Optimization of PLGA microspheres for immunotherapy of tumors in the mouse model

Dissertation

Zur Erlangung des akademischen Grades eines

"Doktors der Naturwissenschaften"

(Dr. rer. nat.)

des Fachbereiches für Biologie

an der Universität Konstanz

vorgelegt von

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Tag der mündlichen Prüfung: 27.07.2010
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Abbreviations

AG	antigen
APC	antigen presenting cell
BMDC	bone marrow derived dendritic cell
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CD	cluster of differentiation
CHS	contact hypersensitivity
CIA	collagen induced arthritis
CpG	cytosin-phosphatidyl-guanosin
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DMEM	Dulbecco's modified eagle medium
DMSO	diemethylsulfoxid
DNA	deoxyribonucleic acid
DNFB	dinitrofluorobenzene
ds	double stranded
DSS	dextransulfate sodium salt
DTH	dihydrotestosterone
EC	endothelial cell
EDTA	ethylenediamine-tetraacetic acid
ER	endoplasmatic reticulum
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FDA	food and drug administration
FITC	fluorescein isothiocyanat
GMCSF	granulocyte macrophage colony stimulating factor
i.p.	intraperitoneal(y)
i.v.	intravenous(ly)
iDC	immature dendritic cell

IFA	incompletes Freud’s Adjuvant
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IMDM	Iscove’s modified dubelccos´s medium
iNKT cells	invariant natural killer
kDa	kilo Dalton
kGy	kilo Gray
LPS	lipopolysaccharide
mAB	monoclonal antibody
mDC	mature dendritic cell
mDC	myeloid dendritic cell
MHC	major histocompatibility complex
moDC	monocyte derived dendritic cell
MS	microspheres
MVA	modified vaccinia Ankara
Mφ	macrophage
N	number in study or group
NK cell	natural killer cell
NKT cell	natural killer T cell
NOD like receptor	nucleotide binding oligomerization domain-like receptor
ODN	oligodeoxynucleotides
ova	ovalbumin
PAMP	pathogen associated molecular pattern
PBS	phosphate buffer saline
pDC	plasmacytoid dendritic cell
PFA	paraformaldehyde
PLGA	poly(lactide-*co*-glycolide)
poly I:C	polymer of inosinic and cytidylic acid
P/S	penicillin/streptomycin
RNA	ribonucleic acid
rVV	recombinant vaccinia virus
s.c.	subcutanous(ly)

ss	single stranded
STZ	streptozotocin
T1D	type I diabetes
TAA	tumor associated antigen
TCR	T cell receptor
TGF	transforming growth factor
Th cell	T helper cell
TIL	tumor infiltrating lymphocyte
TLR	toll like receptor
TNF	tumor necrosis factor
T-reg cell	regulatory T cell
v/v	volume per volume
VLP	virus like particle
w/v	weight per volume
α-C-GalCer	α-C-galactosylceramide
α-C-diPheGalCer	α-C-N-acyl-diPhenyl-galactosylceramide
α-GalCer	α-galactosylceramide
(μ)M	(micro)molar
μm	micro meter

Zusammenfassung / Summary

Zusammenfassung

1971 erklärte der damalige U.S. Präsident Nixon dem Krebs den Krieg, indem er den „National Cancer Act" unterzeichnete. Ungefähr 40 Jahre später, im Jahr 2009, erneuerte U.S. Präsident Barack Obama diese Kriegserklärung, da im Jahr 2007 immer noch ca. 13% der Todesfälle auf Krebs zurückzuführen waren. Krebs wird heutzutage meist opertiv entfernt oder mit Hormontherpie, Chemotherapie, oder Strahlentherpie behandelt. Trotz beträchtlicher Nebenwirkungen ist der Erfolg solcher Therapien jedoch meist gering. Immuntherapie, also eine Aktivierung von $CD4^+$ T Zellen, von B Zellen und / oder cytotoxischer T Zellen zur Therapie von Krebs, ist eine vielversprechende Idee, ist aber leider noch nicht allzu effektiv.

Um kosten- oder arbeitsintensive Methoden zu umgehen, ist es notwendig, dendritische Zellen *in vivo* mit Antigenen zu beladen und zu aktvieren. Dendritische Zellen sind professionelle antigen-präsentierende Zellen, die die Möglichkeit haben, aufgenommene Antigene zusammen mit kostimulierenden Molekülen zu präsentieren. Dadurch lassen sich cytotoxische T Zellen effektiv aktivieren. Biologisch abbaubare PLGA-Mirkosphären (MS) sind eine attraktive Möglichkeit, um die Aufnahme von verkapselten Antigenen und immunstimulierenden Substanzen durch dendritische Zellen zu erreichen. Wir haben nach Verfahren gesucht, diesen experimentellen Ansatz weiter zu verbessern, um den Ansprüchen eines Einsatzes in der Klinik gerecht zu werden.

Kapitel II befasst sich mit der Charakterisierung von PLGA-MS zur Therapie von Tumoren im Mausmodell und vergleicht sie mit dem incomplete Freund's Adjuvant (IFA). Es konnte gezeigt werden, dass PLGA-MS eine Reihe von vorzüglichen Eigenschaften besitzen und sie im Vergleich mit dem „Gold-Standard" IFA sehr gut abschneiden. Kapitel III und IV untersuchen CD1d Liganden auf die Möglichkeit, sie als stimulative oder regulative Modulatoren im PLGA-MS System einzusetzten. Kapitel V setzt unsere vorangegeangenen Ergebisse in einen klinisch relevanten Kontext, indem Tumorzelllysat als „Antigen" verwendet wird. Tumorlysat stellt, z.B. nach Tumoroperationen, eine leicht zugängliche Quelle für eine Vielzahl an verschiedenen Tumorantigenen dar. Grundsätzlich präsentieren sich PLGA-MS als ein flexibles, potentes System. Sie könnten das zeitgemäße

Trägersystem zur *in vivo*-Antigenübertragung sein, das die Immuntherapie so dringend braucht.

Summary

In the year 1971 U.S. President Nixon signed the “National Cancer Act” and thus declared a “war on cancer”. Unfortunately, about 40 years later in 2009, U:S: pesident Barack Obama had to renew this declaration, since in 2007 still about 13% of all human deaths were caused by cancer. Standard therapies for most cancer types are surgery, hormone-, chemo- or radiotherapy, associated with severe side effects but, unfortunately in most cases, only limited success. The idea of immunotherapy, that means therapy of cancer via activation of $CD4^{+}$ T cells, B cells and / or cytotoxic T cells responses against a tumor specific antigen, is promising, but up to date not very effective.

In order to circumvent labor or cost intensive approaches it is necessary to target dendritic cells *in vivo*. They are professional antigen presenting cells, with the ability to present engulfed antigens in the context of co-stimulatory molecules, and thereby efficiently activating cytotoxic T lymphocytes. Biodegradable PLGA-microspheres (MS) are a potent tool to opsonize encapsulated antigens and immunostimulatory molecules for uptake by dendritic cells. Here we investigated, how this approach could be further improved in order to fulfill the requirements for clinical use.

Chapter II deals with the characterization of PLGA-MS in the therapy of tumors in the mouse model and compares PLGA-MS with incomplete Freund’s adjuvant (IFA). It could be shown that PLGA-MS possess a variety of valuable features and compare favorably to the “gold-standard” IFA. Chapter III and IV investigate CD1d ligands for their properties to serve as stimulatory and regulatory modulators in combination with the PLGA-MS system. Chapter V puts our previous findings in a more clinically relevant context, by using tumor cell line lysate as an antigen. Tumor lysate is a source of a variety of tumor antigens, which is easily accessible, e.g., after surgery of tumors. In general, PLGA-MS emerge as a flexible, powerful tool for immunotherapy, which might serve the desperate need for a contemporary *in vivo* antigen delivery device.

Chapter I

Introduction

1. Dendritic cells and immunity

The idea of a "sentinel", patrolling the body and scanning for a necessity for intervention of the immune system is very pictographic, but purposive (1). Indeed, dendritic cells (DC) (2) are scanning the organism for pathogens and signs of inflammation. DCs are antigen-presenting cells (APC) with the unique ability to take up and process antigens (AG) in order to effectively stimulate AG-specific T cells. Thereby, they represent the critical part for the induction of immune responses but also for induction of tolerance or anergy (3-5) (for an overview see figure 1). Murine DCs are characterized by the expression of their linage marker cluster of differentiation (CD) 11c and by several uptake receptors, such as DC-Sign, DEC 205, the scavenger receptor and Fc receptors (7). Originating in the bone marrow, immature DCs (iDC) locate to pathogen exposed tissues like the airways, the skin, or the gastrointestinal mucosa. There, AGs are taken up by pinocytosis or receptor mediated endocytosis and subsequently processed (8).
Tissue resident DCs are in an immature state (iDC). This state is defined by the expression of the CC chemokine receptors (CCR) CCR 1, CCR 2, CCR 5 or CCR 6 (9-11). Maturation is triggert by uptake of AG in combination with a pathogen associated molecular patterns (PAMPS), represented by ligands for the nucleotide binding oligomerization domain-like receptors (NOD-like) (12) or Toll like receptors (TLR) (13). These TLRs are highly expressed by DCs (14) and influence the uptake mechanism of AGs (15) as well as the character of the induced immune response (16-18). Mature DCs (mDC) downregulate the above mentioned CCRs and upregulate the production of proinfammatory cytokines and chemokines like CC chemokine receptor ligand (CCL) 3, CCL4 and CCL5 in order to track iDC, macrophages (Mφ) and monocytes to the site of inflammation. At the same time they upregulate the chemokine receptor CCR7, and thereby gain sensitivity for the lymph node homing chemokines CCL19 and CCL21 (19, 20). The homing of mDC to the lymphatics is crucial for the quality of the following immune response. If high numbers of well stimulated mDC enter the lymph node, DC – T cell interaction is facilitated and competition is reduced (21, 22), whereas presentation by low numbers of sub-optimally stimulated mDC or even iDC may lead to abortive T cell proliferation, tolerance or anergy (23, 24). Another T cell extrinsic mechanism for the induction of peripheral tolerance mediated by iDCs is the activation of regulatory T (T-reg) cells (4).

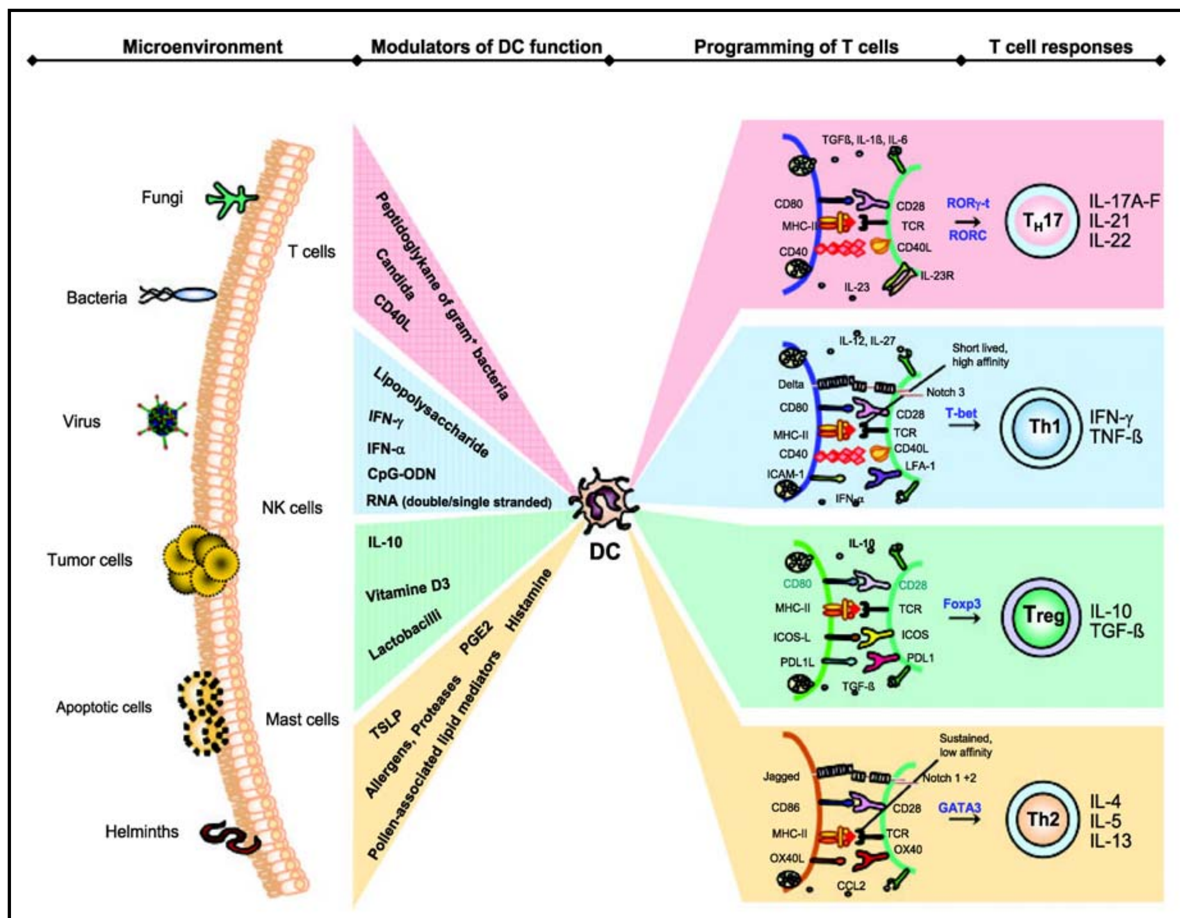

Figure 1: Environmental polarization of dendritic cell functions and programming of T cells: Immature DCs are influenced by the micromilieu, which is built up by environmental factors like microbes, pathogens, epithelial cells or other cells of the immune system. Through this, DCs are activated and express surface molecules or cytokines, which themselves influence the differentiation or activation state of different T cell subtypes. Graphic is adopted from reference (6).

Also going along with maturation of DCs is the upregulation of molecules acquired for antigen presentation, such as major histocompatibility complex (MHC) class I and MHC class II molecules as well as the costimulatory molecules CD80 and CD86, which are essential for proper priming of naive T cells (25). It has been shown that a potent $CD4^+$ T cell response requires a sustained AG presentation by mDCs, playing an important role in immunization attempts and vaccines (26, 27). Further up-regulation of costimulatory molecules, e.g., OX-40 L and 4-1BBL, requires licensing of the DCs mediated by the cross-linking of CD40 on the DCs with CD40L expressed on activated $CD4^+$ T helper (Th) cells (28). This process also triggers the production of interleukin (IL) -12p70, a cytokine, driving the polarization of the $CD4^+$ Th cell response towards a Th1 phenotype required for the efficient activation of CTLs.

AGs, processed by DC are presented on both, MHC class I and class II, dependent on the subset of DC. In the spleen, there can be found mainly two populations of DCs, differing in the expression of CD8 (4, 29, 30). $CD8^-$ DCs are specialized on presentation of exogenous AG “directly” on MHC class II, whereas $CD8^+$ DCs of the spleen are able to process non-replicating, exogenous AG for MHC class I presentation (31). This paradox is termed “cross-presentation” (32, 33) (see figure 2). Cross-presented AG can be soluble or particulate antigen, immune complexes, intracellular bacteria, parasites or, most importantly, cellular AG (34), dependent on the AG dose (35) and costimulation (36). Besides DCs, Mφ are known to be able to cross-present (37).

Interestingly, monocyte-derived DCs (moDC) are not only able to cross-present exogenous AG on MHC class I, but they are also able to “cross-dress” lymph node resident $CD8^+$ / $CD11c^+$ DCs. It could be shown, that lymph node homing monocyte derived DCs engulfed and processed AG and transferred their MHC class I / peptide complex onto lymph node resident DCs, which then in turn efficiently primed $CD8^+$ lymphocytes (38).

Recently, a new subtype of DC emerged, being referred to as blood derived inflammatory DCs. This subtype might be especially important in viral infections or immunizations favoring a strong CTL response. Blood derived inflammatory DCs are recruited to the lymph nodes, dependent on CCR2, but not dependent on CCL2 or CCR7. Most interestingly, they produced large amounts of IL-12p70 and thereby strongly support a Th1 response (40). Thus, they are an attractive target for immunizations, which seek to elicit robust CTL responses.

Of great importance for this work is, that all these DCs subsets are not only able to directly stimulate $CD8^+$ T and B cells. They are also capable of skewing T cell differentiation into different directions like Th1 or Th2, but also Th17 and T-reg (see figure 1). Th2 differentiation is mainly dependent on the cytokines IL-6 and IL-10, whereas the major Th1 cytokines are IFN-γ and IL-12. Dependent on the cytokine environment, $CD4^+$ T cells develop either into Th1 cells, stimulating a CTL response, or into Th2 cells, activating B cells in order to secrete Igs.

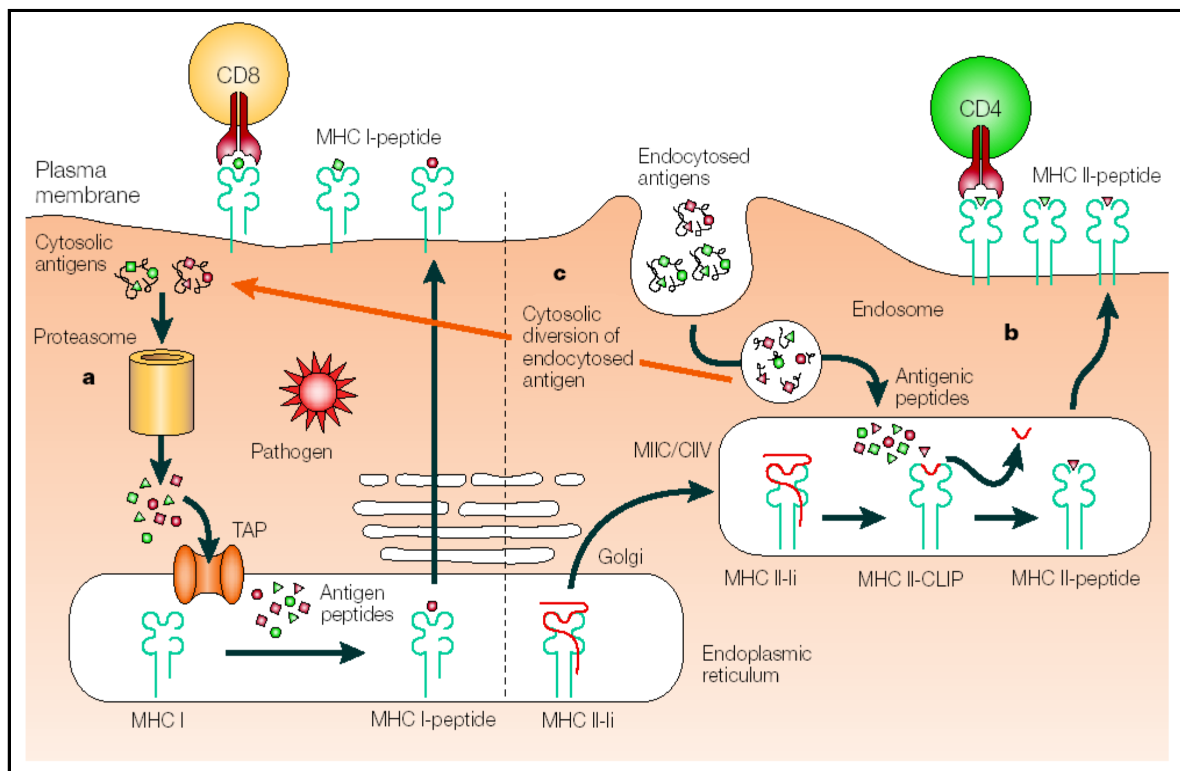

Figure 2: Antigen processing pathways: a) Endogenously synthesized peptides or intracellular pathogen derived peptides are generally presented on MHC class I. These peptides are provided by the proteasome and then transported through the transporters of antigen-processing (TAP) molecules into the endoplasmic reticulum (ER) for loading on MHC class I molecules. b) MHC class II molecules present exogenous proteins, which were taken up by endocytosis. They are degraded in the endosome and subsequently loaded on MHC class II molecules, provided by the ER. c) Dendritic cells are able to endocytose antigens and cross-present them on MHC class I to $CD8^+$ CTLs. The endocytosed material escapes the endosome and enters the MHC class I presentation pathway by being degraded via the proteasome. In most cases, these antigens will also be processed into the MHC class II presentation pathway for recognition by $CD4^+$ Th cells. Graphic is adopted from reference (39).

2. *Principles in immunotherapy*

At present, there are several promising strategies for immunotherapy of tumors. In general the immune system is able to avoid or to cope with tumors. This becomes obvious in spontaneously regressing tumors or the significantly increased susceptibility to tumors in immune suppressed patients. Tumors themselves evade the immune mechanisms by losing targeted tumor antigens, downregulating MHC or costimulatory molecules, by physical exclusion of immune cells or by expression of immune suppressive cytokines, such as IL-10, transforming growth factor (TGF) -β or vascular endothelial growth factor (VEGF), deranging DC

activation or maturation. Even the induction of T cell anergy or the induction of regulatory T (T-reg) cells is part of the immune evading repertoire of tumors. Nevertheless, there are possibilities to effectively stimulate the immune system (41). A central part of all these strategies is the choice of a suitable antigen target.

The innate immune system e.g., can be triggered by the intra-tumoral or systemic application of IFN-γ or IL-12. This activates DCs, which then in turn activate T cells and by their production of IL-12 create a Th1 polarizing environment. An important physiological source of early IFN-γ is the population of NK or NKT cells. Another approach, for the activation of the innate immune system, is to isolate these NK cells from the blood and reinfuse them in an activated state. *In vitro,* activation of NK cell can be achieved by IL-2, which itself is also used in high dose systemic injections, in order to activate the innate immune system in cancer patients. However, all these strategies are limited by severe side effects, most prominently, the excessive induction of tumor necrosis factor (TNF) -α production. The injection of IFN-γ has been proven to be clinically effective. It has been shown to induce tumor regression in renal cell cancer and chronic myelogenous leukemia (reviewed in (41)).

Harnessing the adaptive immune response, e.g., monoclonal antibodies (mAb) are used in modern immunotherapy. One possibility to apply humoral activity in immunotherapy is passive antibody transfer, e.g., against IL-2, CD20 (Rituximab) or CD74 (Mitatuzumab). The latter is used as a standard therapy in combination with chemotherapy in Hodgkin’s lymphoma. These mAbs act either by stimulation of effector cells, by blocking survival signals or by a coupled toxin (reviewed in (42)). Another possibility to make use of the humoral immune response against tumors is to enhance or stimulate B cell activity. This approach must be seen as less promising, since in the last years only one strategy was fairly successful. In this specific study, patients were immunized with an idiotypic immunoglobulin (Ig) of an autologous B cell lymphoma. After boosting these responses with the idiotypic Ig, coupled to the helper protein keyhole limpet hemacyanin (KLH), patients experienced robust anti-tumor responses as well as tumor remission (43).

In general, the induction of cellular responses is more promising. E.g., the use of allogenic tumor cells vaccines. The rank and file had only limited success, but mentionable is GVAX®, a vaccine using tumor cells transfected with granulocyte macrophage colony stimulating factor (GM-CSF), and Melacine®, which consists of

tumor cell lysate, applied in a water-in-oil dispersion and an adjuvant (DETOX®) (44). The second approach to mention in this context is the ex vivo loading of autologous DCs. Patient derived DC are matured and, after being pulsed with peptides or proteins, are reinfused (45, 46). This strategy has been successful in patients with skin cancer or prostate cancer (47-49). The AGs can also be coupled to TLR ligands or other immunostimulatory molecules in order to improve DC maturation or increase their activation state (50, 51). Unfortunately, this procedure is extremely cost and work intensive (52) and externally loaded DCs have been shown to be rather ineffective in the stimulation of T cells due to the rapid peptide turnover (53).

To the present day, the most active field concentrates on adoptive transfer of DCs, presenting tumor associated antigens (TAA) (54, 55), or of T cells, reactive against one or more TAAs (56-58). Another source for tumor reactive T cells are tumor infiltrating T lymphocytes (TIL) from operated tumors, which can be reinfused after *in vitro* expansion. But again, cost and work intensity as well as the need for the vaccine to be “personalized” are on the downside.

To sum up, it is a general consensus that successful immunotherapy is dependent on both, a robust CTL response and a substantial T helper cell activation. Many of the above mentioned strategies make use of immune-stimulants like GM-CSF (59-61), or try to avoid immune regulation by blocking e.g., CTLA-4 (62). The avoidance of functional exhaustion by impeding PD-1 – PD-1L interaction (63) or the down regulation of the anti tumor response by T-reg cells, and thereby by the immune system itself, is also a goal of many immunologists (64). An upcoming idea is to combine immunotherapy with chemotherapy (65).

An appropriate stimulation of $CD4^+$ and $CD8^+$ T cells, a favorable tumor microenvironment, and the reduction of hand-on-time are the main factors to be improved. A promising approach is the *in vivo* loading of APC by adjuvants or carrier systems. *In vivo* delivery of antigens, regardless if it is DNA, proteins or peptides, would circumvent *in vitro* manipulation of lymphocytes and therefore reduce the hand-on-time. An advantage of using DNA or proteins is to be independent of HLA restriction and probably the feeding of multiple epitopes into the MHC class I pathway.

3. Immunostimulatory adjuvants and vaccine delivery systems

Immunostimulatory adjuvants are assigned to either prepare the immune system to the immunization, to enhance immune activation or to mature and activate DCs (66). Examples are, e.g., pre-conditioning the site of injection by a single dose of TNF-α (21), or pretreatment with chloroquine, which positively influences cross-presentation by hindering acidification of the lysosome (67). Also the co-applications of IL-2, IL-12 or GM-CSF are conducted in order to change the organisms microenvironment favoring a strong and long lasting immune response (64, 68, 69).

TLR ligands are widely used as well. There are 11 TLRs known in the mouse, all expressed on APCs, either on their surface (TLR1, 2, 4, 6, 10 and 11) or in the endosomal compartments (TLR3, 5, 7, 8 and 9). They are all dependent on signaling via MyD88, except TLR3, which is signaling via the TIR domain-containing adaptor molecule 1 (TICAM-1). Here I will concentrate on the TLR ligands binding TLR9 and TLR3, namely Cytosine-phosphorothioate-guanine oligodeoxynucleotides (CpG-ODNs) and polyriboinosinic:polyribocitidylic acid (polyI:C).

CpG-ODNs consist of unmethylated, double stranded (ds) DNA and leads to effective stimulation and activation of DC as well as their maturation (70). CpG-ODNs are also known to favor a Th1 biased immune response and to support cross-presentation (16, 18). In vaccination studies it could be shown that CpG-ODNs elicit improved immune responses if they either are used in combination with other TLR ligands (71) or if they are in close proximity to an AG (72, 73), which was generally stated by Blander and Medzhitov in 2006 (74). Today, there are several CpG-ODN sequences available. The most known and most widely used in mouse models is 1826 (5' TCCAGGACTTCTCTCAGGTT 3'), but there exist more, e.g., the 2006 sequence for use in humans (5' TCGTCGTTTTGTCGTTTTGTCGTT 3') or a sequence published in 2008, called KSK (5' TCGTCGTTTTCGTCGTCGTTTT 3'), which has been shown to be effective in mice (75). Another classification identifies CpG-ODNs type A and type B. CpG-ODNs type A (e.g., ODN 2216 and ODN 1585) are characterized by poly G tails and induce high amounts of interferon (IFN) -α and IFN-β in human plasmacytoid dendritic cells (pDCs), strongly activate NK but are relatively weak at activating B cells. CpG-ODNs type B (e.g., ODN 2006) have a phosphorothioate

backbone and lack the poly-G tails. They strongly promote the maturation and activation of human pDCs, but induce only small amounts of IFN-α and –β. B type CpG-ODNs are weak at activating natural killer (NK) cells, but strongly stimulate B cells.

PolyI:C is a double stranded RNA molecule, mimicking a viral infection. Previous experiments revealed that polyI:C also upregulates interferon production, supports cross-presentation (76) and that it can be used in humans successfully (77, 78). Again, like for CpG, there is evidence that its association to an AG increases its beneficial effects (79, 80). Mentionable at this point is that single-stranded (ss) RNA binds to TLR7 in mice and to TLR7/8 in humans. There are ss RNA oligoribonucleotides (ORN) that are approved by the Food and Drug Administration (FDA) for clinical use in humans, such as imiquimod (81, 82). The mode of action is comparable to polyI:C and CpG, leading to up regulation of MHC class I and II, CD80 and CCR7 on DCs and inducing Th1 cytokines like TNF-α and IFN-γ as well as AG specific IgG2a (83, 84). This might be a suitable candidate for further investigation in clinical trials.

In general one could say, that mimicking a viral infection is a promising strategy in order to boost CTL responses *in vivo*. Heterologous boosting of immunizations using variable recombinant viruses is a common and rather safe strategy in many vaccination schedules (85-87). It is noticeable that comparable news about homologous prime-boost strategies are rare (88).

The second part of the adjuvants super-family is what Charles Janeway referred to as “the immunologists dirty little secret” (89): AG carrier systems or delivery devices are crucial preconditions for *in vivo* loading of APC with the AG and immunostimulatory adjuvants of choice. Besides the direct injection of an AG, coupled to an immunostimulatory adjuvant, e.g., CpG (90), the infection with recombinant viruses expressing a TAA (91) or the injection of DNA encoding for an TAA with or without CpG (92), there a more “carrier like” systems available. Liposoms, for example, form a phospholipid bi-layer around an aqueous phase containing immunostimulatory adjuvants and/or AG or DNA. They are one of the first carrier systems, bringing along the advantage to protect their content from lysosomal degradation and to effectively target their content into the MHC class I pathway in APCs (93). Another carrier system, that efficiently targets antigenic content to the MHC class I pathway is virus-like particles (VLP) (94). VLPs are

icosahedral structures consisting of viral structure proteins and a linked AG. This forms a highly repetitive structure, which can be filled or combined with immunostimulatory adjuvants.

The most competent delivery device for AG and adjuvants today is incomplete Freund's adjuvant (IFA). Although having been discovered in the 50s of the last century (95), that is to say nearly 60 years ago, it is still regarded as the "gold standard" for immunizations and is therefore still widely used, even in clinical trials (96). IFA is an emulsion of paraffin oil and the surfactant mannide monooelate. Due to its severe side effects it is lively discussed (97) but inadequately studied (98). Although long term studies reported no incidents of long term side effects (99), more recent studies describe severe local skin reactions or persistent painful granulomas at the injection site, when using a refined formulation of IFA (Montanide ISA-51) in clinical studies (100). Nevertheless, striking immune responses, humoral and cellular, and a good depot effect make IFA still the adjuvant of choice for many researchers and clinical trials. Recent advancements like Ribi DETOX, Quil-A or Alumn face either decreased immunogenicity or have severe side effects, including IgE production, which is connected with allergic reactions. Alumn, for example is approved by the FDA and has good safety records, but leads to decreased Th1 polarization.

4. PLGA-microspheres

There definitely is an onerous necessity for a delivery device, which is well tolerated and effectively targets APCs *in vivo*. We and others make use of a carrier system that provides the possibility to encapsulate AGs and PAMPs for subcutaneous injection. Biodegradable poly(lactide-*co*-glycolide) (PLGA) microspheres (MS), produced by spray drying (other techniques are reviewed in (101)), form micro-sized, spherical structures, which are slowly hydrolyzed in an aqueous environment (for chemical structure see figure 3). Thereby PLGA (resomer RG-502-H; 50:50 - lactate:glycolate) is degraded to non-toxic α-hydroxy-acids, lactic acid and glycolic acid, which are metabolized in the citric acid cycle. This is one reason why PLGA is approved for clinical use and has applications e.g., as biodegradable sutures or for the delivery of drugs (102, 103).

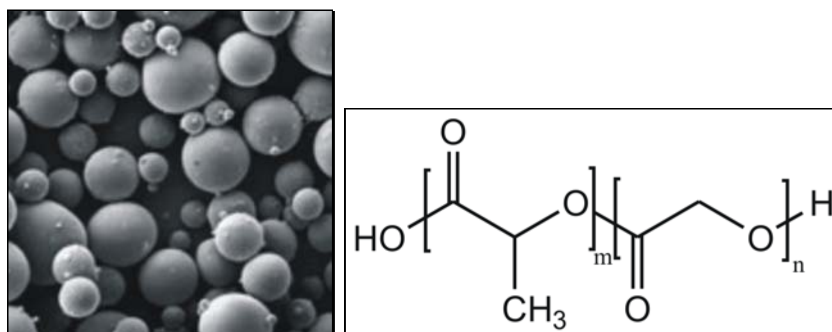

Figure 3: PLGA-microspheres: Left side. Scanning electron micrograph of PLGA-microspheres. Right side: Chemical structure of the 14 kDa molecule poly(lactide-*co*-glycolide). m = lactic acid part; n = glycolic acid part

We produce MS by spray drying technique (104), which enables us to encapsulate virtually any stable AG or TLR ligand, e.g., proteins, peptides or DNA and RNA, in microspheres of about 1-10 µm in size (105, 106) (see figure 3).

