Immunotherapy of Prostate Carcinoma with biodegradable PLGA Microspheres

Dissertation

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Abbreviations

APC	antigen presenting cell
ATP	adenosine triphosphate
BCG	bacillus calmette-guérin
BMDC	bone marrow-derived dendritic cell
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CD	cluster of differentiation
cDC	conventional dendritic cell
CFA	complete Freund´s adjuvant
CLEC	c-type lectin domain family
CpG-ODN	cytosin-phosphatidyl-guanosin oligodeoxynucleotides
CRPC	castration-resistant prostate carcinoma
CTL	cytotoxic T lymphocyte
CTLA	cytotoxic T lymphocyte associated protein
DC	dendritic cell
DMEM	Dulbecco's modified eagle medium
DMSO	dimethylsulfoxid
DNA	deoxyribonucleic acid
EDTA	ethylenediamine-tetraacetic acid
EpCAM	epithelial cell adhesion molecule
ER	endoplasmatic reticulum
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FDA	food and drug administration
FITC	fluorescein isothiocyanate
GM-CSF	granulocyte macrophage colony-stimulating factor
HA	hemagglutinin
HLA	human leukocyte antigen
HSP	heat-shock protein
ICAM	intracellular adhesion molecule
IFN	interferon

Ii	invariant chain
IL	interleukin
imDC	immature dendritic cell
IMDM	Iscove's modified dulbeccos´s medium
i.n.	intranasal
iNKT	invariant natural killer T cell
i.p.	intraperitoneal
ISCOM	immune stimulating complex
i.v.	intravenous
kDa	kilo Dalton
KLH	keyhole limpet hemocyanin
LAIV	live attenuated influenza vaccine
LC	langerhans cell
LPS	lipopolysaccharide
M	matrix protein
MAA	melanoma-associated antigen
mAB	monoclonal antibody
MAGE	melanoma-associated antigen gene
MART	melanoma antigen recognized by T cells
mCRPC	metastatic castration-resistant prostate carcinoma
mDC	mature dendritic cell
MDSC	myeloid-derived suppressor cells
MHC	major histocompatibility complex
MoDC	monocyte derived dendritic cell
MS	microsphere
n	number in study or group
NA	neuraminidase
NK cell	natural killer cell
NKT cell	natural killer T cell
NP	nucleoprotein
PA	polymerase acidic protein
PAP	prostatic acid phosphatase
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffer saline
PCa	prostate carcinoma

PD	programmed cell death
pDC	plasmacytoid dendritic cell
PFA	paraformaldehyde
PLGA	poly(D,L-lactide-*co*-glycolide)
polyI:C	polymer of inosinic and cytidylic acid
PSA	prostate-specific antigen
PSCA	prostate stem-cell antigen
PSMA	prostate-specific membrane antigen
P/S	penicillin/streptomycin
RNA	ribonucleic acid
rVV	recombinant vaccinia virus
s.c.	subcutaneous
STEAP	six-transmembrane epithelial antigen of the prostate
TAA	tumor-associated antigen
TAP	transporter associated with antigen processing
TCR	T cell receptor
TGF	transforming growth factor
Th cell	T helper cell
TLR	toll-like receptor
TNF	tumor necrosis factor
TRAMP	transgenic adenocarcinoma of the mouse prostate
Treg cell	T regulatory cell
TRP	tyrosinase-related protein
TRPM	transient receptor potential melastatin
VLP	virus-like particle
VEGF	vascular endothelial growth factor
v/v	volume per volume
w/v	weight per volume

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Summary

The famous natural scientist Paul Ehrlich had the vision of learning to shoot pathogens with magic bullets, which find their target by themselves. This was long before poly(D,L-lactide-*co*-glycolide) microspheres (PLGA MS) were invented, but one could think he described them.

PLGA MS represent ideal vaccine delivery tools for immunotherapy of cancer and infectious diseases. In contrast to standard vaccinations, PLGA MS are specifically taken up by professional antigen presenting cells resulting in a reduced risk of inducing tolerance. In addition, this high specificity leads to reduced toxicity and can provide long-term effects via immunologic memory. Their mode of action includes a high encapsulation efficiency and a depot effect of the encapsulated cargo.

PLGA MS-based vaccination has been proven to be effective in immunotherapy of syngeneic model tumors in mice. The critical step for the translation to humans is the identification of immunogenic tumor antigens and potent vaccine formulations to overcome immune tolerance.

In the first part of the thesis, HLA-A*0201 transgenic mice were immunized with eight different prostate cancer peptide antigens co-encapsulated with toll-like receptor (TLR) ligands into PLGA MS and analyzed for antigen specific and functional cytotoxic T lymphocyte responses. Only vaccination with $STEAP1_{262-270}$ peptide encapsulated in PLGA MS could effectively crossprime cytotoxic T lymphocytes (CTLs) *in vivo*. These CTLs recognized $STEAP1_{262-270}$ /HLA-A*0201 complexes on human cells and specifically lysed target cells *in vivo*. Vaccination with PLGA MS was much more potent than with incomplete Freund's adjuvant.

The second part of the thesis was focused on chemical modifications of melanoma tumor antigens for improvement of their immunogenicity. Despite the fact that melanoma is one of the most immunogenic tumors with several described tumor antigens, immunotherapies targeting these antigens displayed limited success so far. Substitution of the anchor residues of three melanoma antigen epitopes resulted in increased HLA-A*0201 binding characteristics. However, vaccination of HLA-A*0201 transgenic mice with the heteroclitic peptides coencapsulated with the adjuvants CpG ODN into PLGA MS and co-administered with polyI:C MS revealed some problems posed by the modifications. CTLs induced by the heteroclitic peptides were not cross-reactive to the natural peptide due to high epitope specificity and antigen structure sensitivity of the CTLs and the CTLs were non-lytic. In conclusion, heteroclitic peptides have to be handled with caution and the cross-reactivity and functionality of the elicited CTLs has to be proven for every single heteroclitic analogue.

Current influenza vaccines aim to elicit antibodies directed to viral surface glycoproteins, which, however, are prone to antigenic drift. CTLs have the ability to exhibit a heterosubtypic immunity against most influenza A viruses. In the third part of the study, we encapsulated the

highly conserved immunodominant HLA-A*0201 restricted epitope from matrix protein $M1_{58-66}$ together with TLR ligands in biodegradable PLGA microspheres. Immunization of transgenic mice expressing chimeric HLA-A*0201 molecules with these microspheres induced a strong and sustained CTL response. A heterologous prime-boost vaccination scheme was able to induce both systemic and vigorous CTL responses in the lung which interfered with virus replication, weight loss, and infection related death. Vaccination with well-defined and conserved virus epitopes encapsulated into clinically compatible PLGA microspheres contribute to the control of influenza A virus infections. The promptitude and broad reactivity of the CTL response may help to foreclose pandemic outbreaks of influenza viruses.

In conclusion, PLGA MS are a promising vaccine delivery system with a broad field of application including cancer and infectious diseases. The biggest hurdle, however, remains the identification of suitable, highly specific and immunogenic antigens, which are able to induce robust immune responses.

Zusammenfassung

Der berühmte Naturwissenschaftler Paul Ehrlich hatte die Vision zu lernen, wie man magische Kugeln gießt, die gleichsam den Kugeln des Freischützen nur die Krankheitserreger treffen. Das war lange bevor PLGA MS erfunden wurden, und doch könnte man meinen, er hätte sie beschrieben.

PLGA MS stellen ein ideales Trägersystem für Impfstoffe zur Immuntherapie von Krebs und Infektionskrankheiten dar. Im Gegensatz zu herkömmlichen Impfungen werden PLGA MS speziell von professionell- antigenpräsentierenden Zellen aufgenommen, was das Risiko einer Induktion von Toleranz verringert. Darüber hinaus führt diese hohe Spezifität zu einer reduzierten Toxizität und einer Langzeitwirkung durch das immunologische Gedächtnis. Der Wirkmechanismus der PLGA MS beinhaltet sowohl eine hohe Verkapselungseffizienz, als auch einen Depoteffekt der verkapselten Substanz.

Impfungen, die auf PLGA MS basieren, haben sich in der Immuntherapie von syngenen Modell-Tumoren in Mäusen bereits als wirksam erwiesen. Der kritische Schritt für eine Übertragung des Systems auf den Menschen ist die Identifizierung von immunogenen Tumorantigenen und wirksamen Impfstoff-Zusammensetzungen, um Immuntoleranz zu verhindern. Im ersten Teil dieser Arbeit wurden HLA-A*0201 transgene Mäuse mit acht verschiedenen Prostatakarzinom-Peptidantigenen immunisiert, die zusammen mit TLR Liganden in PLGA MS verkapselt wurden. Anschließend wurden die antigen-spezifischen und

funktionellen zytotoxischen T Zell Antworten analysiert. Nur die Impfung mit dem in PLGA MS verkapselten Peptid STEAP1$_{262-270}$ führte zu einer effizienten Kreuz-Induktion von CTLs *in vivo*. Hierbei erwies sich die Impfung mit PLGA MS im Vergleich zu incomplete Freund's adjuvant als deutlich wirksamer.

Der zweite Teil der Arbeitet befasst sich mit der chemischen Modifikation von Melanoma Tumorantigenen zur Steigerung ihrer immunogenen Aktivität. Obwohl das Melanom als einer der immunogensten Tumore gilt, von dem sogar einige Tumorantigene bekannt sind, waren Immuntherapien, die sich gegen diese Antigene richteten, bisher nicht sehr erfolgreich. Mittels Austausch von Anker-Resten bei drei Melanomepitopen konnte deren Bindung an HLA-A*0201 verbessert werden. Beim Impfen von HLA-A*0201 transgenen Mäusen mit PLGA MS, die sowohl die modifizierten Epitope als auch die TLR Liganden CpG ODN und polyI:C enthielten, zeigten sich jedoch Probleme, die bei der Verwendung dieser heteroklitischen Peptide auftreten können. Die CTLs, die durch Impfung mit den heteroklitischen Peptiden induziert wurden, waren aufgrund ihrer hohen Epitop-Spezifität und der Sensitivität die Antigen-Struktur betreffend nicht kreuzreaktiv mit den natürlichen Peptiden und nicht lytisch. Zusammenfassend kann man sagen, dass heteroklitische Peptide mit Vorsicht zu behandeln sind und die Kreuzreaktivität und Funktionalität der induzierten CTLs für jedes einzelne Peptid getestet werden muss.

Influenza Impfstoffe, die momentan auf dem Markt sind, sollen Antikörper-Antworten gegen Oberflächen-Glykoproteine des Virus hervorrufen, die jedoch anfällig für Antigen-Drift sind. Zytotoxische T Zellen hingegen haben die Fähigkeit heterosubtypische Immunität gegen die meisten Influenza A Viren aufzubauen. Im dritten Teil der Arbeit haben wir ein hoch-konserviertes, HLA-A*0201 bindendes, immunodominantes Epitop des Matrix Proteins M1$_{58-66}$ zusammen mit TLR Liganden in PLGA MS verkapselt. Durch Immunisierung HLA-A*0201 transgener Mäuse mit diesen Mikrosphären konnte eine starke und lang-anhaltende CTL Antwort ausgelöst werden. Mittels eines heterologen Prime-Boost Impfschemas konnten starke und systemische CTL Antworten in der Lunge ausgelöst werden, sowie Virus-Replikation, Infektions-bedingtem Gewichtsverlust und Mortalität einschränken. Man kann daher sagen, dass die Impfung mit den gut definierten und konservierten Virusepitopen in PLGA MS zu einer Kontrolle von Influenza Infektionen beitragen kann. Die Schnelligkeit und das breite Reaktionsspektrum der CTL Antwort könnten auch helfen Influenza Pandemien zu verhindern.

Zusammenfassend kann man sagen, dass PLGA MS ein vielversprechendes Werkzeug zur Verbesserung von Immuntherapien mit einem breiten Anwendungsgebiet sind, das sowohl Krebs als auch Infektionskrankheiten beinhaltet. Die größte Herausforderung jedoch bleibt die

Identifizierung geeigneter, hoch-spezifischer und immunogener Antigene, die robuste Immunantworten auslösen können.

Aim of the Thesis

It was shown that PLGA MS mediated delivery of the model antigen Ovalbumin and of murine tumor cell lysate leads to an induction of anti-tumor CTL responses and tumor eradication in mice [5, 6]. The aim of the first part of the thesis was the transition of the murine model system towards an application in humans. One crucial prerequisite for this attempt is the identification of immunogenic antigens that can be presented on human MHC class I. Therefore, eight different PCa tumor antigen epitopes were encapsulated into PLGA MS and tested for their potential to induce antigen-specific CTL responses in HLA-A*0201 transgenic mice. The elicited CTLs were analyzed for their functional capability to recognize antigen-loaded target cells and to lyse them in an antigen-specific manner. In addition, immune responses elicited by usage of IFA as an alternative vaccine delivery system for the different PCa antigen epitopes was compared to those elicited by PLGA MS.

In the second part of the thesis, anchor residue modification as a strategy to enhance peptide immunogenicity was investigated. Heteroclitic analogues of three known melanoma epitopes were shown to have improved binding capabilities to HLA-A*0201 before [7]. These heteroclitic analogues were compared to the natural epitopes with regard to their potential to induce antigen-specific CTL responses in HLA-A*0201 transgenic mice. The dependence of an increase in CTL induction on improved HLA-A*0201 binding was tested by immunization of non-transgenic mice. In addition, the CTLs induced by the heteroclitic peptides encapsulated into PLGA MS were examined for their cross-reactivity to the natural epitope and their functional ability to lyse target cells in a peptide-specific manner.

Improvement of vaccine delivery systems, however, is not only urgently necessary in the fight against cancer but also for the vaccination against infectious diseases. In the third part of the thesis, the potential of PLGA MS as peptide delivery system for influenza A virus vaccination was tested. Titration of the peptides encapsulated into PLGA MS and analysis of the immune response kinetics after immunization with these PLGA MS allowed the establishment of different vaccination schemes that were tested in infections with a recombinant vaccinia virus encoding one of the epitopes and lethal influenza A virus infection.

1. Introduction

1.1 Dendritic cells

Dendritic cells (DCs) are a heterogeneous cell population, which account for 1-2% of total cell numbers and are situated in most peripheral tissues, especially at the interface of the body with the environment [8]. In the absence of inflammation or immune responses DCs constitutively patrol through the blood, peripheral tissues, lymph, and secondary lymphoid organs, where they take up pathogens, vaccines and self-antigens [9]. Being the most potent and specialized antigen sensing and presenting cells, they can thereupon initiate and orchestrate the type, magnitude and specificity of the immune response [10]. Subsequently, they migrate to secondary lymphoid organs, where they employ their unique ability to prime naïve T cells or activate resting lymphocytes [11].

1.1.1 Dendritic cell subsets

The family of dendritic cells is composed of different subsets diverging in ontogeny, localization and phenotype. Each family member has its specialized immune function concerning interactions with all kinds of immune cells, differential expression of receptors and a distinct production of cytokines and immune modulatory molecules [12]. Both, in human and mouse, DCs can be broadly classified as plasmacytoid DCs (pDC) and conventional DCs (cDC), with the latter being further divided based on their location into lymphoid-resident and migratory DCs [13]. All murine DCs originate from the bone marrow except Langerhans cells (LCs), which derive from embryonic precursors [14, 15]. The murine lymphoid resident $CD8\alpha^+$ cDC and the related migratory $CD103^+$ cDC most closely resemble the human $CD141^+$ (BDCA-3) DC subset found in lymphoid and peripheral tissues [16]. Phenotypically, all three subsets express the chemokine receptor XCR1, the necrotic cell receptor DNGR-1 (CLEC-9A) as well as the Toll-like receptors (TLRs) 1, 2, 3, 6, 7, 8 [17-19]. These DCs can take up and process apoptotic as well as necrotic material and excel at cross-presentation of cellular antigen [16, 17, 20]. Th1 differentiation is promoted through secretion of IL-12 by the murine $CD8\alpha^+$ DCs and $CD103^+$ DCs and IFNλ by human $CD141^+$ DCs, which thereby stimulate $CD8^+$ T cell immunity [21]. For the second murine DC subset, the $CD11b^+$ DCs, a unifying function is so far undiscovered, but a specialized role in the induction of Th2 and Th17 responses as well as in the induction and regulation of $CD4^+$ T cell immunity is emerging [22]. Their

human counterpart, the $CD1c^+$ DCs play a role in the immunity towards extracellular pathogens and are the major Th17 inducing cell type in the lung [23, 24].
Both, murine and human pDCs express high levels of TLR 7 and 9 and are specialized on the production of high amounts of type I interferons (IFNα/β) upon stimulation [25, 26]. After maturation pDCs can efficiently cross-present antigen and induce Th1 and Th2 immunity [27, 28]. Murine Langerhans cells (LCs) are located in the skin and present dermal and epidermal antigens to $CD4^+$ and $CD8^+$ T cells, inducing anergy and thus promoting peripheral tolerance. They can be characterized by the expression of epithelial cell adhesion molecule (EpCam) and the C type lectin Langerin [29]. In human skin two DC subsets have been identified, the epidermal Langerhans cells and the dermal interstitial DCs, which respond to stimuli and inflammation in the skin microenvironment [30].
$CD14^+$ DCs, a subset that is unique to the human immune system, resemble phenotypically most closely monocytes and macrophages. These $CD14^+$ DCs are potent stimulators of B cells and follicular T helper cells [31].

1.1.2 Antigen capture and dendritic cell maturation

Immature DCs are very efficient in antigen capture using several pathways. The first pathway is receptor-mediated endocytosis. Receptors involved in this pathway are receptors for the Fc portions of immunoglobulins for the uptake of immune complexes or opsonized particles [32], specific membrane receptors for the internalization of heat-shock proteins and their associated peptides derived from tumor cells or infected cells [33] or scavenger receptors that are involved in the internalization of various bacteria [34]. In addition, members of the C-type lectin family like macrophage-mannose receptor (MMR) [35], DEC205 [36], CD23 [37], dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN) [38] and Langerin mediate endocytosis in a Ca^{2+} dependent manner [39].
The second pathway is the phagocytosis of apoptotic and necrotic cells as well as viruses, bacteria and intracellular parasites in an actin dependent and receptor mediated manner [40, 41]. The third pathway is constitutive macropinocytosis of large amounts of surrounding fluid by DCs, which occurs in an actin dependent and nonspecific way [35].
To limit the range of antigens that is presented after leaving the peripheral tissues, DCs downregulate their endocytic capacity during maturation. This is on the one hand achieved by decreasing the cell surface expression of most antigen receptors and on the other hand by downregulation of micropinocytosis and phagocytosis [35]. Furthermore, DC maturation leads to changes in morphology, that include a loss of adhesive structures, cytoskeleton reorganization and the acquisition of high cellular motility [42].

Translocation of MHC class II antigen-presenting molecules to the cell surface as well as upregulation of co-stimulatory molecules like CD80 and CD86 and secretion of cytokines are additional events occurring during maturation [43].

Numerous factors trigger DC maturation, including pathogen related molecules that are recognized by DC surface molecules including Toll-like receptors and C-type lectins. DCs express a subset of these molecules including TLR2, recognizing bacterial peptidoglycans and lipopeptides, TLR3, sensing double-stranded RNA, TLR4, responding to LPS of $GRAM^-$ bacteria, TLR5, triggering the response to flagellin and TLR9, binding unmethylated CpG motifs [44, 45]. Another factor that induces DC maturation is cell products whose secretion is triggered by pathogens such as the inflammatory mediators TNF-α, IL-1β, IL-6 and prostaglandin E2 [46]. T cell derived signals like triggering of CD40, Fas and OX40L on DCs by CD40L, FasL and OX40 on T cells, respectively, as well as signals from NK cells, NKT cells and γ/δ T cells also induce functional DC maturation [47-50].

While immature dendritic cells can be defined by the expression of the CC chemokine receptors (CCR) CCR 1, CCR 2, CCR 5 or CCR 6 [51-53], they downregulate these CCRs during maturation and upregulate the production of proinflammatory cytokines and chemokines like CC chemokine receptor ligand (CCL) 3, CCL4 and CCL5 in order to attract immature DC, macrophages and monocytes to the site of inflammation. Consequently, maturing DCs upregulate the chemokine receptor CCR7, leave the inflamed tissues and home via the lymph vessels to the draining lymph node following the gradient of the lymph node homing chemokines CCL19 and CCL21 [54, 55].

1.1.3 Antigen processing and presentation

Captured antigens are processed in distinct intracellular compartments and loaded onto DC antigen-presenting molecules (Fig. 1). Endogenous antigens, most often newly synthesized or cytosolic proteins but also virus-derived antigens are ubiquitinated and cleaved into peptides by the proteasome [56]. In mature DCs the main proteasome type is the immunoproteasome whose subunits LMP2, LMP7 and MECL-1 are induced during maturation, which leads to an altered peptide cleavage and therefore a changed presentation efficiency [57]. The resulting peptides are translocated to the endoplasmatic reticulum via ATP-dependent TAP1/2 transmembrane transporters and loaded on MHC

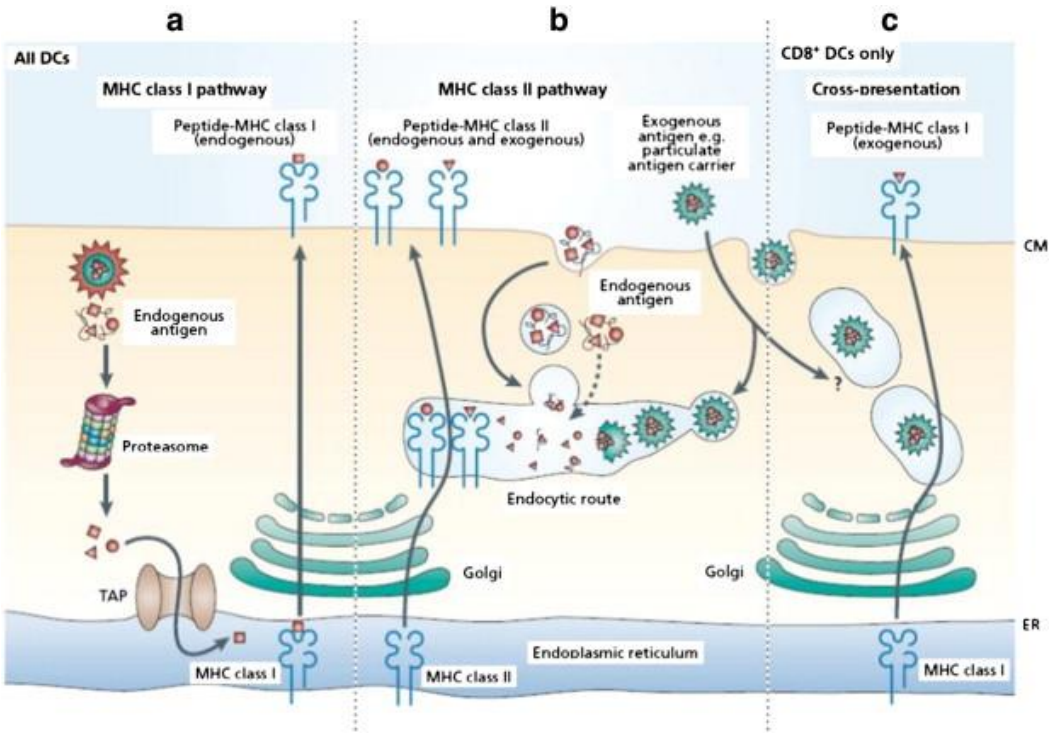

Figure 1. Antigen presentation pathways of dendritic cells.

Endogenous antigens are processed by the proteasome, translocated to the ER and loaded on MHC class I molecules by TAP1/2 transporters. The MHC class I-peptide complex is transported through the Golgi apparatus to the plasma membrane. Exogenous antigens are degraded in the endosomes and lysosomes of the endocytic compartment, the peptides are loaded onto MHC class II molecules and are transferred to the plasma membrane. During cross-presentation exogenous antigens are either processed by the proteasome and loaded onto MHC class I molecules via TAP1/2 in the ER or phagosomes or the antigen is cleaved into peptides by endosomal proteases and bound by MHC class I molecules directly in the endocytic compartment. The image is adopted from [4].

class I molecules. This process is controlled by a loading complex consisting of several ER resident chaperones including tapasin, calnexin and calreticulin [58]. Thereupon, the MHC class I-peptide complex is rapidly transferred through the Golgi apparatus to the plasma membrane. DC maturation increases synthesis and half-life of MHC class I molecules [59].

However, in a special process called “cross-presentation” that is unique to DCs and macrophages, MHC class I molecules are also involved in the presentation of exogenous internalized antigens to stimulate $CD8^+$ T cell immunity [60]. Proteins, which are acquired from other tissue cells by phagocytosis or micropinocytosis are processed through at least two different mechanisms. In the first pathway referred to as “cytosolic pathway”, the antigen is transferred from the phagosome into the cytosol as a consequence of ER membrane fusion with the phagosome during phagocytosis [61]. After hydrolysis of the antigen into oligopeptides by the proteasome, these peptides are loaded onto MHC class I molecules via TAP1/2 in the ER or phagosomes [62]. In the second pathway, which is called “vacuolar pathway”, the protein antigen is cleaved into peptides by endosomal

proteases, mainly cathepsin S, which are then bound by MHC class I molecules probably directly in the endocytic compartment [63]. A third pathway was proposed for the cross-presentation of soluble proteins after degradation by the ER-associated degradation pathway [64].

For presentation on MHC class II, soluble and particulate antigens are efficiently captured by DCs and are degraded in the endocytic pathway by several proteases [65]. A nonamer of three α/β MHC class II dimers and three invariant chains (Ii) leaves the ER after synthesis, passes through the Golgi apparatus to the endosomes and lysosomes of the endocytic pathway [66]. After proteolytic degradation, which is tightly regulated by the ratio between cathepsin S and its endogenous inhibitor cystatin C, antigenic peptides can bind to the MHC class II dimer under the control of two nonpolymorphic MHC class II molecules, namely HLA-DM/HLA-DO in humans and H2-M/H2-O in mice [67, 68]. Maturation of DCs leads to an increase in the activity of cathepsins and of synthesis and translocation of MHC class II-peptide complexes to the plasma membrane where they remain stable for days [69].

In addition, DCs express a third class of MHC molecules, the CD1 family of nonclassical, antigen-presenting molecules. In humans, four different CD1 proteins (CD1a-d) are expressed, whereas in mice only CD1d was identified [70]. CD1a, b and c bind glycolipid antigens of endogenous as well as exogenous origin and stimulate a large repertoire of T cells. CD1d molecules bind α-galactosyloceramides and activate a subset of T cells and NK T cells [71].

1.1.4 T cell priming and activation

After antigen encounter and maturation, DCs rapidly relocalize to the T cell zone of secondary lymphoid organs. DCs have the unique and critical ability to prime naïve $CD4^+$ and $CD8^+$ T cells, which is even increased by the enhanced surface levels of costimulatory and adhesion molecules such as B7.1, B7.2 and B7-DC after DC maturation [72]. The potent immunogenicity of DCs can even break neonatal tolerance [73], peripheral tolerance against soluble antigens [74], transplantation antigens [75], peripheral tissue antigens [76], tumor antigens [77] and viral antigens [78]. On the other hand, DCs are also involved in the induction of central and peripheral tolerance [79]. Presentation of cell-associated exogenous antigen-MHC class I complexes on DCs to naïve CTLs can either result in cross-priming to generate CTLs e.g. to tumors and grafts or cross-tolerization for induction of CTL tolerance to parenchymal antigens [80, 81]. Although DCs were shown to be able to directly activate $CD8^+$ T cells [82], they often

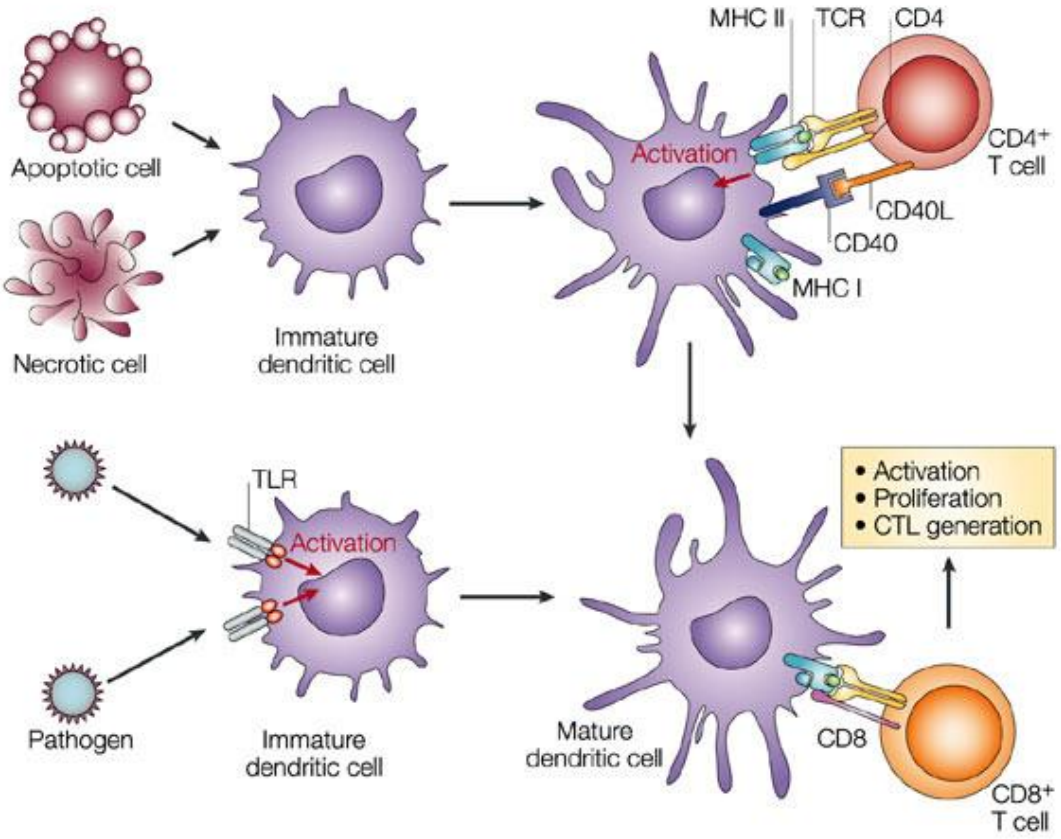

Figure 2. Dendritic cell maturation and T cell stimulation.

DCs take up antigen by phagocytosis of apoptotic or necrotic cells. The phagocytosed antigen is presented to $CD4^+$ T cells on MHC class II, which license the DC through CD40–CD40-L interaction. The activated DC can then promote the $CD8^+$ T-cell response, generating CTLs and T memory cells. Viruses or bacteria can directly stimulate DC maturation by binding to TLRs or by causing the release of inflammatory. The image is adopted from [3].

need to be licensed by $CD4^+$ helper T cells via upregulation of CD40-L on DCs in order to activate CTLs [83].

Successful DC/T cell interaction requires three signals. The first signal is the recognition of MHC/peptide complexes on DCs by antigens-specific T cell receptors which is mediated by several adhesion molecules like integrins and members of the immunoglobulin superfamily [84]. Signal two, namely co-stimulation, is provided by various membrane co-stimulatory molecules, such as CD80/86, CD40, CD70 or inducible T-cell costimulatory ligand (ICOS-L) molecules expressed on DCs and their ligands CD28, CD40L, CD27 and ICOS expressed on T cells. Signal one and two are sufficient for proliferation and cytokine production, but the third signal in terms of cytokines like IL-12 is required for cytotoxic effector function [85].

1.2 Immunotherapy

While cancer represents one of the leading causes of death especially in industrialized countries, infectious diseases are a major burden in developing countries causing a lot of misery and pain [86]. As a new route for combating these diseases there has been a growing focus on immunotherapy. Immunotherapy refers to therapeutic strategies that complement or stimulate the immune system in order to fight diseases and to improve quality of life of the affected individuals [87]. For infectious diseases, prophylactic and palliative vaccines have been proven to be effective since their emergence [88]. Worldwide vaccination programs led to the successful eradication of smallpox and a considerable decline in the incidence of several diseases like poliomyelitis, diphtheria, measles and rubella [89]. Vaccines, however, are not only crucial in the fight against infectious diseases, but also for non-infectious diseases like cancer. As standard therapies such as surgery, chemotherapy and radiation therapy, despite being refined and less invasive nowadays, still have devastating side effects such as nausea, vomiting, alopecia and an increased susceptibility to additional tumors it is urgently necessary to develop alternative treatment methods for cancer patients [90]. Therefore, the major goals are the development of new vaccines or the improvement of existing vaccines using innovative delivery systems and adjuvants to enhance the immunogenicity [91]. There exist two general forms of immunotherapy, namely passive and active immunotherapy. Passive immunotherapy aims at the complementation of the immune system by supplying high amounts of effector molecules [87, 92]. However, this supplementation is rather short-lived and depends on repeated applications [93]. Active immunotherapy on the other hand refers to strategies that activate patients´ immune system following vaccination. Immunotherapies have several advantages over standard therapies including an increased specificity, which results in a reduced toxicity and long term effects owing to immunologic memory [87, 92].

1.2.1 Dendritic cell based immunotherapy

DCs are often called “nature´s adjuvant”, as they provide the unique ability to stimulate naïve T cells. DC can not only induce tumor-specific immune responses but also mediate protection against various types of pathogens. In conclusion, many trials revealed that DC-based immunotherapy is well tolerated with minimal toxicity and is therefore feasible in many malignancies [94]. However, clinical benefit in cancer patients was achieved for only less than 10% [95]. Two different DC based immunotherapeutic approaches can be distinguished, first *ex vivo* DC therapy, using *ex vivo* generated DCs loaded with antigen

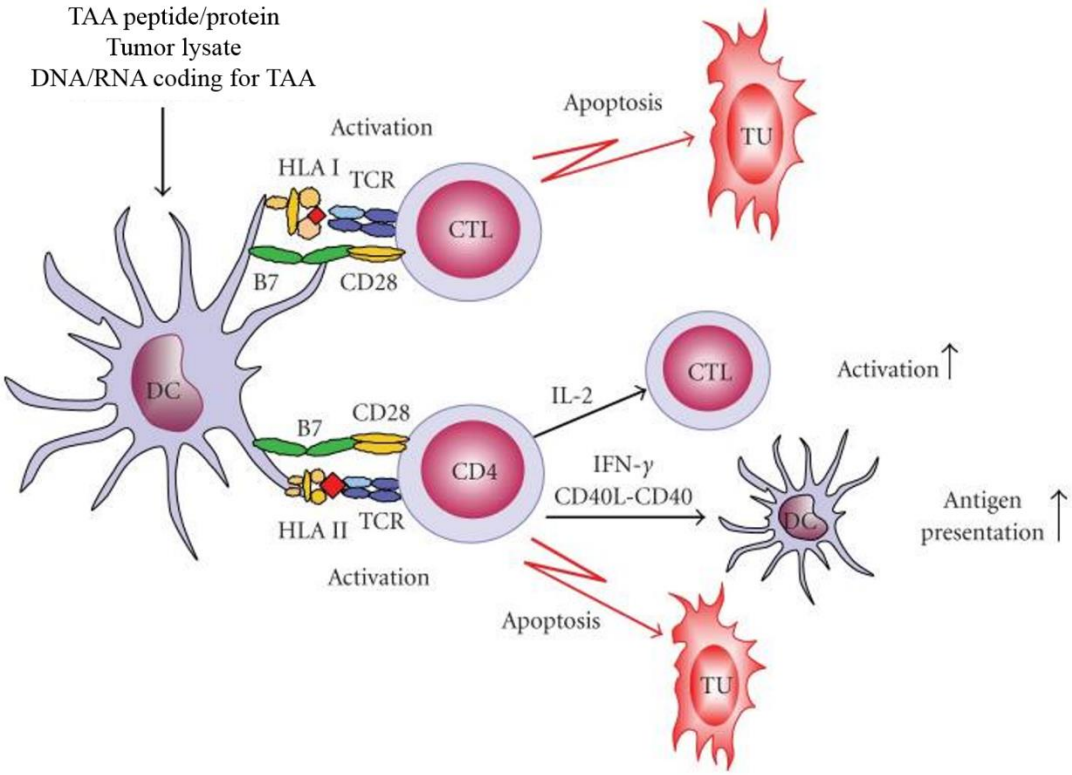

Figure 3. Dendritic cell based immunotherapy.

