a highly organized fashion. Following inoculation into mice, autoantibodies to CCR5 were induced proving that these VLPs could be used to break B-cell tolerance. They then attempted to create additional constructs with other self-peptides in the same way, but were unsuccessful since the chimeric L1 protein did not always assemble into cVLPs. Instead they used another approach where they produced ordinary VLPs from BPV-1, followed by biotinylation of the surface and at the same time created a fusion protein between streptavidin and various peptides of TNF-α (691). This fusion protein was linked to the biotinylated VLPs via a streptavidin-biotin interaction. Again, autoantibodies to the self-peptide were raised in mice, which at least partially protected against collagen-induced arthritis. Later they showed that this ability was not restricted to VLPs from papillomavirus, but was as efficient for VLPs from BKV indicating that acute activation of DCs was not required for breaking B-cell tolerance (692). Using the same approach, they could also break B-cell tolerance in mice to the Aβ peptide, which forms amyloid deposits in the brain of patients with Alzheimer's disease (693). In yet another study, they immunized macaques with cVLPs coated with a simian CCR5 peptide and this resulted in induction of anti-CCR5 autoantibodies. In macaques immunized this way, following challenge with simian immunodeficiency virus, viral loads were lower than in unimmunized controls (694). Recently they also showed that this

[80] *CCR5* (chemokine receptor 5) is probably the most important co-receptor for HIV.

strategy can break B-cell tolerance in mice transgenic for hen egg lysozyme to which B-cell tolerance is maintained by anergy (695).

7.4.3 Anti-tumour studies in primates

We have already touched upon studies in primates when discussing B-cell tolerance, but in this chapter we will focus on studies aimed at inducing a response against tumour antigens using cVLPs. In 2001, Kaufmann *et al* (696) investigated if it was possible to raise a human T-cell response by *in vitro* vaccination. When cVLPs composed of a fusion protein between L1 and an E7 peptide were added to human peripheral blood lymphocytes, the cells started to proliferate. Dendritic cells loaded with cVLPs induced strong proliferation of autologous T cells as well, and the T cells killed target cells in a cytotoxicity assay. A few years later the same group published the results of a randomized double-blind placebo controlled trial of women with HPV16 positive CIN2/3[81] using the same cVLPs (697). As expected, high titres of antibodies against L1 were induced. Interestingly, 38% of the vaccine recipients raised antibodies against E7 despite the fact that this peptide should be located inside the cVLPs. Clinical responses were seen in some patients, although without statistical significance compared to placebo recipients. Recently, two studies in donkeys and horses, respectively, have been published (698, 699). The purpose of both studies was to treat so called equine sarcoids, which are fibroblastic skin tumours where BPV-1 and possibly also BPV-2 have been implicated in the pathogenesis. The animals were vaccinated with cVLPs composed of a fusion protein between BPV-1 L1 and E7. In the study with donkeys, no difference with regard to E7 antibodies was seen between vaccinated and control animals but there was a tendency, although not a statistically significant difference, for a better response in the vaccinated group (698). In 5 out of 12 horses antibodies against E7 were detected although at low titres, whereas all except one developed high anti-L1 antibody titres. Some clinical responses were seen but due to the lack of a control group, these responses were difficult to interpret (699).

[81] *CIN* is an abbreviation for *Cervical Intraepithelial Neoplasia*, which is a premalignant lesion of the cervix.

8 AIMS OF THE THESIS

- To investigate if the major capsid protein, VP1, from murine pneumotropic virus (MPtV) can self-assemble into virus-like particles (VLPs) and, if so, characterize these VLPs with regard to receptor, cell tropism and uptake.

- To examine if VLPs from MPtV can be used in immunotherapy as a complement to VLPs from murine polyomavirus (MPyV).

- To investigate if VLPs based on MPyV and MPtV carrying the tumour antigen Her2/*neu* can protect against outgrowth of transplantable as well as spontaneously arising Her2/*neu*-expressing tumours.

- To optimize the effect of immunization with VLPs carrying Her2/*neu*, and investigate if these protein carrier VLPs can have a therapeutic effect as well as induce immunological memory.

- To study the immune mechanisms responsible for tumour rejection upon immunization with VLPs carrying Her2/*neu*.

9 RESULTS AND DISCUSSION

Development and characterization of MPtV-VLPs (Paper I)

Rationale

Filled with enthusiasm about other groups' promising results, and encouraged by excellent results from our own group (see chapter 7.4.1), we were almost convinced that polyomavirus VLPs could be very potent vectors in gene therapy. Not only did MPyV-VLPs transfer DNA into cells with high efficacy, the lack of pre-existing immunity in humans was a great advantage over e.g. adenoviruses and VLPs based on human polyomaviruses. With these promising results on hand, how come we did not explore MPyV-VLPs any further, but instead decided to turn our focus to a completely different virus that only a pocketful of scientists had showed interest in? Was this a typical move by a scientist, leaving the known and setting off into the unknown, or was there perhaps a clever rationale behind this decision? The cell tropism of MPyV is very broad as described in chapter 2.6.1, while studies had indicated that the tropism of MPtV was mainly restricted to endothelial cells, especially in the lungs, spleen, liver and intestine. As pointed out in chapter 2.7.2, it was unknown at this time whether this apparently restricted tropism was due to limited distribution of the receptor, or to the fact that the virus DNA could be expressed only in certain cell types. Therefore it would also be of great value to try to determine the cell tropism of MPtV-VLPs. If the tropism would be limited to only one or a few cell types, MPtV-VLPs could potentially be used to target genes (as well as proteins as we will see later) to these restricted cell types. A second reason for studying MPtV was that inoculation of both MPyV and MPyV-VLPs into mice, and probably also into humans, induced high antibody titres against the VP1 protein (661, 700, 701). This could potentially reduce the effect of subsequent inoculations with the same VLP in e.g. prime-boost gene or immune therapy. Consequently, if VLPs could be formed by MPtV and such VLPs would not cross-react serologically with MPyV-VLPs, then these two VLPs could be used in combination. Although VLPs from a bunch of polyomaviruses had been obtained when we initiated this study in 2000 (chapter 7.1), it was not known at this time whether VP1 from MPtV could also self-assemble into VLPs.

The construction of a recombinant baculovirus

Before we go into the details of paper I, I think it could be a good idea to briefly describe how VLPs are produced. As mentioned in chapter 7.2, proteins can be expressed in various systems, most commonly in E. coli, yeast and insect cells. Whereas bacteria are easy and cheap to work with and can also produce large amounts of protein in short time, they are prokaryotic organisms and thereby unable to carry out many of the post-translational modifications performed by mammalian cells. Insect cells, on the other hand, are more complicated and time-consuming to work with, but have the advantage of being eukaryotic and can perform post-translational modifications in a manner

similar to mammalian cells. Since MPtV is normally expressed in eukaryotic cells, we decided to use insect cells for expression in order to obtain proteins as identical as possible to the native proteins.

To start with, the gene/s of interest, in this case VP1 of MPtV, is ligated into a special plasmid. The gene is placed under the control of a promoter expressed in the late phase of the viral life cycle to make it possible to produce proteins that are toxic to cells. The next step is to transfect insect cells[82] with this plasmid together with linearized baculovirus DNA. A baculovirus is an enveloped insect cell virus, which contains double-stranded *circular* DNA. Within the insect cell the circular plasmid will be opened up and recombination will occur through ligation of the linear plasmid to the linearized baculovirus DNA. This will result in a recombinant circular baculovirus DNA molecule containing the VP1 gene. Another ingenious feature of this system is the fact that the plasmid contains elements necessary for a viable baculovirus to form. This means that self-ligation of the baculovirus DNA should result in a non-viable virus. Following the creation of a recombinant baculovirus DNA molecule, the baculovirus proteins will be expressed and new viruses will be formed, all containing a viral genome expressing the foreign VP1 gene. These baculoviruses will be secreted into the medium and by simply harvesting the supernatant a solution containing a large number of viruses can be obtained. The supernatant is then used to infect fresh insect cells, which will start to express the genes carried by the baculovirus. If VP1 is expressed, it will (hopefully) self-assemble into pentamers, which in turn will form capsids (VLPs) that can finally be purified.