Whereas empty PLGA-MS were prouved to have no effect on viability, maturation state or stimulatory capacity of DCs *in vitro*, TLR ligand containing PLGA-MS are rapidly taken up within 1 hour and induce effective maturation of DCs (107). It could be shown that the size of about 1-10 µm is favorable for uptake by DCs and commits for well characterized trafficking to lymphatic organs (108, 109). Furthermore it is known that encapsulated AGs are protected from degradation (110) and continuously released over a period of about one month, depending on the resomer composition used (111, 112).

In vivo experiments showed, that PLGA-MS effectively target their contents to DC and Mφ (113, 114). After endosomal escape, the AGs are processed and presented on MHC class I and class II (112, 115). Due to the depot effect that is built up by PLGA-MS, stimulation of T cells is prolonged and could be show to be highly effective (112, 116). Single injections of PLGA-MS containing ovalbumin (ova) and CpG-ODN elicited robust CTL responses and protected from recombinant virus or tumor challenge (72, 73). Endogenous antigens, e.g., tumor lysate (117) or tyrosinase-related protein 2 (TRP2) (118), which were co-encapsulated with TLR ligands displayed similar effects. A former comparison with the FDA approved adjuvant alumn hydroxide revealed clear advantages for PLGA-MS (119).

Taken together, PLGA-MS posses several beneficial characteristics, rendering them interesting for immunotherapy in humans. The method, used for production is save, rapid and enables up-scaling. Production under sterile conditions is also

possible, even though elaborative. Another possibility to produce a drug that meets the criteria for application in humans would be a subsequent sterilization (120, 121). Our laboratory has positive experiences with γ-irradiation of PLGA-MS, which was carried out earlier as well with similar results.

5. Invariant natural killer cells

Natural killer T (NKT) cells are defined as a linage of T cells that expresses markers of natural killer (NK) cells, such as NK1.1 on a C57BL/6 background, along with semi-invariant CD1d restricted αβ T cell receptors (TCRs) (122). They are subdivided into type I and type II. Whereas the former dominates in mice, the latter prevails in humans. Type II NKT cells have a variable TCR V-gene rearrangement and can be stimulated with the self glycolipid 3-sulfated galactosylceramide (sulfatide) (123, 124). Type I NKT cells are also referred to as invariant NKT (iNKT) cells, because they express an invariant TCR encoded by the Vα14Jα18 gene segment combined with a limited number of Vβ chains in mice (125), and correspondingly the Vα24Jα18 and the Vβ11 chains in humans (126). They can be found in significant numbers in the liver, bone marrow and, in reduced numbers, in the spleen (127). NKT cells are capable of producing a large variety of cytokines, such as IFN-γ, IL-2, IL-3, IL-21, IL-10, TGF-β, TNF-α, GM-CSF, or IL-4 (128-132). Depending on the stimulus and the context of stimulation, they commit for different cytokine profiles. Both, Th1, including IFN-γ and TNF-α, or Th2, including IL-4 and IL-13 or even a combination of both, are possible (122, 125, 133). The production of these cytokines and their rapid release influences the activation of cells downstream the activation cascade, such as DCs, NK cells, B lymphocytes, or T cells. Therefore, they are considered as an important bridge between the adaptive and the innate arm of the immune system (127) (for an overview of NKT activation see figure 4, left hand side).
The recognition of glycolipids in the context of CD1d is a key issue during the activation of iNKT cells (134). The non-polymorphic MHC class I like molecule CD1 exists in several isoforms, namely CD1a-e, which are all expressed in humans, whereas in mice only CD1d exists (135). CD1d is found on DCs, macrophages, thymocytes, hepatocytes (122), and tumor cells (136, 137), which in turn are all capable of stimulating iNKT cells.

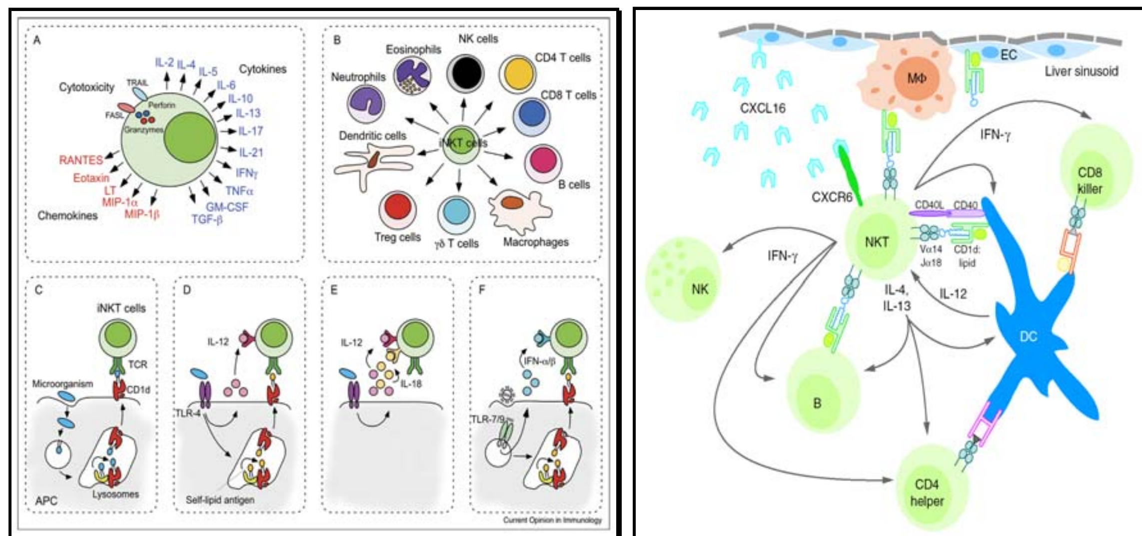

Figure 4: Interaction of NKT cells with their environment: Left hand side: Activated iNKT cells, the 'Swiss-Army knife' of the immune system**.** A) cytokines and chemokines, produced by activated NKT cells. B) Interaction partners of NKT cells. C) Microorganisms directly activate iNKT cells through TCR engagement by glycolipids, presented on CD1d, in the absence of co-stimulation. D) LPS-positive bacteria can activate TLR4-expressing APCs and thereby induce iNKT cell activation through presentation of self-glycolipids in conjunction with IL-12 co-stimulation. Alternatively, E) by an IL-12/IL-18-dependent, CD1d-independent mechanism. F) Finally, intracellular pathogens may stimulate TLR7/9 to induce the presentation of CD1d-restricted, IFN-α/β dependent, self-glycolipids to iNKT cells. Graphic is adopted from reference (127). **Right hand side:** Cellular and molecular network activated by the NKT ligand αGalCer. DCs represent a crucial control center of a cellular network. Upon activation by DCs, NKT cells upregulate CD40L and start the production of Th1 and Th2 cytokines and chemokines, which in turn further activate DCs in order to prime adaptive $CD4^+$ and $CD8^+$ T cell responses. NKT cells can directly induce B cells for antibody production or rapidly activate NK cells. CXCR6/CXCL16 interactions provide essential survival signals for NKT cells. (EC, endothelial cell). Graphic is adopted from reference (122).

There are various ligands for CD1d, the most prominent one is certainly the marine sponge derived glycolipid α-galactosylceramide (α-GalCer) (138) (for NKT interaction upon α-GalCer activation see figure 4, right hand side). The orientation of the ligand in the pocket of CD1d, and therefore the length of its glycosphoingolipids, plays a crucial role for the outcome of the iNKT cells stimulation (139-141). It is under debate, whether a perfect orientation may lead to unphysiologically high cytokine level and therefore less defined cytokine patterns. In this context it becomes interesting, that endogenous ligands have lower affinity to CD1d and tend to more defined cytokine profiles. This may help to explain, why super-activation of iNKT cells by α-GalCer is only occasionally helpful in treatment of autoimmunity models. Given that Th1 and Th2 cytokines are released, they

have opposing and maybe neutralizing effects. In general, the benefit of an activation of iNKT cells in autoimmune diseases strongly depends on and varies with the ligand that is used and the time it is applied (142).

6. CD1d ligands – α-GalCer and its analogues

Since most ligands for CD1d are synthetic, they are excessively numerous and diverse. In principle, they can be divided in two classes, namely endogenous ligands like sulfatide or iGb3, activating type II NKT cells (125), and exogenous ligands. This passage will focus on exogenous ligands, which are most prominently represented by α-GalCer. In 1995, the company Kirin Pharmaceuticals synthesized α-GalCer, a glycolipid based on a glycosphingolipid from an extract of the marine sponge *Agelas mauritianus*, that was collected in the Okinawan sea (143). α-GalCer (for chemical structure see figure 5) was subsequently found to have anti tumor effects when applied to tumor bearing mice (138) and to effectively activate NKT cells (144). It has been shown for α-GalCer that the length of the lipid chains, which is essential for binding into the CD1d binding groove (140, 141), is perfectly orientated and therefore commits for perfect signaling (139). Depending on the cells on which it is presented, α-GalCer leads to a cytokine storm produced by NKT cells. Within minutes α-GalCer leads to the production of IL-4 and IFN-γ, if its presented by DCs (145), but if it is less effectively presented, e.g., by Swann cells, or B cells it induces only IL-4 (146).

In general, NTK cells produce a variety of cytokines upon stimulation with α-GalCer, such as IFN-γ, IL-2, IL-4, IL-10, IL-12, TGF-β, TNF-α, GM-CSF or IL-4 (131, 132). This makes α-GalCer a potent modulator of immune responses but the ability to induce both, Th1 and Th2 polarizing cytokines, make α-GalCer only occasionally helpful in therapy of autoimmunity models (152). In experimental autoimmune encephalomyelitis (EAE), beneficial effect could be observed after repeated injection of α-GalCer. It is lively discussed, if this effect is caused by anergy of the NKT cells, which hinders the production of IFN-γ but not IL-4 (147), or if it is due to an induction of T-reg cells and the subsequent rising of serum IL-10 (148). At least it could be shown that timing of the injection and MHC haplotypes play important roles in this model (149, 150).

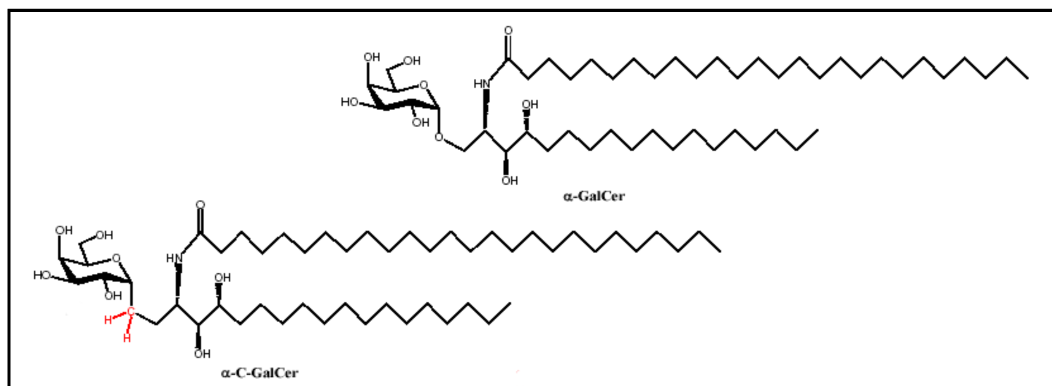

Figure 5: Chemical structures of α-galactosylceramide (α-GalCer) and α-C-galactosylceramide (α-C-GalCer): Differences between the two chemical structures are highlighted in red.

In other models, e.g., CD8+ T cell mediated diabetes or DSS induced colitis, α-GalCer showed beneficial effects like reduced hemorrhage rate, improved survival, or improved weight gain (151).

An analogue of α-GalCer with a more defined cytokine pattern is α-C-galactosylceramide (α-C-GalCer) (153, 154) (for chemical structure see figure 5). The C-glycoside has been published by Frank and colleagues to induce production of substantial amounts of IFN-γ in the almost complete absence of IL-4 and is therefore showing improved modulatory functions. More recent studies confirmed some of their aspects, referring to the cytokine profile (155) or the adjuvant effect (156). In an autoimmune model, α-C-GalCer could be shown to have a beneficial effect in EAE (142), independent of the Th2 cytokine IL-4 or of IL-10. In 2006, another α-GalCer analogue synthesis was described, in which the C26 *N*-fatty acyl tail was replaced by a C8 *N*-fatty acyl tail and a terminal benzene. The interactions formed between the aromatic substitute of the glycolipid and aromatic CD1d side-chain residues contribute additional stability to the complex (157). The new molecule altered the cytokine profile towards Th1 and showed a significantly higher anticancer efficacy than α-GalCer (158).

CD1d ligands with an opposite cytokine pattern, referring to Th1/Th2 bias, are C20:2 (159) or more prominent OCH (160). OCH has a truncated sphingosine chain, and therefore binds ineffectively to CD1d resulting in a more defined cytokine pattern biased towards Th2. Experiments using OCH in autoimmune models for type I diabetes (T1D) (161), STZ induced diabetes, collagen-induced arthritis (CIA) (162) or EAE (160) showed that OCH is a very potent Th2 polarizing agent, leading to reduced symptoms and improved progress of the disease. Also a

very clear Th2 cytokine pattern could be demonstrated for the first OCH analogue in 2009 (163).

This summary of the most studied synthetic CD1d ligands clearly mirrors the high diversity in this field, even though the list is far from being complete. Depending on the required cytokine environment, a variety of ligands exists, more or less specific and more or less studied in immunological models. Predictions about the cytokine pattern induced by a new ligand fail in most cases, for many reasons. On the one hand it is clearly due to the chemical features of the side chains, on the other hand multiple factors of the immune system influence the outcome.

In general it seems evident that stimulation with a synthetic superantigen like α-GalCer is like opening Pandora's box, over-stimulating the cytokine production. Still, it needs to be clarified, whether it is exclusively due to the binding properties of the CD1d ligand. Most likely other factors, like the time point of injection or the context of the stimulus, play a role, too.

7. Aim of thesis

Effective immunotherapy of tumors requires a suitable antigen on the one hand and efficient activation of DCs for proper stimulation of $CD4^+$ and $CD8^+$ T cells on the other. The main topic of this thesis is the characterization and optimization of PLGA-MS as a delivery device for TAA and immunostimulatory adjuvants. This implies the search for an accessible tumor antigen and alternative immunostimulatory adjuvants. Besides further characterization of the system, we wanted to prove the efficacy of PLGA-MS in a broad range of tumor models. Furthermore, we wanted to compare the PLGA-MS to IFA, the best established carrier system in immunizations, in order to be able to estimate their potential in immunotherapy of tumors.

8. References of chapter I:

1. Kubach, J., C. Becker, E. Schmitt, K. Steinbrink, E. Huter, A. Tuettenberg, and H. Jonuleit. 2005. Dendritic cells: sentinels of immunity and tolerance. *Int J Hematol* 81:197-203.
2. Steinman, R.M., and Z.A. Cohn. 1973. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J Exp Med* 137:1142-1162.
3. Banchereau, J., and R.M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature* 392:245-252.
4. Steinman, R.M., D. Hawiger, and M.C. Nussenzweig. 2003. Tolerogenic dendritic cells. *Annu Rev Immunol* 21:685-711.
5. Mellman, I., and R.M. Steinman. 2001. Dendritic cells: specialized and regulated antigen processing machines. *Cell* 106:255-258.
6. Schakel, K. 2009. Dendritic cells--why can they help and hurt us. *Exp Dermatol* 18:264-273.
7. Proudfoot, O., V. Apostolopoulos, and G.A. Pietersz. 2007. Receptor-mediated delivery of antigens to dendritic cells: anticancer applications. *Mol Pharm* 4:58-72.
8. Lanzavecchia, A. 1996. Mechanisms of antigen uptake for presentation. *Curr Opin Immunol* 8:348-354.
9. Sallusto, F., P. Schaerli, P. Loetscher, C. Schaniel, D. Lenig, C.R. Mackay, S. Qin, and A. Lanzavecchia. 1998. Rapid and coordinated switch in chemokine receptor expression during dendritic cell maturation. *Eur J Immunol* 28:2760-2769.
10. Sozzani, S., P. Allavena, G. D'Amico, W. Luini, G. Bianchi, M. Kataura, T. Imai, O. Yoshie, R. Bonecchi, and A. Mantovani. 1998. Differential regulation of chemokine receptors during dendritic cell maturation: a model for their trafficking properties. *J Immunol* 161:1083-1086.
11. Yanagihara, S., E. Komura, J. Nagafune, H. Watarai, and Y. Yamaguchi. 1998. EBI1/CCR7 is a new member of dendritic cell chemokine receptor that is up-regulated upon maturation. *J Immunol* 161:3096-3102.
12. Kaparakis, M., D.J. Philpott, and R.L. Ferrero. 2007. Mammalian NLR proteins; discriminating foe from friend. *Immunol Cell Biol* 85:495-502.
13. Akira, S. 2003. Mammalian Toll-like receptors. *Curr Opin Immunol* 15:5-11.
14. Akira, S. 2006. TLR signaling. *Curr Top Microbiol Immunol* 311:1-16.
15. Blander, J.M., and R. Medzhitov. 2004. Regulation of phagosome maturation by signals from toll-like receptors. *Science* 304:1014-1018.
16. Chu, R.S., O.S. Targoni, A.M. Krieg, P.V. Lehmann, and C.V. Harding. 1997. CpG oligodeoxynucleotides act as adjuvants that switch on T helper 1 (Th1) immunity. *J Exp Med* 186:1623-1631.
17. Ioannou, X.P., S.M. Gomis, B. Karvonen, R. Hecker, L.A. Babiuk, and S. van Drunen Littel-van den Hurk. 2002. CpG-containing oligodeoxynucleotides, in combination with conventional adjuvants, enhance the magnitude and change the bias of the immune responses to a herpesvirus glycoprotein. *Vaccine* 21:127-137.
18. Miconnet, I., S. Koenig, D. Speiser, A. Krieg, P. Guillaume, J.C. Cerottini, and P. Romero. 2002. CpG are efficient adjuvants for specific CTL induction against tumor antigen-derived peptide. *J Immunol* 168:1212-1218.

19. Forster, R., A. Schubel, D. Breitfeld, E. Kremmer, I. Renner-Muller, E. Wolf, and M. Lipp. 1999. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell* 99:23-33.
20. Gunn, M.D., S. Kyuwa, C. Tam, T. Kakiuchi, A. Matsuzawa, L.T. Williams, and H. Nakano. 1999. Mice lacking expression of secondary lymphoid organ chemokine have defects in lymphocyte homing and dendritic cell localization. *J Exp Med* 189:451-460.
21. MartIn-Fontecha, A., S. Sebastiani, U.E. Hopken, M. Uguccioni, M. Lipp, A. Lanzavecchia, and F. Sallusto. 2003. Regulation of dendritic cell migration to the draining lymph node: impact on T lymphocyte traffic and priming. *J Exp Med* 198:615-621.
22. Kedl, R.M., J.W. Kappler, and P. Marrack. 2003. Epitope dominance, competition and T cell affinity maturation. *Curr Opin Immunol* 15:120-127.
23. Bonifaz, L., D. Bonnyay, K. Mahnke, M. Rivera, M.C. Nussenzweig, and R.M. Steinman. 2002. Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral CD8+ T cell tolerance. *J Exp Med* 196:1627-1638.
24. Probst, H.C., K. McCoy, T. Okazaki, T. Honjo, and M. van den Broek. 2005. Resting dendritic cells induce peripheral CD8+ T cell tolerance through PD-1 and CTLA-4. *Nat Immunol* 6:280-286.
25. Reis e Sousa, C. 2001. Dendritic cells as sensors of infection. *Immunity* 14:495-498.
26. Obst, R., H.M. van Santen, R. Melamed, A.O. Kamphorst, C. Benoist, and D. Mathis. 2007. Sustained antigen presentation can promote an immunogenic T cell response, like dendritic cell activation. *Proc Natl Acad Sci U S A* 104:15460-15465.
27. Iezzi, G., K. Karjalainen, and A. Lanzavecchia. 1998. The duration of antigenic stimulation determines the fate of naive and effector T cells. *Immunity* 8:89-95.
28. Schoenberger, S.P., R.E. Toes, E.I. van der Voort, R. Offringa, and C.J. Melief. 1998. T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature* 393:480-483.
29. Liu, Y.J. 2001. Dendritic cell subsets and lineages, and their functions in innate and adaptive immunity. *Cell* 106:259-262.
30. Vremec, D., M. Zorbas, R. Scollay, D.J. Saunders, C.F. Ardavin, L. Wu, and K. Shortman. 1992. The surface phenotype of dendritic cells purified from mouse thymus and spleen: investigation of the CD8 expression by a subpopulation of dendritic cells. *J Exp Med* 176:47-58.
31. Dudziak, D., A.O. Kamphorst, G.F. Heidkamp, V.R. Buchholz, C. Trumpfheller, S. Yamazaki, C. Cheong, K. Liu, H.W. Lee, C.G. Park, R.M. Steinman, and M.C. Nussenzweig. 2007. Differential antigen processing by dendritic cell subsets in vivo. *Science* 315:107-111.
32. Rock, K.L. 1996. A new foreign policy: MHC class I molecules monitor the outside world. *Immunol Today* 17:131-137.
33. Rock, K.L., and L. Shen. 2005. Cross-presentation: underlying mechanisms and role in immune surveillance. *Immunol Rev* 207:166-183.
34. Huang, A.Y., P. Golumbek, M. Ahmadzadeh, E. Jaffee, D. Pardoll, and H. Levitsky. 1994. Role of bone marrow-derived cells in presenting MHC class I-restricted tumor antigens. *Science* 264:961-965.

35. Nelson, D., C. Bundell, and B. Robinson. 2000. In vivo cross-presentation of a soluble protein antigen: kinetics, distribution, and generation of effector CTL recognizing dominant and subdominant epitopes. *J Immunol* 165:6123-6132.
36. Delamarre, L., H. Holcombe, and I. Mellman. 2003. Presentation of exogenous antigens on major histocompatibility complex (MHC) class I and MHC class II molecules is differentially regulated during dendritic cell maturation. *J Exp Med* 198:111-122.
37. Ramirez, M.C., and L.J. Sigal. 2002. Macrophages and dendritic cells use the cytosolic pathway to rapidly cross-present antigen from live, vaccinia-infected cells. *J Immunol* 169:6733-6742.
38. Qu, C., V.A. Nguyen, M. Merad, and G.J. Randolph. 2009. MHC class I/peptide transfer between dendritic cells overcomes poor cross-presentation by monocyte-derived APCs that engulf dying cells. *J Immunol* 182:3650-3659.
39. Heath, W.R., and F.R. Carbone. 2001. Cross-presentation in viral immunity and self-tolerance. *Nat Rev Immunol* 1:126-134.
40. Nakano, H., K.L. Lin, M. Yanagita, C. Charbonneau, D.N. Cook, T. Kakiuchi, and M.D. Gunn. 2009. Blood-derived inflammatory dendritic cells in lymph nodes stimulate acute T helper type 1 immune responses. *Nat Immunol* 10:394-402.
41. Blattman, J.N., and P.D. Greenberg. 2004. Cancer immunotherapy: a treatment for the masses. *Science* 305:200-205.
42. Zafir-Lavie, I., Y. Michaeli, and Y. Reiter. 2007. Novel antibodies as anticancer agents. *Oncogene* 26:3714-3733.
43. Timmerman, J.M., D.K. Czerwinski, T.A. Davis, F.J. Hsu, C. Benike, Z.M. Hao, B. Taidi, R. Rajapaksa, C.B. Caspar, C.Y. Okada, A. van Beckhoven, T.M. Liles, E.G. Engleman, and R. Levy. 2002. Idiotype-pulsed dendritic cell vaccination for B-cell lymphoma: clinical and immune responses in 35 patients. *Blood* 99:1517-1526.
44. Copier, J., S. Ward, and A. Dalgleish. 2007. Cell based cancer vaccines: regulatory and commercial development. *Vaccine* 25 Suppl 2:B35-46.
45. Hsu, F.J., C. Benike, F. Fagnoni, T.M. Liles, D. Czerwinski, B. Taidi, E.G. Engleman, and R. Levy. 1996. Vaccination of patients with B-cell lymphoma using autologous antigen-pulsed dendritic cells. *Nat Med* 2:52-58.
46. Celluzzi, C.M., J.I. Mayordomo, W.J. Storkus, M.T. Lotze, and L.D. Falo, Jr. 1996. Peptide-pulsed dendritic cells induce antigen-specific CTL-mediated protective tumor immunity. *J Exp Med* 183:283-287.
47. Nestle, F.O., S. Alijagic, M. Gilliet, Y. Sun, S. Grabbe, R. Dummer, G. Burg, and D. Schadendorf. 1998. Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. *Nat Med* 4:328-332.
48. Nestle, F.O. 2002. Dendritic cell vaccination for the treatment of skin cancer. *Recent Results Cancer Res* 160:165-169.
49. Waeckerle-Men, Y., E. Uetz-von Allmen, M. Fopp, R. von Moos, C. Bohme, H.P. Schmid, D. Ackermann, T. Cerny, B. Ludewig, M. Groettrup, and S. Gillessen. 2006. Dendritic cell-based multi-epitope immunotherapy of hormone-refractory prostate carcinoma. *Cancer Immunol Immunother*.
50. Susumu, S., Y. Nagata, S. Ito, M. Matsuo, D. Valmori, K. Yui, H. Udono, and T. Kanematsu. 2008. Cross-presentation of NY-ESO-1 cytotoxic T lymphocyte epitope fused to human heat shock cognate protein 70 by dendritic cells. *Cancer Sci* 99:107-112.

51. Wagner, H. 2009. The immunogenicity of CpG-antigen conjugates. *Adv Drug Deliv Rev* 61:243-247.
52. Cerundolo, V., I.F. Hermans, and M. Salio. 2004. Dendritic cells: a journey from laboratory to clinic. *Nat Immunol* 5:7-10.
53. Ludewig, B., K. McCoy, M. Pericin, A.F. Ochsenbein, T. Dumrese, B. Odermatt, R.E. Toes, C.J. Melief, H. Hengartner, and R.M. Zinkernagel. 2001. Rapid peptide turnover and inefficient presentation of exogenous antigen critically limit the activation of self-reactive CTL by dendritic cells. *J Immunol* 166:3678-3687.
54. Carroll, R.G., and C.H. June. 2007. Programming the next generation of dendritic cells. *Mol Ther* 15:846-848.
55. Fontana, R., M. Bregni, A. Cipponi, L. Raccosta, C. Rainelli, D. Maggioni, F. Lunghi, F. Ciceri, S. Mukenge, C. Doglioni, D. Colau, P.G. Coulie, C. Bordignon, C. Traversari, and V. Russo. 2009. Peripheral blood lymphocytes genetically modified to express the self/tumor antigen MAGE-A3 induce antitumor immune responses in cancer patients. *Blood* 113:1651-1660.
56. June, C.H. 2007. Adoptive T cell therapy for cancer in the clinic. *J Clin Invest* 117:1466-1476.
57. Kronig, H., K. Hofer, H. Conrad, P. Guilaume, J. Muller, M. Schiemann, V. Lennerz, A. Cosma, C. Peschel, D.H. Busch, P. Romero, and H. Bernhard. 2009. Allorestricted T lymphocytes with a high avidity T-cell receptor towards NY-ESO-1 have potent anti-tumor activity. *Int J Cancer* 125:649-655.
58. Mateo, L., J. Gardner, Q. Chen, C. Schmidt, M. Down, S.L. Elliott, S.J. Pye, H. Firat, F.A. Lemonnier, J. Cebon, and A. Suhrbier. 1999. An HLA-A2 polyepitope vaccine for melanoma immunotherapy. *J Immunol* 163:4058-4063.
59. Amato, R.J., J. Hernandez-McClain, and H. Henary. 2009. Phase 2 study of granulocyte-macrophage colony-stimulating factor plus thalidomide in patients with hormone-naive adenocarcinoma of the prostate. *Urol Oncol* 27:8-13.
60. Dillman, R.O., S.R. Selvan, P.M. Schiltz, E.F. McClay, N.M. Barth, C. DePriest, C. de Leon, C. Mayorga, A.N. Cornforth, and K. Allen. 2009. Phase II trial of dendritic cells loaded with antigens from self-renewing, proliferating autologous tumor cells as patient-specific antitumor vaccines in patients with metastatic melanoma: final report. *Cancer Biother Radiopharm* 24:311-319.
61. Motohashi, S., K. Nagato, N. Kunii, H. Yamamoto, K. Yamasaki, K. Okita, H. Hanaoka, N. Shimizu, M. Suzuki, I. Yoshino, M. Taniguchi, T. Fujisawa, and T. Nakayama. 2009. A phase I-II study of alpha-galactosylceramide-pulsed IL-2/GM-CSF-cultured peripheral blood mononuclear cells in patients with advanced and recurrent non-small cell lung cancer. *J Immunol* 182:2492-2501.
62. Ansell, S.M., S.A. Hurvitz, P.A. Koenig, B.R. LaPlant, B.F. Kabat, D. Fernando, T.M. Habermann, D.J. Inwards, M. Verma, R. Yamada, C. Erlichman, I. Lowy, and J.M. Timmerman. 2009. Phase I study of ipilimumab, an anti-CTLA-4 monoclonal antibody, in patients with relapsed and refractory B-cell non-Hodgkin lymphoma. *Clin Cancer Res* 15:6446-6453.