DCs provide the unique ability to prime naïve T cells and can stimulate resting T lymphocytes. Therefore, DCs are promising candidates for immunotherapy against cancer. DCs can either be loaded with tumor-associated peptides, proteins and tumor lysate or can be transfected with DNA or RNA encoding for TAAs. DCs efficiently activate and expand $CD8^+$ CTLs, which recognize and lyse tumor cells (TU), and $CD4^+$ T cells, which license DCs, secrete cytokines and help to maintain and expand CTLs. The image is modified from [1].

to stimulate CTLs, and second *in vivo* DC therapy, using antigen linked to DC maturation stimuli.

Most *ex vivo* DC therapies use DCs generated from monocytes or $CD34^+$ hematopoietic stem cells derived from leukocyte apheresis of the patient. Isolated monocytes are cultured in the presence of granulocyte-monocyte colony-stimulating factor (GM-CSF) and IL-4 for differentiation to immature DCs. Maturation is induced with a cocktail of cytokines including IL-1β, IL-6, TNF-α and prostaglandin E2 [46]. Different forms of antigen can be used for loading onto DCs including whole protein, tumor lysates, apoptotic tumor bodies and DCs fused with tumor cells. As the persistence of antigen in *ex vivo* loaded DCs is a key factor for the induction of an effective immune response, endogenous antigen expression is another possibility for antigen loading [96]. Antigen-encoding mRNA can be utilized for transfection of DCs [97], transduction of $CD34^+$

progenitor-derived DCs after cloning into a retroviral vector or transduction of monocyte-derived DCs after cloning into vaccinia or adenoviral vectors [98].

Strategies for modulating DC function *in vivo* are the delivery of antigen to DCs via antibodies targeting specific DC lectin molecules like DEC205, the mannose receptor, DC-SIGN, Langerin and CD40 or the usage of adjuvants such as GM-CSF to attract DCs to the site of antigen deposition [99]. The same mechanism is targeted by the GVAX therapy that employs autologous or allogeneic cancer cells transduced with retro- or adenoviral vectors encoding GM-CSF [100, 101]. *In vivo* maturation of DCs and a Th1 biased immune response can also be achieved by induction of the innate immune system, particularly activation of invariant natural killer T (iNKT) cells with α-Galactosylceramide [102]. In addition, immunotherapy with HSPs is a promising strategy, as HSP-chaperoned tumor-derived peptides are endocytosed by DCs and presented on MHC class I and II molecules for activation of $CD4^+$ and $CD8^+$ T cells [103].

One crucial point in DC-based immunotherapy is the requirement of DC activation for induction of optimal CTL responses. TLRs on DCs can be stimulated to enhance vaccine immunogenicity. Detection of external pathogen products, such as lipopetides (TLR1, 2, 6), LPS (TLR2, 4), flagellin (TLR5) and profilin (TLR11) is performed by TLRs located on the cell surface. TLR3, 7, 8 and 9 are located in the ER and the endosomal/lysosomal compartment, sensing intracellular bacterial or viral nucleic acid like polyI:C (TLR3), R848 (TLR7, 8) and CpG-ODN (TLR9) [104]. The TLR9 ligand CpG-ODN was shown to boost humoral and cell-mediated vaccine responses in clinical trials [105]. PolyI:C and its derivatives Hiltonol and Ampligen, which are well tolerated in humans, lead to an enhancement of cross-presentation and elicit type I IFN responses [106].

1.2.2 Vaccine delivery systems

Vaccine delivery systems help to control the spatial and temporal presentation of antigens to the immune system. Hence, lower vaccine doses can be administered because they can be effectively directed to immune cells and induce immune responses that are stronger, prolonged and more specific.

Liposomes are a spherical, artificial vaccine delivery system that consists of phospholipid bilayers capable of entrapping hydrophilic moieties in the aqueous compartment and hydrophobic moieties in the lipid bilayers. Cellular and subcellular antigens can be entrapped in Liposomes and evoke immune responses upon delivery to the cytoplasm of APCs by membrane fusion [107].

There exist some modified liposomal formulations like the virosomes that have viral envelope proteins anchored in their lipid membrane, transferosomes which are

liposomes consisting of phosphatidylcholine and cholate, archaesomes that are prepared using archaeobacteria membrane lipids, niosomes which stands for non-ionic surfactant vesicles that provide enhanced chemical stability, cochleates that are formed from non-vesicular bilayer sheets consisting of phosphatidylethanolamine, phosphatidylserine and cholesterol intercalated by calcium ions and proteosomes that are prepared by reconstitution of the bacterial outer membrane [108].

In addition, there are emulsion delivery systems that comprise heterogenous liquid systems such as water-in-oil emulsions, oil-in-water emulsions, or more complex systems such as water-in-oil-in-water multiple emulsions, microemulsions or nanoemulsions. The active principle of these systems is the emulsion of antigens dissolved in a water phase in oil leading to a controlled release that is determined by the viscosity of the oil phase, the oil to water ratio and the droplet size of the emulsion. While the depot effect of mineral oils is important for induction of robust immune responses, their administration is accompanied with severe adverse and long lasting side effects [109].

Another delivery system is called ISCOM, which stands for immunostimulatory complexes and describes a supramolecular spherical structure of about 40 nm in diameter, built up by structure-forming and immunomodulating quillaja triterpenoids, lipids and antigens [110].

Virus-like particles (VLP) are another vaccine delivery system consisting of viral envelope proteins that provide the morphology and cell-penetrating ability but lacking the genetic material for infection. Both, cellular and humoral immunity can be stimulated by VLPs [111].

PLGA Microspheres

While worldwide vaccination programs led to a considerable decline in many infectious diseases, these diseases are still leading causes of death, especially in the Third World due to difficulties to attain immunization coverage [112]. First of all, aluminum compounds, which are the only adjuvants used for human vaccines in the US, are unable to induce cell-mediated immunity that is needed for vaccination against intracellular pathogens [113]. Furthermore, alum provides poor adsorption properties for some antigens, is difficult to lyophilize, is unable to elicit mucosal IgA antibody responses and generally needs booster immunizations [113, 114]. Moreover, adverse effects like hypersensitivity reactions occurred in some patients [115]. Calcium compounds and MF59, which are licensed in the EU as adjuvants neither have a high potential to induce cell-mediated immunity [116]. Therefore, single-injection vaccines are desired, as incompletely immunized women cannot pass immunity to newborns leaving them susceptible to infections like tetanus [117].

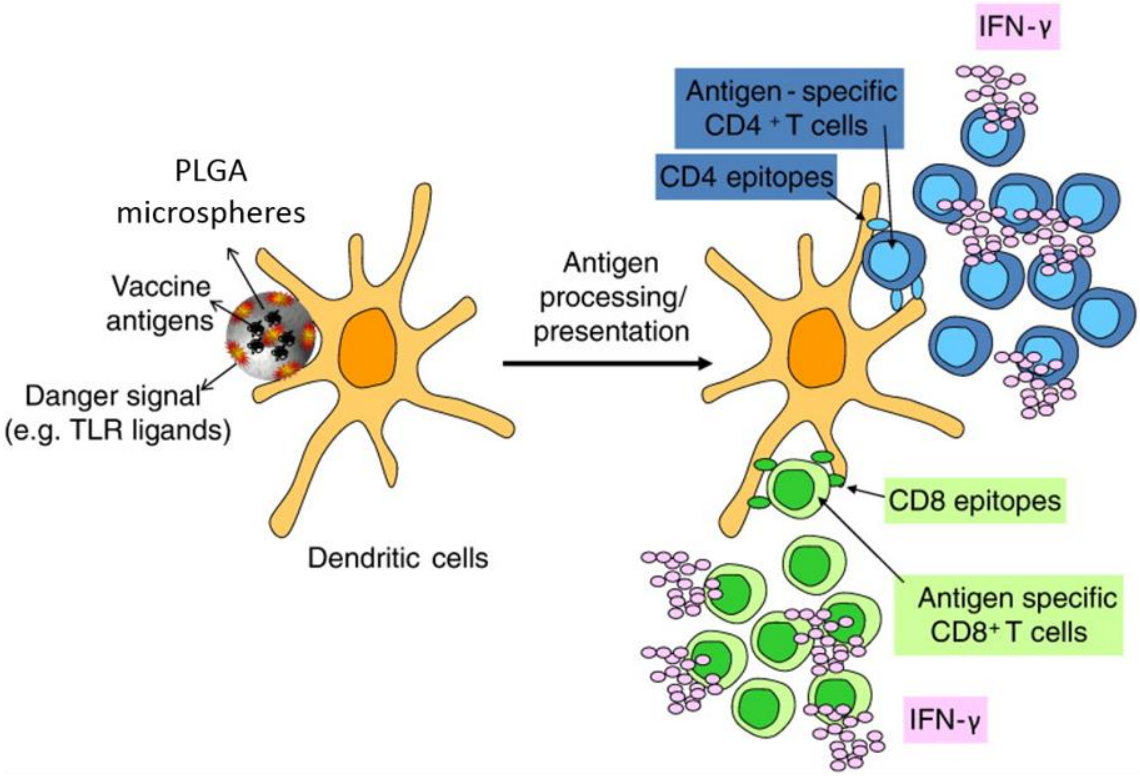

Figure 4. PLGA MS as vaccine delivery system.

PLGA MS are efficiently taken up by DCs, which mature in response to the encapsulated danger signals. The encapsulated antigens are processed and both presented on MHC class II to CD4+ T cells and cross-presented on MHC class I to CD8+ T cells. Presentation of the MHC-peptide complex in combination with co-stimulatory molecules and cytokine release leads to a complete induction of T cells. The image is modified from [92].

In the last twenty years, biodegradable poly(D,L-lactide-co-glycolide) (PLGA), a polymeric ester of the two α-hydroxyacids lactic acid and glycolic acid, has become the most widely studied polymer. Upon hydrolysis of the polymer in aqueous solution the two acids are released and are subsequently metabolized in the citric acid cycle [118]. PLGA polymers have a long safety record, are well characterized and are approved for application in humans [119]. Different compositions of the polymer provide varying properties like the highly crystalline poly(L-lactid acid) in contrast to the amorphous poly(D,L-lactic-co-glycolic acid). Hydrophobicity and release kinetics of the polymer can be influenced by different stoichiometric ratios of lactic and glycolic acids and by molecular mass. A high lactic acid content favors long lag times before release due to polymer mass loss. Discontinuous release of the content is achieved by using medium to high molecular weight polymer and low peptide loading, while low molecular weight polymer and high protein loading favors continuous release [120]. Resomer® RG502H, which was used in our experiments is characterized by a composition of 50% glycolate and 50% lactate and a molecular mass of 14 kDa resulting in a quite hydrophilic polymer

that is almost completely degraded within thirty days in water [118]. This controlled and prolonged release from PLGA microspheres (PLGA MS) bears the possibility to eliminate the need for booster doses.

PLGA MS can be manufactured by different techniques including coacervation, solvent extraction and spray-drying, with the latter being the only technique suited for up-scaling in industry. Spray-drying results in PLGA MS with a defined size of 1.5 to 7 µm. This morphological mimicry of pathogens represents an ideal particle size for the uptake by macrophages and DCs. Phagocytosed MS can travel inside APCs from peripheral sites of injection to secondary lymphoid organs where antigen presentation occurs. Encapsulated proteins are continuously released from these MS at low level with a peak of release during the first day and complete release within around thirty days [121]. Ingestion of PLGA MS and subsequent liberation of lactic and glycolic acids itself leads not to DC maturation and does not affect their maturation capacity and migration, survival, cytokine release or T cell stimulation [122]. Higher specificity for the uptake by DCs as well as an increase in DC maturation can be achieved by surface modification of the PLGA MS with antibodies against several signaling molecules for DC maturation like CD40 and Fcγ receptor [123].

PLGA MS provide a flexible platform for the delivery of various types of antigens such as cell or tumor lysates, proteins, peptides, DNA and RNA to induce either immunogenicity or, for allergologic treatments, tolerance. Other advantages of PLGA MS are the protection of the encapsulated moieties from proteolytic degradation [124], an increase in the efficiency of antigen loading and enhanced antigen release leading to more efficient presentation [125].

Microencapsulated proteins can be processed and presented by APCs on MHC class I and class II molecules to elicit cellular and humoral responses [126]. The induced prominent and sustained antibody response is accompanied by an isotype switch that occurs readily after vaccination [118]. For presentation on MHC class I, the encapsulated proteins undergo cross-presentation, which was shown to depend on proteasome and TAP activity and to be sensitive to Brefeldin A pointing to the cytosolic escape pathway in the context of PLGA MS [125]. *In vitro*, cross-presentation is at least 100-fold more sensitive to antigen encapsulated in PLGA MS compared to soluble antigen. The cellular response to PLGA MS, in particular the $CD8^+$ T cell response however is rather weak and of short duration. Therefore, PLGA MS are applied in combination with one or more immunostimulatory adjuvants like TLR ligands. In particular, CpG motifs binding to TLR9 and polyI:C binding to TLR3 can trigger Th1 responses and thereby enhance CTL responses. TLR ligands and antigen have to be coencapsulated into the same PLGA MS

to reach the same endosome for the generation of potent CTL responses upon a single injection [127].

PLGA MS are therefore a particularly attractive candidate to fight intracellular infections through bacteria, viruses and parasites. In addition, it has been shown that encapsulation of endogenous antigens can break tolerance pointing out that immunization with PLGA MS is also a feasible opportunity for immunotherapy of cancer. Concordant with this finding, PLGA MS mediated delivery of antigenic peptides, proteins and tumor cell lysate leads to an induction of anti-tumor CTL responses and delayed tumor occurrence [5, 128]. A prerequisite for the use of PLGA MS in humans is product sterility. Terminal sterilization by γ-irradiation does not alter their immunstimulatory properties [5]. Displaying high encapsulation efficiency, PLGA MS are also well-suited for multiple antigen delivery and the addition of immunomodulators. Therefore, PLGA MS provide maximum efficacy with minimum number of applications and antigen dose and can be delivered safely and easily.

1.2.3 Antigens

Antigens recognized by immunotherapies should provide several properties. First of all, antigens should be abundantly expressed and be easily accessible. Furthermore, they are supposed to be expressed homogenously, consistently and exclusively on the target. Preferably, antigens are functionally important for the target to avoid escape mechanisms and genetic drift. In contrast, antigen secretion should be minimal. There are multiple reasons for differences in the immunogenicity of an antigen. One reason is the differential efficiency in antigen processing. In addition, the processed peptides provide diverging MHC binding affinities, which can also be influenced by other non-corresponding MHC alleles. Other factors that determine immunogenicity are both recognition of diverse TCR repertoires as well as the unequal ability to induce memory T cells [129-131]. It was also argued, that immunogenicity is basically not amenable to molecular design and is not only triggered by molecular recognition but also for a big part by extrinsic factors such as the individual gene repertoire, self-tolerance and a variety of cellular and regulatory mechanisms. However, antigen processing can be markedly different in cancer cells compared to normal cells. Differing epitopes can be caused by downregulation of (immune-)proteasomal subunits and altered cleavage specificities of the tumor proteasome [132].

A prerequisite for the efficacy of the immunotherapy of cancer is the immunogenicity of the tumor [133, 134].

Pathogens on the other hand can escape the immune system due to mechanisms like antigenic drift or antigenic shift. Then, the immune system often fails to protect against new antigenic variants of the same type or subtype and is rarely cross-reactive against other types [135]. This is especially true for influenza virus, for which annual vaccine reformulation is necessary to maintain immunity against seasonal strains [136, 137].

Peptide antigens

Peptide vaccines are the most precise selection of vaccine components, as they represent the minimal immunogenic region of a protein antigen. With peptides of typically eight to ten amino acid residues for binding to the antigen-binding groove of MHC class I molecules and peptides of thirteen to twenty amino acids binding to MHC class II molecules, selective stimulation of antigen-specific CTLs, B cells or T helper cells can be induced. In contrast, many pathogenic microorganisms are difficult to culture *in vitro*, the production of live attenuated or inactivated vaccines is often impractical and may still contain material that initiates unwanted immune responses. Moreover, vaccines with tumor cells or crude tumor lysates contain predominantly normal proteins that are of no therapeutic benefit. While live attenuated vaccines still bear the risk of reversion or formation of adverse reassortants that can lead to virulence, peptide vaccines are absent of infectious material.

Peptide antigens provide several advantages over these vaccines. The peptides are chemically synthesized in a defined manner and can be economically produced in large scale. In addition, peptides can be stored freeze-dried [138].

Evidence for natural *in vivo* immunogenicity of the peptides is the isolation of virus antigen and TAA peptide-specific T cells from healthy donors and patients [139]. Aichele *et al*. were the first to report induction of a CTL response by a peptide originating from lymphocytic choriomeningitis virus [140]. Moreover, vaccination with MHC class I binding epitopes was able to protect mice against subsequent virus challenge, the outgrowth of transplanted tumor cells and enhanced the lifespan of mice in a lung metastasis model [141-143]. However, vaccination with GP100 and Her2/neu peptides induced only low levels or only short-lived CTL responses, respectively. In contrast, other peptide vaccinations even induced CTL tolerance, leading to enhanced tumor growth [144].

Induction of tolerance can have several reasons. The dose and the route of administration can determine whether a peptide activates or tolerizes CTLs [144]. After subcutaneous administration, the peptides can bind to several cell types besides APCs, which present the peptide without the required co-stimulation thus leading to tolerance. For cancer, some of the peptides originate from deregulated or mutated self-proteins. CTLs that recognize these self-antigens escaped thymic selection and recognize only peptides with low MHC class I binding affinity. The resulting weak interaction between

peptide/MHC complex and T cell receptor is not able to fully activate CTLs [145]. Another disadvantage of the epitopes used for peptide vaccine is the missing or little tertiary structure, which makes them subject to rapid degradation by tissue and serum proteases [146].

Progresses in adjuvant science such as the usage of TLR ligands or ligation of CD40, lipopeptide conjugation, encapsulation into PLGA MS and direct delivery to DCs are some of the approaches to solve these problems. Another possibility is the modification of the peptide antigen itself. One approach is the optimization of the peptides´ binding affinity to the MHC class I complex by site-directed substitution of anchor residues to induce a more stable peptide/MHC class I complex [147]. Site-directed substitution of certain amino acids can also be used to design a peptide that is able to interact more efficiently with the T cell receptor of CTLs [148]. However, these alterations bear the risk, that the T cell receptors do not cross-react with the natural peptide any more [149]. To protect peptide antigens from proteolytic degradation, the naturally occurring α-amino acids can be substituted by their β-amino acid homologues, which leads to an increased overall stability of the peptide [150]. N-acetylation and C-amidation of the peptide can also prevent its degradation by exopeptidases [151].

Enhancement of the CTL response after peptide vaccination can also be achieved by addition of $CD4^+$ T cell epitopes. T helper cells can on the one hand stimulate DC maturation and thus increase antigen presentation. On the other hand T helper cells can directly stimulate CTL expansion by secretion of IL-2 and are involved in the establishment of memory responses [152]. There is no agreement on whether the $CD4^+$ T cell epitope should be derived from the same antigen as the $CD8^+$ T cell epitope or if this is irrelevant and an antigen independent peptide with high binding affinity for MHC class II would be preferable [153].

Another critical factor for the immunogenicity of the peptide vaccines is the length of the peptide. Utilization of longer peptides relies on their correct processing *in vivo* but on the other hand increases the duration of antigen presentation and thus the magnitude of the CTL response [154]. The use of epitope vaccines is restricted to patients of a given HLA haplotype. Therefore, the most effective strategy for vaccination are multiepitope vaccines, that can be presented by many different HLA class I and class II molecules and preclude the case of antigen loss variants or specific downregulation of HLA class I molecules by the target cell [146].

In conclusion, peptide vaccines are simple and cost-efficient in their production, are simple to administer and can induce potent CTL responses especially after administration with vaccine delivery systems and adjuvants.

1.3 Prostate Carcinoma

With 233.000 new cases and 29.480 estimated deaths is prostate cancer (PCa) the second leading cause of cancer related deaths in the United States in 2014 [155]. The majority of patients diagnosed with localized PCa are successfully treated with radiation therapy or radical prostatectomy [156, 157]. However, within ten years around 30% of patients eventually fail on local therapy and progress to advanced-stage or metastatic PCa. Androgen deprivation therapy is the standard care for patients with advanced PCa, although most tumors become resistant to primary hormonal therapy within 14-30 months [158].

For men with metastatic castration resistant prostate cancer (mCRPC) docetaxel chemotherapy in combination with prednisone was the only treatment option for more than a decade with a median overall survival of 16.5 to 19 months [159]. As docetaxel is associated with significant toxicity, the application in patients with asymptomatic or mild symptomatic disease is not favored [159]. New agents including abiraterone acetate, cabazitaxel, enzalutamide and radium-223 dichloride were shown to prolong life for men with advanced PCa for several months [160-163]. Abiraterone acetate blocks CYP17, a critical enzyme in testosterone synthesis. Cabazitaxel is a chemotherapeutic agent belonging to the taxane class of microtubule inhibitors and Enzalutamide is an oral androgen receptor signaling blocker. The introduction of these new agents in the first-line therapies is widening the interval between progression to mCRPC and initiation of chemotherapy.

1.3.1 Immunotherapy of Prostate Carcinoma

Prostate carcinoma represents a typical adenocarcinoma. Expression of a handful of unique antigens both in normal and cancerous prostate cells makes it an interesting target of antigen-specific immunotherapies. The goal of these immunotherapies is the induction of anti-tumor immune responses, decrease in tumor load and to change the course of the disease.

The first approach for immunotherapy of prostate cancer is *ex vivo* loading of DCs with tumor associated antigens (TAA) or TAA-derived peptides. Vaccination of men with CRPC using DCs loaded with peptides derived from prostate specific membrane antigen (PSMA) was well tolerated and elicited antigen-specific cellular immune responses in parts of the patients [164]. Another study used DCs pulsed with peptides derived from prostate specific antigen (PSA), prostate stem cell antigen (PSCA), prostatic acid

phosphatase (PAP) and PSMA to treat men with CRPC. Half of the patients displayed specific T cell responses against all antigens and an increase in PSA doubling time [165]. An additional approach that provides proof of concept for the feasibility of antigen-specific immunotherapies is Sipuleucel-T (Provenge®), an *ex vivo* autologous DC-based immunotherapy product. The target antigen PA2024 is a fusion protein consisting of full-length human PAP fused to full length human GM-CSF. As PAP expression is essentially restricted to the prostate, it is a very specific target for immunotherapy of metastatic prostate cancer [166]. Sipuleucel-T is prepared by culturing autologous leukapheresis peripheral blood mononuclear cells (PBMCs) with PA2014 for approximately 40 h before re-infusion into the patient. This procedure is repeated three times at two-week intervals [167]. APC activation could be detected in the first dose and increased in the second and third dose preparations. Treatment with Sipuleucel-T leads to prolonged median survival indicating that the infused DCs lead to tumor-specific CTL activation. The most common adverse effects associated with treatment were chills, pyrexia, headache, asthenia, dyspnea, vomiting and tremor [168].

GVAX® is a cell-based vaccine for prostate cancer and consists of two immortalized prostate cancer cell lines, PC-3 and LNCaP, which have been transduced to express GM-CSF. GVAX was constructed to break the immune tolerance against a broad spectrum of antigens that are present on prostate cancer cells but many of them also on normal prostate epithelium. The PC-3 cell line is a hormone-refractory cell line derived from a prostate cancer bone metastasis, whereas the LNCaP is a hormone sensitive cell-line developed from a prostate cancer lymph node metastasis, which expresses a number of restricted differentiation antigens. Activation of APCs by GM-CSF has an important function in breaking the tolerance and the establishment of antitumor immune responses [169]. GVAX is well tolerated with minimal adverse effects like local injection site reactions, pruritus and fatigue and shows clinical activity but no advantage in median overall survival in patients with mCRPC [170].

There is also a viral based vaccine for therapy of prostate cancer called ProstVac-VF. Prost-Vac-VF uses two viral vectors, a recombinant vaccinia virus prime and a fowlpox virus boost, both encoding PSA and three T cell costimulatory molecules (TRICOM) B7.1, ICAM-1 and LFA-3. The heterologous prime/boost strategy is used to avoid neutralizating antibody responses to the vector, which are usually more pronounced than those to the plasmid encoded antigen [171]. Clinical safety of the vectors as well as negligible toxicity of the prime/boost schedule could be affirmed, resulting in prolonged overall survival [172].

Another approach for vaccination against prostate cancer is immunization with plasmid DNA encoding tumor associated antigens, which has been shown to induce humoral and

cellular immune responses. The DNA is taken up by host cells that subsequently express the encoded protein. However, only relatively weak initial responses can be elicited, necessitating repetitive immunizations [173]. For prostate cancer, the PAP-encoding plasmid pTVG-HP displayed clinical safety and elicited an antigen-specific T cell response [174].

Additionally, other therapies provide immunomodulatory properties. Besides the direct effect on prostatic epithelial cells, androgen ablation was also reported to result in substantial $CD4^+$ and $CD8^+$ T cell infiltration in the prostate gland [175, 176]. Interestingly, there are also studies showing that androgen ablation reverses age-related thymic involution, thus increasing the output of naïve T cells [177]. Furthermore, androgen ablation augments B cell development and inhibits tolerance to prostatic antigens [178]. With the effect of androgen ablation to boost prostate-specific immune response it is an ideal candidate for combinational therapy together with an immunotherapy. Immunostimulation was also reported for chemotherapy and radiotherapy. In addition to their direct cytotoxic effect on tumor cells, dying tumor cells release molecules that lead to DC maturation. Moreover, antigens from apoptotic tumor cells can be cross-presented on MHC class I for induction of antitumor CTL responses [179].

Moreover, there are also passive immunotherapies for prostate cancer like the antibody-based targeting of checkpoint inhibitors. Cytotoxic T-lymphocyte associated antigen-4 (CTLA-4) and programmed death-1 (PD-1) are immunologic regulators upregulated upon T cell activation [180]. Ipilimumab, an anti-CTLA-4 antibody was reported to modulate antigen specific immune responses [181]. The ligands for PD-1, namely PD-L1 and PD-L2 are often expressed by tumors, thereby inhibiting anti-tumor immune responses. Consistent with this finding, prostate and melanoma tumor infiltrating CTLs expressing high levels of PD-1 were shown to have impaired effector functions. Blocking of PD-1 is associated with antitumor activity [182]. Both, antibodies against CTL-4 and PD-1, demonstrated efficacy, safety and tolerability.

Other antibody based therapies are directed against signals contributing to tumor growth. Cetuximab inhibits cellular growth signals by interaction with the epidermal growth factor receptor [183]. Bevacizumab on the other hand binds specifically to vascular endothelial growth factor (VEGF) thereby interfering with pro-angiogeneic signaling of the tumor [184].

Targeting of immunosuppressors is another point of application for therapy of prostate cancer. Recruitment and expansion of immune suppressor cells like myeloid derived suppressor cells (MDSC) and regulatory T cells (Tregs) can be promoted by the tumor microenvironment to inhibit CTL function [185, 186]. Tasquinimod for example, which targets the S100A9 receptor on MDSCs significantly slowed down disease progression

and improved progression-free survival in men with mCRPC [187]. A low dose of cyclophosphamide in combination with allogeneic GVAX therapy was shown to shift the effector to regulatory T cell ratio towards effector T cells in mice bearing endogenous prostate tumors. This could be due to an inhibition of regulatory T cells and thus stimulation of effector T cells [179].
Immunotherapy for prostate cancer is a promising approach especially for treatment of advanced stage and metastatic disease. Combination therapies with standard therapies could help to improve the quality of life as well as progression free survival for the patients.

1.3.2 Prostate carcinoma antigens

For prostate carcinoma several tumor associated antigens have been identified, representing promising targets for immunotherapy. These TAAs include both, proteins expressed in normal and malignant prostate tissue and molecules which are overexpressed in various tumors including PCa.

Prostate-Specific Antigen (PSA)
PSA is a kallikrein-like serin protease produced almost exclusively in the epithelial cells lining the acini and ducts of the prostate gland. Physiologically, high PSA concentrations can be detected in the seminal fluid where it cleaves high molecular weight proteins responsible for the seminal coagulum into smaller peptides [188]. PSA is expressed under the transcriptional regulation of the androgen receptor and is secreted as a zymogen including a prodomain that must be removed by a protease to get an enzymatically active and mature protein. If the normal prostate glandular architecture is disrupted by cancerous growth and inflammation, increased amounts of PSA are released into the circulation where it can be used as serum marker for diagnosis and monitoring of PCa [189].
There were also hints, that PSA might play a role in pathobiology of prostate cancer as it is involved in alterations of prostate cancer cell invasive capabilities, gene expression, and morphology [190, 191]. PSA is expressed although the cells become more and more undifferentiated and even castration-resistant cells continue to produce PSA, suggesting a role for PSA in development of prostate cancer or its progression from localized to metastatic disease [192]. Furthermore, PSA has some negative effects on DC and T cell activity, like the activation of the immune suppressor TGF-β, inhibition of DC maturation, function and survival as well as adverse influences on lymphocyte proliferation and differentiation [193-195].

Both, in healthy men and patients suffering from chronic prostatitis CTL precursors against PSA have been detectable [196]. Using (oligo)peptide-pulsed or RNA-transfected APCs for *in vitro* stimulation several HLA-A2 and HLA-A3 restricted PSA derived peptides could be identified [197-199]. The most promising attempt for a PSA-based immunotherapy of prostate cancer is ProstVac-VF treatment, which resulted in a longer median survival of the patients but no difference in progression-free survival [167]. A combination of radiotherapy and a poxviral vector vaccine even induced tumor cell killing leading to subsequent epitope spreading [200]. For vaccination with a PSA-expressing vaccinia vector stabilized serum PSA levels and PSA specific CTL responses could be observed in a phase I clinical trial [201]. Moreover, DC-based and liposome-based PSA vaccines were able to induce specific CTLs [202, 203].

Prostate Stem-Cell Antigen (PSCA)

PSCA is a member of the Thy-1/Ly-6 family of glycosylphosphatidylinositol (GPI)-anchored proteins and is expressed on the cell surface of basal and secretory epithelial cells in the normal prostate, but is overexpressed in more than 80% of primary PCa samples particular of high stages [204]. In addition to androgen-dependent and -independent PCa tumors, non-organ confined tumors and PCa bone metastases have been reported to express PSCA but also other tumors including pancreatic adenocarcinoma, renal cell carcinoma and diffuse type gastric cancer [205-207]. Low PSCA protein expression was also found in other organs like bladder, placenta, neuroendocrine cells of stomach and kidney [204]. Neither the physiological role of PSCA in cellular processes nor the function in carcinogenesis is currently completely deciphered. Like other GPI-anchored proteins and Thy-1 family members, PSCA was speculated to be involved in stem-cell survival, T cell activation and proliferation as well as in cytokine and growth factor responses [208, 209]. In addition, the Ly-6 family members have been associated with carcinogenesis, cellular activation and cell adhesion of tumor cells [210, 211].

Both, HLA-A2 and HLA-A24 restricted PSCA derived peptides have been identified, which were able to generate tumor-reactive CTL responses *in vitro* [212, 213]. For elucidation of the therapeutic potential of PSCA, several studies were conducted in transgenic adenocarcinoma of the mouse prostate (TRAMP) mice which express PSCA in the spontaneously developing PCa. Vaccination with PSCA cDNA and viral vectors encoding PSCA induced antigen-specific CTL responses, inhibited PCa progression and increased survival [214, 215]. In addition, anti-PSCA monoclonal antibodies have been exploited in several studies either alone or as conjugates with the toxin maytansinoid or as radioconjugates, all of them displaying promising anti-tumor effects [216-218]. Additionally, a chaperone-based trial using heat-shock protein GRP170 and PSCA led

to a CTL response, inhibition of tumor growth and a prolonged life span in PCa bearing mice [219]. Furthermore, human clinical trials using DCs loaded with PSCA alone or in combination with prostatic acid phosphatase (PAP), PSMA and PSA supported the therapeutic potential of PSCA. The vaccines were well-tolerated and increased the PSA doubling time as well as the overall survival of the patients [165, 220].

Prostatic Acid Phosphatase (PAP)

PAP is a glycoprotein enzyme that is secreted by prostate epithelial cells and is mainly restricted to benign prostate tissue and increases with escalating PCa disease stages in malignant prostate tissue. Non-prostate tissues that reveal low PAP expression are placenta, kidney and testis as well as adenocarcinomas of different tissues such as gastric, breast and colon cancer [221]. There are two forms of PAP, a cellular form present in epithelial cells and a secreted form in the seminal fluid. Both forms are transcribed from the same gene followed by different post-translational modifications. Following puberty PAP is secreted by the prostate gland correlating with testosterone. Physiologically, PAP is reported to enhance the mobility of sperm and to display enzymatic activity [222]. Known substrates of PAP are adenosine monophosphate, phosphotyrosine, phosphocholine, phosphocreatine and ErbB-2 [223]. The interaction of PAP and ErbB-2 regulates the androgen-sensitivity in PCa cells, thus controlling cell proliferation in the castration resistant phenotype. Therefore, active PAP acts as a tumor suppressor [224].

Analysis of pre-existing CTLs in the blood of healthy donors and PCa patients led to the identification of naturally generated peptides binding to HLA-A2, HLA-A3 and HLA-A24 [174, 225]. CTL responses and suppressed tumor growth could be observed after DNA vaccination encoding murine PAP in TRAMP mice [226]. In a human trial, patients were vaccinated with DCs pulsed with murine PAP, which led to cross-reactive CTL responses to human PAP and anti-tumor responses [227]. Promising results could also be observed for another clinical study using a PAP derived peptide that can bind to different HLA subclasses [228]. The most successful PAP based immunotherapy is Sipuleucel-T, which was approved for treatment of asymptomatic or minimal symptomatic CRPC by the food and drug administration (FDA). Patients vaccinated with APCs that were pre-exposed to the GM-CSF-PAP fusion protein experienced an increased overall survival [167].

Transient Receptor Potential Melastatin 8 (TRPM8)

TRPM8 belongs to the transient receptor potential (TRP) family of Ca^{2+} permeable non-selective cation channels, more precisely to the melastatin subfamily. TRPM8 protein consists of six-transmembrane segments with intracellular localization of the amino- and

carboxyterminus. TRPM8 is expressed in trigeminal ganglion neurons and dorsal root ganglion neurons, where it functions as a cold- and menthol-sensitive ion channel [229]. In the prostate, TRPM8 is expressed both in the apical epithelial cells and in the smooth muscle cells and overexpressed in tumors of early stages and low grades [230]. TRPM8 is also expressed in the bladder urothelium and male urogenital tract [231]. Androgens not only regulate TRPM8 expression but also the intracellular localization either in the plasma membrane or in the ER membrane [232]. Functionally, the ER-located TRPM8 modulates the amount of Ca^{2+} in intracellular stores and subsequently regulates cell growth and proliferation. Indeed, reduced basal filling of intracellular Ca^{2+} stores is also the hallmark of the apoptosis-resistant cell phenotype characteristic of advanced prostate cancer [233]. In contrast, plasma membrane located TRPM8 was reported to be involved in prostate cancer cell migration [234]. Moreover, TRPM8 was supposed to be involved in the secretion of citric acid, fibrinolysin, acid phosphatase, several other enzymes and lipids from prostate epithelial cells [235].

