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The Role of Antigen Presentation and Immunodominance for the Induction and Expansion of Cytotoxic T cell Responses with MVA Vector Vaccines

Georg Gasteiger

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Gewidmet
meinem Vater

Georg Gasteiger
(1950-1997)

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Abbreviation List

aa	Aminoacid(s)
ADC	Antigen Donor Cell
APC / pAPC	Antigen Presenting Cell / professional APC
APS	Ammoniumperoxidsulfate
ATP	Adenosintriphosphate
bp	Basepair(s)
BFA	Brefeldin A
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation
CFSE	Carboxy Fluoroscein Succinimidyl Ester
CPE	Cytopathic Effect
CTL	Cytotoxic T Lymphocyte
CVA	Chorioallantois vaccinia virus Ankara
DC / iDC / mDC	Dendritic Cell / immature DC / mature DC
dH$_2$O	Distilled Water
DMSO	Dimethylsulfoxide
dNTP	Desoxyribonucleosidetriphosphate
EDTA	Ethylendiamintetraacetate
EMA	Ethidium Monoazide Bromide
ERAD	ER Asscociated Degradation
ER	Endoplasmatic Reticulum
FACS	Fluorescence Activated Cell Sorting
FITC	Fluoresceinisothiocyanate
FCS	Fetal Calf Serum
FSC	Forward Scatter
gfp, GFP	Green Fluorescent Protein
HLA	Human Leucocyte Antigen
ICS	Intracellular Cytokine Stain
IFN	Interferon
IL	Interleukin
IU	Infectious Units
i.v.	Intravenously
i.p.	Intraperitoneal
L.m. OVA	Listeria monocytogenes expressing ovalbumin
MACS	Magnetic Cell Separation
MHC / pMHC	Major Histocompatibility Complex / Peptide-MHC-Complex
MOI	Multiplicity of Infection
MVA	Modified Vacciniavirus Ankara
OD	Optical Density
ORF	Open Reading Frame
OVA	Chicken Ovalbumin
p.i.	Post Infection
PAGE	Polyacrylamidgel-Electrophoresis
PBMC	Peripheral Blood Monocyte
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction

PE	Phycoerythrin
PerCP	Peridininchlorophyll Protein
PFA	Paraformaldehyde
pfu	Plaque Forming Units
PMSF	Phenylmethylsulfonylfluoride
PO	Peroxidase
rec	Recombinant
RNA	Ribonucleic Acid
RT	Room Temperature
SDS	Sodiumdodecylsulfate
SEM	Standard Error of the Mean
SSC	Sideward Scatter
TAA	Tumor associated Antigen
TAP	Transporter associated with Antigen Processing
TAE	Tris Acetate EDTA
TBS	Tris Buffered Saline
TE	Tris EDTA-Buffer
Tris	Trishydroxymethylaminomethane
Tyr / huTyr	Tyrosinase / human Tyrosinase
Ub	Ubiquitin
UPD	Ubiquitin-Proteasome-Pathway
VACV	Vaccinia Virus
WB	Western-Blot
WT	Wildtype

1 Introduction

1.1 The adaptive immune system and immunotherapy

The immune system has evolved to conserve the integrity of the organism and therefore needs to discriminate between "self" (e.g. cellular proteins), "altered-self" (e.g. proteins derived from transformed genes/malignancies) and "non-self" (e.g. infection, transplant rejection, transmissible tumors). The innate immune system senses danger signals associated with infection or cell destruction and provides very fast defense mechanisms. It also acts as an important interface for the activation of adaptive immune responses. Two hallmarks of (not exclusively) the adaptive immune system are antigen-specificity and memory formation, specified by a faster and more effective reaction upon antigen reencounter. Vaccination approaches try to exploit these features for the immunotherapy of infections and malignancies. Whereas humoral immunity is specialized in clearing pathogens through antibodies when being extracellular, cellular immunity, e.g. mediated by cytotoxic T cells, mainly surveys intracellular pathogens. Most of currently available vaccines are based on inactivated organisms or proteins derived thereof and are protecting vaccinated individuals mainly by inducing neutralizing antibodies. Many of these vaccines are highly effective in preventing bacterial as well as viral infections. The potential of an internationally coordinated use of effective vaccines has been vigorously demonstrated during the smallpox eradication campaign achieving the eradication of variola virus, the causative agent of smallpox. Enormous progress has also been made in fighting poliomyelitis virus. It appears, however, that other infectious diseases remain a big challenge as they cannot be fought by antibody inducing vaccines: HIV envelope proteins are constantly mutating and thus evading the antibody responses detectable in infected individuals (Humbert and Dietrich, 2006). Influenza virus pandemics can arise after antigenic shift, and vaccination with antigens from different strains does not provide cross-protection (Subbarao and Joseph, 2007). Similarly, no effective vaccines are currently available for a number of intracellular pathogens causing millions of deaths especially in third world countries, namely Hepatitis C virus, Mycobacterium Tuberculosis or the malaria inducing Plasmodia (see WHO Global Health Atlas http://globalatlas.who.int/). Therefore novel immunotherapeutic and vaccination strategies aim at the induction of strong CTL immunity to target conserved structures of the mentioned pathogens which during infection are not accessible to antibodies, but can be recognized by cytotoxic T cells. The induction of antigen-specific CTL also bears the potential to detect and to cure tumors or at least to support currently available therapies in a highly specifc manner. In recent years recombinant viral vectors have raised the hope to be effective against the mentioned diseases because of their ability to synthesize heterologous antigens in infected cells and to provide the innate stimuli that are required to activate the adaptive immune system and even to break tolerance. However, clinical vaccination trials are rather disappointing up to date and no recombinant viral vaccine has been licensed for clinical application in humans thus far. While

numerous clinical studies currently evaluate such vaccines basic research is just starting to gain fundamental insights into the induction of T cell immunity with these vectors, which hopefully will improve vaccine efficacy.

1.2 The viral vector MVA

Viruses have evolved highly efficient strategies for infecting cells and exploiting the cellular machinery for production of virally encoded proteins. The immune system is able to sense viral infections resulting in the activation of innate and adaptive reactions. Thus, viral vectors are excellent vehicles for heterologous gene delivery to induce immune responses and are currently studied extensively in preclinical and clinical research (for Review see Brave et al., 2007). Among those viral vectors that have been studied most extensively in humans are live attenuated poxviruses. Modified vaccinia virus Ankara (MVA) is an attenuated strain of vaccinia virus that was developed for the use as a safer vaccine during the last decades of the smallpox eradication campaign. The parental chorioallantois vaccinia virus Ankara (CVA) was serially passaged in primary chicken embryo fibroblast cultures as an attempt to restrict the broad host range of vaccinia virus by mimicking the evolution of other host range restricted Orthopoxviruses. After 371 passages, Mayr and Munz reported that CVA had developed attenuated growth characteristics on the chorioallantois membrane, in tissue cultures and in laboratory animals. After the 516th passage the virus was noted to have a stable, less virulent phenotype than its parental strain and thus was renamed as modified vaccinia virus Ankara (Mayr and Munz, 1964; Sutter and Staib, 2003). Recently, sequencing the complete 177 kb genome of MVA obtained from the 572nd CEF passage confirmed that, during attenuation, MVA lost ~15% of its parental genome (ca. 30 kb) including genes for virus host range regulation and evasion of the host immune response (Antoine et al., 1998). The avirulence of MVA has been documented by inoculation of various animals including newborn, irradiated and SCID mice as well as immune-suppressed macaques (Meyer et al., 1991; Stittelaar et al., 2001; Wyatt et al., 2004). During the smallpox vaccination program, MVA has been safely administered to more than 100,000 humans including individuals considered at high risk for conventional smallpox vaccination (e.g. immunocompromised, elderly, patients with atopic skin diseases) without any report of the adverse effects associated with other vaccinia virus vaccines (Mayr et al., 1978; Stickl et al., 1974). Similarly, the therapeutic administration of high-dose recombinant MVA to HIV-infected individuals without any documented complications further outlines the excellent safety profile of MVA vaccines (Cosma et al., 2003; Dorrell et al., 2006; Harrer et al., 2005). Poxvirus-encoded genes are transcribed in the cytoplasm of the infected cell under strict control of the viral transcription machinery and therefore the risks of genomic integration can be excluded. MVA is unable to productively replicate in humans as well as

most mammalian cells due to a block of morphogenesis at a very late stage of the virus life cycle (Stickl et al., 1974). Importantly, this block to form viral progeny does not affect the expression of viral or heterologous proteins (Sutter and Moss, 1992). Like other poxviruses, MVA induces a cascade-like course of antigen expression with three distinct phases of viral gene expression that are strictly controlled by distinct promoters with early, intermediate and late activity (Moss, 1996, Kastenmuller et al., 2006). The packaging size of the MVA genome for recombinant genes is large, reaching a hypothetical value of ~50kb (Sutter and Staib, 2003) and thus allowing for the expression of full-length and multivalent antigens or the co-expression of adjuvanting molecules. MVA can be handled under safety level 1, recombinant viruses are easy to manufacture and stable over time when frozen or freeze-dried. These features explain why MVA is one of the viral vectors being extensively evaluated for vaccination and immunotherapy. Due to its excellent safety record and immunogenicity replication-deficient vaccinia viruses (VACV), like MVA, are considered as the next generation smallpox vaccines (Earl et al., 2004; Stittelaar et al., 2005; Wyatt et al., 2004): Recombinant MVA are now widely used as vector vaccines in clinical studies (Dorrell et al., 2006; Goonetilleke et al., 2006; Harrop et al., 2006; Imoukhuede et al., 2006; McShane et al., 2004; Meyer et al., 2005; Peters et al., 2007). Furthermore, recMVA are applied in therapeutic and also prophylactic vaccination protocols against infectious diseases (HIV, Malaria and Tuberculosis) and cancer (melanoma, prostate cancer, colon cancer and –using HPV antigens- cervical cancer). For the induction of CD8+ T cell responses, MVA is most frequently used in DNA-MVA prime/boost vaccinations as a strategy to overcome weak T cell priming by MVA and presumed limitations of anti-vector immunity in secondary vaccinations (or Review see Drexler et al., 2004).

1.3   Induction and Expansion of CD8+ T cells

Antigens derived from tumors, viral infections or intracellular parasites can be recognized by cytotoxic T cells (CTL). The induction of strong CTL immunity directed against those antigens is the aim of vaccination and immunotherapy. Effector functions of cytotoxic T cells are mainly mediated by 1) the lysis of recognized cells through the release of perforins and granzymes, 2) FAS-ligand triggered cell death induction and 3) the production of proinflammatory cytokines like IFNγ and TNFα that e.g. interfere with protein synthesis and therefore pathogen replication (Schepers et al., 2005). To develop into cytotoxic T cells naïve precursor T cells need to be primed for their cognate antigen. Antigen-specific T cell responses to virulent pathogens are characterized by three distinct phases. After a brief antigenic stimulation during the priming phase T cells undergo massive clonal expansion and through 15-20 cell divisions increase their number up to 50.000 fold (Schepers et al., 2005; Williams and Bevan, 2007). From day two after the initial antigenic stimulus T cells gain cytotoxic effector function

(van Stipdonk et al., 2003). The peak of the response on day 7/8 is followed by a contraction phase in which most of the effector T cells rapidly undergo apoptosis. The remaining 5-10% of primed T cells form a stable memory pool which can be maintained for years in the absence of antigen and can mediate long-term protection. In recent years it became evident that memory T cells show heterogeneity at least in their effector functions and homing potentials (Schepers et al., 2005; Williams and Bevan, 2007). On a second antigen encounter memory T cells start to divide and to gain immediate effector function more rapidly than during T cell priming (Huster et al., 2006). Very recent studies suggest, that one single naïve precursor T cell can give rise to a complete functional memory and effector T cell pool (Stemberger, Busch et al, personal communication). This is consistent with the concept that in order to be able to respond to potentially any given antigen the immune system maintains only a very low number (in the range of hundreds) of naïve precursor T cells specific for a given antigen (Blattman et al., 2002). These are incredibly low numbers when compared to an estimated $\sim10^{13}$ nucleated cells throughout the different tissues that together compose the human body, and that upon infection or transformation need to be recognized by specific T cells. These numbers illustrate the pivotal role of a well coordinated system that guarantees that naïve T cells will see their antigen (Schumacher, 1999). Specialized bonemarrow-derived professional antigen presenting cells (pAPC) migrate into the tissues and continuously sample antigen to transport it from the periphery to the draining lymph nodes or the spleen (Banchereau and Steinman, 1998). These pAPC, most likely DCs, appear to be essential to report infection and to initiate adaptive immune responses (Huang et al., 1994; Jung et al., 2002; Lenz et al., 2000; Sigal et al., 1999). Immature DC (iDC) can get infected and synthesize e.g. viral antigen. iDC also capture antigen in the periphery by different means of endocytosis, including macropinocytosis, receptor-mediated endocytosis and phagocytosis (Ackerman et al., 2006). DC express so-called pattern-recognition receptors that allow them to sense if the acquired antigen bears additional information (for a recent overview see Kawai and Akira, 2007). For example, toll-like-receptors enable the detection of viral or bacterial DNA (Heil et al., 2004; Hemmi et al., 2000; Medzhitov et al., 1997). These signals drive maturation of DC: they loose the capacity to take up antigen but instead migrate to defined sites in lymphoid organs, especially the paracortical T cell areas where they function to attract naïve T cells via chemokine and cytokine production. During maturation DC upregulate costimulatory molecules and enhance antigen-processing and presentation (Heath et al., 2004). Apparently, only pAPC like DC are able to provide the three signals required for T cell priming: antigen-presentation (MHC with TCR Interaction), costimulation (CD80/86 Interaction with CD28) and cytokines. While DC are generally considered to be essentially required for T cell priming less is known about the secondary expansion of memory T cells. It is controversially discussed, which memory subpopulations preferentially proliferate upon a second antigen encounter, or which cells trigger this expansion. A recent study suggests that DC can maximize the outcome of secondary T cell expansion (Zammit et al., 2005). However, also other pAPC like macrophages might

be able to expand memory T cells (Crowe et al., 2003). While memory T cells appear to be less dependent for costimulation than naïve cells it has not been unambiguously demonstrated or ruled out that T cells can expand in response to peripheral antigen presentation by non-pAPC.

1.4 MHC class I antigen presentation

CD8+ T cells recognize 8-11 amino acids long peptides ("antigenic determinants" or "peptide epitopes") derived from degraded intracellular proteins that are presented by MHC class I molecules on the surface of nucleated cells (Yewdell and Haeryfar, 2005). As peptides for presentation can be generated from virtually any protein, the MHC system enables immunosurveillance of a cells transcriptome and therefore offers the functional basis for the antigen-specific recognition of transformed malignant or infected cells by cytotoxic T cells (Yewdell et al., 2003). For T cell priming, antigenic determinants presented by DC can be derived from intracellular or extracellular sources, since DC can present peptides from proteins synthesized within the infected DC itself ("direct presentation") or from acquired antigen produced by other infected donor cells ("cross-presentation") (Albert et al., 1998; Bevan, 1976).
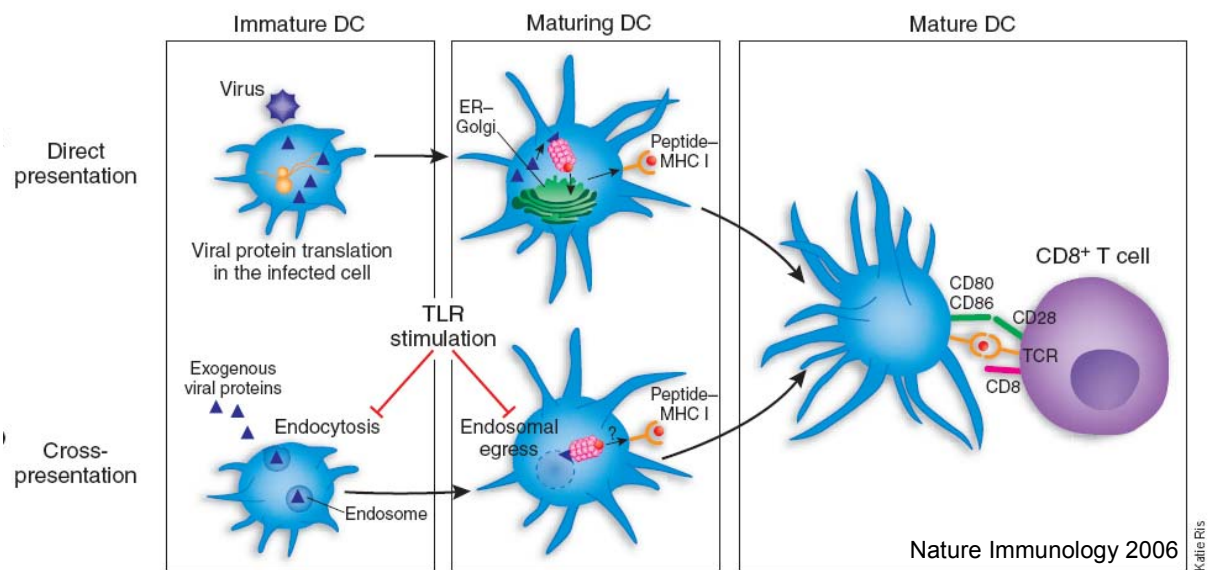
Schematic   Direct presentation and Crosspresentation of viral antigens Antiviral CD8+ T cell responses are induced by DC that present antigen from different sources: Infection of DC leads to synthesis of antigens in the antigen presenting cell ("direct presentation"). Alternativley, DC acquire antigen that has been synthesized by other infected cells and present it to naïve T cells ("crosspresentation") (Hickman-Miller and Yewdell, 2006).

1.4.1 Direct presentation

Several viruses have DC tropism without substantially altering DC viability and antigen presentation. For "direct presentation" viral proteins are synthesized, processed and presented by the infected DC. As in every nucleated cell, the antigens synthesized by DC can be degraded in the cytosol into peptides that (in the case of most peptides) can be transported by the transporter associated with antigen presentation (TAP) into the endoplasmatic reticulum (ER), where they can be further trimmed to fit into the groove of newly synthesized MHC class I molecules (York et al., 2006). The peptide-MHC-complex then is exported to the cell-surface where it can be recognized by the T cell receptor of CD8+ T cells (Ackerman et al., 2006).

1.4.2 Cross-presentation

In contrast to direct presentation, cross-presentation, enables the immune system to prime T cells for antigens that are not expressed in DC (or at least not in the priming DC), e.g. in the case of viruses that do not have DC tropism (like EBV) or that interfere with DC antigen-presentation (like other Herpes viruses). Therefore cross-presentation prevents that viral interference with e.g. antigen presentation would subvert the induction of T cell responses. Cross-presentation might also explain the priming of T cells specific for tumor-associated antigens that are expressed in transformed tissues but not in DC. Shortly after the discovery that viral antigens are presented to cytotoxic T cells by restricting MHC molecules (Zinkernagel and Doherty, 1974), Michael Bevan described CD8^{+} T cell priming by immunization of mice with antigen-expressing cells which lacked the restricting MHC molecules (Bevan, 1976). Generally, exogenous proteins acquired by DC in the periphery are degraded in the phago-lysosome where 10-12mer peptides can be recruited by recycling MHC class II molecules to be presented to CD4+ T cells. Specialized subsets of DC, in mice the non-migratory CD8+ splenic DC (den Haan et al., 2000; Iyoda et al., 2002), can prevent the acquired antigen from lysosomal degradation and preserve it to "cross" it to the classical MHC class I pathway for presentation to CD8+ T cells (Delamarre et al., 2005). It is discussed controversially, if cross-presentation requires the uptake of intact antigen (Norbury et al., 2004; Shen and Rock, 2004; Wolkers et al., 2004), pre-processed intermediates (Blachere et al., 2005; Serna et al., 2003) or chaperoned peptides (Binder and Srivastava, 2005). While different processing pathways have been described several studies found that crosspresentation required re-translocation into the cytosol for degradation by the proteasome and transport of antigenic peptides into the ER by TAP. Additionally, an TAP-independent, cathepsin S requiring vacuolar pathway as well as a phagosome-to-ER/ERAD pathway are discussed (Ackerman et al., 2006; Guermonprez and Amigorena, 2005; Shen and Rock, 2006). Very recent work suggests that in addition there might exist specialized uptake mechanisms that could specifically deliver antigen

into the crosspresentation or classical MHC class II pathway (Burgdorf et al., 2007; Dudziak et al., 2007).

1.4.3   The Ubiquitin-Proteasome-Degradation Pathway

The physiologic function of cells requires tight regulation of cellular proteins. In this regard it is essential to synthesize and to provide proteins when and where they are needed as well as to degrade them e.g. to preclude further action of regulatory proteins or the accumulation of toxic, non-functional and misfolded proteins. Protein degradation is a complex task as there are extremely short-lived, metabolically stable, lowly abundant and also compartmentalized proteins.

The discovery of the proteasome as an abundant cytoplasmic macromolecular structure that is responsible for the selective ATP-dependent degradation of polyubiquitylated protein substrates by Ciechanover, Hershko and Rose was awarded with the Nobel Prize in Chemistry 2004. The so-called 26S proteasome is composed by the 20S catalytic core complex and two 19S-regulator complexes which bind and unfold polyubiquitylated proteins. Ubiquitin is a 76-amino-acid polypeptide that is expressed as a polyubiquitin chain. Cytosolic isopeptidases specifically recognoize amino acid residue G76 and cleave the chain to release mono-ubiquitin. Conjugation of Ubiquitin to a protein substrate requires three steps: first, ubiquitin binds to a ubiquitin-activating enzyme (E1), then it is transferred to a ubiquitin-conjugating enzyme (E2) which together with a protein substrate is recruited by a ubiquitin ligase (E3). E3 ligases determine the specificity of the ubiquitylation process. Interestingly, several viruses have adapted E3 ligases to regulate cellular processes (Chen and Gerlier, 2006). Whereas ubiquitylation was originally regarded solely as a "kiss of death" that targets proteins by K48-linked polyubiquitylation for proteasomal degradation (Bachmair and Varshavsky, 1989; Chau et al., 1989), in recent years it has become evident that ubiquitylation interferes with many cellular processes: for example, K63-linked polyubiquitylation modulates protein-protein interactions and mono-ubiquitylation has been associated with downmodulation of receptors through the endosomal-lysosomal pathway. Recently the control of DNA methylation has been associated with histone-ubiquitylation (Sridhar et al., 2007, for a review on ubiquitylation see Liu et al., 2004).

Hydrolysis by the proteasome is regarded as the key step for the generation of most antigenic peptides (Michalek et al., 1993; Rock et al., 2004). Thereby the proteasome demonstrates a marked selectivity: the carboxy-terminal residues define the capacity of a peptide for entering the class I binding machinery. As cells are rich in aminopeptidases but lack carboxypeptidases peptide fragments created by the proteasome with a suitable carboxy-terminus can be further trimmed at their amino-terminal end, whereas peptides released from the proteasome without a suitable carboxy-terminus cannot be

further processed into binding peptides. The proteasome specificity is a major determinant in the selection of immunogenic peptides (Rock et al., 2004). MHC class I antigen presentation requires the processing of qualitatively and quantitatively sufficient antigenic peptides. It has been estimated that only about 1% of the peptides generated by means of protein breakdown are available for direct antigen presentation. Interestingly, rapid degradation of nascent proteins makes about one third of the intracellular proteolysis. A large pool of these proteins has been found to be ubiquityated and it has been postulated that defective proteins ("defective ribosomal products = DRIPS") constitute a large fraction of these newly synthesized and rapidly degraded proteins (Schubert et al., 2000, Turner et al, 2000). A link to translation would indeed ensure that cytotoxic T cells can "see" antigens before these can exert their specific functions. It is assumed that only such a link would allow lysis of infected cells before they produce viral progeny (for Review see Kloetzel, 2004; Strehl et al., 2005; Rock et al., 2004; Yewdell et al., 2003).

1.4.4 Antigen presentation pathways as targets for vaccination

The outcome of T cell priming is regulated among other factors by the amount of peptide/MHC class I complexes presented on APC and therefore can either enhance or limit T cell responses (Yewdell and Haeryfar, 2005). The expression of some antigens as a stable fusion to monomeric ubiquitin led to polyubiquitylation and rapid proteasomal degradation (Rodriguez et al., 1997; Tobery and Siliciano, 1997). This has been used as a strategy to enhance processing and MHC class I restricted presentation of antigenic peptides with the aim to improve CD8+ T cell responses. Because the immunogenicity of target antigens expressed by VACV has been correlated with the expression of these antigens in DC (Bronte et al., 1997) and because rapid degradation of a model antigen expressed by VACV could overcome a block in antigen-presentation (Townsend et al, 1988), it was hypothesized that targeted degradation of MVA-encoded antigens by ubiquitylation could enhance cytotoxic T-cell responses by enhancing direct antigen presentation. However, MVA bears characteristics that in principle enable direct as well as cross-presentation: MVA has the ability to infect and to efficiently produce viral and recombinant antigens in both pAPC and non-pAPC (Kastenmuller et al., 2006). Interestingly, MVA induces CD8+ T cells which recognize so-called late viral antigens that are not synthesized within infected DC due to an early block of the viral life cycle in these pAPC, and thus appear to be cross-primed (Chahroudi et al., 2006; Di Nicola et al., 2004; Drexler et al., 2003).
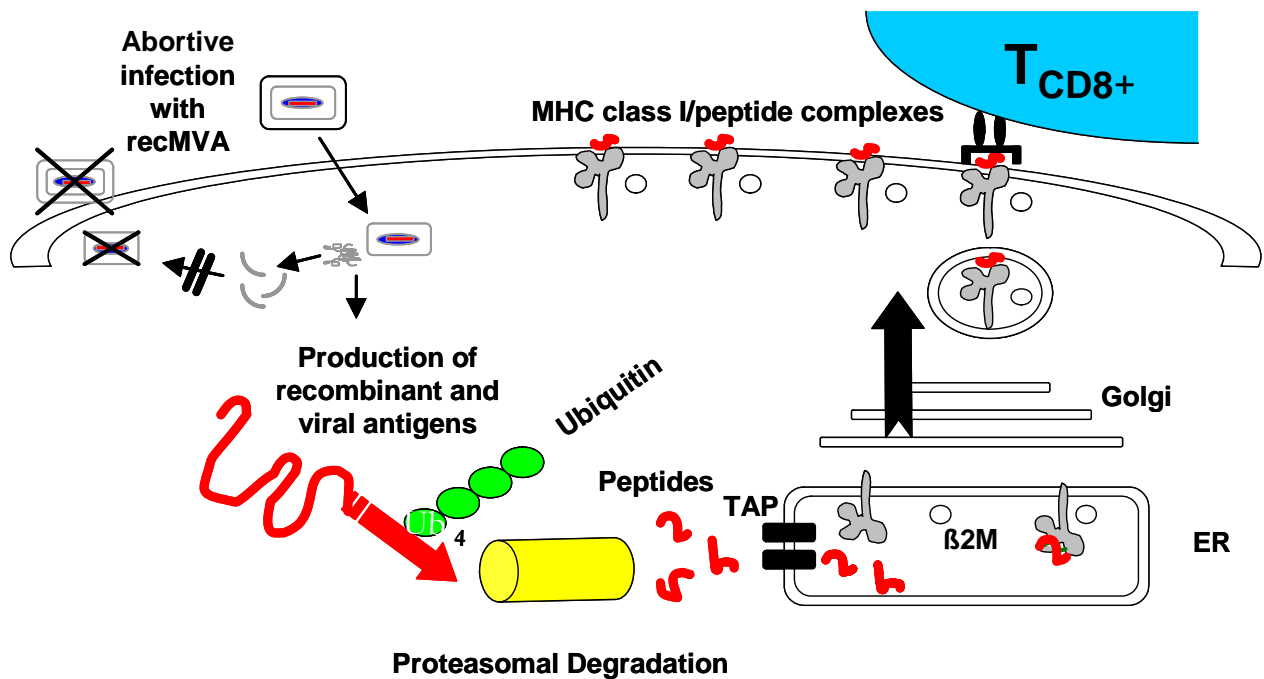
Schematic   Proteasomal degradation and endogenous presentation of antigens delivered by MVA   Abortive infection with MVA induces the synthesis of recombinant and viral antigens. Ubiquitylation targets antigen for proteasomal degradation. Peptides can be transported by TAP into the ER where they can be further trimmed and bind to newly synthesized MHC class I molecules. These peptide/MHC-complexes travel to the surface of infected cells where they can be recognized by CD8+ T cells.

While for some antigens and vectors forced proteasomal degradation indeed enhanced immune responses it remained unclear why in other cases it failed to do so (Wong et al., 2004). Metabolic stability has been discussed as a critical factor for the availability and access of antigen for the two antigen presentation pathways (Norbury and Sigal, 2003). In contrast to earlier reports, several recent studies indicate that stable antigen might be the substrate for efficient cross-priming, whereas the expression of peptides or rapidly degradable protein is thought to enhance endogenous presentation and thereby direct priming (Norbury et al., 2004; Shen and Rock, 2004; Tobery and Siliciano, 1997; Wolkers et al., 2004), (see also 1.4.2.). Consequently, for efficient stimulation of CD8+ T-cells, antigens require distinct features to be presented optimally by a particular presentation pathway. For vaccine development it might be crucial to characterize which antigen presentation pathway is important to induce efficient T-cell immunity with a particular vector.

long-lived antigen   **short-lived antigen / peptides**
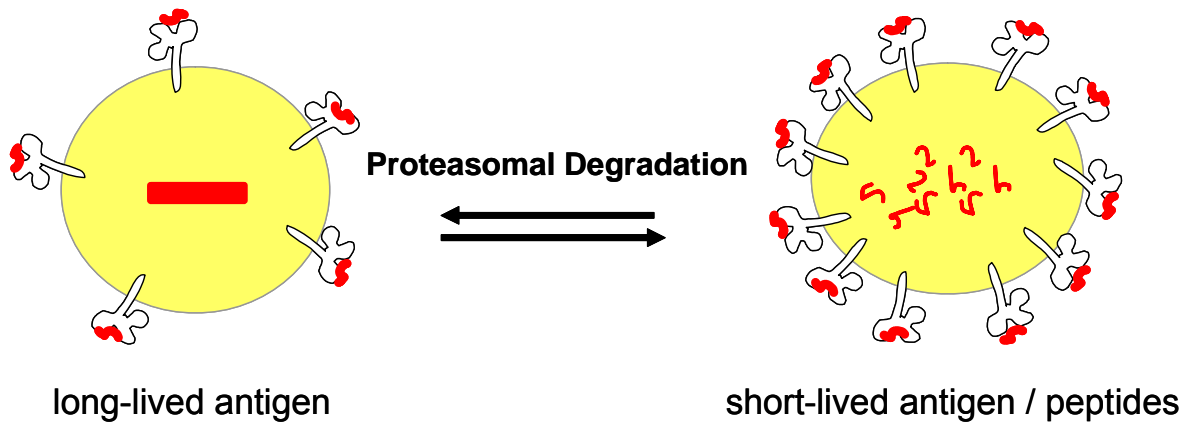
Schematic   The half-live of antigens can influence the availability for different antigen presentation pathways Short-lived antigen and peptides have been shown to enhance loading of MHC molecules via the endogenous route. For crosspresentation DC acquire antigen from other cells and it has been discussed controversially whether long-lived or short-lived proteins are the physiologically relevant source for this antigen presentation pathway. (= MHC class I molecule)

In this regard it is important to distinguish between antigen presentation and T cell priming as the latter might be regarded as an outcome or a consequence of the first. In a presumed scenario of equally and simultaneously occurring direct and crosspresentation of viral antigens, it is evident that other factors like maturation status, location or functional viability (which could differentially affect infected versus non-infected DC) will determine whether the antigen presenting APC will or will not become an T cell priming APC. One key to enhance vaccine efficiency might be to optimally define the properties of target antigens to achieve strong antigen presentation on the priming APC. Therefore it is essential to define for each vector which antigen presentation pathways govern the induction and expansion of CD8+ T cells to enable the selection of efficient antigen formulations (Yewdell and Haeryfar, 2005).

1.5   *Vector Immunity and Immunodominance*

Complex pathogens bear a large number of antigens comprising an enormous amount of peptides that potentially could be presented to CD8+ T cells to induce immune responses. Although the immune system is confronted with such a vast variety of pathogen-specific determinants, T cell responses to viral infections have been found to be directed against a rather small number of antigens. An immunodominance hierarchy reflects the different size of T cell responses against distinct epitopes.

Replication competent vaccinia virus (VACV) encodes ~58.000 amino acids which could theoretically lead to at least 175.000 peptides that could bind to MHC class I molecules (Yewdell 2006). However, CD8+ T cells recognizing as few as ~50 of these potential peptides appear to account for >90% of the total VACV response in C57BL/6 mice (Moutaftsi et al., 2006). In addition, the T cells directed against one single peptide derived from the B8R gene product account for up to the half of the total VACV-specific CD8+ T cells, clearly dominating the VACV-response in C57BL/6 mice (Tscharke et al., 2005). As immunodominant vector-specific responses have been observed in humans and are implicated to account for a limited effectiveness of recombinant vaccines (Smith et al., 2005) a thorough understanding of how immunodominance hierarchies are established might be essential for improved vaccination protocols.