63. Mumprecht, S., C. Schurch, J. Schwaller, M. Solenthaler, and A.F. Ochsenbein. 2009. Programmed death 1 signaling on chronic myeloid leukemia-specific T cells results in T-cell exhaustion and disease progression. *Blood* 114:1528-1536.
64. Knutson, K.L., Y. Dang, H. Lu, J. Lukas, B. Almand, E. Gad, E. Azeke, and M.L. Disis. 2006. IL-2 immunotoxin therapy modulates tumor-associated regulatory T cells and leads to lasting immune-mediated rejection of breast cancers in neu-transgenic mice. *J Immunol* 177:84-91.
65. Zhang, T., and D. Herlyn. 2009. Combination of active specific immunotherapy or adoptive antibody or lymphocyte immunotherapy with chemotherapy in the treatment of cancer. *Cancer Immunol Immunother* 58:475-492.
66. Berinstein, N.L. 2007. Enhancing cancer vaccines with immunomodulators. *Vaccine* 25 Suppl 2:B72-88.
67. Accapezzato, D., V. Visco, V. Francavilla, C. Molette, T. Donato, M. Paroli, M.U. Mondelli, M. Doria, M.R. Torrisi, and V. Barnaba. 2005. Chloroquine enhances human CD8+ T cell responses against soluble antigens in vivo. *J Exp Med* 202:817-828.
68. Ohlschlager, P., M. Quetting, G. Alvarez, M. Durst, L. Gissmann, and A.M. Kaufmann. 2009. Enhancement of immunogenicity of a therapeutic cervical cancer DNA-based vaccine by co-application of sequence-optimized genetic adjuvants. *Int J Cancer* 125:189-198.
69. Simpson-Abelson, M.R., V.S. Purohit, W.M. Pang, V. Iyer, K. Odunsi, T.L. Demmy, S.J. Yokota, J.L. Loyall, R.J. Kelleher, Jr., S. Balu-Iyer, and R.B. Bankert. 2009. IL-12 delivered intratumorally by multilamellar liposomes reactivates memory T cells in human tumor microenvironments. *Clin Immunol* 132:71-82.
70. Sparwasser, T., R.M. Vabulas, B. Villmow, G.B. Lipford, and H. Wagner. 2000. Bacterial CpG-DNA activates dendritic cells in vivo: T helper cell-independent cytotoxic T cell responses to soluble proteins. *Eur J Immunol* 30:3591-3597.
71. Napolitani, G., A. Rinaldi, F. Bertoni, F. Sallusto, and A. Lanzavecchia. 2005. Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells. *Nat Immunol* 6:769-776.
72. Schlosser, E., M. Mueller, S. Fischer, S. Basta, D.H. Busch, B. Gander, and M. Groettrup. 2008. TLR ligands and antigen need to be coencapsulated into the same biodegradable microsphere for the generation of potent cytotoxic T lymphocyte responses. *Vaccine* 26:1626-1637.
73. Heit, A., F. Schmitz, T. Haas, D.H. Busch, and H. Wagner. 2007. Antigen co-encapsulated with adjuvants efficiently drive protective T cell immunity. *Eur J Immunol* 37:2063-2074.
74. Blander, J.M., and R. Medzhitov. 2006. Toll-dependent selection of microbial antigens for presentation by dendritic cells. *Nature* 440:808-812.
75. Cho, H.C., B.H. Kim, K. Kim, J.Y. Park, J.H. Chang, and S.K. Kim. 2008. Cancer immunotherapeutic effects of novel CpG ODN in murine tumor model. *Int Immunopharmacol* 8:1401-1407.
76. Matsumoto, M., and T. Seya. 2008. TLR3: interferon induction by double-stranded RNA including poly(I:C). *Adv Drug Deliv Rev* 60:805-812.
77. Navabi, H., B. Jasani, A. Reece, A. Clayton, Z. Tabi, C. Donninger, M. Mason, and M. Adams. 2009. A clinical grade poly I:C-analogue (Ampligen)

promotes optimal DC maturation and Th1-type T cell responses of healthy donors and cancer patients in vitro. *Vaccine* 27:107-115.
78. Peter, K., Y. Men, G. Pantaleo, B. Gander, and G. Corradin. 2001. Induction of a cytotoxic T-cell response to HIV-1 proteins with short synthetic peptides and human compatible adjuvants. *Vaccine* 19:4121-4129.
79. Schulz, O., S.S. Diebold, M. Chen, T.I. Naslund, M.A. Nolte, L. Alexopoulou, Y.T. Azuma, R.A. Flavell, P. Liljestrom, and C. Reis e Sousa. 2005. Toll-like receptor 3 promotes cross-priming to virus-infected cells. *Nature* 433:887-892.
80. McBride, S., K. Hoebe, P. Georgel, and E. Janssen. 2006. Cell-associated double-stranded RNA enhances antitumor activity through the production of type I IFN. *J Immunol* 177:6122-6128.
81. Bourquin, C., L. Schmidt, V. Hornung, C. Wurzenberger, D. Anz, N. Sandholzer, S. Schreiber, A. Voelkl, G. Hartmann, and S. Endres. 2007. Immunostimulatory RNA oligonucleotides trigger an antigen-specific cytotoxic T-cell and IgG2a response. *Blood* 109:2953-2960.
82. Johnston, D., and J.C. Bystryn. 2006. Topical imiquimod is a potent adjuvant to a weakly-immunogenic protein prototype vaccine. *Vaccine* 24:1958-1965.
83. Wagner, T.L., C.L. Ahonen, A.M. Couture, S.J. Gibson, R.L. Miller, R.M. Smith, M.J. Reiter, J.P. Vasilakos, and M.A. Tomai. 1999. Modulation of TH1 and Th2 cytokine production with the immune response modifiers, R-848 and imiquimod. *Cell Immunol* 191:10-19.
84. Doxsee, C.L., T.R. Riter, M.J. Reiter, S.J. Gibson, J.P. Vasilakos, and R.M. Kedl. 2003. The immune response modifier and Toll-like receptor 7 agonist S-27609 selectively induces IL-12 and TNF-alpha production in CD11c+CD11b+CD8- dendritic cells. *J Immunol* 171:1156-1163.
85. Lu, S. 2009. Heterologous prime-boost vaccination. *Curr Opin Immunol* 21:346-351.
86. Guillonneau, C., J.D. Mintern, F.X. Hubert, A.C. Hurt, G.S. Besra, S. Porcelli, I.G. Barr, P.C. Doherty, D.I. Godfrey, and S.J. Turner. 2009. Combined NKT cell activation and influenza virus vaccination boosts memory CTL generation and protective immunity. *Proc Natl Acad Sci U S A* 106:3330-3335.
87. Kim, S., J.B. Lee, G.K. Lee, and J. Chang. 2009. Vaccination with recombinant adenoviruses and dendritic cells expressing prostate-specific antigens is effective in eliciting CTL and suppresses tumor growth in the experimental prostate cancer. *Prostate* 69:938-948.
88. Schwarz, K., E. Meijerink, D.E. Speiser, A.C. Tissot, I. Cielens, R. Renhof, A. Dishlers, P. Pumpens, and M.F. Bachmann. 2005. Efficient homologous prime-boost strategies for T cell vaccination based on virus-like particles. *Eur J Immunol* 35:816-821.
89. Janeway, C.A., Jr. 1989. Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb Symp Quant Biol* 54 Pt 1:1-13.
90. Heit, A., F. Schmitz, M. O'Keeffe, C. Staib, D.H. Busch, H. Wagner, and K.M. Huster. 2005. Protective CD8 T cell immunity triggered by CpG-protein conjugates competes with the efficacy of live vaccines. *J Immunol* 174:4373-4380.
91. Dreicer, R., W.M. Stadler, F.R. Ahmann, T. Whiteside, N. Bizouarne, B. Acres, J.M. Limacher, P. Squiban, and A. Pantuck. 2009. MVA-MUC1-IL2

vaccine immunotherapy (TG4010) improves PSA doubling time in patients with prostate cancer with biochemical failure. *Invest New Drugs* 27:379-386.

92. Aurisicchio, L., D. Peruzzi, A. Conforti, S. Dharmapuri, A. Biondo, S. Giampaoli, A. Fridman, A. Bagchi, C.T. Winkelmann, R. Gibson, E.R. Kandimalla, S. Agrawal, G. Ciliberto, and N. La Monica. 2009. Treatment of mammary carcinomas in HER-2 transgenic mice through combination of genetic vaccine and an agonist of Toll-like receptor 9. *Clin Cancer Res* 15:1575-1584.
93. Badiee, A., N. Davies, K. McDonald, K. Radford, H. Michiue, D. Hart, and M. Kato. 2007. Enhanced delivery of immunoliposomes to human dendritic cells by targeting the multilectin receptor DEC-205. *Vaccine* 25:4757-4766.
94. Storni, T., and M.F. Bachmann. 2004. Loading of MHC class I and II presentation pathways by exogenous antigens: a quantitative in vivo comparison. *J Immunol* 172:6129-6135.
95. Freund, J. 1951. The effect of paraffin oil and mycobacteria on antibody formation and sensitization; a review. *Am J Clin Pathol* 21:645-656.
96. Speiser, D.E., D. Lienard, N. Rufer, V. Rubio-Godoy, D. Rimoldi, F. Lejeune, A.M. Krieg, J.C. Cerottini, and P. Romero. 2005. Rapid and strong human CD8+ T cell responses to vaccination with peptide, IFA, and CpG oligodeoxynucleotide 7909. *J Clin Invest* 115:739-746.
97. Miller, L.H., A. Saul, and S. Mahanty. 2005. Revisiting Freund's incomplete adjuvant for vaccines in the developing world. *Trends Parasitol* 21:412-414.
98. Billiau, A., and P. Matthys. 2001. Modes of action of Freund's adjuvants in experimental models of autoimmune diseases. *J Leukoc Biol* 70:849-860.
99. Beebe, G.W., A.H. Simon, and S. Vivona. 1972. Long-term mortality follow-up of Army recruits who received adjuvant influenza virus vaccine in 1951-1953. *Am J Epidemiol* 95:337-346.
100. Toledo, H., A. Baly, O. Castro, S. Resik, J. Laferte, F. Rolo, L. Navea, L. Lobaina, O. Cruz, J. Miguez, T. Serrano, B. Sierra, L. Perez, M.E. Ricardo, M. Dubed, A.L. Lubian, M. Blanco, J.C. Millan, A. Ortega, E. Iglesias, E. Penton, Z. Martin, J. Perez, M. Diaz, and C.A. Duarte. 2001. A phase I clinical trial of a multi-epitope polypeptide TAB9 combined with Montanide ISA 720 adjuvant in non-HIV-1 infected human volunteers. *Vaccine* 19:4328-4336.
101. Jain, R.A. 2000. The manufacturing techniques of various drug loaded biodegradable poly(lactide-co-glycolide) (PLGA) devices. *Biomaterials* 21:2475-2490.
102. Johansen, P., Y. Men, H.P. Merkle, and B. Gander. 2000. Revisiting PLA/PLGA microspheres: an analysis of their potential in parenteral vaccination. *Eur J Pharm Biopharm* 50:129-146.
103. Singh, M., X.M. Li, H. Wang, J.P. McGee, T. Zamb, W. Koff, C.Y. Wang, and D.T. O'Hagan. 1997. Immunogenicity and protection in small-animal models with controlled-release tetanus toxoid microparticles as a single-dose vaccine. *Infect Immun* 65:1716-1721.
104. Gander, B. 2005. Trends in particulate antigen and DNA delivery systems for vaccines. *Adv Drug Deliv Rev* 57:321-323.
105. Krishnamachari, Y., and A.K. Salem. 2009. Innovative strategies for co-delivering antigens and CpG oligonucleotides. *Adv Drug Deliv Rev* 61:205-217.

106. Reddy, S.T., M.A. Swartz, and J.A. Hubbell. 2006. Targeting dendritic cells with biomaterials: developing the next generation of vaccines. *Trends Immunol* 27:573-579.
107. Waeckerle-Men, Y., E. Scandella, E. Uetz-Von Allmen, B. Ludewig, S. Gillessen, H.P. Merkle, B. Gander, and M. Groettrup. 2004. Phenotype and functional analysis of human monocyte-derived dendritic cells loaded with biodegradable poly(lactide-co-glycolide) microspheres for immunotherapy. *J Immunol Methods* 287:109-124.
108. Shen, Z., G. Reznikoff, G. Dranoff, and K.L. Rock. 1997. Cloned dendritic cells can present exogenous antigens on both MHC class I and class II molecules. *J Immunol* 158:2723-2730.
109. Manolova, V., A. Flace, M. Bauer, K. Schwarz, P. Saudan, and M.F. Bachmann. 2008. Nanoparticles target distinct dendritic cell populations according to their size. *Eur J Immunol* 38:1404-1413.
110. Panyam, J., W.Z. Zhou, S. Prabha, S.K. Sahoo, and V. Labhasetwar. 2002. Rapid endo-lysosomal escape of poly(DL-lactide-co-glycolide) nanoparticles: implications for drug and gene delivery. *Faseb J* 16:1217-1226.
111. Audran, R., K. Peter, J. Dannull, Y. Men, E. Scandella, M. Groettrup, B. Gander, and G. Corradin. 2003. Encapsulation of peptides in biodegradable microspheres prolongs their MHC class-I presentation by dendritic cells and macrophages in vitro. *Vaccine* 21:1250-1255.
112. Waeckerle-Men, Y., E.U. Allmen, B. Gander, E. Scandella, E. Schlosser, G. Schmidtke, H.P. Merkle, and M. Groettrup. 2006. Encapsulation of proteins and peptides into biodegradable poly(D,L-lactide-co-glycolide) microspheres prolongs and enhances antigen presentation by human dendritic cells. *Vaccine* 24:1847-1857.
113. Waeckerle-Men, Y., and M. Groettrup. 2005. PLGA microspheres for improved antigen delivery to dendritic cells as cellular vaccines. *Adv Drug Deliv Rev* 57:475-482.
114. Malyala, P., D.T. O'Hagan, and M. Singh. 2009. Enhancing the therapeutic efficacy of CpG oligonucleotides using biodegradable microparticles. *Adv Drug Deliv Rev* 61:218-225.
115. Men, Y., R. Audran, C. Thomasin, G. Eberl, S. Demotz, H.P. Merkle, B. Gander, and G. Corradin. 1999. MHC class I- and class II-restricted processing and presentation of microencapsulated antigens. *Vaccine* 17:1047-1056.
116. Men, Y., B. Gander, H.P. Merkle, and G. Corradin. 1996. Induction of sustained and elevated immune responses to weakly immunogenic synthetic malarial peptides by encapsulation in biodegradable polymer microspheres. *Vaccine* 14:1442-1450.
117. Goforth, R., A.K. Salem, X. Zhu, S. Miles, X.Q. Zhang, J.H. Lee, and A.D. Sandler. 2009. Immune stimulatory antigen loaded particles combined with depletion of regulatory T-cells induce potent tumor specific immunity in a mouse model of melanoma. *Cancer Immunol Immunother* 58:517-530.
118. Hamdy, S., O. Molavi, Z. Ma, A. Haddadi, A. Alshamsan, Z. Gobti, S. Elhasi, J. Samuel, and A. Lavasanifar. 2008. Co-delivery of cancer-associated antigen and Toll-like receptor 4 ligand in PLGA nanoparticles induces potent CD8+ T cell-mediated anti-tumor immunity. *Vaccine* 26:5046-5057.

119. Men, Y., C. Thomasin, H.P. Merkle, B. Gander, and G. Corradin. 1995. A single administration of tetanus toxoid in biodegradable microspheres elicits T cell and antibody responses similar or superior to those obtained with aluminum hydroxide. *Vaccine* 13:683-689.
120. Herrero-Vanrell, R., L. Ramirez, A. Fernandez-Carballido, and M.F. Refojo. 2000. Biodegradable PLGA microspheres loaded with ganciclovir for intraocular administration. Encapsulation technique, in vitro release profiles, and sterilization process. *Pharm Res* 17:1323-1328.
121. Montanari, L., F. Cilurzo, F. Selmin, B. Conti, I. Genta, G. Poletti, F. Orsini, and L. Valvo. 2003. Poly(lactide-co-glycolide) microspheres containing bupivacaine: comparison between gamma and beta irradiation effects. *J Control Release* 90:281-290.
122. Bendelac, A., P.B. Savage, and L. Teyton. 2007. The biology of NKT cells. *Annu Rev Immunol* 25:297-336.
123. Behar, S.M., T.A. Podrebarac, C.J. Roy, C.R. Wang, and M.B. Brenner. 1999. Diverse TCRs recognize murine CD1. *J Immunol* 162:161-167.
124. Jahng, A., I. Maricic, C. Aguilera, S. Cardell, R.C. Halder, and V. Kumar. 2004. Prevention of autoimmunity by targeting a distinct, noninvariant CD1d-reactive T cell population reactive to sulfatide. *J Exp Med* 199:947-957.
125. Godfrey, D.I., and M. Kronenberg. 2004. Going both ways: immune regulation via CD1d-dependent NKT cells. *J Clin Invest* 114:1379-1388.
126. Porcelli, S., C.E. Yockey, M.B. Brenner, and S.P. Balk. 1993. Analysis of T cell antigen receptor (TCR) expression by human peripheral blood CD4-8-alpha/beta T cells demonstrates preferential use of several V beta genes and an invariant TCR alpha chain. *J Exp Med* 178:1-16.
127. Matsuda, J.L., T. Mallevaey, J. Scott-Browne, and L. Gapin. 2008. CD1d-restricted iNKT cells, the 'Swiss-Army knife' of the immune system. *Curr Opin Immunol* 20:358-368.
128. Coquet, J.M., K. Kyparissoudis, D.G. Pellicci, G. Besra, S.P. Berzins, M.J. Smyth, and D.I. Godfrey. 2007. IL-21 is produced by NKT cells and modulates NKT cell activation and cytokine production. *J Immunol* 178:2827-2834.
129. Leite-de-Moraes, M.C., M. Lisbonne, A. Arnould, F. Machavoine, A. Herbelin, M. Dy, and E. Schneider. 2002. Ligand-activated natural killer T lymphocytes promptly produce IL-3 and GM-CSF in vivo: relevance to peripheral myeloid recruitment. *Eur J Immunol* 32:1897-1904.
130. Jiang, S., D.S. Game, D. Davies, G. Lombardi, and R.I. Lechler. 2005. Activated CD1d-restricted natural killer T cells secrete IL-2: innate help for CD4+CD25+ regulatory T cells? *Eur J Immunol* 35:1193-1200.
131. Gumperz, J.E., S. Miyake, T. Yamamura, and M.B. Brenner. 2002. Functionally distinct subsets of CD1d-restricted natural killer T cells revealed by CD1d tetramer staining. *J Exp Med* 195:625-636.
132. Singh, N., S. Hong, D.C. Scherer, I. Serizawa, N. Burdin, M. Kronenberg, Y. Koezuka, and L. Van Kaer. 1999. Cutting edge: activation of NK T cells by CD1d and alpha-galactosylceramide directs conventional T cells to the acquisition of a Th2 phenotype. *J Immunol* 163:2373-2377.
133. Kronenberg, M. 2005. Toward an understanding of NKT cell biology: progress and paradoxes. *Annu Rev Immunol* 23:877-900.

134. Spada, F.M., Y. Koezuka, and S.A. Porcelli. 1998. CD1d-restricted recognition of synthetic glycolipid antigens by human natural killer T cells. *J Exp Med* 188:1529-1534.
135. Barral, D.C., and M.B. Brenner. 2007. CD1 antigen presentation: how it works. *Nat Rev Immunol* 7:929-941.
136. Fiedler, T., W. Walter, T.E. Reichert, and M.J. Maeurer. 2002. Regulation of CD1d expression by murine tumor cells: escape from immunosurveillance or alternate target molecules? *Int J Cancer* 98:389-397.
137. Fais, F., F. Morabito, C. Stelitano, V. Callea, S. Zanardi, M. Scudeletti, P. Varese, E. Ciccone, and C.E. Grossi. 2004. CD1d is expressed on B-chronic lymphocytic leukemia cells and mediates alpha-galactosylceramide presentation to natural killer T lymphocytes. *Int J Cancer* 109:402-411.
138. Morita, M., K. Motoki, K. Akimoto, T. Natori, T. Sakai, E. Sawa, K. Yamaji, Y. Koezuka, E. Kobayashi, and H. Fukushima. 1995. Structure-activity relationship of alpha-galactosylceramides against B16-bearing mice. *J Med Chem* 38:2176-2187.
139. Borg, N.A., K.S. Wun, L. Kjer-Nielsen, M.C. Wilce, D.G. Pellicci, R. Koh, G.S. Besra, M. Bharadwaj, D.I. Godfrey, J. McCluskey, and J. Rossjohn. 2007. CD1d-lipid-antigen recognition by the semi-invariant NKT T-cell receptor. *Nature* 448:44-49.
140. McCarthy, C., D. Shepherd, S. Fleire, V.S. Stronge, M. Koch, P.A. Illarionov, G. Bossi, M. Salio, G. Denkberg, F. Reddington, A. Tarlton, B.G. Reddy, R.R. Schmidt, Y. Reiter, G.M. Griffiths, P.A. van der Merwe, G.S. Besra, E.Y. Jones, F.D. Batista, and V. Cerundolo. 2007. The length of lipids bound to human CD1d molecules modulates the affinity of NKT cell TCR and the threshold of NKT cell activation. *J Exp Med* 204:1131-1144.
141. Brutkiewicz, R.R. 2006. CD1d ligands: the good, the bad, and the ugly. *J Immunol* 177:769-775.
142. Coppieters, K., K. Van Beneden, P. Jacques, P. Dewint, A. Vervloet, B. Vander Cruyssen, S. Van Calenbergh, G. Chen, R.W. Franck, G. Verbruggen, D. Deforce, P. Matthys, M. Tsuji, P. Rottiers, and D. Elewaut. 2007. A single early activation of invariant NK T cells confers long-term protection against collagen-induced arthritis in a ligand-specific manner. *J Immunol* 179:2300-2309.
143. Kobayashi, E., K. Motoki, T. Uchida, H. Fukushima, and Y. Koezuka. 1995. KRN7000, a novel immunomodulator, and its antitumor activities. *Oncol Res* 7:529-534.
144. Kawano, T., J. Cui, Y. Koezuka, I. Toura, Y. Kaneko, K. Motoki, H. Ueno, R. Nakagawa, H. Sato, E. Kondo, H. Koseki, and M. Taniguchi. 1997. CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. *Science* 278:1626-1629.
145. Yamamura, T., K. Sakuishi, Z. Illes, and S. Miyake. 2007. Understanding the behavior of invariant NKT cells in autoimmune diseases. *J Neuroimmunol* 191:8-15.
146. Im, J.S., N. Tapinos, G.T. Chae, P.A. Illarionov, G.S. Besra, G.H. DeVries, R.L. Modlin, P.A. Sieling, A. Rambukkana, and S.A. Porcelli. 2006. Expression of CD1d molecules by human schwann cells and potential interactions with immunoregulatory invariant NK T cells. *J Immunol* 177:5226-5235.

147. Parekh, V.V., M.T. Wilson, D. Olivares-Villagomez, A.K. Singh, L. Wu, C.R. Wang, S. Joyce, and L. Van Kaer. 2005. Glycolipid antigen induces long-term natural killer T cell anergy in mice. *J Clin Invest* 115:2572-2583.
148. Chen, Y.G., C.M. Choisy-Rossi, T.M. Holl, H.D. Chapman, G.S. Besra, S.A. Porcelli, D.J. Shaffer, D. Roopenian, S.B. Wilson, and D.V. Serreze. 2005. Activated NKT cells inhibit autoimmune diabetes through tolerogenic recruitment of dendritic cells to pancreatic lymph nodes. *J Immunol* 174:1196-1204.
149. Singh, A.K., M.T. Wilson, S. Hong, D. Olivares-Villagomez, C. Du, A.K. Stanic, S. Joyce, S. Sriram, Y. Koezuka, and L. Van Kaer. 2001. Natural killer T cell activation protects mice against experimental autoimmune encephalomyelitis. *J Exp Med* 194:1801-1811.
150. Jahng, A.W., I. Maricic, B. Pedersen, N. Burdin, O. Naidenko, M. Kronenberg, Y. Koezuka, and V. Kumar. 2001. Activation of natural killer T cells potentiates or prevents experimental autoimmune encephalomyelitis. *J Exp Med* 194:1789-1799.
151. Numata, Y., S. Tazuma, Y. Ueno, T. Nishioka, H. Hyogo, and K. Chayama. 2005. Therapeutic effect of repeated natural killer T cell stimulation in mouse cholangitis complicated by colitis. *Dig Dis Sci* 50:1844-1851.
152. Wu, L., and L. Van Kaer. 2009. Natural killer T cells and autoimmune disease. *Curr Mol Med* 9:4-14.
153. Schmieg, J., G. Yang, R.W. Franck, and M. Tsuji. 2003. Superior protection against malaria and melanoma metastases by a C-glycoside analogue of the natural killer T cell ligand alpha-Galactosylceramide. *J Exp Med* 198:1631-1641.
154. Fujii, S., K. Shimizu, H. Hemmi, M. Fukui, A.J. Bonito, G. Chen, R.W. Franck, M. Tsuji, and R.M. Steinman. 2006. Glycolipid alpha-C-galactosylceramide is a distinct inducer of dendritic cell function during innate and adaptive immune responses of mice. *Proc Natl Acad Sci U S A* 103:11252-11257.
155. Schmieg, J., G. Yang, R.W. Franck, and M. Tsuji. A multifactorial mechanism in the superior antimalarial activity of alpha-C-GalCer. *J Biomed Biotechnol* 2010:283612.
156. Kopecky-Bromberg, S.A., K.A. Fraser, N. Pica, E. Carnero, T.M. Moran, R.W. Franck, M. Tsuji, and P. Palese. 2009. Alpha-C-galactosylceramide as an adjuvant for a live attenuated influenza virus vaccine. *Vaccine* 27:3766-3774.
157. Wu, D., D.M. Zajonc, M. Fujio, B.A. Sullivan, Y. Kinjo, M. Kronenberg, I.A. Wilson, and C.H. Wong. 2006. Design of natural killer T cell activators: structure and function of a microbial glycosphingolipid bound to mouse CD1d. *Proc Natl Acad Sci U S A* 103:3972-3977.
158. Chang, Y.J., J.R. Huang, Y.C. Tsai, J.T. Hung, D. Wu, M. Fujio, C.H. Wong, and A.L. Yu. 2007. Potent immune-modulating and anticancer effects of NKT cell stimulatory glycolipids. *Proc Natl Acad Sci U S A* 104:10299-10304.
159. Yu, K.O., J.S. Im, A. Molano, Y. Dutronc, P.A. Illarionov, C. Forestier, N. Fujiwara, I. Arias, S. Miyake, T. Yamamura, Y.T. Chang, G.S. Besra, and S.A. Porcelli. 2005. Modulation of CD1d-restricted NKT cell responses by using N-acyl variants of alpha-galactosylceramides. *Proc Natl Acad Sci U S A* 102:3383-3388.

160. Miyamoto, K., S. Miyake, and T. Yamamura. 2001. A synthetic glycolipid prevents autoimmune encephalomyelitis by inducing Th2 bias of natural killer T cells. *Nature* 413:531-534.
161. Mizuno, M., M. Masumura, C. Tomi, A. Chiba, S. Oki, T. Yamamura, and S. Miyake. 2004. Synthetic glycolipid OCH prevents insulitis and diabetes in NOD mice. *J Autoimmun* 23:293-300.
162. Chiba, A., S. Oki, K. Miyamoto, H. Hashimoto, T. Yamamura, and S. Miyake. 2004. Suppression of collagen-induced arthritis by natural killer T cell activation with OCH, a sphingosine-truncated analog of alpha-galactosylceramide. *Arthritis Rheum* 50:305-313.
163. Velmourougane, G., R. Raju, G. Bricard, J.S. Im, G.S. Besra, S.A. Porcelli, and A.R. Howell. 2009. Synthesis and evaluation of an acyl-chain unsaturated analog of the Th2 biasing, immunostimulatory glycolipid, OCH. *Bioorg Med Chem Lett* 19:3386-3388.

Chapter II

Eradication of large tumor masses by immunotherapy with biodegradable PLGA microspheres - an alternative to incomplete Freund's adjuvant

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Abstract

The delivery of immune stimulating agents and antigens is a major requirement for successful immunotherapy of cancer. So far incomplete Freund's adjuvant (IFA) was considered to be the "gold-standard" and is used in mice and humans. In this study we compared a mixture of IFA and the model antigen ovalbumin as well as the adjuvants CpG-oligodeoxynucleotides (CpG-ODN) and polyI:C to poly(lactide-*co*-glycolide) (PLGA) microspheres (MS), which contain the antigen and the adjuvant. This study elucidates the potential of PLGA-MS to elicit substantial humoral and cellular immune responses after a single administration. A subsequent injection of recombinant vaccinia virus expressing ovalbumin increases the CTL response significantly. Furthermore, we could show that both, IFA and PLGA-MS, form a depot that accounts for a long-lasting potential to stimulate proliferation of $CD4^+$ and $CD8^+$ T cells, as well as *in vivo* cytotoxicity for at least 3 weeks after the immunization. In several tumor models we could show that PLGA-MS are at least equally efficient than IFA, if not better. In detail, PLGA-MS show a potent protective potential and superior therapeutic efficacy compared to IFA.

Introduction

The quality of an immune response upon immunization is not only dependent on the dose of antigen used, but also on the availability of an adjuvant. Dendritic cells (DCs) are a major factor in inducing immune responses, because they are scanning their environment by the uptake of antigens in the periphery. To acquire their full co-stimulatory capacity and to avoid autoimmunity induced by presentation of self antigens to naïve T cells, DC need to be co-triggered by a danger signal, a so called pathogen associated molecular pattern (PAMP) (1). The most prominent receptors for these, the Toll like receptors (TLR) have various ligands. Here we will focus on cytosine-phosphorothioate-guanine oligodeoxynucleotides (CpG-ODN) (2), binding TLR9, and polyriboinosinic:polyribocitidylic acid (polyI:C), binding TLR3. Both, TLR 9 and TLR 3 are located in the endosome. Modern vaccine delivery systems are challenged by the fact, that these endosomal receptors need to be triggered efficiently (3).

Furthermore, it is known that an antigen and a TLR ligand must be on hand in close proximity (4) or physically associated (5), in order to efficiently activate DCs.
The ability of professional antigen-presenting cells (APCs) to cross-present phagocytosed antigen (6) on major histocompatability complex (MHC) class I is a central phenomenon of tumor-derived antigen based immunotherapy (7, 8). Vaccination with externaly loaded, patient derived DCs yielded promising results in the past years (9, 10), but therapies are expensive and laborious. A major challenge for efficient *in vivo* loading of DCs with an antigen of choice, is to track DCs to the injection site. Several adjuvants are on the market, but so far the gold standard in immunotherapy is the usage of protein or peptide in combination with CpG-ODN and IFA (11, 12).
IFA represents today's most potent adjuvants, but it is also lively discussed for its side effects. Although long term studies reported no incidents of long term side effects (13), more recent studies describe severe local skin reactions or persistent painful granulomas at the injection site, when using an advancement of IFA (Montanide® ISA-51) (14). The reason for these symptoms remain unclear but compared to the enormous potential of IFA or its successors, they appear to be minor. Nevertheless, an alternative method to opsonize an antigen in combination with a TLR ligand to DCs, providing a close proximity of the agents as well as a long lasting depot effect might be of benefit for future studies.
Using biodegradable poly(lactide-*co*-glycolide) (PLGA) microspheres (MS), obtained by spray-drying (15) entails, that encapsulated antigens are taken up, processed and efficiently presented on MHC class I and II (16, 17). PLGA-MS are known to protect the encapsulated antigen and the adjuvant from degradation and thus form a long lasting depot for sustained and prolonged immune responses in vivo (18-20). The particle size of PLGA-MS obtained by spray-drying is about 1-10 µm, which facilitates the uptake of antigens by APCs (21), followed by transport to the secondary lymphoid organs (22). Furthermore, the co-encapsulation of the model antigen ovalbumin and CpG-ODN yields substantial CTL responses as well as striking anti tumor responses (23, 24). Also endogenous tumor antigens were successfully co-encapsulated with CpG-ODN with promising results (25, 26).
In this study we show that a mixture of PLGA-MS co-encapsulating ovalbumin/CpG-ODN and PLGA-MS encapsulating polyI:C yields comparable results to the utilization of IFA as a delivery system.

Materials and methods

Preparation of MS: MS were prepared from 14 kDa PLGA 50:50 carrying hydroxyl and carboxyl-end groups (Resomer RG502H, Boehringer Ingelheim, Ingelheim, Germany). The antigens and TLR ligands were microencapsulated by spray drying as described elsewhere (27). Briefly, 50 mg ovalbumin (Grade V, Sigma) and 5 mg CpG oligodeoxynucleotides with a phosphothioate backbone (CpG-ODN 1826, Microsynth, Balgach, Switzerland) (MS-Ova/CpG) or 0,5 mg polyI:C (Calbiochem, VWR, Dietikon, Switzerland) (MS-polyI:C) were dissolved in 0.5 ml 0.1M $NaHCO_3$ (aqueous phase) and mixed with 1 g of PLGA dissolved in 20 ml of dichloromethane (organic phase). The two phases were subsequently emulsified by ultrasonication (Hielscher, UP200 H, Ampl. 40%) for 10 s on ice. The obtained dispersion was immediately spray-dried (Büchi, Mini Spray-Dryer 191) at a flow rate of 2 ml/min and inlet/outlet temperatures of 40/37 °C. The obtained MS were washed out of the spray-dryer's cyclone with 0.05 % Synperonic (Synperonic®F68, Serva Electrophoresis GmbH, Heidelberg, Germany), collected on a cellulose acetate membrane filter and dried under reduced pressure (20 mbar) for 18 h at room temperature. PLGA-MS were stored at 4 °C. Immediately before use MS were dispersed PBS by ultrasonication for 30 sec.

Mice and immunizations: OT-I were obtained from the Dr. Ying Waeckerle-Men (University of Zurich, Switzerland), OT-II/Thy1.1 mice were obtained from the Prof. Thomas Brocker (LMU Munich, Germany). C57BL/6 mice (H-2b) were originally purchased from Charles River Laboratories. All mice were kept in a specific pathogen-free facility and used at 6–10 wk of age. Animal experiments were approved by the review board of the Regierungspräsidium Freiburg. Mice were immunized either with a mixture of 5 mg PLGA-MS, co-encapsulating ovalbumin (250 µg) and CpG-ODN (25 µg) (MS-Ova/CpG) and 5 mg MS polyI:C (2,5 µg) (MS-polyI:C) or the corresponding amounts of ovalbumin, CpG-ODN and polyI:C in PBS:IFA (1:1) (in IFA). Control mice were either treated with empty microspheres (empty) or left untreated (naïve). All injections were performed in a total volume of 200 µl.

Cell lines and media: EL-4 cells and the ovalbumin expressing transfectant EG-7 were kindly provided by Dr. Wolfram Osen (DKFZ Heidelberg, Germany) and

kept in RPMI medium. MO-5 cells were kindly provided by Dr. Antje Heit (LMU Munich, Germany) and were kept in DMEM medium. All media were purchased from Invitrogen Life Technologies and contained GlutaMAX, 10% Fetal Calf Serum (FCS), and 100 U/ml penicillin/streptomycin (P/S). For the clone Vf10, TrampC2 cells (provided by Dr. Peter Öhlschläger; University of Konstanz, Germany) were transfected with a plasmid encoding for full-length, cytosolic ovalbumin. Vf10 cells were kept in DMEM (-Pyruvate), complemented with 5 % FCS, 5 % Nu-serum (BD Biosciences), 5µg/ml Insulin (Sigma), 10^{-8} M dihydrotestosterone (DTH, Sigma)) and 1% P/S. To maintain the expression of ovalbumin in EG-7, MO-5 and Vf10 cells the media contained geneticin (G418; Sigma) (0,2 mg/ml).