An agonist of TRPM8 was shown to provoke an increased entry of calcium and sodium into the cell and thus induces cell death in TRPM8-expressing tumor cells [236]. A TRPM8-derived HLA-A*0201 binding peptide was identified, which was able to stimulate tumor reactive CTLs *in vitro* [230]. This epitope was also used for vaccination of hormone-refractory prostate cancer patients with peptide-cocktail loaded DCs in a phase I clinical study. In addition, the cocktail included besides TRPM8 also peptides of PSA, PSMA, Survivin and Prostein. Half of the patients displayed clinical and immunological responses with only minimal adverse effects [237].

Prostate-Specific Membrane Antigen (PSMA)

PSMA is a glycoprotein that is expressed in membranes of prostate epithelial cells as well as in the majority of prostate tumors, particularly in undifferentiated mCRPC [238]. In normal physiology, PSMA is also expressed in salivary, brain, small intestine, renal tubular epithelium, breast epithelium, duodenum and the central and peripheral nervous system at low levels [239]. Abundant expression of PSMA can also be found on the new blood vessels that supply most solid tumors, including lung, colon, breast, renal, liver and pancreatic carcinomas as well as sarcomas and melanoma [240]. PSMA is a zinc metalloenzyme with folate hydrolase activity. When located at the jejunal brush border, PSMA is also called folate hydrolase 1 and is involved in folate absorption for transportation to the rest of the body [241]. In the nervous system, PSMA is called NAALADase, which is responsible for hydrolyzing the neurotransmitter N-acetyl aspartylglutamate [242]. The potential function of PSMA in prostate epithelium and renal proximal tubules is the reuptake of folate in the kidneys and release of monoglutamated folates into the seminal fluid [243].

In prostate cancer PSMA expression was found to correlate with cellular folate content, which leads to cellular proliferation and thus an increase in tumor grade [244].
PSMA derived peptides restricted to HLA-A2, -A11, -A24, -A31 and –A33 as well as HLA class II restricted peptides were so far identified [245-247]. Transduction of murine DCs with an adenoviral vector encoding the extracellular domain of PSMA and the T cell co-stimulatory molecule 4-1BBL elicited protective immunity in a murine tumor model [248]. In a clinical trial with PSMA peptide loaded DCs, however, only a partial clinical response was detectable in a minor portion of patients [249]. Two PSMA-derived HLA-A2 peptides loaded onto autologous DCs were used for phase II clinical trials in men with recurrent disease or with hormone-refractory disease. Treatment was well tolerated and led to clinical responses in some patients [250, 251]. Furthermore, a DNA-based immunotherapy approach for treatment of prostate cancer was performed using full-length xenogeneic mouse PSMA. Vaccination was well-tolerated and had an impact on PSA doubling time [252]. Based on the fact that PSMA is a membrane-bound antigen that is present on the cell surface but is not released into the circulation it is also an ideal target for antibody-based vaccination. Various studies using radio-labelled, toxin-conjugated antibodies or anti-PSMA and anti-CD3 bispecific diabodies were conducted displaying promising results in mouse xenograft models [253-255].

Six-Transmembrane Epithelial Antigen of the Prostate 1 (STEAP1)

STEAP1 is predominantly expressed in the plasma membrane of prostate epithelial cells, particularly at cell-cell junctions. At lower transcript levels, STEAP1 can also be detected in colon, liver, ureter, fallopian tubes, uterus, pituitary, pancreas, stomach and breast [256]. In addition, STEAP1 is overexpressed in different stages and metastases of PCa as well as in several other types of human cancer tissues including bladder, colon, pancreas, ovary, testis, breast, cervix, and Ewing sarcoma [256, 257]. *De novo* synthesis of STEAP1 is androgen mediated [258]. Structurally, STEAP1 is a six-transmembrane protein that is thought to act as an ion channel or transporter protein in tight junctions, gap junctions, or in cell adhesion [259]. In line with this function, STEAP1 is also involved in intercellular communication between tumor cells and adjacent tumor stromal cells and therefore plays a key role in tumor growth [260]. One hallmark of an invasive phenotype of PCa cells including the loss of androgen receptor expression is a high level of voltage-gated Na^+ channels [261]. Another parameter that seems to be important for the progression to an apoptotic-resistant, androgen-insensitive stage are Ca^{2+} and K^+ levels [262]. Moreover, STEAP1 can raise the intracellular level of reactive oxygen species leading to increased cell growth [263].
Identification of three naturally processed HLA-A2 restricted peptides capable of inducing CTLs *in vitro* and *in vivo* and in addition three promiscuous $CD4^+$ T cell epitopes

makes STEAP1 to an attractive candidate for PCa immunotherapy [264-266]. Immunization of mice with recombinant DNA prime and modified vaccinia virus Ankara boost encoding STEAP1 resulted in delayed tumor growth in mice challenged with TRAMP-C1 cells [215]. Additionally, two STEAP-specific monoclonal antibodies inhibited STEAP-1–induced intercellular communication and thus inhibited the growth of PCa xenografts in mice [259].

Survivin

Survivin is the smallest member of the Inhibitor of Apoptosis (IAP) family. *Survivin* gene expression is tightly regulated by a large quantity of positive and negative regulators, like oncogenic transcription factors stimulating and tumor suppressors repressing gene expression [267]. Moreover, transcription is strictly cell-cycle dependent, peaking at mitosis but can also be stimulated by growth factors or cytokine stimulation [268, 269]. However, Survivin is not only regulated on transcriptional level, but also via post-translational modifications and regulation of protein stability. Survivin can be found ubiquitously distributed during embryonic and fetal developmental stages but is almost absent in differentiated healthy adult tissues despite cells undergoing self-renewal like hematopoietic precursor cells, keratinocytes, lymphocytes, activated endothelial cells and epithelial cells of the uterine cervix [270]. High overexpression can be detected in many human tumors including breast and lung cancer, prostate, gastric, colon, bladder and esophageal carcinomas, osteosarcomas and lymphomas [271]. Functionally, Survivin participates in the control of mitosis at the spindle checkpoint, has a cytoprotective role by regulation of apoptosis and plays a role in cellular stress response in association with various cellular chaperones [272, 273]. In cancer, Survivin was identified as mediator of chemo- and radioresistance as well as resistance to anti-androgen therapy [274-276].

Various naturally generated Survivin derived peptides were identified binding to HLA-A1, -A2, -A3 and –A11 [277-279]. Additionally, different HLA class II associated peptides have been discovered [280]. DCs on the one hand pulsed with these Survivin peptides or Survivin-containing tumor lysates or on the other hand transduced or transfected with Survivin were proven to elicit CTL responses and anticancer activity *in vitro* [277, 281]. DC based immunotherapy with Survivin peptides was not only successful in a murine lymphoma model [282], but also in clinical studies against different types of solid cancers and metastasis [283-285]. All vaccinations were safe and well tolerated, leading to antigen-specific immunological responses and disease stabilization in some patients. In another phase I/II trial protamine-protected Survivin mRNA was systemically delivered in melanoma patients, where it led to detectable T cell responses [286]. In a mouse model, oral delivery of a DNA vaccine encoding secretory chemokine CCL21 and

Survivin by doubly attenuated *Salmonella* typhimurium led to eradication or suppression of pulmonary metastases of non-small cell lung carcinoma [287].

1.4 Melanoma

The incidence of melanoma is increasing at a dramatic rate worldwide, with already about 76,000 new cases per year just in the United States. Although melanoma represents only less than 5% of skin cancers, it accounts for an overwhelming proportion of skin cancer deaths claiming the death of more than one person per hour in the US [155, 288]. Standard melanoma treatment is the surgical removal of the primary tumor and surrounding normal tissue. During early stage disease this treatment leads to a 5-year survival rate of 98% of the patients. However, melanoma is likely to spread to other parts of the body, decreasing the 5-year survival rate to 16% for regional and distant stage melanoma [289]. For unresectable or metastatic disease, dacarbazine- or hydroxyurea- based chemotherapy were, until 2011, the standard therapy, although they had little impact on overall survival [290]. Moreover, the first cytokine-based adjuvant therapies for melanoma were approved by the FDA. High-dose IL-2 treatment induced inflammation at the tumor site leading to activation of APCs, massive production of chemokines and to the activation of cytolytic activity in monocytes and NK cells. However, the overall response rate was low and the treatment had the potential to be highly toxic, causing capillary leak syndrome, distributive shock and death [291]. For the other early immune-directed therapy, the pleiotropic cytokine IFNα-2b was used, which displays modulating effects on the inflammatory response. In melanoma therapy, IFNα-2b resulted in improved relapse-free and overall survival but was also associated with considerable toxicity including liver dysfunction and myelosuppression [292]. Since 2011, oncogene-targeted therapies are applied successfully for treatment of melanoma. Melanoma has the highest mutational frequency of all tumor pathologies, lots of them affecting the canonical MAPK signaling pathway that promotes survival, growth, migration and resistance to apoptosis. Driver mutations lead to the constitutive activation of the MAPK pathway [293]. Small molecule inhibitors like Vemurafenib and Dabrafenib, which both inhibit the activity of the mutated BRAF kinase, Trametinib, an inhibitor for MEK1 and MEK2, Imatinib, an ATP-competitive RTK inhibitor or the broad spectrum multi-kinase inhibitor Dasatinib led to impressive clinical results in melanoma treatment [294-298].

1.4.1 Immunotherapy of Melanoma

Melanomas are some of the most immunogenic tumors, as they are associated with the highest mutational burden relative to other malignancies most likely resulting from exposure to ultraviolet radiation [299]. Therefore, Melanomas are a good model system for immunotherapies, with the purpose of eliciting long-lasting immunity and controlling micro-metastatic foci.

One strategy for an immune-based therapy is the modulation of cytokines and growth factors. Besides the therapy using IL-2 and IFNα-2b, mentioned above, a long-acting pegylated form of IFNα-2b was approved as adjuvant therapy by the FDA [300]. Moreover, GM-CSF was used in combination with an autologous tumor vaccine to treat patients with metastatic melanoma [301]. In addition, treatment with IL-12 resulted in infiltration of metastatic lesions by tumor-specific CTLs leading to histologic necrosis [302]. Another strategy is the application of antibodies. On the one hand, antibodies can be directed at several cell surface molecules on melanoma cells like the cell-surface ganglioside GM2 or the disialoganglioside GD2, which were reported to generate robust and specific humoral responses in melanoma patients [303, 304]. On the other hand, antibodies can also be used for enhancement of T cell responses by immune checkpoint blockade. CTLA-4 blockade has been shown to stimulate tumor necrosis and lymphocyte and granulocyte infiltration in some metastatic melanoma patients [305]. Similarly, antibody-mediated blockade of the PD-1/PD-1L interaction induced a survival benefit in patients with advanced melanoma [182].

Vaccination with whole cells or tumor cell lysates allows the immune system to interact with a broad antigenic repertoire. One source for these vaccination are autologous tumor cells derived from surgical tumor resection. After irradiation of the cells, they are reinjected with or without immunomodulators into the patient. To increase the immunostimulatory capacity of this vaccine, the hapten dinitrophenyl can be conjugated to proteins on the autologous tumor cells [306]. Irradiated autologous melanoma cells, that were engineered to secrete GM-CSF, led to immune responses in a phase I clinical trial [307]. Allogenic tumor cell vaccines from stable cultured tumor cell lines are the other source, providing the advantage of being more readily applicable. Like for PCa there is also a GVAX vaccine for melanoma, consisting of lethally irradiated GM-CSF secreting, allogenic melanoma cells [308]. VACCIMEL is another vaccine consisting of irradiated allogenic melanoma cells in combination with BCG as an adjuvant [309].

Viral vectors have a long history in the immunotherapy of melanoma. In the beginning direct injection of wildtype vaccinia virus or immunization with vaccinia-infected autologous melanoma cells or allogeneic cell lines were able to induce immune responses and have led to tumor regression [310, 311]. Later on, retroviruses,

adenoviruses and poxviruses have been widely studied as vaccine for the expression of tumor-associated antigens in both homologous and heterologous prime and boost regimens. These viruses have an excellent safety profile and induce both humoral and cellular immune responses [312]. Viruses can also be used to express immune stimulatory molecules like the B7.1 co-stimulatory molecule, leading to improved therapeutic responses due to complete CTL activation and nonspecific adjuvant activity of the virus [313].

Additionally, DNA and RNA vaccines encoding for cytokines, co-stimulatory molecules or melanoma-associated antigens were investigated for immunotherapy of melanoma. For improvement of the antitumor immune response, alphavirus self-replicons of Sindbis virus and Semliki Forest virus have been used [314, 315].

Heat-shock protein vaccines are a further possible treatment option for melanoma. An HSP96-peptide vaccine from autologous tumor has been reported to generate clinical responses in some melanoma patients [316].

Identification of several melanoma-associated antigens has resulted in the use of peptide vaccines. Immuneresponses to peptides were improved by administration with adjuvants, delivery systems or by alteration of the peptide sequence to improve binding affinity to the HLA molecule or the recognition by the T cell receptor [317]. These tumor antigens have also been used for DC based immunotherapy. Immunization of patients with autologous DCs pulsed with a cocktail of five melanoma associated peptides and KLH as adjuvant led to clinical responses and an increased overall survival [318]. In addition, autologous DCs have also been loaded with tumor cell lysates, killed melanoma cells or were electroporated with tumor antigen mRNA [319-321]. An additional immunological approach is the adoptive cell therapy. Autologous tumor infiltrating lymphocytes have been *ex vivo* expanded with IL-2 and anti-CD3 antibody stimulation, prior to reinfusion into the patient where they elicit durable responses [322]. Transfer of T cell clones that were highly selective for melanoma-associated antigens followed by IL-2 treatment demonstrated persistence and migration of T cells to the tumor site [323]. In conclusion, melanoma is a promising target for immunotherapies alone or in combination with standard therapies.

1.4.2 Melanoma tumor antigens

The first melanoma-associated antigens (MAAs) were identified by elution of MHC bound peptides from melanoma-derived MHC-peptide complexes. Based on their pattern of expression, MAAs can be classified into distinct groups. The first group is formed of tumor differentiation antigens. These antigens are expressed by normal melanocytes

during various periods of cell differentiation and are involved in the synthesis of melanin. They are overexpressed in malignant melanoma cells, but not other normal tissues. The members of this group are MART-1, gp100/Pmel17, tyrosinase and tyrosinase-related protein 1 and 2. The second group consists of antigens that are shared by melanoma cells and normal testis tissue and are therefore called cancer testis antigens. These antigens are predominantly expressed by different types of cancer, but not by normal melanocytes or other normal tissues. MAGE-1, MAGE-3, BAGE, NY-ESO-1 and GAGE belong to this group. The third group consists of proteins that often bear mutations in melanoma. Mutations of the members CDK4, β-catenin and CDC27 often results in novel antigens capable of eliciting T cell responses [290, 324].

Melanoma-associated Antigen Gene 1-C2 (MAGE-C2)

The MAGE family was first described by Van der Bruggen *et al.* in 1991 and can be divided in two big subfamilies, MAGE I and MAGE II [325]. The MAGE I subfamily is composed of 24 chromosome X-clustered genes and can be further divided into three subgroups, namely MAGE-A, -B and -C [326]. MAGE-I gene expression is restricted to a small number of normal tissues including spermatocytes, placenta and certain stages of embryonic development. In contrast, MAGE-I is highly overexpressed in melanoma and lung cancer, especially in the squamous cell type, intermediate expression was detected in epithelial cancer types like breast, bladder and prostate cancer and only low expression in lymphomas, leukemias, renal, colon and pancreatic cancers [327]. Proteins of the MAGE family are speculated to function during embryonic development and to be deactivated afterwards on transcriptional level by epigenetic mechanism including DNA methylation and histone deacetylation. In many tumors there occurs widespread demethylation, which could also happen to the promoter of MAGE genes leading to subsequent MAGE expression in tumors [328]. Physiologically, there is growing evidence for a function of MAGE proteins in cell cycle progression and apoptosis [329]. MAGE-C2 in particular was reported to support growth and tumorigenicity of melanoma cells by promoting DNA damage repair [330].

In an animal model, overlapping synthetic long peptides of MAGE-C2 co-administered with CFA and CpG-ODN induced protective immunity against melanoma [331]. Additionally, MAGE-C2 specific CTLs were detectable in the blood of melanoma patients [332]. Furthermore, naturally processed MAGE-C2 derived peptides binding to HLA-A2, -B44 and –B75 have been identified [333-335]. In a phase IB study, autologous DCs co-electroporated with synthetic mRNA encoding CD40L, CD70, constitutively active TLR4 and the melanoma antigens MAGE-A3, MAGE-C2, tyrosinase and gp100 stimulated $CD4^+$ and $CD8^+$ T cell responses in the patients, but induced no objective tumor response [336].

Melanoma Antigen Recognized by T cells (MART1)

MART-1, also called Melanoma Antigen A (Melan-A) was one of the first tumor antigens to be cloned. Its expression is restricted to melanin-producing cells, including normal and transformed skin melanocytes and retinal pigment epithelium [337]. MART1 is a membrane protein with one transmembrane domain [338]. As MART1 expression correlates with melanin content and the pattern and subcellular localization in the endoplasmic reticulum, the trans-Golgi network and the melanosome is quite similar to other melanosomal proteins, a role for MART1 in pigmentation is assumed. Moreover, MART1 forms a complex with Pmel17 and influences its expression, stability, trafficking, and the processing affecting melanosome structure and maturation [339]. MART1 is also expressed in cutaneous melanoma, uveal melanoma and nevus as well as melanoma micrometastases [340]. Vaccination with MART1 antigen can lead to vitiligo, an elimination of normal melanocytes in some areas of the skin [341].

Several MHC class I and MHC class II restricted peptides derived from MART1 have been identified so far [342, 343]. MART1-specific CTLs generated in HLA-A2 transgenic mice were shown to be able to lyse human melanoma cell lines *in vitro* in an HLA-A2-restricted, MART1-specific manner [344]. However, vaccination of stage IV melanoma patients with peptides of gp100, MART1, tyrosinase, and MAGE-3 in Montanide-ISA-720 resulted in no objective clinical responses [345]. The same was true for vaccination with autologous DCs pulsed with HLA-A2 restricted peptides of MART1 and gp100 [346]. Encapsulation of MART1 epitopes into liposomes, however, was reported to enhance their immunogenicity [347]. DCs transduced with an adenovirus encoding the three full-length melanoma antigens tyrosinase, MART1 and MAGE-A6 or MART1 alone have been shown to induce antigen-specific $CD4^+$ and $CD8^+$ T cell responses [348, 349]. A phase 1/2 trial in stage III and IV melanoma patients using a recombinant vaccinia virus encoding HLA-A*0201 restricted epitopes of gp100, MART1 and tyrosinase and CD80 and CD86 costimulatory molecules induced a CTL response [350]. In addition, adoptively transferred MART1 specific CTLs were able to migrate to the tumor, to mediate biological and clinical responses and to establish antitumor immunologic memory [351].

Tyrosinase-Related Protein 2 (TRP-2)

The tissue differentiation antigen TRP-2 is a melanosomal enzyme with dopachrome tautomerase activity, which is associated with the synthesis of melanin in normal melanocytes [352]. Sharing 40% amino acid sequence homology with tyrosinase and TRP-1, TRP-2 belongs to the family of tyrosinase related genes. TRP-2 is expressed both in human melanoma and murine B16 melanoma tumor cells [353]. TRP-2 expression in melanocytes has been shown to be influenced by stress-activated kinase p38 and mitogen-activated protein kinase MEK. In addition, an inverse relationship of

TRP-2 gene regulatory mechanisms to melanocyte growth regulatory pathways was suggested [354]. Microphthalmia-associated transcription tightly regulates transcription of the tyrosinase family genes, but is also involved in the pigmentation, proliferation and survival of melanocytes [355]. Like MART1, TRP-2 is expressed in melanocytes and retina and is overexpressed in melanoma cells, thus, vaccination against these tissue differentiation antigens can induce vitiligo.

Peptides of TRP-2 can bind to HLA-A2, -A3, -A11, -A31, -A33, and -A68 molecules and stimulate peptide-specific CTL responses and to MHC class II molecules for $CD4^+$ T cell responses [356-358]. TRP-2-specific immunization of C57BL/6 mice with established B16 melanomas and a transgenic mouse model of spontaneous skin melanoma induced antigen specific CTL activity that was described to control metastatic progression [359, 360]. In addition, a DC-based genetic vaccination using a recombinant adenovirus or alphavirus-based virus-like replicon particles encoding TRP-2 were shown to inhibit tumor growth and lung metastases in a mouse model [361, 362]. Furthermore, TRP-2 specific T cell transfer, DNA vaccination and targeting of DCs using an anti-alpha-DEC-205 antibody conjugated to TRP-2 are promising tools for TRP-2 based immunotherapy of melanoma [161, 363, 364].

1.5 Influenza A Virus

Influenza virus is a respiratory pathogen belonging to the family *Orthomyxoviridae*. Influenza A viruses are negative-stranded viruses containing eight RNA segments (Fig. 5). Type A viruses can be found in a wide variety of warm-blooded animals and can be further divided into subtypes based on the expression of their surface molecules hemagglutinin (HA) and neuraminidase (NA) [365]. Influenza viruses circulate all over the world with an annual attack rate of 5-10% in adults and 20-30% in children. After entry of the influenza virus into the host cell, the infectious cycle starts with the translocation of the viral ribonucleoproteins into the nucleus, where transcription and replication of the viral genome takes place. Subsequently, the viral ribonucleoproteins are exported from the nucleus and the virus assembles and buds at the host cell plasma membrane [366].

Influenza infection is a serious public health problem that causes worldwide 3 to 5 million cases of severe illness and about 250 000 to 500 000 deaths. Therefore, it is especially recommended for elderly and children to vaccinate against influenza annually [367]. Currently used vaccines stimulate the production of antibodies directed to viral surface glycoproteins, in particular hemagglutinin and neuraminidase [368]. However, due to antigenic drift, these antibodies fail to protect against new antigenic variants of the same

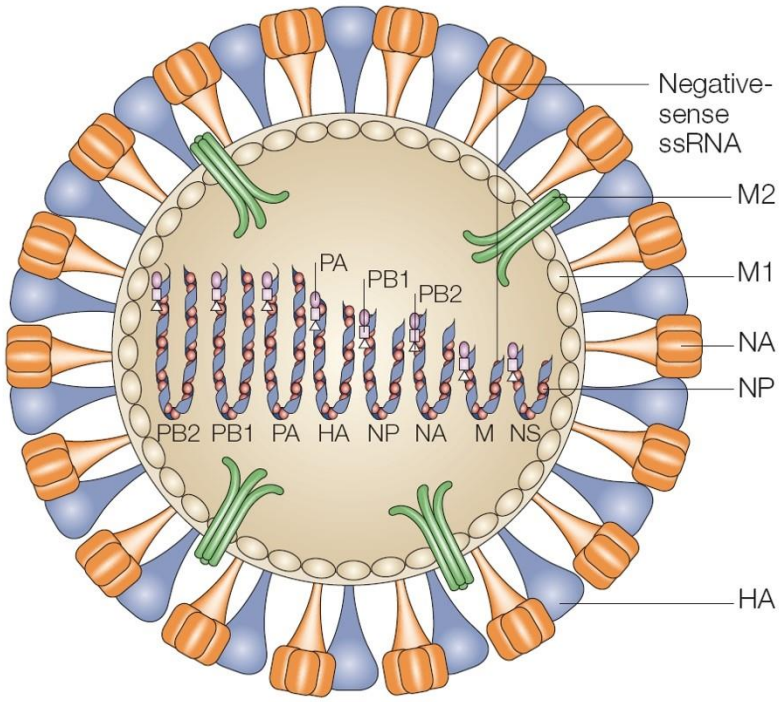

Figure 5. Influenza A virus

Influenza A virus is a negative-stranded RNA virus. The viral envelope contains the highly immunogenic glycoproteins HA and NA. Directly under the envelope the structural protein M1 is located. The ion channels in the envelope are composed of M2 proteins. Influenza A virus contains eight segments of negative-stranded RNA building a complex with the nucleoprotein (NP). Replication of the viral RNA segments is performed by the trimeric RNA-dependent RNA polymerase complex consisting of PA, PB1 and PB2. The image is adopted from [2].

type or subtype and are rarely cross-reactive against other types [135]. Thus, annual vaccine reformulation is necessary to maintain immunity against seasonal influenza viruses ultimately [136, 137]. Clearly, there is a great need for a vaccine that induces heterosubtypic protection against all influenza A viruses. This heterosubtypic immunity is generally mediated by cytotoxic T lymphocytes [369].

1.5.1 Immunotherapy for influenza A virus infection

One possibility for vaccination against influenza are formalin, UV or gamma irradiation inactivated vaccines after propagation of the virus in embryonated hens´ eggs [370]. The vaccine is available as whole vaccine, chemically disrupted split vaccine and subunit vaccine containing purified surface glycoproteins, which are administered intramuscularly or subcutaneously [365]. However, the impact of these vaccines is limited, as they cannot induce CTL responses and mucosal immunity, both of which are necessary to control initial viral replication in the respiratory epithelium [371]. Due to

antigenic drift at the neutralizing antibody combining site, antibodies induced by inactivated vaccines fail to protect against infection with different influenza subtypes or homologous virus strains [372]. The average efficiency of inactivated influenza vaccines is therefore only around 60% [373]. Efficiency can be improved by application of trivalent or quadrivalent inactivated vaccines.

Live attenuated influenza vaccines (LAIV) are an alternative, that more closely resemble the immune response after natural infection. Production of live, attenuated cold-adapted vaccines takes advantage of adaptation of the virus to grow at 25°C and of the segmentation of the virus genome by reassorting a donor strain with a wild-type virus, resulting in a virus that contains the genes encoding the surface glycoproteins HA and NA of the wild-type virus and the six remaining internal segments from the attenuated donor strain [374]. LAIVs are administered intranasally, which results in limited viral replication in the upper respiratory tract but not at core body temperatures and thus inducing cross-protective immunity by stimulating mucosal immunity and CTL responses [375]. Usually, also the LAIVs are administered as trivalent or quadrivalent vaccine. However, the protective effects varies by age and population [376]. Another promising strategy for viral attenuation represent single-cycle replicating viruses. By removing of the HA segment, which functions as receptor binding protein, the virus can infect cells and undergo a single-cycle replication but is incapable to spread after initial infection. This vaccine effectively triggers CTL responses [377].

As virus-like particles are similar to the virus in structure and morphology, they represent a promising platform for presenting surface proteins in a highly immunogenic form. The viral glycoproteins in VLPs are unmodified and presented in a membrane-anchored form mimicking their native conformation while the viral genome is missing. VLPs were reported to induce both, B and T cell responses and can be used to target conserved antigens [378]. Moreover, virus-vectored vaccines were extensively studied for vaccination against influenza virus. All of these vaccine vectors are either replication-defective or cause a self-limiting infection and can readily be manipulated. To enhance the cross-protective capacity of the vaccine, the vectors can be manipulated to induce immune responses against conserved influenza antigens like the matrixproteins, the polymerase or the HA stem. Viral vectors can induce high levels of humoral and cellular immunity, achieving robust and durable protection [379]. In addition, DNA vaccines against influenza virus infection have been established. These DNA vaccines are often based on HA, also trivalent HA, but there are also polyvalent DNA vaccines encoding NA or the less immunogenic matrixproteins 1 and 2 (M1/2) or nucleoprotein (NP) to initiate responses to a broader spectrum of viruses [380-382]. DNA vaccines are fast in

production and can induce B as well as T cell responses but are often insufficient to get protective immunity.

Antibody-based vaccines are a further promising tool against influenza virus infection. Promising targets for antibodies are the HA stem domain, that is higher conserved compared to the globular head domain, or the extracellular domain of M2 (M2e) that is highly conserved among different Influenza A viruses. Both antigens are accessible for antibodies and are therefore good targets for a universal influenza vaccine [383, 384]. In addition, a recombinant M2e protein fused with the TLR5 ligand flagellin was able to potentiate the immune response to the trivalent influenza vaccine in a clinical study [385]. In mice, also peptide-loaded DCs, peptide-conjugated liposomes and protein encapsulated into nanoparticles were successfully tested as vaccine for influenza infection [386-388].

1.5.2 Influenza A virus antigens

Influenza A virus proteins are encoded by the segmented viral genome. These proteins are of diverse immunogenicity, but all of them are potential targets for immunotherapy against influenza virus infection.

Matrix protein 1 (M1)

M1 is encoded on segment seven of the single-stranded RNA genome. M1 is the most abundant protein in the virion and is as major structural protein located directly under the lipid layer. Functionally, M1 binds to the ribonucleoprotein and presumably mediates the transport of ribonucleoproteins to the budding sites as it is required to retain ribonucleoproteins in the cytoplasm after export from the nucleus following replication [389]. Additionally, M1 interacts with the membrane envelope and the cytoplasmic tails of HA and NA and it has been suggested that this interaction ensures the incorporation of M1 into virions [390, 391]. The majority of influenza A virus induced $CD8^+$ cytotoxic T cells are directed against M1, which is highly conserved among different virus subtypes and strains [392, 393]. During infection M1 influences cell death regulation by binding to Hsp70, which disrupts the Hsp70–Apaf-1 complex, resulting in formation of functional apoptosome and activation of caspase-9 [394].

In HLA-A2 positive healthy adults, CTLs against an M1 epitope are detectable and the number increased after vaccination with trivalent inactivated influenza vaccine [395]. Additionally, immunodominant $CD4^+$ T cell responses for M1 were observed in healthy individuals [396]. In a mouse model, a recombinant NP-M1-Hsp60 protein vaccine construct was able to protect the mice against influenza virus challenge [397]. Cross-

protective immunity against lethal influenza virus infection was also reported after vaccination of mice with a plasmid DNA encoding a conserved epitope of M1 or virus-like particles comprised of HA, NA and M1 [398, 399]. Moreover, a CTL response could be elicited by an adenoviral vaccine coexpressing M1 and HA [400]. In humans, a modified vaccinia virus Ankara vector encoding M1 and NP induced potent CTL immunogenicity [137].

Polymerase Acidic protein (PA)

Segment six of the single-stranded RNA genome encodes for PA. Together with PB1 and PB2, PA builds up the trimeric RNA-dependent RNA polymerase complex, which is responsible for the transcription and replication of the viral RNA genome segments [401]. In addition, PA is involved in the conversion of RNA polymerase from transcriptase to replicase [402]. Disruption of the highly conserved protein-protein interaction of the viral polymerase subunits PA and PB1 reduces viral replication efficiency and was thus reported as a novel strategy for virus attenuation [403].

For PA several naturally processed MHC class I binding epitopes have been identified [129, 387]. As part of a multi-peptide vaccine against influenza virus infection, PA was able to induce cross-protection in an HLA-A*2402 human immunity model [387]. Decreased viral load in the lungs and a delay in mortality was also observable after vaccination with a recombinant vaccinia virus containing human T cell epitopes for M1, NS1, NP, PB1 and PA proteins [404].

2. Results

2.1 STEAP1$_{262-270}$ Peptide Encapsulated into PLGA Microspheres Elicits Strong Cytotoxic T Cell Immunity in HLA-A*0201 Transgenic Mice – a New Approach to Immunotherapy against Prostate Carcinoma

CTLs were shown to play a crucial role in the immune response against cancer. Since a number of prostate cancer associated antigens that were able to stimulate CTLs at least *in vitro* was identified in the last years, a growing focus was directed on antigen-specific immunotherapy of prostate cancer. Encapsulation of antigens and TLR ligands into PLGA MS leads to DC maturation, efficient antigen presentation and results in potent and long lasting CTL responses. Therefore, co-encapsulation of PCa antigens and TLR ligands into PLGA MS is a promising strategy for immunotherapy of PCa.

2.1.1 Recombinant expression of Prostate Carcinoma antigens

To elicit tumor antigen specific CTL responses, we recombinantly expressed the antigens in insect cells for subsequent encapsulation into PLGA MS. The Baculo virus system was chosen for the expression of the PCa antigens because of its capacity for large inserts, the high expression levels and the fact that several posttranslational modifications can be accomplished by the system. The DNA sequence of the full-length PSA, PAP and PSCA proteins was cloned into the transfer vector pAcGHLT-B, which contains a 6xHis tag and a glutathione S-transferase (GST) tag upstream of the multiple cloning site. For TRPM8 only the cytoplasmic N-terminal part of the protein was cloned into the transfer vector to preclude solubility problems due to the transmembrane parts of the protein. Co-transfection of the transfer vector and the Baculo virus DNA into *Spodoptera frugiperda* (*Sf*)21 cells allowed recombination between homologous sites, transferring the heterologous PCa gene from the vector to the Baculo virus DNA. Baculo virus infection of *Sf*21 cells resulted in recombinant protein production, which was subsequently purified in a two-step purification via Glutathione GSTrap columns and Ni-NTA HisTrap columns. Purified proteins were concentrated and analyzed by Western

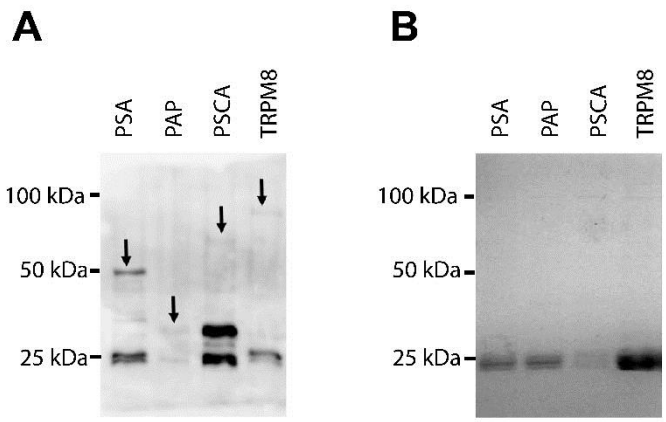

Figure 6. Expression of PCa antigens with the Baculo virus expression system.

Baculo viruses coding for PSA, PAP and PSCA full length proteins as well as the cytoplasmatic N-terminal part of the TRPM8 protein were used for infection of *Sf*21 cells. After lysis of *Sf*21 cells recombinant proteins were purified using GSTrap and His-Trap columns. Samples of the concentrated proteins were boiled with a 10 % β-mercaptoethanol containing SDS-sample buffer and separated on denaturing 12 % SDS-PAGE and analyzed after semi-dry blotting by (A) Western Blot probed with an anti-GST reactive antibody or by direct (B) Coomassie staining of the SDS-PAGE. Calculated protein masses (arrows) for the 6xHis-GST-PCa antigen fusion proteins are 54 kDa for 6xHis-GST-PSA, 37 kDa for 6xHis-GST-PSCA, 71 kDa for 6xHis-GST-PAP and 196 kDa for the 6xHis-GST-N-terminal part of TRPM8.

Blot using an anti-GST reactive antibody after separation on a denaturating SDS PAGE (Fig 6A). Protein Bands could be observed for all antigens at the right size. The calculated sizes for the 6xHis-GST-PCa antigens are 54 kDa for 6xHis-GST-PSA, 37 kDa for 6xHis-GST-PSCA, 71 kDa for 6xHis-GST-PAP and 196 kDa for the 6xHis-GST fusion to the N-terminal part of TRPM8. However, a more prominent protein band was detectable for all antigens at around 25 kDA. This band probably represents only the fusion protein of the 6xHis tag (app. 1 kDa) and the GST tag with a size of around 26 kDa as it is still recognized by the anti-GST antibody, and was most likely caused by a stop of transcription or translation after the tags. In addition, these bands were the only bands visible after Coomassie staining of the SDS-PAGE (Fig 6B). Neither did infection with a lower MOI, at different temperatures and for varying time periods improve the expression of recombinant proteins of the right size, nor did different lysis and purification strategies yield in a higher purification efficiency (data not shown).