Expression of MPtV-VP1 and self-assembly into VLPs

Using the above procedure, we could show that MPtV-VP1 was expressed at high levels in insect cells. This was of course a good start, but did VP1 self-assemble into VLPs? The proteins contained within the insect cells were purified and separated on the basis of density. Following ultracentrifugation, a distinct band was visible in the centrifuge tubes. Analysis by SDS-PAGE revealed that this band contained particles composed of a protein with a size of around 45 kDa, the approximate size of VP1, and electron microscopy showed that the particles had a morphology similar to polyomavirus. MPtV-VLPs had seen the light of day.

Cell tropism, receptor characteristics and cellular uptake

The next step was to determine which cells MPtV-VLPs were able to attach to. Would binding be restricted to endothelial cells or would binding specificity possibly be broader than that? The VLPs were labelled with a fluorescent dye and added to cells. VLPs that did not attach were washed away and the fluorescent intensity was analyzed by FACS analysis as an indicator of cell attachment. Logically, we started by analyzing

[82] In papers I and II Sf9 (Spodoptera frugiperda) cells were used. We later got problems with these cells and changed to Sf21 cells (used in papers III and IV). Strangely enough, Sf9 are derived from Sf21 and not the reverse.

binding to endothelial cells and as expected a strong signal was seen. Thereafter a collection of murine, human and simian cells were analyzed and the VLPs bound to all cell types tested. Of great importance for papers II-IV was the fact that the VLPs attached to both murine and human DCs.

We could subsequently show that MPtV-VLPs did not hemagglutinate erythrocytes in contrast to MPyV-VLPs, indicating that sialic acid was not a part of the receptor. We then attempted to characterize the receptor in further detail. As described in chapter 2.8.1.1, binding of SV40 to cells had been shown to depend on expression of MHC class I, but we could show that MPtV-VLPs attached as efficiently to MHC class I-negative as to MHC class I-positive cells. Cells were thereafter treated with neuraminidase to remove sialic acid from the cell surface, but this did not inhibit MPtV-VLP attachment either, which, in agreement with the lack of hemagglutination, indicated that MPtV-VLPs did not bind to sialic acid. Interestingly, neuraminidase treatment in fact increased binding and it is likely that sialic acid blocks the receptor for MPtV-VLPs. Trypsin and papain treatment abolished binding to all cell types tested with the exception of GMK cells, to which binding was unaffected. The sensitivity of the receptor to trypsin/papain indicates that the receptor, or at least part of it, is a protein. Why GMK behaved differently is unknown and only speculations can be made, and one such speculation could be that MPtV-VLPs bind to different receptors on various cell types.

The results were so far very promising. VLPs were formed and could attach to a range of cell types including DCs. However, attachment is not equal to internalization and attachment without internalization would be worthless. Following incubation of VLPs with cells, trypsin was added in order to remove all VLPs still bound to the surface and thereafter the fluorescent intensity was analyzed. The intensity was almost as high after addition of trypsin as before, indicating that most VLPs had in fact been taken up by the cells. The same phenomenon was shown for a variety of cell types, although only the results for endothelial cells are presented in paper I. As a complement to FACS analysis, uptake was also studied by fluorescence microscopy and it was obvious that at 4^{o}C the VLPs attached to the cell membrane and entered the cells at 37^{o}C (unpublished). We also showed that MPtV-VLPs and MPyV-VLPs did not cross-react serologically, implying that they could be used in combination in a prime-boost regimen.

Gene transfer

So far so good, but one question still remained: Could MPtV-VLPs transfer DNA into cells? A plasmid carrying the gene for enhanced green fluorescent protein (EGFP) was added to sonicated VLPs, whereafter the VLP/DNA complexes were added to cells and the expression of EGFP was studied by fluoresence microscopy. We could show that not more than 0.03% of the cells expressed EGFP *in vitro*, which was much lower than what other groups had reported for e.g. MPyV-VLPs. However, we could not obtain a higher transfection efficacy with DNA complexed with MPyV-VLPs (unpublished). In

addition, we used different packaging methods but in spite of our best efforts there were no improvements in gene transfer (unpublished). We also inoculated mice with MPtV-VLP/DNA complexes. Three weeks later presence of DNA in various tissues was analyzed by PCR[83], and VLP/DNA complexes were superior to naked DNA with regard to persistence *in vivo*. Nonetheless, to perform gene therapy with MPyV-VLPs and MPtV-VLPs was possibly not optimal, neither from our experience or experience from other groups. Our focus now turned to VLPs as carriers of proteins (cVLPs), which seemed to be a much more promising approach.

Conclusions

We showed that MPtV-VP1 could self-assemble into VLPs and that these VLPs could be produced at high levels in insect cells. The MPtV-VLPs attached to all cell types tested indicating that the receptor was broadly distributed. Importantly, they attached to DCs of both murine and human origin and the lack of cross-reactivity with MPyV-VLPs indicated that these two types of VLPs could be used in combination. The reason for the pronounced difference between *uptake* of VLP/DNA complexes into cells and *gene expression* is not completely clear. If we go back to chapter 2.6.4 and the current knowledge of intracellular trafficking of MPyV, a speculation could be that the VLP/DNA complexes are internalized, but that only a minor fraction of the DNA molecules is delivered into the nucleus. However, if the pathways of intracellular trafficking of MPtV are similar to those of MPyV has to the best of my knowledge never been studied. We also know from chapter 7.4.1 that the DNA is probably only partially protected by VLPs and therefore possibly degraded before the VLP/DNA complexes have reached the cells. As noted above, when paper I was published VLPs as a vector in gene therapy was a chapter on the decline, and instead our interest had changed to cVLPs for use in tumour immunotherapy. A major advantage of cVLPs compared to VLPs as a vector in gene therapy, is the fact that there is no need for delivery of cVLPs into the nucleus. What is required is uptake into cells, degradation and subsequent presentation on MHC molecules and in that respect, cVLPs had the potential to be very efficient.

[83] A disadvantage of this kind of *in vivo* study is that you can only prove *presence* of DNA, i.e. whether the DNA is *expressed* is unknown. In theory, the DNA could be located in the extracellular space although this is not very likely since the DNA should be destroyed in that case.

Vaccination against transplantable and spontaneously arising Her2/*neu*-expressing tumours (Papers II, III and IV)

Rationale

Now we put all our efforts into the creation of cVLPs as an anti-cancer vaccine. We decided to switch back to MPyV for a while but MPtV will return later. As reviewed in chapter 7.4.2, several studies had shown successful anti-tumour responses with cVLPs carrying various tumour antigens. We decided to use the antigen Her2/*neu* (chapter 6.4) due to its attractiveness as an antigen in immunotherapy, and the fact that it was already being used in the lab next door.

After the tumour antigen had been selected, we had do decide if it should be attached to the major capsid protein, VP1, or one of the minor capsid proteins, VP2/3. As depicted in chapter 7.4.2, only short peptides could be inserted into VP1 without affecting the structure of the VLP. For HPV-VLPs it had been shown that not more than 60 amino acids could be inserted into L1 (664). Accordingly, if we would like to insert a short peptide, VP1 would be appropriate, while for a large fragment of Her2/*neu*, VP2 or VP3 should be used. At this time the data on immunodominant Her2/*neu* epitopes on H-2^d background was sparse and we decided to use a large fragment. The Her2/*neu* gene was cleaved on the middle and two fusion gene products were created, one between VP2 and the extracellular and transmembrane domains of human Her2/*neu* (amino acids 1-683), and the other between VP2 and the intracellular human Her2/*neu* domain (amino acids 684-1255).