Our knowledge on immunodominance is largely based on experiments investigating Influenza A virus, LCMV (lymphocytic choriomeningitis virus) or Listeria moncytogenes in infection models or by using peptide-pulsed DCs and adoptively transferred TCR transgenic T cells (Yewdell and Bennink, 1999), indicating that T cells can compete at the level of APCs. While the competition of T cells of the same specificity has been clearly demonstrated, competition between T cells of different specificities (cross-competition) is still controversial. There are only a few reports in the literature, which argue for (HSV, Influenza) and against (LCMV) a relevance for cross-competition during the primary induction of an antiviral immune response (Probst et al., 2002, Stock et al., 2006, Thomas et al., 2007, for Review see Kedl et al., 2003)

Several important features for the development of immunodominance hierarchies of T cells have been identified: 1) CD8$^+$ T cells of the same specificity compete for the access to APCs, 2) T cell expansion depends on the precursor frequency, 3) is affected by T cell receptor affinity and 4) can be controlled by APC killing (e.g. LCMV) , or 5) in the absence of APC killing (e.g. Influenza) by downmodulation of antigen from the APC or competition for anti-apoptotic cytokines (Chen et al., 2002, Chen et al., 2004, Yang et al., 2006). Importantly, the immunodominance hierarchy of T cells can dramatically change between primary and secondary immune responses. In the case of Influenza virus this has been imputed to differential antigen presentation during primary and secondary immune responses (Crowe et al., 2003).

Up to now most of the work on immunodominance has been done on viruses with relatively small genomes (10-20 kb). Large DNA viruses like Herpes- or Poxviruses (~ 200 kb) represent a great challenge for the analysis of immunodominance. However, they also offer the possibility to identify crucial mechanisms of immunodominance, since the immune system exceedingly shapes the immune response by trimming it down to reactivity against a few epitopes. Recently identified HLA-A2- and H2-K^b/D^b-restricted poxvirus determinants were derived from a large variety of proteins (Drexler et

al., 2003; Moutaftsi et al., 2006; Oseroff et al., 2005; Pasquetto et al., 2005; Terajima et al., 2003; Tscharke et al., 2005). Usage of these epitopes allowed for the first time a comprehensive analysis of immunodominance in poxviral T cell responses in C57BL/6 and HLA-A2 transgenic mice. The use of MVA for this study bears the advantage of an abortive and largely synchronized infection to minimize an overlap of the phases of viral gene expression by cells, which in the case of replication-competent viruses are infected at different time points during virus spreading.

Aim of thesis

Numerous clinical studies currently evaluate viral vectors as recombinant vaccines albeit very little is known about biological properties of target antigens that are essentially required to induce efficient T cell immunity with these vectors. The present thesis aimed to investigate the immunological mechanisms governing priming and expansion of recombinant and vector-specific T cells with vaccines based on the attenuated vaccinia virus MVA, in order to define basic antigenic requirements for the rational use of target antigens:

1)   As a major question it was asked which antigen presentation pathways mediate the induction of primary and secondary T cell responses when vaccinating with MVA. Mice transgenic for the human HLA-A*0201-molecule or wildtype C57BL/6 mice should be vaccinated with recombinant MVA expressing different antigen formulations of the model antigens ovalbumin and the human tumor-associated antigen tyrosinase to compare the influence of antigen metabolic stability and resulting antigen presentation on the primary CTL induction as well as the secondary expansion of memory T cells *in vivo*.

Therefore, a recombinant MVA should be constructed expressing tyrosinase as a stable fusion to ubiquitin (MVA-Ub/Tyr) with the aim to target tyrosinase for rapid proteasomal degradation and to enhance processing and MHC class I restricted presentation of antigenic peptides. MVA-Ub/Tyr should be characterized *in vitro* and *in vivo* and then be compared to other recombinant viruses encoding for either stable full-length tyrosinase (MVA-Tyr) or expressing the Tyr_{369}-peptide as part of a polytope (MVA-Mini-Tyr). Different experimental approaches should be established to allow delineating the role of different antigen presentation pathways for MVA immunizations. Moreover, it should be tested whether the gained knowledge could help to improve MVA vaccine efficacy.

2)   Anti-vector-immunity and immunodominance of vector-specific T cells has been regarded as one of the major drawbacks of complex viral vectors. To learn about the interdependence of responses specific for recombinant and vector-antigens, different recombinant and knock-out MVA should be used to investigate if and to which extent vector-specific T cells influence the priming and/or the expansion of target specific T cells. Based on these results it should be tested whether it is possible to improve the response to target antigens and to limit the response to vector antigens.

Taken together the aim of this work was to define how target antigens can be used in vaccinations with recombinant MVA in order to enhance target-specific CTL immunity.

2 Materials

2.1 Chemicals

CHEMICAL	MANUFACTURER
2-β-Mercaptoethanol	Sigma *(Munich)*
Acrylamid/Bisacrylamid (30%)	National Diagnostics *(Atlanta,GA, USA)*
Agarose	Gibco/BRL *(Eggenstein)*
Ammoniumperoxidsulfat (APS)	Sigma *(Munich)*
Bacto Agar	Difco Laboratories *(Detroit, MI, USA)*
Brefeldin A	Sigma *(Munich)*
Bromphenolblue	Serva *(Heidelberg)*
Coomassie-Blue G250	Sigma *(Munich)*
^{51}Cr (Na^{51}CrO4)	MP Biomedicals *(Eschwege)*
DMSO	Merck *(Darmstadt)*
DTT	Serva *(Heidelberg)*
EDTA	Sigma *(Munich)*
Ethidiumbromide	Serva *(Heidelberg)*
Glycerol	Roth *(Karlsruhe)*
Monensin	eBiosciences *(San Diego)*
NP-40	Serva *(Heidelberg)*
Reti-Phenol/Chloroform/Isoamylalcohol	Roth *(Karlsruhe)*
Paraformaldehyd (PFA)	Sigma *(Munich)*
Ponceau S	Sigma *(Munich)*
TEMED	Bio-Rad *(Munich)*
Triton X-100	Sigma *(Munich)*
Trypan blue	Biochrom KG *(Berlin)*
Tween 20	Sigma *(Munich)*

2.2 Buffers and Solutions

NAME	COMPOSITION
DNA sample buffer (5x)	50 % TE buffer pH8 (v/v) 50 % Glycerol (v/v) 0.04 % Bromphenol blue (w/v)
FACS buffer pH 7.4	1 % BSA (w/v) 0.02 % NaN_3 from 20% stock (w/v) in 1x PBS
LB agar	1.5 % Agar in LB-Medium
LB medium pH 7.0	1 % casein extract (w/v) 0.5 % yeast extract (w/v) 0.5 % NaCl (w/v) 0.1 % glucose (w/v)
Paraformaldehyd (PFA)	2% Paraformaldehyde (w/v) in PBS buffer
PBS buffer pH 7.4	0.14 M NaCl 2.7 mM KCl 3.2 mM Na_2HPO_4 1.5 mM KH_2PO_4
RIPA buffer pH 7.4	50 mM Tris-HCl 1% NP-40 (v/v) 0.25% Na-deoxycholate (w/v) 150 mM NaCl 1 mM EDTA
SDS-PAGE buffer pH 8.3 (10x)	25 mM Tris 192 mM Glycine 0.1 % SDS (w/v)
SDS-PAGE fixing buffer	50% Methanol (v/v) 40% H_2O (v/v) 10% acetic acid (v/v)
SDS-PAGE loading buffer pH 6.8 (2x)	50 mM Tris 2 % SDS (w/v) 0.04 % Bromphenol blue (w/v) 84 mM 2-Mercaptoethanol

	20 % Glycerol (v/v)
Sucrose 36 % pH 9.0	36% sucrose (w/v) in 10 mM Tris
TAC medium	90% NH_4Cl from 0.16 M stock 10% Tris pH 7.65 from 0.17 M stock
TAE buffer pH 8.0	40 mM Tris/HCl 1 mM EDTA 20 mM sodium acetate
TE buffer pH 8.0	10 mM Tris/HCl 0.1 mM EDTA
TEN buffer pH 7.4 (10x)	100 mM Tris 10 mM EDTA 1 M NaCl
Tris buffer pH 9.0 (1 mM)	
Tris buffer pH 9.0 (10 mM)	
WB stripping buffer pH 6.8	100 mM 2-Mercaptoethanol 2 % SDS (w/v) 62.5 mM Tris/HCl
WB transfer buffer anode pH 8.3	25 mM Tris-Base 192 mM Glycin 20 % Methanol (v/v)
WB transfer buffer cathode pH 8.3	0.5% SDS (w/v) in WB transfer buffer anode
WB-buffer	1% BSA (w/v) in 1x PBS

Unless stated otherwise, buffers were prepared in ultrapure H2O milliQ. The pH was adjusted with HCl or NaOH.

2.3 Cell Culture Media

NAME	COMPOSITION
Freezing Medium	90 % FBS (heat inactivated at 56°C) 10 % DMSO
LB agar	1.5 % Agar in LB-Medium
LB medium	1 % casein extract (w/v) 0.5 % yeast extract (w/v) 0.5 % NaCl (w/v) 0.1 % glucose (w/v)
RIPA Starving Medium	1% Ultraglutamin 1%Pyruvat in DMEM
RPMI 10%/5%/2%	RPMI 1640 supplemented with: 2-10% FCS (heat inactivated at 56°C) 1% Pen-Strep for murine cells medium was supplemented with 50 µM 2-Mercaptoethanol

2.4 Biochemicals

PRODUCT	MANUFACTURER
1 kb DNA Ladder	Invitrogen *(Karlsruhe)*
Ampicillin	Serva *(Heidelberg)*
Bovine serum albumin (BSA)	Sigma *(Munich)*
Desoxyribonucleotides	Roche *(Mannheim)*
Dextransulfate	Sigma *(Munich)*
DMEM	Cambrex, BioWhittaker *(Verviers, Belgium)*
FCS (Fetal Calf Serum)	Biochrom KG *(Berlin)*
GeneRuler 1 kb DNA Ladder	Fermentas *(St. Leon-Rot)*
Geneticin (G418)	Gibco BRL *(Karlsruhe)*
Kanamycine	Serva *(Heidelberg)*

L-[^{35}S] Methionine cell labelling mix	Amersham *(Little Chalfont, UK)*
Lactacystin	Sigma *(Munich)*
LPS (Lipopolysaccharide)	Sigma *(Munich)*
MG132 = Z-Leu-Leu-Leu-al	Sigma *(Munich)*
Na-Pyruvate	Cambrex, BioWhittaker *(Verviers, Belgium)*
Pen-Strep (10.000 U Penicillin/ml, 10 mg/ml Streptomycin)	Cambrex, BioWhittaker *(Verviers, Belgium)*
Phenylmethylsulfonyl fluoride (PMSF)	Sigma *(Munich)*
Prestained Protein Ladder „BroadRange" (6-175 kDa)	Amersham *(Little Chalfont, UK)*
Protein G-Plus Agarose	SC Biotechnology *(Santa Cruz, CA, USA)*
RPMI 1640	Biochrom KG *(Berlin)*
Ultraglutamine (200 mM in 0.85% NaCl)	Cambrex, BioWhittaker *(Verviers, Belgium)*

2.5 Enzymes

PRODUCT	MANUFACTURER
Alcaline Phosphatase	Roche *(Mannheim)*
Calf intestinal Phosphatase	New England BioLabs *(Schwalbach)*
Collagenase VIII	Sigma *(Munich)*
DNAse I	Sigma *(Munich)*
Klenow-Enzyme	Roche *(Mannheim)*
Taq and Pwo DNA Polymerase	Roche *(Mannheim)*
Proteinase K	Sigma *(Munich)*
Restriction enzymes	Roche *(Mannheim)*/NEB BioLabs *(Schwalbach)*
T4-DNA-Ligase	Roche *(Mannheim)*
Trypsin-EDTA	Invitrogen *(Karlsruhe)*
Titan One Tube RT-PCR System	Roche *(Mannheim)*

2.6   Kits

PRODUCT	MANUFACTURER
BD Cytofix/Cytoperm Kit	BD Pharmingen *(Hamburg)*
EndoFree Plasmid Mega Kit	QIAGEN *(Hilden)*
DC Protein Assay Kit	Bio Rad *(Munich)*
Lipofectamin2000	Invitrogen *(Karlsruhe)*
Lumi-Light (Western-Blot Substrat)	Roche *(Mannheim)*
PCR-Master-Mix	Roche *(Mannheim)*
QIAGEN Plasmid Maxi Kit	QIAGEN *(Hilden)*
QIAquick Gel Extraction Kit	QIAGEN *(Hilden)*

2.7   Synthetic Oligonucleotides

Oligonucleotides were synthesized with an ABI oligonucleotide synthesizer and subsequently lyophilized by Mr. Linzer (GSF, Neuherberg).

Name	Short Description	Sequence
Primer 1	BamHI-Ubiquitin	5´-GGG CGG ATC CGA CCA TGC AGA TCT TCG TGA AGA CCC TGAC-3´
Primer 2	Ubiquitin_(15bp Tyr)	5´-CAA AAC AGC CAG GAG CAT CGC ACC TCT CAG GCG AAG GAC CAG-3´
Primer 3	(18bp Ub)-Tyrosinase	5´-CGC CTG AGA GGT GCG ATG CTC CTG GCT GTT TTG TAC TGC CTG- 3´
Primer 4	Tyrosinase-PmeI	5´-GGG CGT TTA AAC TTA TAA ATG GCT CTG ATA CAA GCT GTG GT- 3´
Del III 3' (GS83)	MVA Deletion III 3´	5' GAA TGC ACA TAC ATA AGT ACC GGC ATC TCT AGC AGT 3'
Del III 5' (IIIf1b):	MVA Deletion III 5´	5' CAC CAG CGT CTA CAT GAC GAG CTT CCG AGT TCC 3'
K1Lint-1	K1L-marker gene	5'- TGA TGA CAA GGG AAA CAC CGC -3
K1Lint-2	K1L-marker gene	5'- GTC GAC GTC ATA TAG TCG AGC -3'

2.8 Plasmids

PLASMID	REFERENCE
pIIIΔHR-P7.5	(Staib et al, 2003)
pcDNAI-hTyr	(Drexler et al., 1999)
pSFV1-huTyr	Obtained from I. Drexler

2.9 Synthetic Peptides

All synthetic peptides were purchased from Biosynthan *(Berlin)*. Peptides were diluted in DMSO (1mg/ml, for peptide vaccinations 10mg/ml) and stored at -80°. For peptide coating of cells (ICS and In vivo cytotoxicity assay, see 3.6.4 and 3.6.10) stocks were used 1:1000, resulting in 1µg/ml final concentration.

PEPTIDE	MHC RESTRICTION	AMINOACID SEQUENCE	ORIGIN	REFERENCE
Peptides derived from recombinant antigens:				
Tyr_{1-9}	HLA-A*0201	MLLAVLYCL	Human Tyrosinase	(Wolfel et al., 1994)
Tyr_{369}	HLA-A*0201	YMDGTMSQV	Human Tyrosinase	(Skipper et al., 1996)
OVA_{257}	H2-K^b	SIINFEKL	Chicken Ovalbumin	(Rotzschke et al., 1991)
$H2N_{435}$	HLA-A*0201	ILHNGAYSL	Human Her2/neu	(Rongcun et al., 1999)
Peptides derived from VACV / MVA viral proteins:				
$A6L_6$	HLA-A*0201	VLYDEFVTI	117L-A6L	(Oseroff et al., 2005; Pasquetto et al., 2005)
$B22R_{79}$	HLA-A*0201	CLTEYILWV	189R-B22R	(Terajima et al., 2003)
$C7L_{74}$	HLA-A*0201	KVDDTFYYV	018L-C7L	(Terajima et al., 2003)
$D12L_{251}$	HLA-A*0201	RVYEALYYV	109L-D12L	(Oseroff et al., 2005; Pasquetto et al., 2005)
$H3L_{184}$	HLA-A*0201	SLSAYIIRV	093L-H3L	(Drexler et al., 2003)
$I1L_{211}$	HLA-A*0201	RLYDYFTRV	062L-I1L	(Oseroff et al., 2005; Pasquetto et al., 2005)

A3L$_{270}$	H2-K^b	KSYNYMLL	122L-A3L	(Moutaftsi et al., 2006)
A8R$_{189}$	H2-K^b	ITYRFYLI	119R-A8R	(Moutaftsi et al., 2006)
A42R$_{88}$	H2-D^b	YAPVSPIV	154R-A42R	(Tscharke et al., 2005)
B8R$_{20}$	H2-K^b	TSYKFESV	176R-B8R	(Tscharke et al., 2005)
K3L$_6$	H2-D^b	YSLPNAGDVI	024L-K3L	(Tscharke et al., 2005)
Control Peptides				
ß-Gal$_{96}$	H2-K^b	DAPIYTNV	ß-Galactosidase	(Overwijk et al., 1997)
Flu M1$_{58}$	HLA-A*0201	GILGFVFTL	Influenza Virus Matrix Protein M1	(Bodmer et al., 1989)

2.10 MHC-Multimeres

A2K^b and K^b Multimeres were kindly provided as PE conjugates by Prof. Dirk Busch, Munich.

2.11 Antibodies

SPECIFICITY	CLONE	SPECIES/ ISOTYPE	CONJUGATE	MANUFACTURER/ REFERENCE
Tyrosinase	T 311	Mouse monoclonal IgG2a	-	Novocastra, Newcastle UK
Tyrosinase	C19	Goat polyclonal	-	Santa Cruz, Heidelberg
IFNγ	XMG1.2	Rat IgG1	FITC	BD Pharmingen, Heidelberg
IgG1 isotype	R3-34	Rat IgG1	FITC	BD Pharmingen

CD3ε	145-2C11	Armenian Hamster IgG1	APC/PE	BD Pharmingen
CD8α	5H10	Rat IgG2b	FITC/APC	Caltag/Invitrogen, Karlsruhe
CD11c	HL3	Armenian Hamster IgG1	APC	BD Pharmingen
CD16/32 Fc Block	2.4G2	Rat IgG2b	-	BD Pharmingen
CD62L	MEL-14	Rat IgG2a	PE	Caltag/Invitrogen
CD107a	1D4B	Rat IgG2a	FITC	BD Pharmingen
CD107b	ABL-93	Rat IgG2a	FITC	BD Pharmingen
SIINFEKL/K^b	25-D1.16	Mouse	-	(Porgador et al., 1997)
anti-mouse-IGg F(ab´)$_2$-Fragments		Rat	Alexa Fluor 633	Molecular Probes, Eugene

Appropriate isotype controls were used from the same manufacturers as the relevant antibodies.

Antibodies that were used for purification and analysis of DC are described in 3.6.9

2.12 *Fluorescent Dyes*

Dye	Stock Concentration	Final Concentration	Manufacturer
CFSE (Carboxy Fluoroscein Succinimidyl Ester)	5mM	5µM or 0,5µM	Molecular Probes, Eugene
PI (Propidium Iodide)	10mg/ml	1µg/µl	Molecular Probes, Eugene
EMA (Ethidium Monazide Bromide)	2mg/ml	1µg/ml	Sigma

2.13 Viruses

VIRUS	FULL NAME	REFERENCE
MVA wt	MVA IInew	(Staib et al., 2003)
MVA-Tyr	MVA-huTyr P7.5	(Drexler et al., 1999)
MVA-Ub/Tyr	MVA-Ub/huTyr P7.5	Constructed during this work, (Gasteiger, Kastenmuller et al, *under revision*)
MVA-Mini-Tyr	MVA-pMel P7.5	Obtained from I. Drexler, (Gasteiger, Kastenmuller et al, *under revision*)
MVA-H2N	MVA-huHer-2/neu P7.5	Obtained from I. Drexler, (Gasteiger, Kastenmuller et al, *under revision*)
MVA-OVA	MVA-OVA P7.5	(Kastenmuller, Gasteiger et al, 2007)
MVA-OVA PK1L		(Kastenmuller, Gasteiger et al, 2007)
MVA-OVA P11		(Kastenmuller, Gasteiger et al, 2007)
MVA-SIINFEKL	MVA-MSIINFEKL P7.5	Obtained from I. Drexler, (Gasteiger, Kastenmuller et al, *under revision*)
MVA ΔH3L P7.5 H3L		(Kastenmuller, Gasteiger et al, 2007)
MVA-dB8R		(Kastenmuller, Gasteiger et al, 2007)
MVA-OVA P7.5 dB8R		(Kastenmuller, Gasteiger et al, 2007)
CVA	Chorioallantois vaccinia virus Ankara	Generous gift from A. Mayr, Munich

2.14 Bacteria

E.coli DHB10 were purchased from Gibco BRL, Karlsruhe

Listeria monocytogenes expressing Ovalbumin (L.m. Ova) was kindly provided by Christian Stemberger and Prof. Dirk Busch, Munich

2.15 Cell lines

NAME	DESCRIPTION	ATCC Number / Reference
B-LCL	Human HLA-A*0201 positive lymphoblastoid B cells	Kind gift from Dr. W. Kastenmüller
DC2.4	Murine DC	Kind gift from Dr. KL Rock
NIH3T3	Murine Fibroblasts	CRL-1658
RMA	Murine Thymoma cell line	Kind gift from Dr. F. Lemmonier
RMA-HHD	RMA cells transfected with the HHD (Chimeric HLA-A*0201) molecule	Kind gift from Dr. F. Lemmonier
RMA-S-HHD	RMA-HHD cells that are TAP-deficient	Kind gift from Dr. F. Lemmonier
A375	Human HLA-A*0201 positive melanoma cells	(CRL-1619)

All used CTL lines were generated from splenocytes of MVA or peptide vaccinated HHD mice, weekly restimulated and kindly provided by Ronny Ljiapoci and PD Dr. Ingo Drexler as previously described (Drexler et al., 1999; Drexler et al., 2003)

2.16 Mice

All mice were derived from in-house breeding under specific pathogen-free conditions at the GSF animal facility in Neuherberg following institutional guidelines.

STRAIN	MHC RESTRICTION	REFERENCE
HHD	HLA-A02*01	(Pascolo et al., 1997)
C57BL/6	H2-K^b and H2-D^b	http://jaxmice.jax.org

HHD II is an inbred strain of transgenic mice on a C57BL/6 background. The endogenous H-2 D^b and β2-microglobulin (β2m) gene loci are disrupted and a chimeric human (α1, α2 and mouse α3) HLA-A2.1 heavy chain covalently linked to the human β2m light chain (together called the HHD molecule) is introduced. As the export of MHC molecules to the cell surface requires association with ß2m CD8+ T cells of these mice are educated on and restricted to the HHD molecule. This animal model allows the study of CTL dependent immunity to HLA-A2.1 restricted antigenic determinants in mice (Pascolo et al., 1997).

2.17 Consumables

PRODUCT	MANUFACTURER
3MM-Filter papier	Whatman *(Maidstone)*
Cell culture flasks (T25, T75, T185, T225)	Greiner *(Nürtingen)*, Corning *(New York)* Nunc *(Wiesbaden)*
Cell culture plates 6-, 12-, 24-, 96-well	Corning *(New York)*
Cell lifter	Corning *(New York)*
Cell strainer 100µm	BD Pharmingen *(Hamburg)*
FACS tubes	Bio-Rad *(Munich)*
Falcon tubes (15 ml, 50 ml; PS, PP)	BD Pharmingen *(Hamburg)*
Gene Pulser cuvettes	Bio-Rad *(Munich)*
Gloves	Kimberly-Clark *(Mainz)*
Hyperfilm™ ECL	Amersham *(Little Chalfont)*
LumaPlate™-96	PerkinElmer *(*Waltham)
Nitrocellulose membrane 0,45µM	Bio-Rad *(Munich)*
PCR reaction tubes	Eppendorf *(Hamburg)*
Petri dishes	Nunc *(Wiesbaden)*
ART Pipette tips	Molecular Bioproducts *(San Diego)*
Pipettes 'Cellstar' (1-25 ml) Greiner,	Corning *(New York)*
Reaction tubes (0,5 ml, 1,5 ml, 2 ml)	Eppendorf *(Hamburg)*
Sterile filters (Minisart 0,2-0,45 µm)	Sartorius AG *(Göttingen)*
Syringes (5, 10, 20 ml)	BD Pharmingen *(Hamburg)*
Syringes (Omnifix-F 1 ml)	Braun *(Melsungen)*
Ultracentrifuge tubes (UltraClear)	Beckmann *(Munich)*

2.18 Laboratory Equipment

NAME	TYPE	MANUFACTURER
Centrifuge	Avanti J-25 Megafuge 1.0R Biofuge fresco Biofuge pico	Beckman *(Munich)* Heraeus *(Hanau)* Heraeus *(Hanau)* Heraeus *(Hanau)*
CO_2 Incubator	Function Line Hera Cell 150 Cellstar	Heraeus *(Hanau)* Nunc *(Wiesbaden)*
Contamination monitor	LB 122	Berthold *(Bad Wildbad)*
Cup sonicator	Sonopuls HD200/UW200	Bandelin *(Berlin)*
DNA/RNA Calculator	GeneQuant II	Pharmacia Biotech *(Uppsala, Sweden)*
Electro-blotting System	Fastblot B33/B34	Biometra *(Goettingen)*
Electrotransformator	E. coli Pulser	Bio-Rad *(Munich)*
Film processor	Curix 60	Agfa *(Köln)*
Flow cytometer	FACS Canto	Becton Dickinson *(Hamburg)*
Freezer (-20°C)	Excellence	Bauknecht *(Stuttgart)*
Freezer (-80°C)	Hera freeze Ult 2090	Heraeus *(Hanau)* Revco *(Asheville, USA)*
Fridge (4°C)	UT6-K	Bauknecht *(Stuttgart)*
Gel Dryer	Model 583	Bio-Rad *(Munich)*
Haematocytometer	Neubauer counting chamber	Karl Hecht KG *(Sondheim)*
Horizontal Electrophoresis System	A1 Gator A2 Gator	Owl Scientific *(Portsmouth, USA)*
Ice machine	AF 200	Scotsman *(Milan, Italy)*
Incubation shaker	Innova 4430	New Brunswick Scientific *(Nürtingen)*
Laminar flow	HERAsafe HS 12	Heraeus *(Hanau)*
Magnetic stirrer	Ikamag Reo	IKA Werke *(Staufen)*
Micropipette	Pipetman P10-1000	Gilson *(Middleton, USA)*
Microplate reader	Model 550	Bio-Rad *(Munich)*

Microscope	Kolleg SHB 45 Axiovert 25	Eschenbach *(Nürnberg)* Carl Zeiss *(Oberkochen)*
Microwave	900W	Siemens *(Munich)*
Multi channel pipette	Transferpette-12 (20-200 µl) Calibra 852	Brand *(Wertheim)* Socorex *(Ecublens, Switzerland)*
Nitrogen container	Cryo 200	Forma Scientific *(Waltham, USA)*
PCR Cycler	GeneAmpR PCR System 2700	Applied Biosystems *(Foster City, USA)*
pH-Meter	InoLab pH Level 1	WTW GmbH *(Weilheim)*
Phosphor Imager	Molecular Imager PersonalFX	Bio-Rad *(Munich)*
Phosphor Imager Screen	Imaging Screen-K	Bio-Rad *(Munich)*
Phosphor Screen Eraser	Screen Eraser-K	Bio-Rad *(Munich)*
Pipettor	easy jet pipetman	Eppendorf *(Hamburg)* Gilson *(Middleton, USA)*
Power supply unit	Model 200 / 2.0 Power Pac	Bio-Rad *(Munich)* Biometra *(Goettingen)*
Rotor	Typ 19, SW28, SW 41	Beckmann *(Munich)*
Steam Sterilizer	Varioklav 500E	H+P *(Oberschleißheim)*
Szintillator	TopCount NXT	Packard *(Mediden)*
Thermomixer/ -block	Thermomixer 5436 Comfort	Eppendorf *(Hamburg)* Eppendorf *(Hamburg)*
Ultracentrifuge	Optima LE-8K	Beckmann *(Munich)*
UV Lamp	UVT 2035	Hero Lab *(Wiesloch)*
Vertical Electrophoresis System	P9DS Emperor Penguin™	Owl Scientific *(Portsmouth, USA)*
Vortexer	VF2 Vortex Genie 2	IKA Werke *(Staufen)* Scientific Industries *(Bohemia, USA)*
Waterbath	Assistant VTE Var 3185	Hecht *(Sondheim)*

2.19 Software

PRODUCT	MANUFACTURER
FacsDIVA	Becton Dickinson, Heidelberg
FlowJo 6.4.2	Treestar, Ashland
GraphPadPrism 4	Graph Pad Software, San Diego
Quantity One 4.1.1	Bio Rad, Munich
MS Office	Microsoft, Redmond

3 Methods

3.1 *Mammalian Cell Culture*

Mammalian cells were cultured and handled under sterile conditions. Culture was carried out at 37°C in an incubator providing a 5% CO_2 atmosphere and 95% humidity.

All cell lines used were grown in RPMI medium. Medium was supplemented with 1 % penicillin-streptomycin and 5% or 10% fetal calf serum (FCS), referred to as 5% RPMI and 10% RPMI respectively, depending on the growth rate of cells and the frequency of their use.

Cell lines were either grown in suspension or as monolayers in T185 cell culture flasks. When cells had reached approximately 90 % confluence they were split at a ratio of 1:2 to 1:10 depending on their growth kinetics and intended use. For adherent cell lines medium was removed, the monolayer was washed with PBS and then covered with 3 ml trypsin-EDTA solution and incubated at 37°C for approximately 3 minutes. 7 ml fresh RPMI medium were added to the trypsin-solution and cells were singularized by resuspension and required fractions were transferred into a T185 flask with fresh medium or plated onto cell culture plates.

3.1.1 Cryo conservation of eukaryotic cells

Only cells in their exponential growth phase were subjected to freeze storage. Cells cultivated in a T185 cell culture flask were harvested by trypsination and pelleted for 5 min at 4°C and 1,400 rpm. The cell pellet was carefully resuspended in cold freezing medium and transferred to sterile cryo tubes in 1 ml aliquots. The cells were frozen slowly by storing them over night in slow-cooling containers at 80°C. After 24 h the tubes were transferred to liquid nitrogen (-196 °C) for long term storage.

3.1.2 Thawing of cryo conserved eukaryotic cells

To recultivate cryo conserved cells the cell suspension was thawed in a 37°C water bath and transferred into 10 ml of pre-heated RPMI 10%. The cells were washed once and the cell pellet was resuspended in 10 ml of pre-heated medium. The cell suspension was transferred into a T185 cell culture flask and cultivated at 37°C.

3.2 Bacteriological Techniques

3.2.1 Culture of *E.coli*

E.coli were cultured at 37°C, 5 % CO_2 and grown in liquid culture on a shaker or on agar plates.

Culture techniques used for growth of *E.coli*:

Culture	Medium	Antibiotic	Volume
after transfection (1h pre-culture)	LB-medium	-	0.8 ml
after transfection (over night)	Bacto-Agar Plates	100 µg/ml	
Analytical plasmid preparation	LB-medium	100 µg/ml	2-4 ml
High yield plasmid preparation	LB-medium	100 µg/ml	250 ml
Generation of electrocompetent cells (pre-culture)	LB-medium	-	100 ml
Generation of electrocompetent cells	LB-medium	-	2 x 250 ml

3.2.2 Generation of electro competent bacteria

The production of electro competent bacteria was carried out under sterile conditions using only autoclaved equipment and solutions. About 100 µl of a glycerin culture of the E.coli strain DH10B were spread on an agar plate without selective antibiotic. After over night growth at 37°C in an incubator, a single bacterial colony was picked and transferred to a 5 ml LB-medium pre-culture without antibiotic that was again grown at 37°C over night under vigorous shaking. For the main culture, 500 ml of LB medium without antibiotic were inoculated with 1-2 ml of the pre-culture and incubated at 37°C under vigorous shaking. Bacterial growth was monitored by determining the optical density at a wavelength of 600 nm (OD600) at intervals. The bacteria were harvested in their exponential growth phase at an OD600 of about 0.6. The cell suspension was cooled in ice water for 15 min. The equipment and solutions were precooled to ensure that all the following steps of the protocol could be carried out at nearly 0°C. The bacteria were centrifuged for 15 min at 5000 rpm (Centrifuge Avanti J-25, rotor JA-10), washed three times in 500 ml ddH2O and resuspended in 10 ml 10% glycerin. After centrifugation for 12 min at 5,000 rpm the bacterial sediment was resuspended in

1.5 ml 10% glycerin and shock frozen in liquid nitrogen in 50 µl aliquots in 0.5 ml Eppendorf tubes. The bacteria were stored at –80°C without significant competence loss over several months.

3.2.3 Transformation

To generate bacterial clones expressing the plasmid of interest electro-competent cells were transformed by electroporation. Electrocompetent bacteria (stored at –80°C) were thawed on ice for about 10 min. 50 µl cold MilliQ-water and 5 µl DNA solution purified from a ligation reaction were added to 25 µl bacteria. This mix was applied to a pre-cooled electroporation cuvette and pulsed at 1.8 kV, 200 Ω and 25 µF in the *E.coli Pulser*. Pulsed cells were immediately taken up in 0.8 ml LB medium and incubated in a shaker at 37°C. No antibiotic was used in this pre-culture. After 1 h of incubation 10 % of the bacteria were applied directly onto LB-Agar plates containing ampicillin, the remaining 90 % were carefully pelleted (2 min centrifugation at 3200 rpm), then resuspended in a small amount of fresh LB medium and also plated. All plates were incubated over night at 37°C.

If the colony count was greater on plates derived from transformation with actual ligation reactions than on plates containing the empty vectorcontrol some of these colonies were used for further culture to analyze and finally isolate the plasmid of interest.

3.2.4 Isolation of plasmid DNA

After successful transformation some colonies were picked and further cultured in LB-medium. Plasmid DNA was isolated and analyzed for presence of the plasmid of interest by restriction digestion. One positive clone was selected for a larger culture to isolate plasmid DNA for future use.