Expression of recombinant PCa antigens in *E. coli*

To increase the amount of recombinant protein, an *E. coli* expression system was tested next, as bacteria liquid cultures can be scaled up more easily. For better stability and therefore solubility of the PCa antigens, an expression vector containing a 6xHis-SUMO tag was used and the ER leader sequence of the proteins was removed. *E.coli* strain BL21(DE3) was transformed with the expression plasmid, grown until OD_{600} = 0.6 was reached and then protein expression was induced with 0.4 mM IPTG for different time

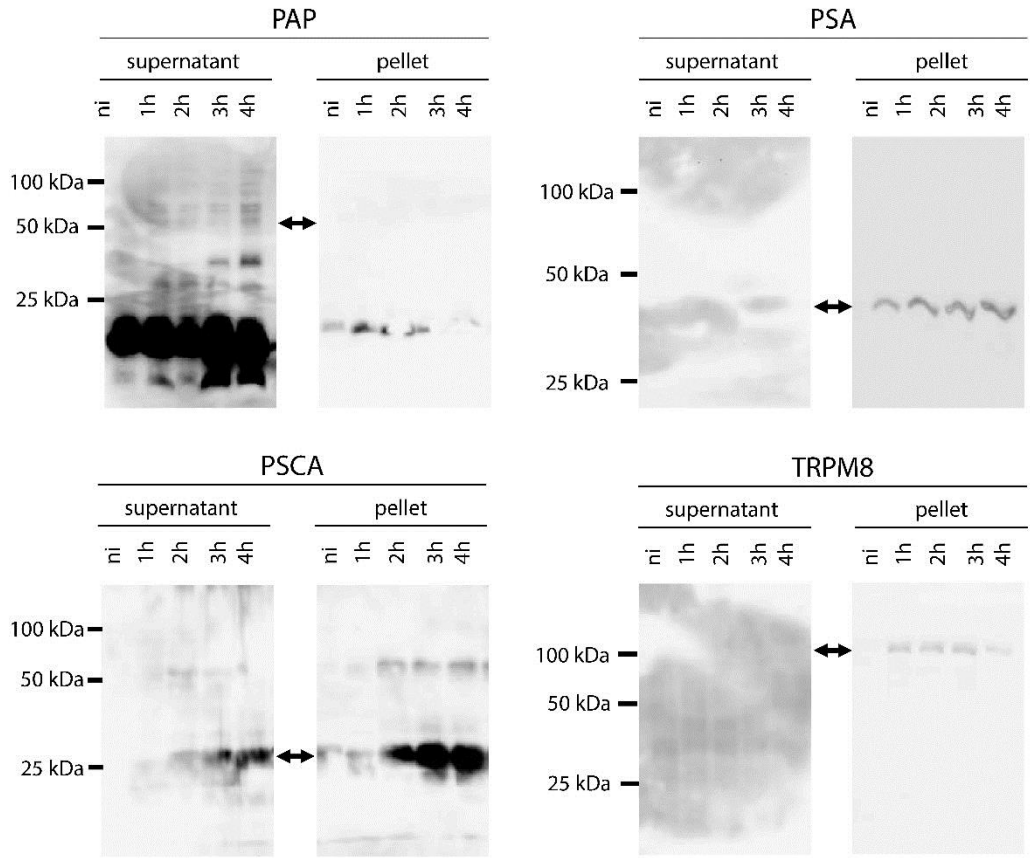

Figure 7. Expression of 6xHis-SUMO PCa antigens in *E. coli*

PSA, PAP and PSCA full length proteins missing the ER leader sequence as well as the cytoplasmatic N-terminal part of the TRPM8 protein were expressed as N-terminally 6-His-SUMO tagged fusion proteins for the indicated time points after induction or as a control without induction (ni = not induced). After bacterial lysis and sonication, supernatants and insoluble pellets were boiled with a 10 % β-mercaptoethanol containing SDS-sample buffer and separated on denaturing 12% SDS-PAGE and after semi-dry blotting analyzed by Western Blot using an anti-His reactive antibody. Calculated protein masses for the 6xHis-SUMO-PCa antigen fusion proteins (arrows) are 38.5 kDa for PSA, 26 kDa for PSCA, 53 kDa for PAP and 90.5 kDa for the N-terminal part of TRPM8.

periods. For analysis of the protein solubility, supernatant and pellet were boiled in SDS sample buffer, separated on a denaturing SDS-PAGE and detected with an anti-His antibody by Western Blot after bacterial lysis (Fig. 7). For 6xHis-SUMO-PAP a size of 53 kDa was calculated. However, only protein bands with a lower molecular weight, presumably a degradation product, was visible. As the 6xHis-SUMO tag has a size of approximately 13 kDa, this band has a too high molecular weight for the tag only. Usage of the E.coli strain BL21(DE3) RIPL that contains genes encoding rare tRNAs neither did result in protein production of the right size (data not shown). For 6xHis-SUMO-PSCA, bands with the right size of 26 kDa could be detected by Western Blot probed with an anti-His antibody. However, more protein could be detected in the insoluble pellet. For 6xHis-SUMO-PAP and 6xHis-SUMO-TRPM8 protein bands at the right size of 38.5 kDa and 90.5 kDa respectively, were detectable only in the insoluble fraction but not in the

supernatant despite improved folding due to the 6xHis-SUMO tag. Attempts to slow down protein synthesis and therefore increase correct folding by decreasing the incubation temperature from 37°C to 20°C or induction of protein expression with lower IPTG concentrations did not result in higher solubility. Moreover, neither minimal growth media like M9 and LB with low salt concentration nor growth media like LB with high salt concentration and HSG medium did influence the solubility. To exclude toxic effects of the proteins on *E.coli*, another strain called BL21(DE3)pLysS, displaying lower background expression was tested but was as well not effective in enhancing the solubility. Different lysis protocols and purification under denaturing conditions with subsequent refolding were shown to be not efficient enough to gain high protein amounts (data not shown).

Recombinant expression of long PCa antigen peptides

An alternative for the usage of full-length tumor associated antigens are long peptides encompassing approximately forty amino acids. They provide several advantages such as a facilitated expression and higher solubility but also some disadvantages like the loss of CTL and helper epitopes, which restrict the immune response. We designed long peptides, including the known HLA-A*0201 epitopes of the PCa antigens PSA, PSCA, PAP and TRPM8 (Tab. 11). All long epitopes were cloned into an *E.coli* expression vector containing a GST-tag. After induction with 0.4 mM IPTG, the bacteria were incubated for 4 h at 37°C. Recombinant long peptides were purified via GSTrap, separated on denaturing SDS-PAGE and analyzed by Western Blot using an anti-GST

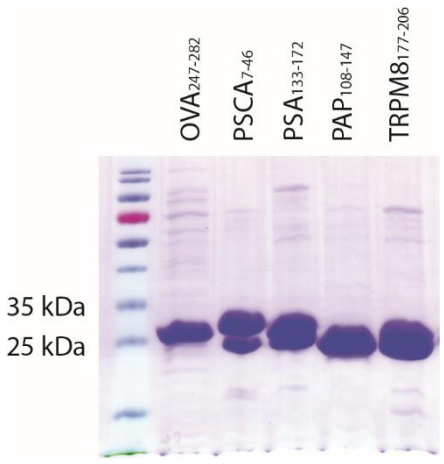

Figure 8. Expression of GST-tagged long PCa antigen peptides in *E. coli*

PSA, PAP, PSCA and TRPM8 long peptides were expressed as N-terminally GST tagged fusion proteins in *E*.coli. After induction of protein expression with 0.4 mM IPTG, bacteria were incubated at 37°C for 4 h. Bacteria were lysed, sonicated and the recombinant long peptides were purified via GSTrap column. Samples of the purified long peptides were boiled with a 10 % β-mercaptoethanol containing SDS-sample buffer and separated on denaturing 12 % SDS-PAGE, which was analyzed by Coomassie. Calculated masses for the GST-PCa antigen long peptide fusion proteins are 31 kDa for GST-PSCA$_{7-46}$, 37 kDa for GST-PSA$_{133-172}$, 31.5 kDa for GST-PAP$_{108-147}$ and 30.3 kDa for GST-TRPM8$_{117-206}$.

antibody (data not shown) and by Coomassie staining of the SDS-PAGE (Fig. 8). All long peptides were detectable at the calculated sizes of 31 kDa for GST-PSCA$_{7-46}$, 37 kDa for GST-PSA$_{133-172}$, 31.5 kDa for GST-PAP$_{108-147}$ and 30.3 kDa for GST-TRPM8$_{117-206}$. However, for PSCA, there was an additional band visible at around 26 kDA, representing probably the GST tag alone. All GST-PCa long peptides could be expressed and purified with high purity (Fig. 8) and in high quantities. For encapsulation into PLGA-MS, 50 mg of each of the long peptides was co-encapsulated with 5 mg CpG-ODN. For comparison, 10 mg of all four PCa long peptides were chemically synthesized and as well co-encapsulated with 5 mg CpG-ODN into PLGA MS. HLA-A*0201 transgenic mice were s.c. immunized with these PLGA MS. On day six after immunization, splenocytes were harvested and restimulated *in vitro* with the known HLA-A*0201 epitopes included in the long peptides and analyzed via intracellular staining of IFN-γ in CTLs. However, neither for the recombinant GST-tagged long PCa antigen peptides nor for the chemically synthesized long peptides a CTL response was measurable in the immunized mice (data not shown).

2.1.2 Synthetic PCa peptide epitopes

One reason, why a vaccine-specific immune response was not detectable with long peptide antigens could be inefficient processing into MHC class I epitopes. Direct encapsulation of PCa epitopes into PLGA MS circumvents this problem and provides additional advantages like the cost-efficient chemical synthesis and most precise vaccine composition. Therefore, we encapsulated known HLA-A*0201 antigen epitopes of the four PCa antigens PSA, PSCA, PAP, and TRPM8 we already used for recombinant protein and long peptide expression (see chapter 2.1.1) and of four additional peptides from the PCa antigens PSMA, Survivin and STEAP1.

PCa peptide antigens are efficiently encapsulated and released from PLGA MS

Characterization of the PLGA MS containing eight PCa antigens PSA$_{154-163}$, PSCA$_{14-22}$, PAP$_{112-120}$, TRPM8$_{187-195}$, PSMA$_{27-35}$, Survivin$_{96-104}$, STEAP1$_{86-94}$ and STEAP1$_{262-270}$ revealed that all peptide antigens were efficiently encapsulated into the PLGA MS (Fig. 9, lanes 2). In addition, all the peptides were released from the microspheres in PBS (Fig. 9, lanes 3). The time interval for the release in aqueous medium mimics the timespan of six days between immunization and analysis used in our *in vivo* immunization protocol. Furthermore, no overt differences in encapsulation efficiency and release were observable between the eight different PCa peptide antigens.

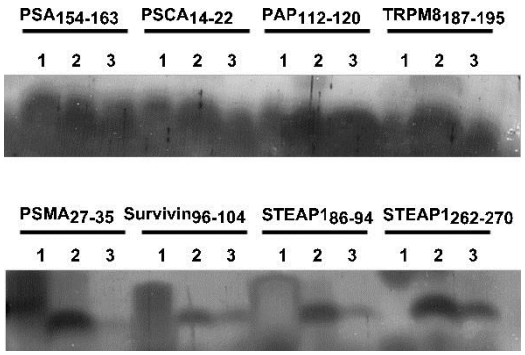

Figure 9 Characterization of PLGA encapsulated prostate carcinoma antigens by *in vitro* release.

For the characterization of PLGA-MS, the encapsulation efficiency and release of the indicated peptides was analyzed. As control, 50 µg of the respective peptide was dissolved in a final volume of 250 µl (1). Encapsulation efficiency (2) was determined by dissolving 5 mg of the respective microspheres in 50 µl DMSO. After 30 min of incubation, samples were diluted with 200 µl 0.1% NaOH solution. For analysis of the release from MS (3), 5 mg of the indicated MS were dispersed in 250 µl PBS and incubated at 37°C under agitation for 6 days. Peptide amounts were estimated from silver stained Tris-tricine PAGE. The experiment has been repeated twice with similar outcome.

PCa peptide antigens bind and stabilize HLA-A*0201

One of the most important features of a peptide antigen is a strong binding and thus stabilization of MHC class I molecules. In silico analysis with the publically available epitope prediction programs SYFPEITHI (http://www.syfpeithi.de/) and BIMAS (http://www-bimas.cit.nih.gov/molbio/hla_bind/) assigned excellent binding properties to all eight peptide antigens for HLA-A*0201.

Peptide	Sequence	Concentration to stabilize MHC (µM)	Off-kinetics (h)
$FluM1_{58-66}$	GILGFVFTL	< 0.5	>> 6
$PSA_{154-163}$	VISNDVCAQV	2	3
$PSCA_{14-22}$	ALQPGTALL	< 0.5	>> 6
$PAP_{112-120}$	TLMSAMTNL	1	>> 6
$TRPM8_{187-195}$	GLMKYIGEV	1	>> 6
$PSMA_{27-35}$	VLAGGFFLL	< 0.5	>> 6
$Survivin_{96-104}$	LTLGEFLKL	1	3
$Steap_{86-94}$	FLYTLLREV	1	>> 6
$Steap_{262-270}$	LLLGTIHAL	1	>> 6

Figure 10. HLA-A*0201 binding characteristics of the PCa peptide epitopes.

For the stability assay, T2 cells were incubated at room temperature overnight with the indicated peptides over a range of peptide concentrations from 0.5 to 10 µM in the presence of 1 µg/ml β2-microglobulin. The concentrations of the respective peptides needed to stabilize HLA-A*0201 on the cell surface are provided. For the off- kinetics, T2 cells were incubated overnight in the presence of 10 µM peptide, followed by an incubation at 37°C in the presence of 50µg/ml cycloheximide. Loss of HLA-A*0201 molecules from the cell surface (off-kinetics) was monitored by flow cytometry after 1, 2, 3, 4 and 6 h, respectively, as compared to cells incubated without peptide. The experiments have been performed twice with similar outcome.

Stabilization of the HLA-A*0201 peptide complex on the cell surface of T2 cells was compared to the immunodominant, high affinity HLA-A*0201 ligand influenza virus matrix peptide $M1_{58-66}$. Like the M1 reference peptide, $PSCA_{14-22}$ and $PSMA_{27-35}$ stabilized HLA-A*0201 even at concentrations below 0.5 µM (Fig. 10). All of the other antigens were also able to stabilize HLA-A*0201 already at low concentrations. $PSA_{154-163}$ and $Survivin_{96-104}$ were the only epitopes that could not stabilize HLA-A*0201 as efficiently. In conclusion, all of the eight PCa peptide antigens provide good or superb HLA-A*0201 binding properties even at low concentration and can stabilize the complex on the cell surface with low off rates.

Superior induction of a $STEAP1_{262-270}$-specific CTL response by immunization of HLA-A*0201 transgenic mice with PLGA MS as compared to IFA

Immunogenicity of the eight different PCa peptide antigens was assayed *in vivo* using AAD mice [405]. AAD mice express a transgene encoding an MHC class I fusion protein consisting of the peptide binding α1 and α2 domain of the human HLA-A*0201 and the CD8 binding α3 domain of mouse H-$2D^d$. Corresponding amounts of PCa peptide as well as of the adjuvants CpG-ODN and polyI:C were either encapsulated into PLGA-MS or emulsified in IFA. Vaccination with $STEAP1_{262-270}$, both in PLGA MS and IFA, induced peptide specific CTLs. A brief restimulation of these CTLs with $STEAP1_{262-270}$ peptide *in vitro* triggered a robust IFN-γ response, which was significantly higher for the PLGA MS compared to IFA (Fig. 11A+B). Vaccination with only the adjuvants CpG-ODN and polyI:C encapsulated into PLGA-MS served as negative control. Restimulation of the splenocytes from these control mice with $STEAP1_{262-270}$ peptide induced no IFN-γ response. Surprisingly, vaccination with $PSA_{154-163}$, $PSCA_{14-22}$, $PAP_{112-120}$, $TRPM8_{187-195}$, $PSMA_{27-35}$, $Survivin_{96-104}$ and $STEAP1_{86-94}$ peptides co-encapsulated with CpG-ODN in PLGA MS and co-administered with polyI:C PLGA MS induced no peptide specific CTLs. This lack of a detectable CTL response was not only observed after PLGA MS based vaccination but also when mice were vaccinated with the same peptides emulsified in IFA (Fig. 11A+B). The failure to elicit CTL responses for these seven PCa peptide antigens could therefore not be attributed to problems with PLGA MS specific encapsulation or release but most likely to marginal immunogenicity of these PCa peptide antigens in AAD mice.

PLGA-MS induced CTLs specifically lyse peptide-loaded target cells *in vivo*

Next, the functionality of the generated $STEAP1_{262-270}$ specific CTLs in terms of their ability to lyse target cells in a peptide-specific manner was assayed. For vaccination with

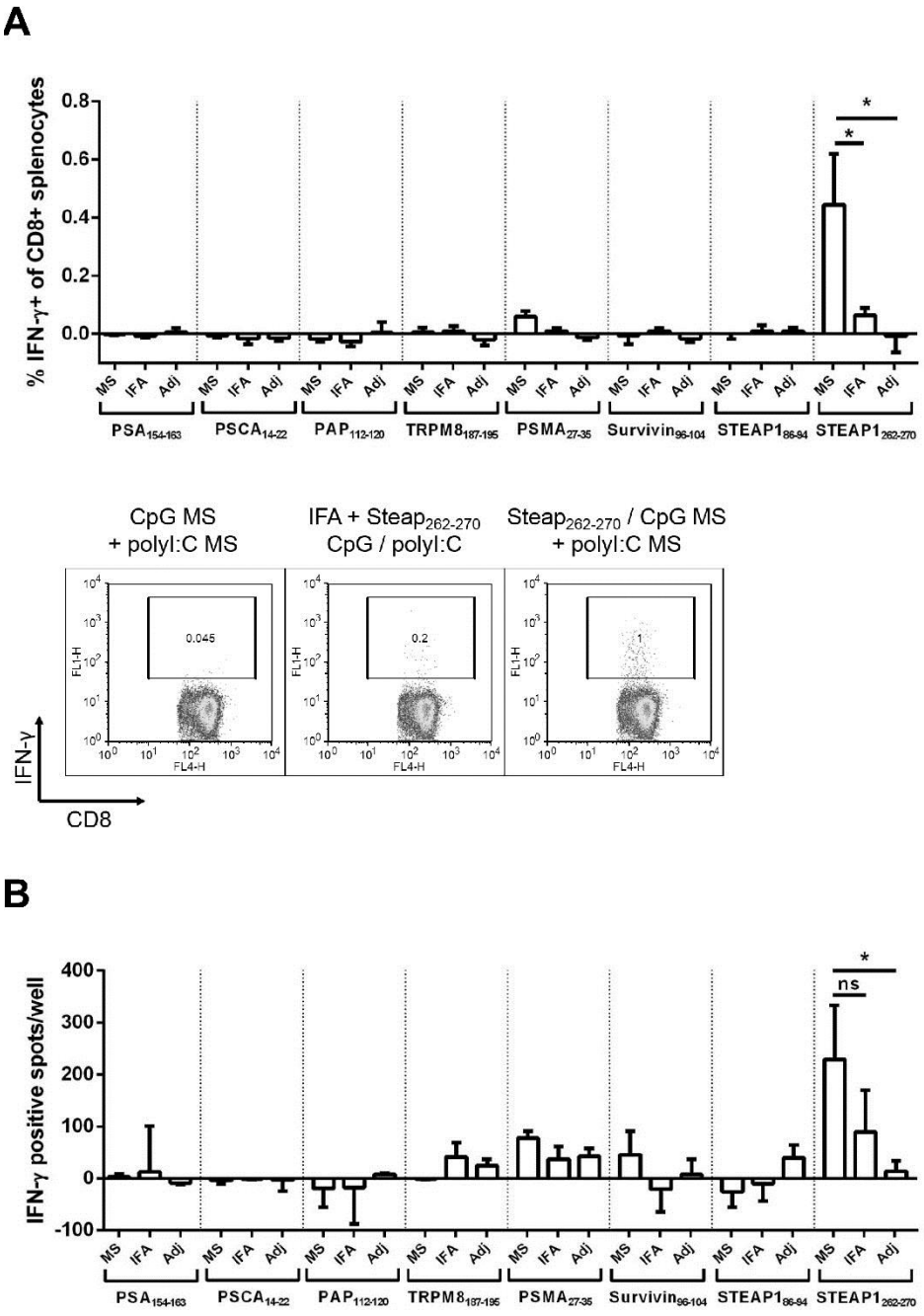

Figure 11. Comparison of the CTL response after immunization with PCa peptide antigens encapsulated into PLGA MS or emulsified in IFA.

Male HLA-A*0201 transgenic AAD mice (n=3) were immunized s.c. with 5 mg peptide/CpG MS and 5 mg polyI:C MS per mouse (containing 50 µg peptide, 25 µg CpG, 2.5 µg polyI:C). Alternatively, equivalent amounts of peptide, CpG and polyI:C were emulsified in IFA and used for vaccination. As control, adjuvants containing MS (Adj) incorporating only CpG and polyI:C were used. On day 6 after immunization splenocytes were isolated and analyzed for peptide specific $CD8^+$ T cell responses by intracellular cytokine staining (ICS) for IFN-γ and flow cytometry (A) as well as ELISPOT assay (B). Representative examples of dot plots of the indicated ICS experiments are shown in (A) below the bar graph. Background levels (no peptide) were subtracted. Values (mean ± SEM) in (A) are given in percent IFN-γ^+ of $CD8^+$ lymphocytes. The p values of (A) were calculated by an unpaired t-test with Welsch´s correction (*p = 0.0218; *p = 0.0108). Values (mean ± SEM) in (B) are given as IFN-γ specific spots/well. The p value was calculated by an unpaired t-test with Welsch´s correction (*p = 0.0233). The experiments have been performed three times with similar outcome.

STEAP1$_{262-270}$/CpG MS and polyI:C MS the *in vivo* cytotoxicity assay showed robust responses. At day 6 after immunization STEAP1$_{262-270}$ pulsed target cells were specifically lysed whereas unpulsed control cells were not affected in the same mice (Fig. 12). In control mice immunized with CpG MS and polyI:C MS neither the peptide pulsed nor the unpulsed cells were lysed. In conclusion, CTLs induced by vaccination with PLGA MS containing STEAP1$_{262-270}$ were not only able to elicit an IFN-γ response upon restimulation (Fig. 11) but also killed the target cells in a peptide-specific manner (Fig. 12). In contrast, CTLs of mice that were immunized with TRPM8$_{187-195}$/CpG MS and polyI:C MS did not lyse TRPM8$_{187-195}$ pulsed target cells, which is consistent with the finding that specific CTL responses could not be elicited after PLGA MS or IFA based vaccination (Fig. 11).

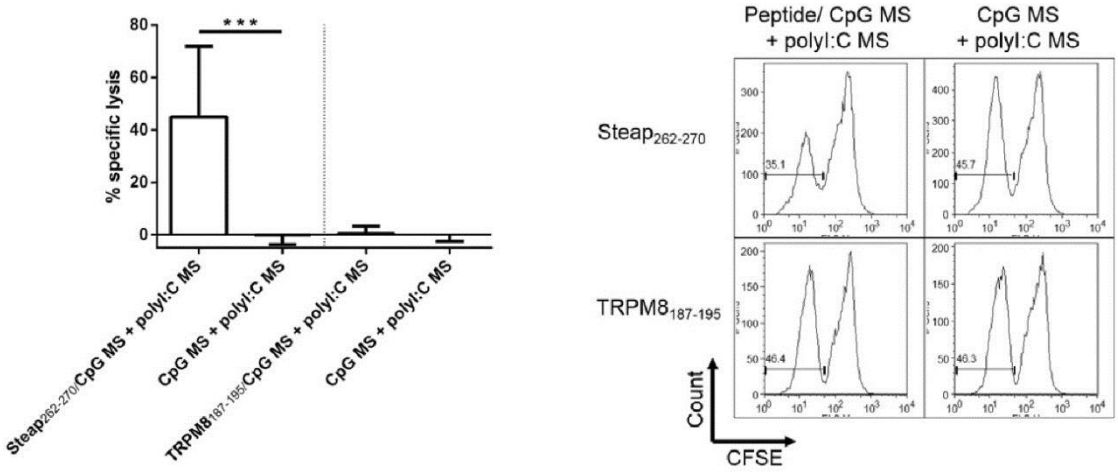

Figure 12. CTL mediated *in vivo* cytotoxicity elicited by vaccination with PLGA MS.

Male HLA-A*0201 transgenic AAD mice (n = 6) were immunized with 5 mg STEAP1$_{262-270}$ /CpG MS or TRPM8$_{187-195}$/CpG MS and 5 mg polyI:C MS per mouse. As controls adjuvants microspheres bearing respective amounts of CpG and polyI:C were used. On day 6 after immunization half of the splenocytes from naïve mice were left untreated, the other half was pulsed with 10^{-6} M of the respective peptide for 1 h at 37°C. Afterwards, untreated and peptide pulsed cells were stained with 1 µM or 10 µM CFSE, respectively. A mixture of 1 x 10^7 pulsed and unpulsed cells was injected i.v. in the tail vein of the immunized mice. After 24 h splenocytes of the immunized mice were analyzed for CFSE labeled cells by flow cytometry. Representative examples of flow cytometry histograms of CFSE labeled target cells from the mice immunized as indicated are shown to the right side the graph. Values (mean ± SEM) are given in percent specific lysis. The p value was calculated by an unpaired t-test with Welsch´s correction (***p = 0.0010). The experiments have been performed three times with similar results.

PLGA MS induced CTLs recognize human HLA-A*0201/STEAP1$_{262-270}$ complexes

The first signal for the activation of primed T cells is provided through the T cell receptor which interacts with peptide/MHC complexes on the surface of antigen presenting cells.

Therefore, CTLs induced by vaccination with PLGA MS including STEAP1$_{262-270}$ should recognize and respond to human APCs presenting STEAP1$_{262-270}$ peptide on HLA-A*0201. Indeed, splenocytes of vaccinated mice did not only respond upon peptide restimulation but also recognized and responded to peptide pulsed human HLA-A*0201 positive T2 cells (Fig. 13). Peptide specificity was analyzed by comparison to stimulation with unpulsed T2 cells, which resulted in a significantly lower IFN-γ release. The same result was observable for CTL stimulation with human monocyte-derived dendritic cells (MoDCs) raised from HLA-A*0201 positive donors after uptake of STEAP1$_{262-270}$/CpG MS leading to maturation of the MoDCs and to HLA-A*0201-mediated peptide presentation on the cell surface. Stimulation with mature MoDCs after CpG MS uptake induced significantly less IFN-γ producing CTLs. These data show that STEAP1$_{262-270}$ specific CTLs raised in HLA-A*0201/H-2D^{d} chimera expressing AAD mice recognize this peptide also on human HLA-A*0201 and that human MoDC can be readily charged with the STEAP1$_{262-270}$ peptide by PLGA MS.

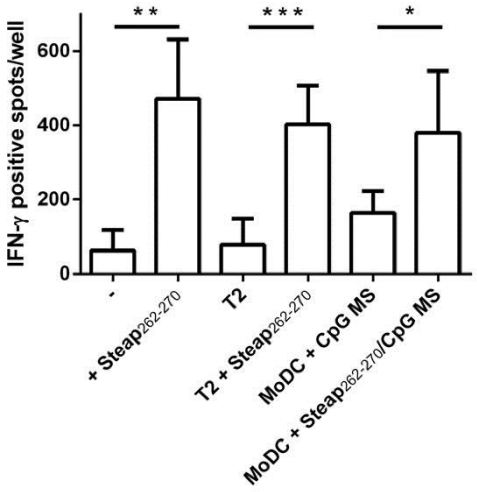

Figure 13. STEAP1$_{262-270}$/HLA-A*0201 recognition on PLGA MS-pulsed human dendritic cells by peptide-specific CTLs.

Male HLA-A*0201 transgenic AAD mice (n = 6) were immunized with 5 mg STEAP1$_{262-270}$ /CpG MS and 5 mg polyI:C MS per mouse. On day 6 after immunization splenocytes were isolated and stimulated by addition of either +/- STEAP1$_{262-270}$ peptide alone, by T2 cells externally pulsed or not with STEAP1$_{262-270}$ peptide, or by human HLA-A*0201^{+} MoDC charged with CpG MS alone or with STEAP1$_{262-270}$/CpG MS. Peptide specific CD8^{+} T cells were detected by ELISPOT assays. Values (mean ± SEM) are given as IFN-γ specific spots/well. The p values were calculated by an unpaired t-test with Welsch´s correction (**p = 0.0010; ***p = 0.0002; *p = 0.0241). The experiments have been performed twice with similar outcome.

2.2 Immunotherapy of Malignant Melanoma with optimized Melanoma-Associated Antigen Epitopes encapsulated into biodegradable PLGA Microspheres

With an increasing understanding of immune responses, the idea of cancer immunotherapies evolved. However, despite melanoma being one of the most immunogenic tumors with several described tumor antigens, immunotherapy theory outstripped performance. There is a clear need for raising the immunogenicity by modification of tumor antigens and the combination with adjuvants and vaccine delivery systems. Therefore, we tested the immunostimulatory capability of the three naturally processed, HLA-A*0201-restricted melanoma antigen epitopes MAGE $C2_{336-244}$, $MART1_{26-35}$, and TRP-$2_{180-188}$ in comparison to their modified analogues. The modifications are described in more detail below. These analogues, a heteroclitic analogue of $MART1_{26-35}$, modified MAGE $C2_{336-244}$ and TRP-$2_{180-188}$ epitopes including unnatural derivatives were designed to have an improved HLA-A*0201 binding capacity and to provide a higher stability [7].

Equal encapsulation capacity and release of natural melanoma epitopes and their modified analogues from PLGA MS

Comparison of the encapsulation and release characteristics of the three natural melanoma epitopes MAGE $C2_{336-244}$, $MART1_{26-35}$ and TRP-$2_{180-188}$ and their modified analogues from PLGA MS revealed no differences between the peptides. All peptides were encapsulated into the PLGA MS with the same efficiency (Fig. 14 lanes 2). Incubation of the PLGA MS in PBS for six days resulted in the release of all peptides from the PLGA MS (Fig. 14 lanes 3). The time interval for the release in aqueous medium mimics the timespan of six days between immunization and analysis used in our *in vivo* immunization protocol.

TRP-$2_{180-188}$ analogue modified with unnatural derivatives does not elicit cross-reactive CTLs

For improved stability and binding to HLA-A*0201, the natural TRP-$2_{180-188}$ epitope SVYDFFVWL was changed to [D-alpha-methyl-phenylglycine][L-norvaline]YDFFVW[L-Porpargylglycine] (TRP-2 mod1). Firstly, we analyzed whether improved binding to HLA-A*0201 subsequently gives raise to increased CTL responses in HLA-A*0201 transgenic AAD mice. AAD mice were immunized with PLGA MS containing either the natural TRP-2 epitope or TRP-2 mod1 in combination with CpG-ODN. PolyI:C was added in separate

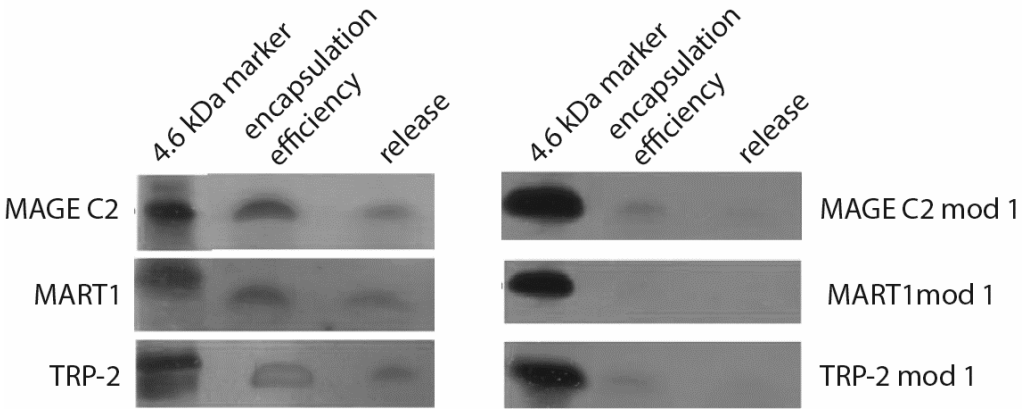

Figure 14. Comparision of encapsulation efficiency and release of natural and modified epitopes from PLGA MS *by in vitro* release.

For the characterization of PLGA-MS, the encapsulation efficiency and release of the indicated peptides was analyzed. Encapsulation efficiency (2) was determined by dissolving 5 mg of the respective microspheres in 50 µl DMSO. After 30 min of incubation, samples were diluted with 200 µl 0.1% NaOH solution. For analysis of the release from MS (3), 5 mg of the indicated MS were dispersed in 250 µl PBS and incubated at 37°C under agitation for 6 days. Peptide amounts were estimated from silver stained Tris-tricine PAGE. The experiment has been repeated twice with similar outcome.

PLGA MS. After six days, splenocytes of the mice were restimulated with the epitopes to assess the number of IFN-γ producing CTLs. Immunization of AAD mice with the natural TRP-2 epitope resulted in reproducible CTL responses measured both in ICS and ELISPOT assay. A significantly increased CTL response compared to the natural TRP-2 peptide could be detected after vaccination with TRP-2 mod1 encapsulated into PLGA MS, but only after restimulation with the modified TRP-2 peptide (white bar). In contrast, the CTLs generated by vaccination with TRP-2 mod1 PLGA-MS could not be restimulated with the natural TRP-2 peptide (black bar) (Fig. 15A/B).

To prove the HLA-A*0201 dependence of the effects observed in AAD mice, TRP-2 and TRP-2 mod1 PLGA MS were also used to immunize C57BL/6 mice, because the peptides were reported to bind also to H2-K^b. Immunization of C57BL/6 mice led to the induction of a robust CTL response that was detectable both by ICS and ELISPOT assay. In contrast, vaccination with empty MS or PLGA MS encapsulating the adjuvants only, induced no specific immune stimulation. In accordance to the results observed in AAD mice, vaccination with TRP-2 mod1 in PLGA MS elicited as well a significantly higher CTL response in C57BL/6 mice, but only after restimulation with the modified peptide (white bar). Restimulation with the natural peptide (black bar) resulted in an even lower CTL response as compared to vaccination with the natural peptide (Fig. 15C/D). Summarizing, T cell receptors of the CTLs induced with TRP-2 mod1 MS did not cross-react with the natural peptide any more. Moreover, the benefit in the induction of CTL responses gained by usage of the modified peptide cannot clearly be attributed to improved HLA-A*0201 binding, as this effect was also observable in C57BL/6 mice.