Vaccination against a transplantable human Her2/*neu*-positive tumour

We constructed two recombinant baculoviruses both expressing MPyV-VP1 and either of the two fusion proteins above. When expressing these proteins in insect cells, we could not obtain any VLPs with the fusion protein containing the intracellular domain. The reason for this is unknown, but we assumed that the intracellular domain was toxic to the cells. Fortunately, VLPs were obtained for the other construct but…at this stage we could not be sure that these VLPs in fact contained the fusion protein. Could it be that VP1 had self-assembled into VLPs alone, leaving out the fusion protein? To our satisfaction, the VLPs indeed contained human Her2/*neu* although the number of fusion proteins per VLP was not more than approximately 3. We could also show that these VLPs, in the following referred to as $Her2_{1\text{-}683}$PyVLPs, could enter cells (unpublished).

Some people were wondering if the Her2/*neu* protein was really located within the VLPs or were parts of it exposed on the outside? I am unable to give a thorough answer to this question but when analyzing the VLPs by electron microscopy, they looked completely normal. For other cVLPs people have tried to bind mAbs to the foreign protein and an inability to bind would indicate localization of the protein within the VLPs (164). This might seem as a clever strategy, but reliable results from such experiments are difficult to obtain, since the experiments require the use of an antibody

that specifically binds to the exposed parts of the protein. As we will come back to, these cVLPs did not seem to induce an antibody response *in vivo* against Her2/*neu*, which also indicated that Her2/*neu* was not exposed.

We initially tested the efficacy of $Her2_{1-683}$PyVLPs in a classical tumour transplantation model. Mice were immunized subcutaneously (s.c.) with $Her2_{1-683}$PyVLPs, and approximately two weeks later[84] challenged with human Her2/*neu*-positive tumour cells (D2F2/E2), and followed for tumour outgrowth. We were satisfied to see that the mice were consistently and completely, or almost completely, protected against outgrowth.

Vaccination against spontaneously arising rat Her2/*neu*-positive tumours in BALB-neuT mice

Critical reviewing is a cornerstone in the education of a PhD student. Although the results from the transplantable model were successful, they were easy to criticize due to the use of heterologous antigens (human and rat Her2/*neu*) in a mouse, meaning that tolerance did not have to be broken. It would therefore be of great value to investigate if these cVLPs could also prevent tumour outgrowth in a tolerant model. At a conference about Her2/*neu* vaccines at Karolinska Institutet we listened to a presentation by professor Guido Forni from Turin, Italy. He presented results on vaccination in a rat Her2/*neu* transgenic mouse model he and his colleagues had created, the BALB-neuT mice (chapter 6.5). We discussed with him in a coffee break and this was the starting point of a very fruitful collaboration. When the BALB-neuT mice were given one single vaccination with $Her2_{1-683}$PyVLPs at 6 weeks of age[85], 5 out of 6 mice were completely protected against breast carcinoma development. We also attempted to give one single immunization of $Her2_{1-683}$PyVLPs when the mice were either 10 or 14 weeks old, and while immunization at 10 weeks induced a delay in outgrowth, immunization at 14 weeks resulted in no effect at all.

When paper II was published in 2005, the anti-tumour effect seen after $Her2_{1-683}$PyVLPs immunization at 6 weeks of age was the most potent effect seen so far in this model following one single immunization. With regard to the immune mechanisms mediating the effect, the cellular response was not studied at all at the time, however no anti-Her2/*neu* antibodies were detected. We concluded that the effect on tumour inhibition was most likely mediated by a T cell response. Probably T cells were raised against human Her2/*neu*, which cross-reacted with rat Her2/*neu* expressed by the tumour cells. As we will see in paper IV, it is possible that it was much more intricate

[84] The observant reader might have noticed that the time point of tumour cell challenge was not very consistent, i.e. sometimes 13 and sometimes 21 days after immunization in paper II. This inconsistency was due to the human factor. In the following, we have challenged the mice 14 days after immunization as a rule.

[85] If you have sharp eyes you might have noticed that the BALB/c mice were immunized by the s.c. route, while the BALB-neuT mice by the i.p. route. The purpose was to use s.c. immunization also in the BALB-neuT mice, but due to miscommunication the cVLPs were given i.p. in these mice. However, since this route turned out to work very well, we decided not to change routes..

than that. This was, and to the best of my knowledge still is, the only study where an antibody-independent response has been demonstrated in BALB-neuT mice. As I have written in chapter 6.7.5.2, I have no really good explanation for the fact that cell-mediated anti-tumour responses are uncommon in this model, but a speculation could be that few protein-based vaccines have been tested.

Planning of further studies

Often in research, a few answers lead to a multitude of new questions. We had shown that $Her2_{1-683}$PyVLPs were efficient not only in a non-tolerant transplantable tumour model, but also in mice that were tolerant to rat Her2/*neu*. We now sat down at the table and brainstormed about further studies that would be interesting to perform, and decided to focus on the following questions:

- Was the remarkable response seen in BALB-neuT mice due to the use of human rather than rat Her2/*neu* (as mentioned in chapter 6.5 BALB-neuT mice are generally considered tolerant to rat but not human Her2/*neu*)? In relation to this, we were interested in the cross-reactivity between human and rat Her2/*neu* in the transplantable model.
- Could the response following immunization be improved even further by the addition of adjuvants, or by loading DCs with cVLPs?
- Would it be possible to obtain protection also in a therapeutic setting, i.e. when immunizations were performed after tumour cell challenge?
- Could the cVLPs induce immunological memory?
- What components of the immune system mediated tumour protection? We could not detect any anti-Her2/*neu* antibodies following cVLP immunization, but could demonstrate an IFNγ response by ELISPOT in the transplantable model (see below). This indicated, but did not prove, that a cellular response mediated protection, and we aimed at studying this response in more detail.

MPtV-VLPs as carriers of Her2/*neu*

An attempt was made to construct MPtV-VLPs carrying the extracellular and trans-membrane domains of either human or rat Her2/*neu*. In order to reduce the size of the fusion protein and hopefully be able to get incorporation of a larger number of fusion proteins in each VLP, we decided to use the 48 C-terminal amino acids of VP2/3 in-stead of the full-length VP2 protein. This region contains the VP1 binding domain and the strategy had earlier been shown by Abbing *et al* (688) to be successful (chapter 7.4.2.4). To be able to study the cross-reactivity between human and rat Her2/*neu*, four new cVLPs were constructed, two of them based on MPyV and two on MPtV. Two cVLPs contained the extracellular and transmembrane domains of human Her2/*neu*, and two contained the same domains of the rat protein. All four cVLPs were success-fully constructed as demonstrated by electron microscopy (unpublished). However, the reduction in size of the VP2-Her2/*neu* protein did not increase the number of fusion proteins per cVLP. It is possible that the main factor limiting the number of molecules

is not the size of the protein, but rather the presence of the hydrophobic transmembrane domain of Her2/*neu*. We now assume that this domain may affect the structure of the cVLPs, and that the incorporation of a large number of fusion proteins into the same cVLP would result in cVLP disruption.

Considering protection against D2F2/E2, we observed that cVLPs containing human Her2/*neu* protected against tumour outgrowth regardless of if they were based on MPyV or MPtV. Accordingly, we had now proved that also cVLPs based on MPtV could be used as an anti-tumour vaccine. Then the cVLPs containing rat Her2/*neu* were tested against a rat Her2/*neu*-expressing tumour (TUBO), and also in this case statistically significant protection against outgrowth was seen with cVLPs from both MPyV and MPtV[86].