Isolation of plasmid DNA for analytical purposes (Mini-Prep)

For plasmid isolation 2-4 ml of ampicillin-containing LB-medium were inoculated with bacteria picked from one colony grown on agar plates. Bacteria were cultured overnight in a shaker at 37°C and 200 rpm. The next morning 1200 µl culture were transferred to a 1.5 ml reaction vial and centrifuged at 9000 rpm for 3 min to pellet the bacteria. Supernatant was discarded and successively 300 µl each of buffer P1, P2 and P3 were added. After addition of the RNAse- and the membrane-destabilizing chelator EDTA-containing buffer P1 the solution was vortexed to resolubilize the pellet; after P2 addition the solution was mixed an incubated at room temperature for 5 minutes. Buffer P2 contains SDS and NaOH and leads to alkaline lysis of bacteria. After addition of neutralizing buffer

P3 the solution was mixed and incubated on ice for 5 min. Cellular debris and proteins were visible as a white fluffy precipitate, which was removed by two 20 minute centrifugation steps (13000 rpm, 4°C). In-between centrifugations supernatant was transferred into a new reaction tube to improve separation from the precipitate. After the second centrifugation supernatant was again transferred to a fresh reaction tube; DNA was precipitated by addition of 600 µl isopropanol, 5 min incubation at room temperature and centrifugation (20 min, 13000 rpm, 15°C). Supernatant was discarded and tubes were inverted on a paper tissue to better remove residual fluid. The precipitated DNA was washed with 500 µl ethanol (75%) and centrifuged for 15 min at 4°C with maximum speed. Supernatant was then removed followed by a second careful washing with 400 µl ethanol which were promptly removed afterwards. The reaction tube was carefully inverted to remove fluid and the pellet was air-dried for approximately 20 min. DNA was then dissolved in 45 µl H_2O and a fraction (2-10µl) was used for restriction digestion, in order to determine whether the analyzed clone carried the plasmid with the insert in correct orientation.

High yield isolation of plasmid DNA (Maxi-Prep)

For high yield plasmid isolation 250 ml antibiotic-containing LB-medium were inoculated with 700 µL culture from an analytical preparation known to be positive for the vector in question. The culture was shaken (110 rpm) at 37°C over night in a big culture flask with indents to provide optimal oxygen saturation. The next morning 700 µl of this culture were removed, covered with glycerin (50%) and stored at –80°C as a back-up stock.

Plasmid isolation was conducted using the Qiaquick Maxi Kit according to the manufacturers instructions. Isolated DNA was taken up into 500 µl of TE buffer and left to completely dissolve overnight, then the DNA concentration was measured. Restriction digestion was performed to ensure that the correct plasmid was isolated and a sample was sent for commercial sequencing.

3.3 Molecular Biology

3.3.1 PCR

The Polymerase chain reaction (PCR) was used to specifically amplify target genes or gene fragments. Reaction conditions and temperature settings were adapted depending on the template length but were typically as follows:

Composition of a standard PCR reaction:

Reagents	Volume	Stock Conc.	Final Conc.
PCR master mix	25 µl or 50 µl	2x	1x
Primer 1	5 µl	5 pmol/µl	
Primer 2	5 µl	5 pmol/µl	
Template DNA	1µl	~1µg/µl	10^5-10^6 copies
sterile ddH$_2$O	add final volume		
Final volume	50 or 100 µl		

Standard PCR cycle profile: The annealing temperature was determined individually for each primer pair. Elongation time was adjusted to the length of the desired product, e.g. for Ub/Tyr 3 minutes were choosen.

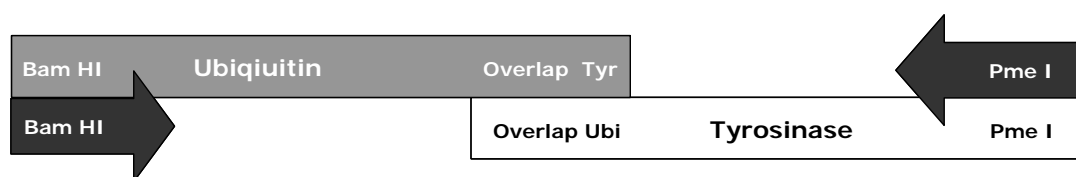|	Temperature	Time	Cycle No.
Initial Denaturation	94°C	2 min	1 x
Denaturation	94°C	30 s	
Annealing	45 - 58°C	60 s	30 x
Elongation	72°C	1 - 3 min	
Final Elongation	72°C	7 min	1 x

3.3.2 Construction of the Ub/Tyr fusion gene by hybridization PCR

The ubiquitin/tyrosinase fusion gene was generated using a hybridisation-PCR-technique allowing to fuse the murine ubiquitin (Ub) gene in frame with the human tyrosinase (Tyr) gene without linker sequences. For more information about the design of the Ub/Tyr fusion gene please **see chapter 4.2.**

First, the ubiquitin cDNA (Gene Bank Accession No. 908748) was cloned and amplified from a RNA preparation of murine B16 melanoma cells (CRL-6475) using a standard reverse-transcriptase-PCR method (Titan One Tube RT-PCR System, Roche) according to the manufacturers instructions. The primers 1 (5´-GGG CGG ATC CGA CCA TGC AGA TCT TCG TGA AGA CCC TGAC-3´) and 2 (5´-CAA AAC AGC CAG GAG CAT CGC ACC TCT CAG GCG AAG GAC CAG-3´) were chosen in order to create a *Bam*HI restriction site (underlined) at the 5´-end of the resulting fragment and a 15 bp overlap to Tyr at the 3´-end. Additionally, the above primers served to mutate the ubiquitin residue G76 to A76 (coding sequence shaded), see also **schematic in Figure 3 and 4**.

In a second step, human tyrosinase was amplified by standard PCR (Roche) from the plasmid pcDNAI-hTyr (Drexler et al., 1999). The primers 3 (5´-CGC CTG AGA GGT GCG ATG CTC CTG GCT GTT TTG TAC TGC CTG- 3´) and 4 (5´-GGG CGT TTA AAC TTA TAA ATG GCT CTG ATA CAA GCT GTG GT- 3´) extended the Tyr cDNA with an 18 bp overlap to ubiquitin at the 5´-end which also contained the mutated sequence for the ubiquitin residue A76 (coding sequence shaded) and a *Pme*I restriction site at the 3´-end (underlined), see also **schematic in Figure 5**.

The resulting fragments were purified (PCR-purification-kit, Qiagen) and used as templates in a hybridization-PCR to construct the Ub/Tyr fusion gene. During a pre-PCR without primers, complementary gene sequences of the fusion partners are able to anneal and serve as their own primers in the following elongation. Due to the absence of primers, the 3'→5' single strands were not amplified. After the pre-PCR, primers 1 and 4 were added to amplify the ubiquitin/tyrosinase fusion gene (Ub/Tyr).

Schematic of Hybridization PCR Ubiquitin and Tyrosinase templates bind through a homologous overlap that has been constructed in the previous PCR reactions

	Temperature	Time	Cycle No.
Initial Denaturation	94°C	6 min	
Pre-Annealing	40°C	2 min 30 s	1 x
Pre-Elongation	72°C	3 min	
Addition of primers			
Denaturation	94°C	1 min	
Annealing	42 °C	1 min	30 x
Elongation	72°C	3 min	
Final Elongation	72°C	7 min	1 x

Cycle profile of hybridization PCR. The annealing temperature of the pre-PCR depended on the complementary gene sequences of the fusion partners, the annealing temperature during the actual amplification cycles was determined for the used primer combination.

3.3.3 Analytical Gelelectrophoresis

To verify the sizes of PCR products or fragments resulting from restriction digestion electrophoresis in 1% agarose gels was used. Gels were prepared using 1x TAE buffer and the fluorescent, DNA-intercalating dye ethidiumbromide (5 µg/100ml gel) was added for visualization of DNA. A minimal volume of 12 µl was used for analysis, containing the DNA solution and approximately 10% loading buffer. If less than 10 µl DNA solution were to be used the volume was adjusted using TE-buffer. 6 µl of a premixed 1 kb ladder was used as mass standard and for estimation of DNA concentration of vector and insert prior to ligation reactions. Electrophoresis was conducted at 75-85 V for 30-50 minutes. The gel was then removed from the electrophoresis chamber, analyzed and photographed under UV-excitation (312 nm) to enable assessment of fragment size, band intensity and integrity.

3.3.4 DNA Purification from agarose gels

Gel electrophoresis was also used to separate one DNA species from another or to clean DNA from other reaction components such as enzymes prior to further use. To this end the fragment of interest was excised from the agarose gel after electrophoresis with a clean scalpel and DNA was extracted from the gel using the QIAquick Gel Extraction Kit according to the manufacturer's instructions. If DNA was to be used in ligation reactions H_2O was used for elution instead of EB-buffer in order to prevent inhibition of ligase by incorrect salt concentrations.

3.3.5 Restriction Digestion

Restriction digestions of vectors and inserts were performed at 37°C for 1 hour (control digestions after plasmid preparation) or 2 hours. Reaction mixtures varied depending on the amount of DNA and on the restriction enzymes used. All enzymes were used with the buffer and BSA concentration recommended by the manufacturer. If a combination of enzymes was used the buffer allowing for the highest possible efficiency of both enzymes was selected. Generally, 1 U enzyme was used to digest 1 µg of DNA. The volume of the enzyme solution did never exceed 10% of the total reaction volume.

After digestion reaction vessels were incubated at 65°C for 10 minutes in order to stop the reaction. To prevent unspecific cleavage DNA was either purified using the QIAquick PCR purification kit or purified by gel electrophoresis and DNA extraction from the gel.

3.3.6 Dephosphorylation

Vector DNA intended for ligation reactions was dephosphorylated after the restriction digestion had been stopped. Removal of the free phosphate at the 3' end of the vector prevents religation of the linearized vector. 3µl (9 U) alkaline phosphatase (AP) and 10µl 10x AP buffer were added to the vector restriction reaction. Water was added to achieve a total volume of 100µl. The reaction was incubated at 37°C for 1 h. After 30 minutes incubation, another 3µl of AP were added. To stop the dephosphorylation 5µl EDTA were added and the reaction tubes were incubated at 70°C for 20 min. To achieve a complete inhibition of the reaction vector DNA was immediately purified by gel electrophoresis and DNA extraction from the gel.

3.3.7 Ligation

Ligations of fragments were combined at a vector to insert ratio of 1:4. Concentrations were estimated from agarose gels under consideration of the different sizes of vector and insert. Per 1 µg of DNA 1 U ligase was used. Ligation reactions were always carried out in 20µl reaction volume containing 2µl 10x ligase buffer. Negative control reactions contained only vector and no insert DNA. Ligations were carried out overnight while floating in a melting ice-water mix. 1U ligase was added in the morning and the reaction incubated for 60 min at RT. The ligation reaction was stopped by addition of 5µl EDTA and a 10 minute heating step at 65°C. Ligated DNA was subsequently precipitated by addition of 40µl H_2O, 4µl 3 M NaAc and 150µl EtOH, incubated for 30 min at –80°C to increase DNA precipitation, then purified using two centrifugation steps (25 min and 15 min at 4°C and maximum speed), washing the pellet with 500µl 75% EtOH in-between the centrifugations. The pellet was air-dried, dissolved in 15µl H_2O and used for transformation of electro-competent bacterial cells.

3.3.8 Determination of DNA Concentration

Concentrations of solutions containing double stranded DNA were photometrically measured by their adsorption of light at a wavelength of 260 nm. Depending on the expected DNA content the concentration of the solution or a dilution were assessed by double measurement against H_2O (or the agent in which the DNA had been solubilized) as reference. Since an adsorption of 1 equals a concentration of 50µg/ml dsDNA, the concentration of the solution was calculated as follows:

$$Adsorption \ at \ OD_{260} \ x \ 50 \ \mu g/ml \ x \ dilution \ factor \ = \ concentration \ of \ dsDNA$$

3.4 Protein Analysis

3.4.1 Western Blot

Western Blotting is an antibody-based method that can be used to detect and quantify proteins that have been separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to their molecular weight.

Preparation of cell lysates

To isolate proteins cell monolayers were removed from the culture dish using a cell scraper and transferred into 2 ml Eppendorf tubes. The tubes were centrifuged at 2000 rpm for 5 minutes to pellet cells. Supernatants were removed and pellets resuspended in 80 µl lysis buffer, freeze-thawed three times, sonicated for three times one minute and centrifuged for 1 min at maximum speed, then supernatants were used for analysis. In order to apply the same amount of protein for each sample in SDS-PAGE with subsequent Western blot analyses, sample protein concentrations were determined according to Lowry using the DC-Protein Assay Kit (Bio-Rad) following the manufacturers instructions. Absorption of the dyed samples was measured at 655 nm in a microplate reader (Bio-Rad) and the protein concentration was calculated with respect to likewise treated BSA standards (20 µg, 40 µg, 60 µg).

SDS-Page

Up to 100 µl of the cell lysates were mixed with an appropriate amount of 5x protein loading buffer and incubated for 5 min at 95°C to denature proteins and break disulfide bonds. After a short centrifugation to clear condensed fluid from the top of the reaction vessels, samples were applied to the pockets of the stacking gel for migration into the gel at 50 V in a vertical electrophoresis chamber. The same procedure was applied for the protein molecular weight marker. After the samples had entered the resolving gel the voltage was turned up to 180 V if short (5 hour) gel running time was needed or left at 50 V if proteins were left to separate over night.

resolving Gel 8%	50 ml	stacking Gel	20 ml
Bis/Acrylamide 30%	13.3 ml	Bis/Acrylamide 30%	3.4 ml
H_2O milliQ	23.2 ml	H_2O milliQ	13.1 ml
Tris pH 8.8 1.5 M	12.5 ml	Tris pH 6.8 1 M	2.5 ml
SDS 10%	0.5 ml	SDS 10%	0.2 ml
APS 10%	0.5 ml	Ponceau S Solution	0.5 ml
TEMED	0.02 ml	APS 10%	0.2 ml
		TEMED	0.02 ml

Separation was stopped when the visible loading buffer front had nearly reached the lower edge of the gel. The gel was removed from the electrophoresis chamber and transferred into transfer buffer after the stacking gel had been cut off. The gel and a nitrocellulose membrane (0.45µl pore size) of the same size as the gel were equilibrated for 10 minutes in transfer buffer in separate containers. Gel and membrane were placed between 8 layers of whatman-paper and into a pre-cooled, semi-dry-blotting apparatus. Blotting voltage was set to be a maximum of 0.5 mA/cm^2 gel size. Blotting was carried out for 20 minutes.

After blotting, the nitrocellulose membrane was incubated in blocking buffer for 2 hours at room temperature or over night at 7°C. After blocking the membrane was washed 3 times for 10 minutes in 1x TBS-T and then incubated for 2 hours with the 1st antibody (T311) diluted 1:100 in blocking buffer. Unbound antibody was removed by washing as described above. The membrane was then incubated for 1 hour with the secondary anti mouse antibody diluted 1:3000 in blocking buffer without NaN$_3$ and subsequently washed again. Depending on the size of the membrane 2-3 ml substrate solution (a 1:1 mix of Lumi-Light solution A and B) were used to cover the membrane. The membrane was incubated for 5 minutes between plastic covers and then the detection solution was removed using paper tissues. Protein-specific signals were detected exposure to a photographic film.

3.4.2 Metabolic Labeling and Immunoprecipitation

To monitor the half life of proteins within infected cells pulse-chase experiments using radio-active sulphur (^{35}S) were conducted.

For each time point 3x10^6 RMA cells were infected at a density of 10^7 cells/ml and a MOI 10. Cells were incubated on ice for 20 min and then shifted to 37° and diluted with fresh media to a concentration of 10^6 cells/ml. After four hours post infection cells were washed twice and starved for 20 min with met/cys free DMEM containing ultraglutamin and pyruvat 1% each (RIPA starvation medium). Cells were then adjusted to a density of 10^{7}cells per 100µl starving medium and transferred into prewarmed eppendorf tubes. To achieve a 10min pulse, 150µCi of ^{35}S-labeled methionine/cystein was added per 100µl cell suspension and cells were immediately incubated for 10 min at 37° under continuous mixing. During the pulse cellular protein biosynthesis should lead to incorporation of radioactively labeled methionine into newly synthesized proteins. To stop the pulse 800µl ice-cold RPMI was added and the cells were immediately washed. Cells were diluted to 3x10^{6}cells/ml RPMI 10% FCS, transferred into a T35 culture flask for incubation at 37°. At the indicated chase times cells

were resuspended and equal aliquots of cells were taken, spun and lysed with the western-blot lysis buffer and subsequently frozen on dry-ice. After taking the last sample, lysates were freeze-thawed twice and subjected to immunoprecipitation with 10µl of anti-tyrosinase mAb C-19 and 30 µl Protein-G-Plus-Sepharose in 900µl RIPA immunoprecipitation buffer. Precipitates were boiled in loading buffer and separated by 8% SDS-PAGE. Gels were fixed for 20 min in fixation buffer and then dried. Analysis of radioactivity was visualized on a phosphor imager plate.

3.5 *Virological Methods*

3.5.1 *In vitro* **infection of cells with MVA**

For MVA infection of adherent cell lines cells were grown to 80% confluence in tissue-culture plates. Virus (stored at –80°C) was thawed at room temperature and sonicated for 30 seconds to singularize viral particles. For each well the chosen MOI was dissolved in 1 ml RPMI. Medium was removed from the monolayer and 1 ml RPMI/MVA was carefully added to each well. Cells were then incubated at 37°C as usual.

When infecting cell lines growing in suspension cells were adjusted the night before to a maximum density of $5x10^5$ cells/ml to be in exponential growth phase the next day. Cells were infected at a density of 10^7 cells/ml at the desired MOI. Cells were incubated on ice for 20 min and then shifted to 37° and diluted with fresh media to a concentration of 10^6 cells/ml.

3.5.2 **Generation of recombinant MVA**

To construct MVA Ub/Tyr, the Ub/Tyr fusion gene was cloned into the MVA transfer plasmid pIIIΔHR-P7.5 for the insertion into the Deletion III of MVA (Staib et al, 2003).

The MVA transfer vector contains two MVA sequences, Del III flank-1 and Del III flank-2, that are identical to the neighboring sequences of the deletion III. By intergenomic homologous recombination, the gene sequences between the flanks can be stably integrated at the deletion III locus of the MVA genome (see schematic). Since the transcription of poxviral genes in the cytoplasm of infected cells is controlled by the viral transcription machinery, recombinant genes have to be placed under the control of a vaccinia virus promoter. The vaccinia virus promoter P7.5 integrated in the vector plasmid pIIIΔHR-P7.5 controls the transcription of the recombinant gene and allows for early and late gene expression (Sutter and Moss, 1992; Wyatt et al., 1996). To be able to isolate recombinant MVA viruses, the insertion cassette contains the vaccinia virus gene K1L that transiently extends the host range specificity of MVA to rabbit kidney cells (RK13) (Staib et al, 2003). Since wt MVA does not proliferate productively on this cell line, only recombinant viruses containing the K1L gene, and thus the entire insertion cassette, are able to replicate.

The marker gene K1L is flanked by two homologous sequences originating from the LacZ gene that allow for its elimination from the newly generated recombinant virus. By serial passages on MVA permissive CEF-cells, the selective pressure is removed and the K1L gene can be deleted effectively from the viral genome by intra genomic homologous recombination of these LacZ flanks. The target

gene and a single LacZ sequence remain in the recombinant viral genome. The schematic illustrates the two steps of homologous recombination for the generation of recombinant MVA-Ub/Tyr:
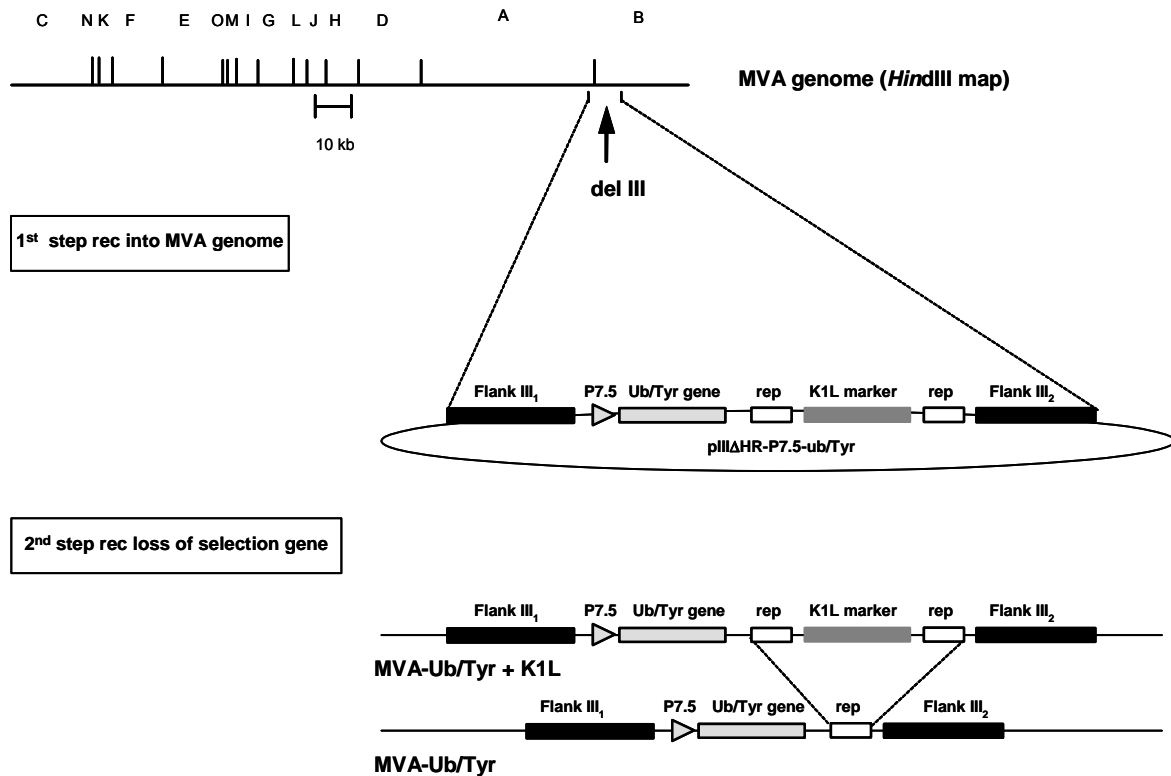
Schematic   Construction of recombinant MVA expressing an ubiquitin/tyrosinase fusion gene (MVA-Ub/Tyr) Sites of the restriction endonuclease HindIII within the genome of MVA are indicated. (Step 1), after homologous recombination with the pIII-ΔHR-P7.5-Ub/Tyr vector plasmid the expression cassette containing the Ub/Tyr gene, lacZ gene flanking sequences (rep), the K1L host range selection gene and flanking MVA-DNA sequences were integrated into deletion III of the MVA genome. The final recombinant virus, MVA-Ub/Tyr, was obtained after deletion of the K1L marker gene during a second step of homologous recombination involving synthetic repetitive sequences (rep).

3.5.2.1 *In vitro* transfection for homologous recombination

To generate recombinant viruses by homologous recombination permissive CEF cells were infected and transfected with the MVA transfer vector pIII-ΔHR-P7.5-Ub/Tyr.

Infection with MVA wt was carried out at MOI 0.05 as described. Then tansfection reagents were prepared using separate 14 ml polystyrene tubes for each well. Mix A and B were prepared as listed below, incubated for 5 minutes, then mixed together and incubated at room temperature for 20

minutes. During the incubation time the lipofectamine 2000 transfection reagent forms DNA-containing microspheres that can transfect cells by membrane-fusion.

Solutions used for transfection of CEF cells with plasmid DNA

Mix A	Plasmid DNA	4 µg
	RPMI-only	250 µl
Mix B	Lipofectamine 2000	10 µl
	RPMI-only	250 µl

One hour after infection cells were washed with 2 ml RPMI per well. 1 ml RPMI was added to each well prior to addition of the transfection solution. Plates were gently rocked and culture was continued under standard conditions. After 3 to 5 hours incubation time the transfection medium was removed and replaced by 1-2 ml fresh RPMI 10 %. Transfected cells were harvested 48 hours post transfection using a cell scraper. Viruses were released from the cells by freeze-thawing and sonication.

3.5.2.2 Plaque purification on RK13 cells

The vaccinia virus host range gene K1L of the insertion cassette allows for isolation of recombinant MVA on RK13 cells by plaque purification. Only those recombinant MVA viruses that have genomically integrated the insertion cassette, and thus both K1L and the gene of interest, are able to grow on RK13 cells. The virus suspension obtained from transfection and infection of CEF cells was diluted in 10^{-1} steps in RPMI 10% to result in dilutions of 10^{-1} to 10^{-4}. RK-13 cells were grown in 12-well plates to 80% confluence. Growth medium was removed and the cells were infected with the virus suspension by adding 1 ml of each dilution to three wells, respectively. After 48 to 72 h growth at 37°C recombinant viruses induced typical plaques in the cell layer. Under the microscope, plaques were isolated with a 20 µl pipette tip, transferred into a Eppendorf tube and resuspended in 500 µl RPMI 10% medium. The isolated cells were freeze-thawed thrice and sonicated three times for one minute. To obtain wildtype free recombinant virus preparations, the isolated plaques were passaged on RK-13 cells up to 5 times. To screen for wildtype free recombinant virus, viral DNA was isolated and analyzed by a Deletion III PCR.

3.5.2.3 Selection on CEF cells

RK-13-Isolates of recombinant MVA were passaged on permissive CEF-cells to allow the loss of the K1L gene by a second step of homologous recombination. To obtain pure K1L-free recombinant virus clones, the isolated plaques were passaged on CEF-cells cells 3 times. Then cell lysates were serially

diluted and plated on 96-well plates with CEF cells. Single clones were isolated by harvesting complete wells from a dilution that reached a maximum of 30% of infected wells (as judged by CPE) per 96-well plate. Clones were amplified on CEF cells, viral DNA was isolated and analysed by PCR.

3.5.2.4 Extraction of DNA from infected cells

Tissue culture cells were infected at a MOI 10 in a 6-well-dish. Cells were harvested with a cell scraper and pelleted at 2.000 rpm for 2 min. The pellet was resuspended in 400µl of 1xTE buffer (pH 7.6) and freeze-thawed three times. 50µl of 10x TEN buffer (pH 7.4), 50 µl of proteinase K and 23 µl of SDS 20% were added to the virus suspension, the sample was mixed and incubated for 2 to 4 hours at 56°C. The DNA was extracted with 600µl of phenol-chloroform. The samples were shaked and centrifuged at 14,000 rpm for 10 min at room temperature. The aqueous upper phase was transferred into a fresh tube. The extraction procedure was repeated up to three times. The DNA was precipitated by adding 1/10 volume of 3 M sodium acetate and 2 volumes of ultrapure ethanol absolute. The sample was frozen at -80°C for 30 min to aid precipitation and DNA pelleted by centrifugation at 14,000 rpm for 15 min at 4°C. The pelleted DNA was washed with 250µl of ethanol 70% and centrifuged at 14,000 rpm for 3 min at room temperature. The pellet was air dried for 30 min or lyophilized for 10 min at room temperature and resuspended in 50µl of 1x TE buffer (pH 7.6).

3.5.3 Virus amplification and crude stock preparation

For large scale MVA virus preparations primary CEF cells were cultivated in 10-40 T225 cell culture flasks at 37°C. After 2-3 days, the medium was changed and the 80% confluent cell layer was infected at an MOI of 0.3 by adding the calculated volume of virus suspension diluted in 1 ml medium to each flask. The cells were cultivated for another 2-3 days until a cytopathic effect was recognizable. The cells were harvested by freezing at -80°C and thawing subsequently. During the thawing process, the flasks were shaken regularly to completely abrade the cell layer with the pieces of frozen medium. The cell suspensions were transferred to 250 ml ultra centrifugation cups and centrifuged for 90 min at 4°C and 13 500 rpm (Optima LE 80K, rotor: type 19). The cell pellet was resuspended in a suitable volume of 10 mM Tris buffer pH 9.0 (max. 30 ml for further purification and 6 ml for crude stock preparations), freeze-thawed three times and sonicated three times for one minute in 50% ice water. This crude stock virus preparation was either stored at -80°C or further purified.

3.5.4 Virus purification

MVA crude stock virus preparations were purified from cellular debris and proteins by saccharose cushion ultra-centrifugation. To break down the cells and to separate virus particles from the cell wall, crude stock preparations were homogenized on ice by 30 strokes in a 40-ml douncer with a pestle. Cellular debris was pelleted by centrifuging the suspension for 5 min at 4000 rpm and 4°C. The virus containing supernatant was transferred to a new 50 ml Falcon tube and the pellet was resuspended in 25 ml of cold 10 mM Tris buffer pH 9.0 and returned to the douncer. The procedure was repeated a total of three times and resulted in a maximum of 80 ml of virus suspension. Ultracentrifugation cups for Beckman rotor SW28 were prepared with 25 ml of sucrose 36% in 10 mM Tris buffer pH 9.0 and the virus suspension was carefully lain onto the cushion. Virus particles were pelleted by ultracentrifugation for 90 min at 13.500 rpm and 4°C (Optima LE 80K, rotor: SW28). The supernatant was removed and the pellets resuspended in a maximum volume of 12 ml 1 mM Tris pH 9.0. For the second purification step, ultracentrifugation cups for Beckman rotor SW41 were prepared with 9.5 ml of sucrose 36% in 10 mM Tris buffer pH 9.0 and the purified virus suspension was again carefully laid onto the cushion. Virus particles were pelleted by ultracentrifugation for 90 min at 13.500 rpm and 4°C (Optima LE 80K, rotor: SW41). The supernatant was removed carefully and the obtained virus pellet was resuspended in 3-6 ml 1 mM Tris pH 9.0 and stored at -80°C.

3.5.5 Virus titration and growth kinetics

The infectivity of a MVA suspension was determined by titration and plaque formation frequency. Virus material was freeze-thawed thrice (-80° and 37° water bath) and sonicated three times for one minute in 50% ice water in a cup sonicator. The virus suspension was serially diluted in RPMI 2% medium to result in dilutions from 10^{-1} to 10^{-11}. Primary CEF cells were harvested by trypsination, split 1:10, and resuspended in a final volume of 10 ml RPMI 2%. 100 µl of cell suspension per well were plated into a flat bottom 96-well plate. Each time 100µl of a virus dilutions were added per well to a total of 16 wells/dilution. Cells were then incubated at 37°C for 7 days, analyzed for cytopathic effect by microscopy and the virus titer was calculated based on the number of CPE per dilution.

To test whether recombinant viruses had similar growth kinetics as wildtype virus CEF cells were infected at MOI 0.01 and harvested at different time points (whereas t=0 is hasrvested immediately to control the viral input). Titration was carried out for each time point.

3.6   Immunological Methods

3.6.1   Preparation of splenocytes

Spleens were removed and homogenated with a syringe plunger over a cell strainer into RPMI 10% medium. After centrifuging the homogenate (5', 1500rpm) the erythrocytes were lysed with 3ml ACT buffer (2', 37°) and washed with 40 ml RPMI 1%. The cells were again filtered over a Nylon filter and counted.

3.6.2   Preparation of PBMC

Mice were bled via tail veins and the blood was collected into tubes containing 100µl PBS and 10µl heparin to inhibit blood clotting. Erythrocytes were lysed in 10ml ACT buffer for 10 minutes at room temperature. Thereafter, cells were washed once with PBS (1500rpm, 5', 4°C) and used for staining.

3.6.3   Cell counting

Cells were counted at a 1:40 dilution. 50µl of cell suspension was mixed with 450µl PBS. From this 1:10 dilution 50µl were mixed with 50µl of Trypan blue solution (0.4%, Sigma) and 100µl PBS to result in a 1:40 dilution (total). Cells were counted in a Neubauer counting device. Two quadrates were counted and the cell number was calculated using the following formula:

$$n \text{ (cells/ml)} = \text{mean of two quadrates x dilution factor x } 10^4$$

3.6.4   Intracellular Cytokine Staining (ICS)

Peptide stimulation of lymphocytes

For peptide stimulation for each sample 200µl RPMI 10% containing 4×10^6 splenocytes were transferred to flat-bottomed 96-well plates. For each peptide a mastermix containing 5 µl vortexed and sonicated peptide (from 1mg/ml stock) and 5 µl of brefeldin A (1mg/ml stock) per 1 ml RPMI 10% medium was prepared and thoroughly mixed. 50 µl of this mastermix were added to each well and mixed. Cells were incubated with the peptides for 5 hours at 37°C in a 5% CO_2 atmosphere.

EMA-Staining and Fc-Block

Cells were transferred to a 96well V-bottom plate, washed and then incubated with 50µl Fc block (1:100) and EMA (1:1000), (20', under light) to block Fcγ receptors and stain dead cells, respectively. Cells were then washed twice with FACS buffer in a total volume of 200µl (2', 1500rpm).

The anti-CD16/CD32 antibody, added in excess, serves to block cellular Fc receptors. This blockade prevents unspecific signals generated by antibodies that fail to specifically bind their target and are instead bound by the Fc receptors on leucocytes.

EMA staining is used for live/dead discrimination, since this photo-activated molecule can enter only dead or damaged cells that no longer have intact membranes. Upon entering these cells EMA can form stable links to nucleic acids present in the cell. This reaction requires the presence of visible light and is irreversible, allowing specific detection of EMA stained cells by excitation with a 488 nm laser.

Surface markers and intracellular cytokine staining

After EMA stain and Fc-Block, washed cells were stained with 50µl of the surface markers CD8a and CD62L (30', in the dark on ice). After that the cells were again washed three times with FACS-buffer. Cells were then treated with 100µl Cytofix/Cytoperm to permealize the cell walls (15', in the dark on ice). Cells were then washed three times with PermWash puffer before they were stained with 50µl of intracellular antibodies (1:500 dilution of a αIFNγ FITC-labeled antibody in 50 µl Perm-Wash buffer, 30', in the dark on ice). Finally, cells were washed again three times, fixed with 1% PFA and stored until used for analysis.