Intracellular cytokine staining (ICS) and tetramer staining: For intracellular cytokine staining mice were immunized by s.c. injection of MS in 200 µl PBS at the base of the tail. Six days later, splenocytes were isolated and incubated with or without 10 µM SIINFEKL peptide (Eurogentec, Cologne, Germany) in the presence of brefeldin A (10µg/ml, Sigma-Aldrich) for 5 h at 37 °C. After washing cells were stained with PE-Cy5-conjugated anti-mouse CD8α antibody (BD Biosciences Pharmingen, Clone 53-6.7) for 20 min at 4 °C. The cells were washed again and fixed with 4 % paraformaldehyde in PBS for 10 min at room temperature. After washing the cells twice with PBS, cells were labeled intracellularly with FITC-conjugated rat-anti-IFN-γ antibody (clone XGM1.2, diluted in PBS/0.1% Saponin) at 4 °C overnight. The next day cells were washed and resuspended in PBS for flow cytometric analysis. Background values for each sample (without peptide) were subtracted. The H-2Kb/SIINFEKL tetramers used in this study were kindly provided by Prof. Thomas Brocker (LMU Munich, Germany). For MHC tetramer staining, splenocytes were stained with PE-labeled SIINFEKL/H-2Kb-tetramer for 20 min at 37 °C and subsequently with PE-Cy5-conjugated rat anti-CD8 -IgG for 20 min at 4 °C. Cells were washed twice and measured by flow cytometry.
Data were acquired using a FACScan Flowcytometer (BD Biosciences) and analyzed with FlowJo software (Treestar).

In vivo cytotoxicity assay: Cytotoxic activity of $CD8^+$ cells *in vivo* was assessed as described elsewhere (28). Briefly, splenocytes from naive mice were pulsed

with 10 µM SIINFEKL peptide for 1 h at 37 °C or left untreated. Pulsed cells were stained with 20 µM carboxyfluorescein succinimidyl ester (CFSE), while unpulsed cells were stained with 2 µM CFSE. Immediately before injection, both cell populations were mixed in equal amounts, and a total number of 1×10^7 cells per mouse were injected i.v.. After 18 h, splenocytes were prepared and analyzed by flow cytometry. 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)] × 100.

Chromium release assay: C57BL/6 mice were immunized either by a mixture of PLGA-MS or the corresponding components in IFA. Six days later splenocytes were isolated and used as effectors in a primary chromium release assay as previously described (29). EL-4 (H-2b) (negative control) or EG-7 cells served as target cells. After 4 h of coincubation at 37°C supernatants were analyzed by enumerating the γ-counts per minute.

Ex vivo cytotoxicity assay: For measurement of ex vivo cytolytic activity a time-resolved fluorimetric assay was used. C57BL/6 mice were immunized either by a mixture of PLGA-MS or the corresponding components in IFA. Six days later splenocytes were isolated and used as effectors in a primary cytotoxicity assays as previously described (30) using DELFIA® EuTDA cytotoxicity reagents (PerkinElmer).

Briefly, 5×10^4 Vf10 cells served as target cells and were labeled with BaTDA. After 4 h of co-incubation, supernatants were mixed with Europium solution for 1 h and subsequently measured at excitation/emission 340/615 nm and a lag time of 200 nsec. Specific cytolysis was calculated as follows: [(counts for immunized mouse - counts for naïve mouse) * 100)] / (counts for total lyses).

ELISA: For detection of ovalbumin specific immunoglobulin (Ig) blood samples were taken at indicated time points after immunization, either by a mixture of PLGA-MS or the corresponding components in IFA, using a heparin coated microvette (Sarstedt, Nümbrecht, Germany) and spun down for 5 min at 1000 rpm. Serum was stored at –80 °C. 96 well ELISA plates were coated with 0,1 mg/ml

ovalbumin in PBS over night at 4 °C. The next day plates were wash 3 times with PBS-Tween20 (0,05 %) and serum samples were applied for 2 h at room temperature. For detection of mouse Ig isotypes the mouse-Ig-isotyping-kit (BD Bioscience) was used according to the manufacturers instructions. For detection of IL-4 and IFN-γ in supernatants of cell cultures the respective kit (mouse IFN-γ/IL-4 ELISA set, BD Bioscience) was used, following the manufacturers instruction.

Proliferation assay: Proliferation of CD8 and CD4 lymphocytes was measured via CFSE dilution. Mice, immunized either by a mixture of PLGA-MS or the corresponding components in IFA, received $1x10^7$ CFSE (10µM) labeled splenocytes derived from either OT-I mice or OT-II/Thy1.1 mice. For transfer of CD8 lymphocytes, OT-I splenocytes treated with erythrocyte-lyses buffer (155 mM NH_4CL, 0,01 M Tris) for 5 min at room temperature. Cells were magnetically sorted for $CD8^+$ surface expression, labeled with CFSE and injected i.v. into Thy1.1 recipient mice. Three days later spleens of Thy1.1 recipient mice were stained for $Thy1.2^+$ cells (PE-Cy5-conjugated anti-mouse CD90.2 (Thy1.2), eBioscience, Clone 53-2.1) for 20 min at 4 °C washed with PBS and analyzed by flow cytometry for CFSE fluorescence.
For transfer of OT-II/Thy1.1 splenocytes, spleens of OT-II/Thy1.1 mice were homogenized and treated with erythrocyte-lyses buffer for 5 min at room temperature. Cells were labeled with CFSE and injected i.v. into C57BL/6 recipient mice. Three days later spleens of C57BL/6 recipient mice were harvested, homogenized and stained for Thy1.1 (PE-Cy5-conjugated anti-mouse CD90.1 (Thy1.1) Ig, eBioscience, Clone HIS51) and for CD4 (PE conjugated anti mouse CD4 Ig, eBioscience, Clone RMA4-5) for 20 min at 4 °C. CD4/Thy1.1 double positive cells were analyzed for CFSE fluorescence.

Results

Humoral response after a single injection of MS-Ova-CpG. In order to determine the humoral immune response to PLGA-MS over a time course of 4 weeks, mice received a single injection of MS-Ova/CpG. The ovalbumin specific Ig isotype concentrations shown in figure 1 indicate a robust IgM, IgG1 and IgG2a response in the absence of IgE. Empty MS showed no significant increase of Ig isotypes

within 4 weeks (data not shown). The maximum concentration of about 12,5 µg/ml ovalbumin specific IgG1 was reached after 2 weeks and stayed consistent until the end of the experiment after four weeks. The relative IgG1 to IgG2a response indicates a rather balanced Th1 to Th2 response, with slight bias to Th2.

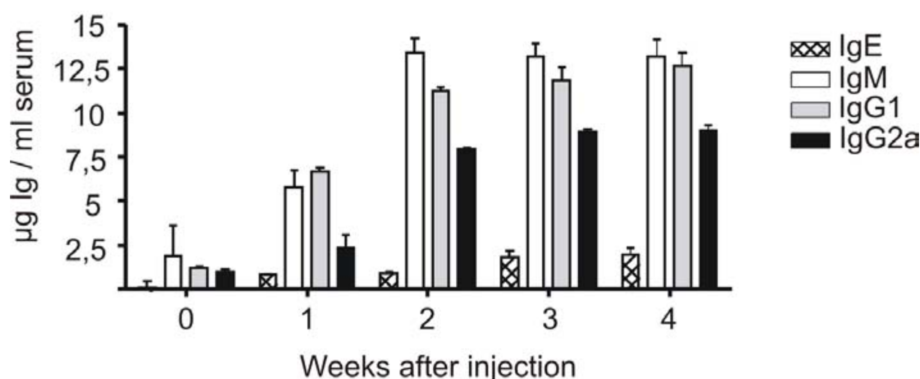

Figure 1: Serum levels of Ovalbumin specific Ig after a single injection of PLGA-MS. C57BL/6 mice (n=5) were immunized with 5mg of PLGA-MS, containing ovalbumin (50mg/g) and CpG oligonucleotides (5mg/g). Serum was obtained from capillary blood at day 0 and weekly for 4 weeks. Serum was analyzed for ovalbumin specific IgE (crossed bars), IgM (empty bars), IgG1 (doted bars), IgG2a (black bars). Given values are µg/ml ovalbumin specific Ig. One out of two experiments with the same outcome is shown.

Pre-treatment with TNF-α and repeated injections of PLGA-MS. We tested two possibilities to optimize the CTL response to PLGA-MS. On the one hand, a pre-treatment of the injection site with TNF-α, on the other hand a booster immunization. We immunized mice with MS-Ova/CpG 2 to 10 hours after a pre-conditioning injection of 200 ng TNF-α. After six days we analyzed splenocytes for improved ovalbumin specific CTL responses by ICS. As shown in figure 2A, a pre-treatment of the injection site with 200ng TNF-α at least 6 hours prior to the immunization increases the CTL response drastically. Clearly, treatment of less than 6 hours in advance let to no advantage compared to mice that were not pre-treated. Still 10 hours after the pre-treatment the immunization benefited from the conditioned environment. The second strategy we investigated was the effect of booster immunizations. As figure 2B shows, a homologous prime boost strategy, with an interval of 4 weeks, was not able to increase CTL responses but a heterologous boost using recombinant vaccinia virus, expressing full length ovalbumin, clearly enhanced the ovalbun specific CTL response. This finding did not reflect our expectations so that we analyzed for SIINFEKL/H-2Kb tetramer

positive T lymphocytes after the same injection regiment (figure 2C). Surprisingly, although the homologous treatment was not able to further increase the ovalbumin-specific CD8$^+$/IFN-γ$^+$ population in the spleen, the SIINFEKL/H-2Kb-tetramer$^+$/CD8$^+$ population could obviously be increased. A homologous as well as a heterologous prime boost strategy resulted in a significant increase compared to a single administration of PLGA-MS when stained for SIINFEKL/H-2Kb-tetramer$^+$/CD8$^+$ cells.

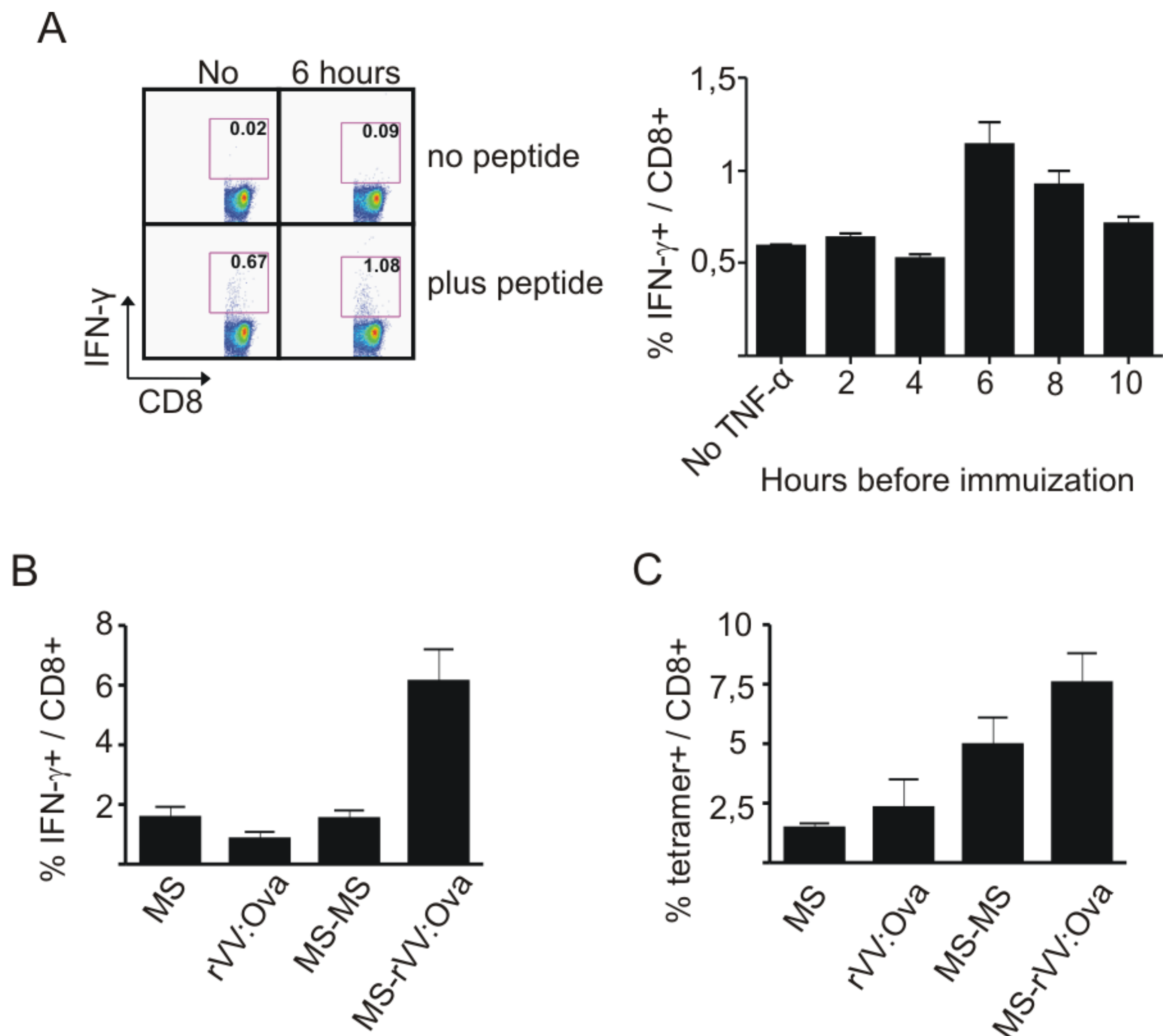

Figure 2: (A) Pre-treatment of the injection site. C57BL/6 mice (n=3) were pre-treated with 200ng TNF-α at the site of injection 2h, 4h, 6h, 8h or 10h prior to immunization with 5mg of microspheres, containing ovalbumin (50mg/g) and CpG-ODN (5mg/g). Control animals were left untreated. Six days after immunization splenocytes were isolated and analyzed for intracellular IFN-γ by ICS. Left side shows dot plots for exemplary animals. Right side shows a quantitative representation of one out of three similar experiments. Values are given in percent IFN-γ$^+$ cells of the CD8$^+$ lymphocyte population, +/- SEM. Background signals (no peptide) were subtracted. One out of three similar experiments is shown. **(B&C) Booster immunizations.** C57BL/6 mice (n=2) received either single injections of microspheres (MS) or recombinant vaccinia virus expressing ovalbumin (rVV:Ova), or additional booster immunizations after 4 weeks (MS-MS or MS-rVV:Ova).

After six days splenocytes were isolated and analyzed for intracellular IFN-γ by ICS **(B)** or SIINFEKL/H-2Kb-tetramer staining (C). In each case a quantitative analysis of one out of 3 experiments is shown. Values for (B) are given in percent IFN-γ^+ cells of the $CD8^+$ lymphocyte population, +/- SEM. Background signals (no peptide) were subtracted. Values in **(C)** are given in percent SIINFEKL/H-2Kb-tetramer$^+$ cells of the $CD8^+$ lymphocyte population, +/- SEM. The background (naïve mouse) was subtracted. One representative out of three experiments is shown.

Il-4 and IFN-γ produced by $CD4^+$ splenocytes. To further characterize the bias of Th1 to Th2 after immunization we measured cytokine levels of IL-4 and IFN-γ in the supernatant of splenocytes derived from mice, either immunized with PLGA-MS or the according components in IFA. Therefore, splenocytes were stimulated by plate bound anti-CD3 / anti-CD28 antibodies, six days after the immunization for 24 hours. Subsequently, supernatants of both groups were analyzed by ELISA for levels of IFN-γ and IL-4. As figure 3 shows, in both groups we found predominantly IFN-γ and little IL-4, proposing a robust Th1 bias.

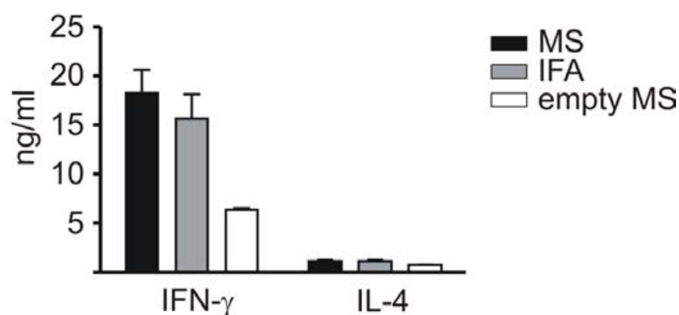

Figure 3: Production of IFN-γ and IL-4 by $CD4^+$ cells after PLGA-MS immunization. C57BL/6 mice (n=2) were immunized with a mixture of PLGA-MS (black bars), the corresponding amounts of ovalbumin, CpG-ODN and polyI:C in IFA (gray bars), or empty PLGA-MS (white bars). After six days splenocytes were isolated and magnetically sorted for $CD4^+$ cells. $5x10^5$ $CD4^+$ cells were restimulated by plate-bound Ig anti-CD3 and Ig anti-CD28 for 18 hours. Supernatants were analyzed for IL-4 and IFN-γ by ELISA. Values are given in ng/ml, +/- SEM. The graph shows one out of three similar experiments.

In vivo proliferation after injection of PLGA-MS. Since the proliferation of antigen specific CTLs is crucial for a successful of immunotherapy we investigated the potential of PLGA-MS to induce proliferation of $CD8^+$ and $CD4^+$ cells *in vivo*. Figure 4A shows that PLGA-MS are, comparable to IFA, able to induce detectable $CD8^+$ cell proliferation for at least 21 days. The same is true for $CD4^+$ cell proliferation induced by PLGA-MS, which is even more persistent compared to $CD8^+$ cells (Figure 4B).

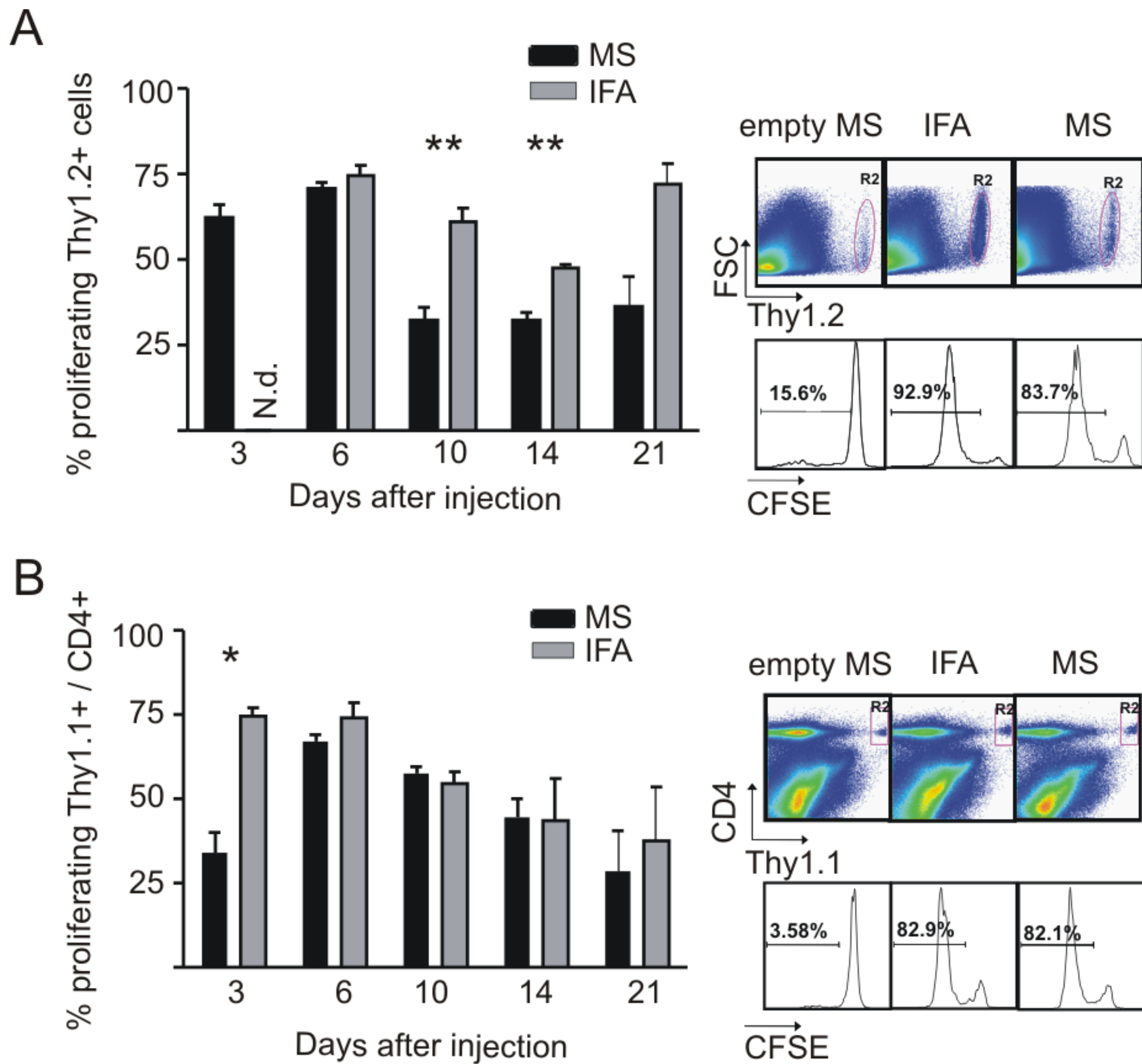

Figure 4: (A) Potential to stimulate proliferation of CD8+ cells. Thy1.1 mice (n=3) were immunized with a mixture of PLGA-MS (black bars), the corresponding amounts of ovalbumin, CpG-ODN and polyI:C in IFA (gray bars). Three days before mice were sacrificed, $1x10^7$ magneticaly sorted CD8+, CFSE labeled splenocytes, derived from OT-I mice were injected i.v.. After three days spleens of the recipient mice were taken and the Thy1.1+ cells were analyzed for CFSE fluorescence. On the right side a quantitative analysis is shown for day 3, 6, 10, 14, and 21 (one out of two experiments with similar results). Values are given in percent proliferating Thy1.1+ cells, +/- SEM. Background values (PBS treated mice) were subtracted. On the right side representative gating is shown for day six.

(B) Potential to stimulate proliferation of CD4 positive cells. C57BL/6 mice (n=2) were immunized with microspheres (black bars) or IFA (gray bars). Three days before mice were sacrificed, $2x10^6$ CFSE labeled splenocytes from OT-II/Thy1.1 mice were injected i.v.. After three days spleens of the recipient mice were taken and Thy1.1/CD4 double positive cells were analyzed for CFSE fluorescence. On the right a quantitative analysis is shown for day 3, 6, 10, 14, and 21 (one out of two experiments with similar results). Values are given in percent proliferating

Thy1.1/CD4 positive cells, +/- SEM. Background values (PBS treated mice) were subtracted. On the right side representative gating is shown for day six.

In vivo and ex vivo cytotoxicity. In order to show the cytotoxic effect of the generated CTLs *in vivo*, we performed *in vivo* and *ex vivo* cytotoxicity assays. Figure 5A shows an *ex vivo* 51-chromium cytotoxicity assay, 6 days after immunization either with a mixture of PLGA-MS or the corresponding components in IFA. EL-4 thymoma cells served as control cells, whereas EL-4 cells, stably transfected with a plasmid encoding for full-length, cytosolic ovalbumin (EG-7 cells) served as target cells.

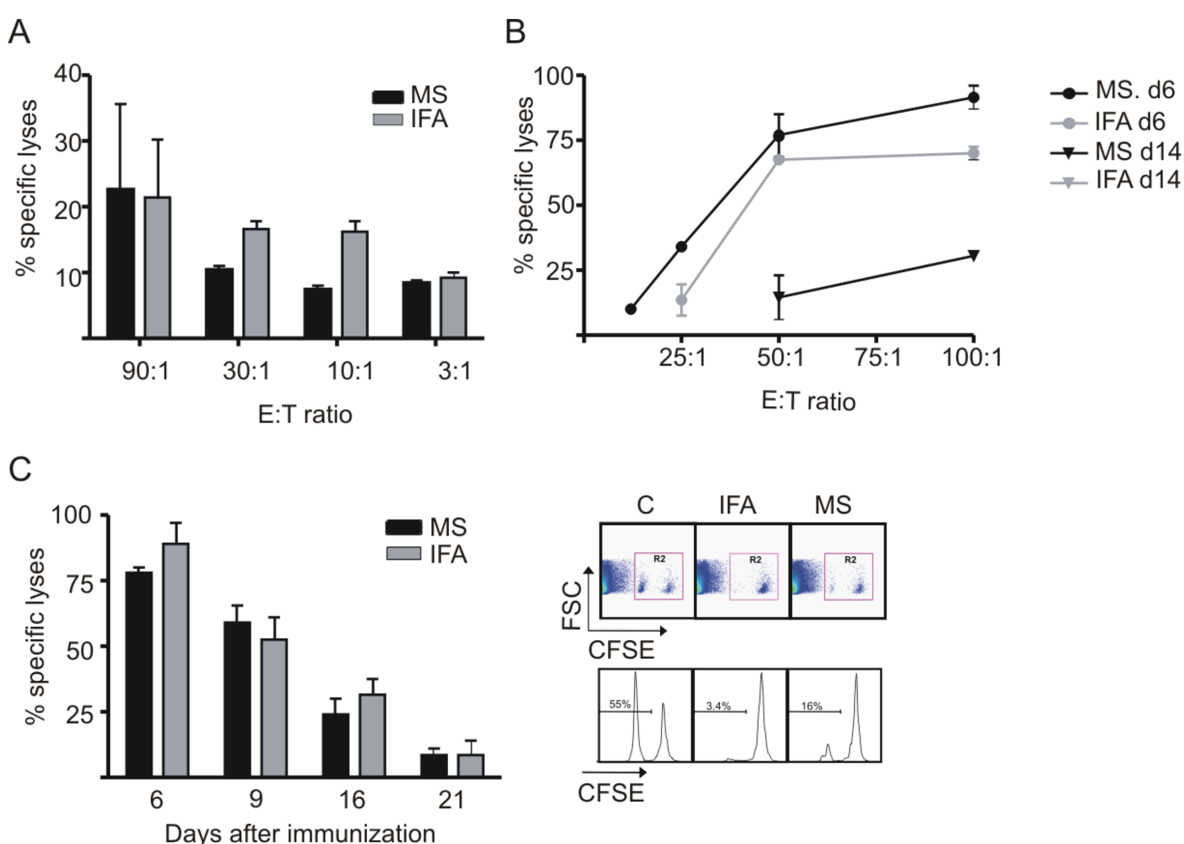

Figure 5: (A&B) Ex vivo cytotoxicity of PLGA-microspheres induced CTLs. (A) 51-chromium release assay. C57BL/6 mice (n=2) were immunized with a mixture of PLGA-MS (black bars), the corresponding amounts of ovalbumin, CpG-ODN and polyI:C in IFA (gray bars). On day six, splenocytes were co incubated with 51-chromium labeled EL-4 or ovalbumin expressing EG-7 cells, for four hours. Supernatants were than measured by γ-count. Values are given in percent specific lyses of $5x10^4$ target cells, +/- SEM. One out of two similar experiments is shown. (B) Ex vivo BaTDA-cytotoxicity assay. C57BL/6 mice (n=2) were immunized with a miture of PLGA-MS (black lines) or equal amounts of ovalbumin and CpG oligonucleotides in IFA (gray line). Spleens were removed after 6 days (circles) or after 14 days (triangles) and co-incubated with BaTDA labeled TrampC2 cells that express ovalbumin for four hours. Supernatants were than mixed with

Europium solution and incubated on a shaker for one hour prior to measurement at ex./em. 340/615nm. Values are given in percent specific lyses +/- SEM of $5x10^4$ target cells with effector to target ratios of 100, 50, 25 and 12,5 to one. One out of three experiments is shown. **(C) In vivo cytotoxicity assay.** C57BL/6 mice (n=3) were immunized with microspheres (black bars) or IFA (gray bars). *In vivo* cytotoxicity was analyzed for day 6, 9, 16, and 21. (Left side) Quantitative representation of one out of three experiments is shown, +/- SEM. (Right side) Flow cytometry dot plots and histograms of representative animals. The lymphocyte population was analyzed for different CFSE fluorescence intensities. One representative out of three experiments is shown.

In this case, IFA elicits a slightly superior *ex vivo* cytotoxicity in the 51-chromium cytotoxicity assay referring to the magnitude as well as the effector to target ratio. In contradiction to this, the EuBATDA cytotoxicity assay in Figure 4B implies that PLGA-MS elicite better *ex vivo* cytotoxicity responses, since they yield a detectable response even after 14 days post immunization. In this assay the prostate tumor derived cell line TrampC2, stably transfected with a plasmid encoding for full-length, cytosolic ovalbumin (Vf10) served as target cells. *In vivo* cytotoxicity assays performed in figure 5c showed robust responses in both cases after 6 six days, being detectable for up to 21 days. Taken together, all cytotoxicity assays clearly show a strong cytotoxic potential for both systems, IFA as well as PLGA-MS.

Anti tumor effects. Since we wanted to compare the anti-tumor potential of the PLGA-MS in respect to IFA, we tested two models of solid, subcutaneous tumors. EL-4 lymphoma cell derived tumors (Figure 6A) and the very aggressive and fast growing B16F10 melanoma tumors (Figure 6B). The correspondent ovalbumin transfectant, EG-7 and MO-5, was used in either a protective setting (left side), where therapy was started 6 days before challenge, or in a therapeutic setting (right side), where mice were immunized when palpable tumors were already established. Subsequently, tumor sizes were measured daily until they reached 15mm in mean size of 2 orthogonal measurements. If this abort criterion was fullfilled, mice were sacrificed and percentualy substracted from the total sum.
All mice being treated with PLGA-MS in a protective setting survived tumor free in protective models using EG-7 (figure 6A, left side) and MO-5 (figure 6B, left side), whereas 20 % of mice being treated with IFA developed tumors in the EG-7 model and 60 % in the MO-5 model. The therapeutic settings showed, that both delivery systems were able to cure all mice from EG-7 tumors (figure 6A, right side). For

MO-5 (figure 6B, right side) however, 20 % of the PLGA-MS group and 30% of the IFA group survived tumor free.

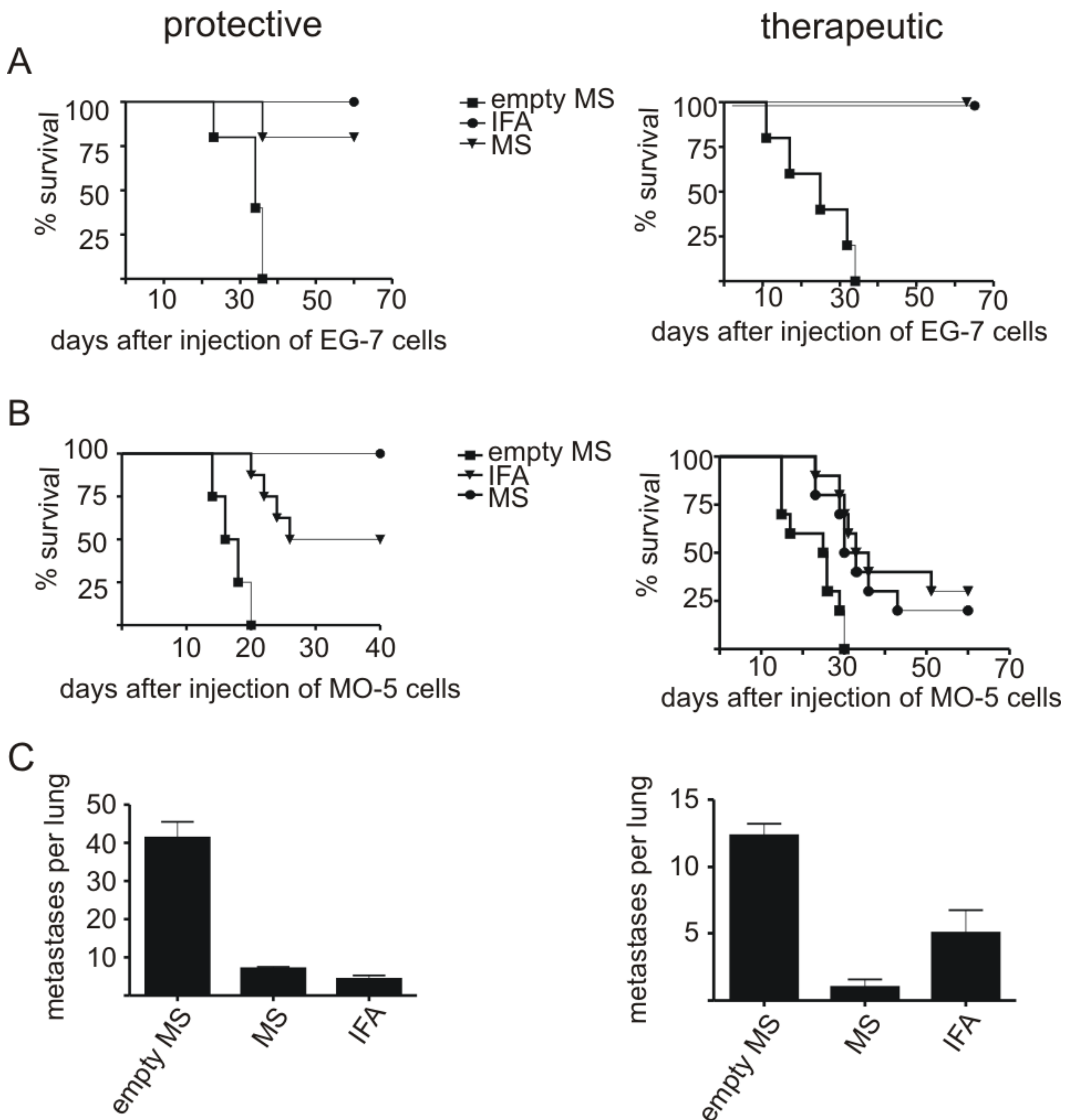

Figure 6: Tumor challenge with (A) EG-7 & (B) MO-5. Left side (protective setting): C57BL/6 mice (n=5) were immunized with PLGA-MS (circles), IFA (triangles) or empty PLGA-MS (squares). After six days mice were challenged with $5x10^5$ MO-5 cells s.c.. Tumor sizes were measured daily until they reached 15mm in mean size of 2 orthogonal measurements. Right side (therapeutic setting): C57BL/6 mice (n=10) were challenged with $5x10^5$ MO-5 cells s.c.. As soon as palpable tumors occurred mice were immunized with a mixture of PLGA-MS (circles), the corresponding amounts of ovalbumin, CpG-ODN and polyI:C in IFA (triangles) or empty PLGA-MS (squares). Tumor sizes were measured daily until they reached 15mm in mean size of 2 orthogonal measurements. **(C) Metastatic tumor challenge.** Left side (protective setting) C57BL/6 mice (n=3) were immunized with a mixture of PLGA-MS (MS), the corresponding amounts of ovalbumin, CpG-ODN and polyI:C in IFA (IFA) or empty PLGA-MS (empty MS). After six days mice were challenged

with $5x10^4$ melanin expressing MO-5 cells i.v.. After two weeks mice were sacrificed and lungs were removed. Visible metastases were counted and are given in means +/- SEM. Right side (therapeutic setting). C57BL/6 mice (n=3) were challenged with $2x10^4$ melanin expressing MO-5 cells i.v.. After one week mice were immunized with a mixture of PLGA-MS (MS), the corresponding amounts of ovalbumin, CpG-ODN and polyI:C in IFA (IFA) or empty PLGA-MS (empty MS). After seven days mice were sacrificed and lungs were removed. Visible metastases were counted and are given in means +/- SEM. All graphs show one representative experiment out of three.