Cross-reactivity between human and rat Her2/*neu*

cVLPs based on MPyV did not confer protection against a tumour expressing the heterologous Her2/*neu* protein, i.e. such cVLPs carrying human Her2/*neu* did not protect against rat Her2/*neu*-positive TUBO cells and vice versa. Interestingly, VLPs from MPtV did confer protection also against tumours expressing the heterologous Her2/*neu* protein, although not as efficiently as against tumours expressing the homologous protein. This indicated that MPtV-VLPs were most likely more efficient than those based on MPyV, and we therefore decided to primarily use cVLPs from MPtV in future studies. Cross-reactivity was only analyzed by studying the effect on tumour outgrowth, and cross-reactive T cells were not analyzed by ELISPOT. As reviewed in chapter 6.7.5.1, few studies have investigated cross-reactivity in transplantable tumour models between human and rat Her2/*neu*, and in these studies complete cross-reactivity, partial cross-reactivity, and no cross-reactivity[87] have all been shown.

Potentiation of the immune response and protection in the therapeutic setting

So far we had only studied the effect of cVLPs in a prophylactic setting. Although it is a theoretical possibility to vaccinate all people at high risk of developing Her2/*neu*-positive cancer, this is not a very likely scenario. It is more probable that immunization will be used to treat already established tumours and to prevent relapse. Therefore we wanted to explore if cVLPs could be effective also when given *after* tumour cell challenge. MPtV-VLPs carrying human Her2/*neu* given alone were efficient when inoculated two days before but not two days after challenge. In an attempt to improve the response we added the adjuvant CpG. A combination of cVLPs and CpG protected against tumour outgrowth when given up to 6 days after challenge and in some cases already established tumours regressed. Another study from our group showed that the

[86] When I wrote this part of the thesis I got somewhat puzzled when I looked at table III in paper III. I realized that VLPs from MPyV carrying rat Her2/*neu* (neuMPyVLPs) only protected 53% of mice against TUBO outgrowth. Could this really be a statistically significant result with a p-value of less than 0.001? I recalculated and there is no doubt about the fact that this value is correct.

[87] *Complete cross-reactivity* in this context means complete protection against outgrowth of a tumour expressing the heterologous antigen. In line with this, *no cross-reactivity* means no protection at all.

immune response could be enhanced by loading DCs with $Her2_{1-683}$PyVLPs (643). In the same study experiments were performed to analyze the potential of prime-boost-immunization with cVLPs based on MPyV and MPtV, respectively. Contrary to our expectations, it was here shown that one inoculation of MPyV-VLPs lacking Her2/*neu* did not reduce the efficacy of a subsequent immunization with MPyV-VLPs carrying Her2/*neu*. Although only 5 µg rather than 50 µg VLPs were used, reduced but still high anti-VP1 antibody titres were induced. Accordingly, the antibody response induced by VLPs seems to affect repeated immunizations much less than initially anticipated. One possibility could in fact be that the antibodies induced by inoculation of empty VLPs coat the Her2/*neu*-cVLPs and promote the uptake by immune cells.

Immunological memory

Next we wanted to explore if MPtV-VLPs containing human Her2/*neu* could induce immunological memory. Protection against outgrowth of D2F2/E2 was obtained when immunization with cVLPs alone was performed at least up to 6 weeks before challenge and, if combined with CpG, at least up to ten weeks. Accordingly, the cVLPs induced a long-term immunological response.

Returning to the BALB-neuT mice

When BALB-neuT mice were immunized with cVLPs based on either MPyV or MPtV carrying rat Her2/*neu*, a delay in tumour outgrowth was seen when the cVLPs were given at either 6 or 10 weeks, but none of the mice were completely protected. This means that there was a considerable difference between cVLPs containing human and rat Her2/*neu*, respectively. However, the results showed that tolerance to rat Her2/*neu* could be broken with cVLPs carrying the rat protein. Only a few studies have looked at cross-reactivity between human and rat Her2/*neu* in these mice. As written in chapter 6.7.5.2, two studies have shown that DNA vaccines encoding human Her2/*neu* had no effect at all (580, 581), and one study showed a minor delay in outgrowth with an adenovirus encoding the human protein (311).

Effects on dendritic cells

As mentioned in chapter 7.3, VLPs based on some viruses induce maturation of DCs. A previous study from our group (643) had shown that MPyV-VLPs did not induce up-regulation of maturation markers on the surface of either human or murine DCs, although a slightly increased production of IL-12 was observed. As noted above, we had seen a somewhat better immunization effect with cVLPs based on MPtV than on MPyV, at least in the transplantable tumour model. Could it be that the superior efficacy of MPtV-VLPs was due to the fact that they induced maturation of DCs? However, we did not see any difference between the two VLPs, i.e. MPtV-VLPs induced production of IL-12 by DCs to the same extent as MPyV-VLPs, and no upregulation of maturation markers could be demonstrated.

The complex nature of the anti-tumour response (Papers II, III, IV)

The potent anti-tumour effect of cVLPs in both the transplantable and transgenic models prompted us to ask which immune mechanisms that were responsible. The main question is, or at least was when the studies presented in paper IV were initiated: Was the response mediated by $CD8^+$ CTLs or by antibodies? However, as we have discussed before, nature is rarely black or white, but rather makes up a sophisticated complexity, which can only partially be understood by experiments. This is of course interesting, but at the same time makes life difficult for me as a scientist as we will see in this very last part of the thesis. We will here try to delineate the immune mechanisms responsible for the anti-tumour effect and will focus on the transplantable tumour model.

We can start by summarizing what is known about the anti-tumour response induced by cVLPs. In 1998, it was shown that cVLPs from HPV could prevent tumour outgrowth in normal and in MHC class II-deficient mice, but not in mice lacking perforin (674). Furthermore, depletion of NK cells did not impair tumour protection. This indicated that the anti-tumour response occurred independently of $CD4^+$ T cells and NK cells. Studies have also shown that VLPs based on parvovirus can be cross-presented and cross-prime T cells (670, 672). On the basis of this, one would maybe assume that the same holds true for polyomavirus VLPs, but this should not be taken for granted, since the viruses use different receptors and thereby might affect the immune system in different ways. We have earlier touched upon the fact that an antibody response against Her2/*neu* should not be induced due to the localization of Her2/*neu* within the VLPs[88]. However, as mentioned above one clinical trial has actually demonstrated that antibodies can be raised (against E7) (697). It was also shown that intact HPV16 L1/L2-E7 cVLPs did not induce an antibody response against E7 in mice, but anti-E7 antibodies were raised following denaturation of the cVLPs (674).

The approach

In order to obtain as comprehensive understanding as possible of the underlying immune mechanisms, we decided to study this issue from several angles. By ELISPOT we could measure the IFNγ response either to the Her2/*neu* protein (paper II) or to single immunodominant $CD8^+$ T-cell epitopes (papers III and IV). Anti-Her2/*neu* antibodies were measured by FACS analysis through incubation of sera with Her2/*neu*-positive cells. To in more detail analyze the effect of various cell types, both BALB/c and C57Bl/6 mice were depleted of $CD4^+$ T cells, $CD8^+$ T cells and NK cells either alone or in combination. As as complement, tumour rejection was also studied in knockout mice on C57Bl/6 background.

[88] An antibody response could in fact be induced if parts of Her2/*neu* are exposed on the outside of the cVLPs, or if the cVLPs are broken down before they reach the cells of the immune system.