3.6.5 Tetramer Stain

For tetramer staining 8×10^6 cells were used per sample and the staining was performed in a 96 well V-bottom plate. First the cells were incubated with 50µl Fc block (1:100) and EMA (1:1000), (20', under light) to block Fcγ receptors and stain dead cells, respectively. Cells were then washed once with FACS buffer in a total volume of 200µl (2', 1500rpm). First, cells were incubated with 30µl FACS buffer containing 1µl of the respective tetramer (30′ on ice, in the dark). Then 20µl FACS buffer containing 0,5µl of antibody (e.g. anti-CD8-FITC) was added and cells were incubated for additional 30′. The samples were washed three times with FACS buffer, fixed in 1% PFA, and stored in the dark at 4°C before further analysis.

3.6.6 Chromium Release Assays

Specific lysis by A*0201-restricted murine CTL reactive against defined peptides was determined in a 6h standard [^{51}Cr]-release assay. HLA-A*0201-positive A375 cells were infected for 2h at MOI=10, washed and labeled for 1h at 37°C with 100 μCi Na^{51}CrO4, and then washed four times. Labeled target cells were plated in U-bottomed 96-well plates at 1 x 10^4 cells/well and incubated with effector cells at various E:T ratios. Using a topcount scintillation counter the specific ^{51}Cr release was determined in supernatants which were taken at different time points post coincubation for kinetic analysis (Drexler et al., 1999).

3.6.7 Degranulation Assay

Splenoytes were prepared and stimulated with peptides as described for ICS. FITC-conjugated anti-CD107a/b (BDPharmingen) and 1:1000 monensin (eBiosciences) was added during the stimulation time. Upon degranulation, lysosomal proteins (LAMP 1 and 2) are transiently exposed on the cell surface. Antibodies can bind to these proteins and are then taken up by endocytosis. Monensin inhibits Golgi export and additionally endosomal acidification to prevent destruction of the FITC fluorochrome (Betts et al., 2003). After stimulation and simultaneous staining for degranulation, CTL were then stained with EMA and surface markers as described for ICS.

3.6.8 FACS-based antigen-presentation Assays

RMA-HHD cells or freshly isolated DC were infected for 2h at MOI 10 and then washed. For *ex vivo* assays purified splenic DC were isolated at the indicated time post vaccination. APC were co-cultured at different ratios with antigen-specific CTL lines in the presence of 1mg/ml BFA (Sigma) for 5h. Staining and analysis for intracellular IFNγ-production was carried out as described above. When DC were used as APC CD11c and CD3 antibodies were included in the ICS staining protocol.

To detect k^b-SIINFEKL-complexes, infected DC2.4 cells were stained with the 25-D1.16 antibody (Porgador et al., 1997) and then labeled with Alexa-Fluor-633-conjugated goat anti-mouse-IGg F(ab´)$_2$-Fragments (Molecular Probes).

3.6.9 Purification and analysis of DC

To mature DC *in vivo* mice were treated with 20ng CpG i.v. the day before DC isolation. To isolate splenic DC spleen suspensions were digested for 30 min at 37°C with collagenase VIII and DNAse I (Sigma) and then treated for 5 min with EDTA. Splenocytes were then incubated with CD11c MicroBeads (Miltenyi, Bergisch Gladbach). CD11c+ DC were isolated by magnetic beads isolation according to the manufacturer's recommendations (MiniMACS, Miltenyi). Purity was confirmed to be >80% by FACS-analysis. DC were stained with antibodies specific for CD11c (HL3), CD80 (16-10A1), CD86 (G/1), CD54 (3E2), I-A^b (M5/114.15.2) and HLA-A2 (BB7.2) (all BD Pharmingen). Fluorochrome-conjugated isotype-matched mAbs were used as controls. Propidium iodide (Molecular Probes) was added immediately befor analysis for live/dead-discrimination.

3.6.10 In vivo Cytotoxicity

Splenocytes were prepared as described above. After cell counting, cells were divided equally and incubated for one hour with a relevant or a control peptide (1µg/ml). Cells were washed three times with PBS and incubated with CFSE (10', 37°C). One cell pool stained with a high concentration of CFSE (5µM), the other cell pool with a low concentration of CFSE (0.5µM). To bind excessive CFSE the reaction was stopped by adding RPMI 10% medium and incubated on ice for 5 minutes. After three washing steps with PBS, both cell pools were mixed, resuspended in PBS, and injected intravenously. After the indicated time points, the mice were bled and the blood was prepared as described before. Splenocytes were prepared as above.

The Percentage of antigen specific cell lysis was calculated as followed:

$$\%lysis = 100 - ((\%CFSE^{high}/\%CFSE^{low} \times 100) \times (\%CFSE^{low}/\%CFSE^{high} \text{ of naïve control}$$

3.6.11 Listeria monocytogenes challenge

Mice were challenged i.v. with different doses of L. m. OVA to assess CTL-mediated protective immunity. To quantify the number of bacteria in spleens of challenged mice spleens were harvested by dissociation through a wire in RPMI 10% and diluted in water containing 0.1% Triton X-100. Serial dilutions were plated on brain heart infusion agar plates, incubated for 24h and colony forming units were counted.

3.6.12 Flow cytometry

Flow cytometry allows for analysis of cells on a single cell level. By aspiration through a fine needle and hydrodynamic focusing cells successively enter a detection channel, where they pass through a set of laser beams. Analysis of cell size, granularity and protein expression is based on the forward light scatter (FSC), the sideward light scatter (SSC) and the emission of light by laser-activated fluorochromes, respectively. The usage of several lasers and different fluorochromoes with distinct emission spectra allows for the simultaneous analysis of a variety of different markers. Analysis is possible for proteins expressed at the cell surface, as well as for intracellular proteins after permeabilization and fixation of the cell. The optical readout from analyzed cells is converted to digital information in a detector system and can be visualized and analyzed using specific software such as FACS-Diva or FloJo. Since emission spectra of some fluorochromes show partial overlaps each experiment contained single color samples for each used fluorochrome be able to define cells that are truly positive and to adjust instrument settings in order to subtract signal overlaps for each detection channel.
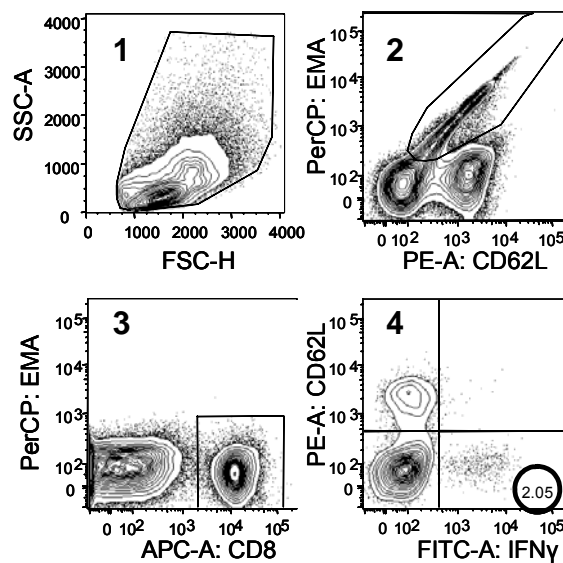
Schematic: FACS-Analysis. A representative gating strategy for ICS samples is depicted. Analyzed cells were identified by typical FSC/SSC parameters (1), discriminated from dead cells (2: EMA-) and then analyzed for surface (3: CD8+) and expression markers (4: IFNγ+).

3.7 Immunizations

Only female mice between 8-12 weeks of age were used. Vaccination schedule, injection route and dose varied depending on type of vaccination and between prime and boost settings.

When indicated, mice received 20ng of synthetic phosphorothioated CpG1668 (TIB-Molbiol, Berlin, Germany) in 100 µl PBS (i.v.) the day prior to vaccination for *in vivo* maturation of DC.

For vaccination with infected cells, 10^6 cells per mouse were infected for 2h at MOI 10 and washed extensively. DCs were incubated with 1µg/ml synthetic peptide (Biosyntan, Berlin, Germany) and extensively washed. 10^6 peptide-coated or infected DC were injected into the tail-vein of CpG-treated or control mice.

For peptide vaccination, mice were immunized s.c. at the tail-base with 0.1 mg peptide and 10 ng of synthetic CpG1668.

Vaccine preparations and application routes

Vaccine	Dose	Volume	Application Route	Time Point
Cardiotoxin	50 µg in 1x PBS	50 µl	i.m. in tibialis anterior	8 days before prime with DNA-vaccine
pSFV1 replicon - *huTyr*	45 µg in 1x PBS	50 µl	i.m. in tibialis anterior	Prime
Peptide-preparation - *huTyr$_{1-9}$* - *huTyr$_{369}$*	100 µg in 1x PBS containing 10 nmol Oligo CpG as adjuvant	100 µl	s.c. at the tail-base	Prime
MVA	10^5-10^8 IU in 1x PBS	20µl 50µl 100µl 500µl	i.d. i.m. i.v. i.p.	Prime or Boost

3.8 Statistical analysis

All statistical analysis was performed using GraphPad Prism4 Software. If not stated otherwise, results are generally expressed as mean±SEM. Differences between groups were analyzed for statistical significance using two-tailed student t-test.

4 Results

4.1 MVA has a tropism for pAPC

4.1.1 MVA efficiently infects DC resulting in expression of recombinant antigen

Previous work has shown that MVA has the ability to infect human DC and to express viral and recombinant antigens (Kastenmuller et al., 2006). To characterize infection of murine DC *in vivo* matured splenic DC (mDC) (see also 3.6.9) were isolated and infected with MVA expressing green fluorescent protein (MVA-GFP). FACS-analysis revealed that MVA efficiently infected DC leading to a strong GFP expression (**Figure 1**). Infection of DC did neither reduce the surface expression of MHC class I or II glycoproteins, nor that of costimulatory molecules CD80 and CD86.
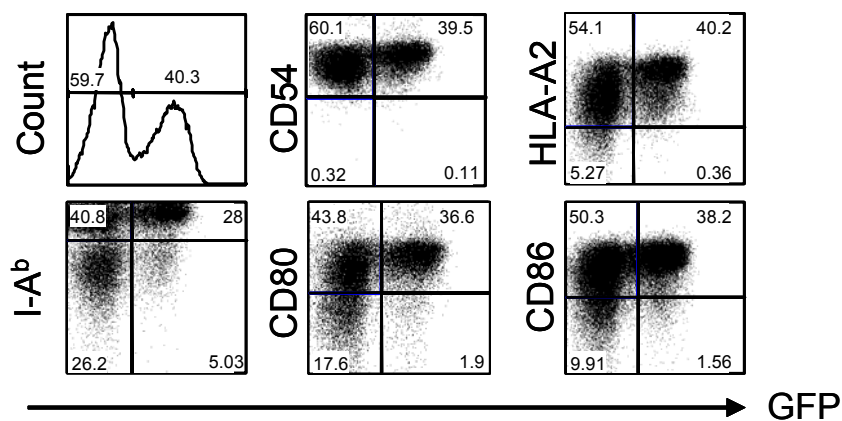
Figure 1　　**MVA efficiently infects DC** FACS analysis of MVA-GFP-infected matured splenic DC (mDC). Surface expression of MHC class I (HLA-A2), MHC class II (I-A^b), costimulatory molecules B7.1 (CD80) and B7.2 (CD86) and ICAM-1 (CD54) was compared for infected and non-infected mDC 6h post infection.

4.1.2 MVA-infected DC allow for MHC class I restricted antigen presentation

To test whether MVA-infected DC were able not only to synthesize MVA-encoded antigens, but also to process and to present antigenic peptides to CD8+ T cells in a MHC class I restricted manner splenic mDC were isolated, infected with MVA expressing tyrosinase (MVA-Tyr) and then were coincubated with a tyrosinase-specific T cell line. FACS-analysis of IFNγ-production showed that

infected DC specifically stimulated tyrosinase-specific CD8+ T cells. This data confirmed the expression and processing of recombinant tyrosinase as well as peptide-loading onto MHC-class-I-molecules. Experiments using bone marrow-derived murine DC yielded similar results (data not shown).
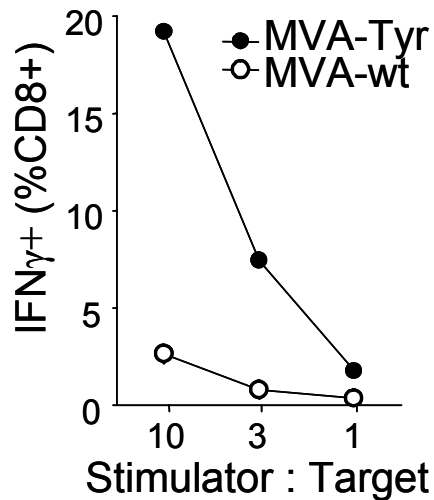
Figure 2 **MVA-infected DC present antigenic peptides** IFNγ-production of Tyr$_{369}$-specific CTL after stimulation with MVA-Tyr-infected splenic mDC.

The findings depicted in Figure 1 and 2 closely reflected MVA infection of human DC (Chahroudi et al., 2006; Kastenmuller et al., 2006). The observation that MVA efficiently infected DC and allowed for peptide-loading onto MHC class I suggested a potential role for direct priming in the induction of CTL-immunity with MVA vaccines. In addition, the immunogenicity of target antigens expressed by VACV has been correlated with the expression of these antigens in DC (Bronte et al., 1997). Therefore it seemed reasonable that one strategy to enhance the immunogenicity of target antigens expressed by MVA could be to specifically increase the presentation of the respective antigenic peptides by MVA-infected DC. This can be achieved by increasing proteasomal turnover of these antigens which in turn should enhance peptide generation and MHC class I restricted presentation and thus improve the induction of CD8+ T cells (see also 1.4.4).

Therefore a recombinant virus was constructed with the aim to target a model antigen for rapid proteasomal degradation by the expression of a stable fusion to ubiquitin.

4.2 Construction and generation of MVA-Ub/Tyr

The ubiquitin gene encodes for a polyubiquitin-chain consisting of repetitive head-to-tail fusions of the 76-amino-acid ubiquitin polypeptide. Upon translation of this gene cytosolic isopeptidases specifically recognoize amino acid residue G76 and cleave the chain to release monomeric ubiquitin. These peptidases do also cleave ubiquitin when expressed in fusion with other proteins. The effectiveness of this process has been exploited to use ubiquitin fusion and subsequent removal to synthesize target genes with amino-acids other than a start-codon-defined methionine in order to manipulate the half-live of the resulting proteins according to the so-called "N-End-rule" (Bachmair et al., 1986). This study, in contrast, aimed at the expression of a protein as a stable fusion to ubiquitin which requires the prevention of ubiquitin cleavage. This can be achieved by mutating the glycine in position 76 to alanine (Rodriguez et al., 1997).

Tyrosinase was chosen as a model-antigen for ubiquitination. Tyrosinase is a key enzyme in melanin synthesis. As a melanocyte differentiation antigen, tyrosinase is commonly expressed in malignant melanoma (Chen et al., 1995; Halaban, 2002; Jimenez et al., 1988). It is a well characterized tumor-associated antigen that is recognized by cytotoxic T cells from melanoma patients (Brichard et al., 1993; Yee et al., 1996). In addition, tyrosinase-specific CTL could be primed *in vitro* from PBMC of melanoma patients by stimulation with autologous monocyte-derived DC that were infected with a recombinant MVA expressing tyrosinase (MVA-Tyr) (Drexler et al., 1999). This vector has also been used in clinical studies for vaccination with the live-vaccine or vector-transduced DC (Di Nicola et al., 2004; Meyer et al., 2005). These studies were not able to detect strong CTL immunity directed against tyrosinase. Immune responses appeared to be directed mainly against the the viral backbone, which underscores the importance of enhancing immmunogenicity of target antigens delivered by MVA and of improving the target-to-vector-ratio of induced responses. In HLA-A02*01-transgenic mice vaccinated with MVA-Tyr, CD8+ T cell responses against both the recombinant as well as vector antigens can be studied (Drexler et al., 1999; Drexler et al., 2003; Pascolo et al., 1997). In the case of tyrosinase, CTL are reactive against the Tyr_{369} internal peptide which is contained within the mature protein. Tyr_{369} is generated by proteasomal degradation and loaded onto MHC-class-I-molecules in a TAP-dependent manner (Skipper et al., 1996). Therefore the targeted enhanced proteasomal degradation of tyrosinase should increase the presentation of Tyr_{369} peptides.
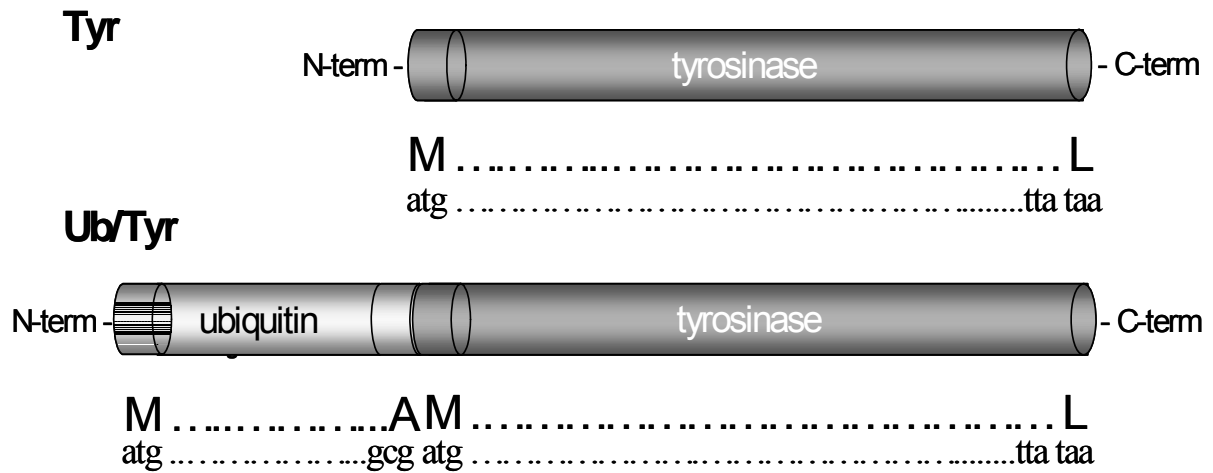
Tyr

Ub/Tyr

Figure 3 **Schematic map of the ubiquitin/tyrosinase fusion gene (Ub/Tyr)** Upper panel (Tyr) and lower panel (Ub/Tyr), the N- (M) und C-terminal amino acid components (L) of full length tyrosinase (Tyr) and ubiquitin/tyrosinase (Ub/Tyr) are indicated as well as the respective codons of the corresponding open reading frame. For Ub/Tyr, the in frame transition from the ubiquitin part (A) to the tyrosinase part (M) is also depicted including the corresponding base pairs, below.

4.2.1 Construction of the MVA transfer vector pIIIΔHR-P7.5-Ub/Tyr

As a first step, the ubiquitin cDNA (Gene Bank Accession No. 908748) was cloned and amplified from a RNA preparation of murine B16 melanoma cells using a standard reverse-transcriptase-PCR method. The primers 1 and 2 were designed in order to create

1) a *Bam*HI restriction site at the 5′-end of the resulting fragment to allow the ligation into the MVA transfer vector pIIIΔHR-P7.5

2) a15 bp overlap to Tyr at the 3′-end which was designed to align to an homogous overlap of the tyrosinase fragment used in a hybridization PCR

3) a mutation of the ubiquitin residue G76 to A76 by PCR mutagenesis
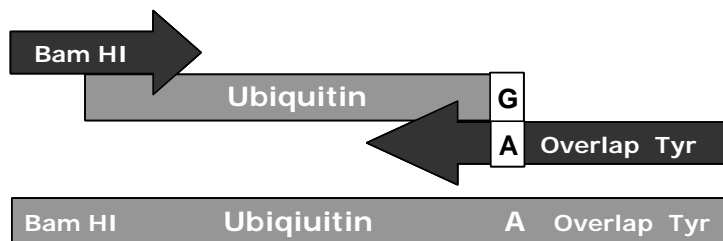
Figure 4 **Schematic of Ubiquitin RT-PCR** Ubiquitin cDNA was cloned and amplified from a RNA preparation of murine B16 melanoma cells by a standard reverse-transcriptase-PCR. The primers were chosen in order to create a *Bam*HI restriction site at the 5′-end of the resulting fragment and a 15 bp overlap to Tyr at the 3′-end. Additionally, the above primers served to mutate the ubiquitin residue G76 to A76 to prevent deubiquitination of the Ub/Tyr-fusion protein by cytosolic isopeptidases.

In a second step, human tyrosinase was amplified by standard PCR from the plasmid pcDNAI-hTyr (Drexler et al., 1999). The primers 3 and 4 were chosen in order to

1) extend the Tyr cDNA with an 18 bp overlap to ubiquitin at the 5´-end (which also contained the mutated sequence for the ubiquitin residue A76) to allow annealing to the ubiquitin fragment in a hybridization PCR

2) create a *Pme*I restriction site at the 3´-end to allow the ligation into the MVA transfer vector pIIIΔHR-P7.5
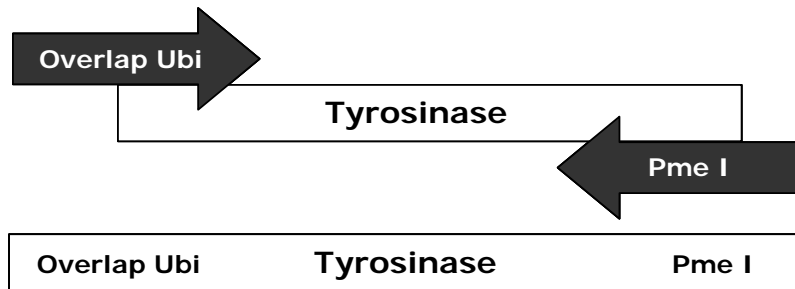
Figure 5   Schematic of Tyrosinase PCR Human tyrosinase was amplified by standard PCR from the plasmid pcDNAI-hTyr. Primers extended the Tyr cDNA with an 18 bp overlap to ubiquitin at the 5´-end which also contained the mutated sequence for the ubiquitin residue A76 (see also Figure 4) and a *Pme*I restriction site at the 3´-end

The resulting fragments were purified and used with primers 1 and 4 as templates in a hybridization-PCR to construct the Ub/Tyr fusion gene (see also 3.3.2):
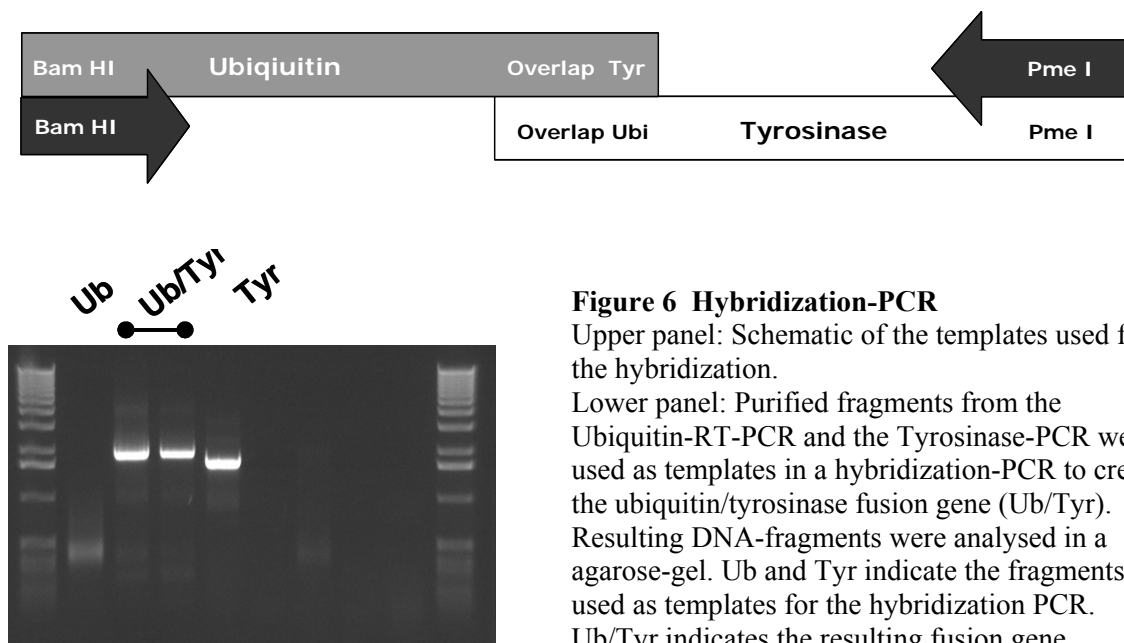
Figure 6 Hybridization-PCR
Upper panel: Schematic of the templates used for the hybridization.
Lower panel: Purified fragments from the Ubiquitin-RT-PCR and the Tyrosinase-PCR were used as templates in a hybridization-PCR to create the ubiquitin/tyrosinase fusion gene (Ub/Tyr). Resulting DNA-fragments were analysed in a agarose-gel. Ub and Tyr indicate the fragments used as templates for the hybridization PCR. Ub/Tyr indicates the resulting fusion gene.

The MVA transfer vector pIIIΔHR-P7.5-Ub/Tyr was generated by inserting the ubiquitin/tyrosinase fusion gene (Ub/Tyr) between the unique *Bam*HI and *Pme*I restriction sites of pIIIΔHR-P7.5 placing it under control of the P7.5 VACV-specific early/late promoter. The vector pIIIΔHR-P7.5-Ub/Tyr was then transferred into Escherichia coli DH10B by electroporation and selected through resistance to ampicillin. Plasmid-DNA was amplified and prepared. Before further use the transfer vector was subjected to a control digest to confirm the integration of the Ub/Tyr fusion gene. Plasmids derived from two different bacterial clones were further sent for commercial sequencing to detect eventual mutations. After confirmation of the presence of an intact Ub/Tyr fusion gene by *in silicio* alignment of the analyzed sequences one plasmid was chosen for the generation of recombinant MVA-Ub/Tyr.
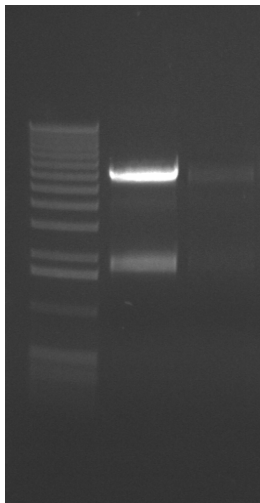
Figure 7   Control digest The MVA transfer vector pIIIΔHR-P7.5-Ub/Tyr was digested with BamHI and PmeI to determine the size of the integrated gene. The resulting DNA-fragments were analyzed in an agarose-gel. Left lane: Marker (DNA-ladder), Right lane: digested plasmid DNA

4.2.2 Generation and isolation of recombinant MVA-Ub/Tyr

To generate MVA-Ub/Tyr CEF cells were transfected with pIIIΔHR-P7.5-Ub/Tyr and simultaneously infected with MVA wt to allow for homologous recombination. Recombinant MVA were isolated through plaque purification by passaging on RK13 rabbit kidney cells. Plaques were harvested and amplified on CEF cells. Then DNA was extracted and genomes of recombinant viruses were analyzed by PCR.
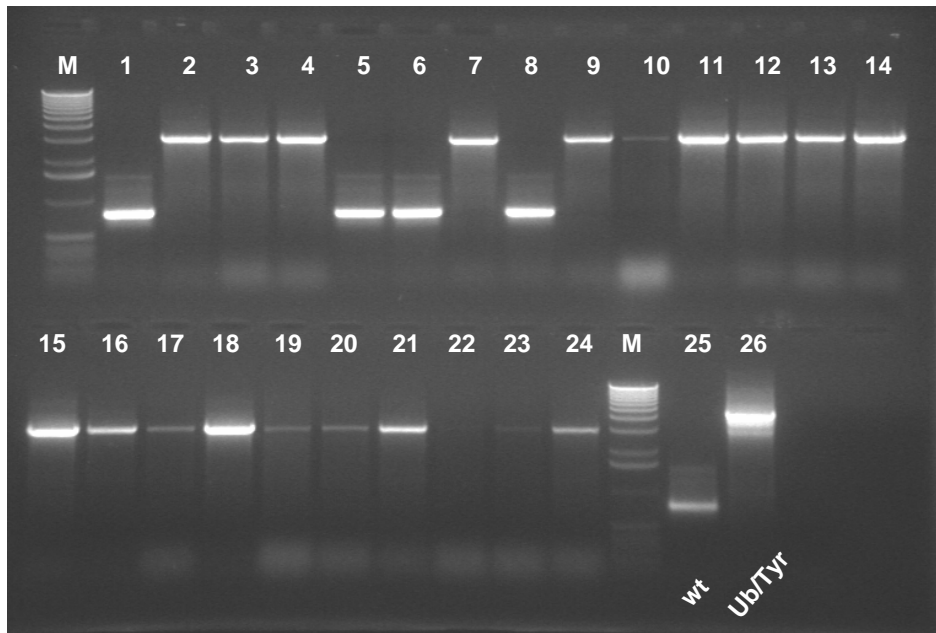
Figure 8 **PCR-Analysis of Plaque-isolates** Plaques were isolated from RK-13 cells, amplified on CEF cells and DNA was prepared. A Deletion-3-PCR was performed. DNA isolated from MVA wt infected cells (lane 25) and the plasmid pIII-ΔHR-P7.5-Ub/Tyr (lane26) served as control templates. Resulting DNA fragments were analyzed in a agarose-gel for their size. Clones 1, 5, 6 and 8 are not recombinant. All other clones have integrated the Ub/Tyr fusion gene into Deletion 3. M = Marker (DNA-ladder)

Viral clones 12 and 18 were chosen and passaged on CEF cells. After 3 passages single clones were isolated by limiting dilution on CEF 96well plates. Single clones were picked, amplified and DNA was isolated. Viral genomes were analyzed for the recombinant insert by Deletion-3-PCR. A K1L-PCR was performed to detect recombinant clones that had lost the K1L-marker-gene by a second step of homologous recombination.
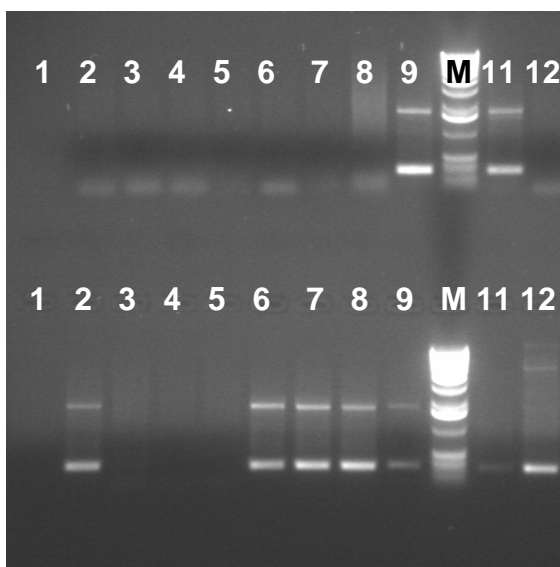
Figure 9 **K1L-PCR** Viral genomes of single clones were analysed for the presence of the K1L marker-gene. Lane 11 and 12 on the lower panel represents wt and plasmid control template DNA, respectively. Clones 1, 4 and 7 from the upper panel and 1, 4 and 5 from the lower panel were chosen for amplification and named A, B, C, D, E and F, respectively.

Based on these PCR analyses recombinant and K1L-free clones were chosen and further amplified on CEF cells. PCR-analysis of viral genomes was repeated before the large-scale production of viral stocks (**Figure 10**) to confirm that only recombinant viruses that had lost the K1L-marker cassette were amplified.
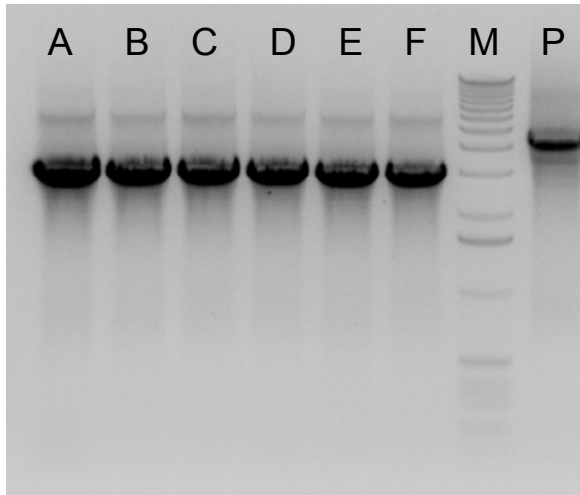

Figure 10　　**Deletion-3-PCR**　　Viral genomes of were analysed for the sequences integrated in Deletion 3. All clones show bands consistent with the expected size of ~ 2900 bp. Lane P shows DNA amplified from the plasmid pIII-ΔHR-P7.5-Ub/Tyr which in addition contains the K1L gene (~1100 bp) and 300bp from the LacZ repeats that are depleted when K1L is lost upon second homologous recombination.

PCR-analysis confirmed the correct insertion of the transgene into the MVA genome, the loss of the K1L marker gene as well as the absence of contaminating wildtype MVA. Clone B was chosen and a viral stock was prepared and purified. As MVA-Ub/Tyr should be compared to MVA-Tyr a viral stock of this recMVA was prepared in parallel. The resulting stocks were titrated on CEF cells. Titers were calculated to be 4×10^{10} (MVA-Ub/Tyr) and 7×10^{10} (MVA-Tyr). Repeated titrations yielded similar results.