In experiments, using melanin producing MO-5 cells injected i.v. in order to establish metastases in the lung, both IFA and PLGA-MS were able to clearly reduce the numbers of metastases in a protective setting. A therapeutic setting showed a clear benefit for the group being treated with PLGA-MS compared to the IFA group. Taken together, we could show that PLGA-MS are a potent tool to effectively stimulate a robust immune response in order to eradicate not only solid tumors, but also metastases.

Discussion

In the present study, we were able to show that a mixture of PLGA-MS encapsulated antigen and CpG-ODN, together with encapsulated polyI:C is able to elicit robust CTL responses that can even be improved by preconditioning the injection site with TNF-α (figure 1A) or by subsequent booster immunizations (figure 2B&C). Interestingly, we could demonstrate, that homologous booster immunizations indeed are able to improve the antigen specific $CD8^+$ T cell response to the model antigen ovalbumin, like it has been reported in earlier studies (31) but the level of activated $CD8^+$/IFN-γ^+ stayed unaffected. The reason for this remains unclear. Nevertheless, heterologous booster immunizations elevated both, number of specific CTLs and the number activated CTLs as previously shown for other vaccines in mice (32) and humans (33, 34).

Furthermore, a single injection of PLGA-MS elicits substantial humoral responses (figure 1), which in detail proposes, by its IgG2a (Th1) to IgG1 (Th2) ratio, a rather balanced Th1/Th2 bias with a tendency towards a Th2 biased $CD4^+$ T cell response. This could be a disadvantage, because in vivo DC loading with PLGA-MS aims strongly on a CTL response in order to eradicate tumors. Contradictory to that, we found a clear induction of IFN-γ but less IL-4 when investigating sorted

$CD4^+$ splenocytes 6 days after injection of the PLGA-MS, clearly proposing a Th1 bias (figure 3). Jegerlehner *et. al.* showed in 1997 that the induction of IgG2a class switching is mainly induced via CpG-mediated engagement of TLR9 on B cells, and not generally by the induction of Th1 responses and IFN-γ (35). Since both techniques, the IgG2a:IgG1 ratio and secretion of IFN-γ/IL-4 by $CD4^+$ T cells, are used to address that question (36, 37) our case remains unsolved. The fact that CpG-ODN are known to favor a Th1 bias (38) is supporting the data set showing a robust the induction of IFN-γ production by $CD4^+$ lymphocytes. Considering these aspects, we think that our PLGA-MS induced immune responses are mainly Th1 biased.

The second part of the study was designed to compare PLGA-MS to IFA. Both are antigen carriers that mimic a pathogen infection and are well-established delivery system for antigens and CpG-ODNs. Furthermore, both delivery systems show comparable capacities to induce proliferation of T cells. Even though IFA seems to be more successful in stimulating proliferation of $CD8^+$ T cells at later time-points (figure 4A). Nevertheless, PLGA-MS are able to induce detectable proliferation for at least 21 days after their injection. In case of $CD4^+$ T cells the two delivery systems gave the same results, indicating that both systems procure a strong depot effect and excellent immune stimulative properties (figure 4B). It is known, that encapsulated antigens show a remarkable potential to stimulate proliferation of $CD4^+$ and $CD8^+$ T cells *in vitro* and *in vivo* (39), but we could show for the first time, that this potential lasts for a time span of at least 3 weeks after immunization. The *ex vivo* and *in vivo* cytotoxicity as well as the therapeutic tumor experiments are indicating a robust and long lasting immune response (figure 5). The protective properties of the encapsulation of antigens and adjuvants in PLGA-MS become obvious in the protective tumor settings (figure 6A&B, left side). Both experiments display encapsulation as superior. In contrast to that, the model for metastasized tumors indicate, that PLGA-MS show a comparable efficacy to IFA referring to protection but is clearly more successful in the therapeutic setting (figure 6C). Reasons for that might be an promising effect of the PLGA particles themselves, leading to better uptake by APCs and efficient processing of the encapsulated antigens. On the other hand, adjuvants might be more accessible when using IFA as a delivery system, what might be an explanation for its side effects like skin reactions and granulomas. Taken together PLGA-MS show a great potential in

delivering antigens and adjuvants to the immune system. PLGA-MS seem to be at least as powerful as the “gold-standard” delivery device IFA, but without showing any side effects. Therefore it might be an alternative to IFA and a promising candidate for clinical trials in the near future.

References of chapter II:

1. Akira, S. TLR signaling. Curr Top Microbiol Immunol, *311:* 1-16, 2006.
2. Miconnet, I., Koenig, S., Speiser, D., Krieg, A., Guillaume, P., Cerottini, J. C., and Romero, P. CpG are efficient adjuvants for specific CTL induction against tumor antigen-derived peptide. J Immunol, *168:* 1212-1218, 2002.
3. Mutwiri, G. K., Nichani, A. K., Babiuk, S., and Babiuk, L. A. Strategies for enhancing the immunostimulatory effects of CpG oligodeoxynucleotides. J Control Release, *97:* 1-17, 2004.
4. Blander, J. M. and Medzhitov, R. Toll-dependent selection of microbial antigens for presentation by dendritic cells. Nature, *440:* 808-812, 2006.
5. Yarovinsky, F., Kanzler, H., Hieny, S., Coffman, R. L., and Sher, A. Toll-like receptor recognition regulates immunodominance in an antimicrobial CD4+ T cell response. Immunity, *25:* 655-664, 2006.
6. Guermonprez, P. and Amigorena, S. Pathways for antigen cross presentation. Springer Semin Immunopathol, *26:* 257-271, 2005.
7. Carbone, F. R. and Bevan, M. J. Class I-restricted processing and presentation of exogenous cell-associated antigen in vivo. J Exp Med, *171:* 377-387, 1990.
8. Rock, K. L. and Shen, L. Cross-presentation: underlying mechanisms and role in immune surveillance. Immunol Rev, *207:* 166-183, 2005.
9. Nestle, F. O. Dendritic cell vaccination for the treatment of skin cancer. Recent Results Cancer Res, *160:* 165-169, 2002.
10. Waeckerle-Men, Y., Uetz-von Allmen, E., Fopp, M., von Moos, R., Bohme, C., Schmid, H. P., Ackermann, D., Cerny, T., Ludewig, B., Groettrup, M., and Gillessen, S. Dendritic cell-based multi-epitope immunotherapy of hormone-refractory prostate carcinoma. Cancer Immunol Immunother, 2006.
11. Speiser, D. E., Lienard, D., Rufer, N., Rubio-Godoy, V., Rimoldi, D., Lejeune, F., Krieg, A. M., Cerottini, J. C., and Romero, P. Rapid and strong human CD8+ T cell responses to vaccination with peptide, IFA, and CpG oligodeoxynucleotide 7909. J Clin Invest, *115:* 739-746, 2005.
12. Moss, R. B., Diveley, J., Jensen, F., and Carlo, D. J. In vitro immune function after vaccination with an inactivated, gp120-depleted HIV-1 antigen with immunostimulatory oligodeoxynucleotides. Vaccine, *18:* 1081-1087, 2000.
13. Beebe, G. W., Simon, A. H., and Vivona, S. Long-term mortality follow-up of Army recruits who received adjuvant influenza virus vaccine in 1951-1953. Am J Epidemiol, *95:* 337-346, 1972.
14. Toledo, H., Baly, A., Castro, O., Resik, S., Laferte, J., Rolo, F., Navea, L., Lobaina, L., Cruz, O., Miguez, J., Serrano, T., Sierra, B., Perez, L., Ricardo, M. E., Dubed, M., Lubian, A. L., Blanco, M., Millan, J. C., Ortega, A., Iglesias, E., Penton, E., Martin, Z., Perez, J., Diaz, M., and Duarte, C. A. A phase I clinical trial of a multi-epitope polypeptide TAB9 combined with Montanide ISA 720 adjuvant in non-HIV-1 infected human volunteers. Vaccine, *19:* 4328-4336, 2001.
15. Gander, B. Trends in particulate antigen and DNA delivery systems for vaccines. Adv Drug Deliv Rev, *57:* 321-323, 2005.
16. Men, Y., Thomasin, C., Merkle, H. P., Gander, B., and Corradin, G. A single administration of tetanus toxoid in biodegradable microspheres elicits T cell

and antibody responses similar or superior to those obtained with aluminum hydroxide. Vaccine, *13:* 683-689, 1995.

17. Men, Y., Gander, B., Merkle, H. P., and Corradin, G. Induction of sustained and elevated immune responses to weakly immunogenic synthetic malarial peptides by encapsulation in biodegradable polymer microspheres. Vaccine, *14:* 1442-1450, 1996.
18. Audran, R., Peter, K., Dannull, J., Men, Y., Scandella, E., Groettrup, M., Gander, B., and Corradin, G. Encapsulation of peptides in biodegradable microspheres prolongs their MHC class-I presentation by dendritic cells and macrophages in vitro. Vaccine, *21:* 1250-1255, 2003.
19. Newman, K. D., Elamanchili, P., Kwon, G. S., and Samuel, J. Uptake of poly(D,L-lactic-co-glycolic acid) microspheres by antigen-presenting cells in vivo. J Biomed Mater Res, *60:* 480-486, 2002.
20. Waeckerle-Men, Y., Allmen, E. U., Gander, B., Scandella, E., Schlosser, E., Schmidtke, G., Merkle, H. P., and Groettrup, M. Encapsulation of proteins and peptides into biodegradable poly(D,L-lactide-co-glycolide) microspheres prolongs and enhances antigen presentation by human dendritic cells. Vaccine, *24:* 1847-1857, 2006.
21. Shen, Z., Reznikoff, G., Dranoff, G., and Rock, K. L. Cloned dendritic cells can present exogenous antigens on both MHC class I and class II molecules. J Immunol, *158:* 2723-2730, 1997.
22. Manolova, V., Flace, A., Bauer, M., Schwarz, K., Saudan, P., and Bachmann, M. F. Nanoparticles target distinct dendritic cell populations according to their size. Eur J Immunol, *38:* 1404-1413, 2008.
23. Heit, A., Schmitz, F., Haas, T., Busch, D. H., and Wagner, H. Antigen co-encapsulated with adjuvants efficiently drive protective T cell immunity. Eur J Immunol, *37:* 2063-2074, 2007.
24. Schlosser, E., Mueller, M., Fischer, S., Basta, S., Busch, D. H., Gander, B., and Groettrup, M. TLR ligands and antigen need to be coencapsulated into the same biodegradable microsphere for the generation of potent cytotoxic T lymphocyte responses. Vaccine, *26:* 1626-1637, 2008.
25. Goforth, R., Salem, A. K., Zhu, X., Miles, S., Zhang, X. Q., Lee, J. H., and Sandler, A. D. Immune stimulatory antigen loaded particles combined with depletion of regulatory T-cells induce potent tumor specific immunity in a mouse model of melanoma. Cancer Immunol Immunother, *58:* 517-530, 2009.
26. Hamdy, S., Molavi, O., Ma, Z., Haddadi, A., Alshamsan, A., Gobti, Z., Elhasi, S., Samuel, J., and Lavasanifar, A. Co-delivery of cancer-associated antigen and Toll-like receptor 4 ligand in PLGA nanoparticles induces potent CD8+ T cell-mediated anti-tumor immunity. Vaccine, *26:* 5046-5057, 2008.
27. Waeckerle-Men, Y., Gander, B., and Groettrup, M. Delivery of tumor antigens to dendritic cells using biodegradable microspheres. Methods Mol Med, *109:* 35-46, 2005.
28. Barber, D. L., Wherry, E. J., and Ahmed, R. Cutting edge: rapid in vivo killing by memory CD8 T cells. J Immunol, *171:* 27-31, 2003.
29. Schwarz, K., van Den Broek, M., Kostka, S., Kraft, R., Soza, A., Schmidtke, G., Kloetzel, P. M., and Groettrup, M. Overexpression of the proteasome subunits LMP2, LMP7, and MECL-1, but not PA28 alpha/beta, enhances the presentation of an immunodominant lymphocytic choriomeningitis virus T cell epitope. J Immunol, *165:* 768-778, 2000.

30. Blomberg, K., Hautala, R., Lovgren, J., Mukkala, V. M., Lindqvist, C., and Akerman, K. Time-resolved fluorometric assay for natural killer activity using target cells labelled with a fluorescence enhancing ligand. J Immunol Methods, *193:* 199-206, 1996.
31. Schwarz, K., Meijerink, E., Speiser, D. E., Tissot, A. C., Cielens, I., Renhof, R., Dishlers, A., Pumpens, P., and Bachmann, M. F. Efficient homologous prime-boost strategies for T cell vaccination based on virus-like particles. Eur J Immunol, *35:* 816-821, 2005.
32. Shephard, E., Burgers, W. A., Van Harmelen, J. H., Monroe, J. E., Greenhalgh, T., Williamson, C., and Williamson, A. L. A multigene HIV type 1 subtype C modified vaccinia Ankara (MVA) vaccine efficiently boosts immune responses to a DNA vaccine in mice. AIDS Res Hum Retroviruses, *24:* 207-217, 2008.
33. McShane, H., Pathan, A. A., Sander, C. R., Keating, S. M., Gilbert, S. C., Huygen, K., Fletcher, H. A., and Hill, A. V. Recombinant modified vaccinia virus Ankara expressing antigen 85A boosts BCG-primed and naturally acquired antimycobacterial immunity in humans. Nat Med, *10:* 1240-1244, 2004.
34. Gudmundsdotter, L., Nilsson, C., Brave, A., Hejdeman, B., Earl, P., Moss, B., Robb, M., Cox, J., Michael, N., Marovich, M., Biberfeld, G., Sandstrom, E., and Wahren, B. Recombinant Modified Vaccinia Ankara (MVA) effectively boosts DNA-primed HIV-specific immune responses in humans despite pre-existing vaccinia immunity. Vaccine, *27:* 4468-4474, 2009.
35. Jegerlehner, A., Maurer, P., Bessa, J., Hinton, H. J., Kopf, M., and Bachmann, M. F. TLR9 signaling in B cells determines class switch recombination to IgG2a. J Immunol, *178:* 2415-2420, 2007.
36. Mann, J. F., Shakir, E., Carter, K. C., Mullen, A. B., Alexander, J., and Ferro, V. A. Lipid vesicle size of an oral influenza vaccine delivery vehicle influences the Th1/Th2 bias in the immune response and protection against infection. Vaccine, *27:* 3643-3649, 2009.
37. Habjanec, L., Halassy, B., and Tomasic, J. Immunomodulatory activity of novel adjuvant formulations based on Montanide ISA oil-based adjuvants and peptidoglycan monomer. Int Immunopharmacol, *8:* 717-724, 2008.
38. Ioannou, X. P., Gomis, S. M., Karvonen, B., Hecker, R., Babiuk, L. A., and van Drunen Littel-van den Hurk, S. CpG-containing oligodeoxynucleotides, in combination with conventional adjuvants, enhance the magnitude and change the bias of the immune responses to a herpesvirus glycoprotein. Vaccine, *21:* 127-137, 2002.
39. Hamdy, S., Elamanchili, P., Alshamsan, A., Molavi, O., Satou, T., and Samuel, J. Enhanced antigen-specific primary CD4+ and CD8+ responses by codelivery of ovalbumin and toll-like receptor ligand monophosphoryl lipid A in poly(D,L-lactic-co-glycolic acid) nanoparticles. J Biomed Mater Res A, *81:* 652-662, 2007.

Chapter III

α-Galactosylceramide and its C-glycoside α-C-galatosylceramide show no significant differences in activation of NKT cells

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Abstract

Since α-C-galactosylceramide was reported to show an advantageous cytokine profile, compared to α-galactosylceramide in the past years, we decided to investigate both glycolipids for their Th1 polarizing capacity. An improved cytokine environment is known to positively influence immunotherapeutical approaches. Here, we investigated putative beneficial effects of both, the C and the O glycoside of α-galactosylceramide in an immunization strategy based on poly(lactide-*co*-glycolide) microspheres (PLGA-MS). Therefore, α-C-galactosylceramide was synthesized in our laboratory and microencapsulated into PLGA-MS or used in a soluble form. Surprisingly, α-C-galactosylceramide did neither show any superiority to α-galactosylceramide wit respect to the activation of NKT cells or DCs nor did it lead to altered levels of IFN-γ or IL-4. In particular, we were not able to reproduce the previously reported Th1 polarizing effects of α-C-galactosylceramide. We investigated the potential of both substances to improve poly(lactide-*co*-glycolide) microspheres induced cytotoxic T lymphocyte responses, *in vivo* cytotoxicity and anti tumor responses, but we failed to confirm an improved anti tumor response by α-C-galactosylceramide as compared to α-galactosylceramide.

Introduction

Invariant natural killer T (iNKT) cells are a subtype of NK cells, which express an invariant T cell Vα14/18 (mouse/human) receptor that recognizes glycolipid antigens in the context of the non-polymorphic MHC class I like molecule CD1d (1, 2). They can be found in significant numbers in the liver, bone marrow, and in reduced numbers in the spleen (3). INKT cells are identified by their reactivity to α-galactosylceramide (α-GalCer) (4), a synthetic glycolipid, efficiently binding to CD1d. α-GalCer was found to stimulate proliferation of NKT cells, maturation of DC, up-regulation of costimulatory molecules and production of IL-12 (5), as well as cytokine production of e.g., IFN-γ, IL-2, IL-10, TGF-β, TNF-α, GM-CSF or IL-4 (6, 7). Furthermore, NKT cells are attested to support cross-priming of antigens by DC activation (5) and thus forming a cross-link between the native and the adaptive immune system (8).

Consequently, α-GalCer has been shown to influence the Th1/Th2 bias of an immune response (9) and to be effective in anti-tumor responses (10), therapies against melanoma metastases of liver and lung, e.g., using α-GalCer pulsed DCs (11-13), as well as infectious diseases (14). Several studies stated that α-GalCer can act as an adjuvant (15) and cooperates with TLR stimulation (16, 17). More recently the C-glycoside analogue of α-GalCer, α-C-galactosylceramide (α-C-GalCer) was reported to improve immune-modulatory functions (18, 19), due to the preferential induction of Th1 cytokines produced by NKT cells and DCs. Recent studies confirmed some of these findings, in particular the cytokine profile (20) and the adjuvant effect (21).

α-GalCer shows the highest efficacy if it is loaded directly onto DCs. Therefore, a potent *in vivo* loading of DCs with α-GalCer or α-C-GalCer would facilitate their usage for immunotherapy of tumors or infectious diseases. We, and others, have recently shown that poly(lactide-*co*-glycolide) (PLGA) microspheres (MS) are a powerful device to target APCs *in vivo* and induce substantial CTL responses resulting in tumor regression (22, 23). PLGA is a well-defined, biodegradable polymer, which is approved for clinical use and is widely used e.g., for sutures or implants. It is known to protect microencapsulated materials from degradation, thus forming an antigen depot (24). Prior studies showed, that PLGA-MS, ranging from 1-10 µm in size, are effectively taken up at the site of injection (25) by APCs which efficiently present the delivered antigen on MHC class I and class II (26). These features make PLGA-MS an interesting candidate for further studies involving the in vivo loading of DCs with CD1d ligands in order to activate NKT cells. These NKT cells could provide useful assistance by a rapid and robust production of cytokines and the establishment of a Th1 polarizing environment.

In order to test α-C-GalCer as an additional immune-stimulator together with PLGA-MS, containing co-encapsulated ovalbumin (Ova) and CpG-oligodeoxynucleotides (CpG-ODN) (MS-Ova/CpG), we synthesized α-C-GalCer according to the protocol of Wipf and Pierce (27). Surprisingly, we found only minor differences between α-C-GalCer and α-GalCer, when we tried to reproduce the key experiments of two previous reports. Similarly, the encapsulated glycolipids did neither improve the CTL response nor anti tumor activity.

Material and methods

Glycolipids: α-C-GalCer [(3S,4S,5R)-1-(a-D-galactopyranosyl)-3-(N-hexacosanoylamino)-4,5-nonadecanediol] (18) was synthesized following a protocol by Wipf *et al*. (27). The key reactions of this synthesis route are a diastereoselective alkenylalane addition to a C-glycosidic α-galactosyl N-tert-butanesulfinyl imine derivative, followed by epoxidation and carbamate ring opening to generate an amino diol stereotriad. The resulting α-C-Galactosylsphingosine was coupled to the fatty acid chloride to obtain the required α-C-GalCer.
α-GalCer [(2S,3S,4R)-1-O-(a-D-galactopyranosyl)-2-(N-hexacosanoylamino)-1,3,4-octadecanetriol] was synthesized according to reference (40). All glycolipids were dissolved in Dimethyl sulfoxide DMSO (0,5mg/ml) and diluted in PBS immediately before i.v. injection.

Mice and immunizations: C57BL/6 mice (H-2b) were originally purchased from Charles River Laboratories. All mice were kept in a specific pathogen-free facility and used at 6–10 weeks of age. Animal experiments were approved by the review board of the Regierungspräsidium Freiburg, Germany.

ELISA: For the IFN-γ, IL-4, TNF-α and IL-12 cytokine determination, the corresponding BD-ELISA Kit (BD OptEIATM, BD Bioscience) was used, following the manufacturer’s instructions. Absorbance at 450-570 nm was measured, using a SpectrafluorPlus plate reader (Tecan).

ELISPOT Assay: For IFN-γ and IL-4 ELISPOT the respective ELISpot Kit (Cellular Technology Ltd.) was used according to the manufacturers instructions. Plates were read using an ELISpot plate reader (Cellular Technology Ltd.).

Intracellular cytokine staining (ICS): Splenocytes were isolated and restimulated with or without 10 µM SIINFEKL peptide (Eurogentec, Cologne, Germany) in the presence of brefeldin A (10 µg/ml, Sigma-Aldrich) for 5 h at 37 °C. Subsequently, cells were stained with anti-mouse-CD8α-PE-Cy5 antibody (BD Biosciences Pharmingen, Clone 53-6.7) or anti-mouse NK1.1-PerCP-Cy5.5 (BD Biosciences Pharmingen) and anti-mouse-CD11c APC conjugated (BD Biosciences Pharmingen) for 20 min at 4°C. After fixation with 4%

paraformaldehyde in PBS for 10 min at room temperature, cells were labeled intracellularly with rat-anti-IFN-γ-FITC antibody (clone XGM1.2, diluted in PBS/0.1% Saponin) or anti-mouse-IL-12-PE antibody (BD Biosciences Pharmingen) at 4 °C overnight. The next day cells were analyzed by flow cytometry. Background values for each sample (without restimulation) were subtracted.
Data were acquired using a FACScan flow cytometer (BD Biosciences) and analyzed with FlowJo software (Treestar).

In vivo cytotoxicity assay: Splenocytes from naive mice were pulsed with 10 μM SIINFEKL peptide for 1 h at 37 °C or left untreated. Pulsed cells were stained with 20 μM carboxyfluorescein succinimidyl ester (CFSE), while unpulsed cells were stained with 2 μM CFSE. Immediately before injection, both cell populations were mixed in equal amounts, and a total number of $1×10^7$ cells were injected i.v.. After 18 h, splenocytes were prepared and analyzed for CFSE fluorescence by flow cytometry. 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)] × 100.

Preparation of PLGA-microspheres: Microspheres (MS) were prepared from 14 kDa poly(D,L-lactide-*co*-glycolide) (PLGA) 50:50 carrying hydroxyl and carboxyl-end groups (Resomer RG502H, Boehringer Ingelheim, Ingelheim, Germany) by spray drying. Briefly, 50 mg ovalbumin (Grade V, Sigma) and 5 mg CpG oligodeoxynucleotides with a phosphothioate backbone (CpG-ODN 1826, Microsynth, Balgach, Switzerland) or 0,5 mg polyI:C (Calbiochem, VWR, Dietikon, Switzerland) (MS polyI:C) were dissolved in 0.5 ml 0.1 M $NaHCO_3$ (aqueous phase) and mixed with 1 g of PLGA dissolved in 20 ml of dichloromethane (organic phase). Optionally 0.5mg α-GalCer (MS-α-GalCer) or α-C-GalCer (MS-α-C-GalCer) were dissolved in 160μl methanol / chloroform and mixed with 1 g of PLGA dissolved in 20 ml of dichloromethane. The two phases were subsequently emulsified by ultrasonication (Hielscher, UP200 H, Ampl. 40%) for 10 s on ice. The obtained w/o dispersion was immediately spray-dried (Büchi, Mini Spray-Dryer 191) at a flow rate of 2 ml/min and inlet/outlet temperatures of 40/37 °C. The

obtained MS were washed out of the spray-dryer's cyclone with 0.05 % Synperonic (Synperonic®F68, Serva Electrophoresis GmbH, Heidelberg, Germany), collected on a cellulose acetate membrane filter and dried under reduced pressure (20 mbar) for 18 h at room temperature. PLGA-MS were stored at 4 °C. Immediately before use MS were dispersed in PBS by ultrasonication for 30 sec.

5mg MS-Ova/CpG (containing 250 μg Ova, 25 μg CpG-ODN) or indicated amounts of MS-α-GalCer and MS-α-C-GalCer were injected in PBS s.c. at the base of the tail.

Results and discussion

We analyzed serum cytokine levels of IL-4, IFN-γ, TNF-α and IL-12 in mice, injected with graded doses of either α-GalCer or α-C-GalCer. To that aim, mice were immunized i.v. with 20 ng, 200 ng, or 2000 ng of either α-GalCer or α-C-GalCer. Peripheral blood was drawn at different time points post injection and serum was stored at –80 °C for cytokine quantification by ELISA. As sown in figure 1A, differences in the abundance of IL-4 could only be detected at lower doses of the two ceramides. At a dose of 2000 ng no significant difference could be detected. After the initial burst of IL-4 secretion, a prolonged phase of IFN-γ production has been described for α-C-GalCer (19). This effect, and the complete absence of IL-4, as previously reported, could not be observed by us. The cytokine profiles for IL-12 and TNF-α were in accordance with the literature (19).

The source of IFN-γ detected in the serum of α-GalCer or α-C-GalCer treated mice is presumably the NKT cells population. In order to characterize the sources of IFN-γ and IL-12 we injected mice i.v. with 1 μg of either α-GalCer or α-C-GalCer and $CD11c^{+}$ splenocytes were magnetically isolated 6 hours post injection. $CD11c^{+}$ splenocytes were stained and gated for the expression of $CD11c^{high}$ and $NK1.1^{+}$ as shown in figure 1B (top graph). The $NK1.1^{+}$ population in gate R2 produced mainly IFN-γ (left panel) but only little IL-12 (right panel), whereas the $CD11c^{high}$ population (gate R3) was positive for IL-12.

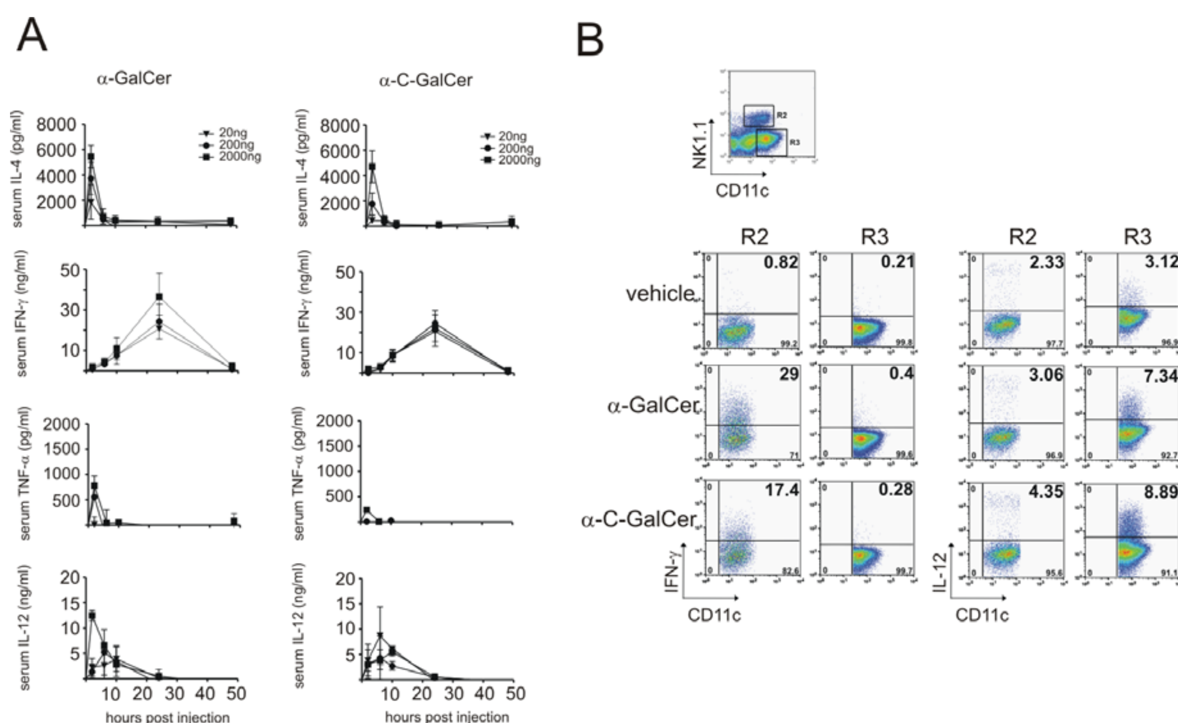

Figure 1: Serum cytokine levels and cytokines produced by CD11c$^+$ splenocytes. (A) Mice (n=3) were injected i.v. with indicated doses of either α-GalCer (left panel) or α-C-GalCer (right panel) and bled after 2, 6, 10, 24 and 48 hours. IFN-γ, IL-4, TNF-α, and IL-12 cytokine levels in the sera (mean values +/- SEM) were determined after injections of indicated amounts of the respective glycolipid. One out of three similar experiments is shown. (B) Mice (n=2) were injected i.v. with 1 µg of either α-GalCer or α-C-GalCer. After 6 hours, splenocytes were prepared, magnetically sorted for CD11c, and stained for CD11c and NK1.1. Shown in the top dot plot for CD11c^{high} DCs (gate R3) and NK1.1$^+$ cells (gated as R2). Both populations were stained for intracellular IFN-γ (left side) or IL-12 (right side). Representative dot plots for each group are shown from one, out of three independent experiments with similar outcome.

Interestingly we found that α-C-GalCer was slightly superior in the stimulation of IL-12 produced by DC, but clearly less effective than α-GalCer with regard to the stimulation of NK1.1$^+$ cells to produce IFN-γ.