Her2/*neu*-specific CD8$^+$ T cells, but no anti-Her2/*neu* antibodies were demonstrated

By ELISPOT analysis we could demonstrate a Her2/*neu*-specific IFNγ response by splenocytes from immunized BALB/c mice. The immunodominant epitopes on H-2^d background had not yet been identified when paper II was in preparation, and for stimulation in the ELISPOT assay we used cells instead (Her2/*neu*-positive D2F2/E2 cells and, as negative control, Her2/*neu*-negative D2F2 cells) or Her2/*neu*-cVLPs. A Her2/*neu*-specific response could be shown by both kinds of stimulation. Thanks to the work by Gallo *et al* (311) the immunodominant CD8$^+$ T-cell epitopes are now known and could be used in papers III and IV. Logically it might seem more appropriate to use stimulatory agents that contain the full-length Her2/*neu* protein (or at least the extracellular and transmembrane domains that are included in the cVLPs) instead of single peptides. However, both with cells and cVLPs we had a problem with high background levels. An IFNγ response was raised not only against the Her2/*neu* protein but also against the VP1 and VP2 proteins. Remember that each cVLP contains approximately three VP2-Her2/*neu* fusion proteins, implying that >98% of the protein contents of a cVLP is made up of VP1. With regard to D2F2 and D2F2/E2 cells, these cell lines produce IFNγ making the measurement of "Her2/*neu*-specific" IFNγ extremely tricky. Using immunodominant epitopes, we obtained a much lower background and the ELISPOT analysis was more reproducible. An IFNγ response specific for Her2/*neu* CD8$^+$ T-cell epitopes could be demonstrated for Her2/*neu*-cVLPs based on both MPyV and MPtV. Notably, the response was stronger for cVLPs from MPtV than from MPyV, which is in line with the results on protection against tumour outgrowth. No anti-Her2/*neu* antibodies were ever detected despite repeated measurements.

When analyzing the effect of a vaccine, the most important issue is of course whether it can confer protection *in vivo* against the tumour, virus, bacteria or whatever you immunize against. Nevertheless, the analysis of a response *in vitro* has several advantages. Different responses/cell types can be separated from each other and the effect of a vaccine can be analyzed more rapidly compared to using e.g. effect on tumour outgrowth as an end-point. However, the correlation between immune responses *in vitro* and *in vivo* is often poor. This was something we observed when using CpG. While the addition of CpG improved the anti-tumour response we could not detect an increased number IFNγ-secreting cells. Moreover, different *in vitro* assays have different advantages as well as disadvantages. A drawback of ELISPOT is that you analyze *production* of a particular cytokine, in our case IFNγ, and by contrast to e.g. a cytotoxicity assay you do not measure *killing* of target cells. Additionally, it is not clear which cells that produce the cytokine contrary to e.g. intracellular cytokine staining, where secretion specifically by CD8$^+$ T cells can be studied. However, if using immunodominant CD8$^+$ T-cell epitopes, an IFNγ response should be strongly indicative of a CD8$^+$ T-cell response (Th1 response), rather than some kind of "general" IFNγ production. Nonetheless, could it be that the used epitopes are also CD4$^+$ T-cell epitopes and in that case a mixture of a CD4$^+$ and a CD8$^+$ T cell response would be detected? Since the Her2$_{63\text{-}71}$

and neu_{66-74} peptides are short (9 amino acids) it is unlikely that they would be capable of stimulating a $CD4^+$ T-cell response (MHC class II peptides are normally 10-15 amino acids as discussed in chapter 4.2.1). Importantly, Gallo and colleagues in fact showed that these peptides were not immunodominant $CD4^+$ T-cell epitopes (311). Thus, we can conclude that we were indeed analyzing a Her2/*neu*-specific $CD8^+$ T-cell response.

Depletion of $CD4^+$ and $CD8^+$ T cells as well as NK cells

Ultimately, to truly investigate which parts of the immune defence that are involved in Her2/*neu*-induced tumour rejection, we have to analyze the effect on tumour outgrowth *in vivo*. This can be performed in mice after depletion of specific subsets of immune cells, or in knockout mice lacking certain components of the immune system. In paper IV, we started by depleting BALB/c mice of $CD4^+$ and $CD8^+$ T cells, either alone or in combination, by injecting mAbs against these cell populations. When studying the effect of depletion of $CD4^+$ and $CD8^+$ T cells during the immunization phase[89], mice were protected in the absence of either subset but not after depletion of both subsets simultaneously.

As a complement to table 1 in paper IV, the results of the two experiments with depletion of immune cells in the effector phase are shown in the diagrams in figure 16. Following depletion in the effector phase, there was still a statistically significant protection against tumour outgrowth compared to unimmunized mice after depletion of either $CD4^+$ or $CD8^+$ T cells, although $CD4^+$ T-cell depletion impaired the response to some extent. Why depletion of $CD4^+$ T cells resulted in reduced tumour protection is not clear to me. It is also intriguing that the tumours in these mice developed at a very late time point in both experiments. Whether these late occuring tumours had lost expression of Her2/*neu* was not studied. Depletion of $CD4^+$ and $CD8^+$ T cells in combination resulted in loss of tumour protection, at least there was no statistically significant difference compared to unimmunized mice. When looking at the diagrams in figure 16, one could argue that the effect was not lost completely, since there is indeed a gap between the curves for mice depleted of $CD4^+/CD8^+$ T cells and unimmunized mice. As we will return to, it is possible that NK cells had some impact, and in the second experiment (lower diagram) the curves representing unimmunized mice and mice depleted of all three subsets are more or less overlapping. The fact that it is impossible to achieve 100% depletion might also explain some of the differences. In both experiments selective depletion of NK cells impaired, although not completely abrogated protection.

[89] Sometimes referred to as the *induction phase*.