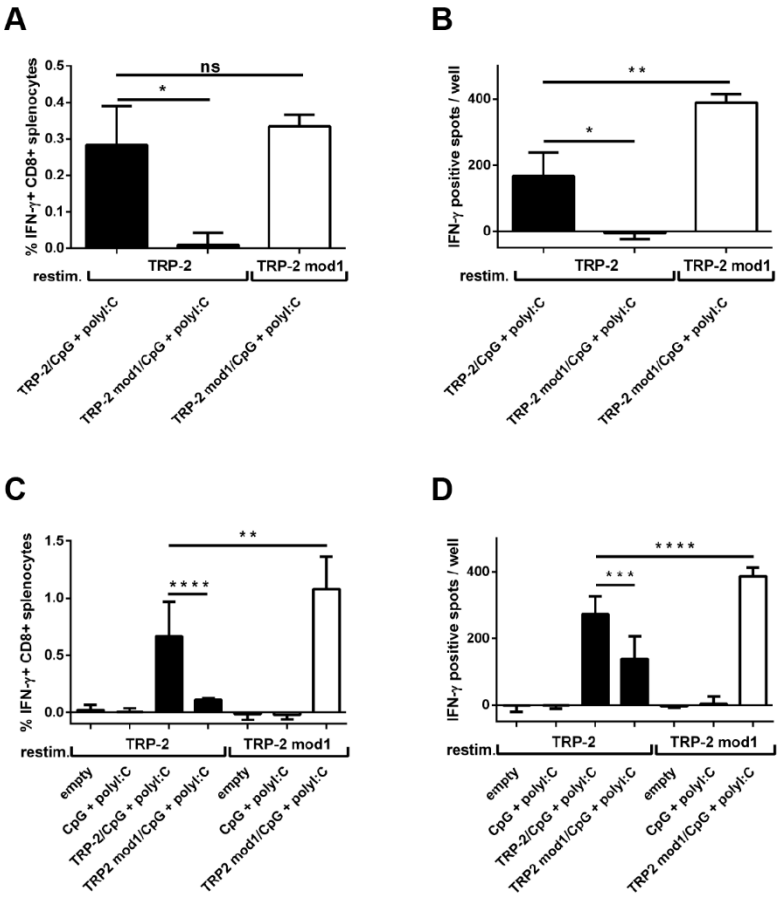

Figure 15. Comparison of the immunostimulatory capacity of TRP-2 and TRP-2 mod1 encapsulated into PLGA MS.

(A/B) HLA-A*0201 transgenic AAD mice (n=4) or (C/D) C57BL/6 mice (n=5) were immunized with 5 mg peptide/CpG MS and 5 mg polyI:C MS per mouse. As control, empty MS or adjuvants containing MS incorporating only CpG and polyI:C were used. On day 6 after immunization splenocytes were isolated, restimulated with TRP-2 peptide (black bars) or TRP-2 mod1 peptide (white bars) and analyzed for peptide specific $CD8^+$ T cell responses by intracellular cytokine staining (ICS) for IFN-γ and flow cytometry (A/C) as well as ELISPOT assay (B/D). Background levels (no peptide) were subtracted. Values (mean ± SEM) in (A/C) are given in percent IFN-γ+ of CD8+ lymphocytes. The p values of (A/C) were calculated by an unpaired t-test with Welsch´s correction ((A) *p = 0.0106) ((C) **p = 0.0088; ****p = <0.0001). Values (mean ± SEM) in (B/D) are given as IFN-γ specific spots/well. The p values were calculated by an unpaired t-test with Welsch´s correction ((B) *p = 0.136; **p = 0.0048) ((D) ****p = <0.0001; ***p = 0.0001). The experiments have been performed at least two times with similar outcome.

Comparison of the immunogenicity of MAGE-$C2_{336-244}$ epitope and its modified form including unnatural derivatives

The natural MAGE-$C2_{336-244}$ epitope ALKDVEERV was modified by replacement of the first two amino acids by the unnatural derivatives L-S-methyl-cysteine and L-2-aminoctanoic acid (MAGE C2 mod1) to improve stability and HLA-A*0201 binding capacity.

For evaluation of the immunogenicity of the two forms of MAGE $C2_{336-244}$ epitope, AAD mice were immunized with PLGA MS containing either the natural or the modified form

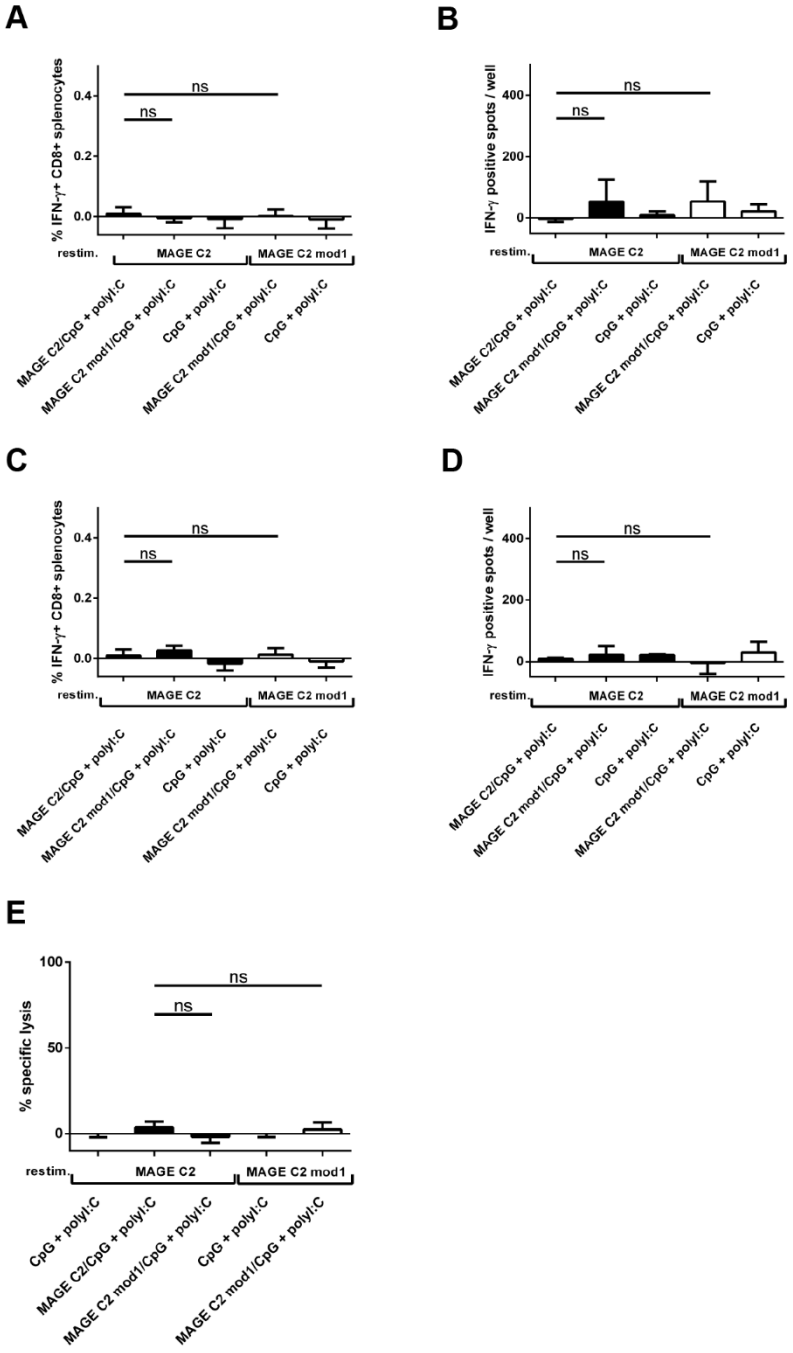

Figure 16. Comparison of the immunogenicity of MAGE C2 and MAGE C2 mod1 encapsulated into PLGA MS.

(A/B) HLA-A*0201 transgenic AAD mice (n=6) or (C/D) C57BL/6 mice (n=3) were immunized with 5 mg peptide/CpG MS and 5 mg polyI:C MS per mouse. As control adjuvants containing MS incorporating only CpG and polyI:C were used. On day 6 after immunization splenocytes were isolated, restimulated with MAGE C2 peptide (black bars) or MAGE C2 mod1 peptide (white bars) and analyzed for peptide specific $CD8^+$ T cell responses by ICS for IFN-γ and flow cytometry (A/C) as well as ELISPOT assay (B/D). Background levels (no peptide) were subtracted. Values (mean ± SEM) in (A/C) are given in percent IFN-γ^+ of $CD8^+$ lymphocytes. The p values of (A/C) were calculated by an unpaired t-test with Welsch´s correction. Values (mean ± SEM) in (B/D) are given as IFN-γ specific spots/well. The p values were calculated by an unpaired t-test with Welsch´s correction. In (E) AAD mice (n = 4) were immunized with 5 mg peptide/CpG MS and 5 mg polyI:C MS per mouse. As controls adjuvants microspheres bearing respective amounts of CpG and polyI:C were used. On day 6 after immunization splenocytes from naïve were left untreated or were pulsed with 10^{-6} M MAGE C2 peptide (black bars) or MAGE C2 mod1 peptide (white bars) and stained with 1 µM or 10 µM CFSE, respectively. A mixture of pulsed and unpulsed cells was injected i.v. into the immunized mice. Analysis for CFSE labeled cells was performed by flow cytometry. Values (mean ± SEM) are given in percent specific lysis. The p value was calculated by an unpaired t-test with Welsch´s correction. The experiments have been performed twice with similar results.

of the epitope. *In vitro* restimulation of the splenocytes after six days resulted in no measurable CTL induction for both, the natural and the modified epitope by ICS (Fig. 16A). In the more sensitive ELISPOT assay, a slight CTL response was observable after vaccination with MAGE C2 mod1 and restimulation with either the natural (black bar) or the modified peptide (white bar). This signal was only scarcely over detection limit, but clearly over the unspecific background signal induced by vaccination with PLGA MS containing the adjuvants only (Fig.16B). This slight increase can definitely be ascribed to the improved HLA-A*0201 binding ability, as the effect was not observable after vaccination of C57BL/6 mice neither by ICS nor by ELISPOT assay (Fig. 16C/D).

Despite a reported H2-K^b binding competence of the natural MAGE $C2_{336-244}$ epitope, no CTL induction was detectable after vaccination of C57BL/6 mice with PLGA MS incorporating the peptide.

Nonetheless, the small number of CTLs induced after vaccination with MAGE C2 mod1 MS could have functional influence on immunity to melanoma in AAD mice. Therefore, CTL functionality was tested in an *in vivo* cytotoxicity assay. Six days after vaccination no specific lysis of peptide-loaded target cell was detectable, neither for mice immunized with MAGE C2 MS nor for mice immunized with MAGE C2 mod1 MS. The ratio of unpulsed target cells to peptide pulsed target cells in the spleen was the same for vaccination with each of the peptides encapsulated into PLGA MS as well as for vaccination with adjuvants MS (Fig. 16E). In conclusion, vaccination with MAGE C2 mod1 PLGA MS induced a detectable CTL response, which was either too small or not functional for specific lysis of target cells.

Superior capacity of a heteroclitic $MART1_{26-35}$ analogue compared to the natural epitope to induce specific CTL responses

For improvement of HLA-A*0201 binding, the natural EAAGIGLTV $MART1_{26-35}$ epitope was changed to ELAGIGLTV. Vaccination of AAD mice with PLGA MS encapsulating the natural $MART1_{26-35}$ epitope induced no measurable specific CTL response, neither in ICS nor in ELISPOT assay. In contrast, immunization with MART1 mod2 MS resulted in a robust CTL induction, measurable after restimulation with both, the modified peptide (white bar) and, albeit lower, the natural peptide (black bar). For the control immunization with adjuvants MS, no specific immune responses were elicited (Fig. 17A/B). This improvement due to modification of the $MART1_{26-35}$ epitope is a clearly HLA-A*0201 dependent phenomenon, as no immune responses were measurable in C57BL/6 mice, neither by ICS nor by ELISPOT (Fig.17 C/D).

To prove the functionality of the induced CTLs, an *in vivo* cytotoxicity assay was performed. As expected, no specific lysis of peptide pulsed target cells was observable after immunization with adjuvants MS or MS incorporating the natural MART1 epitope.

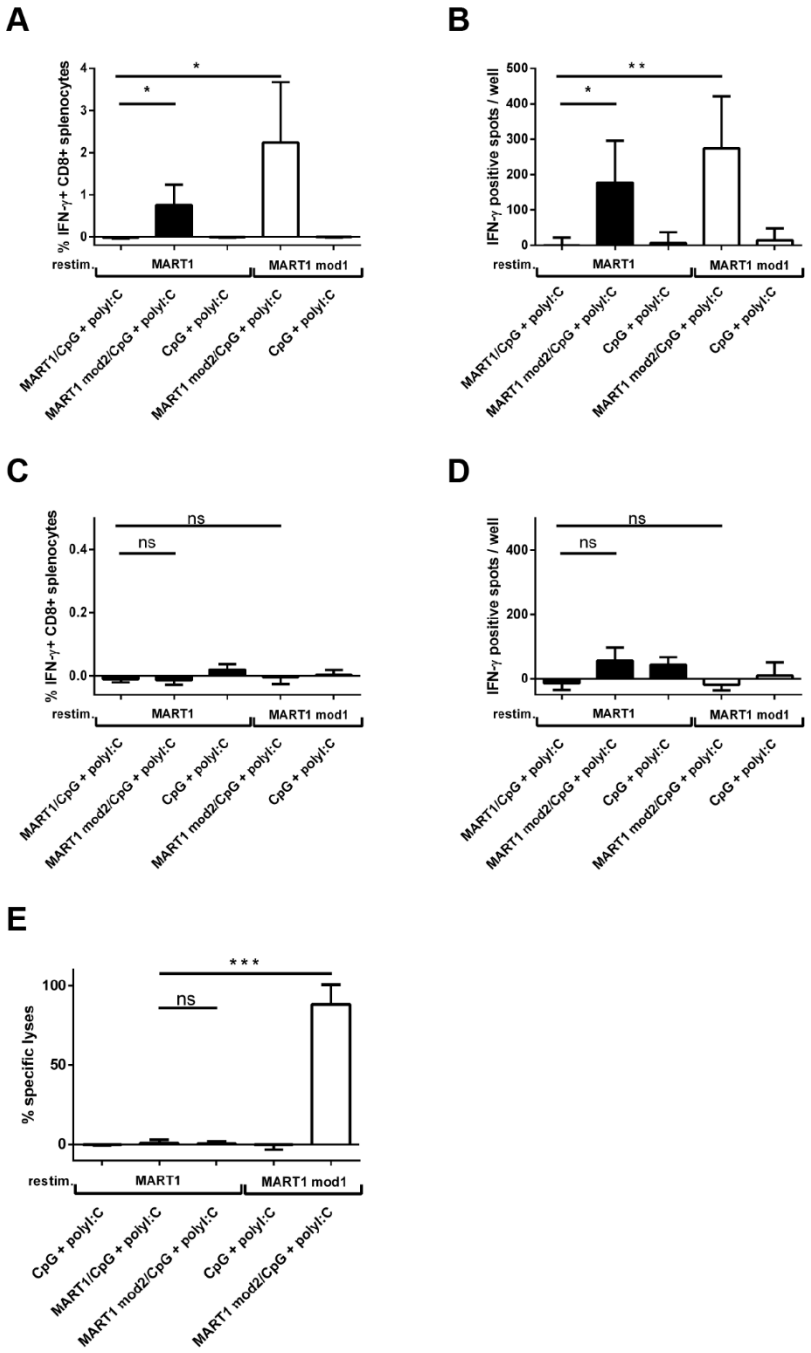

Figure 17. Induction of antigen specific CTL responses by MART1 and its heteroclitic analogue

(A/B) HLA-A*0201 transgenic AAD mice (n=6) or (C/D) C57BL/6 mice (n=3) were immunized with 5 mg peptide/CpG MS and 5 mg polyI:C MS per mouse. As control adjuvants containing MS incorporating only CpG and polyI:C were used. On day 6 after immunization splenocytes were isolated, restimulated with MART1 peptide (black bars) or MART1 mod2 peptide (white bars) and analyzed for peptide specific $CD8^+$ T cell responses by ICS for IFN-γ and flow cytometry (A/C) as well as ELISPOT assay (B/D). Background levels (no peptide) were subtracted. Values (mean ± SEM) in (A/C) are given in percent IFN-γ^+ of $CD8^+$ lymphocytes. The p values of (A/C) were calculated by an unpaired t-test with Welsch´s correction ((A) *p = 0.0102; **p = 0.0117). Values (mean ± SEM) in (B/D) are given as IFN-γ specific spots/well. The p values were calculated by an unpaired t-test with Welsch´s correction ((B) *p = 0.0145; **p = 0.0057). In (E) AAD mice (n = 4) were immunized with 5 mg peptide/CpG MS and 5 mg polyI:C MS per mouse. As controls adjuvants microspheres bearing respective amounts of CpG and polyI:C were used. On day 6 after immunization splenocytes from naïve mice were left untreated or were pulsed with 10^{-6} M MART1 peptide (black bars) or MART1 mod2 peptide (white bars) and stained with 1 µM or 10 µM CFSE, respectively. A mixture of pulsed and unpulsed cells was injected i.v. into the immunized mice. Analysis for CFSE labeled cells was performed by flow cytometry. Values (mean ± SEM) are given in percent specific lysis. The p value was calculated by an unpaired t-test with Welsch´s correction (***p = 0.0006). The experiments have been performed twice with similar results.

In addition, CTLs induced by MART1 mod2 MS lysed target cells that were pulsed with the modified peptide. Surprisingly, these CTLs induced by MART1 mod2 MS though did not lyse target cells pulsed with the natural peptide (Fig. 17E). This could be due to a lack of functionality or the instability of the MART1-HLA-A*0201 complex on the cell surface. However, the complex was stable enough to restimulate specific CTLs whose TCRs were shown to be cross-reactive to the natural peptide.

2.3 Cytotoxic T cell vaccination with PLGA microspheres interferes with influenza A virus replication in the lung and suppresses infectious disease

Influenza virus infections cause several million cases of severe illness worldwide and about half a million deaths per year. Every year expensive vaccination programs are conducted with newly designed vaccines aimed at eliciting neutralizing antibodies but the antigenic drift of influenza A virus often enables the virus to escape from immune protection. Our PLGA MS-based approach aims at eliciting cytotoxic T cell mediated immunity against conserved viral antigens, leading to immunity to emerging strains of new influenza A viruses that are prone to escape conventional vaccination regimen.

High immunogenicity of PLGA MS-based immunization with the influenza A virus epitope $M1_{58-66}$

Initially, the immunogenicity of an immunization with the conserved $M1_{58-66}$ epitope of influenza virus matrix protein encapsulated into biodegradable PLGA microspheres was addressed. For this purpose HLA-A*0201 transgenic AAD mice were s.c. immunized with a mixture of $M1_{58-66}$/CpG MS and polyI:C MS. Six days after the immunization, a strong induction of antigen specific CTLs was measureable in the spleen (Figure 18A/B). While immunization with microspheres containing only the adjuvants CpG ODN and polyI:C induced only a minor response due to unspecific stimulation, no IFN-γ secretion was detectable after immunization with empty microspheres. In contrast to the immunization of AAD mice, no IFN-γ producing, antigen-specific CTLs could be induced in C57BL/6 mice as evidence for the HLA-A*0201 restriction of the $M1_{58-66}$ epitope (Figure 18C). These data show the high immunogenic potential of a PLGA MS-based immunization with the influenza virus epitope $M1_{58-66}$ and the Th1 polarizing TLR ligands CpG ODN and polyI:C.

MS induce strong and sustained immune responses *in vivo*

To determine the optimal peptide amount for vaccination with PLGA MS, AAD mice received a single injection of MS containing 12.5, 25, 50 or 100 µg $M1_{58-66}$ peptide coencapsulated with 25 µg CpG ODN in a mixture with polyI:C MS (2.5 µg). The lowest peptide concentration already induced a robust immune response in the spleens of

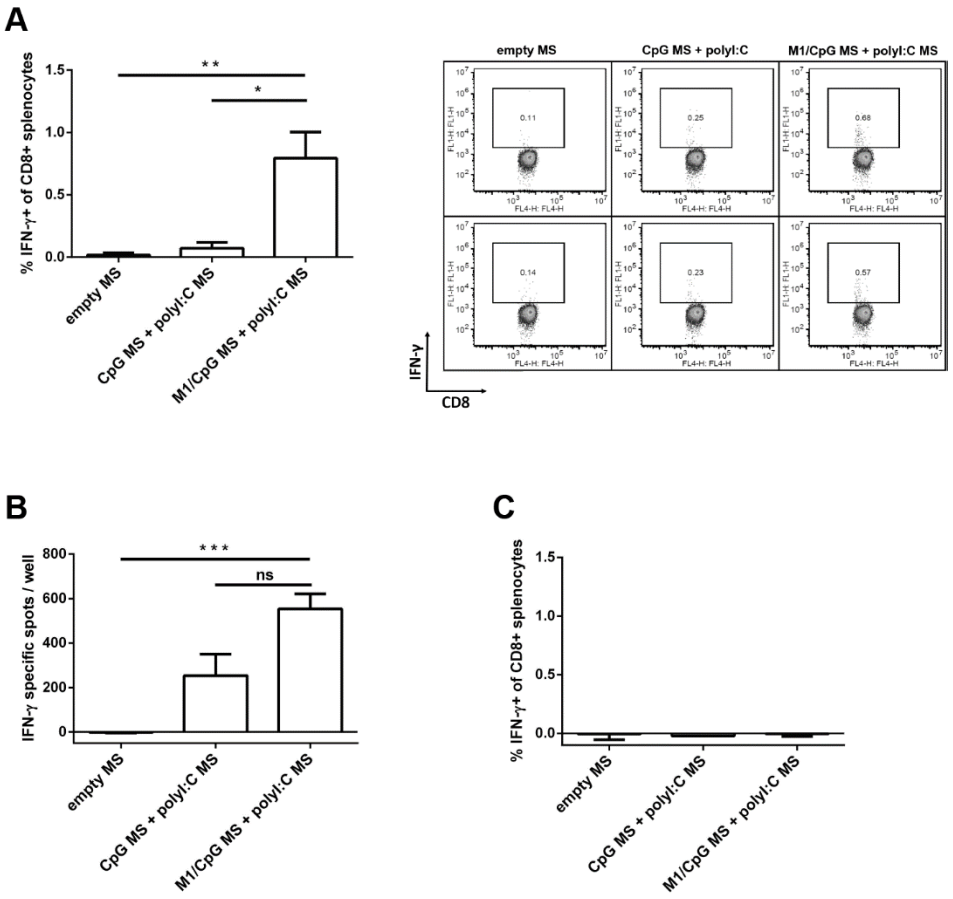

Figure 18. Production of IFN-γ by CD8⁺ T cells after Immunization with M1/CpG MS and polyI:C MS

AAD mice (A/B) (n = 7/6) or C57BL/6 mice (C) (n = 3) were immunized with 5 mg MS containing 50 µg $M1_{58-66}$ peptide and 25 µg CpG ODN and 5 mg MS containing 2.5 µg polyI:C. Control groups were immunized with corresponding amounts of encapsulated CpG and polyI:C (n(A/B) = 6/2; n(C) = 2) or empty MS (n(A/B) = 4/2; n(C) = 2) in 5 mg MS respectively. After 6 days, splenocytes were isolated and analyzed for IFN-γ production after peptide restimulation by ICS (A/C) / ELISPOT (B). Background levels (no peptide) were substracted. Values (mean ± SEM) in (A/C) are given in percent IFN-γ⁺ of CD8⁺ lymphocytes. The p values of (A) were calculated by an unpaired t-test with Welsch´s correction (**p = 0.0098; *p = 0.0129). Dot plots show IFN-γ⁺ (y axis) of CD8⁺ (x axis) splenocytes for two representative samples of each group. Values (mean ± SEM) in (B) are given as IFN-γ specific spots/well. The ρ value was calculated by an unpaired t-test with Welsch´s correction (***p = 0.0004). The experiments have been performed twice with similar results.

immunized mice after 6 days (Fig. 19A/B). The number of $M1_{58-66}$-specific, IFN-γ secreting CD8⁺ T cells increased corresponding to the peptide amount encapsulated in the MS. Maximal response was reached at a peptide concentration of 50 µg per mouse. The MS containing 100 µg $M1_{58-66}$ peptide did not induce a higher immune response, which could be due to a limited encapsulation efficacy or a peptide saturation of the T cells. As a further characteristic of the vaccination with MS the immune response kinetics were analyzed (Figure 19C). Therefore, AAD mice were immunized with the previously identified optimal peptide concentration of 50 µg $M1_{58-66}$ peptide per mouse. On day 4 after immunization, no peptide specific CTLs were detectable. The peak of the immune response could be measured between day 6 and day 8. There was a decreasing but

persistent CTL response at least until the last time point of measurement on day 35. Taken together, the best vaccination protocol was found to be an immunization of the AAD mice with MS containing 50 µg $M1_{58-66}$ peptide and CpG ODN (25 µg) in combination with polyI:C MS (2.5 µg).

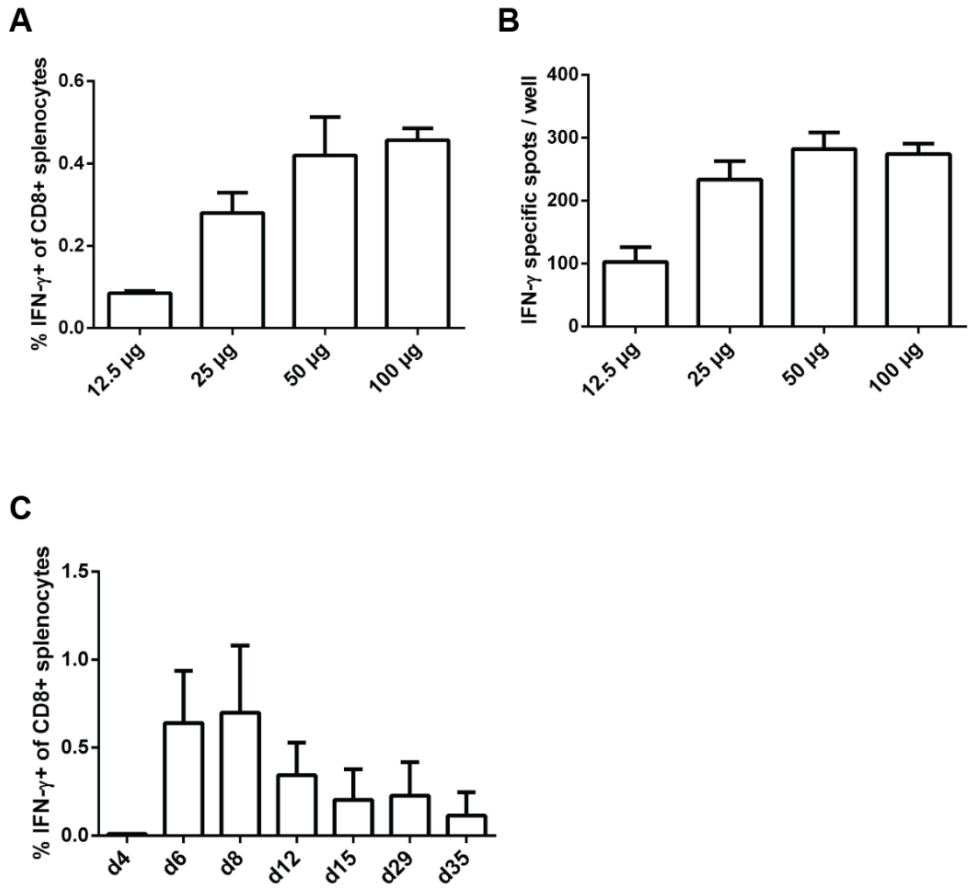

Figure 19. Titration of the peptide amount and kinetics of the CTL response after immunization with M1/CpG MS and polyI:C MS

(**A/B**) AAD mice (n = 3) were immunized s.c. with 5 mg MS containing 100 µg; 50 µg; 25 µg or 12.5 µg $M1_{58-66}$ peptide and 25 µg CpG ODN and 5 mg MS containing 2.5 µg polyI:C. After 6 days, splenocytes were isolated and analyzed for IFN-γ production after peptide restimulation by ICS (A) or ELISPOT (B). Background levels (no peptide) were substracted. Values (mean ± SEM) are given in percent IFN-γ^+ of $CD8^+$ lymphocytes. (**C**) AAD mice were immunized s.c. with 5 mg MS containing 50 µg $M1_{58-66}$ peptide and 25 µg CpG ODN and 5 mg MS containing 2.5 µg polyI:C. After 4 (n = 2); 6 (n = 5); 8 (n = 5); 12 (n = 5), 15 (n = 5), 29 (n=4) and 35 (n = 4) days splenocytes were isolated and analyzed for IFN-γ production after peptide restimulation by ICS. Background levels (no peptide) were substracted. Values (mean ± SEM) are given in percent IFN-γ^+ of $CD8^+$ lymphocytes.

Co-encapsulation of two peptide antigens does not diminish the elicited immune response

Immune escape by antigenic drift reduces the reliability of vaccination against influenza virus. Therefore, it is advantageous to encapsulate more than one peptide antigen into MS. As it was shown before, that antigen and toll-like receptor ligand need to be co-

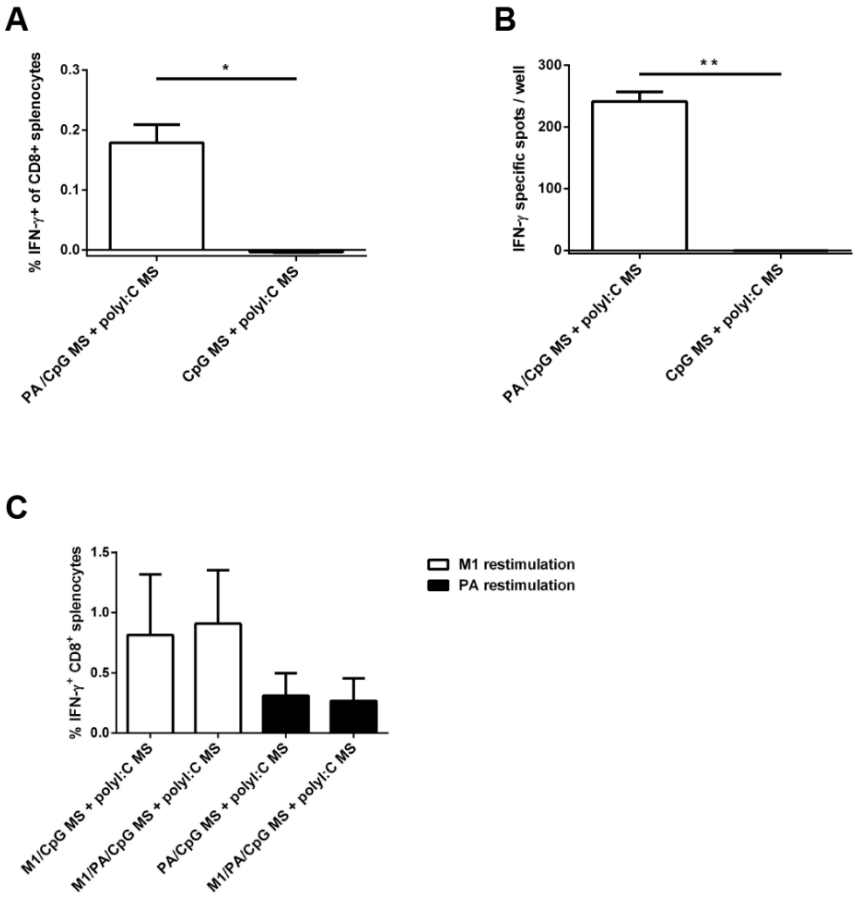

Figure 20. CTL responses elicited after immunization with PA/CpG MS and polyI:C MS or co-encapsulated M1/PA/CpG MS and polyI:C MS

(**A/B**) AAD mice (n = 3) were immunized s.c. with 5 mg MS containing 50 µg PA_{46-54} peptide and 25 µg CpG ODN and 5 mg MS containing 2.5 µg polyI:C. The control group was immunized with corresponding amounts of encapsulated CpG and polyI:C in MS (n = 2). After 6 days, splenocytes were isolated and analyzed for IFN-γ production after peptide restimulation by ICS (A) / ELISPOT (B). Background levels (no peptide) were substracted. Values (mean ± SEM) in (A) are given in percent IFN-γ^+ of $CD8^+$ lymphocytes. The ρ value of (A) was calculated by an unpaired t-test (*p = 0.0184). Values (mean ± SEM) in (B) are given as IFN-γ specific spots/well. The p value was calculated by an unpaired t-test (** p = 0.0013). (**C**) AAD mice (n = 3) were immunized s.c. with 5 mg MS containing 50 µg PA_{46-54}, 50 µg $M1_{58-66}$ peptide and 25 µg CpG oligonucleotides and 5 mg MS containing 2.5 µg polyI:C. The control group was immunized with corresponding amounts of encapsulated M1 or PA and CpG in 5 mg MS and polyI:C MS. After 6 days, splenocytes were isolated and analyzed for IFN-γ production after restimulation with M1 or PA peptide by ICS. Background levels (no peptide) were substracted. Values (mean ± SEM) are given in percent IFN-γ^+ of $CD8^+$ lymphocytes.

encapsulated into one MS [127] we tested if two antigens can be encapsulated together with CpG ODN into one MS with the same efficacy. As second antigen PA_{46-54} was used, which is, like $M1_{58-66}$, a naturally processed, HLA-A*0201 restricted and highly conserved epitope of the influenza virus [129]. First of all, we could show that PA_{46-54}/CpG MS mixed with polyI:C MS were highly immunogenic in AAD mice and induced a strong peptide specific CTL response in the spleens of vaccinated mice (Figure 20A/B). Next, AAD mice were immunized with MS encapsulating $M1_{58-66}$ peptide (50 µg), PA_{46-54} peptide (50 µg) and CpG ODN (25 µg) mixed with polyI:C MS (2.5 µg). Splenocytes were restimulated *ex vivo* with either $M1_{58-66}$ peptide or PA_{46-54} peptide and compared to splenocytes of AAD

mice injected with MS containing only one of the peptides. Both, $M1_{58-66}$ and PA_{46-54} single encapsulated MS induced CTL responses with $M1_{58-66}$ MS being more potent. The MS encapsulating both peptide antigens elicited comparable CTL responses after restimulation with the respective peptides as the MS encapsulating only one peptide (Figure 20C). Accordingly, co-encapsulation of two peptide epitopes into one MS did not diminish the elicited immune response to one of them.

Vaccination with PLGA-MS protects from infection with a recombinant vaccinia virus

In the first instance, we compared the potency of a PLGA-MS based vaccination to an immune response elicited by a viral infection. Therefore, AAD mice were immunized with MS encapsulating $M1_{58-66}$ peptide and CpG ODN mixed with polyI:C MS or were infected with a recombinant vaccina virus coding for M1 (rVV M1) or $M1_{58-66}$ (rVV-ESM1). While immunization with PLGA-MS induced a strong and robust $M1_{58-66}$ specific CTL response, almost no response was measurable after infection with recombinant vaccinia virus (Figure 21A).

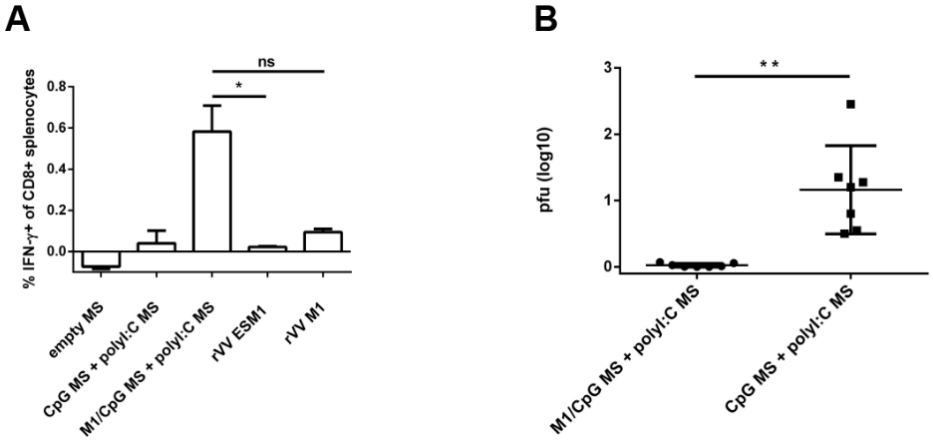

Figure 21. Comparison of the CTL response after immunization with M1/CpG MS and polyI:C MS or infection with recombinant vaccinia virus and induction of protective immunity

(**A**) AAD mice (n = 5) were immunized s.c. with 5 mg MS containing 50 µg $M1_{58-66}$ peptide and 25 µg CpG ODN and 5 mg MS containing 2.5 µg polyI:C or infected i.p. with 2 x 10^6 pfu rVV ESM1 or rVV M1. Control groups were immunized with corresponding amounts of encapsulated CpG and polyI:C in MS (n = 3) or empty MS (n = 3). After 6 days, splenocytes were isolated and analyzed for IFN-γ production after peptide restimulation by ICS. Background levels (no peptide) were substracted. Values (mean ± SEM) are given in percent IFN-γ^+ of $CD8^+$ lymphocytes. The ρ value was calculated by an unpaired t-test with Welsch´s correction (*p = 0.0114). (**B**) AAD mice (n = 7) were immunized s.c. with either 5 mg MS containing 50 µg $M1_{58-66}$ peptide and 25 µg CpG ODN and 5 mg MS containing 2.5 µg polyI:C or 5 mg CpG MS and 5 mg polyI:C MS containing corresponding amounts. Six days after vaccination, mice were challenged i.p. with 2 x 10^6 pfu rVV ESM1, and 4 days later ovaries were recovered for determination of the viral titer. Individual mice and the median values per group are shown. The p value was calculated by an unpaired t-test with Welsch´s correction (**p = 0.0040). The experiments have been performed twice with similar results.