These findings are in disagreement with recent work performed with α-C-GalCer synthesized by R. Frank and colleagues (18, 19, 28). They described α-C-GalCer as a strong stimulator of IFN-γ but not IL-4, presumably produced by NKT cells. In accordance with reference (19), we could confirm that α-C-GalCer is a strong inducer of DC mediated IL-12 production, but we did not find a more potent stimulation of NKT cells to produce IFN-γ. Importantly, we could not reproduce the claimed reduction of IL-4 in the sera of α-C-GalCer immunized mice (19).

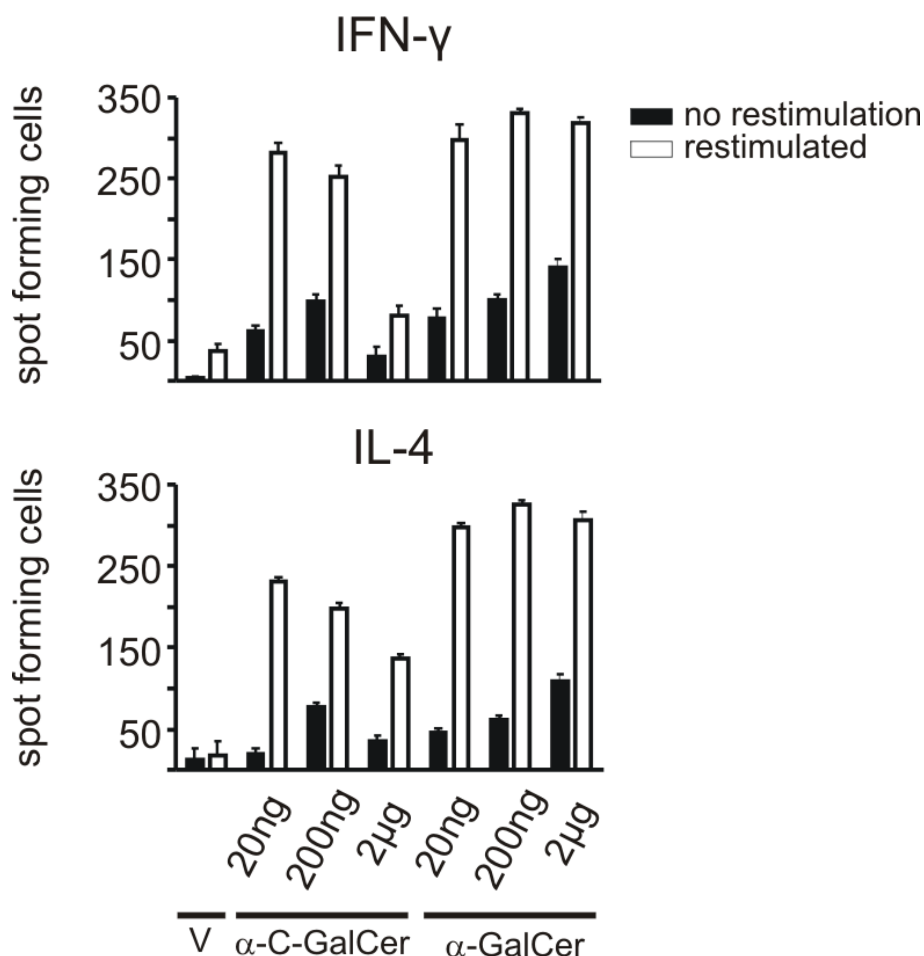

Figure 2: ELISPOT for IFN-γ and IL-4. Mice (n=2) were immunized with vehicle (V) or indicated doses of α-GalCer or α-C-GalCer. Two days later the splenocytes were isolated. $3x10^5$ cells were either restimulated with α-GalCer (100 ng/ml) (white bars) or left untreated (black bars) for 16 hours in precoated ELISPOT plates at 37 °C. IFN-γ (upper graph) or IL-4 (lower graph) specific spots were counted and are given +/- SEM. The graph represents one out of three independent experiments.

In order to gain deeper insight into the activation of NKT, we performed another experiment previously described by Fuji *et al.* (19). We immunized mice with vehicle or different concentrations of either α-GalCer or α-C-GalCer. Two days later we isolated the splenocytes and restimulated them with α-GalCer (100 ng/ml) for 16 hours. As shown in figure 2, α-GalCer is able to prime NKT cells even when low doses are applied. Our data even suggest that α-C-GalCer primes splenocytes a bit less effectively for the production of IL-4 and IFN-γ than α-GalCer.

Since we could confirm the identity of the ceramides used in this study by NMR-spectra (not shown) a difference in chemical composition can not account for the discrepancy of our results and those published by Fuji *et al.* (39). In general, an altered interaction of the aliphatic chains of the N-acyl and the sphingosine tail with

amino acids of the lipid chain pocket of the CD1d molecule could be possible. This would, as a consequence, alter the immune-stimulatory features of the substance (29), and thereby influence the cytokine production. Crystal structures of α-GalCer bound to CD1d showed that in addition to the galactose 2'-OH hydrogen and the 3'-OH of the sphingosine the glycosidic 1'-O of α-GalCer is also involved in binding to CD1d (30). According to structural considerations, a replacement of the 1'-O by CH_2 in the C-glycoside is not expected to stabilize the binding to NKT cells because the 1'-O atom seems to be important for the correct positioning of the CD1d ligand for recognition by the invariant TCR of NKT cells. This notion would be consistent with our results (figure 1 and 2).

Loading of DCs with α-GalCer has previously been shown to be effective in anti cancer therapies (31, 32) but it is also well known that *ex vivo* loading of DCs is laborious and expensive (33). With PLGA-MS we make use of a tool that targets antigens and adjuvants to DCs *in vivo* upon s.c. injection (34, 35). To investigate the potential of α-GalCer or α-C-GalCer charged PLGA-MS for immune stimulation we immunized mice with PLGA-MS containing ovalbumin and CpG oligonucleotides (MS-Ova/CpG) and PLGA-MS containing either α-GalCer (MS-α-GalCer) or α-C-GalCer (MS-α-C-GalCer). Six days later we performed an in vivo cytotoxicity assay. As shown in figure 3 we could not find any improvement of our original vaccination strategy when the α-GalCer or α-C-GalCer containing PLGA-MS were co-administrated.

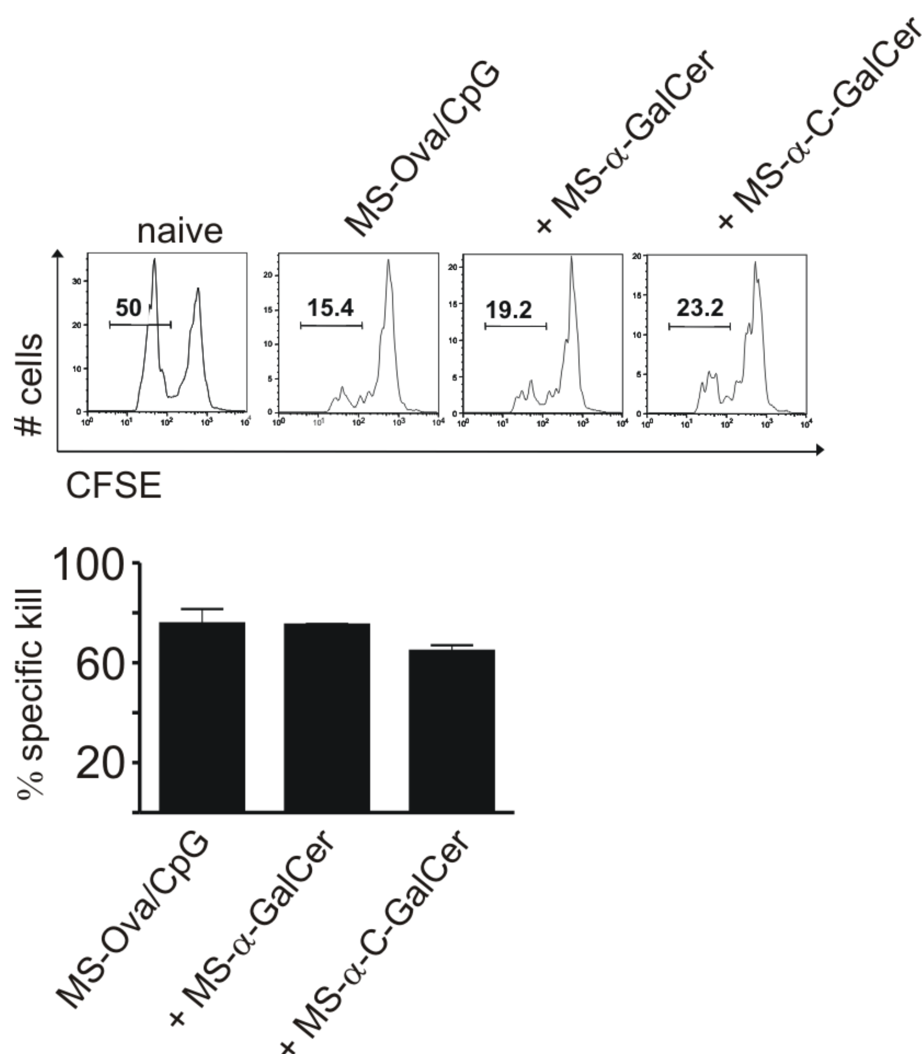

Figure 3: In vivo cytotoxicity assay. Mice (n=2) were immunized either with 5mg MS-Ova/CpG or with 5mg MS-Ova/CpG in combination with 1mg MS α-GalCer (indicated as +MS-α-GalCer) or 1mg MS α-C-GalCer (indicated as +MS-α-C-GalCer). After six days, mice received $1x10^7$ SIINFEKL pulsed (low CFSE) or unpulsed (high CFSE), CFSE-labeled C57BL/6 splenocytes. 18 hours later splenocytes were analyzed for CFSE fluorescence. The upper graph shows a representative histogram for one animal of each group, the lower graph shows one quantitative analysis of three experiments, values are given in percent specific lysis, +/- SEM.

The loading of DC with α-GalCer has been shown to improve NKT proliferation and IFN-γ production (11) but also the maturation of DCs and their potential to stimulate $CD4^+$ and $CD8^+$ T cells via CD40 binding (36, 37). This suggests that the activation of DCs through the uptake of antigen and adjuvants in PLGA-MS microencapsulated form is highly effective and could not be further promoted by the supply of the investigated CD1d ligands.

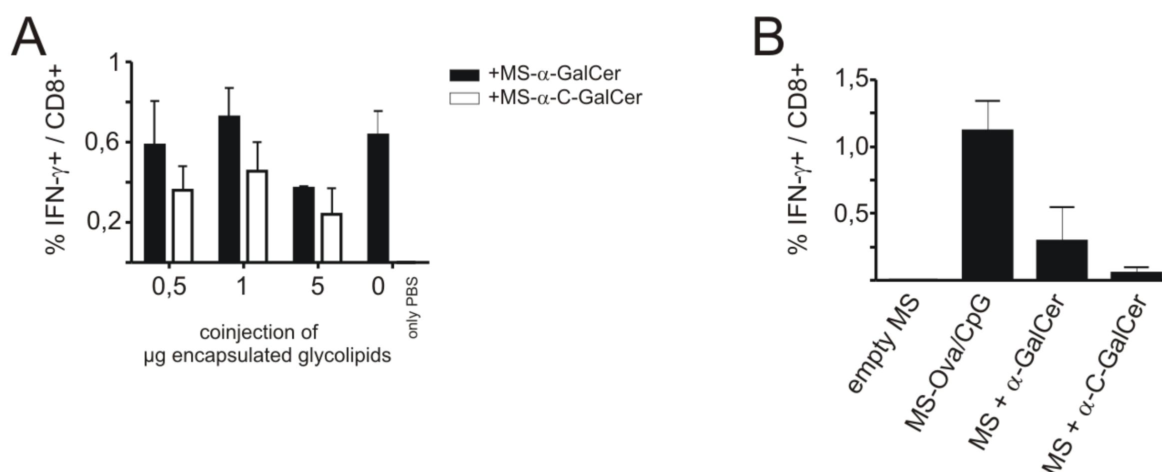

Figure 4: CTL response after co-injection of encapsulated or soluble glycolipids and MS-Ova/CpG. Mice (n=2) were immunized with 5 mg MS-Ova/CpG and (A) indicated amount of MS-α-GalCer (black bars) or MS-α-C-GalCer (white bars) s.c. or (B) with or without 1 µg soluble glycolipids (indicated as MS + α-GalCer and MS + α-C-GalCer) i.v.. After six days splenocytes were isolated and analyzed for IFN-γ^+/CD8$^+$ lymphocytes, +/- SEM. Both experiments were conducted three times with similar results. One representative of each is shown.

To confirm this finding we analyzed the CTL response to MS-Ova/CpG and the co-injection of either MS-encapsulated or soluble glycolipids. Respective kinds of PLGA-MS were mixed and injected s.c. followed by analysis of splenocytes at day 6 after injection. Figure 4A shows a titration of encapsulated glycolipids in combination with MS-Ova/CpG. Compared to an injection of MS-Ova/CpG alone neither the addition of α-GalCer nor of α-C-GalCer showed any enhanced IFN-γ response. In contrast to our expectation, the co-delivery of encapsulated glycolipids negatively affected the generation of CTL responses at higher doses. This is actually enhanced if the glycolipids are not encapsulated but injected i.v., dissolved in PBS. We injected 1 µg of either α-GalCer or α-C-GalCer i.v. together with a s.c. injection of MS-Ova/CpG and measured the Ova specific CTL response on day six after injection. As shown in figure 4B, we found that a co-injection of both glycolipids strongly reduced the percentage of SIINFEKL specific, CD8$^+$/IFN-γ^+ lymphocytes.

Taken together these experiments clearly show, that the co-delivery of a putative CD1d ligand does not foster the establishment of a strong CTL response but has adverse effects, at least in our system. It has been shown for α-GalCer, that it can act as an adjuvant in combination tumor cells (38), proteins or peptides (16) or Ova and TLR ligands (17). An adjuvantic activity has also been shown for α-C-GalCer (19). It is known, that DCs provide cytokines, which serve as co-

stimulatory signals that can avoid NKT cell anergy (11). This indicates a dependency on a close interaction of the glycolipid and an antigen that is not given when the glycolipid is applied systemically. Nevertheless, it is possible to further increase the potential of PLGA-MS by the co-injection of encapsulated polyI:C (data not shown), implying that *in vivo* loading of DCs with α-GalCer or α-C-GalCer is either ineffective or activates a negative feedback loop (39).
Since various reports describe α-GalCer as a potent agent in protection from tumors and its adjuvantic activity in therapeutic tumor models we decided to compare α-GalCer, α-C-GalCer and MS-Ova/CpG in therapeutic and protective tumor models.

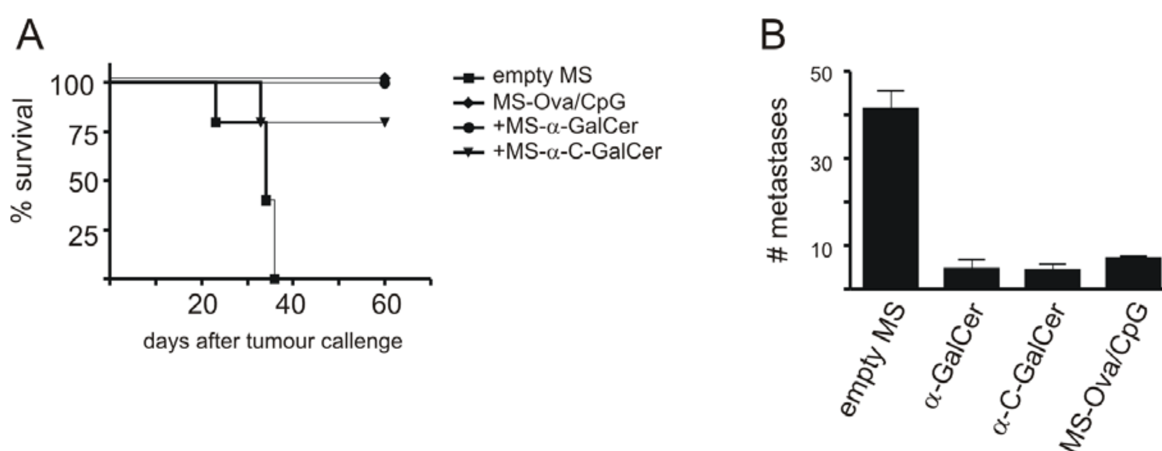

Figure 5: Tumor therapy and protection from lung metastases. (A) Mice (n=5) were challenged with $5x10^5$ EG-7 cells s.c. into the right flank. As soon as palpable tumors emerged, mice were immunized with 5 mg empty MS (squares), 5 mg MS-Ova/CpG (diamonds) or with 5mg MS-Ova/CpG and 0,5 µg encapsulated α-GalCer (circles) or α-C-GalCer (triangles). Tumors were measured daily until the mean of two orthogonal measurements has reached 15mm. Values are given in percent survival. One representative experiment out of two is shown. (B) Mice (n=3) were injected s.c. with empty MS, 5 mg MS-Ova/CpG, 2 µg soluble α-GalCer i.v., or 2 µg soluble α-C-GalCer i.v.. Two days later mice were challenged i.v. with $1x10^5$ melanin expressing MO-5 cells. After two weeks, visible lung metastases were counted and are given in mean numbers of counted metastases, +/- SEM. The experiment as shown was conducted three times with similar results.

In figure 5A a therapeutic setup is shown, using an ova expressing EG-7 cell line. $5x10^5$ cells were injected into the right flank and as soon as palpable tumors occurred, mice were immunized s.c with MS-Ova/CpG alone or in combination with 0,5 mg MS-α-GalCer or MS-α-C-GalCer. Subsequently, tumor sizes were measured daily until they reached 15mm in mean size of 2 orthogonal measurements. If this abort criterion was fulfilled, mice were sacrificed. There is only a non-significant difference between α-GalCer and α-C-GalCer treated

groups, while all animals treated with MS-Ova/CpG alone survived tumor free. In order to employ a more sensitive assay we performed a lung metastases formation experiment, which revealed a significant difference between α-GalCer and α-C-GalCer in the past (18). We injected 1 μg of either α-GalCer or α-C-GalCer i.v. or MS-Ova/CpG s.c. and two days later mice were challenged with $1x10^5$ melanin-positive ova expressing MO-5 cells. After two weeks visible lung metastases were counted as documented in figure 5B. Interestingly, we were again not able to find any difference between α-GalCer and α-C-GalCer treated groups and only minor differences between the PLGA-MS treated group and the groups having been treated with the respective glycolipid.

Taken together, we can state that an additional activation of the innate arm of the immune system does not further enhance the potency of our system. We could demonstrate that PLGA-MS based immunotherapy is a powerful tool that yields substantial activation of the immune system (figure 5). There was no evidence, that α-C-GalCer shows any superiority compared to α-GalCer (figures 1 and 2). Repeating the key experiments of previous studies (18, 19) we only obtained minor differences between the O- and the C-glycoside, if any. A preferential application of α-C-GalCer is therefore not warranted.

Nevertheless, both substances are potent inducers of DCs and NKT cells and account for rapid induction of early IFN-γ and IL-12 (figure 2), as well as the protection from tumor growth (figure 5). α-C-GalCer, as well as α-GalCer, might be possible candidates to support less effective therapeutic strategies by their NKT cell-activating features.

References of chapter III:

1. Kawano, T., J. Cui, Y. Koezuka, I. Toura, Y. Kaneko, K. Motoki, H. Ueno, R. Nakagawa, H. Sato, E. Kondo, H. Koseki, and M. Taniguchi. 1997. CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. *Science* 278:1626-1629.
2. Spada, F.M., Y. Koezuka, and S.A. Porcelli. 1998. CD1d-restricted recognition of synthetic glycolipid antigens by human natural killer T cells. *J Exp Med* 188:1529-1534.
3. Matsuda, J.L., T. Mallevaey, J. Scott-Browne, and L. Gapin. 2008. CD1d-restricted iNKT cells, the 'Swiss-Army knife' of the immune system. *Curr Opin Immunol* 20:358-368.
4. Morita, M., K. Motoki, K. Akimoto, T. Natori, T. Sakai, E. Sawa, K. Yamaji, Y. Koezuka, E. Kobayashi, and H. Fukushima. 1995. Structure-activity relationship of alpha-galactosylceramides against B16-bearing mice. *J Med Chem* 38:2176-2187.
5. Fujii, S., K. Shimizu, C. Smith, L. Bonifaz, and R.M. Steinman. 2003. Activation of natural killer T cells by alpha-galactosylceramide rapidly induces the full maturation of dendritic cells in vivo and thereby acts as an adjuvant for combined CD4 and CD8 T cell immunity to a coadministered protein. *J Exp Med* 198:267-279.
6. Gumperz, J.E., S. Miyake, T. Yamamura, and M.B. Brenner. 2002. Functionally distinct subsets of CD1d-restricted natural killer T cells revealed by CD1d tetramer staining. *J Exp Med* 195:625-636.
7. Singh, N., S. Hong, D.C. Scherer, I. Serizawa, N. Burdin, M. Kronenberg, Y. Koezuka, and L. Van Kaer. 1999. Cutting edge: activation of NK T cells by CD1d and alpha-galactosylceramide directs conventional T cells to the acquisition of a Th2 phenotype. *J Immunol* 163:2373-2377.
8. Bendelac, A., P.B. Savage, and L. Teyton. 2007. The biology of NKT cells. *Annu Rev Immunol* 25:297-336.
9. Yoshimoto, T., A. Bendelac, C. Watson, J. Hu-Li, and W.E. Paul. 1995. Role of NK1.1+ T cells in a Th2 response and in immunoglobulin E production. *Science* 270:1845-1847.
10. Kobayashi, E., K. Motoki, T. Uchida, H. Fukushima, and Y. Koezuka. 1995. KRN7000, a novel immunomodulator, and its antitumor activities. *Oncol Res* 7:529-534.
11. Fujii, S., K. Shimizu, M. Kronenberg, and R.M. Steinman. 2002. Prolonged IFN-gamma-producing NKT response induced with alpha-galactosylceramide-loaded DCs. *Nat Immunol* 3:867-874.
12. Ishikawa, A., S. Motohashi, E. Ishikawa, H. Fuchida, K. Higashino, M. Otsuji, T. Iizasa, T. Nakayama, M. Taniguchi, and T. Fujisawa. 2005. A phase I study of alpha-galactosylceramide (KRN7000)-pulsed dendritic cells in patients with advanced and recurrent non-small cell lung cancer. *Clin Cancer Res* 11:1910-1917.
13. Fuji, N., Y. Ueda, H. Fujiwara, T. Toh, T. Yoshimura, and H. Yamagishi. 2000. Antitumor effect of alpha-galactosylceramide (KRN7000) on spontaneous hepatic metastases requires endogenous interleukin 12 in the liver. *Clin Cancer Res* 6:3380-3387.
14. Wilson, M.T., A.K. Singh, and L. Van Kaer. 2002. Immunotherapy with ligands of natural killer T cells. *Trends Mol Med* 8:225-231.

15. Gonzalez-Aseguinolaza, G., L. Van Kaer, C.C. Bergmann, J.M. Wilson, J. Schmieg, M. Kronenberg, T. Nakayama, M. Taniguchi, Y. Koezuka, and M. Tsuji. 2002. Natural killer T cell ligand alpha-galactosylceramide enhances protective immunity induced by malaria vaccines. *J Exp Med* 195:617-624.
16. Silk, J.D., I.F. Hermans, U. Gileadi, T.W. Chong, D. Shepherd, M. Salio, B. Mathew, R.R. Schmidt, S.J. Lunt, K.J. Williams, I.J. Stratford, A.L. Harris, and V. Cerundolo. 2004. Utilizing the adjuvant properties of CD1d-dependent NK T cells in T cell-mediated immunotherapy. *J Clin Invest* 114:1800-1811.
17. Hermans, I.F., J.D. Silk, U. Gileadi, S.H. Masri, D. Shepherd, K.J. Farrand, M. Salio, and V. Cerundolo. 2007. Dendritic cell function can be modulated through cooperative actions of TLR ligands and invariant NKT cells. *J Immunol* 178:2721-2729.
18. Schmieg, J., G. Yang, R.W. Franck, and M. Tsuji. 2003. Superior protection against malaria and melanoma metastases by a C-glycoside analogue of the natural killer T cell ligand alpha-Galactosylceramide. *J Exp Med* 198:1631-1641.
19. Fujii, S., K. Shimizu, H. Hemmi, M. Fukui, A.J. Bonito, G. Chen, R.W. Franck, M. Tsuji, and R.M. Steinman. 2006. Glycolipid alpha-C-galactosylceramide is a distinct inducer of dendritic cell function during innate and adaptive immune responses of mice. *Proc Natl Acad Sci U S A* 103:11252-11257.
20. Schmieg, J., G. Yang, R.W. Franck, and M. Tsuji. A multifactorial mechanism in the superior antimalarial activity of alpha-C-GalCer. *J Biomed Biotechnol* 2010:283612.
21. Kopecky-Bromberg, S.A., K.A. Fraser, N. Pica, E. Carnero, T.M. Moran, R.W. Franck, M. Tsuji, and P. Palese. 2009. Alpha-C-galactosylceramide as an adjuvant for a live attenuated influenza virus vaccine. *Vaccine* 27:3766-3774.
22. Schlosser, E., M. Mueller, S. Fischer, S. Basta, D.H. Busch, B. Gander, and M. Groettrup. 2008. TLR ligands and antigen need to be coencapsulated into the same biodegradable microsphere for the generation of potent cytotoxic T lymphocyte responses. *Vaccine* 26:1626-1637.
23. Heit, A., F. Schmitz, T. Haas, D.H. Busch, and H. Wagner. 2007. Antigen co-encapsulated with adjuvants efficiently drive protective T cell immunity. *Eur J Immunol* 37:2063-2074.
24. Waeckerle-Men, Y., E.U. Allmen, B. Gander, E. Scandella, E. Schlosser, G. Schmidtke, H.P. Merkle, and M. Groettrup. 2006. Encapsulation of proteins and peptides into biodegradable poly(D,L-lactide-co-glycolide) microspheres prolongs and enhances antigen presentation by human dendritic cells. *Vaccine* 24:1847-1857.
25. Men, Y., C. Thomasin, H.P. Merkle, B. Gander, and G. Corradin. 1995. A single administration of tetanus toxoid in biodegradable microspheres elicits T cell and antibody responses similar or superior to those obtained with aluminum hydroxide. *Vaccine* 13:683-689.
26. Waeckerle-Men, Y., B. Gander, and M. Groettrup. 2005. Delivery of tumor antigens to dendritic cells using biodegradable microspheres. *Methods Mol Med* 109:35-46.
27. Wipf, P., and J.G. Pierce. 2006. Expedient synthesis of the alpha-C-glycoside analogue of the immunostimulant galactosylceramide (KRN7000). *Org Lett* 8:3375-3378.

28. Grajewski, R.S., A.M. Hansen, R.K. Agarwal, M. Kronenberg, S. Sidobre, S.B. Su, P.B. Silver, M. Tsuji, R.W. Franck, A.P. Lawton, C.C. Chan, and R.R. Caspi. 2008. Activation of invariant NKT cells ameliorates experimental ocular autoimmunity by a mechanism involving innate IFN-gamma production and dampening of the adaptive Th1 and Th17 responses. *J Immunol* 181:4791-4797.
29. Silk, J.D., M. Salio, J. Brown, E.Y. Jones, and V. Cerundolo. 2008. Structural and functional aspects of lipid binding by CD1 molecules. *Annu Rev Cell Dev Biol* 24:369-395.
30. Koch, M., V.S. Stronge, D. Shepherd, S.D. Gadola, B. Mathew, G. Ritter, A.R. Fersht, G.S. Besra, R.R. Schmidt, E.Y. Jones, and V. Cerundolo. 2005. The crystal structure of human CD1d with and without alpha-galactosylceramide. *Nat Immunol* 6:819-826.
31. Shimizu, K., and S. Fujii. 2009. DC therapy induces long-term NK reactivity to tumors via host DC. *Eur J Immunol* 39:457-468.
32. Fukushima, S., S. Hirata, Y. Motomura, D. Fukuma, Y. Matsunaga, Y. Ikuta, T. Ikeda, T. Kageshita, H. Ihn, Y. Nishimura, and S. Senju. 2009. Multiple antigen-targeted immunotherapy with alpha-galactosylceramide-loaded and genetically engineered dendritic cells derived from embryonic stem cells. *J Immunother* 32:219-231.
33. Cerundolo, V., I.F. Hermans, and M. Salio. 2004. Dendritic cells: a journey from laboratory to clinic. *Nat Immunol* 5:7-10.
34. Men, Y., B. Gander, H.P. Merkle, and G. Corradin. 1996. Induction of sustained and elevated immune responses to weakly immunogenic synthetic malarial peptides by encapsulation in biodegradable polymer microspheres. *Vaccine* 14:1442-1450.
35. Newman, K.D., P. Elamanchili, G.S. Kwon, and J. Samuel. 2002. Uptake of poly(D,L-lactic-co-glycolic acid) microspheres by antigen-presenting cells in vivo. *J Biomed Mater Res* 60:480-486.
36. Hermans, I.F., J.D. Silk, U. Gileadi, M. Salio, B. Mathew, G. Ritter, R. Schmidt, A.L. Harris, L. Old, and V. Cerundolo. 2003. NKT cells enhance CD4+ and CD8+ T cell responses to soluble antigen in vivo through direct interaction with dendritic cells. *J Immunol* 171:5140-5147.
37. Fujii, S., K. Liu, C. Smith, A.J. Bonito, and R.M. Steinman. 2004. The linkage of innate to adaptive immunity via maturing dendritic cells in vivo requires CD40 ligation in addition to antigen presentation and CD80/86 costimulation. *J Exp Med* 199:1607-1618.
38. Liu, K., J. Idoyaga, A. Charalambous, S. Fujii, A. Bonito, J. Mordoh, R. Wainstok, X.F. Bai, Y. Liu, and R.M. Steinman. 2005. Innate NKT lymphocytes confer superior adaptive immunity via tumor-capturing dendritic cells. *J Exp Med* 202:1507-1516.
39. Minami, K., Y. Yanagawa, K. Iwabuchi, N. Shinohara, T. Harabayashi, K. Nonomura, and K. Onoe. 2005. Negative feedback regulation of T helper type 1 (Th1)/Th2 cytokine balance via dendritic cell and natural killer T cell interactions. *Blood* 106:1685-1693.
40. Figueroa-Perez, S., and R.R. Schmidt. 2000. Total synthesis of alpha-galactosyl cerebroside. *Carbohydr Res* 328:95-102.

Chapter IV

α-C-N-acyl-diPhenyl-galactosylceramide - A new analogue of α-C-galactosylceramide shows effective Th2 polarization and is highly potent in therapy of models for Th1 dependent autoimmunity

Marc Mueller, Mirko Zierke, Joshua Pierce, Peter Wipf
and Marcus Groettrup

Abstract

The cytokine environment is one of the most important parameters in balancing out an immune response. A potent source of early cytokines is the natural killer T (NKT) cell population. These NKT cells are effectively stimulated by glycolipids, presented on the MHC class I like molecule CD1d. Depending on the glycolipid, the stimulated NKT cells produce a variety of cytokines supporting T helper (Th) 1, Th2 or Th17 responses. α-C-galactosylceramide (α-C-GalCer), a C-glycoside of the synthetic CD1d ligand α-galactosylceramide (α-GalCer), has gained increasing attention because of its putative Th1 polarizing effect. This effect is explained to be due to the stimulation of IFN-γ and IL-12 production in the absence of Th2 cytokines like IL-4. We investigated a Di-phenyl analogue (α-C-diPheGalCer) of α-C-GalCer and found striking Th2 polarizing conditions in response to this novel compound. α-C-diPheGalCer, applied intravenously leads to rapid production of IL-4 but only marginal IFN-γ levels in blood serum. Furthermore, we could show that a single injection of 2 μg of this substance entails significant protection from experimental autoimmune colitis, diabetes and contact hypersensitivity. *In vitro* studies suggest that α-C-diPheGalCer shows no increased toxicity compared to the vehicle used. In conclusion, α-C-diPheGalCer is a potent Th2 polarizer and a promising drug with potential for the treatment of autoimmune diseases.

Introduction

During the past decade, invariant natural killer (iNKT) cells have been characterized as an important bridge between the adaptive and the innate arms of the immune system (1). They are also known as Type I NKT lymphocytes and are characterized by the expression of an invariant TCR utilizing Vα14/18 segments in mice and humans respectively, which recognizes glycolipid antigens in the context of the non-polymorphic MHC class I like molecule CD1d (2, 3). Upon stimulation with the marine sponge derived glycolipid α-galactosylceramide (α-GalCer) they produce a vast variety of both T helper (Th) 1 and Th2 cytokines, such as IFN-γ, IL-2, IL-3, IL-21, IL-10, TGF-β, TNF-α, GM-CSF or IL-4 (4-6). Thereby they show a great potential to modulate arising or ongoing immune responses. Based on the assumption that the affinity of the ligand to the CD1d molecule is an important

determinant of the kind of cytokine profile evoked (7), several different CD1d ligands are used in modern immunotherapy research (8-11).