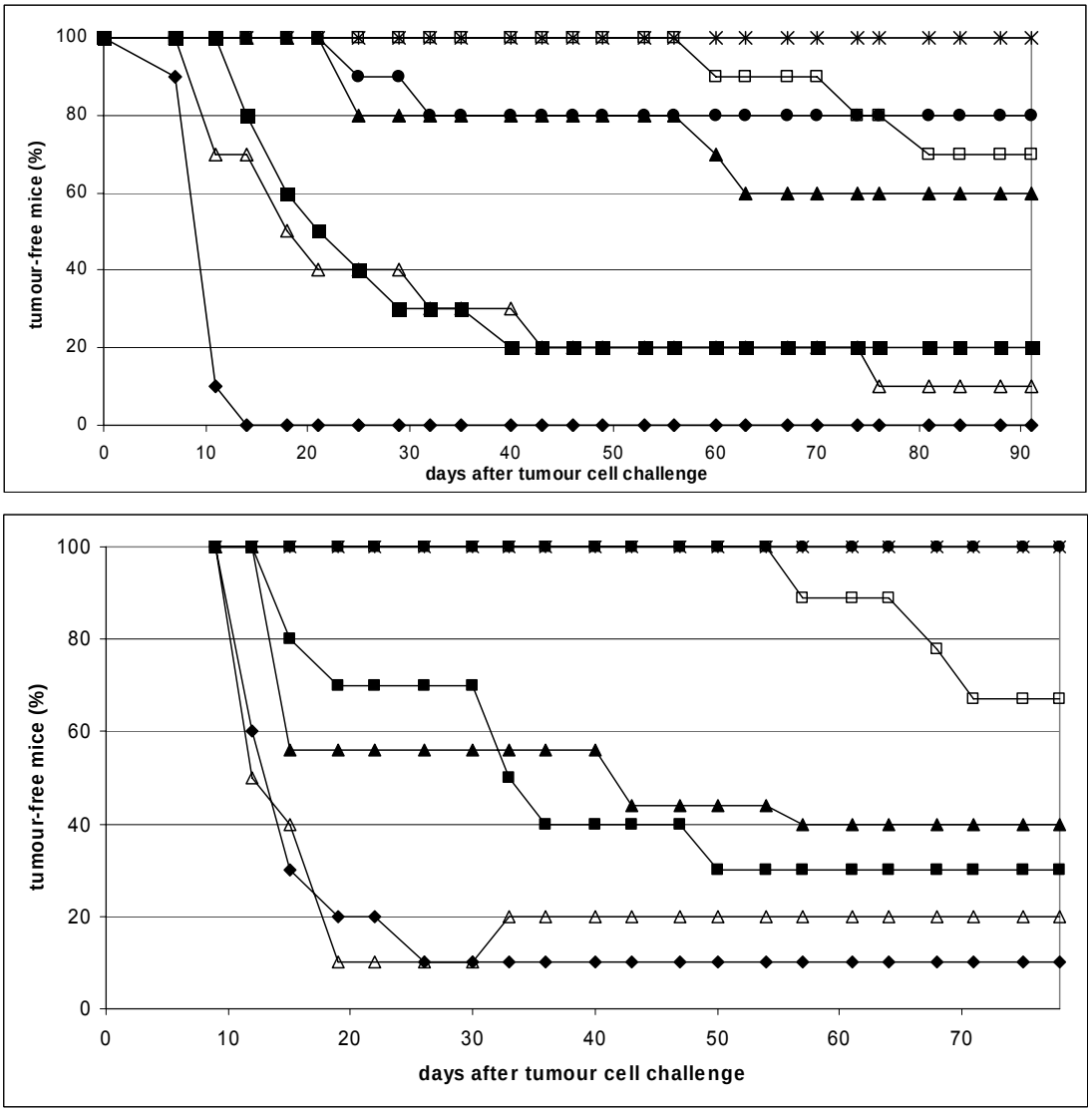


Figure 16. Depletion of $CD4^+$ and $CD8^+$ T cells as well as NK cells either alone or in combination in the effector phase in BALB/c mice. Results from two independent experiments are shown. Mice immunized with Her2MPtVLPs were depleted of (□) $CD4^+$ T cells, (●) $CD8^+$ T cells, (■) both $CD4^+$ and $CD8^+$ T cells, (▲) NK cells or (Δ) all three cell types, respectively. Unimmunized mice (♦) and non-depleted Her2MPtVLP immunized mice (*) were included as respective negative and positive controls. Mice were challenged with $5x10^4$ D2F2/E2 cells two weeks after immunization.

In conclusion, tumour rejection was obtained after depletion of either $CD4^+$ or $CD8^+$ T cells, but not after complete depletion of both types during either the immunization or effector phase. However, depletion of cells selectively during the immunization phase is difficult to obtain, considering the long-lived effect of the antibodies. This means that even if the injections are terminated just after immunization, depletion persists into the effector phase. Due to this we can not conclude if the abolished protection after combined depletion in the immunization phase was due to the depletion of $CD4^+$ and $CD8^+$ T cells during the effector or immunization phase. However, since the results of depletion of either $CD4^+$ or $CD8^+$ T cells during both phases still resulted in tumour protection, we can conclude that mice were protected against tumour outgrowth regardless of if depletion was performed during the immunization or effector phase.

Studies in knockout mice

As a complement to the depletion studies we also aimed at studying tumour rejection in knockout mice lacking various components of the immune system. Due to the absence of appropriate knockout mice on BALB/c background at our animal department, we used mice on C57Bl/6 background. This makes the results from the two different mouse models not fully comparable. In addition, the C57Bl/6 model was not as reproducible as the BALB/c model, due to the used transplantable tumour, making the results even more difficult to interpret. An important difference between depletion of cellular subsets by antibodies, and the use of knockout mice is that depletions make it possible to study the effect of subsets of cells during specific phases of the response, while a knockout strain is devoid of a particular immune compartment during the entire response.

In line with the results from BALB/c mice, $CD8^{-/-}$ mice were protected against tumour outgrowth while $CD4^{-/-}CD8^{-/-}$ mice were not. In contrast, protection was lost in $CD4^{-/-}$ mice. When normal C57Bl/6 mice were depleted of $CD4^+$ and/or $CD8^+$ T cells, the results were somewhat different. Depletion of only $CD4^+$, only $CD8^+$ or $CD4^+/CD8^+$ T cells in combination all abrogated protection.

Knockout mice versus mice depleted of immune cells

Since there was a difference between $CD8^{-/-}$ mice and mice depleted of $CD8^+$ T cells in the C57Bl/6 model, one might wonder what the functional difference is between these two types of "CD8-negative" mice. The CD8 molecule is expressed on the cell surface either as an αα homodimer or as an αβ heterodimer. Surface expression of CD8β is dependent on expression of the α-chain, since the β-chain will otherwise be retained within the ER and degraded. This implies that a mouse with a $CD8\alpha^-$ genotype has a $CD8\alpha^-\beta^-$ phenotype (702). The first $CD8\alpha^-$ mouse strain was created in 1991 and resulted in complete absence of $CD8^+$ T cells, and cytotoxic responses to both viral antigens and alloantigens were dramatically decreased, while the $CD4^+$ T-cell population seemed to be unaffected. The authors concluded that "CD8 is necessary for the development of functional MHC class I-restricted T cells" (703). Another study confirmed these results by showing that it was not possible to obtain cytotoxicity to viruses in this strain (704). Concurrently, the same scientists created mice lacking CD4 resulting in normal development of $CD8^+$ T cells and decreased Th cell activity for antibody responses, while the cytotoxic activity against viruses was normal or only slightly affected (705). These two strains were thereafter crossed to generate double-negative mice. Surprisingly, these mice contained significant numbers of αβT cells that recognized MHC class I antigens, and they here showed that alloreactive CTLs could be induced in the absence of CD8 (706). Soon a study showed that $CD8^{-/-}$ mice could indeed reject skin grafts mismatched for MHC class I as efficiently as normal mice, and depletion of $CD4^+$ cells had no effect, while depletion of $CD4^+$ and $CD8^+$ T cells

resulted in delayed rejection[90]. The authors suggested that double-negative cells were responsible for the rejection and that T cells without CD8 could be functional. Apparently, in this study there was a difference between $CD8^{-/-}$ mice and mice depleted of $CD8^+$ T cells (707). Some interesting studies on this topic have also been performed using MPyV. In 1998, it was shown that adult C57Bl/6 $\beta2m^{-/-}$ mice were highly susceptible to MPyV tumour development, while $CD8^{-/-}$ mice showed much lower susceptibility. It was suggested that MHC class I-restricted antiviral CTLs were functional in the absence of CD8 (708). It was later shown that adult C57Bl/6 $CD8^{-/-}$ mice controlled MPyV infection as efficiently as normal mice and generated a virus-specific MHC class I-restricted T-cell response, although these cells were short-lived and had a reduced capacity to produce IFNγ (709). To conclude this section, the results are, as they often are in research, contradictory. The available data point to the fact that there is a difference between e.g. $\beta2m^{-/-}$ mice and $CD8^{-/-}$ mice, as well as between $CD8^{-/-}$ mice and mice depleted of $CD8^+$ T cells. In addition, it seems like CTL responses can be induced in mice lacking CD8. On the basis of this, diverging results from $CD8^{-/-}$ mice and mice depleted of $CD8^+$ T cells as seen in our study are not very surprising.

Summary and interpretation of the results

So what components of the immune system actually mediate tumour protection? We have assumed, as proposed in papers II and III, that the anti-tumour response following Her2/*neu*-cVLP immunization was mediated by $CD8^+$ T cells. This proposal was based on the presence of a Her2/*neu*-specific $CD8^+$ T-cell response as shown by ELISPOT in combination with the absence of anti-Her2/*neu* antibodies. In paper IV, as could be expected, we showed that tumour protection could occur in the absence of $CD4^+$ T cells during the immunization phase. This result could easily be explained and deduced by the fact that cVLPs from other viruses can be cross-presented, which could be the case for cVLPs from polyomavirus as well. However, we were somewhat surprised by the finding that tumour protection was obtained in BALB/c mice depleted of $CD8^+$ T cells, as well as in $CD8^{-/-}$ mice. This indicates that $CD4^+$ T cells can mediate tumour protection independent of $CD8^+$ T cells and antibodies. Below we will go through possible explanations for this unexpected result, and in the end summarize and try to draw some conclusions. Finally, as could be expected, tumour protection was lost in the absence of both $CD4^+$ and $CD8^+$ T cells.