Infection with vaccinia virus is a good model for viral protection, as it depends on the activity of CTLs. Hence, we compared the antiviral capacity of mice immunized with $M1_{58-66}$/ CpG containing MS and polyI:C MS with control mice that received CpG MS and polyI:C MS. Mice were challenged with 2 x 10^6 pfu vaccinia virus on day 6 after immunization, viral titers were determined 4 days later in the ovaries. Vaccination with $M1_{58-66}$/ CpG MS and polyI:C MS led to an almost complete protection against vaccinia virus (Figure 21B). In contrast, control mice showed high rVV-ESM1 titers in the ovaries. Taken together, PLGA-MS can induce viral protection against vaccinia virus in an antigen-specific manner.

Single vaccination with PLGA-MS does not allay influenza virus infection

Protective immunity against influenza virus in vaccination and natural infection is primarily mediated by antibody responses against surface proteins. However, reduced disease severity can be achieved with strong T cell responses to internal viral proteins [406]. After having successfully demonstrated the antiviral capacity of PLGA-MS in a vaccinia virus infection model, we investigated whether vaccination with PLGA-MS can also temper influenza virus infection. To test this, mice were immunized either with PLGA-MS containing $M1_{58-66}$/ CpG and polyI:C MS or as a control for the antigen-specificity with CpG MS and polyI:C MS. On day 6 after immunization mice were inoculated intranasally with 5 × 10^4 pfu H1M1pdm09 influenza virus. After 48 h lungs of both groups were collected and virus titers were determined by plaque assay. There was no reduction of virus titer in the lungs of mice treated with MS containing $M1_{58-66}$ compared to the control group (Figure 22A). Both groups showed a continuous loss of bodyweight starting from day 2 (Figure 22B). Onset of clinical symptoms was at day 4 post infection evolving to score four (death) in both groups (Figure 22C). Although s.c. vaccination with PLGA-MS has a high antiviral capacity during vaccinia virus infection it does not allay influenza virus infection *in vivo*.

PLGA-MS can induce mucosal and systemic immunity

Systemic vaccination might not be suitable to induce CTLs against influenza virus at the site of infection, namely the respiratory tract. To overcome these limitations, we evaluated two alternative immunization strategies with the aim to generate both, mucosal and systemic immunity. For the first vaccination scheme AAD mice were primed s.c. with 5 mg peptide (50µg)/CpG (25 µg) MS and 5 mg polyI:C (2.5 µg) MS in a volume of 200 µl and boosted after 14 d i.n. with half the amount of MS in a volume of 50 µl. For the second vaccination strategy, mice were immunized i.n. with peptide (25 µg)/CpG (12.5 µg) MS and polyI:C (1.25 µg) MS. The same immunization protocols were applied to the

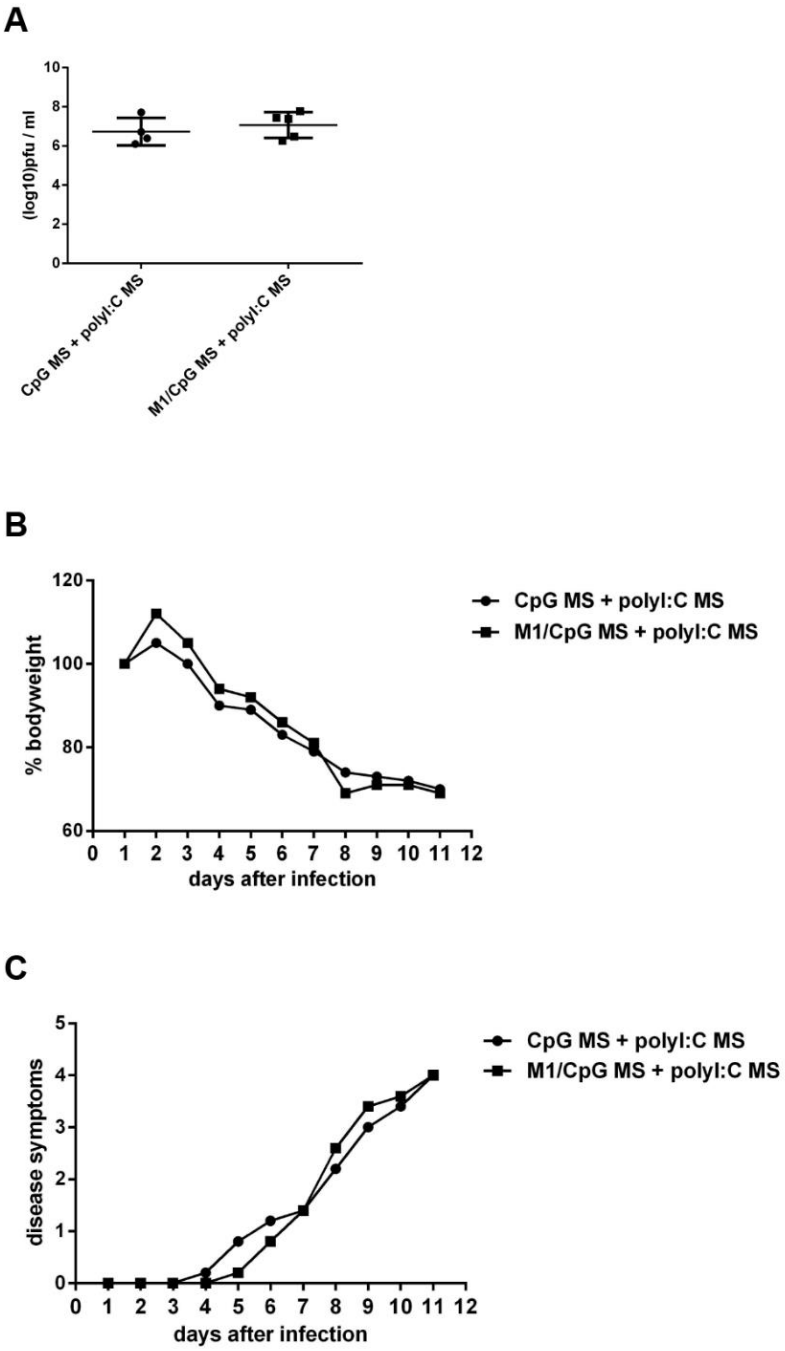

Figure 22. Failure to induce Influenza virus protective immunity solely by subcutaneous PLGA MS-based vaccination

AAD mice (n = 5) were immunized s.c. with 5 mg MS containing 50 µg $M1_{58-66}$ peptide and 25 µg CpG ODN and 5 mg MS containing 2.5 µg polyI:C. The control group was immunized with corresponding amounts of encapsulated CpG and polyI:C. Six days after vaccination, mice were challenged i.n. with 5×10^4 pfu RB1 H1N1pdm09 influenza virus (RB1). (A) 48 h after infection, lungs were recovered for determination of the viral titer. Individual mice and the median values per group are shown. Disease symptoms (B) and bodyweight (C) were monitored during a time period of 11 d after infection. Median values per group are shown.

control groups that received CpG MS and polyI:C MS. Six days after the last immunization the numbers of antigen-specific CTLs were analyzed in spleen (Figure 23A) and lung (Figure 23B). In the spleen, the highest number of antigen-specific CTLs

was found for the group that received a single subcutaneous immunization (Figure 23A). A strong CTL response was also observable in the spleens of mice immunized with $M1_{58-66}$, PA_{46-54}, or $M1_{58-66}$/ PA_{46-54} in the prime-boost setting. In contrast, only a minor response

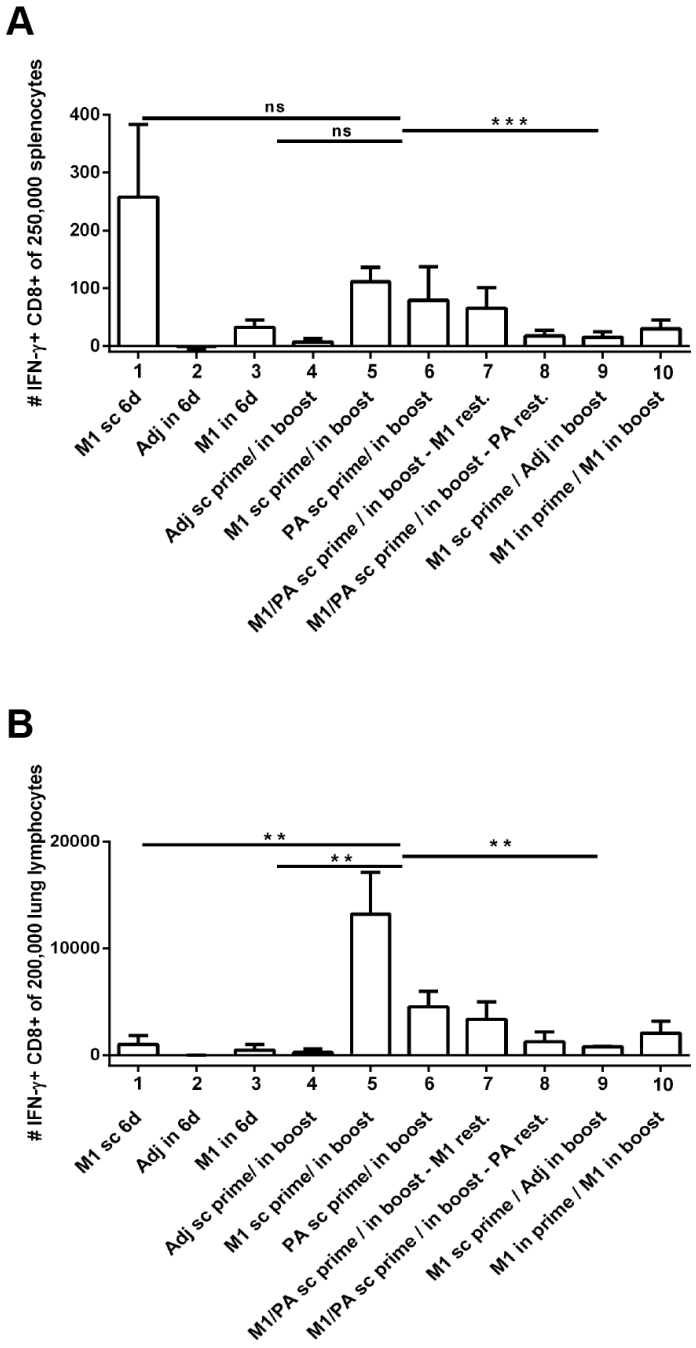

Figure 23. Subcutaneous priming and intranasal boosting with PLGA-MS yields strong CTL responses to influenza virus epitopes in the lung

(A/B) For priming, AAD mice were s.c. immunized with M1/CpG MS (n=6), PA/CpG MS or M1/PA/CpG MS each and 5 mg polyI:C MS. The control group was immunized with corresponding amounts of CpG MS and polyI:C MS (n = 4). Boost and single i.n. immunizations were performed with 2.5 mg of the corresponding MS. Six days after the last immunization, splenocytes (A) and lung cells (B) were isolated and analyzed for IFN-γ production after peptide restimulation by ICS. Background levels (no peptide) were substracted. Values (mean ± SEM) are given in (A) as absolut number of IFN-γ^+ $CD8^+$ lymphocytes per 2.5 x 10^5 counted splenocytes and in (B) as absolut number of IFN-γ^+ $CD8^+$ cells per 2 x 10^5 lung leukocytes. The ρ values were calculated by an unpaired t-test with Welsch´s correction (A) (***ρ = 0.0004), (B) (** p = 0.0017, **p = 0.0017, **p = 0.002). The experiments have been performed twice with similar results.

could be detected for the group that has received a single i.n. immunization (lane 3). For both new vaccination protocols no antigen specific CTLs were detectable for the control groups immunized with adjuvants MS. Furthermore, a combination of a s.c. prime vaccination with $M1_{58-66}$/CpG MS and polyI:C MS and an i.n. boost vaccination with adjuvants MS just induced a negligible number of antigen-specific CTLs (lane 9). A completely different picture could be observed in the lungs of the immunized mice (Figure 23B). Here, the highest number of antigen-specific CTLs was measured for $M1_{58-66}$/CpG MS and polyI:C MS applied with the prime-boost vaccination setting (lane 5). In addition, PA_{46-54} and $M1_{58-66}$/ PA_{46-54} administered according to the prime-boost vaccination setting could induce a M1 specific CTL response, too (lane 7). In contrast, there was only a low response after single s.c. or single i.n. vaccination with $M1_{58-66}$ MS and no measurable response for the control groups. Interestingly, an s.c. antigen-specific prime followed by an unspecific, inflammatory stimulus in the lung also led to no CTL response in the lung (lane 9) thus indicating that a successful i.n. boost relies on antigen to accumulate specific CTLs in the lung.

Heterologous Vaccination with PLGA MS leads to Protective Immunity against Influenza Virus Infection

Next, we wanted to investigate if the systemic and mucosal immunity induced by the heterologous prime-boost vaccination enables protection against challenge with a lethal dose of influenza virus. Single i.n. immunization with $M1_{58-66}$/CpG MS and polyI:C MS as well as prime-boost immunization with adjuvants microspheres or PA_{46-54}/CpG MS and polyI:C MS displayed no protective capacity against pandemic influenza virus H1N1pdm09 (Figure 24A). High virus titers measured 48 hours after infection in the lung correlated with a tremendous weight loss of all three groups between day five and six and subsequent infection related death (Figure 24 B/C). Both, mice vaccinated with $M1_{58-66}$, PA_{46-54} and CpG co-encapsulated into one microsphere mixed with polyI:C MS and mice vaccinated with $M1_{58-66}$/CpG MS and polyI:C MS according to the prime-boost setting not only showed systemic and vigorous CTL responses in the lung (Figure 23B) but were also protected from influenza virus infection (Figure 24 B/C). In accordance to a significantly reduced virus titer in the lung, no weight loss was observable. In summary, vaccination with the conserved and well defined epitopes $M1_{58-66}$ and PA_{46-54} co-encapsulated together with the TLR 9 ligand CpG ODN into clinically compatible PLGA microspheres and mixed with microspheres encapsulating the TLR 3 ligand polyI:C enable control of influenza A virus infections.

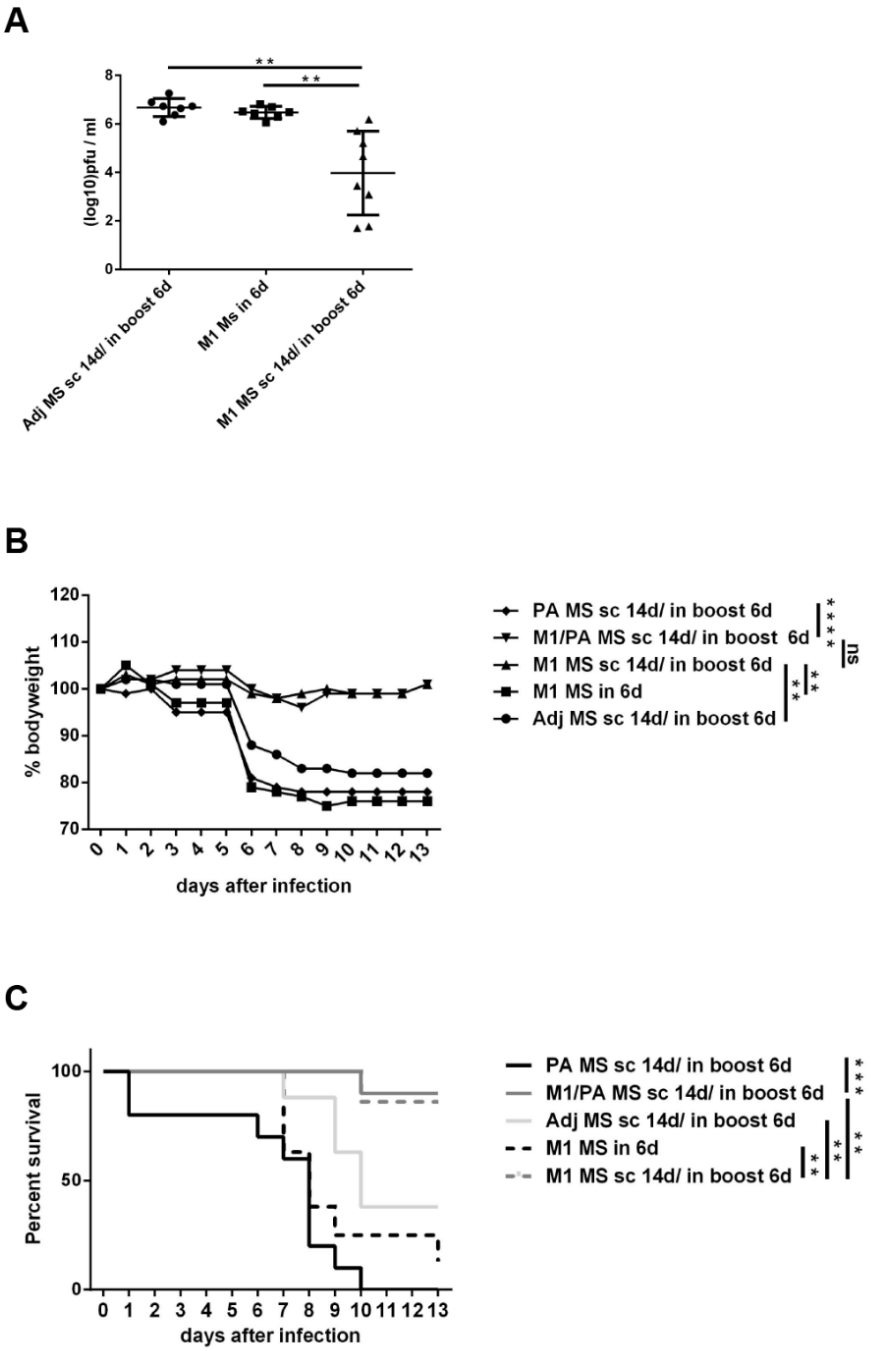

Figure 24. Induction of Influenza virus protective immunity by PLGA MS based vaccination

For priming, AAD mice were s.c. immunized with 5 mg M1/CpG MS (n=8), PA/CpG MS or M1/PA/CpG MS each and 5 mg polyI:C MS. The control group was immunized with corresponding amounts of CpG MS and polyI:C MS (n = 7). Boost and single i.n. immunizations were performed with 2.5 mg of the corresponding MS. Six days after vaccination, mice were challenged i.n. with 5×10^4 pfu RB1 H1N1pdm09 influenza virus. (A) 48 h after infection, lungs were recovered for determination of the viral titer. Individual mice and the median values per group are shown. The p value was calculated by an unpaired t-test with Welsch´s correction (**p = 0.0028; **p = 0.0045). Bodyweight (B) and survival (C) was monitored during a time period of 13 d after infection. Median values per group are shown. The p values were calculated by a paired t-test ((B) ***p = 0.0009; ***p = 0.0003; ****p = <0.0001); ((C) *p = 0.0401; **p = 0.0089; **p = 0.0040; ***p = 0.0002).

Heterologous vaccination induces sustained immune responses

Immunity against influenza virus should ideally last for at least one influenza season. After vaccination according to our heterologous vaccination scheme, immune responses were detectable both in spleen and lung for four weeks. Moreover, specific CTLs in lung and spleen could be restimulated with an additional intranasal boost immunization after decay of the initial immune response (Fig. 25A/B).

In the spleen, the number of specific CTLs more than doubled between weeks one and two after the boost immunization, probably due to migration from the lung to the spleen. Between weeks two and four the number of CTLs in the lung remained stable until a strong decrease in week eight (Fig. 25A). In the lung, equal numbers of specific CTLs were observable during the first three weeks. After eight weeks, no specific CTLs were measurable in the lung anymore (Fig. 25B). Re-boost after eight weeks induced robust CTL responses both in lung and spleen indicating an efficient formation of T memory cells.

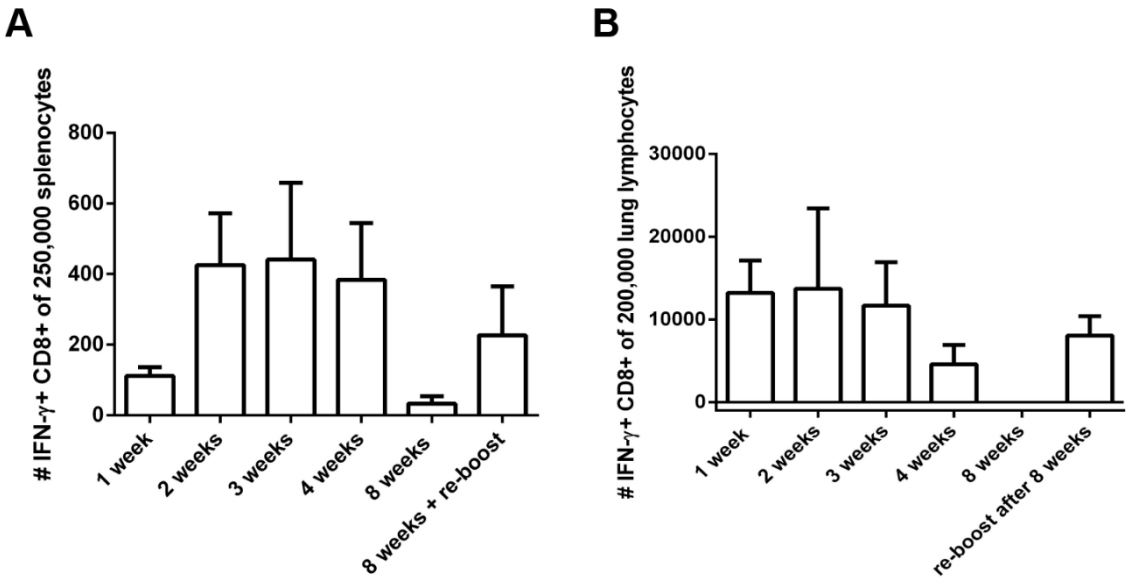

Figure 25. Subcutaneous priming and intranasal boosting with PLGA-MS induces sustained immune responses in spleen and lung

(**A/B**) For priming, AAD mice were s.c. immunized with M1/CpG MS (n=3) MS and polyI:C MS. Boost and re-boost i.n. immunizations were performed with 2.5mg M1/CpG MS. 1, 2, 3, 4 and 8 weeks after the boost immunization, and 6 d after the re-boost (week 9) splenocytes (A) and lung cells (B) were isolated and analyzed for IFN-γ production after peptide restimulation by ICS. Background levels (no peptide) were substracted. Values (mean ± SEM) are given in (A) as absolut number of IFN-γ^+ $CD8^+$ lymphocytes per 2.5 x 10^5 counted splenocytes and in (B) as absolut number of IFN-γ^+ $CD8^+$ cells per 2 x 10^5 lung leukocytes.

3. Discussion

Recombinant expression of PCa antigens

Soluble protein is a prerequisite for encapsulation into PLGA MS, but also one of the major challenges as protein solutions have to be highly concentrated for encapsulation. Preparations enriched of a specific protein are rarely easily obtained from natural host cells. In contrast, whole tumor lysates have the advantage of simultaneously inducing immune responses against multiple TAAs. However, autologous tumor lysate is only available after recurrence of the disease and the tissue has to be stored in the meantime. In addition, measurement of effector T cell responses to maybe even unknown TAAs is difficult to achieve [407]. Additionally, tumor lysates contain predominantly normal self-proteins that are not beneficial for the immunotherapy or even contain material that potentially induces further malignancy. Administration of TAAs is therefore more desirable than crude tumor preparations [138]. Despite a great number of recombinant protein expression systems, major obstacles, especially solubility problems, are encountered in the expression of many proteins.

There is no universal strategy to improve the level of expression or to solve partial or non-expression, but several strategies can be tested like changing the vector, changing the host or the expression strain, changing the culture parameters of the recombinant host strain such as reduced temperature or induction conditions, co-expression of other genes such as molecular chaperones, folding modulators or solubility-enhancing fusion-tags and changing the gene sequences, which may help increase expression and the proper folding of desired protein [408, 409].

There are many hosts used for the production of recombinant protein but the most prevalent one that is used is *E. coli* due to its easier culture, short life cycle, well-known genetics, and easy genetic manipulation. The baculovirus expression system in contrast provides a eukaryotic environment capable of performing proper folding, disulfide bond formation, oligomerization and post-translational modification such as signal- and proteolytic cleavage, N- and O-glycosylation, amidation, phosphorylation, prenylation and carboxymethylation [410].

However, no efficient recombinant expression of the four PCa antigens PSA, PSCA, PAP and TRPM8 could be achieved neither in *Pichia pastoris* or human embryonic kidney (HEK) cells (A. Aichem, BITG, Swiss, unpublished data), nor with baculovirus (Fig. 6) or *E. coli* (Fig. 7) expression systems. All the above mentioned strategies to improve protein expression were tested, like changing the vector from transfer vector pAcGHLT-B to pAcGHLT-C in the baculovirus system. For *E.coli*, different expression strains were

examined. To exclude toxic effects of the recombinant protein on *E.coli*, BL21(DE3)pLysS strain, which suppresses basal expression was employed, as well as strain BL21(DE3)RIPL coding for rare tRNAs that could otherwise interrupt transcription. Additionally, different temperatures, lower MOI or IPTG concentrations for induction and varying expression time periods were tested (data not shown). However, none of these strategies nor co-expression with the solubility-enhancing 6xHis-SUMO or 6xHis-GST-fusion tags (Fig. 7) resulted in higher solubility.

Furthermore, we tested a two step protocol during which protein biosynthesis was inhibited with chloramphenicol to permit chaperone mediated refolding of misfolded and aggregated proteins *in vivo.* This chaperone-based procedure was earlier reported to increase yields of soluble proteins produced in *E.coli* [411], but did not improve solubility of the PCa antigens. The only fine-tuning that could still be done is codon optimization of the antigens for expression in *E.coli*. However, this attempt is not very promising, as usage of the BL21(DE3)RIPL strain resulted in no improvement.

As a high amount of protein is needed for encapsulation into PLGA MS (50 mg/g PLGA), purification under denaturating conditions and subsequent refolding was not efficient enough for our purpose (data not shown). The same problems were reported by other groups that tried to express PSA in mammalian cells, insect cells or *Pichia pastoris*, who also gained only low protein yields after refolding of the protein from inclusion bodies and multiple column chromatography steps [412-414]. Acevedo et al. and other groups even tried to isolate PSA from seminal plasma with limited success [415].

Expression and immunogenicity of PCa long peptide antigens

Long peptide vaccines have evolved as a simple solution for the many problems that have surfaced with the expression of whole protein vaccines. These long peptides can include single or multiple CTL epitopes, but also T helper cell epitopes. The T helper cell activation can directly induce proliferation of CTLs. Additionally APCs are activated that subsequently license CTLs and control the induction of T memory cells [416]. Overlapping long peptides of the oncogenic proteins of HPV16 were already successfully tested for vaccination therapy of patients with advanced or recurrent HPV16-induced gynecological carcinoma in a phase II clinical trial [417]. Expression of GST-tagged PCa long peptide antigens in E.coli and subsequent purification with GSTrap columns yielded in high protein amounts and high purity (Fig. 8).

However, vaccination of AAD mice with PLGA MS containing the GST-long (250 µg/mouse) peptides and CpG ODN in combination with polyI:C MS induced no measureable CTL response (data not shown). Induction of robust therapeutic T cell responses requires long-lived presentation of antigenic epitopes by MHC class I

molecules on fully activated DCs. PLGA MS were shown to fully activate DCs via release of the encapsulated TLR ligands and to efficiently cross-present the encapsulated antigens on MHC class I in a sustained manner [418]. Prolonged presentation is also mediated by the long peptides themselves, as they require processing into small epitopes of 8-10 amino acids before presentation. On the other hand, this required processing can also be a disadvantage if it is not accurate due to tissue-specific processing or altered cleavage by tumor cells [138].

To rule out that processing was perturbed by the GST tag, chemically synthesized long peptides were encapsulated into PLGA MS and tested in the AAD mice system, but neither induced a specific CTL response (data not shown). However, immunogenicity is not only triggered by molecular recognition but also by extrinsic factors such as individual gene repertoire, self-tolerance and a variety of cellular regulatory mechanisms [419]. Still, inaccurate processing of the long peptides could not be ruled out despite using directly the CTL epitopes for encapsulation into PLGA MS and subsequent immunization of AAD mice.

PLGA MS based tumor antigen peptide vaccination against PCa

T-cell based immunotherapy of cancer is a promising non-invasive option for treatment of minimal residual disease, prevention of metastatic spread and delay of recurrence without compromising quality of life. One of the major obstacles when not using tumor lysate or whole proteins is the identification of possible antigenic peptide epitopes that are capable of initiating effective antitumor T-cell responses. Ideal target epitopes are exclusively expressed in nonvital tissues, highly expressed in metastatic disease and accessible to therapeutic modalities [420].

All eight PCa epitopes used in this study scored very well for *in silico* predictions of HLA-A*0201 binding. Positive prognosis for their potential as antigenic epitopes by ‘reverse immunology’ approaches was confirmed by the characterization of the HLA-A*0201 binding properties using the HLA-A*0201 positive human T2 cell line (Fig. 10). However, both the *in silico* prediction and the usage of the human TAP-deficient T2 cell line only determine the HLA-A*0201 binding characteristics. Neither the transport of the peptides to the ER nor TAP binding and translocation efficiency are taken into account in these assays, although they were described as being relevant factors for the immunostimulatory ability of peptide antigens [421]

The efficacy of tumor immunotherapy is highly dependent on breaking the tolerance of the specific $CD8^+$ T cell repertoire against the non-mutated self-tumor antigens [422]. Furthermore, cancer often coincidences with an immunosuppressive microenvironment within and around the tumor, characterized by the prevalence of immunosuppressive cytokines and high infiltration of regulatory T cells (Treg) [423, 424]. TLR agonists are

being widely used to break immunological tolerance towards autologous TAAs and activate anti-cancer immune responses [425, 426].

The utilization of CpG ODN and polyI:C in our immunizations generates a strong Th1 polarizing condition, which in combination with the usage of a peptide exclusively presented on HLA-A*0201 to $CD8^+$ cytotoxic T cells, counteracts an unwanted Treg induction. Stimulation of TLR3 and TLR9 with polyI:C and CpG ODN on human natural killer cells, respectively, induces their release of IFN-γ and TNF-α and up-regulation of their cytolytic activity against tumor cells [427]. Additionally, polyI:C was shown to prolong survival of tumor-bearing rodents [428]. This effect is mediated by an increase in the proliferation and function of $CD8^+$ T cells during the primary response and an enhanced memory $CD8^+$ T cell response leading to antitumor immunity [429]. Furthermore, CpG ODN were identified as effective adjuvants in tumor antigen immunization with comparable protection from tumor challenge but less toxicity than IFA [430].

In the last decade, much work has been devoted to the investigation of DC based immunotherapies. However, cultivation and antigen pulse of human DC *in vitro* and injection of these cells into patients is a very labor and cost intensive approach, which yielded in largely disappointing results [165, 431-433]. The ideal tool for the improvement of DC based immunotherapies is the usage of PLGA MS as antigen delivery system. PLGA MS are rapidly and efficiently taken up by immature DCs *in vitro* and *in vivo* and release the encapsulated contents intracellularly in a sustained manner for processing and presentation on MHC class I and II molecules [122, 418, 434]. In addition, encapsulation of protein antigens and tumor lysate into PLGA MS have already been proven to be efficacious in inducing anti-tumor immune responses that led to eradication of the tumors [5, 6].

All PCa tumor antigens used in this study could be efficiently encapsulated into PLGA MS and were released during incubation in an aqueous environment (Fig. 9). In addition, the peptides were not harmed by the spray-drying process, as only one single band was visible after release of the peptides from the PLGA MS. Another antigen delivery system that has been used in numerous studies is incomplete Freund´s adjuvant (IFA), a water-in-oil emulsion that can be mixed with antigens and pattern molecules. However, IFA has - in contrast to PLGA MS - some severe side effects like local skin reactions, inflammations, sterile abscesses and cysts as well as persistent painful granulomas at the site of injection [435].

In our study, we showed that PLGA MS were even superior to IFA-based vaccination with $STEAP1_{262\text{-}270}$ peptide antigen and the TLR ligands CpG ODN and polyI:C in HLA-

A*0201 transgenic AAD mice (Fig. 11). Previously, our group could already demonstrate the superior ability of PLGA MS in comparison to IFA in eradication of preexisting tumors and suppression of lung metastases in a mouse model [6]. Unexpectedly, for all the other PCa antigen epitopes tested in this study neither PLGA MS nor IFA were able to induce peptide-specific CTLs. This result cannot be attributed to differences in the quality or purity of the used peptides as their identity was confirmed by mass spectrometry and their purity by HPLC analysis (data not shown). Moreover, only the $PSA_{154-163}$ peptide contains a cysteine which can be oxidized over time and hence become less immunogenic (Tab. 1). The immunogenic $STEAP1_{262-270}$ peptide has a high hydropathicity score (listed in Tab. 1) but so does $PSMA_{27-35}$ which did not yield a CTL response in AAD mice. Yu and colleagues suggested that poor immunogenicity of self/tumor antigens derives from peptide/MHC-I instability rather than from tolerance [436], which we

Table 1. Properties of the PCa Epitopes. Comparison of the human PCa epitopes with their murine homologues. Differences are marked with capital letters. The grand average hydropathicity was calculated as sum of the hydropathy values of all amino acids based on the Kyte-Doolittle scale.

	Murine epitope	Human epitope	Grand average of hydropathicity
$PSA_{154-163}$	IisndvcNqv *	visndvcaqv	1.010
$PSCA_{14-22}$	alHpgAalQ	alqpgtall	0.978
$PAP_{112-120}$	tlmsamtnl	tlmsamtnl	0.833
$TRPM8_{187-195}$	glmkyigev	glmkyigev	0.544
$PSMA_{27-35}$	vlaLTGTFl	vlaggffll	2.467
$Survivin_{96-104}$	ltVSeflkl	ltlgeflkl	1.056
$STEAP1_{86-94}$	flytllreI	flytllrev	0.933
$STEAP1_{262-270}$	lllgtVhal	lllgtihal	1.911
	* transmembrane protease serine 11G (highest homology to human PSA)		

consider unlikely for these antigens as they all provide good HLA-A*0201 binding and stabilization characteristics (Fig. 10).