The most widely used CD1d ligand is α-GalCer (12). It has been extensively studied over the past years and was shown to be able to influence the Th1/Th2 bias of immune responses (13) and to have immune-stimulatory functions in anti tumor responses (10) or in infectious diseases (9). Interestingly, the ability of α-GalCer to influence the adaptive immune response has been exploited for the regulation of autoimmune diseases as well, a concept supported by the finding that NKT deficient mice are susceptible to autoimmune diseases (14, 15). As demonstrated in models of autoimmune encephalomyelitis (EAE) (16) or spontaneous type 1 diabetes (T1D) in NOD mice (17), α-GalCer can be a beneficial stimulus. Most likely due to the rather undefined cytokine profile elicited by α-GalCer, it also may augment undesired effects as described for arthritis models and experimental colitis (18) (19).

At present it is under debate, whether ligands with lower affinity for CD1d are closer to physiology and hence create a more balanced cytokine environment (20). The α-GalCer analogue OCH, for example, has a shortened sphingosine tail and shows a better defined cytokine pattern (21). It mainly activates the production of IL-4 and therefore promotes a Th 2 environment. In 2006 an α-GalCer analogue was described, in which the C26 *N*-fatty acyl tail was replaced by a C8 *N*-fatty acyl tail and a terminal benzene. The interactions formed between the aromatic substituent of the glycolipid and aromatic CD1d side-chain residues contribute additional stability to the complex (22). The new molecule altered the cytokine profile towards Th1 and showed a significantly higher anticancer efficacy than α-GalCer (23). This is ascribed to an improved stability of the ceramide/CD1d complex which could potentially enhance the Th1 response by a prolonged NKT cell stimulation (24). Here we investigate the *N*-(5-*para*biphenylpentanoyl) analogue (α-C-diPheGalCer), of α-C-galactosylceramide (α-C-GalCer), that was synthesized by the group of P. Wipf at the University of Pittsburgh (unpublished data).

A second aim of this study was the investigation of a novel mode of application of CD1d ligands. We made use of biodegradable poly(lactide-*co*-glycolide) (PLGA) microspheres (MS), a powerful tool to target APCs *in vivo* and induce substantial CTL responses as well as striking tumor regression (25, 26). PLGA is a well-

defined polymer, which is approved for clinical use e.g., for surgical sutures or implants. It is known to protect its contents from degradation and thus forms a long lasting depot *in vivo* (27). Since it is known, that repeated injection of CD1d ligands lead to anergy of iNKT cells resulting in the production of IL-4 alone (28), we wanted to elucidate, whether a depot of a strong Th2 polarizing agent would further improve its performance.

Materials and methods

Glycolipids: α-GalCer [(2S,3S,4R)-1-O-(a-D-galactopyranosyl)-2-(N-hexacosanoylamino)-1,3,4-octadecanetriol] was synthesized according to (29) and was provided by Prof. R Schmidt (University of Konstanz).
The α-C-GalCer analogue α-C-diPheGalCer [(3S,4S,5R)-1-(a-D-galactopyranosyl)-3-(N-5-parabiphenylpentanoyl)] was synthesized and provided by P. Wipf (University of Pittsburgh). All glycolipids were dissolved in dimethyl sulfoxide (DMSO) (0,5 mg/ml) and diluted in PBS immediately before i.v. injection.

Mice: C57BL/6 mice (H-2b) were originally purchased from Charles River Laboratories. All mice were kept in a specific pathogen-free facility and used age and sex matched at 6–10 wk of age. Animal experiments were approved by the review board of the Regierungspräsidium Freiburg, Germany.

ELISA: For the IFN-γ and IL-4 cytokine determination, the corresponding BD-ELISA Kit (BD OptEIATM, BD Bioscience) was used, following the manufacturer's instructions. Absorbance at 450-570 nm was measured, using a SpectrafluorPlus plate reader (Tecan).

Quantitative real-time RT-PCR: Real-time RT-PCR was used to quantify transcription factor expression levels in $CD4^+$ mouse splenocytes. Total RNA was extracted from magnetically sorted (CD4 T cell isolation kit, mouse; Miltenyi Biotec) $CD4^+$ splenocytes using a NucleoSpin® RNA II extraction kit (Macherey-Nagel). 1 µg of total RNA was reverse-transcribed using oligo(dT) primers or 18S rRNA RT primers (5'-GAGCTGGAATTACCGCT-3') and the reverse transcription system (Promega). Quantitative PCR was performed with the LightCycler® instrument (Roche Applied Science) using the LightCycler® Fast Start DNA

Master SYBR Green I reaction mix (Roche Applied Science) with the following primers: GATA-3 specific forward (5'-CTGGAGGAGGAACGCTAATG-3'); GATA-3 specific reverse (5'-AGATGTGGCTCAGGGATGAC-3') and T-bet specific forward (5'-GGACCCAACTGTCAACTGCT-3'); T-bet specific reverse (5'-AACTGTGTTCCCGAGGTGTC-3'). Mouse 18S was used as a reference gene with the following primers. 18S rRNA forward (5'-GAGGTAGTGACGAAAAATAACAAT-3') and 18S rRNA reverse (5'-TTGCCCTCCAATGGATCCT-3'9. Primers were purchased from Microsynth (Balgach, CH).

Intracellular cytokine staining (ICS): Splenocytes were isolated and restimulated with or without 10 µM SIINFEKL peptide (Eurogentec, Cologne, Germany) in the presence of brefeldin A (10 µg/ml, Sigma-Aldrich) for 5 h at 37°C. Subsequently, cells were stained with anti-mouse-CD8α-PE-Cy5 antibody (BD Biosciences Pharmingen, Clone 53-6.7) for 20 min at 4 °C. After fixation with 4% paraformaldehyde in PBS for 10 min at room temperature, cells were labeled intracellularly with rat-anti-IFN-γ-FITC antibody (clone XGM1.2, diluted in PBS/0.1 % Saponin) at 4°C overnight. The next day, cells were analyzed by flow cytometry. Background levels of each sample (without restimulation) were subtracted.

Preparation of PLGA-microspheres: Microspheres (MS) were prepared from 14 kDa poly(D,L-lactide-*co*-glycolide) (PLGA) 50:50 carrying hydroxyl- and carboxyl-end groups (Resomer RG502H, Boehringer Ingelheim, Ingelheim, Germany) by spray drying. Briefly, 50 mg ovalbumin (Grade V, Sigma) and 5 mg CpG oligodeoxynucleotides with a phosphothioate backbone (CpG-ODN 1826, Microsynth, Balgach, Switzerland) or 0,5 mg polyI:C (Calbiochem, VWR, Dietikon, Switzerland) (MS polyI:C) were dissolved in 0.5 ml 0.1M $NaHCO_3$ (aqueous phase) and mixed with 1 g of PLGA dissolved in 20 ml of dichloromethane (organic phase). Optionally 0.5mg α-C-diPheGalCer was dissolved in 160 µl methanol / chloroform (MS-α-C-diPheGalCer) and mixed with 1 g of PLGA dissolved in 20 ml of dichloromethane. The two phases were subsequently emulsified by ultrasonication (Hielscher, UP200 H, Ampl. 40%) for 10 s on ice. The obtained dispersion was immediately spray-dried (Büchi, Mini Spray-Dryer 191) at a flow rate of 2 ml/min and inlet/outlet temperatures of 40/37 °C. The obtained MS were washed out of the spray-dryer's cyclone with 0.05 % Synperonic

(Synperonic®F68, Serva Electrophoresis GmbH, Heidelberg, Germany), collected on a cellulose acetate membrane filter and dried under reduced pressure (20 mbar) for 18 h at room temperature. PLGA-MS were stored at 4°C. Immediately before use MS were dispersed in PBS by ultrasonication for 30 sec.
5 mg MS-Ova/CpG (containing 250 µg Ova, 25 µg CpG-ODN) and 5 mg MS-polyI:C (containing 2,5 µg polyI:C) (MS mix) or 4 mg MS-α-C-diPheGalCer (containing 2 µg MS-α-C-diPheGalCer) were injected in PBS s.c. at the base of the tail.

DSS-induced colitis: Mice were treated with a 2,2 % solution of dextrane sulfate sodium salt (DSS, MP Biomedicals, Solon, Ohio, USA) *ad libitum* via the drinking water for five days. At day four mice were inoculated with α-C-diPheGalCer either encapsulated into PLGA-MS s.c. or dissolved in PBS i.v.. Control groups were treated with the corresponding volumes of DMSO in PBS. The percentage of body weight loss was calculated as following: (((body weight at day x) X 100)) / (body weight at day 0))-100.

DNFB induced contact hypersensitivity: Contact hypersensitivity (CHS) was induced as described in reference (30). Briefly, mice were sensitized with 5 µl of a 1 % solution of 2,4-dinitrofluorobenzene (DNFB; Sigma) in acetone:olive oil (4:1) epicutanously on the left ear. Five days later the right ear was challenged with 5 µl of a 1 % solution DNFB in acetone:olive oil (4:1) and after 24 hours ear swelling was assed with a micrometer.

Low-dose streptozotocin induced diabetes: Male mice were treated daily with 40 mg/kg streptozotocin (STZ; Sigma) in freshly prepared Na-Citrate buffer (0,1M) i.p. for 5 days. On day four mice were injected with α-C-diPheGalCer either encapsulated into PLGA-MS (s.c.) or dissolved in PBS (i.v.). Control groups were treated with the corresponding volumes of DMSO in PBS. Blood glucose levels were monitored using a blood glucose analyzer (Contour, Bayer). Increase in blood glucose level on day 7 and day 9 was calculated as follows: (blood glucose level on day x) - (blood glucose level on day 0).

Propidium Iodide (PI) / AnnexinV staining: Splenocytes were incubated in medium containing increasing doses of α-C-diPheGalCer dissolved in DMSO or

with the same volume of DMSO. After 24 hours, cells were washed twice with cold PBS and resuspended in AnnexinV binding buffer (FITC AnnexinV Apoptosis Detection Kit I, BD Pharmingen). Cells were stained with PI and AnnexinV following the manufacturers protocol and analyzed by flow cytometry. AnnexinV^{+} cells were classified as apoptotic, cells that were AnnexinV^{+}/PI^{+} were counted as dead cells.

Results

α-C-diPheGalCer was synthesized according to literature protocols (31) to give amino diol 1 (Figure 1). This intermediate served as the starting material for the synthesis of α-C-diPheGalCer as follows: Deprotection of the Boc group with HCl in dioxane, and coupling to 5-([1,1'-biphenyl]-4-yl)pentanoic acid to provide the desired *N*-acyl diPhe derivative in high yield. Benzyl protective groups were subsequently removed by H_2/Pd, resulting in **2** (Figure 1). Detailed information of this synthesis and spectroscopic data will be published separately (J. Pierce and P. Wipf, unpublished).

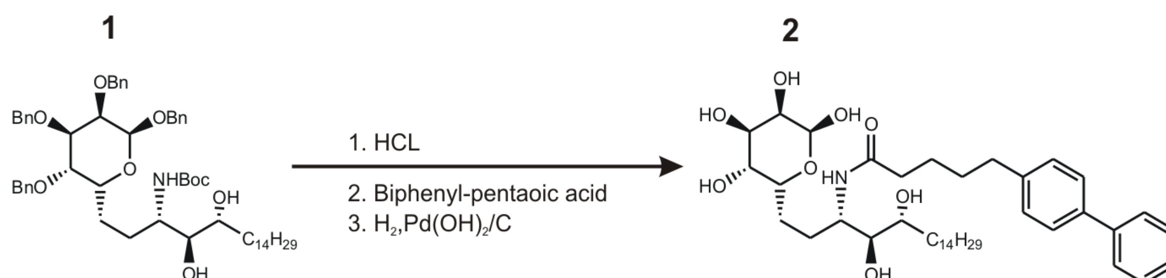

Figure 1: Synthesis of α-C-diPheGalCer. The synthesis started with an amino diol **1**, representing an intermediate of the previously described α-C-GalCer synthesis (31). The Boc group was removed and replaced by 5-parabisphenyl acid to receive α-C-diPheGalCer **2**.

In an initial experiment we wanted to characterize the cytokine pattern, induced by a single i.v. injection of 2 µg α-C-diPheGalCer. We treated mice with 2 µg of either α-C-diPheGalCer (diPheGal) or 2 µg of α-GalCer (α-GalCer), both dissolved in DMSO (0,5 mg/ml) and diluted in PBS to a final concentration of 10 µg/ml. α-C-diPheGalCer induced an initial burst of IL-4 release (figure 2; upper graph), peaking at 2 hours post injection. Compared to α-GalCer, α-C-diPheGalCer yielded lower quantities of IL-4, but in both cases IL-4 in the serum had vanished 10 hours after injection. Interestingly, there was virtually no release of IFN-γ detected (figure 2; lower graph) after the injection of α-C-diPheGalCer. Unlike for

α-GalCer, where we found robust production of IFN-γ, which peaked 24 hours after injection and was still detectable after 48 hours. Thus, α-C-diPheGalCer shows a clear Th2 polarizing cytokine pattern, characterized by substantial amounts of early IL-4 in the absence of IFN-γ.

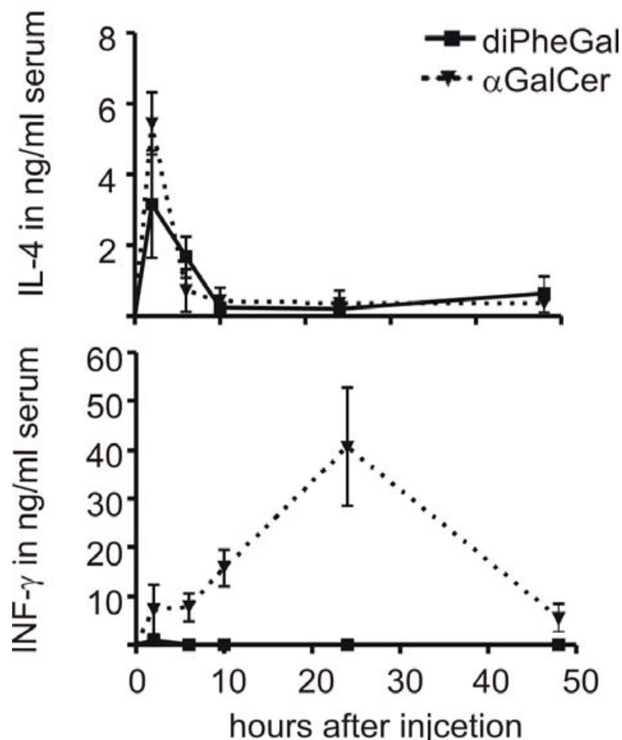

Figure 2: Serum cytokine levels after injection of 2 µg α-C-diPheGalCer or α-GalCer. Mice (n=3) received i.v. injections of either α-C-diPheGalCer or α-GalCer. At indicated time point blood samples were taken and analyzed by standard sandwich ELISA for IL-4 (upper graph) and IFN-γ (lower graph). Mean values are given in ng/ml serum +/- SEM. The experiment was conducted 3 times with the same outcome. One representative experiment is shown.

To further strengthen the initial data on the Th2 polarizing capacity of α-C-diPheGalCer we investigated the expression of the transcription factors GATA-3 and T-bet upon treatment with α-C-diPheGalCer. Therefore we made use of the Th1 dependent murine disease model DSS-induced colitis. We induced colitis by feeding mice a 2,2 % DSS solution for five days and monitored weight loss until day 8. On day four, mice were treated with either 2 µg α-C-diPheGalCer (diPheGal) or with the vehicle alone. At day 8 the average weight loss of the vehicle treated groups ranged between 13,5 % to 14,5 % whereas the α-C-diPheGalCer treated groups showed a decreased weight loss of about 8 % to 8,5 %. Splenocytes of these mice where subsequently sorted for $CD4^+$ lymphocytes, followed by RNA isolation. The real-time RT-PCR analysis, shown in figure 3,

revealed a significant reduction in the expression of T-bet in the α-C-diPheGalCer treated groups, whereas GATA-3 levels were not affected. This clearly indicates a shift Th1 vs. Th2 differentiation by α-C-diPheGalCer in favor of Th2.

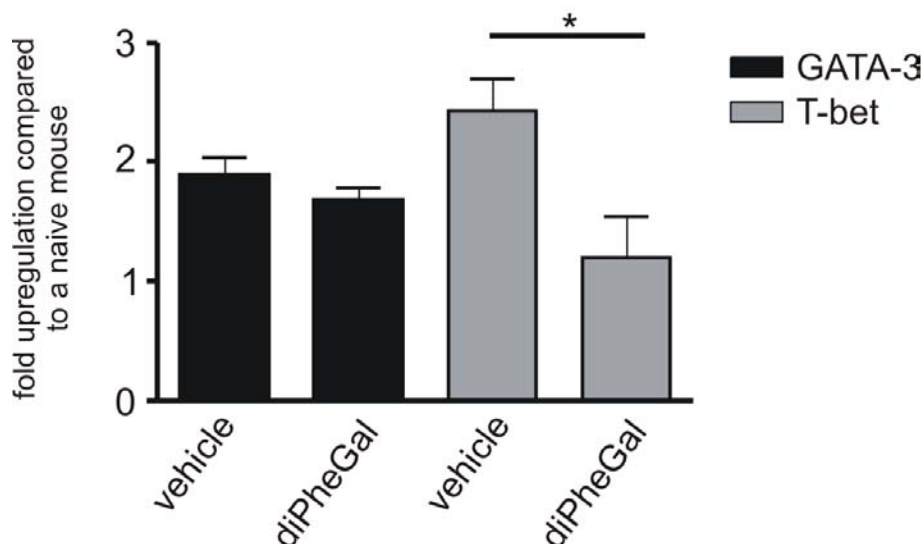

Figure 3: Quantification of GATA-3 and T-bet mRNA levels in colitic mice, treated with α-C-diPheGalCer. Mice (n=9) were treated with 2,2 % DSS for a period of five days. On day four mice were treated either with 2 µg dissolved α-C-diPheGalCer in PBS (diPheGal) or with the vehicle alone (vehicle). At day 8 splenocytes were isolated and magnetically sorted for $CD4^+$ lymphocytes. Expression levels of GATA-3 and T-bet mRNA were assessed by real-time PCR using SYBR GREEN technology. Mean values were normalized for 18S rRNA and are given in fold upregulation compared to a naïve mouse, +/- SEM. Real-time RT-PCR analysis was performed twice for each sample. The graph shows a compilation of three independent experiments with the same outcome. Differences between groups were assessed by an unpaired students t-test (p=0,0106)

To further investigate the ameliorating effects of α-C-diPheGalCer in DSS induced colitis over a longer period, we again challenged mice for five days with a 2,2 % solution of DSS in drinking water. On day four, mice were treated with a single injection of either 2 µg of dissolved α-C-diPheGalCer in PBS i.v. (indicated as dissolved) or with 2 µg of PLGA-MS encapsulated α-C-diPheGalCer in PBS s.c. (indicated as encapsulated). Control animals were treated with corresponding volumes of DMSO in PBS (vehicle). The observed change in body weight is given in figure 4A as percentage of body weight change.

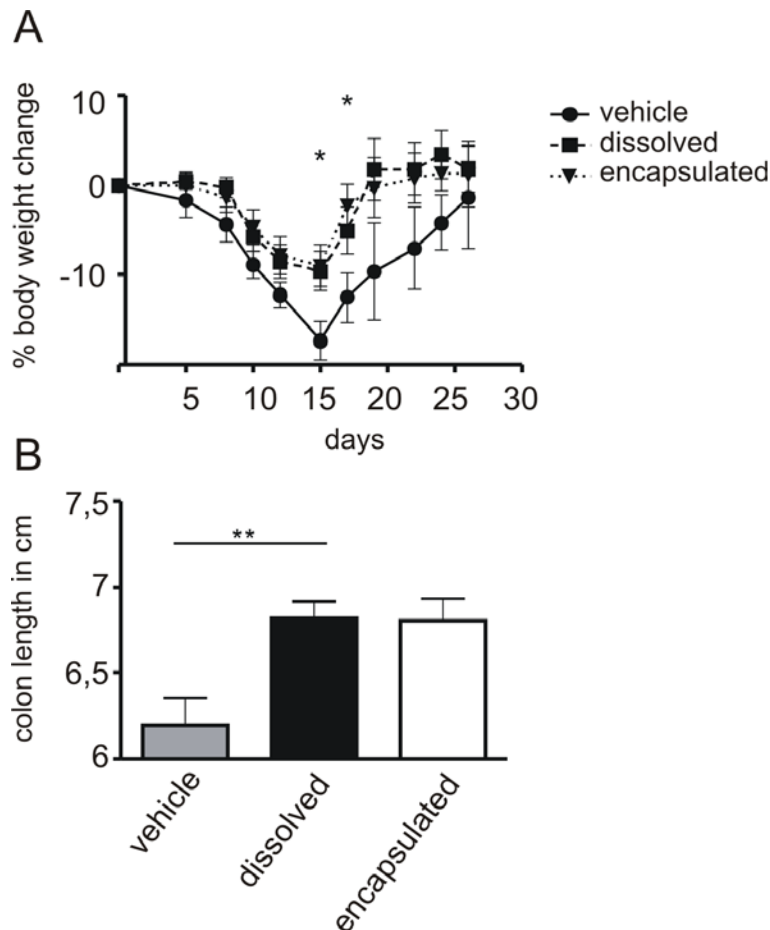

Figure 4: DSS-induced colitis. (A) Mice (n=25) were treated with 2,2 % DSS for a period of five days. On day four, mice were treated either with 2 µg dissolved α-C-diPheGalCer in PBS (dissolved; square), 2 µg encapsulated α-C-diPheGalCer (encapsulated; triangles) or DMSO in PBS (vehicle; circles). The body weight was assessed at indicated time points and body weight loss was calculated, which is given in percent body weight loss, +/- SEM. The graph shows a compilation of five independent experiments with similar results. Differences between groups were assessed by one-way analyses of variance (day 15: p=0,0165; day 17: p=0.0238). (B) Experiments were conducted as in (A). At day 11, mice (n=12) were sacrificed and colon length was measured. Values are given in colon length in cm, +/- SEM. The graph represents a compilation of 3 independent experiments with similar results. Differences between groups were assessed by one-way analyses of variance (p=0,0238).

A significant benefit of the treatment could be detected at day 15 and day 17. In general it could be stated, that a single injection of α-C-diPheGalCer applied early after onset of the disease lead to decreased body weight loss of the treated groups and to faster recovery compared to the vehicle treated group. This significant benefit is also reflected by the colon length of α-C-diPheGalCer treated animals 11 days after onset of the experiment (figure 4B). The excised colons, starting at the appendix, ending at the rectum, were measured with a ruler. Again it could be shown, that α-C-diPheGalCer treated mice significantly benefit from an

early injection. The reduction in colon length, indicating an ongoing inflammation, was clearly reduced in α-C-diPheGalCer treated groups compared to the vehicle treated groups. Encapsulation of α-C-diPheGalCer in PLGA-MS does not further improve the long-term outcome of the experiment, compared to soluble α-C-diPheGalCer.

Another model for Th1 driven autoimmunity is contact hypersensitivity (CHS). Here, we sensitized ears of mice with 5 µl NDFB (1 % in acetone : olive oil) and challenged the opposing ears after five days again with 5 µl DNFB (1 % in acetone : olive oil). Immediately after challenge, mice were treated with either 2 µg of dissolved α-C-diPheGalCer in PBS i.v. (indicated as dissolved), with 2 µg of encapsulated α-C-diPheGalCer in PBS s.c. (indicated as encapsulated) or with the vehicle alone (vehicle). 24 hours post challenge, the ear thickness was measured (figure 5).

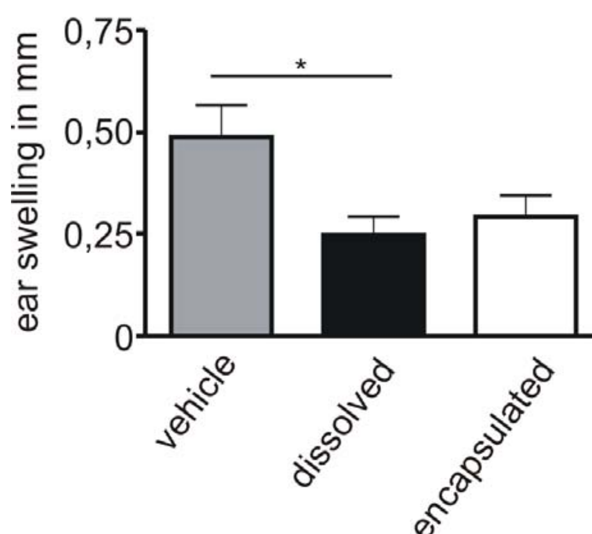

Figure 5: DNFB induced contact hypersensitivity. Mice (n=13) were sensitized at the left ear with 5 µl of a 1% DNFB in acetone:olive oil (4:1) mixture on day 0. Five days later, mice were challenged at the right ear with 5 µl of 1 % DNFB. Ear swelling, compared to the naive ear at day 0, was measured after 24 hours. Values are given as mean ear swelling in µm, +/- SEM. The graph represents a composition of 3 independent experiments with similar results. Differences between groups were assessed by one-way analyses of variance (p=0,0222).

As shown in figure 5, significant differences between α-C-diPheGalCer treated groups and the vehicle treated group could be observed. Of note, there is a slight benefit for the group being treated with dissolved α-C-diPheGalCer i.v., compared to the group having received encapsulated α-C-diPheGalCer s.c..

To further characterize the therapeutic potential of α-C-diPheGalCer in Th1 driven autoimmunity, we treated mice with low-dose streptozotocin (STZ) i.p. for 5 five days. STZ is a sugar analog which is taken up by pancreatic Langerhans-islet cells causing apoptosis. The reduced number of insulin secreting cells leads to diabetic blood glucose level. On day four, mice were immunized with either 2 µg of dissolved α-C-diPheGalCer in PBS i.v. (indicated as dissolved), with 2 µg of PLGA-MS encapsulated α-C-diPheGalCer in PBS s.c. (indicated as encapsulated), or with the vehicle alone (vehicle). Every two to three days, blood was analyzed for glucose levels. Figure 6 shows a kinetic of the blood glucose levels in mg glucose per dl blood. In line with the other models we have used, early treatment with α-C-diPheGalCer resulted in a significant reduction in the susceptibility to develop diabetes. For later time points an additional benefit for the group with encapsulated α-C-diPheGalCer can be seen, compared to the group being treated with soluble α-C-diPheGalCer.

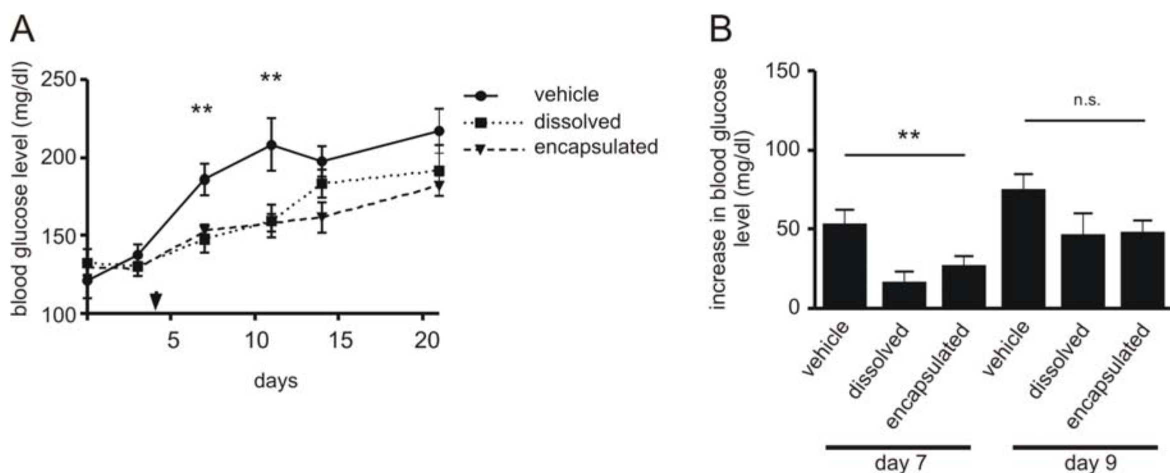

Figure 6: Low-dose streptozotocin induced diabetes. (A) Mice (n=15) were treated daily with 40 mg streptozotocin / kg bodyweight i.p. for a period of five days. On day four, mice were treated either with 2 µg of soluble α-C-diPheGalCer in PBS (dissolved; square), 2 µg of encapsulated α-C-diPheGalCer (encapsulated; triangles) or DMSO in PBS (vehicle; circles). Blood glucose levels were measured at indicated time points and are given in mg glucose / dl blood, +/- SEM. The graph shows a composition of three independent experiments with similar results. Differences between groups were assessed by one-way analyses of variance (day 7: p=0,0071; day 11: p=0.0025) (B) Increase of blood glucose levels was calculated for the same mice as in (A) for day 7 and day 9. Values are given in increase in blood glucose levels compared to day 0, +/- SEM. The graph represents a composition of 3 independent experiments with similar results. Differences between groups were assessed by one-way analyses of variance (day 7: p=0,0082).

In order to further characterize our new compound we investigated the *in vitro* toxicity of α-C-diPheGalCer on primary cells. To this aim, splenocytes of naïve

mice were co-incubated with increasing doses of dissolved α-C-diPheGalCer or corresponding volumes of DMSO for 24 hours. After 24 hours the percentage of dead cells was assessed by PI/AnnexinV staining and analyzed by flow cytometry. Furthermore, the viable lymphocyte population was assessed by FSC/SSC gating. As figure 6 shows, α-C-diPheGalCer itself cannot be claimed to cause cell death, since the detected cell death is also apparent in the vehicle control curve. Only minor differences could be detected, implying a vehicle mediated toxicity. Hence, α-C-diPheGalCer shows no signs of toxicity in the concentration, used in our studies.

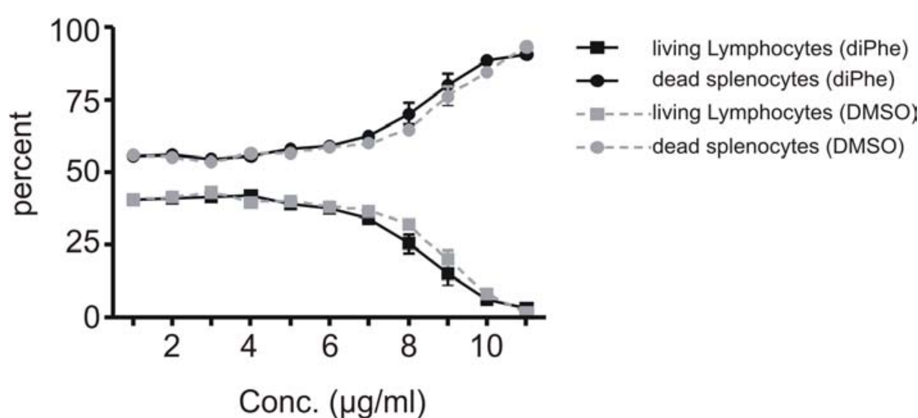

Figure 7: *In vitro* toxicity of α-C-diPheGalCer: Splenocytes of a naïve mouse were incubated in medium, containing increasing doses of α-C-diPheGalCer dissolved in DMSO (black) or corresponding volumes of DMSO (gray). After 24 hours, cells were stained for PI/AnnexinV and cell death was analyzed by flow cytometry. Values are given in percent total dead cells (circles) or % living lymphocytes (squares), +/- SEM. The graph shows a compilation of three independent experiments with similar outcome.

Furthermore, we conducted *in vivo* toxicity studies, in order to test toxicity of α-C-diPheGalCer in the spleens of mice treated with 2 µg of α-C-diPheGalCer i.v.. 24 hours after injection we could not detect any reduction of $CD4^+$, $CD8^+$, $NK1.1^+$ or $CD11c^+$ splenocytes, compared to naïve mice (data not shown). In none of the experiments, the injection of α-C-diPheGalCer had any visible, adverse effects on the mice referring to fitness or health.
Studies with α-GalCer or α-C-GalCer showed, that under certain conditions, a co-injection of these ceramides can stimulate robust CTL responses (32). In contrast to that we could show in chapter III (figure 4) that a co-injection of α-GalCer or α-C-GalCer leads to a clear reduction of the CTL response. In order to investigate, whether this effect can be seen for α-C-diPheGalCer as well, we co-injected a

mixture of PLGA-MS, containing either ovalbumin/CpG or polyI:C (MS mix) together with either encapsulated α-C-diPheGalCer s.c. or dissolved α-C-diPheGalCer i.v.. At day 6 after immunization splenocytes were analyzed for SIINFEKL specific CTL response (figure 8). Interestingly, i.v. administration of α-C-diPheGalCer significantly enhanced the number of SIINFEKL specific $CD8^+$/IFN-γ^+ lymphocytes, whereas a s.c. co-injection of encapsulated α-C-diPheGalCer did not.