Could a low number of antibodies be an explanation for the anti-tumour effect in the absence of $CD8^+$ T cells?

If an anti-tumour response following vaccination is not mediated by $CD8^+$ T cells, one could still postulate that it is mediated by antibodies. Could it be that there are low titres of anti-Her2/*neu* antibodies undetectable by our assay? Yes, but such antibody titres must be extremely low. Repeated attempts have been made by several members of our own as well as other groups to measure anti-Her2/*neu* antibodies, and such antibodies

[90] The fact that rejection finally occurred was assumed to be due to repopulation of $CD8^+$ T cells after the injections of depletion antibodies had been terminated.

have never been detected. As a positive control, we have used sera from mice immunized with an adenovirus encoding Her2/*neu*, and such sera gave a strong response. Since we obtained protection also in mice depleted of $CD4^+$ T cells, the antibody response, if existing, should be CD4-independent. In addition, in case the response would be antibody-mediated, there should be no difference between mice depleted of $CD4^+$ T cells only, and mice depleted of both $CD4^+$ and $CD8^+$ T cells. Therefore I would like to conclude that it is highly unlikely that antibodies in mice lacking $CD8^+$ T cells mediated the anti-tumour response.

Could incomplete eradication of immune cells be an explanation for the anti-tumour effect in the "absence" of $CD8^+$ T cells?

The degree of depletion was tested four days after one single injection of anti-CD4 and anti-CD8 mAbs and was for both cell types >96%. Injections were thereafter given every four to five days until about day 55 after challenge. Since the hybridomas used for production of the mAbs are a fusion between a murine myeloma cell and a *rat* plasma cell, anti-rat antibodies could be raised and thereby reduce the efficacy of repeated injections. At the day of termination of the study, the degree of depletion was again tested and was 70-80%. However, it should be noted that the last injection was given more than three weeks before the day of termination. This means that the reduced depletion could either be due to the presence of anti-rat antibodies, or simply due to repopulation as a consequence of the long time period since the last injection. If $CD4^+$ T cells are depleted, no such anti-rat antibodies should be raised (or at least only very low titres unless the antibodies develop independently of $CD4^+$ T cells), implying that the effect of repeated injections should not be diminished in CD4-depleted or CD4/CD8-depleted mice but only in CD8-depleted mice. However, no difference was seen between the various groups of mice with regard to the degree of depletion of the cell types. This speaks against a major role for anti-rat antibodies.

Concerns have also been raised whether depletion of $CD4^+$/$CD8^+$ cells is a "FACS artefact", which could either be due to down-regulation of the CD4/CD8 molecules, i.e. the cells are still there but can not be detected by FACS analysis, or the depletion antibodies remain bound to the CD4/CD8 molecules preventing binding to the same molecules by the antibodies used for FACS staining. However, it should be noted that the antibodies used in this thesis have been used extensively in different laboratories and to the best of my knowledge no such "problems" have been reported. We have also analyzed the presence of remaining $CD8^+$ T cells by a functional assay, ELISPOT, and could show that the IFNγ response was severely impaired following depletion of $CD8^+$ or CD4/$CD8^+$ T cells. Since "only" 96% of the $CD4^+$ and $CD8^+$ T[91] cells were depleted, it is theoretically possible that the remaining 4% $CD8^+$ T cells were sufficient to protect against tumour outgrowth. However, I consider this as quite far-fetched. Additionally,

[91] It should however be pointed out that in most cases the degree of depletion was higher (96% was the minimum percentage of depletion). At the same time, it can be mentioned that the IFNγ response was not reduced to really the same extent (paper IV, figure 2).

only a minor fraction of these $CD8^+$ T cells were Her2/*neu*-specific, implying that the total number of Her2/*neu*-specific $CD8^+$ T cells was extremely low. In summary, in my opinion it would be unlikely that tumour protection was a consequence of remaining $CD8^+$ T cells.

What about $CD4^+$ cytotoxic T cells?

As reviewed in chapter 4.3.1.4, $CD4^+$ CTLs can kill target cells either in an MHC class I- or class II-dependent manner without the need for $CD8^+$ T cells. This means that a direct cytotoxic effect can be exerted by T cells also in the absence of the classical $CD8^+$ CTLs. Is then MHC class II expressed by D2F2/E2 and EL4-Her2 cells? The parental cell line D2F2 is derived from a mouse mammary tumour and most likely D2F2/E2 cells do not express MHC class II. What kind of cells EL4 is, is not clear to me. According to American Type Culture Collection EL4 is a T-cell lymphoma line and this is also stated in most publications, but I do not know where this has been shown. Going back to the original publication on this cell line by Gorer from 1960 (710), the cells were initially referred to as a lymphogenous leukosis[92], then there were speculations about lymphatic leukemia and also lymphosarcoma. Assuming that at least D2F2/E2 is class II negative, the $CD4^+$ CTLs (if they exist) must be MHC class I-restricted. I have not been able to find any mouse study where it has been convincingly shown that the anti-tumour response has been mediated by MHC class I-restricted $CD4^+$ CTLs. Nonetheless, as mentioned in chapter 4.3.1.4.2, $CD4^+$ CTLs can be identified in mice following anti-tumour vaccination. However, the presence of such cells has usually been demonstrated by *in vitro* assays. In addition, as I have also written above it has been suggested that the $CD4^+$ CTLs do not kill target cells directly but rather through indirect mechanisms, e.g. through stimulation of monocytes and macrophages.

What about NK cells?

Tumour protection was impaired, although not completely abrogated after depletion of NK cells. Considering the fact that only 70-80% of these cells were depleted, what would happen following the complete depletion of NK cells? I would like to suggest that it is definitely possible that the anti-tumour response was at least partially mediated by NK cells. However, it is unlikely that the response was completely NK-cell mediated, since depletion of $CD4^+$ and $CD8^+$ T cells in combination abrogated the response. Moreoever, the finding that mice immunized with $Her2_{1\text{-}683}$PyVLP were not protected against the Her2/*neu*-negative cell line D2F2 (paper II) shows the Her2/*neu*-specificity of the response, which also argues against a role for NK cells only.

It should be noted that anti-asialo GM_1-serum can deplete other cell types such as $CD4^+$ and $CD8^+$ T cells. In C57Bl/6 mice, NK cells express the surface marker NK1.1 making depletion of NK cells easy by the use of the commercially available monoclonal anti-NK1.1 antibody. In BALB/c mice however, expression of NK1.1 is absent,

rendering NK cell depletion somewhat trickier. Instead, serum against the cell-surface molecule asialo-GM_1 is generally used. For long it was thought that this molecule was unique to NK cells. However, as often in science, things are not what you think they are. Today we know that other immune cells such as $CD4^+$ and $CD8^+$ T cells express asialo-GM_1. The percentage of asialo-GM_1 positive cells among $CD4^+$ and $CD8^+$ T cells under normal conditions differs between studies and is probably also dependent on the mouse strain. Up to 30% of murine $CD8^+$ T cells can be asialo-GM_1 positive (711-713) and up to 20% of murine $CD4^+$ T cells (712). Moreover, infections with viruses like LCMV and respiratory syncytial virus increase the expression of asialo-GM_1 and >90% of virus-specific $CD4^+$ and $CD8^+$ T cells can be positive (712, 714)

Concluding remarks

In summary, the above data point to the fact that $CD4^+$ T cells can protect against tumour outgrowth in the absence of both $CD8^+$ T cells and antibodies. This is a highly interesting finding and has been the topic of intense discussion both inside and outside our group. So far nobody has been able to give a valid explanation. It seems like $CD4^+$ and $CD8^+$ T cells can compensate for each other in tumour protection. It is possible that as long as $CD8^+$ T cells are present, they are the main mediators of protection and can be activated through cross-priming if $CD4^+$ T cells are lacking. However, how can $CD4^+$ T cells kill tumour cells in the absence of both $CD8^+$ T cells and antibodies? I can think of three possible mechanisms: 1.) Direct killing of target cells in an MHC class I-dependent manner, 2.) Direct killing of target cells in an MHC class II-dependent manner and 3.) Indirect killing of target cells, i.e. $CD4^+$ T cells affect other cells, which in turn kill the targets. Although direct killing of tumour cells by MHC class I- or II-restricted $CD4^+$ T cells cannot be completely excluded, I personally would like to consider such a scenario as a little bit far-fetched. I suggest it is more likely that the $CD4^+$ T cells kill the tumour cells through an indirect mechanism as described above.