4.3   In vitro characterization of MVA-Ub/Tyr

4.3.1   MVA-Ub/Tyr has normal viral growth kinetics

To control that the insertion of the Ub/Tyr fusion gene did not affect the ability of the resulting recombinant virus to infect cells and to promote viral gene expression viral growth kinetics were analyzed and compared to MVA wt and MVA-Tyr.
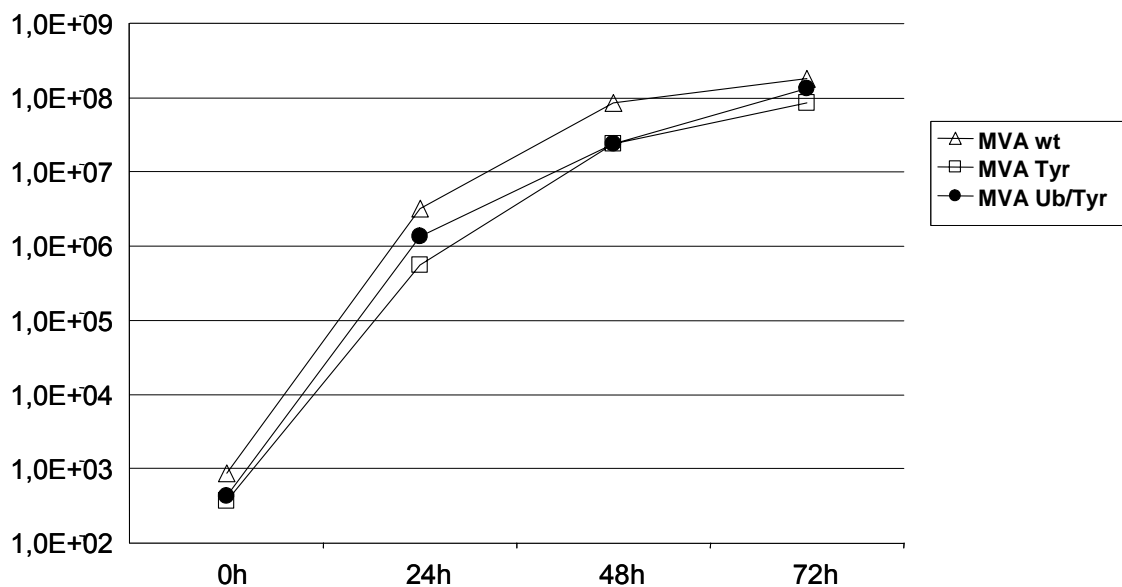

Figure 11 **MVA-Ub/Tyr shows normal viral growth kinetics** Multiple step growth curves. Permissive CEF cells were infected with the indicated viruses and harvested after the indicated time. For each time point viral load was determined by titration.

4.3.2   Expression of ubiquitylated tyrosinase leads to rapid proteasomal degradation

In western blot analysis (**Figure 12**), ubiquitylated tyrosinase produced by MVA-Ub/Tyr was slightly bigger in size than authentic tyrosinase expressed by MVA-Tyr, in line with the fusion to the 8kDa-ubiquitin. Notably, ubiquitylated tyrosinase expressed by MVA-Ub/Tyr was only detectable in the presence of specific proteasome inhibitors. Under these conditions, the total amount of accumulating protein was comparable for both viruses. Without proteasome inhibition the amount of tyrosinase in MVA-Ub/Tyr infected cells was below detection limit, indicating that ubiquitylation of tyrosinase resulted in rapid proteasomal degradation.

Figure 12 **Expression of ubiquitylated tyrosinase leads to efficient proteasomal degradation**
Westernblot analysis of NIH cells infected with MVA-wt, MVA-Tyr or MVA-Ub/Tyr in the presence
(+) or absence (-) of specific proteasome inhibitors (10μM Lactacystin and 20μM MG-132). At 0, 12
or 24 h post infection, cell lysates were resolved by SDS-Page.

Pulse-chase-experiments (as described in 3.4.2) showed that ubiquitylated tyrosinase was subject to
rapid degradation with a half-life of less than 30 min (**Figure 13**). In contrast, authentic tyrosinase
which is known to have a half-life of more than 10h (Jimenez et al., 1988) was stable over the entire
observation period. These experiments also confirmed that comparable amounts of tyrosinase protein
are synthesized by MVA-Tyr and MVA-Ub/Tyr.
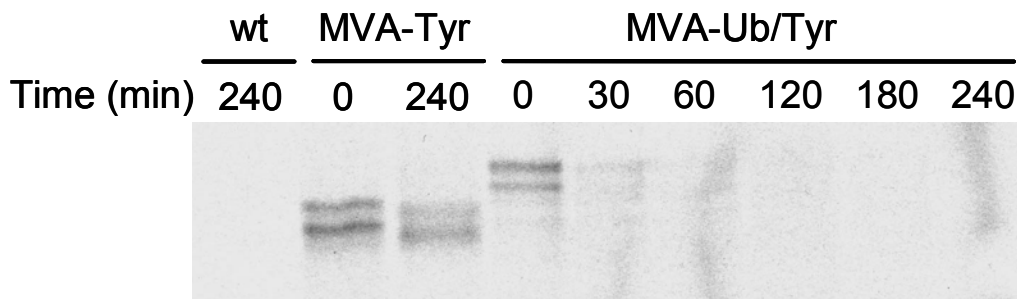
Figure 13 **Ubiquitylated tyrosinase has a drastically reduced half-life** Pulse-chase-labeling of
RMA cells infected with MVA-wt, MVA-Tyr or MVA-Ub/Tyr. After a brief pulse with ^{35}S-labeled
methionine, cells were further incubated. At indicated time points after pulsing, immunoprecipitation
was performed.

4.3.3 Ubiquitylation of tyrosinase enhances MHC class I peptide loading of pAPC and non-pAPC

One of the aims of the construction of MVA-Ub/Tyr was to study whether enhanced antigen presentation could serve to improve CTL immunity against target antigens. Therefore it was tested whether rapid proteasomal degradation of ubiquitylated tyrosinase indeed increased peptide generation and loading onto MHC-class-I-molecules. For this purpose it was decided to assess presentation of the Tyr_{369} internal peptide which is contained within the mature tyrosinase protein and generated by proteasomal degradation. (Skipper et al., 1996; Wolfel et al., 1994, Wolfel et al., 2000). In chromium-release assays, expression of ubiquitylated tyrosinase resulted in significantly enhanced Tyr_{369}-specific CTL recognition of infected target cells as compared to MVA-Tyr-infected cells. MVA-Ub/Tyr-infected A375 cells were lysed at lower Effector:Target ratios and earlier during the viral infection (**Figure 14**). As a control, infected target cells assayed against VACV-specific T-cells ($B22R_{79}$) showed comparable lysis for both viruses. These results indicated that rapid degradation of tyrosinase leads to enhanced peptide processing and presentation of peptide/MHC-class-I-complexes. Similar results were obtained with A*0201-transfected mouse target cells (data not shown).
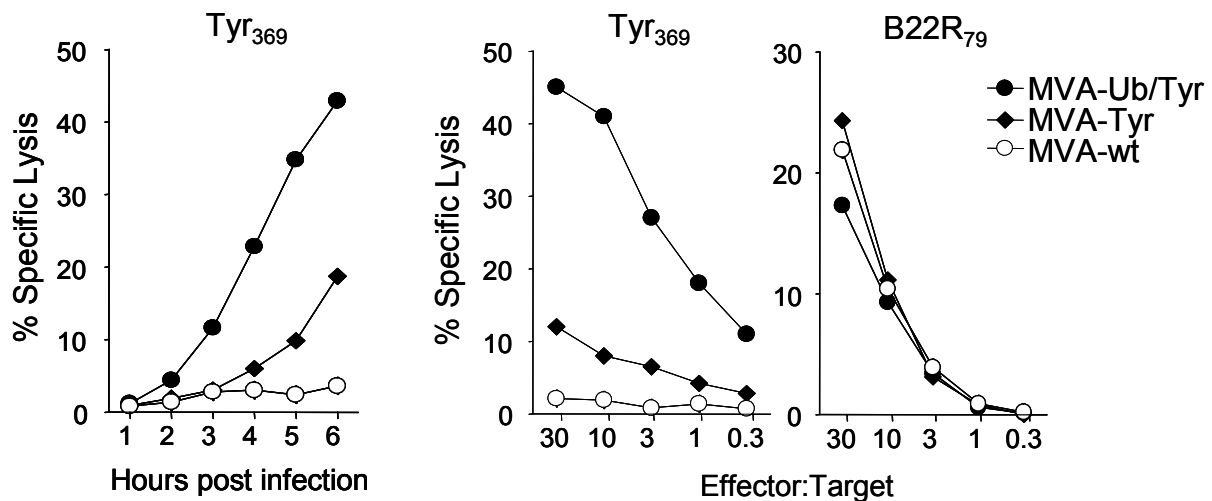

Figure 14   MVA-Ub/Tyr infected cells are more efficiently recognized by CTL ^{51}Cr release assay of infected A375 target cells. Tyr_{369}-specific lysis is shown after indicated time points post infection (left panel) or for different Effector:Target ratios (middle and right panel).

Additionally, MVA-Ub/Tyr-infected pAPC consistently had a superior capacity to stimulate Tyr_{369}-specific degranulation and IFNγ-production (**Figure 15**). As the intracellular staining for IFNγ was

conducted with saturating concentrations of fluorochrome-conjugated anti-IFNγ-antibody the specific increase in the mean fluorescence intensity could be used to detect the relative increase in the amount of synthesized IFNγ per cell. This analysis revealed that MVA-Ub/Tyr infected DC not only stimulated more cells to produce IFNγ but also that the amount of IFNγ produced per activated CD8+ T cell increased. Together these experiments indicate that both pAPC and non-pAPC present increased amounts of Tyr$_{369}$/MHC class I complexes on their surface when infected with MVA-Ub/Tyr and therefore more efficiently activate CTL *in vitro*.
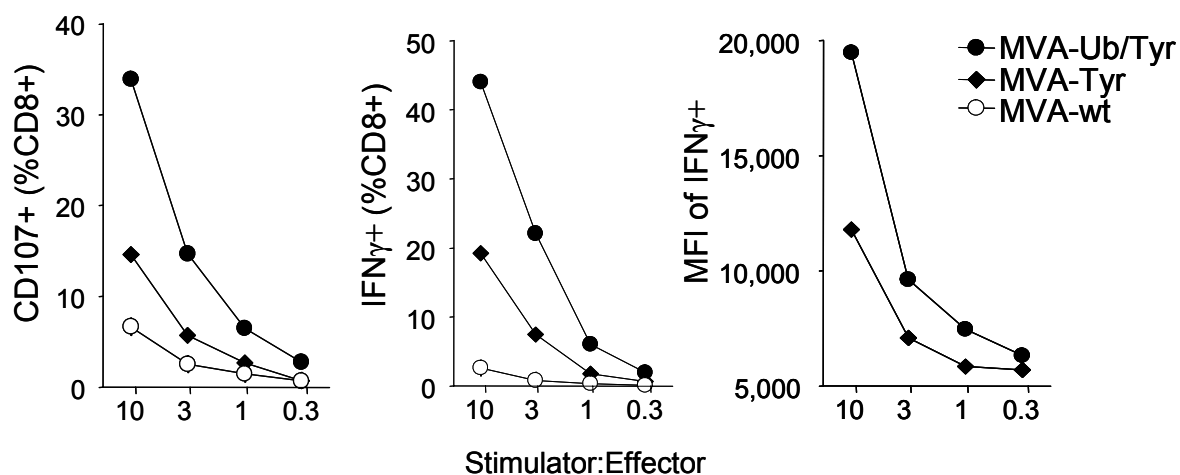
Figure 15   **MVA-Ub/Tyr infected DC have an increased ability to activate CTL.** FACS-analysis of staining of Degranulation marker CD107a/b (left panel) or IFNγ-production (middle and right panel) of Tyr$_{369}$-specific CTL after coincubation with *in vitro* infected splenic mDC (MOI=10) at indicated Stimulator:Effector ratios. MFI= mean fluorescence intensity

4.4 Crosspriming of Cytotoxic T cells Dictates Antigen Requisites for MVA Vector Vaccines

4.4.1 Rapid degradation of MVA-delivered antigen impairs T cell priming

As infection of DC with MVA-Ub/Tyr resulted in enhanced presentation of Tyr_{369} peptides as compared to MVA-Tyr-infected DC, it was expected that vaccination with MVA-Ub/Tyr would enhance CD8+ T cell priming, if direct priming by infected DC dominated the CD8+ T cell response. Unexpectedly, the induction of tyrosinase-specific CD8+ T cells was dramatically reduced (by more than 80%) in mice that received MVA-Ub/Tyr as compared to mice that were vaccinated with MVA-Tyr (**Figure 16**). Notably, frequencies of CD8+ T cells directed against determinants derived from viral proteins were comparable in both groups, indicating that the differences observed for tyrosinase-specific CD8+ T cells were merely due to the altered metabolic stability of tyrosinase. Route-specific effects could be ruled out, since similar results were obtained with intramuscular, intravenous and intradermal administration of the vaccine (**Figure 17**).
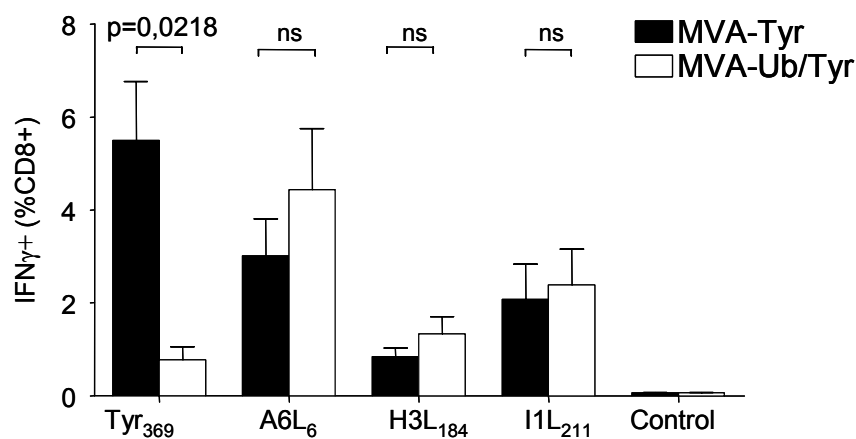

Figure 16 **Rapid degradation of antigen impairs CD8+ T cell priming.** Groups of A*0201-mice (n=4) were vaccinated i.p. with MVA-Tyr or MVA-Ub/Tyr. Tyrosinase- and vector-specific CD8+ T cell responses on day 8 post vaccination are indicated as the percentage of CD8+ splenocytes producing IFNγ in response to the indicated peptides. (ns=not significant)
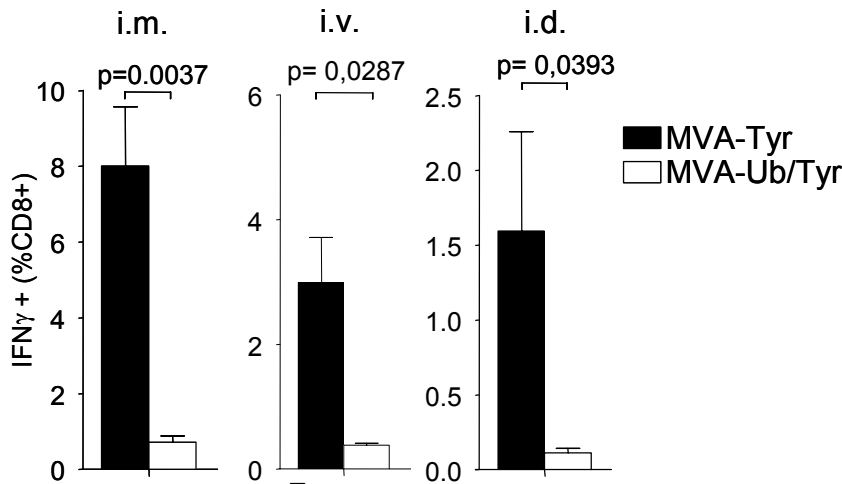
Figure 17 Different routes of vaccination. The same experiment as depicted in Figure 16 was repeated but mice were vaccinated by different routes: i.m. (left), i.v. (middle) or i.d. (right). Tyr_{369}-specific responses are indicated. No significant differences in the response to vector-specific peptides were detected (data not shown).

To exclude the possibility that the observed impairment of MVA-Ub/Tyr to efficiently induce CTL immunity was due to an ability of MVA to infect DC *in vitro* (**Figure 1**) but potentially not *in vivo*, mice were vaccinated intravenously with MVA-GFP and splenic DC were purified 8h post vaccination and subsequently analyzed for GFP-expression. FACS-analysis showed that 3-5% of the $CD11c^{bright}$ DC were infected (**Figure 18**). To test whether *in vivo* infected DC were able to stimulate T-cells and if enhanced degradation of Ub/Tyr would also increase presentation of tyrosinase peptides *in vivo*, mice were vaccinated with MVA-Tyr and MVA-Ub/Tyr and again splenic DC were purified 8h post vaccination. Then purified DC were coincubated with T cell lines and activation of the T cells was assessed by ICS. DC from vaccinated mice were able to stimulate VACV-$B22R_{79}$- as well as Tyr_{369}-specific T cells (**Figure 18**). DC isolated from groups of mice that received MVA-Ub/Tyr efficiently activated Tyr_{369}-specific T cells which confirmed the results obtained from *in vitro* infected cells (**Figure 15**).
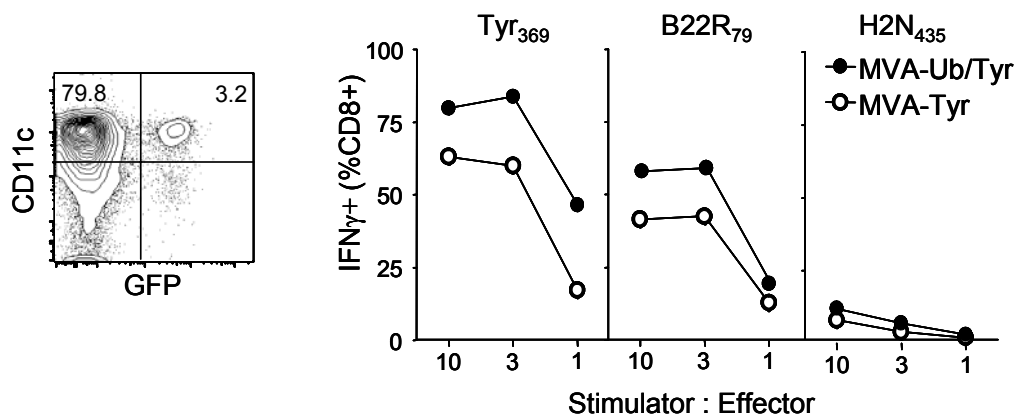

Figure 18   DC are infected and present MVA-delivered antigen on MHC-class-I *in vivo*. Mice were vaccinated intravenously with MVA-GFP. After 8h, CD11c-sorted splenic DC were analyzed for purity and GFP-expression (left). Mice were vaccinated intravenously with MVA-Tyr or MVA-Ub/Tyr. After 8h splenic DC were purified and coincubated with T-cell-lines reactive against the indicated peptides. Specific activation of T cells was detected by analysis of IFNγ-production (right)

These experiments indicated, that DC were indeed infected *in vivo* when vaccinating with MVA and that the expression of ubiquitylated tyrosinase in these DC also enhanced direct presentation and the capacity to stimulate CTL *ex vivo*. However, this antigen-presentation by infected DC did not correlate with the primary T cell response. As there was no apparent inhibition of direct antigen-presentation *in vitro* and *in vivo*, infected DC were further analyzed for the expression of co-stimulatory molecules. No down-regulation of costimulatory molecules was detected when infecting DC *in vitro* (**Figure 1**), however *in vivo* infected DC expressed lower amounts of CD80 and CD86 as compared to uninfected DC (GFP negative) (**Figure 19**).
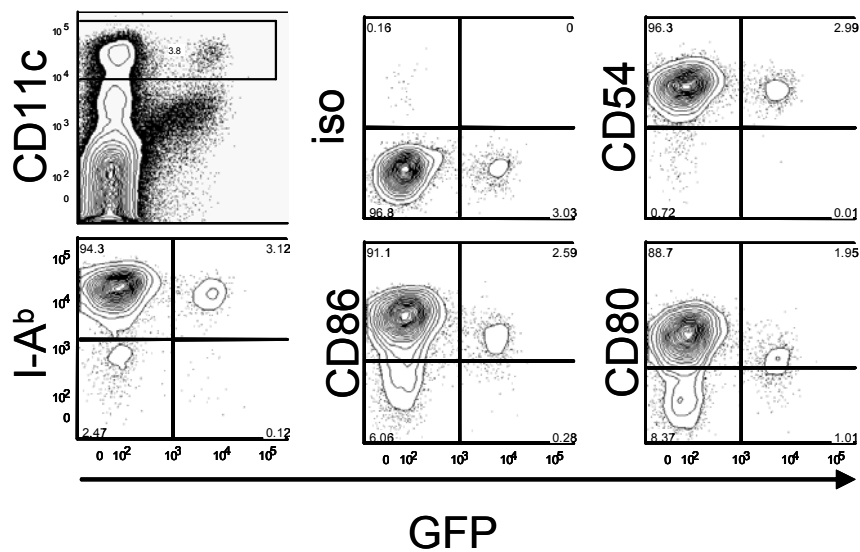

Figure 19 *In vivo* **infected DC express low amounts of costimulatory molecules CD80 and CD86.** Mice received i.v. MVA-GFP. 8h post vaccination spleens were harvested and digested and stained with the indicated surface markers. A representative FACS-analysis of *in vivo* infected splenic DC is depicted. After live/dead discrimination with propidium iodide blots were gated on CD11c+ cells (gate shown in the upper left blot)

These observations led to the hypothesis that the primary CTL response induced by MVA vaccination does not depend on antigen presentation by directly infected DC but rather is induced by DC that acquire antigen from other infected cells and cross-present it to naïve T cells. This hypothesis was addressed with the next experiments.

4.4.2 Cross-presentation of MVA-encoded antigen is sufficient to prime CD8+ T cells

To test this hypothesis a vaccination approach that prevented direct presentation of viral and recombinant antigens was used. Mice were vaccinated with TAP-deficient RMA-S-HHD cells that were infected with recombinant MVA. In these experiments, CD8+ T cell responses are induced by cross-presentation of antigen synthesized in the infected non-pAPC. Vaccination with MVA-infected cells yielded T-cell responses comparable to those induced by the live vaccines, with regard to size and immunodominance hierarchy of CD8+ T cells specific for viral and recombinant determinants (**Figure 20**). To exclude that residual infectivity was inoculated with these cells, we titrated aliquots of the vaccine preparations. The amount of transferred IU was reduced by more than two logs which proved to be insufficient to prime detectable CD8+ T cell responses when given as a live vaccine (data not shown). Comparable results were obtained with analogous experiments using HLA-mismatched NIH/3T3 cells (**Figure 20**). Similar to TAP-deficient or MHC-mismatched non-pAPC, vaccination with syngenic DC induced a strong T-cell response against Tyr_{369} only when DC were infected with MVA-Tyr expressing long-lived antigen. When DC where infected with MVA-Ub/Tyr, T cell priming against Tyr_{369} was markedly reduced (**Figure 20**), despite the strong direct T cell stimulatory capacity of these DC (**Figure 15** and **18**). These experiments demonstrated that cross-presentation alone is sufficient to elicit a strong T cell response to MVA vaccines. Importantly, cross-presentation of tyrosinase did only induce a strong CD8+ T cell response when the MVA-infected cells expressed long-lived antigen and not ubiquitylated tyrosinase, indicating a reduced suitability of short-lived antigen for cross-presentation in the context of MVA vaccines.
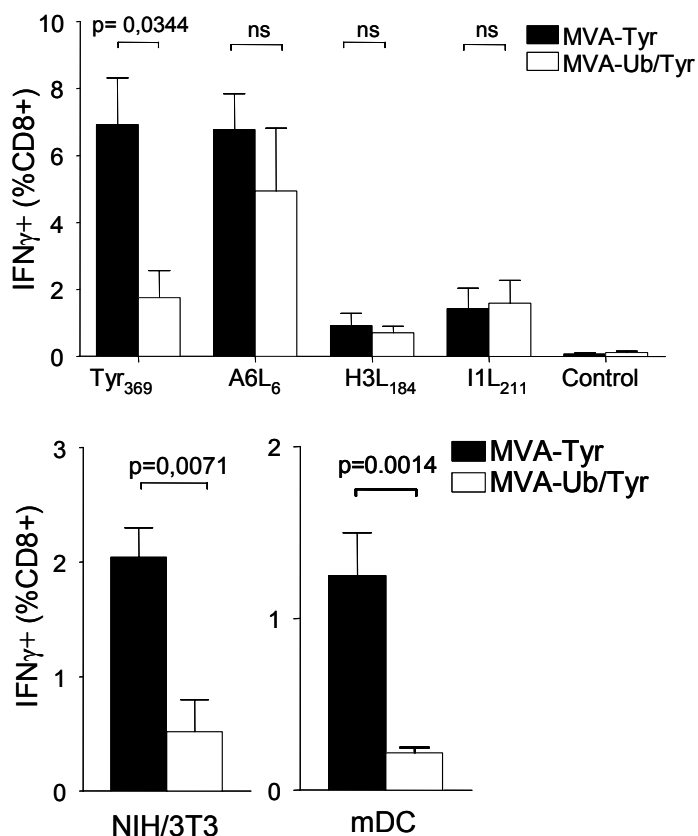

Figure 20 Cross-presentation of MVA-encoded antigen is sufficient to prime CD8+ T cells

TAP-deficient RMA-S-HHD cells were infected with MVA-Tyr or MVA-Ub/Tyr for 2h, washed extensively and used to vaccinate groups of A*0201 mice (n=4). Figures indicate tyrosinase- and vector-specific CD8+ Tcell responses on day 8 post vaccination. (upper panel)

The same experiment was repeated with NIH/3T3 (lower left panel) or syngenic bone-marrow derived mDC (lower right panel). Tyr_{369}-specific responses are indicated. No significant differences in the response to vector-specific peptides were detected (data not shown).

4.4.3 *In vivo* maturation of DC abrogates CD8+ T cell priming with MVA vaccines

In the previous experiments exclusive cross-priming induced a CD8+ T cell response that was similar to that using MVA as a live vaccine, which should allow for both antigen presenting pathways. In the next experiments the cross-presentation pathway was down-regulated and MVA-induced T-cell responses were analyzed under these conditions. To interfere with cross-presentation DC were matured *in vivo* by the treatment with CpG. This approach has recently been described to down-regulate the uptake of exogenous antigen, an essential step for cross-presentation (Wilson et al., 2006). In animals treated with CpG prior to infection, priming of CD8+ T cells for recombinant and viral antigenic determinants was reduced by more than 90% as compared to untreated MVA-infected mice (**Figure 21**). As a control, the capacity of antigen-presenting DC to prime CD8+ Tcell responses in CpG-treated mice was analyzed. *In vivo,* peptide-pulsed DC primed comparable amounts of antigen-specific CD8+ T cells in CpG-pretreated or untreated mice (**Figure 22**). This confirmed that the abrogation of T cell priming was specifically due to the inhibition of cross-presentation and not caused by a general impairment of T-cell induction through CpG treatment. Interestingly, vaccination with *ex vivo*-infected DC did only induce a Tyr_{369}-specific CD8+ T cell response in untreated mice, suggesting an impaired ability of infected DC to prime CD8+ T cells, if cross-presentation was disabled (**Figure 22**).
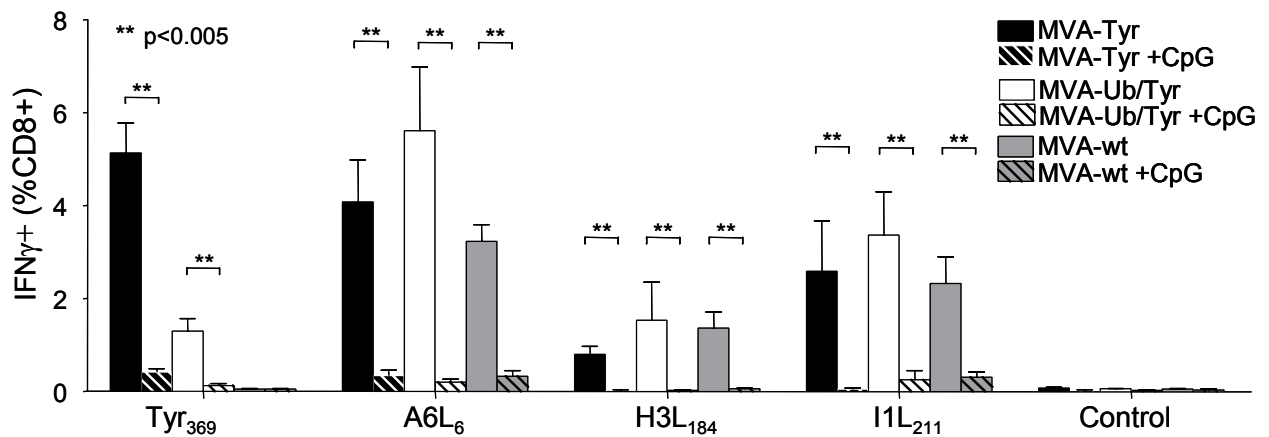

Figure 21 *In vivo* **maturation of DC abrogates CD8+ T cell priming with MVA vaccines.** Groups of A*0201-mice (n=4) were either CpG-treated or left untreated one day prior to vaccination with MVA-wt, MVA-Tyr or MVA-Ub/Tyr. On day 8 post vaccination tyrosinase- and vector-specific T_{CD8+} responses were analyzed by intracellular cytokine staining in splenocytes (**=p<0.005).
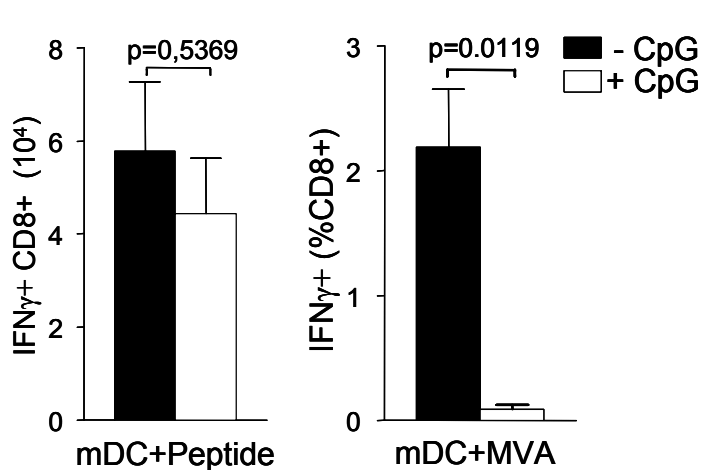
Figure 22 **Peptide-coated but not MVA-infected DC prime T cells after CpG treatment** CpG-pretreated or untreated mice (n=4) were immunized with Tyr_{369} peptide-coated *in vivo* matured splenic mDC. Total numbers of Tyr_{369}-specific CD8+ T cells on day 8 post vaccination are indicated (left panel). *In vivo* matured Splenic mDC were infected with MVA-Tyr, washed extensively and used to vaccinate groups of A*0201-mice (n=4) that either had received CpG-treatment the day before or were left untreated. Figures indicate Tyr_{369}-specific CD8+ T cell responses on day 8 post vaccination.

To exclude that CpG-treatment would alter the capacity of DC to endogenously express, process or present recombinant and viral antigens *in vivo*, splenic DC were isolated 6-8h post immunization and coincubated with CTL-lines reactive against viral and recombinant antigens. Independently, whether mice received CpG-treatment 15h before vaccination or not, splenic DC were fully capable to stimulate antigen-specific IFNγ-production in CD8+ T cells (**Figure 23**). Although direct presentation was detected in both groups this appeared to be inefficient to prime T cells. The interference with cross-presentation, however, abrogated T cell priming. From these experiments it was concluded that cross-presentation is the dominating pathway for the priming of CD8+ T cells when immunizing with MVA.
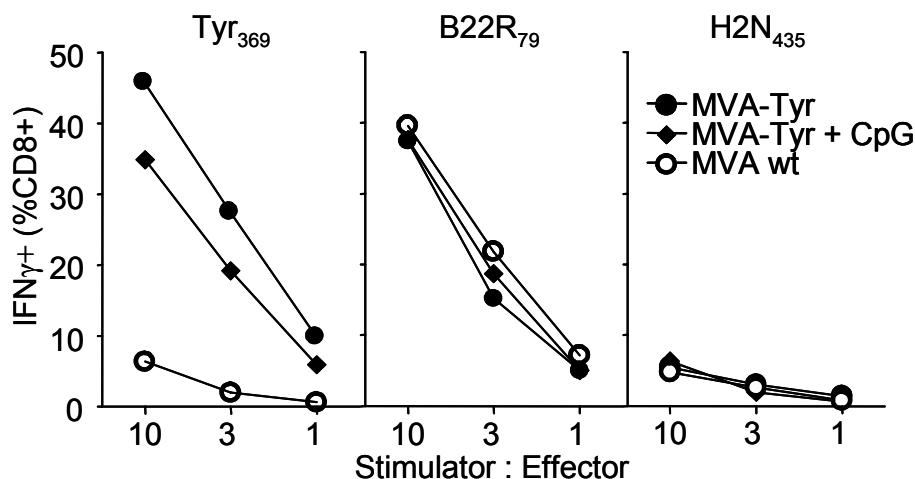

Figure 23 *In vivo* **matured DC express, process and present viral antigens in vivo.** Groups of A*0201-mice (n=5) were either CpG-treated or left untreated one day prior to vaccination with MVA-wt or MVA-Tyr. 7h post vaccination, splenic mDC were isolated and coincubated with T cell lines specific for Tyr_{369}, VACV-$B22R_{79}$ or $H2N_{435}$. Specific activation of T cells was detected by intracellular staining for IFNγ-production.