In contrast to our observation, $PSCA_{14-22}$ and $PSA_{154-163}$ loaded DCs were shown to induce immune responses and prolong overall survival in patients with hormone and chemotherapy-refractory disease in a phase I/II trial [220]. In concordance with this result, Feyerabend *et al.* were able to increase PSA doubling time in hormone-sensitive PCa patients with biochemical recurrence after primary surgical treatment by vaccination with a cocktail of fourteen PCa antigen epitopes emulsified in montanide combined with

imiquimod, GM-CSF, mucin-1-mRNA/protamine complex, local hyperthermia or no adjuvant. This cocktail included amongst others $PSA_{154-163}$, $PSCA_{14-22}$, $Survivin_{96-104}$, $TRPM8_{187-195}$ and two additional MHC class II restricted T helper cell epitopes [437].
In contrast to our study, Alves *et al*. reported that both peptides, $STEAP1_{86-94}$ and $STEAP1_{262-270}$ were immunogenic *in vivo* after emulsification in IFA and vaccination of HLA-A*0201 transgenic HHD mice [266]. In our study, $STEAP1_{86-94}$ induced neither encapsulated into PLGA MS nor emulsified in IFA a peptide-specific CTL response. Furthermore, $TRPM8_{187-195}$ and $PSMA_{27-35}$ specific CTLs generated from human blood were able to lyse LNCaP tumor cells *in vitro* [230, 246]. Specific T cell reactivity for $Survivin_{96-104}$ could be detected in leukemia and melanoma patients [278]. However, none of this peptides encapsulated into PLGA MS induced a CTL response in the AAD mouse system.

As some of the antigens like $PAP_{112-120}$ display complete and others like $PSMA_{27-35}$ only minor conservation between human and mouse sequences neither the induction of xeno-CTLs nor self-tolerance seems to play a crucial role for the immunogenicity of the peptides (Tab. 1). The only immunogenic epitope $STEAP1_{262-270}$ differs in a single conserved amino acid between the murine epitope (LLLGTVHAL) and its human homologue (LLLGTIHAL) used in this study. CTLs raised against human $STEAP1_{262-270}$ have even been shown to cross-react with mouse $STEAP1_{262-270}$ [264]. This suggests that a reduced tolerance induction against the human $STEAP1_{262-270}$ epitope in AAD mice cannot explain the superior CTL response to this peptide (Fig. 11). One could argue that the exclusive response to $STEAP1_{262-270}$ in AAD mice is due to a much higher CTL precursor frequency in the T cell repertoire of these mice. While a bias in the T cell repertoire can certainly contribute to this phenomenon, it seems unlikely that CTL precursors specific for seven other PCa epitopes are lacking in AAD mice given that they react to numerous other HLA-A*0201 epitopes [405].
Since the T cell repertoire in humans likely varies among individuals this parameter cannot easily be taken into consideration. *In vitro* generation of CTL clones specific for the eight PCa epitopes from blood of different HLA-A*0201 positive donors would be the only possibility to address this issue. Of note, the CTL response to the $STEAP1_{262-270}$ epitope was HLA-A*0201 specific as no immune response was detectable after immunization of C57BL/6 mice (data not shown). Vaccination with peptide epitopes obviously excludes the possibility of differences in antigen processing efficiencies, too. Differences in TAP binding and translocation efficiencies cannot be ruled out though.

Machlenkin and coworkers previously reported on the CTL inducing capability of a cell based prime-boost vaccination with $STEAP1_{262-270}$ peptide-pulsed DCs in HLA-A2.1/H-$2D^b$-$\beta_2$microglobulin monochain transgenic HHD mice *in vivo* and peptide induced CTL activity *in vitro* [264]. In agreement with these results we were able to monitor *in vivo* cytolytic activity of the peptide specific CTLs, but already after a single vaccination with peptide-loaded PLGA MS (Fig. 12). The $STEAP1_{262-270}$ response elicited by MS was so strong, that its detection by IFN-γ ICS or ELISPOT did not require by *in vitro* CTL expansion. Previously, it was shown that a CTL line specific for a modified version of another epitope of STEAP1 ($STEAP1_{292-300}$) could recognize HLA-A*0201$^+$ and STEAP1$^+$ prostate cancer and other cell lines after *in vitro* restimulation [420]. We could show that $STEAP1_{262-270}$ specific CTLs, after a single immunization with PLGA MS containing the epitope, were not only able to recognize the epitope presented on HLA-A*0201 by T2 lymphoblastoid cells but also on human MoDCs that present the epitope after PLGA MS uptake. STEAP1 is an ideal target for cancer immunotherapy as it is strongly expressed in multiple tumor types like prostate cancer, melanoma, colon cancer and Ewing´s sarcoma, it has a restricted expression in normal tissues, especially at the cell surface [420]. Its localization at the cell-cell junctions of the secretory epithelium taken together with the six-transmembrane topology, suggests a function of STEAP1 as channel/transporter protein in cell-cell junctions and an involvement in tumor growth [259] which may render an elimination of STEAP1 from PCa cells less likely. In addition, STEAP1 is overexpressed in different stages and metastases of PCas. Immunotherapy targeting STEAP1 would therefore be beneficial over a long period of PCa progression including metastatic, hormone-insensitive stages, which is the major application for immunotherapies. Further improvement of the PLGA MS vaccination system could be reached by surface modification of the PLGA MS like conjugation with the DC specific antibodies anti-CD11c and anti-DEC205, which was shown to enhance the uptake of the PLGA MS by DCs, while modification of the surface with anti-CD40 and anti-Fcγ antibodies induces superior DC maturation [92].

Further experiments that should be taken into account for this project are for example the *in vitro* generation of CTL clones specific for the eight PCa epitopes from blood of different HLA-A*0201 positive donors. With this experiment, variances between the human and murine T cell repertoire could be addressed. In addition, the effectiveness of a vaccination with $STEAP1_{262-270}$ peptide encapsulated into PLGA MS in immunotherapy of PCa should be demonstrated either *in vitro* or *in vivo*. *In vivo*, AAD mice could be inoculated with a TRAMP cell line stably transfected with the AAD construct or by breeding of AAD mice with TRAMP mice. *In vitro*, the cytotoxic potential of CTLs induced

by vaccination with PLGA MS incorporating STEAP1$_{262-270}$ peptide could also be assessed by co-culture with the human LNCaP prostate adenocarcinoma cell line.

Vaccine design against melanoma with chemically modified CTL epitopes encapsulated into PLGA MS

Melanoma is one of the most immunogenic tumors with several described tumor antigens. However, despite feasibility and safety of cancer immunotherapy using CTL epitopes of cancer antigens, efficacy has mostly been limited. Immunogenicity of the peptides can be improved by modification of tumor antigens and the combination with adjuvants and vaccine delivery systems. Another disadvantage of the epitopes used for peptide vaccine is the missing or little tertiary structure, which makes them subject to rapid degradation by tissue and serum proteases [146].

There are several modifications reported to incorporate resistance against proteases. Steer *et al.* could show that already a single amino-acid substitution of a naturally occurring α-amino acid with the homologous β-amino acid has a great impact on the overall stability of the peptide [150]. Other methods used to achieve protease resistance include peptide backbone modifications, D-amino acids or retroinversion of sequences by converting all amino-acids to D-amino-acids and reversion of the sequence [138, 438]. Moreover, N-acetylation and C-amidation can also prevent degradation of the peptide by exopeptidases [151]. However, improvement of the biostability of the vaccine epitopes with these modifications often results in reduced MHC affinity and reduced immunogenicity.

Peptide vaccination can even induce CTL tolerance, which depends on type and length of the peptide as well as dose, route of administration or usage of one or multiple peptides [144]. As the peptides for vaccination originate from deregulated or mutated self-proteins and CTLs specific for these self-antigens escaped thymic selection and recognize only peptides with low MHC class I binding affinity. The resulting weak interaction between peptide/MHC complex and T cell receptor is not able to fully activate CTLs [145].

For full activation of the CTLs, the peptide has to be presented at sufficient levels but also has to display adequate MHC affinity. Stability of peptide-MHC complexes was shown to directly correlate to immunogenicity of the vaccine and therefore higher frequencies of specific CTLs [439]. Interaction of the epitope with the allele-specific peptide-binding cleft of the MHC molecule depends on the peptide´s shape, size and electrostatic complementarity with the MHC residues [440].

For the peptide, interaction with the MHC molecule is mediated by so called anchor residues which are located in close proximity to the N- and C-termini [441]. Substitution

of primary and secondary anchor residues with non-proteogenic amino-acids can improve MHC class I binding and enhance TCR activation.

For anchor residue P_1, substitution with aromatic amino acids in D-conformation were proven to be favorable for binding to HLA-A*0201. This substitution was applied for the design of TRP-2 mod1, for which the serine at P_1 was replaced by D-alpha-methyl-phenylglycine (Tab. 2). The improved binding affinity can be explained by the π-π and cation-π interactions of the aromatic ring in combination with the formation of a strong hydrogen bonding network between the NH_2 group and the HLA α-chain.

The HLA-A*0201 binding affinity can be further improved by substitution of anchor residue P_2. Hoppes *et al*. reported that replacement of the alanine at P_2 of MART 1_{26-35} by a leucine increased the HLA binding score from 18% to 58% [7]. MAGE-C2 mod1 and TRP-2 mod1 were also modified at the P_2 position with L-S-methyl-cysteine and L-2-aminoctanoic acid, respectively (Tab. 2). Improved HLA-A*0201 binding can be explained by more hydrophobic contact between the extended alkyl side-chains and the hydrophobic pocket of the HLA-A*0201 peptide binding cleft. Additionally, TRP-2 mod1 was C-terminally modified by substitution of the lysine by the unsaturated amino-acid L-Porpargylglyine on position P_C (Tab. 2) [7].

Other modifications aim at the improvement of the interaction with the TCRs of CTLs, which is mediated by the middle part of the peptide that extrudes out of the MHC binding groove [442]. Modifications in the TCR interacting region often result in analogues that can result in hyperstimulation of CTLs and can also break T cell tolerance, which can be explained by increased avidity and residence time of the TCR-peptide-MHC complex [443, 444]. On the other hand, usage of these modified epitopes can lead to T cell exhaustion and an abrogated TCR interaction [445].

Table 2. Natural and modified melanoma peptide antigens

	Natural peptide	**Modified peptide**
Mart-1_{26-35}	EAAGIGILTV	ELAGIGLTV
MAGE-$C2_{336-344}$	ALKDVEERV	[L-S-methyl-cysteine][L-2-aminoctanoic] KDVEERV
TRP-$2_{180-188}$	SVYDFFVWL	[D-alpha-methyl-phenylglycine] [L-norvaline]YDFFVW[L-Porpargylglycine]

There were no altered characteristics concerning encapsulation into PLGA MS observable comparing the natural HLA-A*0201-restricted melanoma antigen epitopes MAGE $C2_{336-244}$, MART1_{26-35} and TRP-$2_{180-188}$ to their modified analogues (Fig.14).

Determination of the HLA binding affinity of TRP-2 and TRP-2 mod1 by a fluorescence polarization assay was previously reported to be comparable. A difference was

detectable in terms of an extended CTL stimulation measurable after co-incubation of CTLs and human T2 cells pulsed with the epitope by pMHC multimer staining [7].
Vaccination of AAD mice with the natural TRP-$2_{180-188}$ epitope encapsulated into PLGA MS resulted in a robust induction of peptide-specific CTLs. This CTL response was even more potent after immunization with PLGA MS incorporating TRP-2 mod1, but only when restimulated with the modified peptide. In contrast, CTLs that have been induced with TRP-2 mod1 MS were not able to recognize the natural peptide anymore (Fig. 14). As the TRP-2 peptide was modified with three big substitutions including an aromatic amino acid, an extended alkyl side-chain and an unsaturated amino-acid, the peptide may have undergone conformational changes that impede recognition by the TRP-2 specific CTLs. Small structural changes in peptides presented by MHC molecules often result in large changes in immunogenicity, indicating that T cell receptors are exquisitely sensitive to antigen structure. Studies of human CTL responses to natural viral antigens show that immunodominant $CD8^+$ T cells are finely restricted in epitope specificity [446]. Lee *et al.* even demonstrated that single amino acid changes applied to a HIV protein peptide sequence, abolished functional recognition for both specific T cell clones and for uncloned T cell responses in the blood of different HIV-infected patients [447]. Specific antigen recognition is a crucial factor in cell mediated immunity, as it leads to activation and expansion of mature T cells responding with precise and adapted immune responses. Cross-reactivity of vaccine-boosted T cells can be both, advantageous and disadvantageous according to the situation.
On the one hand, cross-reactive T cells are able to recognize multiple viral variants and broaden the ability to recognize the infinite variety of antigens with a relatively limited number of distinct specificities in the T cell repertoire [448]. On the other hand, cross-reactivity of T cells can lead to undesirable auto- reactivity [449].
As the AAD mice are constantly back-crossed with C57BL/6 wildtype mice, they contain both H2-D^d and HLA-A*0201 MHC class I epitopes. Vaccination of C57BL/6 mice with TRP-2 or TRP-2 mod1 encapsulated into PLGA MS induced even higher immune responses in the wildtype mice as compared to the AAD mice. The enhanced immune response to the modified peptide can therefore not clearly be attributed to improved binding to the HLA-A*0201 molecule (Fig. 15C/D).

A study by Appay *et al*. elucidated the cross-reactivity of CTLs after peptide vaccination. Therefore, melanoma patients were immunized with the Mart1 mod2 epitope emulsified in IFA together with CpG ODN, which was shown to induce expansion of MART1 mod2 specific $CD8^+$ T cells *in vivo*. *Ex vivo* stimulation of PBMCs with a collection of predicted MART1 cross-reactive peptides showed that MART1 mod2 reactive $CD8^+$ T cells are

usually constituted of widely cross-reactive naïve cells prior to vaccination. Peptide vaccination though resulted in expansion of memory T cells whose reactivity was predominantly restricted to Mart1 mod2, probably due to selective activation from the pool of precursors [450]. In addition, vaccination of patients with Mart1 mod2 generally displayed a stronger reactivity for the heteroclitic modified peptide than for the native $Mart1_{26-35}$ peptide [343].

This could be confirmed by vaccination of AAD mice with either the native $Mart1_{26-35}$ peptide or Mart1 mod2 encapsulated into PLGA MS. While no CTL induction was observable for the natural peptide, CTL responses were observable after vaccination with PLGA MS containing the heteroclitic analogue. *Ex vivo* restimulation with the natural peptide induced reduced IFN-γ secretion by the specific CTLs as restimulation with the heteroclitic peptide (Fig. 17A/B).

Other studies already demonstrated that this inability to recognize anchor-modified variants can already occur with very subtle structural differences [451]. Differential T cell recognition of native and modified MART1 peptide was also reported to be influenced by peptide-dependent MHC dynamics, which can impact TCR recognition by altering the barriers for conformational adjustments, influencing the entropic costs for receptor binding and shifting the populations of binding-competent states. Varying flexibility in the MHC molecules due to binding of heteroclitic peptides therefore has implications for specificity, cross-reactivity and antigenicity in cellular immunity [452]. In contrast to the lacking cross-reactivity of the CTLs induced by vaccination with the modified MART1 peptide encapsulated into PLGA MS, immunization with PLGA MS incorporating the modified MAGE C2 epitope induced CTLs that could be restimulated with both the natural and the modified peptide (Fig. 16A/B). The improved immunogenicity of both, MART1 mod2 and MAGE C2 mod1 can clearly be attributed to the better HLA-A*0201 binding characteristics, as none of the modified peptides encapsulated into PLGA MS elicited immune responses in C57BL/6 mice (Fig. 16C/D; 17C/D).

Stuge *et al.* raised the question whether the factors that shape the peptide-specific T cell repertoire after vaccination may be different from those that affect the endogenous response [453]. As the presence of tumor antigen specific CTLs elicited after vaccination often does not correlate with clinical responses, it was also shown by other groups that vaccine-elicited CTLs respond to native peptide with predominantly low recognition efficiency. Additionally, these CTLs were diverse in T cell receptor variable chain beta expression. One hypothesis was that vaccination with heteroclitic peptides results in high antigen densities on APCs that could drive CTLs of low recognition efficiency and interferes with elicitation of high recognition-efficiency CTLs either by direct competition for antigen on APC surface or down-modulation of peptide/MHC complexes [454].

Furthermore, CTL populations induced by vaccination were shown to be significantly different from endogenous responses in terms of the ability to lyse tumor cells. While nearly all CTL clones from an endogenous response were efficient at melanoma cell lysis, most CTLs elicited by vaccination were inefficient in lysis or required up to 1000-fold higher peptide concentrations for similar levels of lysis [453]. This induction of non-lytic CTL clones by heteroclitic peptide vaccines resulting from differential recognition of native and heteroclitic peptides by many T cells was also reported by other groups [455, 456].

In fact, the same lack of functionality following vaccination with heteroclitic peptides encapsulated into PLGA MS was observed by us. While vaccination with MAGE mod1 and MART mod2 peptides encapsulated into PLGA MS induced CTLs that were cross-reactive with the natural epitopes, no specific lysis of target cells pulsed with the natural peptides was measurable (Fig. 16D/17D). This points towards a substantially different requirement of antigen specific CTLs for cognate peptides for efficient target lysis [457]. However, reduced HLA-A*0201 binding capability of the natural peptide compared to the modified peptide and therefore a decreased stability of the MHC class I/peptide complex on the surface of the target cells could be another reason for the loss of functionality during the *in vivo* cytotoxicity assay. A chromium release assay that requires only five hours of antigen presentation instead of 24 hours for the *in vivo* cytotoxicity assay could lead to clarification of this issue. Additionally, a B16 melanoma cell line could be stably transfected with both, human MART1 and the AAD construct. Inoculation of AAD mice with this cell line, would be an opportunity to examine the cytotoxic potential of the CTLs induced after vaccination with MART1 mod2 peptide encapsulated into PLGA MS *in vivo*.

Induction of a broadly reactive immune response against influenza A viruses

The most pivotal task in the fight against a recurrence of an influenza virus pandemic is the development of a vaccine that induces heterosubtypic protection against all influenza A viruses. Heterosubtypic immunity is mediated by serotype cross-reactive CTLs specific for conserved internal proteins like the matrix protein [458, 459]. Induction of CTL responses in contrast to antibody responses is indispensable for the suppression of initial viral replication in the respiratory epithelium [371] and furthermore can provide partial [460, 461] or complete protection of mice from infection with heterosubtypic influenza viruses [462, 463]. CTLs induce apoptosis of virus infected cells after recognizing viral peptides bound to MHC class I molecules and secrete antiviral cytokines, notably IFN-γ TNF [464]. Following natural influenza A virus infection the CTL response of HLA-

A*0201^{+} individuals (i.e. approx. 50% of the caucasian population) is exquisitely focused on the immunodominant $M1_{58-66}$ peptide [465, 466].

In our study, we investigated the potency of PLGA MS-based vaccination with $M1_{58-66}$ peptide. We could show that PLGA MS containing $M1_{58-66}$ peptide and the TLR ligand CpG mixed with polyI:C MS were capable of inducing robust CTL responses in HLA-A*0201 transgenic mice after a single subcutaneous vaccination without the need to induce antigen-specific T helper cells (Fig. 18A/B). This CTL response was clearly HLA-A*0201 restricted as no response was detectable after vaccination of C57BL/6 mice (Fig. 18C). Peptide vaccines provide several advantages over the currently used inactivated viral vaccines. Peptides can be easily produced in large scale and bear no risk of infectious particles. In addition, PLGA MS protect the encapsulated peptides from proteolytic degradation, thus a lower antigen dose is required. In our experiments, the maximum CTL response was reached after vaccination with PLGA MS containing 50 µg peptide per mouse (Fig. 19A/B). It was shown before that an increase of the peptide dose leads to an increased number of antigen-specific T cells in the primary response [467, 468]. However, peptide dose also influences the quality of the T cell response in terms of functional avidity, as high peptide concentrations result in apoptosis of high avidity T cells via a TNF-α mediated mechanism [469]. Low peptide concentrations polarized CD8^{+} T cells towards high avidity populations that were more efficacious in their ability to reduce viral infection [470]. In contrast, low avidity CTLs induced by high peptide concentrations were able to prevent outgrowth of murine tumors by recognition of high antigen levels on tumor cells while ignoring lower levels of the same antigen on non-cancerous tissue [471].

Furthermore, the use of PLGA MS as antigen delivery device provides a depot effect and therefore a prolonged antigen release [124, 165]. This leads to a CTL response that peaks on day 6 and reaches even beyond day 35 after immunization (Fig. 19C). The promptitude of the CTL response is of special interest, as early viral clearance after influence A virus infection is required for both, significant reduction in the severity of symptoms and the prevention of lethal viral pneumonia [472, 473]. Direct pulsing of DCs with MHC class I restricted influenza virus epitopes without usage of a vaccine delivery system was reported to induce potent antiviral CTL responses though leading to a delayed clearance of an influenza virus challenge [386]. Another advantage of peptide vaccines is the fact that they are flexible in their design and can therefore contain more than one antigen, which increases the clonality of the elicited T cell response [132]. As immune escape by antigenic drift is a threat to the potency of a vaccination against

influenza virus, it is advantageous to use such a multi-epitope vaccine. The only limitation, however, is the number of different peptides that can be encapsulated into one microsphere. Co-encapsulation of the influenza epitopes $M1_{58-66}$ and PA_{46-54} though led to an equally strong CTL response as single encapsulation of each peptide (Fig. 20C). Ichihashi *et al*. previously reported, that three influenza antigen peptides need to be co-conjugated in liposomes in the presence of CpG ODN to get CTL responses. In contrast, we could show that both, $M1_{58-66}$ and PA_{46-54} single encapsulated into PLGA MS with CpG ODN already induced robust CTL responses without the need of an additional antigen peptide (Fig 18A/B; 20A/B) [387].

Being dependent on CTL activity, protection against vaccinia virus is a good model to study vaccination efficiency. In fact, we were not only able to show an effective CTL induction by measuring the IFN-γ release of antigen specific $CD8^+$ splenocytes but we could also evidence their functionality in viral protection after systemic vaccination with PLGA MS bearing the viral epitope $M1_{58-66}$. However, systemic immunization with $M1_{58-66}$ containing PLGA MS did neither ease the symptoms nor lead to an extended survival after infection with influenza A virus.

It was previously reported that systemic administration of vaccines generally fails to induce mucosal immunity. In contrast, mucosal immunizations have the ability to induce both local and systemic immunity and therefore induce local long-term immunological memory at the site of entry [474]. In addition, s.c. immunization does not lead to an instant elimination of the infected airway epithelial cells by the induced CTLs even though i.v. injected target cells are destroyed rapidly [387]. Intranasal immunization with PLGA MS is a feasible approach, as PLGA was shown to be not toxic, neither for cilia nor for mucosal epithelial cells, and therefore likely does not damage the airway mucosa [475, 476]. Furthermore, i.n. administration of antigens incorporated in nanoparticles composed of PLGA led to over 100 fold increased antibody responses in comparison with aqueous solution of parainfluenza virus proteins in mice [477]. Amidi *et al*. already demonstrated that i.n. vaccination with N-trimethyl chitosan nanoparticles loaded with an influenza subunit vaccine resulted in higher IgG responses as compared to intramuscular (i.m.) administration [478].

However, i.n. administration of $M1_{58-66}$ microspheres neither induced specific CTLs in the spleen nor in the lung of the immunized mice. In contrast, Wells *et al.* reported that transferred lymphocytes from previously infected mice migrated to the lungs of the host mice during influenza virus infection and led to an earlier and greater reduction in pulmonary virus titer. On the other hand, transfer of naive lymphocytes to infected mice resulted in a very late reduction of the pulmonary virus titer on day 21 [472]. Additionally,

a systemic prime was needed prior to mucosal immunization with microparticles containing simian immunodeficiency virus (SIV) to induce protective immunity in macaques challenged with SIV [479].

A systemic prime vaccination could also contribute to the establishment of long-term memory as it was shown before that the memory T cell population in the lung airways is dynamic and depends on a systemic source of T cells that is continually recruited from the circulation. This implicates a significant connection of memory T cell pools in the secondary lymphoid organs and those in peripheral tissues [480]. In fact, systemic prime vaccination and intranasal boost vaccination resulted in a prominent CTL infiltration of the lung including a significantly higher number of $M1_{58-66}$ specific CTLs (Fig. 23B). Vaccination with the two well-defined and conserved virus epitopes $M1_{58-66}$ and PA_{46-54} encapsulated into clinically compatible PLGA MS contributed to the control of influenza A virus infections and interferes with virus replication, weight loss, and infection related death (Fig. 24).

As i.n. vaccination does not require injection it is less painful and has therefore a high patient compliance. Application would also not require trained medical personnel, which is a great benefit in mass vaccination programs or in countries with poor health care. Interestingly, neither a single i.n. immunization nor a single s.c. vaccination was sufficient to induce a robust CTL response in the lung (Fig. 23B). These results indicate that both, mucosal and systemic immunity are needed to induce protective immunity in the lung. Single s.c. immunization might not be sufficient, because injected vaccines are generally poor inducers of mucosal immunity. Single i.n. vaccination alone on the other hand could induce tolerance as mucosal surfaces are adapted to the presence of foreign microorganisms. In addition, an inflammatory stimulus in terms of an adjuvants boost after a peptide specific prime vaccination also does not lead to a cross-protective immunity through migration of antigen-specific cells from the lymphoid organs to the lung mucosa.

Ninomiya *et al.* even stated that both MHC class I and class II are necessary after vaccination with a MHC class I restricted influenza virus epitope encapsulated in liposomes and anti-CD40 mAb to induce protective immunity against influenza A virus in mice [481]. However, protection against influenza A virus infection through humoral and cell-mediated immunity was also reported after s.c. or i.p. immunization of mice with HA split-product virus vaccines encapsulated into poly(gamma-glutamic acid)-graft-L-phenylalanine nanoparticles [388].

The usage of CpG ODN and polyI:C as adjuvants activates innate signaling pathways and helps the vaccine to be distinguished from commensal bacteria. Furthermore, these

two TLR ligands generate a strong Th1 polarizing condition, minimizing the probability of unwanted tolerance induction. PLGA MS themselves also help to circumvent tolerance as they are specifically taken up by professional antigen presenting cells. A study by Maroof *et al*. showed, that i.n. vaccination with a TLR4 agonist and a detergent split-influenza antigen promotes antigen-specific antibody responses but also polyfunctional antigen-specific Th17 cells leading to an increased weight loss and morbidity during early stages of disease [482]. The utilization of CpG ODN and polyI:C in our vaccination protocol, in contrast, generates a strong Th1 polarizing condition, which in combination with the usage of a peptide exclusively presented on HLA-A*0201 to $CD8^+$ cytotoxic T cells, excludes the induction of an unwanted Th17 cell mediated, vaccine-induced IL-17 response.

Consistently, no increased weight loss or morbidity was observable for our vaccination (Fig. 24). Similarly, Ichihashi *et al.* already could demonstrate that immunization of HLA-A*2420 transgenic mice with liposome-conjugated HLA-A*2420 restricted influenza virus peptides in the presence of CpG-ODN protected from lethal influenza virus challenge. In contrast to our study, a single peptide was not able to mediate this effect, but three epitopes were necessary to induce protection [387].

Additionally, CpG ODN were shown to provide assistance for a whole inactivated influenza virus vaccine after i.n. administration in recruiting DCs to the nasal epithelium and forming trans-epithelial dendrites to capture luminal viruses [483]. This formation of trans-epithelial dendrites might also be beneficial for the uptake of PLGA MS by DCs of the airways. Furthermore, influenza infection and or i.n. administration of vaccine with polyI:C results in up-regulation of TLR3 but not TLR4 expression in the NALT. Another reason for the usage of CpG ODN as adjuvant is the reported enhancement of viral immunization effects in aged mice, as influenza A virus infection is especially harmful for elder people [484].

The combination of vaccine and polyI:C mimics the state of viral infection and induces protective immune responses including the expression of IFN-α/-β and Th1 and Th2 related cytokines such as IFN-γ, IL-12, p40 and IL-4. Moreover, safety of polyI:C to the central nervous system was demonstrated by direct intracerebral injection [485]. McNally et al. demonstrated that intranasal administration of polyI:C induces local Th1 chemokine production in the lungs and airways, which generates a biphasic and sustained migration of T lymphocytes to the airways and stimulates low levels of T cell proliferation in the draining lymph nodes approximately four to six days after treatment [486]. Especially in elder mice, intervention of the anti-inflammatory milieu by i.n. administration of polyI:C or CpG ODN reduced the mortality after influenza A virus infection [487].

With our prime/boost vaccination scheme we were able to successfully perform the first step of a potent vaccination, namely the conversion of naïve T cells to effector CTLs. The heart of a long-lasting protection, however, is the induction of memory T cells that can fast and efficiently fight recurrent infections. In the majority of healthy individuals, $CD4^+$ and $CD8^+$ memory T cell populations can be observed that are broadly cross-reactive to the internal proteins M1 and NA of various influenza A strains [488]. For reactivation, T memory cells need to localize to the draining lymph nodes near the infection side and interact with virus-laden bone-marrow derived DCs. Reactivated memory T cells migrate to the lung and mediate protection by acceleration of the clearance in the lung [489, 490].

However, the contributions of different $CD8^+$ memory T cell subsets, that are heterogenous in terms of phenotype, function and anatomical distribution, to recall responses at mucosal sites after recurrent infection or infection after vaccination are not fully understood so far. It was proposed that effector–memory T cells that accumulate in peripheral sites such as the lung mediate early and nonsustained responses to the influenza virus challenge, whereas central–memory cells in secondary lymphoid organs mediate a late but sustained proliferative response to the challenge [491].

Application of the prime-boost vaccination scheme induced $CD8^+$ T memory cells both in the spleen and the lung (Fig. 25A/B). Which subset of $CD8^+$ T memory cells mediated this memory still has to be investigated. In the lung, the CTL response remained stable over three weeks and declined then. The CTL response could be reactivated by another i.n. boost vaccination. The reactivation was measured 6 days after re-boost (Fig. 25B). As there are also reports that influenza specific $CD8^+$ T memory cells are in a rather activated state and can display effector function within 6 h of antigen contact [492], shorter time points for analysis of reactivation could be considered. Additionally, there are reports suggesting an inverse correlation between activation status and proliferative potential, such that cells with the most rested phenotype have the capacity to mediate the strongest recall responses [491].

Indeed, the response measured on day 6 after re-boost was not as prominent as one week after the first boost vaccination. In the spleen, the CTL response increased in the first two weeks after immunization, remained stable until week four and could be reactivated with another i.n. boost (Fig.25A). In the spleen, the CTL response on day 6 after re-boost was higher compared to the response one week after the first boost. This points to an effector response that starts in the lung epithelium, expands to a systemic response and is being converted into a memory response that can rapidly be reactivated in the secondary lymphoid organs.

Eyles *et al*. revealed that i.n. vaccination of mice with a volume of 50 µl, almost half of the vaccine was found in the lung, around one third ended up in the gastrointestinal tract and the rest remained in the nasal passages [493]. With regard to the common mucosal immune system, it would be interesting to monitor uptake of the PLGA MS after i.n. vaccination e.g. by using PLGA MS containing quantum dots. The NALT has already been assumed to be the site of uptake of microparticles from the nasal cavity [474]. In the lung, PLGA MS were shown to be taken up by alveolar macrophages and deposit to the lung periphery [494]. Additionally, inhaled PLGA MS are believed to encounter the wide spread DC network that can be found throughout the conducting airways, lung interstitium, lung vasculature, pleura, and bronchial lymph nodes [495]. In the murine lung, there are at least five different subsets of DCs, namely resident DCs, plasmacytoid DCs, alveolar DCs, inflammatory DCs and interferon-producing killer DCs [496]. DC subsets in the human lung are less explored, but were shown to be composed of both pDCs and mDCs [497].
One possibility to enhance the pulmonary delivery by PLGA MS is the modification of the surface with chitosan. Retention of chitosan modified PLGA MS because of improved adhesion to the bronchial mucus and lung tissue leads to a sustained drug release, which is probably mediated by opening of the intracellular tight junctions [498].

Taken together, we have established a safe and powerful vaccination against influenza virus infection that interferes with virus replication, weight loss and infection related death in a mouse model, which very closely reflects the human situation. This applicable approach could help to foreclose pandemic outbreaks of influenza viruses, especially in under-developed countries. In further experiments, the DC subsets that take up the PLGA MS after i.n. immunization and their migration should be further characterized by usage of PLGA MS incorporating quantum dots. In addition, influenza specific $CD8^+$ T memory cells should be analyzed for their ontogeny, localization and activation status. Moreover, infection of mice vaccinated according to our prime-boost vaccination protocol with other influenza A virus strains would enable conclusions about the cross-protective potential of the induced CTLs.

Concluding remarks

In conclusion, PLGA microspheres represent an ideal vaccine delivery tool for immunotherapy of cancer and infectious diseases. For the broad field of application, the PLGA MS systems allows a range of variations and optimizations such as the dose, the way of application, vaccination schemes and surface modifications to maximize the elicited immune response. Additionally, they bear the advantage of simultaneous adjuvants delivery, to induce DC maturation and to break tolerance. The biggest

challenge remains the identification of highly specific and immunogenic antigens, especially as peptide modification was shown not to result in the expected advantages.

4. Material and Methods

4.1 Cloning of prostate carcinoma antigens

4.1.1 Cloning into Baculo virus expression vector

In order to express PCa antigens for encapsulation into PLGA MS, PSA, PAP, PSCA full length genes and the N-terminal, cytosolic part of TRPM8 corresponding amino acids 1 to 688, were cloned into Baculo virus transfer vector pAcGHLT-B (BD biosciences, Heidelberg, Germany). Templates were kindly provided by Dr. Annette Aichem (BITg, Switzerland). All PCa antigens were PCR amplified using KAPA HIFI^{TM} polymerase kit (Peqlab, Erlangen, Germany) according to manufacturer´s instructions (Tab. 5) in the PCR thermocycler T3 (Biometra, Göttingen, Germany). All primer pairs were synthesized by Microsynth AG (Balgach, Switzerland) (Tab. 4).

Table 3. Primer for cloning of PCa antigens into pAcGHLT-B transfer vector

Gene	Sense primer (5´→ 3´)	Antisense primer (5´→ 3´)
PSA	CCGCTCGAGGAATCCTG TCTCGGATTGTGGGAG	GGACACCATCGTGGCCA ACCCCTGAGGTACCCCG
PSCA	CCGCTCGAGGACTGCTG TGCTACTCCTGCAAAGC	CGCCAGCGGGGCCCAT GCCTGAGGTACCCCG
PAP	CCGCTCGAGGAAAGGAGT TGAAGTTTGTGACTTTGG	GGTACTGAGGACAGTAC AGATTAGGGTACCCCG
TRPM8	CCGCTCGAGGAATGTCCT TTCGGGCAGCCAGGCTC	GCAATGGTATGGAGAGATT TCCCGATGAGGTACCCCG

After purification of the DNA with the NucleoSpin® Gel and PCR Clean-up Kit (Machery-Nagel, Düren, Germany), PCR product and plasmid were digested using the restriction enzymes XhoI and KpnI (Fermentas, Schwerte, Germany) and subsequently purified again with the PCR Clean-up Kit. After dephosphorylation of the vector pAcGHLT-B with Antarctic phosphatase (NEB, Frankfurt, Germany), plasmid vector and insert DNA were ligated using T4 ligase (NEB) according to the manufacturer´s instructions.

Table 4. PCR program for PCa antigen cloning

Cycle	Temperature	Time	Step
1	94°C	5 min	Initial denaturation
10	94°C	30 s	Denaturation
	68°C	30 s	Primer annealing
	72°C	PSA/PSCA → 2 min PAP/TRPM8 → 4 min	Elongation
20	94°C	30 s	Denaturation
	50°C	30 s	Primer annealing
	72°C	PSA/PSCA → 2 min PAP/TRPM8 → 4 min	Elongation
1	72°C	8 min	Final elongation

4.1.2 Cloning into *E.coli* expression vector

For improvement of protein expression, quantity and solubility ER leader sequences were removed and PSA_{32-386}, PAP_{20-261}, $PSCA_{20-123}$ and $TRPM8_{1-688}$ were cloned into pET-His6-SUMO vector (Invitrogen, Darmstadt, Germany). All PCa antigens were PCR amplified using KAPA HIFI™ polymerase kit (Peqlab) according to manufacturer´s instructions (Tab. 7) in the PCR thermocycler T3 (Biometra). All primer pairs were synthesized by Microsynth AG (Tab. 6).