Future perspectives

The following studies would be of interest to perform in order to better delineate the mechanisms responsible for tumour protection:

- The C57Bl/6 model is far from optimal since it is problematic to obtain a significant difference between immunized and unimmunized wildtype mice. Therefore it would be of great value to perform experiments in BALB/c knock-out mice lacking B cells, MHC class I (β2m), MHC class II, CD4, CD8, CD4/CD8, perforin, RAG-1/2 and IFNγ. Unfortunately, presently few of these strains are available at our animal department.

- It would be valuable to analyze the activity of $CD4^+$ T cells in *in vitro* and *in vivo* cytotoxicity assays. In relation to this, MHC class II expression should be analyzed on D2F2/E2 and EL4-Her2 cells. Even if *in vitro* analysis would show

[92] Probably we would today call this *lymphocytosis*.

absence of MHC class II, it could be claimed that MHC class II is upregulated *in vivo*.

- Following depletion of either $CD8^+$ or $CD4^+/CD8^+$ T cells, we have shown that the IFNγ response is severely impaired, but we have not shown that the *cytotoxic* response is lost. It could still be that the remaining cells are able to kill target cells, and such an issue could be explored by e.g. an *in vivo* cytotoxicity assay with and without depletion of immune cells.

- It would be of interest to deplete immune cells after immunization in a therapeutic context to explore if the immune mechanisms responsible for tumour rejection in this setting are different from those in a prophylactic setting.

- Finally, it would be of great interest to analyze if injection of anti-asialo GM_1-serum in addition to depletion of NK cells also affects $CD4^+$ and $CD8^+$ T cells, especially following cVLP immunization.

10 CONCLUSIONS, GENERAL DISCUSSION AND FUTURE STUDIES

In my opinion, the main take-home-message of this thesis is that cVLPs compose a very efficient anti-tumour vaccine, at least in a pre-clinical context. They can prevent tumour outgrowth in a prophylactic as well as therapeutic setting, induce immunological memory and break tolerance to Her2/*neu*. The induced tumour protection can probably be mediated both by $CD8^+$ T cells, and by a yet not fully clarified mechanism involving $CD4^+$ T cells independent of both antibodies and $CD8^+$ T cells.

The mysteriously high efficacy of cVLPs

How can cVLPs be so remarkably efficient? How can as little as 800 ng of Her2/*neu* protein[93] completely protect against outgrowth of 10^5 tumour cells? This is a question that we have been discussing in our group throughout the years and we cannot give any definitive answer. However, we have mainly been thinking along three lines. Firstly, cVLPs from MPyV and MPtV are efficiently and rapidly taken up by cells, which implies that efficient delivery into the cells of Her2/*neu* occurs. Secondly, the Her2/*neu* protein is protected by the cVLP and will not be destroyed before it reaches the cells of the immune system. This is in contrast to if free/naked protein would be inoculated since much of it would probably be broken down rapidly. Thirdly, a possibility could be that the cVLPs work as an adjuvant and this seems reasonable to me but we still do not know in what way.

Could it be that the cVLPs themselves are in fact not very efficient and that the potent effect is rather mediated by some other substance in the cVLP preparations? One initial concern was presence of LPS. Since the VLPs are produced in insect cells and not bacteria the LPS contents should be very low. Additionally, when particles such as plasmids are purified by CsCl gradient centrifugation, most LPS is normally lost. Furthermore, we have measured the concentration of LPS and it turned out to be below the level of detection (unpublished).

Further studies on Her2/*neu*-cVLPs

The following studies are in the pipeline or at the planning stage in our lab:

- To study the immune mechanisms in more detail, especially by the use of knockout mice on BALB/c background as outlined above.

- To improve the efficacy of Her2/*neu*-cVLP immunization even further by the use of other, potentially more potent adjuvants. In particular it would be

[93] This is the approximate amount of Her2/*neu* protein in 50 μg cVLPs.

interesting to analyze if even more long-lived immunological memory could be obtained.

- When the immunization protocol has been optimized, it would be of interest to pursue a clinical trial. It could be argued that cVLPs are very complicated and expensive to manufacture. However, one should keep in mind that HPV-VLPs are produced in enormous amounts and used in clinical practice today, meaning that efficient methods for large-scale production and purification of VLPs are available.

- To use VLPs carrying other tumour antigens such as prostate-specific antigen, and these have been constructed and are now ready for *in vivo* testing.

- To construct cVLPs containing Her2/*neu* that lacks the hydrophobic, and potentially "VLP-toxic", transmembrane domain. This could possibly result in incorporation of an increased number of VP2-Her2/*neu* fusion proteins into each cVLP.

The future of immunotherapy against cancer

Despite several decades of research on immunotherapy against cancer the results from clinical trials have mainly been discouraging. It should however be pointed out that most clinical trials have been performed in patients with advanced disease, often resistant to conventional therapies. Better response rates could probably be obtained in patients with less advanced disease. In addition, generally immunotherapy as single therapy has been tested. As briefly discussed in chapter 6.7, from mouse experiments it is known that active immunotherapy could be efficacious in combination with other therapies such as chemotherapy and passive administration of antibodies, and it is possible that the same would be true in humans. Many people claim, opposite to the professor in chapter 6.1, that the function of the immune system is not to defend us against cancer, and although immunotherapy is efficient in mice it will hardly work in patients. However, I would argue that several observations speak in favour of immunotherapy as a promising treatment for cancer. Today it is well established that mAbs have an effect in cancer patients and trastuzumab is only one example in a row of such antibodies. Although the mechanism of action is still not fully known, and it is unclear whether there is any role for the immune system (see chapter 5.5.1), it is obvious that these antibodies do work and this is of course the important point. Accordingly, if antibodies could be induced by active vaccination there are reasons to assume that these antibodies will also indeed have an anti-tumour effect. However, a difficulty is to induce high titres of these antibodies. With regard to T cells, it has been demonstrated that adoptively transferred lymphocytes can induce regression of metastatic cancer showing proof-of-concept. Also in the case of T cells, it is troublesome to obtain a large number of such cells with high affinity for the malignant cells, but future studies will most likely solve this problem.

We have now reached the end of this thesis and so has soon my time as a PhD student. An exciting, interesting but also very tough period of my life is over. So how should I end the thesis? With some clever words of course. Initially I considered putting together some clever words on my own, but then I asked myself why I should do that, since so many clever things have been said before. Eventually I chose the following quick-witted and suitable quotation by a former PhD student at our department: “I do know the impact of science on my life. Wherever my future position will be, in the clinic or in a laboratory, I hope to preserve the feeling of humbleness to the complexity of biological knowledge and to the short-lived nature of truth” (715). It is now time to leave research, at least temporarily, and do what my mum has always wanted her son to do: start a *real* job. Maybe I should have my hair cut and a new pair of jeans as well.

Fin

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