4.4.4 The dominating pathway of antigen presentation dictates antigen requisites

The data obtained so far suggested that the underlying pathway for CTL priming has major consequences for antigen formulations contained in MVA-based vaccines (**Figure 24**). To test this prediction it was analyzed whether mice vaccinated with MVA-Tyr were able to prime CD8+ T cell responses to a second tyrosinase peptide (Tyr_{1-9}) which, in contrast to Tyr_{369}, is derived from the signal sequence and is presented TAP-independently (Wolfel et al., 2000). Peptides located in the signal sequence can be efficiently presented via the classical endogenous route, but are not cross-presented (Wolkers et al., 2004). Consistently, target cells infected with MVA-Tyr were also recognized by Tyr_{1-9}-specific CTL (**Figure 24**), confirming efficient endogenous processing and presentation of Tyr_{1-9}. Mice vaccinated with MVA-Tyr mounted strong CD8+ T cell responses to Tyr_{369}, but failed to prime CTL specific for Tyr_{1-9}. Importantly, Tyr_{1-9}-specific T cells could be induced by peptide vaccination (**Figure 24**).
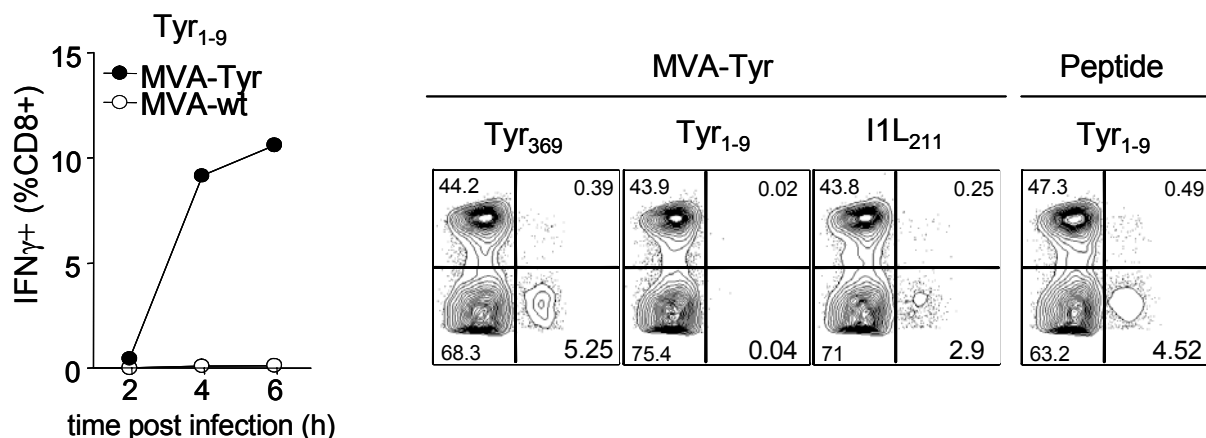

Figure 24   Cross-priming of CD8+ T cells dictates antigen requisites. RMA-HHD cells were infected with MVA-Tyr or MVA-wt and coincubated with Tyr_{1-9}-reactive CTL. Specific activation of CTL was assessed by intracellular staining for IFNγ-production (left panel). Groups of A*0201-mice (n=4) were vaccinated with MVA-Tyr or with the Tyr_{1-9} peptide. Representative plots from intracellular IFNγ-staining on day 8 post vaccination are depicted (right panel).

Next, the efficiency of polytope or minigene formulations to prime T cell responses was investigated, because these antigen formulations are commonly used in MVA-based vaccines and currently evaluated in several clinical studies. The data obtained so far indicated a reduced capacity of minigene-constructs to prime CD8+ T cells as compared to vaccines expressing the mature protein. A recombinant MVA expressing Tyr_{369} as part of a polytope (MVA-Mini-Tyr) was used for this

experiments. MVA-Mini-Tyr-infected target cells presented the Tyr_{369} peptide, but vaccination with MVA-Mini-Tyr completely failed to prime Tyr_{369}-specific T cells (**Figure 25**). To confirm this data using a well-established model antigen MVA expressing full-length OVA (MVA-OVA) or the Ova_{257} peptide SIINFEKL as a minigene (MVA-SIINFEKL) were compared. Surface staining of k^b/SIINFEKL complexes showed that direct presentation was enhanced when non-pAPC (data not shown) or pAPC were infected with MVA-SIINFEKL as compared to MVA-OVA (**Figure 26**). When vaccinating C57BL/6 mice with the respective viruses both vaccines induced a comparable vector-specific response (data not shown), but MVA expressing the long-lived antigen primed about four times more SIINFEKL-specific CTL than MVA encoding the SIINFEKL minigene (**Figure 26**).
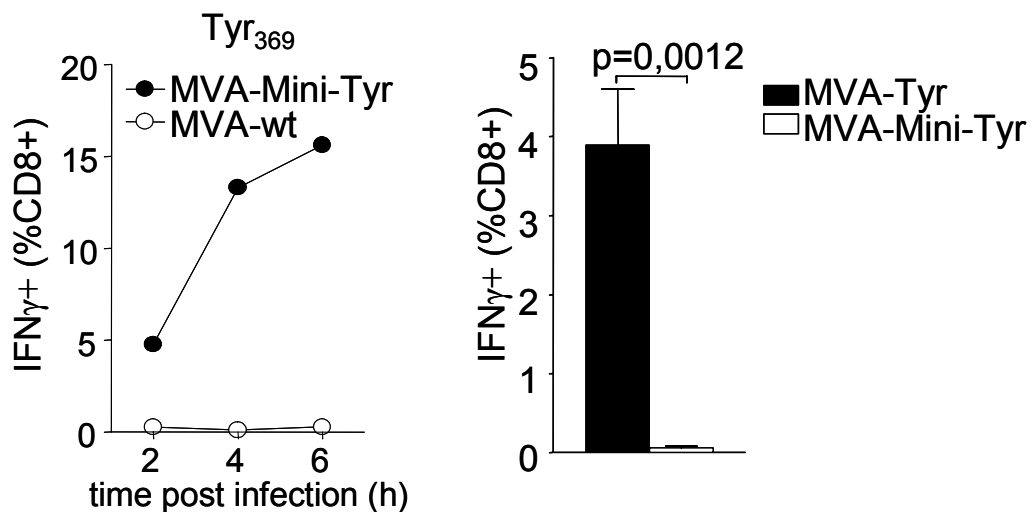

Figure 25   **The dominating pathway of antigen presentation can be targeted by the antigen formulation (I)** RMA-HHD cells were infected with MVA expressing the tyrosinase peptide Tyr_{369} encoded in a minigene (MVA-Mini-Tyr) or MVA-wt and were coincubated with Tyr_{369}-reactive CTL. Specific activation of CTL was assessed by intracellular staining for IFNγ-production (left panel). Groups of A*0201-mice (n=4) were vaccinated with MVA-Mini-Tyr or MVA expressing full-length tyrosinase (MVA-Tyr) and analyzed on day 8 post vaccination for Tyr_{369}-specific IFNγ-production (right panel).
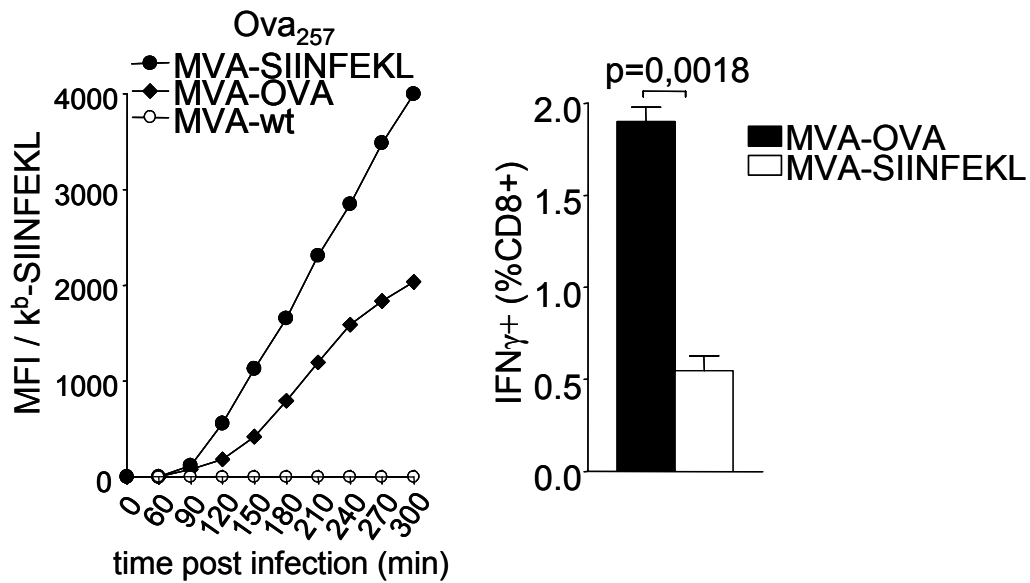
Figure 26 **The dominating pathway of antigen presentation can be targeted by the antigen formulation (II)** DC2.4 were infected with MVA-wt, MVA expressing full-length OVA or the Ovalbumin peptide SIINFEKL as a minigene. At indicated times post infection cells were analyzed for the surface expression of k^b/SIINFEKL complexes. Data are shown as mean fluorescence intensity (MFI). Note that MVA-wt infected cells define background staining (left panel). Groups of C57BL/6-mice (n=4) were vaccinated with MVA-OVA and MVA-SIINFEKL. CD8+ T cell responses on day 8 post vaccination are indicated (right panel).

Taken together, this data suggested that the induction of CTL immunity with vaccines based on MVA strongly, if not exclusively, depends on cross-priming and therefore requires vaccines to be designed for the expression of long-lived antigens that are suitable for cross-presentation.

4.5 Cross-competition of CD8⁺ T cells shapes the immunodominance hierarchy during boost vaccination

4.5.1 Priming of T cells of different specificities occurs independently

While the use of different antigen formulations allowed to graduate the ensuing T cell response against a recombinant target gene from non detectable (MVA-Mini-Tyr or MVA wt) to a low (MVA-Ub/Tyr or MVA-SIINFEKL) or to a robust response (MVA-Tyr or MVA-OVA) it was observed that the responses for MVA-specific antigens remained unaltered. There was apparently no influence of the strength of the primary CTL response induced against one antigen on the priming of T cells of different specificities. Therefore it was hypothesized that T cell priming occured independently of T cell competition during MVA vaccinations. To test this hypothesis it was further analyzed whether the quantity of induced T cells would be influenced by additional introduction or removal of immunodominant epitopes. At d8 post infection of HLA-A2 transgenic (HHD) mice with MVA wt, T cells recognizing epitopes from late gene products ($A6L_6$, $H3L_{184}$ and $I1L_{211}$) were dominating the response (**Figure 16 and 27**). T cells recognizing epitopes derived from early proteins were subdominant ($B22R_{79}$), or close to detection limit (< 0.1% of CD8⁺: $C7L_{74}$, $D12L_{251}$). Recombinant viruses additionally expressing human tyrosinase (MVA-Tyr) or human Her-2/neu (MVA-Her-2/neu) induced a strong Tyr_{369}- or moderate $Her-2/neu_{435}$-specific response. However, the vector-specific response remained unchanged compared to MVA wt. To exclude a mouse strain specific effect, C57BL/6 mice were analyzed. In these mice the cellular immune response against MVA wt is highly dominated by $B8R_{20}$-specific T cells, which recognize a determinant derived from an early gene product, followed by $A3L_{270}$- (late gene product), $K3L_6$- (early gene product) and $A8R_{189}$- (early gene product) specific T cells. Again, a recombinant virus expressing ovalbumin (MVA P7.5 OVA) induced a strong additional OVA_{257}-specific response in these mice without altering the frequencies of vector-specific T cells (**Figure 27**)). In addition, a mutant virus (MVA ΔB8R) with a deleted *B8R* gene was used. MVA ΔB8R did not induce a $B8R_{20}$-specific T cell response, and other vector-specific T cells did not compensate for that loss (**Figure 27**). From these experiments it was concluded that the primary induction of vector-specific T cells using MVA is not influenced by the priming of other vector-induced T cells. The time of expression (early vs late) of viral antigens did not correlate with their position within the immunodominance hierarchy.
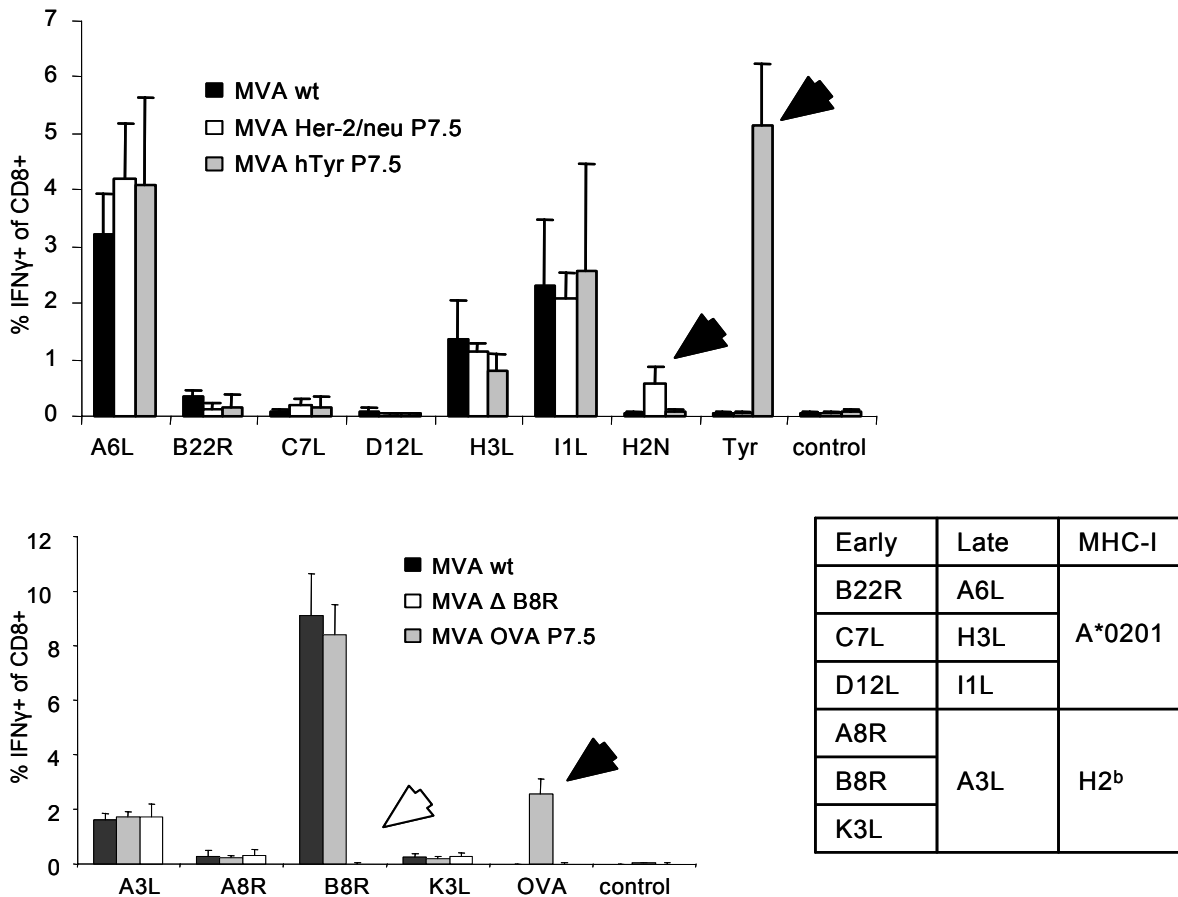
Figure 27 **Priming of T cells is independent of T cell competition** HHD mice were vaccinated i.p. (10^7 IU) with rec MVA or MVA wt (upper panel). C57BL/6 mice were vaccinated i.p. (10^8 IU) with MVA wt, MVA P7.5 OVA or MVA deletion mutant (MVA ΔB8R) (lower panel). Table shows early and late viral antigens and MHC class I restriction of the respective epitopes. On day 8 post vaccination, tyrosinase-, her-2/neu-, ova- and vector-specific CD8$^+$ T cell responses were analyzed by intracellular cytokine staining of splenocytes after a brief incubation with the indicated peptides. Filled arrows indicate additional, open arrows missing responses as compared to MVA wt.

4.5.2 Immunodominance hierarchy after secondary immunization correlates with viral gene expression

Next it was analyzed whether the timing of antigen expression (early vs late viral genes) was relevant for the reactivation of memory T cells. When boosting HHD mice with MVA wt, memory T cells recognizing epitopes derived from late gene products such as $H3L_{184}$ and $I1L_{211}$ did not expand (**Figure 28**). $A6L_6$-specific T cells were not amplified compared to the primary response. Interestingly, early gene product $B22R_{79}$-specific T cells expanded vigorously during the secondary immunization and became the dominant T cell population among the tested epitope specificities. This severe switch is surprising since it contradicts the prediction that T cells dominating the primary response should also dominate the secondary response, if memory precursor frequencies were merely

the critical factor for the immunodominance hierarchy of recall responses. In C57BL/6 mice the primary response is dominated by T cells specific for $B8R_{20}$, which is derived from an early gene product. If early antigen expression supports recall expansion, boosting of C57BL/6 mice with MVA wt should still support the recall expansion of $B8R_{20}$-specific T cells. Indeed, boosting with MVA wt led to a strong expansion of $B8R_{20}$-specific T cells (**Figure 28**). From these experiments it was hypothesized that i) T cells specific for early viral proteins might be able to suppress the expansion of other virus-specific T cells, and ii) T cells specific for late viral proteins should have a disadvantage to proliferate and expand during recall responses.
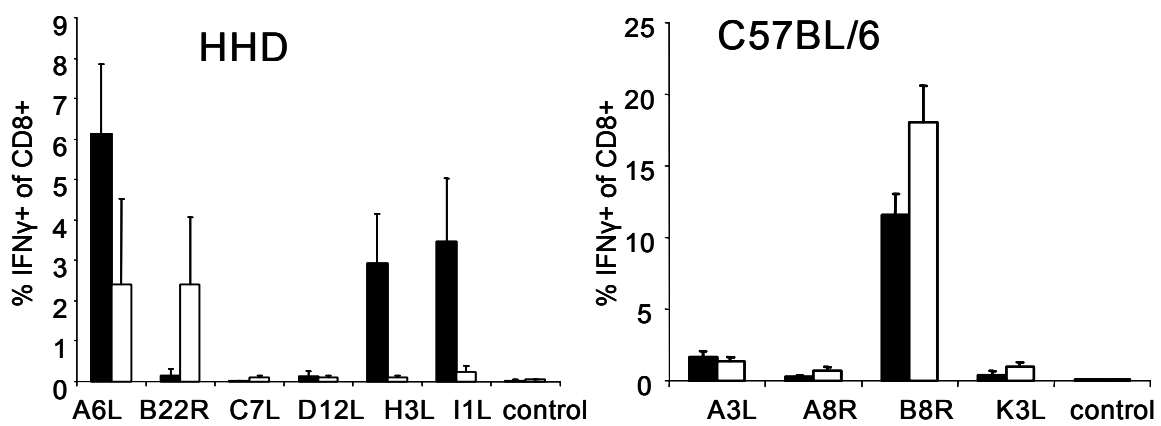

Figure 28   Immunodominance hierarchy is changed after secondary immunization in HHD mice. HHD or C57BL/6 mice were analyzed on d8 post prime (■) or boosted at d35 post prime and analyzed six days later (□). Mice were vaccinated i.p. with 10^8 IU. In HHD mice only $B22R_{79}$-specific T cells increase in frequency, $A6L_6$-, $H3L_{184}$- and $I1L_{211}$-specific T cells do not proliferate during secondary immunizations (left). In C57BL/6 mice $B8R_{20}$-specific T cells dominate the primary and secondary response (right).

One possible explanation for these observations was, that the poor proliferation of T cells specific for late viral antigens during recall responses was due to the lack of replication of MVA *in vivo*. Comparative analysis of the immune response at d8 post prime using MVA or the replication-competent parental strain CVA revealed a similar immunodominance hierarchy for both viruses (**Figure 28 and 29**).

Also in CVA immunized mice, $A6L_6$ was immunodominant followed by $I1L_{211}$-and $H3L_{184}$-specific responses. Interestingly, a substantial response against $B22R_{79}$ was detected at d8 post immunization with CVA. At d6 after boosting, again a suppression of T cell responses against late viral epitopes was observed using this replication-competent virus (**Figure 29**).
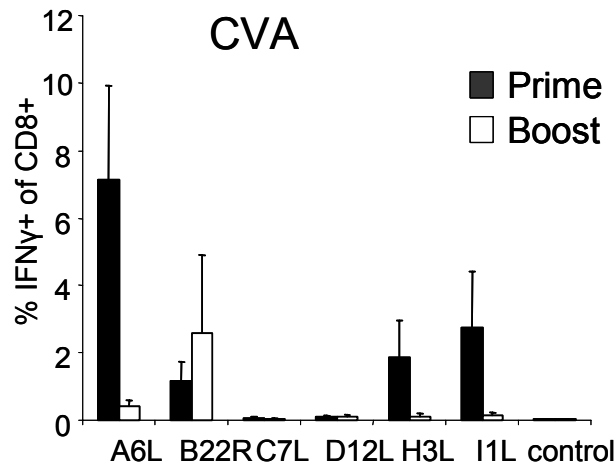

Figure 29 **Replication-competent virus CVA shows a similar shift in the immuno-dominance hierarchy as MVA.** Frequencies of IFNγ-producing CD8+ T cells in the spleen are shown on d8 (Prime) or d6 (Boost) post vaccination with replication competent VACV

To exclude that the lack of recall proliferation was due to a general malfunction of T cells specific for epitopes derived from late gene products, MVA primed mice were boosted with the respective peptides. As seen in **Figure 30**, $H3L_{184}$-specific T cells could be readily amplified *in vivo* when mice were re-vaccinated with $H3L_{184}$ peptide. Also $B22R_{79}$-specific T cells were expanded, when boosting with the respective peptide, yet to a lower extent. The frequency of $C7L_{74}$-specific T cells did not exceed that of peptide primed mice. In that respect, the expansion of MVA primed T cells by single peptide re-vaccination resulted in a pattern, which resembles the immunodominance hierarchy induced by MVA in the primary response. This indicates that VV-specific T cells, which did not proliferate in recall responses to the virus, possibly lacked antigen-specific stimulation. Impairment of viral antigen processing or presentation might account for reduced or inhibited proliferation of T cells recognizing late gene product-derived epitopes during VV recall responses. To further analyze this issue, it was decided to focus on the *H3L* late gene product.
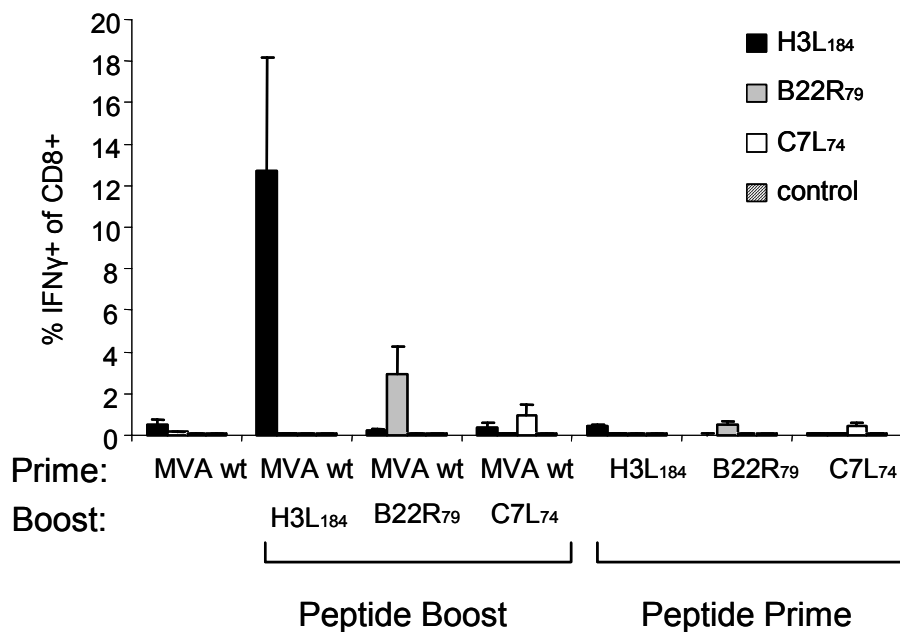
Figure 30   T cells specific for early and late viral gene products can be efficiently expanded with peptide vaccination. INFγ-production of VACV specific T cells from freshly isolated splenocytes of MVA wt (10^7 IU) immunized HHD mice. Mice were analyzed at d35 post prime, or boosted with $H3L_{184}$, $B22R_{79}$ or $C7L_{74}$ specific peptides and analyzed 5 days later. To be able to distinguish between priming and boosting of T cells induced by the peptide vaccination naïve mice were primed with $H3L_{184}$, $B22R_{79}$ or $C7L_{74}$ specific peptides and analyzed 5 days later.

4.5.3   Antigen presentation of late viral proteins is substantially delayed

The viral life cycle of VACV can be divided into three distinct phases:early, intermediate and late viral gene expression. The interval between these phases is only about one hour on the transcriptional level (Kastenmuller et al., 2006). To test if differences in viral transcription led to a relevant delay of the presentation of viral antigens, several T cell lines specific for VACV early or late protein determinants were used to perform detailed analyses of the ability of MVA infected stimulator cells to trigger peptide/MHC-specific T cell stimulation. As shown in **Figure 31**, already at 2h post infection the majority of $B22R_{79}$-specific T cells were stimulated (95%). $A6L_6$- and $I1L_{211}$-specific T cells did not get activated by infected cells even at 8h post infection, despite similar functional affinity for peptide-pulsed target cells. Coincubation of T cells with stimulators that had been infected for a longer period (up to 14h) did not lead to higher stimulation rates (data not shown). $H3L_{184}$-specific T cells were activated as early as 6h post infection (20%) and about 40% of the T cells produced IFN-γ at 8h post infection. Notably, this T cell line has the highest functional affinity. From these data it was concluded that presentation of determinants derived from late viral gene products to $CD8^+$ T cells is generally reduced and delayed for several hours as compared to early viral gene products.
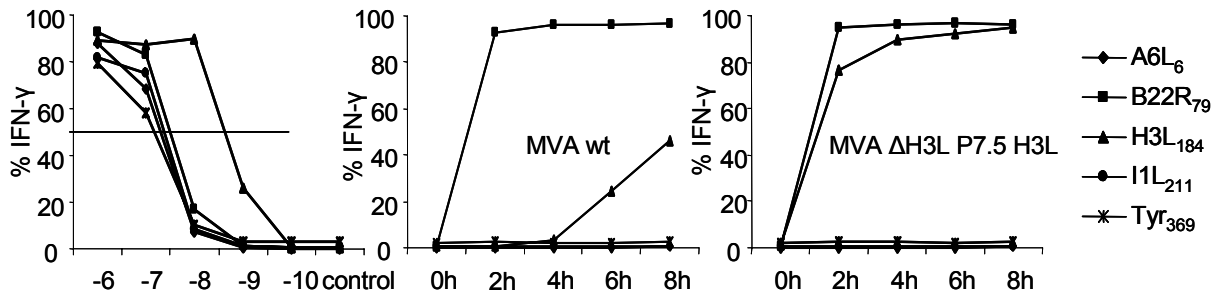
Figure 31 **Antigen presentation of late viral proteins is delayed.** Peptide titration shows similar affinity of T cell lines except $H3L_{184}$-specific T cells showing a higher affinity (left panel). Infected LCL (MOI 10) were used for a kinetic analysis to stimulate IFNγ-production in several VV-specific T cell lines. MVA wt infected cells (middle panel) stimulate $B22R_{79}$- (early gene) specific T cells (■) already 2h post infection. $H3L_{184}$- (late gene) specific T cells (▲) get stimulated 6h post infection. $A6L_6$- (♦), $I1L_{211}$- (●) specific T cells (both late) or control cell line (✱) are not stimulated by MVA wt infected cells. LCL infected by recombinant virus MVA ΔH3L P7.5 H3L expressing the *H3L* gene under an early/late promoter rapidly induce IFNγ-production in $H3L_{184}$-specific T cells (right panel).

4.5.4 Timing of viral antigen expression regulates T cell expansion

To test whether the reduced presentation of determinants from late viral antigens could be overcome by the expression of such antigens under the control of a early viral promoter a recombinant MVA in which the natural *H3L* gene has been knocked-out and was re-inserted into the viral genome under the control of a the P7.5 promoter with early/late activity (MVA ΔH3L P7.5 H3L) was used. In *vitro*, MVA ΔH3L P7.5 H3L infected target cells induced IFN-γ in $H3L_{184}$-specific T cells very early post infection (**Figure 31**). Importantly, $H3L_{184}$-specific T cells were readily expanded in the secondary response when boosting with MVA ΔH3L P7.5 H3L (**Figure 32**).
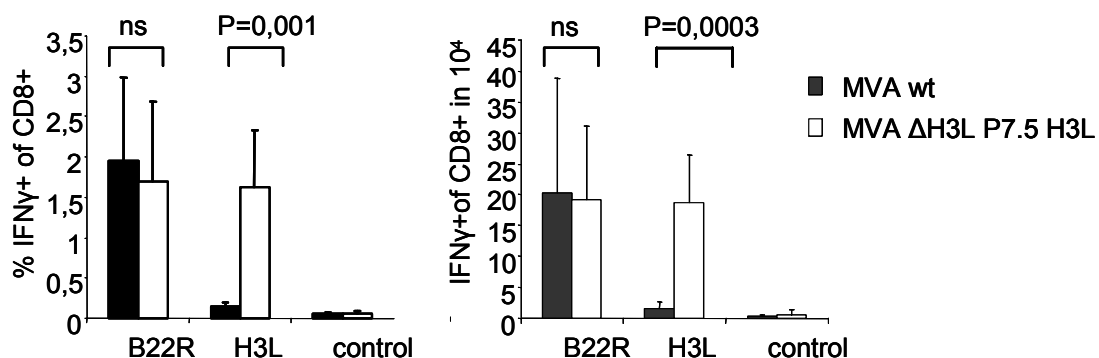

Figure 32 **MVA ΔH3L P7.5 H3L amplifies H3L specific T cell responses.** HHD mice were primed i.p. with 10^8 IU of MVA wt and then boosted with MVA wt (■) or MVA ΔH3L P7.5 H3L (□). MVA ΔH3L P7.5 H3L induces a significant expansion of $H3L_{184}$-specific INFγ-producing T cells as compared to MVA wt, without altering the T cell response against $B22R_{79}$. Relative (A) and absolute numbers (B) of INFγ-producing $H3L_{184}$-specific T cells measured in the spleen compared to MVA wt.

Thereby, the absolute numbers of H3L$_{184}$-specific T cells reached similar levels as B22R$_{79}$-specific T cells. Importantly, the responses against other epitopes (A6L$_6$, I1L$_{211}$ and B22R$_{79}$) were comparable for both viruses.