Table 5. Primer for cloning of PCa antigens lacking the ER leader sequence into pET-SUMO expression vector

Gene	Sense primer (5´→ 3´)	Antisense primer (5´→ 3´)
PSA	CCAGTGGGTCTCAGGTGGTA TCCTGTCTCGGATTGTGGG	CCGCTCGAGTCAGGGGT TGGCCACGATGGTGTCC
PSCA	CCAGTGGGTCTCAGGTGGTC TGCTGTGCTACTCCTGCAAAG	CCGCTCGAGCTATAGCT GGCCGGGTCCCCAGAG
PAP	CCAGTGCACCTGCATATGGTGGGA AGGAGTTGAAGTTTGTGACTTTGG	CCGCTCGAGCTAATCTG TACTGTCCTCAGTACC
TRPM8	CCAGTGCACCTGCATATGGTGG GTCCTTTCGGGCAGCCAGGCTC	CCGCTCGAGTCATCGG GAAATCTCTCCATACC

Table 6. PCR program for cloning of PCa antigens lacking the ER leader sequence

Cycle	Temperature	Time	Step
1	95°C	3 min	Initial denaturation
18	95°C	20 s	Denaturation
	72°C → PSA/PSCA 76°C → PAP/TRPM8	15 s	Primer annealing
	72°C	PSA/PSCA → 30 s PAP/TRPM8 → 1 min	Elongation
15	95°C	20 s	Denaturation
	60°C	15 s	Primer annealing
	72°C	PSA/PSCA → 30 s PAP/TRPM8 → 1 min	Elongation
1	72°C	5 min	Final elongation

After purification of the DNA with the NucleoSpin® Gel and PCR Clean-up (Machery-Nagel), PCR product and plasmid were digested using the restriction enzymes XhoI and BsaI for PSA and PSCA or XhoI and AarI for PAP and TRPM8 (all Fermentas) and subsequently purified again. After dephosphorylation of the vector pET-SUMO with Antarctic phosphatase (NEB), plasmid vector and PCR product were ligated using T4 ligase (NEB) according to the manufacturer´s instructions.

4.2 Cloning of prostate cancer antigen long peptides

As expression quantity and solubility of full-length and ER leader sequence lacking PCa antigens was not efficient enough to get the protein amounts necessary for encapsulation into PLGA MS, long PCa antigen peptides were cloned for expression in *E. coli*. $PSA_{133-172}$, $PAP_{108-147}$, $PSCA_{7-46}$ and $TRPM8_{117-206}$ were cloned into pGEX-4T-2 vector (GE Healthcare, München, Germany). All PCa antigen long peptides were PCR amplified using Phusion® High Fidelity PCR Polymerase Kit (NEB, Frankfurt, Germany) according to manufacturer´s instructions (Tab. 9) in the PCR thermocycler T3 (Biometra). All primer pairs were synthesized by Microsynth AG (Tab. 8). After purification of the DNA with the NucleoSpin® Gel and PCR Clean-up (Machery-Nagel), PCR product and plasmid were digested using the restriction enzymes XhoI and EcoRI (both Fermentas) and subsequently purified again. After dephosphorylation of the vector pGEX-4T-2 with

Antarctic phosphatase (NEB), plasmid vector and PCR product were ligated using T4 ligase (NEB) according to the manufacturer´s instructions.

Table 7. Primer for cloning of PCa antigen long peptides into pGEX-4T-2 *E.coli* expression vector

Gene	Sense primer (5´→ 3´)	Antisense primer (5´→ 3´)
$PSA_{133-172}$	CCGAATTCCTGGG GCAGCATTGAAC	GCTCGAGTCACAT GAACTTGGTCACC
$PSCA_{7-46}$	CCGAATTCCGC CTGTTGATGGC	GCTCGAGTCA CTCCCCCAGC
$PAP_{108-147}$	CCGAATTCCGAC GTTGACCGGAC	GCTCGAGTCAAGG AACTGTGTGCAC
$TRPM8_{117-206}$	CCGAATTCCGCTT GGATTCTCACGG	GCTCGAGTCACT CTGAACTCCTGC

Table 8. **PCR program for cloning of PCa antigen long peptides**

Cycle	Temperature	Time	Step
1	98°C	30 s	Initial denaturation
	98°C	10 s	Denaturation
18	63°C	20 s	Primer annealing
	72°C	20s	Elongation
	98°C	10 s	Denaturation
15	56°C	20 s	Primer annealing
	72°C	20 s	Elongation
1	72°C	8 min	Final elongation

4.3 Agarose gel electrophoresis

TAE buffer

40 mM	Tris (Invitrogen)
20 mM	Acetic acid (VWR)
1 mM	EDTA (Roth)

6 x DNA loading buffer

12% (w/v)	Glycerol (VWR)
60 mM	EDTA (Roth)
0.0003%(w/v)	Bromphenol blue (Applichem)

1 % agarose gels were prepared with TAE-buffer and 1 µl Ethidium Bromide (Sigma-Aldrich) per 50 ml gel. Smart Ladder or Smart Ladder SF (Eurogentec, Cologne, Germany) were used for size determination. For separation, the gels were run in TAE-buffer at 90 V. Analysis was performed using the Molecular Imager® Gel Doc™ (BioRad, München, Gemany).

4.4 Preparation of chemically competent *E. coli*

LB medium

1% (w/v)	Tryptone (BD)
0.5% (w/v)	Yeast extract (BD)
1% (w/v)	NaCl (Roth)

LB Agar

	LB medium
1.5% (w/v)	Agar (BD)

$CaCl_2$/glycerol solution

100 mM	$CaCl_2$ (Roth)
10% (v/v)	Glycerol (VWR)

The *E. coli* strains DH5α, BL21(DE3), BL21(DE3)pLysS and BL21(DE3)RIPL were struck on a LB agar plate and incubated over night at 37°C. A starter culture of 5 ml LB medium was inoculated with a single colony and was grown over night at 37°C. The next day, 1 ml of the starter culture was used for inoculation of 100 ml LB medium. The culture was allowed to grow at 37°C until an OD_{600} of 0.6-0.7 was reached (measured using SmartSpec™ Plus, BioRad). Subsequently, the cells were put on ice for 30 min, spun down at 4000 x g at 4°C and re-suspended in chilled $CaCl_2$/glycerol solution. Aliquots were stored at -80°C until further use.

4.5 Transformation of chemically competent bacteria

Chemically competent *E. coli* were thawed on ice. 5 µl of plasmid DNA was added to the bacteria, which were subsequently incubated for 30 min on ice followed by a heat shock at 42°C for 30 s. After addition of 500 µl LB medium, the bacteria were shaken at 37°C for one hour to allow phenotypic expression. Subsequently, the bacteria were plated on LB agar plates containing appropriate antibiotics (100 µg/ml ampicillin, 50 µg/ml kanamycin, 34 µg/ml chloramphenicol (all Sigma-Aldrich)) allowing selection of successfully transformed bacteria.

LB-agar plates were incubated overnight at 37 °C. The next day, 5 ml LB-medium containing the appropriate antibiotics were inoculated with single colonies. After an overnight incubation at 37 °C and 250 rpm plasmid DNA was purified from the bacteria using the NucleoSpin® Plasmid MiniPrep kit (Machery-Nagel) according to the manufacturer's protocol. Cloning success was confirmed by control restriction enzyme digests and by sequence analysis (GATC Biotech, Konstanz, Germany).

4.6 Cell lines

Spodoptera frugiperda (*Sf*21) insect cells were cultured in Insect-XPress medium (Lonza, Cologne, Germany) containing 1% (v/v) penicillin/streptomycin (P/S) (Gibco, Darmstadt, Germany) at 27°C/ 5% CO_2. The TAP-deficient, HLA-A*0201 positive T2 cell line was maintained in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco) containing 10% (v/v) FCS (Gibco) and 1% P/S (Gibco) and was cultured at 27°C/ 5% CO_2. For determination of vaccinia virus titer BSC-40 cells were cultured in Minimal Essential Medium (MEM) (Gibco) supplemented with 5% (v/v) FBS and 1% (v/v) P/S at 37°C/ 5% CO_2. Madin-Darby canine kidney (MDCK II)-cells for the Influenza virus plaque assay were grown in MEM medium supplemented with 0.2% (v/v) FBS 1% (v/v) P/S at 37°C/ 5% CO_2.

4.7 Baculo Virus recombinant protein expression system

Insect cell lysis buffer

20 mM	MOPS (Sigma)
100 mM	KCl (Merck)
20 mM	Imidazol (Sigma)
1 x	Protease inhibitor cocktail (complete Mini EDTA-free, Roche)

Recombinant Baculo viruses were generated by co-transfection of *Sf*21 cells with wild-type AcNPV virus DNA and the recombinant Baculo virus transfer vector DNA using the BaculoGold™ Transfection Kit (BD biosciences) according to the manufacturer´s instructions. After four days, growth medium was replaced by new medium and the transfected *Sf*21 cells were infected with 1 ml of the infectious supernatant. After two additional rounds of infection, virus titers in the supernatants reached the optimal MOI for infection and expression of recombinant proteins. Infectious supernatants were

stored at -4°C for further infections. Cells were centrifuged at 1000 x g and 4°C for 5 min. Cell pellets were lysed with insect cell lysis buffer and centrifuged again. Recombinant protein was subsequently purified from supernatants.

4.8 *E.coli* recombinant protein expression system

Table 9. E.coli strains for recombinant protein expression

E.coli strain	Antibiotic resistance	Company
BL21(DE3)		Promega
BL21(DE3)pLysS	Chloramphenicol	Promega
BL21(DE3)RIPL	Chloramphenicol; Streptomycin/Spectinomycin	Promega

LB low salt	
1% (w/v)	Tryptone (BD)
0.5% (w/v)	Yeast extract (BD)
0.5% (w/v)	NaCl (Roth)

HSG	
1.35% (w/v)	Tryptone
0.7% (w/v)	Yeast extract
1.49% /w/v)	Glycerine (Sigma)
42.7 mM	NaCl
5.7 mM	K_2HPO_4 (Merck)
11 mM	KH_2PO_4 (Roth)
0.56 mM	$MgSO_4$ x $7H_2O$ (Merck)

M9	
M9 salts	
42 mM	Na_2HPO_4 (Sigma)
22 mM	KH_2PO_4 (Roth)
17 mM	NaCl (Roth)
10 mM	NH_4Cl (Sigma)
20% (v/v)	M9 salts
0.2% (v/v)	1M $MgSO_4$ (Merck)
2% (v/v)	20% Glucose (Riedel- de Haen)
0.01% (v/v)	1M $CaCl_2$ (Roth)

After transformation, single colonies were used to inoculate 5 ml LB precultures containing the appropriate antibiotics. After an overnight incubation at 37 °C and 250 rpm, the 500 ml LB containing the appropriate antibiotics were inoculated with the precultures. The culture was incubated at 37°C and 250 rpm until the OD_{600} reached 0.6 – 0.7. After induction of recombinant protein expression with 0.4 mM IPTG, cultures were incubated for another five hours at 37°C and 250 rpm. To improve protein folding, IPTG concentration was decreased to 0.1 mM IPTG, temperature was reduced from 37°C to 20°C for the five hours of incubation and fresh LB medium with or without 200 µg/ml

Chloramphenicol (Sigma-Aldrich) was added to the culture for another two hours after the five hours incubation. Afterwards, the cultures were harvested and centrifuged for 10 minutes at 7000 rpm and 4°C.
The bacterial pellets were resuspended in 4 ml PBS. After addition of 10 mg/ml lysozyme and an incubation for 30 minutes on ice, the suspensions were sonicated five times for 20 seconds at 70 % power. 1 % triton-x 100 was added to the suspension and after another incubation for 30 min at 4°C, the suspension was sonicated under the same conditions as before. After a final centrifugation at 4 °C and 15000 rpm for 15 minutes, supernatants were used for protein purification.

PBS pH 7.4

137 mM	NaCl (Roth)
2.7 mM	KCl (Merck)
10 mM	$NaHPO_4$ (Sigma)
1.8 mM	KH_2PO_4 (Roth)

4.9 Protein purification

HisTrap binding buffer pH 7.4

20 mM	Na_3PO_4 (Sigma)
0.5 M	NaCl (Roth)
5 mM	Imidazole (Sigma)

HisTrap elution buffer pH 7.4

20 mM	Na_3PO_4
0.5 M	NaCl
0.5 M	Imidazole

GSTrap binding buffer pH 7.4

1 x	PBS

GSTrap elution buffer pH 8.0

50 mM	Tris-HCl (Invitrogen)
10 mM	reduced GSH (Sigma)

GST-tagged and 6xHis-tagged recombinant proteins were purified with 1 ml GSTrap FF or HisTrap FF affinity chromatography columns (both GE healthcare) according to the manufacturer´s instructions, respectively. A flow rate of 1 ml/min was applied for both columns. At first, the columns were washed with five column volumes destilled water before they were equilibrated with ten column volumes of the respective binding buffer. After loading of the supernatant, unbound proteins were washed off the column with ten column volumes binding buffer. Subsequently, recombinant protein was eluted from the column with the respective elution buffer. Bacterial lysate, washing steps and the eluted fractions were analyzed by SDS-PAGE, Coomassie or silver staining and Western blot.

The eluted solutions were concentrated using Amicon Ultra centrifugal filters (Millipore, Schwalbach, Germany).

4.10 SDS PAGE

10 x running buffer		**4 x stacking gel buffer**		**4 x separating gel buffer**	
0.25 M	Tris pH 8.3 (Sigma)	0.5 M	Tris pH 6.8	1.5 M	Tris pH 8.8
1.92 M	Glycine (Roth)	0.4% (w/v)	SDS	0.4% (w/v)	SDS
1% (w/v)	SDS (Serva)				

SDS-PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis) was carried out with Mini Protean® cells (BioRad) under denaturing conditions. Gel electrophoresis and the respective buffers are described elsewhere [499]. Acrylamide (4K solution, 30%) was purchased from AppliChem. Polymerisation was induced with 10% ammoniumpersulfate and TEMED (both Sigma-Aldrich). PAGE-Ruler Prestained Protein Ladder (Thermo Scientific) served as molecular weight marker. During entry of the samples into the stacking gel, gels were run at 80 V followed by separation at 120 V.

4.10.1 Tricine SDS PAGE

10 x Anode buffer pH 8.9		**10 x Cathode buffer pH 8.25**		**3 x Gel buffer pH 8.45**	
1 M	Tris (Sigma)	1 M	Tris	3 M	Tris
0.225 M	HCl (Riedel-de Haën)	1 M	Tricine (Sigma)	1 M	HCl
		1% (w/v)	SDS	0.3% (w/v)	SDS

For separation of peptides smaller than 5 kDa, Tricine SDS PAGE was performed with Mini Protean® cells (BioRad) as described elsewhere [500]. Polymerisation was induced with 10% ammoniumpersulfate and TEMED (both Sigma-Aldrich). Spectra Multicolor Low Range Protein Ladder (Thermo Scientific) served as molecular weight marker. During entry of the samples into the stacking and spacer gel, gels were run at 30 V followed by separation at 80 V.

4.11 SDS PAGE staining

Coomassie fixing solution	
50% (v/v)	Methanol (Sigma)
10% (v/v)	Glacial acetic acid (Roth)

Coomassie staining solution	
0.1% (w/v)	Coomassie Brilliant Blue R-250 (Serva)
10% (v/v)	Glacial acetic acid

Coomassie destaining solution	
40% (v/v)	Methanol
10% (v/v)	Glacial acetic acid

Coomassie storage solution	
5% (v/v)	Glacial acetic acid

For visualization of protein bands on SDS PAGE or Tricine SDS PAGE, either Coomassie or silver staining of the gels was performed. For Coomassie staining gels were first incubated in fixing solution for 1h or overnight with gentle agitation. Afterwards, gels were stained in Coomassie staining solution for 20 min under gentle agitation. For destaining, gels were incubated in destaining solution until the background was fully destained. Destained gels were stored in storage solution.
Silver staining of the gels was performed with the Pierce™ Silver Stain Kit (Thermo Fisher Scientific, Schwerte, Germany) according to the manufacturer´s instructions.

4.12 Western Blot

Blotting buffer	
25 mM	TRIZMA (Sigma)
200 mM	Glycine (Roth)
10% (v/v)	Methanol (Sigma)

Western Blots were performed with the semi-dry Fastblot B34 system (Biometra, Göttingen, Germany) according to the manufacturer´s instructions. Whatman membranes (GE healthcare) where soaked with 1 x blotting buffer. Protein transfer onto Protran BA85 nitrocellulose membranes (pore size 0.45 µm, GE healthcare) was carried out for 45 min at 100 V.
Membranes were blocked with 1 x PBS, 0.02% (v/v) Tween-20, 3% (w/v) BSA (blocking buffer) for one hour at room temperature prior to incubation with the indicated primary antibodies (Tab. 10) diluted in 1 x PBS, 3% (w/v) BSA, which were incubated for one hour at room temperature or overnight at 4°C. After washing, membranes were incubated

with horse-radish peroxidase (HRP) coupled secondary antibody for one hour at room temperature. Western blots were developed using SuperSignal West Pico (Thermo Scientific) according to the manufacturer's instruction using the ChemiDoc system in conjunction with Quantity One Software (both Bio-Rad).

Table 10. Antibodies for Western Blot detection

Antibody	Host	Company
anti-6HIS-HRP	mouse	Roche
anti-GST	rabbit	Thermo Scientific
anti-rabbit-HRP	swine	Dako

4.13 Mouse strains

C57BL/6 ($H2^b$) mice were originally purchased from Charles River Laboratories. HLA-A/H2-D (AAD) mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Both mouse strains were further bred in the animal facilities of the University of Konstanz. AAD mice express a recombinant class I MHC molecule, which consists of peptide binding regions of human HLA-A*0201 linked to the CD8 binding domain of murine H2-Db, thus recognizing HLA-A*0201 restricted peptide epitopes. All mice were kept in a specific pathogen free facility and kept under pathogen-free conditions. Experimental animals were used at 8 –12 weeks of age.

4.14 AAD mice genotyping PCR

After digestion of the tail biopsies in 600 µl 50 mM NaOH (Sigma) at 96°C and 900 rpm for 45 min, the digest was neutralized with 50 µl 1 M Tris pH 8.0. After centrifugation at 11000 x g, 2 µl of the supernatant were used as PCR template. PCR was performed with GoTaq polymerase (Promega) according to manufacturer's instructions (Tab. 13) using T3 Thermocycler (Biometra). The primer pairs including an internal control displayed in Table 11 were used, which were all synthesized by Microsynth (Tab. 12).

Table 11. Primer for AAD mice genotyping PCR

	Sense Primer (5´→3´)	Antisense Primer (5´→3´)
AAD	ACGGAAAGTGAAGGCCCACTC	GCAGCCATACATCCTCTGGACG
Control	CAAATGTTGCTTGTCTGGTG	GTCAGTCGAGTGCACAGTTT

Table 12. PCR program for genotyping of AAD mice

Cycle	Temperature	Time	Step
1	94°C	3 min	Initial denaturation
	94°C	30 s	Denaturation
35	67°C	1 min	Primer annealing
	72°C	1 min	Elongation
1	72°C	2 min	Final elongation

4.15 Preparation of PLGA Microspheres

PLGA MS were prepared from 14 kDa PLGA 50:50 carrying hydroxyl- and carboxyl-end groups (Resomer® RG502H, Evonik Röhm GmbH, Darmstadt, Germany). The peptides (Tab. 13) and TLR ligands were microencapsulated by spray drying as described elsewhere [501]. All short peptides were synthesizes by AG Henklein, Charité Berlin, Germany), all modified peptides were synthesized by AG Ovaa (HNK Instituut, Amsterdam, NL) and all long peptides were synthesized by S. van Kasteren (Leiden University, NL) (Tab. 14).

Briefly, 10 mg of short and long peptides or 50 mg of GST-long peptides and 5 mg CpG oligodeoxynucleotides with a phosphothioate backbone (CpG-ODN 1826, Microsynth, Balgach, Switzerland) or 0.5 mg polyI:C (Sigma-Aldrich, Schnelldorf, Germany) were dissolved in 0.5 ml 0.1 M $NaHCO_3$ (aqueous phase) and mixed with 1 g of PLGA dissolved in 20 ml dichloromethane (organic phase). Coencapsulation of two peptides was performed by co-dissolving 10 mg of each peptide with 5 mg CpG-ODN in 0.5 ml of 0.1 M $NaHCO_3$ and 1 g PLGA in 20 ml dichloromethane. The aqueous and the organic phase were homogenized under ultrasonication (Hielscher, UP200 H, Ampl. 40%) for 10 s on ice. The obtained water phase/organic phase dispersion was immediately spray dried (Büchi, Mini Spray-Dryer 191, Büchi Flawil, Switzerland) at a flow rate of 2 ml/min

and inlet/outlet temperatures of 40°C/37°C. The obtained MS were washed out of the spray-dryer´s cyclone with 0.05% poloxamer 188 (Synperonic®F68, Serva Electrophoresis, Heidelberg, Germany), collected on a cellulose acetate membrane filter and dried under reduced pressure (20 mbar) for 18 h at room temperature. MS were stored at 4°C. Immediately before use, MS were dispersed in PBS by ultrasonication (Transsonic DigitalS, Elma Schmiedbauer, Singen, Germany, 100%) for 30 s to obtain a homogenous MS solution.

Table 13. Peptides encapsulated into PLGA MS

Peptide	Sequence
$FluM_{58-66}$	GILGFVFTL
PA_{46-54}	FMYSDFHFI
$PSA_{154-163}$	VISNDVCAQV
$PSCA_{14-22}$	ALQPGTALL
$PAP_{112-120}$	TLMSAMTNL
$TRPM8_{187-195}$	GLMKYIGEV
$PSMA_{27-35}$	VLAGGFFLL
$Survivin_{96-104}$	LTLGEFLKL
$STEAP_{86-94}$	FLYTLLREV
$STEAP_{262-270}$	LLLGTIHAL
Mart-1_{26-35}	EAAGIGILTV
Mart-1_{26-35} mod2	ELAGIGLTV
MAGE-$C2_{336-344}$	ALKDVEERV
MAGE-$C2_{336-344}$ mod1	[L-S-methyl-cysteine][L-2-aminoctanoic] KDVEERV
TRP-$2_{180-188}$	SVYDFFVWL
TRP-$2_{180-188}$ mod 1	[D-alpha-methyl-phenylglycine][L-norvaline]YDFFVW[L-Porpargylglycine]
$PSA_{133-172}$	WGSIEPEEFLTPKKLQCVDLHVISNDVCAQVHPQKVTKFM
$PAP_{108-147}$	DVDRTLMSAMTNLAALFPPEGVSIWNPILLWQPIPVHTVP
$PSCA_{7-46}$	ALLMAGLALQPGTALLCYSCKAQVSNEDCLQVENCTQLGE
$TRPM8_{117-206}$	AWILTGGTHYGLMKYIGEVVRDNTISRSSE

4.16 Characterization of the PLGA-MS

For the characterization of the PLGA MS, encapsulation efficiency and release of the different peptides was analyzed. 50 µg of the respective peptide was dissolved in a volume of 250 µl, as peptide control. For the determination of the encapsulation efficiency, 5 mg MS were dissolved in 50 µl DMSO (Sigma) and incubated for 30 min at room temperature. Afterwards, 200 µl 0.1 M NaOH was added to the solution. Release of the peptides from the PLGA-MS was identified by dispersing 5 mg PLGA-MS in 250 µl PBS by ultrasonication (Transsonic DigitalS, Elma Schmiedbauer) for 30 s. After 6 days, samples were spun down and the supernatant was loaded onto a Tris-tricine SDS-PAGE. Peptides in the gel were visualized by silver staining.

4.17 Peptide Binding Assay

Peptides were tested for concentration-dependent HLA-A*0201 stabilization on T2 cells. T2 cells were washed twice in serum-free IMDM and incubated at room temperature overnight with the candidate peptides over a concentration range from 0.5 to 10 µM in the presence of 1 µg/ml β2-microglobulin (BD). T2 cells were washed twice and stained with an APC conjugated anti-human HLA-A2 antibody clone BB7.2 (eBioscience). Incubation with PBS served as negative control. For the off kinetics, T2 cells were incubated over night at room temperature with 10 µM of the respective peptide followed by an incubation at 37°C in the presence of 50 µg/ml cycloheximide. After 1, 2, 3, 4 and 6 h the loss of HLA-A*0201 molecules from the surface was monitored by flow cytometry. Flow cytometric analyses were performed on BD FACS Calibur or Accuri C6 flow cytometer (both BD). Data analysis was performed with FlowJo (TreeStar Inc., Ashland, USA).

4.18 Immunizations

For subcutaneous vaccination with PLGA MS, a mixture of 5 mg MS containing short/long peptide (50 µg) or GST-long peptide (250 µg) and CpG-ODN (25 µg) and 5 mg polyI:C MS (2.5 µg) were injected in a total volume of 200 µl at a single site at the base of the tail. Control groups received 5 mg CpG-ODN MS (25 µg) and 5 mg polyI:C MS (2.5 µg) or empty MS. For comparison of MS with IFA (Sigma) respective amounts of short peptide (50 µg), CpG ODN (25 µg) and polyI:C (2.5 µg) were emulsified in PBS:IFA (1:1). For intranasal immunizations mice were anesthetized by i.p. injection of

150 µl of a ketamine (Sanofi)-rompun (Bayer)-solution (equal volumes of a 2%-rompun-solution and a 10%-ketamin-solution were mixed at the rate of 1:10 with PBS). Intranasal vaccination was performed with a mixture of 2.5 mg MS loaded with $M1_{58-66}$ and/or PA_{46-54} (25 µg) and CpG-ODN (12.5 µg) and 2.5 mg polyI:C MS (1.25 µg) in 50 µl PBS (25 µl per nostril). Control groups received 2.5 mg CpG-ODN MS (12.5 µg) and 2.5 mg polyI:C MS (1.25 µg).

4.19 Isolation of cells

Splenocytes were isolated by mechanical disruption. For isolation of lung lymphocytes, lungs were washed with PBS, cut into small pieces and digested at 37°C in IMDM containing 0.2 mg/ml of collagenase D (Roche), 0.2 mg/ml of DNase I (Roche), 10 % FCS, 100 U/ml P/S for 45 min at 37°C under agitation. Cells were gently pressed through a mesh, washed and re-suspended in 36% Percoll® in PBS (Sigma-Aldrich).The cell suspension was gently overlaid onto 70% Percoll® and centrifuged for 30 min at 1.350 x g at 4°C. Lymphocytes were collected from the interface.

4.20 Intracellular Cytokine Staining

FACS buffer	
1 x	PBS
2% (v/v)	FCS
2 mM	EDTA
0.02 mM	NaN_3 (Riedel-de Haen)

Permeabilization buffer	
1 x	FACS buffer
0.1% (w/v)	Saponin (Sigma)

Intracellular cytokine staining (ICS) was performed to detect intracellular interferon gamma (IFN-γ) as a measure of CTL activation. Isolated splenocytes or lung lymphocytes were incubated with or without 10 µM of the respective peptide in the presence of 10 µM/ml brefeldin A (Sigma-Aldrich) for 5 h at 37°C. After washing, the cells were stained with APC-conjugated rat anti-mouse CD8α antibody clone 53-6.7 (eBioscience) for 20 min at 4°C in FACS buffer. The cells were washed twice with FACS buffer before they were fixed with 4% paraformaldehyde (Thermo Fisher) in PBS for 5 min at 4°C. Afterwards, cells were washed twice with PBS/0.1% saponin (Sigma-Aldrich) for permeabilization and were then labeled intracellularly with FITC-conjugated rat anti-IFN-γ antibody (clone XGM1.2) (PD Basler, Konstanz University) in permeabilization buffer overnight at 4°C. The next day, cells were washed twice and were resuspended

in FACS buffer for flow cytometry. Background values of each sample (no restimulation with peptide) were subtracted. Flow cytometric analyses were performed on BD FACS Calibur or Accuri C6 flow cytometer (both BD). Data analysis was performed with FlowJo software (TreeStar Inc., Ashland, USA).

4.21 ELISPOT Assay

A commercially available antibody pair (BD Biosciences) for the detection of IFN-γ producing cells was used according to the manufacturer´s protocol. ELISPOT 96 well plates were coated with anti-IFN-γ antibody and subsequently blocked. Splenocytes were incubated with or without 10 µM of the respective peptide overnight. After incubation with a biotinylated antibody for two hours, a streptavidin-alkaline phosphatase enzyme conjugate was added for 40 minutes at room temperature. Spots of the dried plate were counted using an ImmunoScan instrument (C.T.L., Cellular Technology Ltd., USA).

4.22 *In Vivo* Cytotoxicity Assay

Red blood cell lysis buffer

1.54 M	NH_4Cl (Sigma)
0.14 M	$NaHCO_3$ (Sigma)
1 mM	EDTA (Roth)

The cytotoxic activity of CTLs *in vivo* was analyzed exactly as described elsewhere [502]. In brief, splenocytes from naïve mice were depleted from erythrocytes. Half of the cells was left untreated, the other half was pulsed with 10^{-6} M peptide for 1 h at 37°C. Afterwards, untreated and peptide pulsed cells were stained with 1 µM or 10 µM CFSE (Sigma), respectively, for 10 min at 37°C. A mixture of 1 x 10^7 pulsed and unpulsed cells in a total volume of 200 µl was injected i.v. in the tail vein of mice that were immunized with peptide/CpG MS and 5 mg polyI:C MS or control mice that received 5 mg CpG MS and 5 mg polyI:C MS 5 d before. After 24 h splenocytes of the immunized mice were analyzed for CFSE labeled cells by flow cytometry. Flow cytometric analyses were performed on BD FACS Calibur (BD) and data analysis with FlowJo software (TreeStar Inc.). The percentage of specific cytolysis was calculated as follows: 100 – [(% peptide pulsed cells in vaccinated mice/% unpulsed cells in vaccinated mice)/ (% peptide pulsed cells in control mice/% unpulsed cells in control mice)] x 100.

4.23 Peptide/HLA-A*0201 Recognition Assay

MACS buffer

1 x	PBS
0.5 % (v/v)	FCS
2 mM	EDTA

Mice were immunized s.c. with a mixture of 5 mg $STEAP_{262-270}$/CpG MS and 5 mg polyI:C MS. After 6 days, splenocytes were isolated by mechanical disruption. The isolated splenocytes were coincubated with $STEAP_{262-270}$ peptide or $STEAP_{262-270}$ presenting human HLA-A*0201 positive cells overnight. T2 cells were incubated at 37°C for 1 h with or without 10^{-6} M $STEAP_{262-270}$ peptide, washed twice and then used for the recognition assay. MoDCs were generated from human peripheral blood mononuclear cells (PBMC) as previously described [503]. In brief, heparinized blood was taken from HLA-A*0201 positive donors to obtain PBMC by density gradient centrifugation on Ficoll-Paque1 (GE healthcare) at 1,800 rpm for 20 min. After magnetic sorting for $CD14^+$ monocytes (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer´s instructions, cells were further cultured for 5 d in AIM-V (Gibco) containing 1,000 U/ml of GM-CSF and 800 U/ml of IL-4 (both Peprotech, Hamburg, Germany). On day five, 0.25 mg $STEAP_{262-270}$/CpG MS or CpG MS were added to 1 x 10^6 immature MoDCs. After two days, MoDCs were washed twice with PBS and were then used for the recognition assay. For the recognition assay, 1 x 10^5 antigen presenting cells were co-incubated overnight with 100 µl of the spleen suspension. Analysis was performed by ELISPOT assay.

4.24 Vaccinia virus infection and protection assay

Crystal violet solution

3.7% (v/v)	Paraformaldehyde (Thermo Fisher)
0.05% (w/v)	Crystal violet (Merck)

To assay viral protection, AAD mice were immunized s.c. with a mixture of 5 mg MS loaded with $M1_{58-66}$ peptide (50 µg) and CpG-ODN (25 µg) and 5 mg polyI:C MS (2.5 µg). On day 6 after immunization mice were challenged i.p. with 2 x 10^6 pfu recombinant vaccinia virus coding for $M1_{58-66}$ rVV-ESM1 (kind gift of Dr. Yewdell, NIH Bethesda, USA). Four days later, ovaries were taken, single cell suspensions were prepared by

physical disruption in MEM, 5% FCS and analyzed by plaque assay after six freeze-thaw cycles. Suspensions were titrated and added to confluent BSC-40 cells. After infection, BSC-40 cells were incubated for 24 h at 37°C, 5% CO_2. After removal of the medium, plaques were visualized by addition of crystal violet solution for 1 h, counted, and the pfu were calculated.

4.25 Influenza virus infection

Influenza virus infection, observation of the mice and the plaque assay were performed by Prof. Oliver Planz (University of Tübingen, Germany). Hereafter, the methods are explained in brief. On day 6 after the last MS immunization, mice were anesthetized by i.p. injection of 150 µl of a ketamine (Sanofi)-rompun (Bayer)-solution (equal volumes of a 2%-rompun-solution and a 10%-ketamin-solution were mixed at the rate of 1:10 with PBS), before i.n. inoculation with the influenza A virus strain A/Regensburg/D6/09 (H1N1pdm09, RB1). For infection, a 10 x 50% mouse lethal dose (MLD_{50}) was used (10 x MLD_{50} = 5×10^4 pfu in C57BL/6 mice). For determination of the clinical score following disease symptoms were found and defined: ruffled fur, teeth crunching, ataxia, dyspnea and conjunctivitis. If mice showed one of the aforementioned symptoms they received one score; 2 symptoms = score 2; 3 and more symptoms = score 3; death = score 4. Note, score 4 was kept throughout the observation period. Score represents the mean value of the group. Furthermore, mice were weighted daily. When mice were euthanized at the clinical endpoint of 75% of the initial bodyweight, the bodyweight at the endpoint was kept throughout the observation period.

4.26 Influenza virus plaque assay

To assess the number of infectious particles in the lungs, a plaque assay using AVICEL® (FMC BioPolymer, Sandvika, Norway) was performed in 96-well plates as described previously [41] 48 h after infection. Briefly, Madin-Darby canine kidney (MDCK II)-cells were grown to confluency in 96-well dishes. Then they were washed with PBS and infected with serial dilutions of the supernatants in PBS/BSA for 30 min at 37°C. After incubation, cells were overlaid with overlay-medium [1:1, MEM-medium containing 0.2% BSA, antibiotics and 2.5% AVICEL® -Medium (FMC BioPolymer)] for 24 h. Afterwards, virus-infected cells were immunostained by incubating for 1 h with a monoclonal antibody specific for the influenza A virus nucleoprotein (Serotec, Puchheim, Germany), followed by 30 min incubation with peroxidase-labeled anti-mouse antibody (DIANOVA,

Hamburg, Germany) and 10 min incubation with True Blue™ peroxidase substrate (KPL, Gaithersburg, USA). Stained plates were scanned on a flatbed scanner and the data were acquired by Corel DRAW 9.0 software (Corel Corporation, Ottawa, Canada). Viral titers are shown as the logarithm to the base 10 of the mean values.

4.27 Statistics

For statistical analyses, groups from similar experiments were pooled and analyzed for significant differences as indicated in the graph. The p values for experiment composites are given in the figure legends. All statistical analyses were performed using Prism software (GraphPad Software, Inc., La Jolla, USA).

Record of contribution

I designed and performed all experiments except for

the results depicted in Figure 15 C/D, 16 C/D and 17 C/D which were obtained in the context of the master thesis of Julia Körner, which was completely performed under my supervision.

the infection of the mice, determination of the virus titer and monitoring of disease symptoms, body weight and survival in Figure 22 and 24 which were performed in the context of a collaboration with Prof. Oliver Planz and Carmen Hartmayer at the University of Tübingen.

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