4.5.5 Cross-competition of T cells regulates T cell expansion

Although epitopes derived from late viral proteins were presented with a strong delay on the surface of infected cells, one would expect that T cells recognizing these epitopes would eventually get stimulated and could proliferate. *In vivo,* however, H3L$_{184}$- and I1L$_{211}$-specific T cells were not amplified during recall responses (**Figure 28 and 29**). Based on this observation, it was speculated that in addition to the delayed presentation of viral late proteins, T cells with different specificities compete for infected APCs. Particularly, T cells recognizing VACV epitopes from early proteins should have an advantage to cross-compete compared to T cells recognizing VACV epitopes from late proteins. Since peptide immunizations in HHD mice are very efficient, this question was experimentally addressed by priming naïve mice simultaneously with pairs of peptides. Cohorts of mice were immunized with H3L$_{184}$ together with either B22R$_{79}$ or C7L$_{74,}$ which are both derived from early proteins, or a control peptide derived from human tyrosinase (Tyr$_{369}$) (**Figure 33**). At d35 post prime, T cell frequencies in the peripheral blood were determined by tetramer analysis. Thereafter, mice were boosted either with MVA wt or a recombinant virus expressing human tyrosinase under control of an early/late promoter (MVA hTyr). Five days later, the specific T cell frequencies in the blood were measured again by tetramer staining. To compensate variations of peptide-induced T cell frequencies within individual mice, the ratio of the frequencies before and after boosting with MVA vaccines was calculated as a relative measure of T cell proliferation. When H3L$_{184}$-specific T cells were induced together with C7L$_{74}$- or B22R$_{79}$-specific T cells, they did not proliferate after boosting with MVA wt. Importantly, when H3L$_{184}$-specific T cells were accompanied by irrelevant Tyr$_{369}$-specific T cells, they were readily amplified. However, when peptide-specific CD8$^+$ T cells were boosted with MVA hTyr expressing human tyrosinase under the control of an early/late promoter, the H3L$_{184}$-specific response was again suppressed. These data demonstrate that the proliferation of H3L$_{184}$-specific T cells strongly depends on the ability of participating T cells of other specificity to cross-compete.
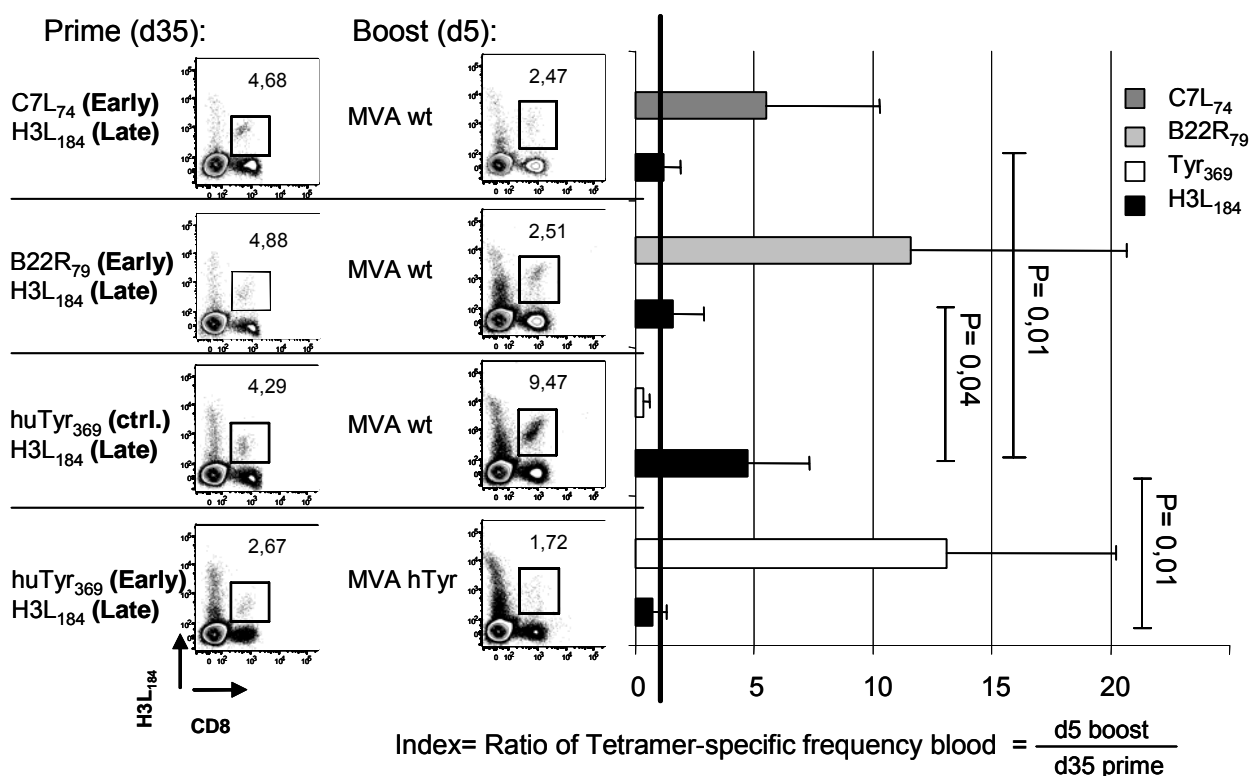
Figure 33   Cross-competition between T cells recognizing early and late determinants. HHD mice were simultaneously primed with pairs of peptides. At d35 post prime, frequencies of respective tetramer specific $CD8^+$ T cells in the peripheral blood were determined. Then mice were boosted i.p. with 10^8 IU of MVA wt or MVA hTyr. Five days later, tetramer specific $CD8^+$ T cells were again measured in the blood. Numbers within dot plots indicate frequencies of $H3L_{184}$-specific T cells before (left column) and after boosting (right column). Index shows relative increase of tetramer specific T cells after boosting. Black line indicates that frequencies before and after boost are equal (index =1). In contrast to control T cells (Tyr_{369}), the presence of $B22R_{79}$- or $C7L_{74}$-specific T cells (both early genes) significantly suppresses the expansion of $H3L_{184}$- (late gene) specific T cells after boosting with MVA wt. However, when MVA hTyr is used the expansion of $H3L_{184}$-specific T cells is again suppressed but Tyr_{369}-specific T cells are readily amplified.

4.5.6 T cells cross-compete early after priming

Since cross-competition between T cells was observed only during boost vaccinations, one possible explanation was that this phenomenon is linked to the presence of T cells with fully developed effector function. Naïve T cells gain effector function early (around d3) during primary responses (van Stipdonk et al., 2001). Furthermore, by using Listeria monocytogenes, it has been demonstrated previously that a second infection during the priming phase can induce typical recall responses (Busch et al., 2000). Therefore, mice were re-immunized during the primary MVA response with the aim to define a time-point after priming when T cells are able to execute cross-competition. For a detailed

kinetic analysis, mice were primed and subsequently re-vaccinated once from d1 to d6 post prime (**Figure 34**). Six days after re-vaccination, mice were sacrificed and the epitope dominance pattern was determined and compared to mice, which had received MVA only once. Interestingly, when mice were re-immunized on d1 or d2 post prime, the epitope pattern did not change. When re-immunizing at 1 day after prime $A6L_6$-, $I1L_{211}$- and $H3L_{184}$-specific $CD8^+$ T cells even increased (**Figure 34** and data not shown). Re-immunization on d2 did not alter the frequencies or absolute numbers of the different T cell populations. However, when boosting on d3 or later, $A6L_6$-, $I1L_{211}$-and $H3L_{184}$-specific T cells frequencies were reduced, whereas $B22R_{79}$-specific T cells expanded. From d4 on or later, $B22R_{79}$-specific T cells were significantly amplified as seen in secondary responses after 35 days. To confirm that T cells were able to cross-compete early after primary infection, MVA wt primed mice were re-vaccinated with MVA wt or MVA ΔH3L P7.5 H3L. Indeed, similar to boosting at d35, $B22R_{79}$- and $H3L_{184}$-specific T cells expanded when mice were boosted early (d5) with MVA ΔH3L P7.5 H3L (**Figure 35**).
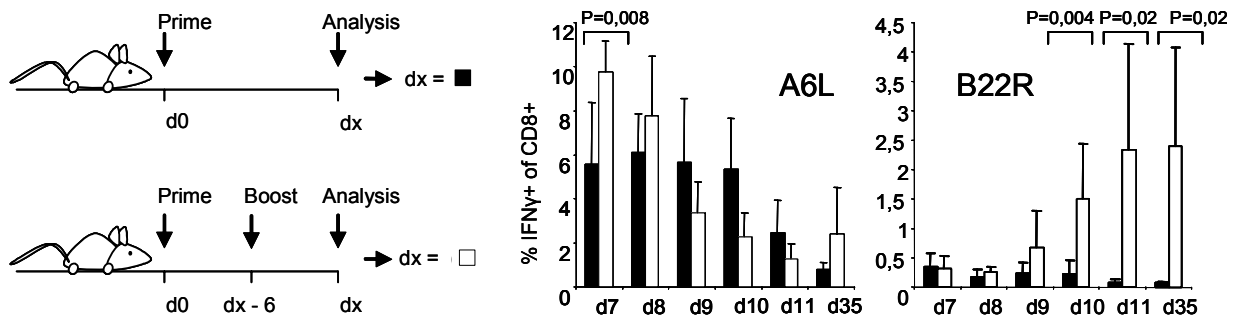

Figure 34 **Competition between T cells occurs early after priming (I).** HHD mice were primed with MVA wt and boosted with the same virus early at indicated days after priming Schematic of prime-boost regimen (left). Intracellular cytokine staining of splenocytes, comparing the VV-specific $CD8^+$ T cell responses after priming (■) or 6 days post boosting (□) (right). $B22R_{79}$-specific T cells are significantly increased when boosting at d4 post prime or later.
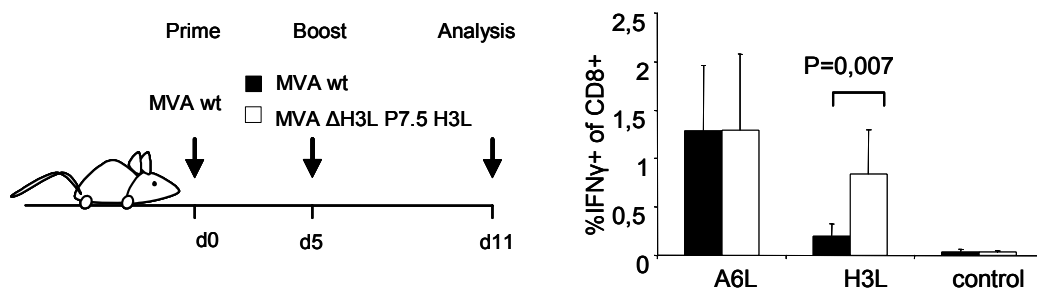

Figure 35 **Competition between T cells occurs early after priming (II).** HHD mice were primed with MVA wt and boosted with d5 with MVA wt (■) or MVA ΔH3L P7.5 H3L (□). Schematic of prime-boost regimen. $H3L_{184}$-specific T cell responses can be significantly amplified when using MVA ΔH3L P7.5 H3L (□) as compared to MVA wt (■).

4.5.7 Cross-competition between T cells specific for early viral determinants

Thus far, the data showed that T cell cross-competition between T cells recognizing early or late viral products is functionally important for shaping the immunodominance hierarchy during recall responses. As during boost vaccinations T cells recognizing early epitopes efficiently expanded the next question was whether T cells recognizing early epitopes also cross-compete with each other. In C57BL/6 mice the $B8R_{20}$-specific T cell response dominates both the primary and secondary response against VV. To analyze, if $B8R_{20}$-specific T cells were cross-competing with T cells of other specificities and impaired their expansion, MVA $\Delta B8R$ was used for prime/boost immunizations (**Figure 36**). Despite the absence of $B8R_{20}$-specific T cells the expansion of $A3L_{270}$-specific T cells was suppressed in the recall response, presumably by cross-competing $K3L_6$- and $A8R_{189}$-specific T cells, which rapidly expanded. Again a switch in the immunodominance hierarchy favouring the proliferation of T cells recognizing epitopes derived from early gene products was found. In contrast, in a prime-boost regimen using MVA wt, $K3L_6$- and $A8R_{189}$-specific T cells were not amplified to the same level as compared to MVA $\Delta B8R$.
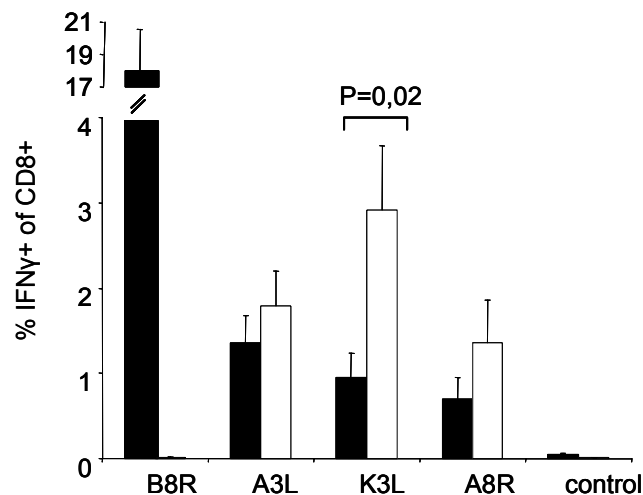

Figure 36 **Early virus-specific T cells suppress the expansion of early and late virus-specific T cells.** Intracellular cytokine staining of splenocytes comparing MVA $\Delta B8R$ ($\square$) with MVA wt ($\blacksquare$) at d6 post homologues boost (d35+6). Immunodominance hierarchy is changing in MVA $\Delta B8R$ immunized mice favouring the expansion of $K3L_6$- and $A8R_{189}$- (both early genes) specific T cells over $A3L_{270}$- (late gene) specific T cells in the absence of $B8R_{20}$-specific T cells.

To further characterize T cell cross-competition between T cells recognizing early viral products, short interval prime/boost immunizations were performed. The $K3L_6$- and $A8R_{189}$-specific T cell response induced by MVA $\Delta B8R$ were defined as the baseline and the $A3L_{270}$-specific T cell response was considered as an internal control. Then the $K3L_6$- and $A8R_{189}$-specific T cell response induced by recombinant viruses which contain an additional moderate (MVA OVA $\Delta B8R$ – OVA_{257}) or highly (MVA wt – $B8R_{20}$) immunogenic epitope or both (MVA OVA) was compared. As shown in **Figure 37**, this stepwise addition of cross-competing T-cells specific for early viral epitopes ($OVA_{257}/B8R_{20}$) leads to a gradually increased suppression of other T cells also specific for early viral epitopes ($K3L_6/A8R_{189}$), while T cells specific for late viral epitopes ($A3L_{270}$) remain fully suppressed. This data demonstrated that cross-competition is also functional among T cells specific for early viral proteins during recall responses.
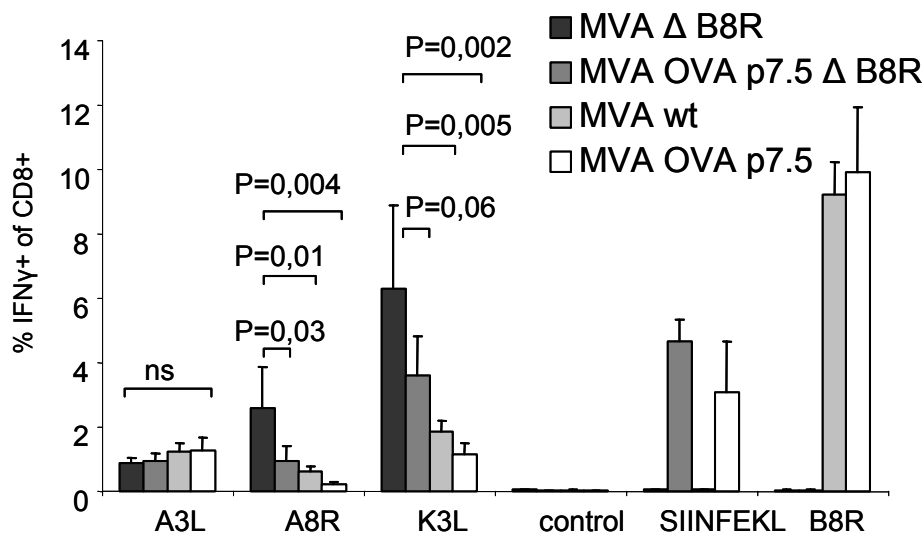

Figure 37   Cross-competition is functional among T cells specific for early viral antigens during recall responses Intracellular cytokine staining of splenocytes comparing MVA $\Delta B8R$ (■) with MVA OVA P7.5 $\Delta B8R$ (▨), MVA wt (▢) and MVA OVA P7.5 (□) at d6 post homologues boost (d5+6) (B). The expansion of $K3L_6$- and $A8R_{189}$- (both early genes) specific T cells is successively suppressed by gradual increase of cross-competing $B8R_{20}$- and OVA_{257}- (both early genes) specific T cells, while $A3L_{270}$- (late gene) specific T cells remain fully suppressed.

4.5.8   Timing of viral antigen expression is crucial for vaccination strategies

In order to investigate the insights obtained so far could be exploited to induce strong CTL immunity against a desired recombinant antigen, two viruses expressing the model antigen ovalbumin driven by a promoter with exclusive early (MVA K1L OVA) or late (MVA P11 OVA) activity were used. When measuring the processing and presentation of ovalbumin by specific staining of Ova_{257}-loaded MHC-I

complexes on infected RMA cells, a clear correlation with the time of expression was found. Already 2h post infection a significant amount of H2-K^b Ova$_{257}$ complexes could be detected on the surface of MVA K1L OVA infected cells with a maximum at about 12h post infection. In contrast, minimal amounts of Ova$_{257}$ on the surface of MVA P11 OVA infected RMA cells were not detected before 12h post infection. *In vivo*, however, a comparable OVA-specific T cell response in the spleen of MVA P11 OVA and MVA K1L OVA immunized mice was found (**Figure 38**). This confirmed that during priming neither the amount of presented peptide/MHC-I complexes nor the time after infection at which viral antigen expression occurs, determines the outcome of the T cell response and therefore is consistent with the notion that crosspriming dominates the primary T cell response to MVA. However, during secondary immunizations only MVA K1L OVA immunized mice readily amplified Ova$_{257}$-specific T cells, while MVA P11 OVA failed to expand Ova$_{257}$-specific T cells (**Figure 38**). Importantly, the T cell responses against virus-specific antigens were comparable for both viruses (data not shown).
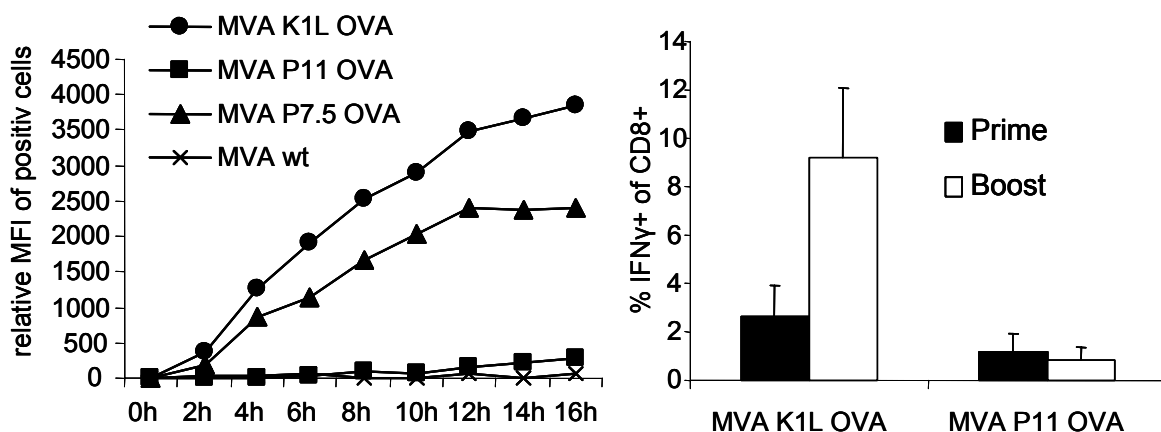

Figure 38   Timing of viral antigen expression is crucial for vaccination strategies. For relative quantification of SIINFEKL-K^b complexes on infected cells, 25-D1.16 antibody was used. Mean fluorescence intensity of positive cells is shown (left panel). Intracellular cytokine staining of splenocytes comparing MVA K1L OVA with MVA P11 OVA d8 (■) post prime or d6 (□) post boost after priming with MVA P7.5 OVA (right panel).

This data clearly demonstrated that for the induction of strong CTL immunity during boost vaccinations it is crucial to express recombinant antigens under a viral promoter with early activity. To test if these conclusions would also apply to the induction of protective immunity against a lethal pathogen short interval prime/boost vaccinations were followed by a challenge of a lethal dose of Lysteria monocytogenes expressing Ovalbumin (L.m.-Ova). This system was chosen because protection against L.m.-Ova depends on Ova-specific CD8+ T cells. Only MVA constructs expressing

ovalbumin early during the viral life cycle were able to fully protect mice when challenged with lethal doses of L.m.-Ova (**Figure 39**). Mice immunized with MVA P11 OVA could reduce the bacterial load only slightly better than mice immunized with MVA wt, indicating that late expression of OVA does not confer significant CTL immunity. This is most likely because OVA-specific T cells were outcompeted by other vector-specific T cells. When OVA was expressed early during the viral life cycle OVA-specific T cells were able to expand (**Figure 38**) and to acquire effector functions to a extent that conferred protective immunity (**Figure 39**).
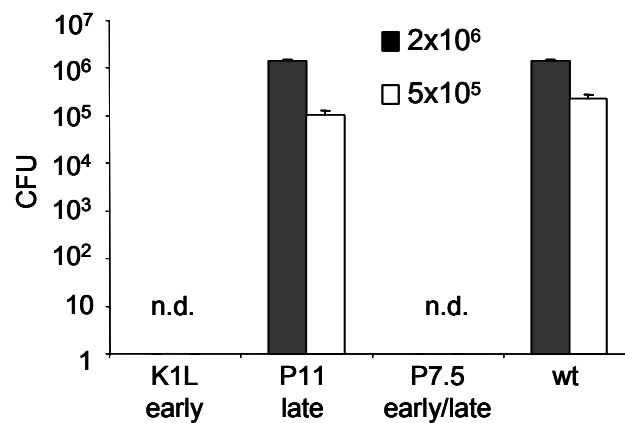

Figure 39 **The induction of heterologous protective CTL im munity with MVA prime/boost vaccinations requires early expression of a target antigen** C57BL/6 mice were primed and boosted with either MVA expressing OVA under the control of an early (K1L), late (P11) or early and late (P7.5) promoter. On day 6 post boost mice were challenged with lethat doses of L.m. OVA. The next day the bacterial load in liver (data not shown) and spleen was determined. Only those vaccines that expressed OVA early during the viral life cycle were able to protect mice. *n.d. = non detectable*

These experiments strongly support the conclusion that the secondary response to MVA is dominated by T cells recognizing early gene products, with T cells specific for late gene products being boosted weakly or not at all. The expansion of T cells being able to mediate protective immunity requires the expression of recombinant antigens under the control of a viral promoter with early activity. Only then these T cells are able to execute cross-competition and to expand.

4.6 Direct presentation can be targeted for the efficient expansion of memory CD8+ T cells

In the first part of this thesis it was noted that the primary induction of T cells with MVA-based vaccines requires efficient cross-presentation of antigens. In the second part it was found that T cells specific for some of the antigens that induce robust primary responses are not expanded during secondary vaccinations. It was observed that T cells cross-compete and by this limit the proliferation of T cells for other specificities. Additionally, it could be demonstrated that the early expression of antigens (regulated by different viral promoters) correlated with the presentation of antigenic peptides and was essentially required to allow T cells specific for these antigens to compete and to proliferate. In the first part of this thesis, recombinant viruses were used that expressed target antigens under the same viral promoter and therefore at the same time during the viral infection. This allows analyzing whether the expansion of memory CD8+ T cells correlates with the ability of infected cells to generate and to directly present antigenic peptides. In the following experiments this issue was addressed in the context of homologous prime/boost vaccinations (2x recMVA) and when using heterologous prime/boost regimens (using DNA or peptide priming). The heterologous regimens were of special interest as these correspond to the use of recMVA vaccines in current clinical studies and as the influence of direct presentation on the expansion of memory CD8+ T cells could be investigated under experimental conditions where no T-cells specific for other viral antigens compete during the secondary vaccinations.

4.6.1 Antigen formulations that fail in primary vaccinations efficiently activate memory T cells

MVA-Ub/Tyr and MVA-SIINFEKL failed to induce strong CTL-immunity because short-lived antigen (Ub/Tyr) or minigenes (Mini-Tyr, SIINFEKL) had a reduced suitability for the cross-presentation pathway which dominated the primary induction of T cells in the context of MVA vaccines (**Figure 20, 25** and **26**). However, peptides derived from these antigens were efficiently loaded onto MHC class I molecules of infected cells and presented to T cells *in vitro* and *in vivo* (**Figure 15, 18, 25** and **26)**. HHD mice were vaccinated with MVA-Tyr and then separated into three groups which 30 days later received MVA-Mini-Tyr, MVA-Tyr or MVA-Ub/Tyr as booster vaccination. The expansion of Tyr_{369}-specific T cells strongly correlated with the ability of infected cells to present antigenic peptides. MVA-Ub/Tyr was most efficient to boost Tyr_{369}-specific memory T cells. Similarly, MVA-SIINFEKL primed about four times less OVA_{257}-specific T cells but efficiently expanded OVA_{257}-specific memory T cells to a comparable extent as MVA-OVA (**Figure 40**)
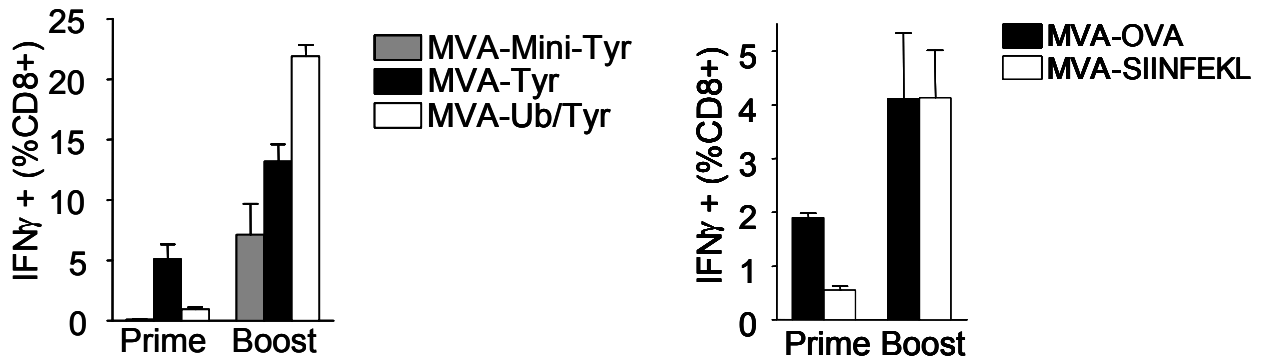
Figure 40   Antigen formulations that fail to prime naïve T cells can be used for the expansion of memory CD8+ T cells HHD (left) or C57BL/6 mice (right) were primed with the indicated viruses and the frequency of Tyr_{369}- or OVA_{257}-specific T cells were analyzed on day 8 post prime (left and right panel, respectively). To analyze the ability of the indicated viruses to expand memory T cells, groups of mice were either primed with MVA-Tyr (n=12) (left panel) or MVA-OVA (n=8) (right panel) and then boosted with the indicated viruses (n=4). Tyr_{369}- or OVA_{257}-specific T cells were analyzed on day 6 post boost (left and right panel, respectively).

Next it was tested whether targeting a recombinant antigen for rapid degradation to enhance direct presentation could also improve vaccination regimens that are frequently used in current clinical trials. Therefore mice were primed by peptide vaccination (Tyr_{369}-Peptide + CpG s.c.) or by DNA vaccination (DNA-Tyr i.m.) and boosted with MVA-Tyr or MVA-Ub/Tyr on day 30 post prime. Again, MVA-Ub/Tyr expanded more Tyr_{369}-specific memory T cells than MVA-Tyr. Importantly, in this experimental setup an influence of T cells specific for other antigens (DNA-Prime) or other peptides (Peptide-Prime) on the outcome of memory T-cell expansion can be excluded. Accordingly, the enhanced expansion of T cells with MVA-Ub/Tyr appeared to be independent of an influence of tyrosinase antibodies or antigen-specific CD4+ T cell help, as these arms of the immune system should not be activated through Tyr_{369} peptide priming.
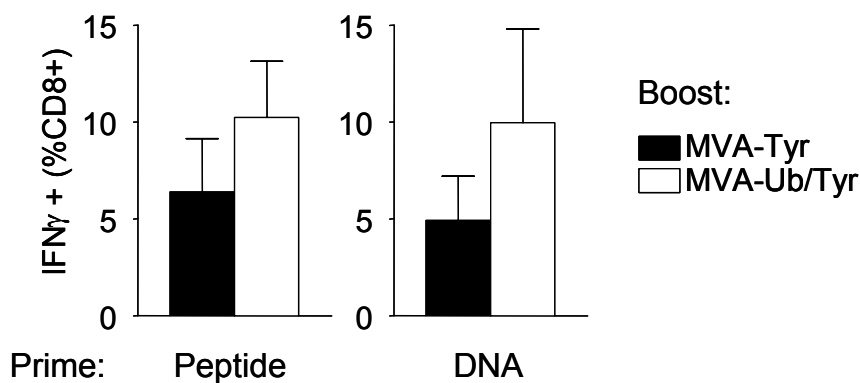

Figure 41   Enhanced expansion of CD8+ T cells in heterologous prime/boost vaccinations

HHD mice were primed with Tyr_{369} peptide or tyrosinase DNA and then boosted on day 30 with the indicated viruses. Analysis was performed by ICS on day 6 post boost

4.6.2 Direct presentation is sufficient to expand memory CD8+ T cells

Consistent with the finding that T cell priming with MVA depends on cross-priming, no induction of CD8+ T cells specific for the leader-sequence derived antigenic determinant Tyr_{1-9} was found (**Figure 24**). Peptides derived from signal-sequences are not cross-presented but can be presented via the endogenous route (Wolkers et al., 2004). Accordingly, cells infected with MVA-Tyr were able to directly activate Tyr_{1-9}-specific T cells (**Figure 23**). Therefore it was tempting whether MVA-Tyr would be able to expand Tyr_{1-9}-specific memory T cells. To achieve priming of these T cells mice were vaccinated with Tyr_{1-9} peptide. After 30 days mice were boosted with MVA-Tyr. To be able to differentiate between the secondary expansion of Tyr_{1-9}-specific T cells and memory frequencies after the peptide prime, control animals were boosted with MVA wt. MVA-Tyr specifically expanded Tyr_{1-9} specific T cells. This experiment showed that direct presentation is sufficient to boost memory CD8+ T cells.
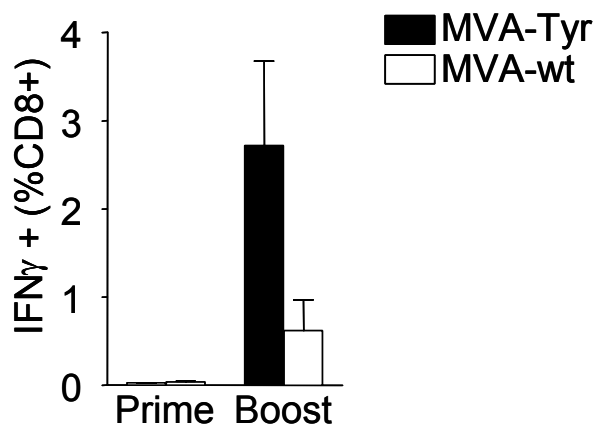

Figure 42 Direct presentation is sufficient to expand memory CD8+ T cells

HHD mice were vaccinated with Tyr_{1-9} peptide. In the memory phase mice were vaccinated with MVA-Tyr. To be able to differentiate between the secondary expansion of Tyr_{1-9}-specific T cells and memory frequencies after the peptide prime, control animals were boosted with MVA wt

4.6.3 Enhanced *in vivo* cytotoxicity after MVA-Ub/Tyr boost

The results obtained so far indicated that enhancing direct presentation e.g. by targeted degradation of a desired antigen improves the outcome of MVA-boost regimens when analyzing IFNγ-producing T-cells. To test if the increased amount of effector T cells would reflect also an enhanced functionality *in vivo,* the capacity of vaccinated mice to lyse peptide-presenting cells was tested. The assessment of *in vivo* cytotoxicity is regarded as a good correlate of vaccine efficiency when targeting CTL responses. Mice were transferred with syngenic splenocytes that had been labeled with the Tyr_{369} or the irrelevant $H2N_{435}$ peptide. To be able to distinguish cells by FACS analysis, cells were stained with different concentrations of fluorescent CFSE. At the peak of the secondary response (day 6 post boost) mice were transferred i.v. with 10 Mio splenocytes/peptide. After 4 hours PBMC and splenocytes were harvested and the percentage of Tyr_{369}-labelled and control cells was determined by FACS analysis.

MVA-Ub/Tyr boosted mice showed higher specific lysis in the peripheral blood as well as the spleen (**Figure 43**). While MVA-Tyr vaccinated mice had a reduced *in vivo* cytotoxicity when T cell priming occurred at a lower dose, MVA-Ub/Tyr boosted mice showed only a slight reduction in the periphery but still reached a maximum specific cytotoxicity in the spleen.
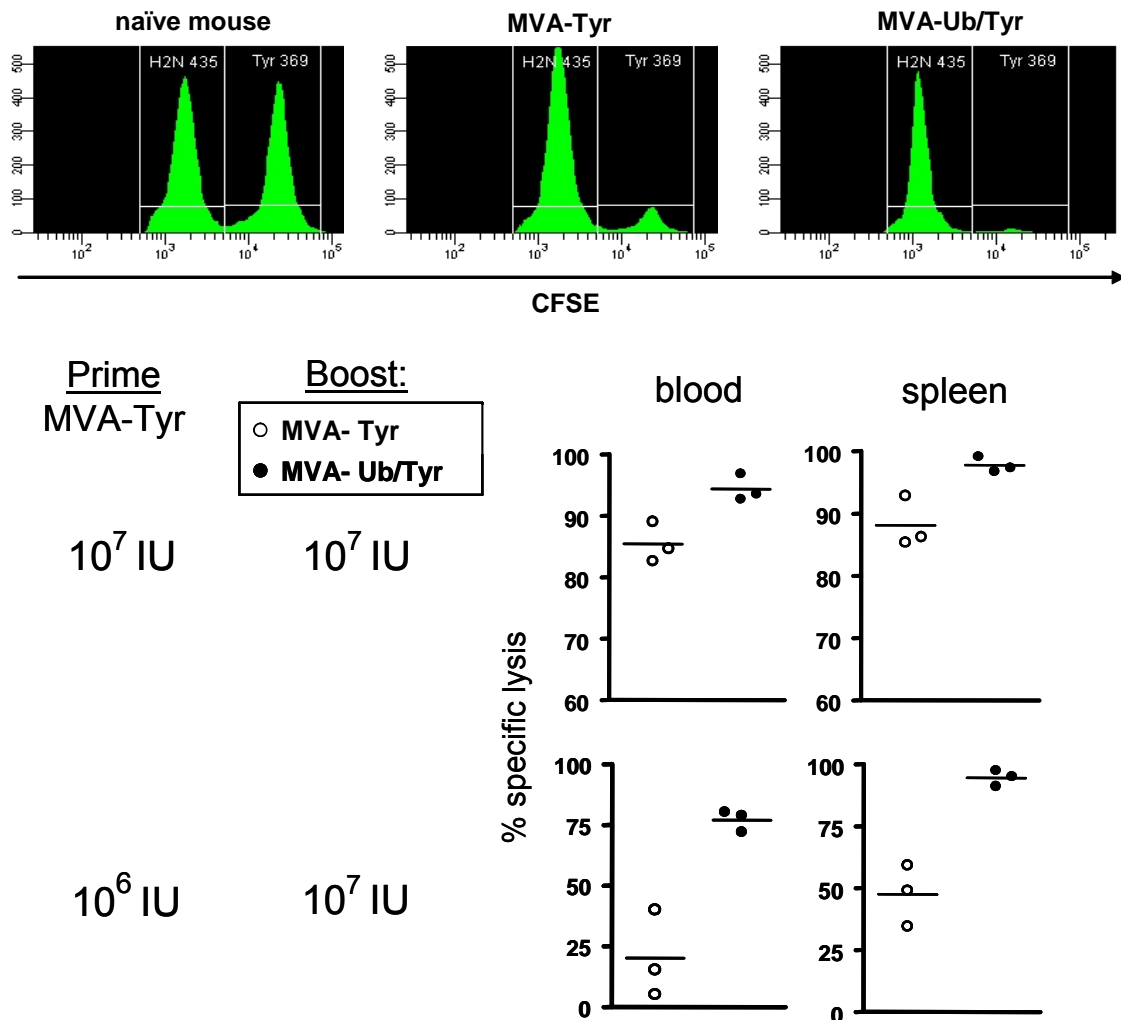

Figure 43   *In vivo* **cytotoxicity is enhanced by MVA-Ub/Tyr boost vaccination** Mice were vaccinated with different doses of MVA-Tyr. On day 30 post prime, mice were boosted with either MVA-Tyr or MVA-Ub/Tyr. On day 6 post boost *in vivo* cytotoxicity was measured. Data represent specific lysis of Tyr_{369} coated splenocytes in a 4h assay.

In all the experiments performed the expansion of memory T cells correlated with the ability of antigens to get presented by infected cells via the endogenous route. These data showed that enhancing direct presentation by targeting a full-length antigen for rapid proteasomal degradation is a powerful approach to enhance the efficiency of MVA boost vaccinations.

5 Discussion

5.1 Crosspriming of cytotoxic T cells dictates antigen requisites

Recently, substantial progress has been made in the characterization of the antigen presentation pathways for MHC-class-I-restricted determinants. Yet, for many vectors it is still unknown which pathways contribute to the primary induction of CD8+ T cell responses. This is of particular interest, because the biological properties of an antigen allowing efficient direct or cross-presentation seem to differ (Blachere et al., 2005; Norbury et al., 2004; Shen and Rock, 2004; Tobery and Siliciano, 1997; Wolkers et al., 2004). The present work confirmed that a short half-life of endogenous antigens delivered by a viral vector leads to efficient processing and direct presentation of antigenic determinants. On the other hand, rapid degradation of antigen limited the availability for the cross-presentation pathway, which efficiently exploited mature protein. It could be shown that the infection with the viral vector MVA comprises central features which in principle would allow for direct CD8+ T cell priming. MVA-infected DC exhibited strong antigen expression and presentation *in vitro* and *in vivo*. A role for direct priming in MVA immunity therefore seemed to be likely. Strikingly, however, CD8+ T cell priming was found to be dominated by cross-presentation when using MVA-based vaccines. This notion is supported by several lines of evidence: 1) Recombinant MVA expressing rapidly degradable protein enhanced direct presentation *in vitro* and *in vivo,* but failed to prime strong CTL immunity. 2) Cross-priming experiments using infected TAP-deficient or MHC-class-I–mismatched non-pAPC donor cells evoked a similar response regarding CTL frequencies and the immunodominance hierarchy as vaccination with MVA when given as a live vaccine. 3) Inhibition of cross-presentation by *in vivo* maturation of DC almost completely abrogated priming of MVA-specific CTL. Importantly, systemic maturation of DC did neither inhibit their capacity for endogenous expression, processing or presentation of antigens nor their ability to directly prime T cells *in vivo*. 4) None of the performed experiments showed a correlation of the size of the primary T cell response with the generation of antigenic peptides or direct presentation of these determinants by infected cells. The priming of CTL instead required the expression of antigenic determinants as a substrate suitable for cross-presentation. Accordingly, no T cell priming was observed against an epitope derived from a signal peptide (Wolkers et al., 2004). CD8+ T cell responses strongly correlated with the steady-state level of long-lived antigen which was found to be the substrate for efficient cross-presentation when delivered by MVA. Importantly, these findings are independent of the route of vaccination and apply to different model antigens tested in different mouse strains.

Crosspriming dominates primary T cell induction

Based on the presented data it was concluded that the functionally relevant pathway to induce CD8+ T cell responses with MVA vaccines *in vivo* is cross-priming. Although a minor role for direct priming cannot be excluded, the experimental data clearly suggest that this potential contribution to T cell priming may not be relevant for vaccine design with this vector. Residual responses observed upon vaccination with MVA encoding rapidly degradable antigen or minigene constructs could also be explained by cross-presentation of peptides or polypeptides which has been found to be quite inefficient (Norbury et al., 2004; Shen and Rock, 2004; Wolkers et al., 2004). Therefore these findings possibly indicate a functionally exclusive role of cross-presentation for the induction of primary CTL responses with MVA vaccines. In the past, this has only been postulated for those viruses that either do not infect DC (Sigal et al., 1999; Subklewe et al., 2001) or that considerably interfere with DC antigen-presentation (Yewdell and Hill, 2002). Therefore the present study appears to provide first experimental evidence that cross-priming can dominate the induction of CTL to a virus that efficiently infects DC and allows strong antigen-presentation in these pAPC.

Transfer of pre-proteasomal antigen enables efficient crosspriming

The experimental data strongly support the hypothesis that the transfer of substrates for the proteasome (Norbury et al., 2004; Shen and Rock, 2004; Wolkers et al., 2004) rather than postproteasomal products (Binder and Srivastava, 2005; Blachere et al., 2005; Serna et al., 2003) enables efficient T cell cross-priming. In accordance with studies conducted with replication-competent VACV the expression of rapidly degradable proteins abrogated T cell priming when infected cells were used as crosspriming vaccines. In addition, it was observed that T cells were efficiently cross-primed also against viral antigens.

Differences to replicating vaccinia viruses

However, the experiments revealed considerable differences for immunizations with the attenuated MVA strain compared to replication-competent VACV. Although cross-presentation of VACV-derived antigens has been observed (Basta et al., 2002; Larsson et al., 2001; Serna et al., 2003; Shen et al., 2002), several studies demonstrated a functional role of direct priming for the response to VACV infection (Basta et al., 2002; Norbury et al., 2002; Shen et al., 2002). Consistently, the delivery of destabilized antigens or minigenes by immunizing with VACV had no disadvantage or even enhanced T-cell priming (Norbury et al., 2004; Tobery and Siliciano, 1997). When contained in MVA vaccines, in contrast, these antigen formulations failed to induce strong CTL responses and in this regard resembled data obtained with Semliki-Forest-Virus, a vector that is unable to infect DC and therefore CTL are thought to be induced via cross-priming (Huckriede et al., 2004).

Possible explanations for inefficient direct priming

Beyond replication resulting in sustained antigen expression (versus abortive infection with a single round of antigen expression) there are other explanations that could account for the dominating role of cross-presentation in MVA responses as compared to VACV. During its host range adaptation, MVA lost multiple genes, including at least two viral proteins with proposed anti-apoptotic functions (Antoine et al., 1998; Aoyagi et al., 2007; Dobbelstein and Shenk, 1996). Accordingly, a recent study showed that DC undergo apoptosis earlier when infected with MVA than VACV and that MVA infection leads to a accelerated shutdown of host cell protein synthesis in DC (Chahroudi et al., 2006). MVA infected DC are unable to mature even when treated with cytokines (Kastenmuller et al., 2006). In addition, VACV including MVA have been reported to impair the capacity of DC to migrate and to adequately respond to chemokines (Humrich et al., 2007). Therefore an important question is whether the inability of MVA-infected DC to prime T cell could result from an altered functional plasticity and possibly the incapacity to form immunological synapses. One could speculate that the severe shutdown of host protein synthesis would be sufficient to prevent several steps required for T-cell priming. As stable interactions between T cells and APC start to form after 8 hours during priming (Mempel et al., 2004) the rapid induction of apoptosis in MVA-infected DC is likely to contribute to the observed inefficient direct priming.

Consequences for vaccine design

Extending the above observations, the data suggest that MVA-infected DC used in transfer vaccination protocols (Di Nicola et al., 2004) could function mainly as a carrier for antigen to be cross-presented by endogenous pAPC (Allan et al., 2006; Racanelli et al., 2004). Primary T-cell responses elicited by MVA-infected DC did not correlate with direct presentation of antigenic peptides, but similar to vaccinations with the live vaccine depended on the availability of mature protein within these pAPC. Since DC restrict VACV gene expression to the early viral life cycle (Kastenmuller et al., 2006), the amount of antigen available for cross-presentation may even be limited. Further evaluation is needed whether other cell types which allow for extended target gene expression and which might be easier to obtain from patients, could improve such vaccination protocols.

The insights gained here open the door for a variety of potential targets to improve vaccine efficacy by improving cross-presentation. In this respect, localizing antigen to a certain subcellular compartment, the coexpression of cytokines or molecules that enhance cross-presentation, or the use of adjuvants should be studied. Most importantly, the data show that it is essential to express recombinant antigens as long-lived proteins when inducing primary T cell responses with vaccines based on MVA. This is of considerable interest, as clinical trials evaluating MVA vector vaccines frequently use polytope

antigens. The data obtained here could help to explain, why these vectors failed to induce primary T cell responses (see also final conclusions).

5.2 T cell cross-competition shapes the immunodominance during secondary vaccinations

With the aim to dissect the interdependence between target- and vector-specific responses T cell cross-competition was found to be responsible for the dramatic switch in the epitope dominance patterns of VACV-specific $CD8^+$ T cells comparing primary with secondary infections. Changes in immunodominance hierarchies of T cells have also been reported for other pathogens like influenza, herpes viruses or LCMV (Belz et al., 2000; Nugent et al., 1995; Probst et al., 2003). In the LCMV infection model these changes have been attributed primarily to T cell exhaustion. In contrast, upon influenza virus infection the changing epitope pattern has been attributed to differential antigen expression, reflecting the capacity of memory T cells to respond to non-dendritic cells (Crowe et al., 2003). Another group has recently challenged this interpretation (Chen et al.,2004), and came to the conclusion that also cross-presentation could account for changes in dominance patterns. Furthermore, this study demonstrated that immunodominance hierarchies were independent of perforin or Fas-mediated lysis in the secondary response and therefore were not connected to APC killing or $CD8^+$ T cell cross-competition. La Gruta et al. elegantly shifted epitopes within the same viral infection context to study immunodominance (La Gruta et al., 2006). They found that the epitope hierarchies were a result of antigen dose and the size of the pre-existing T cell pool. Competitive interactions as demonstrated in the present work seemed to have only little impact in their model. Removal or addition of immunogenic epitopes in influenza virus had no effect during priming but led to compensation or suppression of other T cells during boosting, respectively (Jenkins et al., 2006; Webby et al., 2003). The authors attributed their findings to effects of antibodies or limiting amounts of antigen. The presented data, however, links the immunodominance pattern arising during secondary vaccinations to the presence of primed T-cells. A reason for potential differences between VACV and other infectious agents could be the high level gene expression promoted by VACV leading to high antigen amounts. Hence competition mediated by limited antigenic resources seems to be unlikely. Consistent with that view, a mathematical model of T-cell competition recently predicted that a large set of different epitopes, as found for VV infection, indeed should decrease T-cell competition (Scherer et al., 2006). However, under these conditions, high affinity of T cells and high expression levels of single epitopes would increase the chances for T cell competition. Hence, when immunizing with VV, the APC itself or resources of the APC other than the peptide/MHC complexes may become

the limiting factor for competing T cells. Besides costimulatory or adhesion molecules this could involve cytokines or access to APC. Another possibility is that memory T cells could be able to silence APCs after a certain number of interactions. Further work will be necessary to elucidate the cellular and molecular basis of T cell cross-competition.

Surprisingly, T cell cross-competition has been rarely documented so far. Marrack's group demonstrated T cell cross-competition by using peptide-pulsed DC and transferred TCR transgenic T cells (Kedl et al., 2002). They found that the degree of competition depended on the affinity of responding T cells, but was much less efficient than competition among T cells with the same epitope specificity (Willis et al., 2006). In the LCMV model, T cell cross-competition is believed to be functionally not important (Probst et al., 2002). However, in this particular report cross-competition was only analyzed during the priming phase. In contrast, in the present work it was found that cross-competition towards VACV is active during the secondary response and depends on immediate T cell effector function (presence of primed T cells). This notion is supported by Kedl et al., who succeeded to demonstrate T cell cross-competition by using primed T cells (Kedl et al., 2002). Additionally, performing second vaccinations early after the prime vaccination demonstrated that T cells were able to cross-compete with the beginning of d3 after priming. This correlates with the development of cytotoxicity of naïve T cells after exposure to APCs, which was detectable after 48h and was fully present after 72h (van Stipdonk et al., 2001). In a bacterial model, Wong and Pamer showed that CTL activity developed within 72hr and that these T cells probably eliminated APCs, thereby regulating antigen presentation and consequently T cell priming and expansion (Wong and Pamer, 2003). Further work will be necessary to define, which features of effector T cells are crucial to execute cross-competition. Interestingly, Belz et al. recently showed that during secondary influenza infections, T-cells terminate the antigen presentation in a perforin dependent manner (Belz et al., 2007). However, in the influenza model neither perforin, granzyme B, nor FAS/FASL interaction seemed to influence the immunodominance hierarchy (Chen et al., 2002, Chen et al., 2004). Others showed that early onset of IFN-γ production by $CD8^+$ T cells correlated with the immunodominant responses (Liu et al., 2004).

The primary response to the replication-competent VACV strain CVA was similar as compared to MVA, apart from a substantial response against $B22R_{79}$. Interestingly, an increased $B22R_{79}$-specific response was also induced and detected when performing short interval prime boost experiments with MVA. This in a way imitates replication due to repeated infections during the priming phase. In that respect, the primary response to a replication-competent VACV could be interpreted as a dynamic process of priming and boosting of T cells. This is in contrast to persistent chronic viral infections where T cell exhaustion is a factor for changing immunodominance hierarchies. As recently shown, using LCMV as a model for a chronic viral infection, $CD8^+$ T cells undergo extensive peptide-

dependent division independent of IL-7 or IL-15. This suggests that these $CD8^+$ T cells go through a fundamentally different pattern of differentiation compared to memory $CD8^+$ T cells that develop after an acute infection (Shin et al., 2007). In addition to A*0201 transgenic mice, the suppression of secondary T cell responses against late viral epitopes by T cells recognizing early viral epitopes could also be confirmed in C57BL/6 mice, and seems to be a characteristic feature of VACV infection. A similar connection between immunodominance and the kinetics of viral protein expression has been observed using LCMV (Probst et al., 2003; Tebo et al., 2005). Indeed, in an elegant model using hemi-splenectomized mice and analysis of TCR-ß motifs, Bousso et al. found that the timing of recruitment of individual T cell clones contributes more to the immune responses than their precursor frequency (Bousso et al., 1999).

In contrast to T cells specific for $H3L_{184}$ or $I1L_{211}$, which don't proliferate during secondary responses, the expansion of T cells specific for $A6L_6$ is not fully suppressed when the boost is performed late in the memory phase (d35). This might be explained by comparatively higher T cell numbers against $A6L_6$ which cannot be controlled as easily by competing T cells recognizing early epitopes. After short interval prime boost experiments (d5), the $A6L_6$-specific T cell response is fully suppressed, possibly because during priming the T cell size is increased as compared to the memory phase (d35) and therefore competition is enhanced. Nevertheless, it could be that the amount of $B22R_{79}$-specific T cells induced after priming is probably too low to completely out-compete $A6L_6$-, $H3L_{184}$- and $I1L_{211}$-specific T cells. Additionally, about 2% $B22R_{79}$-specific T cells cannot fully account for the total VV specific response after secondary immunization, which is about 30-40% of all $CD8^+$ T cells (Moutaftsi et al., 2006). Therefore it is intriguing to speculate that one or more yet unidentified HLA-A*0201 restricted epitopes might exist.

It can be expected that T cells recognizing epitopes derived from early viral proteins confer better protection against infection. Since VACV replication is completed at about 8h post infection, T cells specific for late viral proteins are likely to be activated too late to confer any protection due to delayed antigen presentation in infected cells. In this context, T cell cross-competition and the subsequent preferential expansion of T cells specific for early proteins reflects the host's ambition to rapidly clear viral infections. One could speculate that this finding might also apply to other large DNA viruses like herpes viruses. Interestingly, a recent report analyzing T cells in CMV-infected humans described that the number of IE-1- (immediate early protein 1) and not pp65- (a late viral protein) specific T cells correlated with protection from disease (Bunde et al., 2005).

Consequences for vaccine design

In summary, the presented data suggest that the expansion of virus-specific CD8$^+$ T cells is regulated by T cell cross-competition favouring T cells which are able to rapidly detect infected cells. Therefore, the outcome of this competition was heavily influenced by the timing of antigen expression, but independent of the route of vaccination or the ability of a virus to replicate. In immunotherapy using recombinant viral vectors a successful expansion of the desired T cell response can be strongly impaired by cross-competing vector-specific T cells. The data show that this impairment can be compensated by expressing target antigens early during the viral life cycle enabling antigen-specific T cells to successfully cross-compete. This allowed improving the T cell responses against target antigens and simultaneously decreasing the vector-specific response. The identification of other factors determining the outcome of T cell cross-competition and ultimately the elucidation of the molecular and cellular mechanism behind T cell cross-competition should help to design improved vaccines.

5.3   *Direct presentation can be targeted for the expansion of memory T cells*

The activation of naïve CD8+ T cells has been studied far more extensively than the reactivation of memory T cells (Belz et al., 2006, Yewdell and Haeryfar, 2005). The latter is of special interest because multiple parameters can change in secondary as compared to primary vaccinations. For example, antigen-specific antibodies could target antigens to Fc-receptor mediated antigen-presentation. Memory CD4+ T cells (including the regulatory subsets) could influence the expansion of CD8+ T cells. Generally, CD8+ T cells that can recognize a defined antigen will be present at different quantity (more memory T cells than naïve precursors), quality (differentiated memory T cells versus naïve T cells) and location (e.g. effector memory cells in peripheral tissues, liver, bonemarrow). In contrast to primary vaccinations with MVA the magnitude of T cell responses following boost vaccinations showed a clear correlation with the capacity of infected cells to endogenously present antigenic peptides. While MVA-Ub/Tyr failed to induce strong primary responses, T cells were expanded much better as compared to MVA-Tyr. The enhanced capacity to boost CTL immunity also translated into a more efficient killing of target cells *in vivo*. Even those antigen-formulations that did not induce T cell priming could be used to expand memory T cells: MVA-Mini-Tyr expanded Tyr$_{369}$-specific cells. Vaccination with MVA-Tyr could not induce a primary response directed against the signal-sequence derived peptide Tyr$_{1-9}$. However, when Tyr$_{1-9}$ specific T cells were primed by peptide-vaccination, MVA-Tyr was able to expand those cells. Therefore it was concluded, that direct

presentation is at least sufficient to expand memory T cells. An influence of antibodies or CD4+ T cells could be excluded as MVA-Ub/Tyr had a consistently higher boost efficacy also when mice had been primed with peptide-vaccination. The same outcome of vaccination was found after DNA-priming. A change of the dominant antigen presentation pathway from cross-presentation in prime to direct presentation in boost vaccinations could be explained by differential stimuli that are required to expand memory versus naïve T cells. Memory T cells are less dependent on costimulation and therefore might be able to respond to antigen presented by pAPC other than DC or even non-pAPC. Interestingly, ~80% of the cells that are infected in the spleen after i.v. application of MVA and VACV appear to be macrophages (data not shown and Norbury et al., 2002) and it is not clear which role these cells play for CTL immunity to MVA-encoded antigens. One concept could be that these cells synthesize antigen and transfer it to DC for crosspriming (Allan et al., 2006; Racanelli et al., 2004). This would not preclude that the same cells could be able to activate memory T cells by direct presentation. While infected pAPC rapidly undergo apoptosis before they fully mature and activate naïve T cells their endogenous antigen presentation, cytokine production and short life-span could still be sufficient to drive memory T cell expansion. This concept would predict that 1) memory T cells indeed require less costimulation or are independent thereof and 2) that the dynamic interactions between T cell and APC occur faster and earlier during memory T cell activation than for priming of naïve cells (Mempel et al., 2004).

The knowledge gained from these experiments indicates that targeted degradation of a recombinant antigen provides a suitable strategy to use full-length antigens for the efficient expansion of memory CD8+ T cells. It will be tempting to see if these findings can help to improve vaccination of tumor-patients or the *in vivo* expansion of adoptively transferred T cells.

Final Conclusions

The results obtained in this thesis contribute to the understanding of the immunological mechanisms driving priming and expansion of naïve and memory CD8+ T cells with viral vectors. The study provides a basis for rationally adapting antigens to induce strong CTL immunity with optimized prime/boost protocols. As MVA efficiently infected DC *in vivo* it was unexpected that the primary response to MVA vaccines is mediated by cross-presentation. As further shown, this has major consequences for the biological properties of target antigens which require expression as mature long-lived proteins in order to optimally feed the cross-presentation pathway to stimulate naïve T cells. In contrast, upon boost vaccinations T cell responses correlated with the capacity of recombinant antigens to get efficiently presented via the endogenous route, which could be targeted by rapidly degraded antigen. Consequently, rapid degradation of a full-length antigen impaired naïve T cell priming but proved to be a suitable strategy to efficiently expand memory T cells. Importantly, these data appear to provide first experimental evidence that cross-priming can dominate the induction of CTL to a virus that efficiently infects DC and allows strong antigen-presentation in these pAPC.

Primary vaccinations

The data underscore the requirement for a more detailed understanding of how candidate vaccines for immunotherapy elicit T cell responses. As demonstrated, it is essential and possible to adjust the properties of target antigens to the delivering vector and the underlying pathway of antigen presentation. In the case of vectors that work via direct presentation, such as lentiviral vectors (He et al., 2006), targeting proteins for rapid degradation should enhance CTL immunity. On the other hand, the immunization experiments confirmed that the preferred substrate for cross-priming *in vivo* is stable mature protein. Therefore, the synthesis of high amounts of long-lived antigen needs to be optimized for vectors that rely on cross-priming, such as MVA. These vector-specific antigen requirements might help to explain the somehow disappointing results of a recent series of clinical studies in which antigen-specific T cell responses were not detectable *ex vivo* after primary vaccination with MVA-based HIV-1 polytope vaccines (Goonetilleke et al., 2006; Peters et al., 2007). Similarly, only the protein portion but not a multi-epitope string simultaneously produced by a MVA malaria vaccine proved to be immunogenic in another clinical trial (McConkey et al., 2003), indicating that these findings may also apply to humans.

The results obtained here should facilitate future vaccine design as the proposed strategies allow the differential use of antigens in prime and boost vaccinations: since MVA-based vectors can easily accommodate large inserts, the expression of full-length antigens (stable in prime and targeted for degradation in boost vaccinations) could overcome some of the limitations of frequently used polytope vaccines: 1) the low immunogenicity in primary vaccinations, 2) the need to define protective epitopes

for the MHC-allels of vaccine recipients and 3) the risk of microbial or tumor escape from CTL immunity by eliciting narrow T cell responses directed only against a limited number of antigenic determinants.

Secondary vaccinations

Vector-specific immunity is regarded as a major limitation for the use of several viral vectors. The present work could demonstrate a functional role of T cell competition in a complex viral model. T cell cross-competition was found to be a major regulator of the expansion of T cells in boost vaccinations with MVA. It could be demonstrated that the early expression of target antigens is crucial to enhance responses against a recombinant antigen while simultaneously limiting vector-specific responses.

Consistent with that data, the expansion of memory T cells could be linked to direct antigen presentation. This has broad implications for the current use of MVA as boost-vaccines in heterologous prime/boost regimens. The presented data also suggest differential requirements when vaccinating naïve individuals in a prophylactic setting ("prime") as opposed to infected individuals or tumor patients, when the immune system has potentially been activated by the cognate pathogen or malignancy and therefore vaccination might rather represent a second antigen encounter ("boost"). Similarly, for adoptive transfer protocols the efficient *in vivo* expansion of memory T cells is a great challenge. The presented data suggest that in such conditions the *in vivo* expansion of T cells with MVA-based vaccines could be optimized by targeting direct antigen presentation, for example through rapid degradation of antigens.

As MVA vector vaccines are increasingly tested in human trials, the insights gained here could be rapidly translated into clinical evaluation and hopefully enhance vaccine efficacy.

Summary

CD8$^+$ T cells play a pivotal role for the clearance of viruses and intracellular pathogens as well as for tumor-immunosurveillance. Recombinant vaccines based on modified vaccinia virus Ankara (MVA) have an excellent safety record and are currently being evaluated in numerous clinical studies for immunotherapy of infectious diseases and cancer. Despite the use in humans, knowledge about the biological properties of target antigens to efficiently induce MVA vaccine-mediated immunity *in vivo* is sparse. In addition, it is unknown how T cell responses directed against recombinant or viral antigens interdepend. Therefore this study asked, if the modulation of recombinant antigens can enhance the desired response and, if the response to vector-specific antigens can be influenced. It was sought to define by which pathways MVA-encoded antigens are presented in order to prime naïve or to expand memory CD8+ T cells. Based on these findings, it should be studied whether certain antigen formulations can be used to improve the outcome of vaccination. Moreover, it was analyzed whether T cell responses to vector antigens and to recombinant antigens are influenced by each other and if this knowledge could be exploited to improve the target-to-vector ratio of induced T cell responses.

To address these questions a recombinant virus was constructed to target the human tumor-associated antigen tyrosinase for rapid proteasomal degradation which led to enhanced generation and presentation of antigenic determinants by infected cells. This virus was compared to the immunogenicity of other recombinant viruses in HLA-A*0201 transgenic mice. In addition, T cell responses to the MVA vector and to ovalbumin as a second model-antigen were studied in C57BL/6 mice. It was observed that DC are infected *in vivo* upon vaccination of mice with MVA. These DC were able to efficiently express recombinant and viral antigens and to present these to cytotoxic T cells. Unexpectedly, it was found that antigen presentation of infected DC did not result in the efficient induction of CTL immunity which in contrast strongly depended on the uptake and cross-presentation of antigens synthesized by other infected cells. These data provide first experimental evidence that cross-priming can dominate the induction of CTL to a virus that efficiently infects DC. Antigen produced by MVA as long-lived mature protein was found to be the preferred substrate for efficient CD8+ T cell crosspriming. In contrast, several of the antigen formulations that failed in T cell priming served for the efficient expansion of memory CD8+ T cells, which could be specifically improved by targeting antigens for efficient proteasomal degradation and endogenous presentation.

The performed study further showed that the induction and strength of primary CD8$^+$ T cell responses against various vaccinia-specific epitopes in naïve hosts occurred largely independent from each other. However, during boost vaccinations T cell cross-competition was a major regulator of the expansion of virus-specific T cells. Particularly, T cells recognizing determinants derived from late viral proteins had a clear disadvantage to proliferate during secondary responses. To enable the amplification of

target-specific T cells it was essentially required to synthesize recombinant antigens under the control of promoters which are active early during the viral life cycle. Early expression of antigens strongly enhanced the efficiency of boost vaccinations and conferred protective immunity against a lethal challenge with *Listeria monocytogenes*. Moreover, the obtained data appear to be the first experimental evidence of a functional role of T cell cross-competition for shaping the immunodominance hierarchy in response to a complex pathogen.

Overall, these findings underscore that it is essential to adjust antigen formulations to the applied vector system and vaccination regimen in order to achieve the induction and expansion of effective CTL immunity. The vector-specific antigen requirements that were found might help to explain the somehow disappointing results of a recent series of clinical studies with MVA vectors as HIV or malaria vaccines. The presented data suggest a differential use of antigen formulations in prime and boost vaccinations to improve the T cell responses against target antigens while simultaneously decreasing the vector-specific response. As clinical protocols already apply MVA as vector vaccines, it should be feasible to rapidly investigate these issues in humans and to potentially enhance vaccine efficacy against infectious diseases and cancer.

Zusammenfassung

CD8+ T-Zellen sind von zentraler Bedeutung für die Erkennung und Bekämpfung von Viren, intrazellulären Pathogenen oder auch Tumorzellen. Rekombinante Impfstoffe auf der Basis des modifizierten Vaccinia Virus Ankara gelten als sicher und nebenwirkungsarm. Die Immunogenität solcher Impfstoffe wird derzeit in zahlreichen Studien zur Immuntherapie von Infektionskrankheiten und Tumorerkrankungen erprobt. Trotz der Anwendung im Menschen ist nur wenig darüber bekannt, welche Eigenschaften rekombinante Zielantigen haben müssen, um im Kontext des viralen Vektors MVA immunogen zu sein. Unklar ist auch, ob und wie sich T-Zellantworten gegen rekombinante oder virale Antigene gegenseitig beeinflussen. Darum befasste sich die vorliegende Forschungsarbeit mit der Frage, ob es möglich ist, durch die gezielte Modulation der Qualität von rekombinanten Antigenen die gewünschte Immunantwort zu verstärken. Eine zentrale Frage war dabei, über welche Antigenpräsentations-Wege die Impfung mit MVA zur Primärinduktion von naïven T-Zellen oder zur sekundären Expansion von Gedächtnis-T-Zellen führt. Auf der Basis von diesen Untersuchungen sollte getestet werden, ob bestimmte Antigen-Formulierungen die resultierende Immunantwort selektiv verbessern können. Darüber hinaus stellte sich die Frage, ob sich T-Zellantworten verschiedener Spezifitäten (z.B. gegen rekombinante oder virusspezifische Antigene) beeinflussen, und ob sich daraus Strategien ableiten lassen, um Antworten gegen Zielantigene zu verbessern und vektorspezifische Antworten zu reduzieren.

Um diese Fragen adressieren zu können, wurde ein rekombinantes MVA konstruiert, mit dem Ziel, das humane tumorassoziierte Antigen Tyrosinase einer schnellen proteasomalen Degradation zuzuführen. Dies verstärkte die HLA-A*0201-restringierte Präsentation von antigenen Tyrosinase-peptiden. Die Immunogenität dieses und anderer rekombinanter Viren wurde in HLA-A*0201-transgenen Mäusen verglichen. Außerdem wurden T-Zellantworten gegen ein zweites Modellantigen (Ovalbumin) sowie Vektorantigene in C57BL/6 Wildtyp-Mäusen untersucht. Es konnte gezeigt werden, dass dendritische Zellen (DC) durch die Impfung mit MVA sehr effizient infiziert werden. *In vivo* infizierte DC waren in der Lage, sowohl rekombinante als auch Virusantigene zu synthetisieren, auf endogenem Wege Peptide daraus zu generieren und diese zytotoxischen T-Zellen zu präsentieren. Überraschenderweise führte diese Art der Antigenpräsentation nicht zu einer effizienten Primärinduktion von naïven T-Zellen. Dies geschah stattdessen über die Kreuzpräsentation von Antigenen, die von anderen infizierten Zellen produziert und von nicht infizierten DC aufgenommen wurden. Diese Ergebnisse erbrachten einen ersten experimentellen Beleg für die Hypothese, dass die CD8+ T-Zellantwort gegen ein Virus, das sehr effektiv dendritische Zellen infiziert, von der Kreuzpräsentation abhängig sein kann. Es zeigte sich, dass nur solche Antigene, die von MVA als stabiles Protein produziert werden, als geeignetes Substrat zur Induktion einer starken Primärantwort dienten. Interessanterweise konnten Antigenformulierungen, die nicht zur Primärinduktion von naïven

T-Zellen geeignet waren, erfolgreich für die sekundäre Expansion von Gedächtnis-T-Zellen genutzt werden. Diese Daten legen nahe, dass für Zweitimpfungen vor allem die direkte Antigenpräsentation von endogenen Peptiden wichtig ist. In Übereinstimmung hierzu konnte eine Steigerung der Effizienz von Zweitimpfungen zum Beispiel durch die gezielte Destabilisierung von Tyrosinase und damit der Verstärkung der direkten Antigenpräsentation erreicht werden.

Die Studie konnte weiterhin zeigen, dass sich Primärantworten gegen verschiedene viruskodierte Antigene zunächst nicht gegenseitig beeinträchtigen. Erst bei Zweitimpfungen wird die Expansion virusspezifischer T-Zellen durch Mechanismen der T-Zell-Kompetition reguliert. Im Besonderen haben die T-Zellen, welche antigene Peptide von späten viralen Proteinen erkennen, entscheidende Nachteile und proliferieren, wenn überhaupt, deutlich schwächer. Um die Amplifikation von T-Zellen mit gewünschter Spezifität zu ermöglichen, war es nötig, die entsprechenden Antigene unter die Kontrolle eines viralen Promotors zu stellen, der eine frühe Aktivität im Kontext des viralen Lebenszyklus besitzt. Dadurch konnte die Effizienz von Zeitimpfungen deutlich erhöht und eine protektive Immunität in einem Listerien Belastungsversuch erreicht werden. Die vorliegenden Daten konnten außerdem den ersten experimentellen Nachweis für eine funktionelle Bedeutung der T-Zell-Kompetition in der Immunantwort auf ein komplexes Pathogen erbringen.

Zusammenfassend konnte im Verlauf dieser Forschungsarbeit gezeigt werden, dass es für die effiziente Induktion und Expansion von zytotoxischen T Zellen essentiell ist, die Antigen-Formulierung an das gewählte Vektor-System und Impfschema anzupassen. Diese vektorspezifischen Anforderungen an das Antigen könnten helfen, die bisher eher enttäuschenden Ergebnisse von klinischen Studien zur Erprobung von MVA Vektoren als Impfstoffe für HIV oder Malaria zu verstehen. Die vorliegenden Daten legen die differenzierte Anwendung von Antigen-Formulierungen in Primär- und Sekundärimpfungen nahe, um die T-Zellantwort gegen Zielantigene selektiv zu Verstärken und die Vektorantwort gleichzeitig zu begrenzen. Da MVA in zahlreichen Studien als Vektor-Vakzine getestet wird, sollte es möglich sein, die hier gewonnenen Erkenntnisse rasch im Menschen zu erproben, und damit hoffentlich zu einer verbesserten Immunogenität dieser Vektoren beizutragen.

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