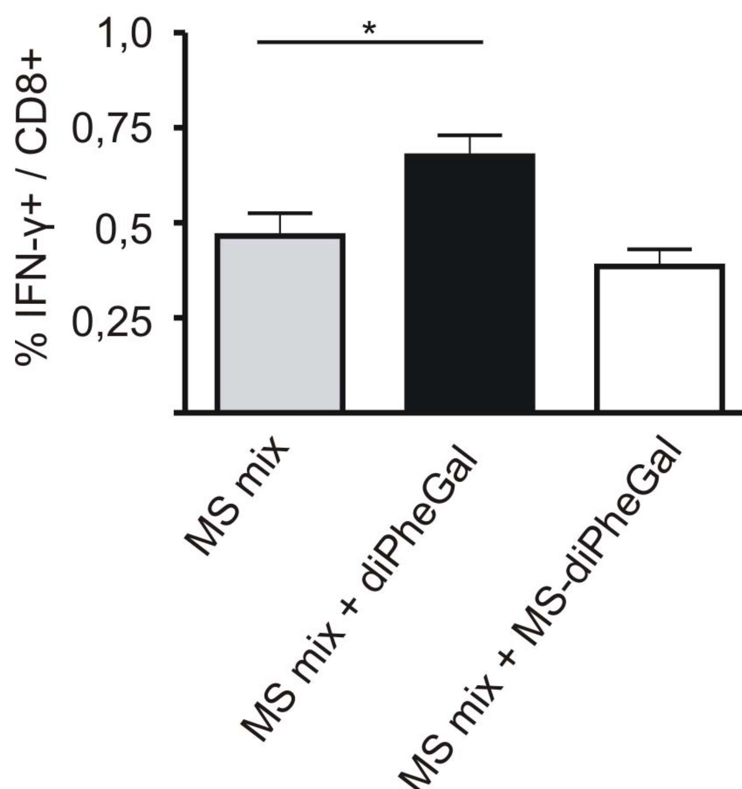

Figure 8: Co-injection of dissolved or encapsulated α-C-diPheGalCer with a mixture of PLGA-MS containing ovalbumin and CpG or polyI:C. Mice (n=12 for group MS mix and MS mix + diPheGal; n=9 for MS mix + MS-diPheGal) were immunized with a mixture of PLGA-MS containing either ovalbumin and CpG or polyI:C (MS mix), MS mix plus 2 µg dissolved α-C-diPheGalCer in PBS (MS mix + diPheGal) or with MS mix and 2 µg encapsulated α-C-diPheGalCer (MS mix + MS-diPheGal). On day six after immunization mice were sacrificed and homogenized splenocytes were analyzed for $CD8^+$/IFN-γ^+ cells by flow cytometry. Values are given in percent SIINFEKL specific $CD8^+$/IFN-γ^+ splenocytes, +/- SEM. Background values (not restimulated) were subtracted. The graph shows a composition of 4 independent experiments with comparable results. Differences between groups were assessed by an unpaired students t-test (p=0,0216).

Discussion

Treatment of autoimmune diseases with ligands for CD1d has been a rapidly growing field. The finding that a new analogue of α-GalCer induces IL-4 but only little IFN-γ raised our interest to exploit α-C-diPheGalCer for the treatment of Th1 dependent autoimmunity. The new compound OCH was effective in the therapy of EAE (21), experimental colitis (8), collagen induced arthritis (CIA) (33), or type I diabetes (T1D) in NOD mice (34). Here we investigated a substance, namely α-C-diPheGalCer, that induces a Th2 but not a Th1 cytokine pattern. The absence of IFN-γ in the serum of α-C-diPheGalCer treated mice (figure 2) in combination with the down regulation of the transcription factor T-bet in α-C-diPheGalCer treated mice, suffering from colitis (figure 3), can be evidenced as the bias for Th2 differenciation.

The efficacy of α-C-diPheGalCer in activation of NKT cells, calls for the necessity to investigate the toxicity of this new substance. Our first data indicate only minor toxicity of the substance *in vivo*, as it did not affect the numbers of CD4, CD8, NK1.1 or CD11c positive splenocytes 24 hours after injection of α-C-diPheGalCer i.v. (data not shown), or *in vitro* (figure 7). Undesired cytotoxic effects of the substance are most likely due to the used vehicle. DMSO possesses a great potential as a solvent for ceramides and is widely used in the field. Chemical studies about α-C-diPheGalCer revealed that it can be dissolved in a 1 : 1 (v/v) ethanol : DMSO mixture in a concentration of <1 mg / ml. A vehicle of a 1 : 1 (v/v) mixture of ethanol : DMSO is approved for clinical use and therefore the solution of 0,5 mg α-C-diPheGalCer per ml ethanol:DMSO could be used in clinical trials.

In order to characterize the new substance in *in vivo* autoimmune disease models, we conducted experiments using DSS as an inducer of experimental colitis in mice. Our experiments show that an early injection of α-C-diPheGalCer is significantly reducing the symptoms of the induced disease, as revealed by a reduced weight loss and accelerated recovery (figure 4). It has been shown for α-GalCer that similar effects in EAE are dependent on IL-4 and IL-10 release or, in general, a reduced inflammatory cytokine environment (35). Comparable results could be achieved using OCH in T1D (34) and for EAE (35). In all cases there was a clear dependency on NKT cells, which could be convincingly demonstrated in NKT cell deficient mice. This is, most likely, also true for α-C-diPheGalCer, but this remains to be experimentally investigated. The need for an early modulation of the

cytokine environment also is consistent with our finding that the encapsulation of α-C-diPheGalCer did not yield a better protection in the investigated models for colitis and diabetis. The slow release profile of PLGA-MS seems not to be needed to exert protection.

Another model we investigated is CHS. It is assumed, that $CD4^+$ and $CD8^+$ T cell responses emerge 24 hours post challenge with DNFB, in which $CD8^+$ T cells were shown to have effector functions and $CD4^+$ T cells appear to be regulatory (36). As a consequence, α-C-diPheGalCer must be available immediately in order to show an alleviative effect in CHS. This is consistent with our data that show a slight benefit for dissolved α-C-diPheGalCer compared to encapsulated α-C-diPheGalCer (figure 5). Release studies with PLGA-MS showed that within 24 hours approximately 20 % of their content has been released (37). This might not be sufficient to stimulate NKT cells effectively. Consequently the PLGA-MS must have been taken up and processed by DCs very effectively in order to account for the therapeutic benefit that is still obvious.

Interestingly, co-injection of encapsulated α-C-diPheGalCer has no beneficial effect on the CTL response. In contrast, dissolved α-C-diPheGalCer increased the CTL responses, when injected i.v., simultaneously to the s.c. injection of a MS-Ova/CpG / MS-polyI:C mixture (figure 8). Although this effect is not understood, it might represent an interesting strategy to promote CTL responses in anti-tumor immunizations.

Taken together, we think that the new compound deserves to be tested in further experimental models of autoimmune disease. So far we could show that α-C-diPheGalCer is highly effective in polarizing the cytokine environment towards Th2, it is effective at a dose which entails no obvious toxicity and thereby provides an excellent platform for further therapeutic development.

References for chapter IV:

1. Bendelac, A., P.B. Savage, and L. Teyton. 2007. The biology of NKT cells. *Annu Rev Immunol* 25:297-336.
2. Kawano, T., J. Cui, Y. Koezuka, I. Toura, Y. Kaneko, K. Motoki, H. Ueno, R. Nakagawa, H. Sato, E. Kondo, H. Koseki, and M. Taniguchi. 1997. CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. *Science* 278:1626-1629.
3. Spada, F.M., Y. Koezuka, and S.A. Porcelli. 1998. CD1d-restricted recognition of synthetic glycolipid antigens by human natural killer T cells. *J Exp Med* 188:1529-1534.
4. Leite-de-Moraes, M.C., M. Lisbonne, A. Arnould, F. Machavoine, A. Herbelin, M. Dy, and E. Schneider. 2002. Ligand-activated natural killer T lymphocytes promptly produce IL-3 and GM-CSF in vivo: relevance to peripheral myeloid recruitment. *Eur J Immunol* 32:1897-1904.
5. Jiang, S., D.S. Game, D. Davies, G. Lombardi, and R.I. Lechler. 2005. Activated CD1d-restricted natural killer T cells secrete IL-2: innate help for CD4+CD25+ regulatory T cells? *Eur J Immunol* 35:1193-1200.
6. Coquet, J.M., K. Kyparissoudis, D.G. Pellicci, G. Besra, S.P. Berzins, M.J. Smyth, and D.I. Godfrey. 2007. IL-21 is produced by NKT cells and modulates NKT cell activation and cytokine production. *J Immunol* 178:2827-2834.
7. Godfrey, D.I., and M. Kronenberg. 2004. Going both ways: immune regulation via CD1d-dependent NKT cells. *J Clin Invest* 114:1379-1388.
8. Ueno, Y., S. Tanaka, M. Sumii, S. Miyake, S. Tazuma, M. Taniguchi, T. Yamamura, and K. Chayama. 2005. Single dose of OCH improves mucosal T helper type 1/T helper type 2 cytokine balance and prevents experimental colitis in the presence of valpha14 natural killer T cells in mice. *Inflamm Bowel Dis* 11:35-41.
9. Wilson, M.T., A.K. Singh, and L. Van Kaer. 2002. Immunotherapy with ligands of natural killer T cells. *Trends Mol Med* 8:225-231.
10. Fujii, S., K. Shimizu, M. Kronenberg, and R.M. Steinman. 2002. Prolonged IFN-gamma-producing NKT response induced with alpha-galactosylceramide-loaded DCs. *Nat Immunol* 3:867-874.
11. Fuji, N., Y. Ueda, H. Fujiwara, T. Toh, T. Yoshimura, and H. Yamagishi. 2000. Antitumor effect of alpha-galactosylceramide (KRN7000) on spontaneous hepatic metastases requires endogenous interleukin 12 in the liver. *Clin Cancer Res* 6:3380-3387.
12. Morita, M., K. Motoki, K. Akimoto, T. Natori, T. Sakai, E. Sawa, K. Yamaji, Y. Koezuka, E. Kobayashi, and H. Fukushima. 1995. Structure-activity relationship of alpha-galactosylceramides against B16-bearing mice. *J Med Chem* 38:2176-2187.
13. Yoshimoto, T., A. Bendelac, C. Watson, J. Hu-Li, and W.E. Paul. 1995. Role of NK1.1+ T cells in a Th2 response and in immunoglobulin E production. *Science* 270:1845-1847.
14. Taniguchi, M., M. Harada, S. Kojo, T. Nakayama, and H. Wakao. 2003. The regulatory role of Valpha14 NKT cells in innate and acquired immune response. *Annu Rev Immunol* 21:483-513.
15. van der Vliet, H.J., J.W. Molling, N. Nishi, A.J. Masterson, W. Kolgen, S.A. Porcelli, A.J. van den Eertwegh, B.M. von Blomberg, H.M. Pinedo, G.

Giaccone, and R.J. Scheper. 2003. Polarization of Valpha24+ Vbeta11+ natural killer T cells of healthy volunteers and cancer patients using alpha-galactosylceramide-loaded and environmentally instructed dendritic cells. *Cancer Res* 63:4101-4106.

16. Pal, E., T. Tabira, T. Kawano, M. Taniguchi, S. Miyake, and T. Yamamura. 2001. Costimulation-dependent modulation of experimental autoimmune encephalomyelitis by ligand stimulation of V alpha 14 NK T cells. *J Immunol* 166:662-668.
17. Naumov, Y.N., K.S. Bahjat, R. Gausling, R. Abraham, M.A. Exley, Y. Koezuka, S.B. Balk, J.L. Strominger, M. Clare-Salzer, and S.B. Wilson. 2001. Activation of CD1d-restricted T cells protects NOD mice from developing diabetes by regulating dendritic cell subsets. *Proc Natl Acad Sci U S A* 98:13838-13843.
18. Kim, H.Y., H.J. Kim, H.S. Min, S. Kim, W.S. Park, S.H. Park, and D.H. Chung. 2005. NKT cells promote antibody-induced joint inflammation by suppressing transforming growth factor beta1 production. *J Exp Med* 201:41-47.
19. Ronet, C., S. Darche, M. Leite de Moraes, S. Miyake, T. Yamamura, J.A. Louis, L.H. Kasper, and D. Buzoni-Gatel. 2005. NKT cells are critical for the initiation of an inflammatory bowel response against Toxoplasma gondii. *J Immunol* 175:899-908.
20. Sakuishi, K., S. Oki, M. Araki, S.A. Porcelli, S. Miyake, and T. Yamamura. 2007. Invariant NKT cells biased for IL-5 production act as crucial regulators of inflammation. *J Immunol* 179:3452-3462.
21. Miyamoto, K., S. Miyake, and T. Yamamura. 2001. A synthetic glycolipid prevents autoimmune encephalomyelitis by inducing Th2 bias of natural killer T cells. *Nature* 413:531-534.
22. Wu, D., D.M. Zajonc, M. Fujio, B.A. Sullivan, Y. Kinjo, M. Kronenberg, I.A. Wilson, and C.H. Wong. 2006. Design of natural killer T cell activators: structure and function of a microbial glycosphingolipid bound to mouse CD1d. *Proc Natl Acad Sci U S A* 103:3972-3977.
23. Chang, Y.J., J.R. Huang, Y.C. Tsai, J.T. Hung, D. Wu, M. Fujio, C.H. Wong, and A.L. Yu. 2007. Potent immune-modulating and anticancer effects of NKT cell stimulatory glycolipids. *Proc Natl Acad Sci U S A* 104:10299-10304.
24. Liang, P.H., M. Imamura, X. Li, D. Wu, M. Fujio, R.T. Guy, B.C. Wu, M. Tsuji, and C.H. Wong. 2008. Quantitative microarray analysis of intact glycolipid-CD1d interaction and correlation with cell-based cytokine production. *J Am Chem Soc* 130:12348-12354.
25. Schlosser, E., M. Mueller, S. Fischer, S. Basta, D.H. Busch, B. Gander, and M. Groettrup. 2008. TLR ligands and antigen need to be coencapsulated into the same biodegradable microsphere for the generation of potent cytotoxic T lymphocyte responses. *Vaccine* 26:1626-1637.
26. Heit, A., F. Schmitz, T. Haas, D.H. Busch, and H. Wagner. 2007. Antigen co-encapsulated with adjuvants efficiently drive protective T cell immunity. *Eur J Immunol* 37:2063-2074.
27. Waeckerle-Men, Y., E.U. Allmen, B. Gander, E. Scandella, E. Schlosser, G. Schmidtke, H.P. Merkle, and M. Groettrup. 2006. Encapsulation of proteins and peptides into biodegradable poly(D,L-lactide-co-glycolide) microspheres prolongs and enhances antigen presentation by human dendritic cells. *Vaccine* 24:1847-1857.

28. Parekh, V.V., M.T. Wilson, D. Olivares-Villagomez, A.K. Singh, L. Wu, C.R. Wang, S. Joyce, and L. Van Kaer. 2005. Glycolipid antigen induces long-term natural killer T cell anergy in mice. *J Clin Invest* 115:2572-2583.
29. Figueroa-Perez, S., and R.R. Schmidt. 2000. Total synthesis of alpha-galactosyl cerebroside. *Carbohydr Res* 328:95-102.
30. Weigmann, B., J. Schwing, H. Huber, R. Ross, H. Mossmann, J. Knop, and A.B. Reske-Kunz. 1997. Diminished contact hypersensitivity response in IL-4 deficient mice at a late phase of the elicitation reaction. *Scand J Immunol* 45:308-314.
31. Wipf, P., and J.G. Pierce. 2006. Expedient synthesis of the alpha-C-glycoside analogue of the immunostimulant galactosylceramide (KRN7000). *Org Lett* 8:3375-3378.
32. Silk, J.D., I.F. Hermans, U. Gileadi, T.W. Chong, D. Shepherd, M. Salio, B. Mathew, R.R. Schmidt, S.J. Lunt, K.J. Williams, I.J. Stratford, A.L. Harris, and V. Cerundolo. 2004. Utilizing the adjuvant properties of CD1d-dependent NK T cells in T cell-mediated immunotherapy. *J Clin Invest* 114:1800-1811.
33. Chiba, A., S. Oki, K. Miyamoto, H. Hashimoto, T. Yamamura, and S. Miyake. 2004. Suppression of collagen-induced arthritis by natural killer T cell activation with OCH, a sphingosine-truncated analog of alpha-galactosylceramide. *Arthritis Rheum* 50:305-313.
34. Mizuno, M., M. Masumura, C. Tomi, A. Chiba, S. Oki, T. Yamamura, and S. Miyake. 2004. Synthetic glycolipid OCH prevents insulitis and diabetes in NOD mice. *J Autoimmun* 23:293-300.
35. Singh, A.K., M.T. Wilson, S. Hong, D. Olivares-Villagomez, C. Du, A.K. Stanic, S. Joyce, S. Sriram, Y. Koezuka, and L. Van Kaer. 2001. Natural killer T cell activation protects mice against experimental autoimmune encephalomyelitis. *J Exp Med* 194:1801-1811.
36. Gorbachev, A.V., and R.L. Fairchild. 2001. Regulatory role of CD4+ T cells during the development of contact hypersensitivity responses. *Immunol Res* 24:69-77.
37. Jiang, W., R.K. Gupta, M.C. Deshpande, and S.P. Schwendeman. 2005. Biodegradable poly(lactic-co-glycolic acid) microparticles for injectable delivery of vaccine antigens. *Adv Drug Deliv Rev* 57:391-410.

Chapter V

Co-encapsulation of tumor lysate and CpG-ODN in PLGA-MS for anti-tumor immunotherapy – A proof of principle

Marc Mueller and Marcus Groettrup

Abstract

The success of an immunotherpeutic approach is dependent on several factors, for example the immunological status of the patient, the carrier system, the antigen and the immunostimulatory adjuvants. We have established a well characterized carrier system, which can be used to microencapsulate several Toll like receptor ligands, in our case CpG oligodeoxynucleotides (CpG-ODN) and polyI:C. Biodegradable poly(lactide-*co*-glycolide) (PLGA) microspheres (MS) represent a potent carrier system which is very effective in tumor immunotherapy in mice (Chapter II). The choice of the antigen is a more complex issue. So far, we have worked with ovalbumin, a well studied model antigen, with a variety of tumor models and well established read out systems. Here we tested our approach in a more physiological model by using tumor lysate. Although cell lysate cannot be easily characterized and is scantly defined, the clinical availability is a clear advantage. Here we show, that PLGA-MS loaded with tumor cell line derived lysates and CpG-ODN, mixed with PLGA-MS containing polyI:C elicits *ex vivo* detectable CTL responses and show anti tumor activity in tumor bearing mice.

Introduction

Prostate cancer is one of the most common cancers in men beyond 60 years of age. In about 80 % of all men above 70 years of age, at least latent prostate tumors could be detected in situ (1). The survival of patients diagnosed with prostate cancer is about 80-90 % if the tumor is limited to the prostate gland itself, but only 30 % for metastatic tumors. Diagnosis of prostate cancer is reliant on the Gleason score, prostate specific antigen (PSA) levels or the level of early prostate cancer antigen - 2 (EPCA-2). Modern standard therapy for tumors of the prostate include radical surgery, radio therapy, or hormone ablation therapy. At least in some cases, hormone therapy decelerates progression or accounts for stable disease. Unfortunately, incidences of hormone resistance almost always appear after several months of hormone ablation therapy (2). Cryo-conservation of the removed primary tumor can be used for saving patient specific antigenic material for lysate preparation in cases of recurrence.

So far the best model to study treatment of prostate tumors is the transgenic adenocarcinoma mouse prostate (TRAMP) mouse. TRAMP mice are transgenic

for the SV40 large T antigen under the control of the rat probasin regulatory element (3). They remain healthy until puberty (week 4 to 5), but during the following weeks all TRAMP mice progressively develop prostate intraepithelial neoplasia, with documented progression to invasive carcinoma of epithelial origin and metastasis (4), closely resembling the human pathology (5).

It has been shown that biodegradable poly(lactide-*co*-glycolide) (PLGA) microspheres (MS) bearing encapsulated antigens are taken up by dendritic cells very efficiently *in vitro* and *in vivo*. Furthermore, it is known that the encapsulation of an antigen into PLGA-MS protects the antigen from degradation and provides a long lasting depot for sustained and prolonged immune responses in vivo (6-8). The particle size of PLGA-MS obtained by spray-drying is about 1-10 µm, which facilitates the uptake of antigens by APCs (9), followed by transport to the secondary lymphoid organs (10). We and others could show that vaccination approaches involving the co-encapsulation of the model antigen ovalbumin (ova) and CpG oligodeoxynucleotides (ODN) yield substantial cytotoxic T lymphocyte (CTL) responses as well as striking anti tumor responses (11, 12). These are excellent prerequisites for efficient *in vivo* loading of professional antigen presenting cells (APC) and thus for the priming of CTL mediated anti tumor responses.

In 2007 it was shown, that encapsulation of tumor cell lysate and subsequent injection leads to delayed tumor occurrence, even without the usage of any immunostimulatory adjuvant (13). Other laboratories could show that encapsulation of endogenous antigens can break tolerance (14) and that co-encapsulated tumor lysates with CpG-ODN lead to impaired tumor growth in mice (15). We could further improve this system by adding a second Toll like receptor (TLR) ligand, separately encapsulated. This has been shown to influence DCs in terms of T helper (Th) cell response polarization (16).

In chapter II we have shown that PLGA-MS as a delivery system is at least as potent as IFA, which has been used in clinical trials with melanoma patients (17). Furthermore, the spray-drying-technique is rapid, highly reproducible and easy to scale up. Therefore, a clinical trial using PLGA-MS encapsulated tumor lysate in combination with two encapsulated TLR ligands might be a suitable and feasible approach for clinical use.

Material and methods

Preparation of MS: MS were prepared from 14 kDa PLGA 50:50 carrying hydroxyl and carboxyl-end groups (Resomer RG502H, Boehringer Ingelheim, Ingelheim, Germany). The tumor cell line lysate and TLR ligands were microencapsulated by spray drying. Lysate of ~ $1x10^8$ TrampC2 cells (in 0.75 ml 0.1M $NaHCO_3$) stably transfected with full-length cytosolic ovalbumin (figures 2 and 3) or EG-7 cell lysate (figure 4), and 5 mg CpG oligodeoxynucleotides with a phosphothioate backbone (CpG-ODN 1826, Microsynth, Balgach, Switzerland) dissolved in 0.25 ml 0.1M $NaHCO_3$ (MS Lysate/CpG) or 0,5 mg polyI:C (Calbiochem, VWR, Dietikon, Switzerland) dissolved in 0.5 ml 0.1M $NaHCO_3$ (MS polyI:C) (aqueous phase) were mixed with 1 g of PLGA dissolved in 20 ml of dichloromethane (organic phase). Alternatively the aqueous phase contained 50 mg ovalbumin (Grade V, Sigma) and 5 mg CpG (MS-Ova/CpG).

The two phases were subsequently emulsified by ultrasonication (Hielscher, UP200 H, Ampl. 40%) for 10 s on ice. The obtained dispersion was immediately spray-dried (Büchi, Mini Spray-Dryer 191) at a flow rate of 2 ml/min and inlet/outlet temperatures of 40/37 °C. The obtained MS were washed out of the spray-dryer's cyclone with 0.05 % Synperonic (Synperonic®F68, Serva Electrophoresis GmbH, Heidelberg, Germany), collected on a cellulose acetate membrane filter and dried under reduced pressure (20 mbar) for 18 h at room temperature. PLGA-MS were stored at 4°C. Immediately before use MS were dispersed in PBS by ultrasonication for 30 sec.

γ-Irradiation of PLGA-MS with a dosage of 25 kGy was performed by the company Gamma-Service (Radeberg, Germany).

Mice and immunizations: C57BL/6 mice (H-2b) and TRAMP mice (3) were originally purchased from Jackson Laboratories. All mice were kept in a specific pathogen-free facility and used at 6–10 wk of age if not other information is provided. Animal experiments were approved by the review board of the Regierungspräsidium Freiburg. Mice were immunized with a mixture of 5 mg MS-Ova/CpG or MS-Lysate/CpG together with 5 mg MS-polyI:C. Control mice were treated with empty microspheres (empty) or left untreated (naïve). All injections were performed in a total volume of 200µl s.c. at the base of the tail.

Cell lines and media: EL-4 thymoma cells and the ovalbumin expressing transfectant EG-7 were kindly provided by Dr. Wolfram Osen (DKFZ Heidelberg, Germany) and kept in RPMI medium. Media were purchased from Invitrogen Life Technologies and contained GlutaMAX, 10% Fetal Calf Serum, and 100U/ml penicillin/streptomycin. For the clone Vf10, TrampC2 cells (provided by Dr. Peter Öhlschläger; Univerity of Konstanz, Germany) were transfected with a plasmid encoding for full-length, cytosolic ovalbumin. Vf10 cells were kept in DMEM (-Pyruvate), complemented with 5 % FCS, 5 % Nu-serum (BD Biosciences), 5µg/ml Insulin (Sigma), 10^{-8} M dihydrotestosterone (DTH, Sigma)) and 1% P/S. To maintain the expression of ovalbumin in EG-7, MO-5 and Vf10 cells the media contained geneticin (G418; Sigma) (0,2 mg/ml).

Intracellular cytokine staining: For intracellular cytokine staining (ICS), mice were immunized with PLGA-MS s.c. at the base of the tail. Six days later, splenocytes were isolated and incubated with or without 10 µM SIINFEKL peptide (Eurogentec, Cologne, Germany) in the presence of brefeldin A (10 µg/ml, Sigma-Aldrich) for 5 h at 37°C. After washing, cells were stained with PE-Cy5-conjugated anti-mouse CD8α antibody (BD Biosciences Pharmingen, Clone 53-6.7) for 20 min at 4°C. The cells were washed again and fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. After washing the cells twice with PBS, cells were labeled intracellularly with FITC-conjugated rat-anti-IFN-γ antibody (clone XGM1.2, diluted in PBS/0.1% Saponin) at 4°C overnight. The next day, cells were washed and resuspended in PBS for flow cytometry. Background values for each sample (without peptide) were subtracted.
Data were acquired using a FACScan flow cytometer (BD Biosciences) and analyzed with FlowJo software (Treestar).

***Ex vivo* cytotoxicity assay:** For measurement of ex vivo cytolytic activity a time-resolved fluorometric assay was used. C57BL/6 mice or TRAMP mice were immunized with PLGA-MS. At the indicated time points splenocytes or blood lymphocytes were isolated and used as effectors in a primary cytotoxicity assay as previously described (18) using DELFIA® EuTDA cytotoxicity reagents (PerkinElmer).

Briefly, $5x10^4$ Vf10 cells served as target cells and were labeled with BaTDA. After 4 h of coincubation, supernatants were mixed with Europium solution for 1 h and subsequently measured at excitation/emission 340/615 nm and a lag time of 200 nsec. Specific cytolysis was calculated as follows: [(counts for immunized mouse - counts for naïve mouse) * 100)] / (counts for total lyses).

Therapeutic EG-7 tumor setting: C57BL/6 mice were challenged with $5x10^5$ EG-7 tumor cells in PBS, s.c. at the right flank. As soon as tumors were palpable, treatment with PLGA-MS was initiated. Subsequently, tumor growth and survival was monitored. Tumor sizes were measured daily until they reached 15mm in mean size of 2 orthogonal measurements. If this abort criterion was fulfilled, mice were sacrificed.

Results

A central parameter for all vaccine formulations is, whether production under sterile conditions is possible. Since production of clinical grade, sterile PLGA-MS would require a sterile GMP facility not frequently accessible, we decided to γ-irradiate PLGA-MS. In order to elucidate, whether γ-irradiation affects the immunostimulatory properties of PLGA-MS we immunized C57BL/6 mice (n=3) with γ-irradiated or non-sterile MS-Ova/CpG in combination with MS-polyI:C and performed an ICS for IFN-γ, six days post injection. As shown in figure 1, we could not detect a significant influence of γ-irradiation on PLGA-MS.

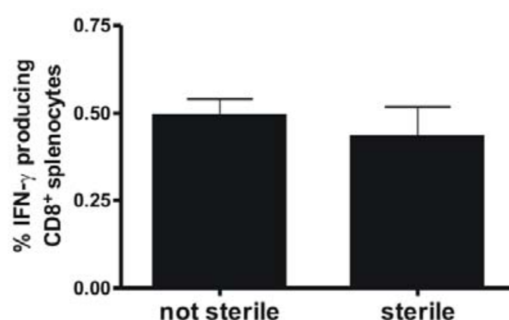

Figure 1: γ-irradiation of PLGA-MS does not alter the outcome of CTL-targeted immunization: Mice (n=3) were immunized with 5 mg MS containing Ova/CpG in combination with 5 mg MS-polyI:C, either γ-irradiated with a dosage 25 kGy (sterile) or not sterilized (not sterile). Six days after immunization an ICS for IFN-γ was performed to monitor the success of the immunization. Values are given in percent $CD8^+$ / IFN-$γ^+$ lymphocytes, +/- SEM. No significant

difference could be observed. The experiment was conducted three times, with similar results. One representative experiment is shown.

In an initial experiment, we established an appropriate read out system for this new approach in our laboratory. We immunized C57BL/6 mice with MS containing either Ova/CpG (gray line; MS-Ova/CpG) or Lysate/CpG (black line; MS-Lysate/CpG) in combination with MS-polyI:C. After six days we performed a DELFIA® EuTDA cytotoxicity assay, using splenocytes derived from the immunized mice as effector cells and ovalbumin transfected TrampC2 cells (clone Vf10) as target cells. As figure 2A shows, splenocytes from both groups were potent in killing Vf10 target cells directly *ex vivo* in a 4 hour assay. As shown in figure 2B this potential for killing BaTDA labeled target cells lasted for at least 14 days after immunization. On day 21 cytotoxic activity was detected only at the highest effector to target ratio. The group, which was immunized 14 days prior to the assay showed substantial killing for effector to target ratios of 100:1 and 50:1, but not for 25:1, the lowest effector to target ratio used. Immunization 6 days prior to the assay reproduced well the data obtained in figure 2A.

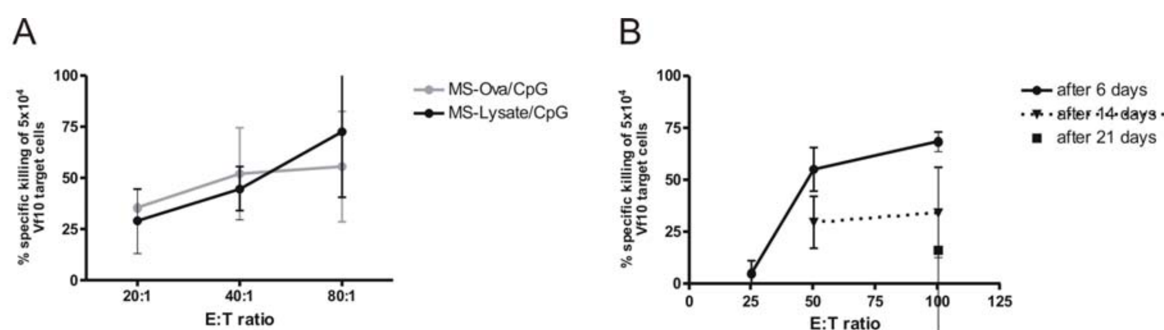

Figure 2: *Ex vivo* cytotoxicity of C57BL/6 derived splenocytes after immunization with MS-Lysate/CpG and MS-polyI:C: A) C57BL/6 mice (n=2) were immunized with 5 mg MS-polyI:C and either 5 mg MS-Lysate/CpG (MS-Lysate/CpG) or with 5 mg MS-Ova/CpG (MS-Ova/CpG). At day six post immunization, splenocytes were monitored for ex vivo cytotoxicity in a DELFIA® EuTDA cytotoxicity assay. Values are given in percent specific killing of $5x10^4$ Vf10 target cells for the indicated effector to target ratios. The graph shows a summary of three independent experiments with the same outcome. B) C57BL/6 mice (n=2) were immunized with 5 mg MS-polyI:C and 5 mg MS-Lysate/CpG. At day six (black circles, solid line), at day 14 (black triangle, dotted line) and at day 21 (black square) after immunization, splenocytes were monitored for ex vivo cytotoxicity in a DELFIA® EuTDA cytotoxicity assay. Values are given in percent specific killing of $5x10^4$ Vf10 target cells for the indicated effector to target ratios. The graph shows a summary of three independent experiments with the same outcome.

Figure 3 shows an initial experiment using TRAMP mice. Mice were primed at 10 weeks of age and boosted after 4 weeks with MS containing Lysate/CpG in combination with MS-polyI:C. Six days after the primary immunization (black squares; solid line) and the booster immunization (black triangles; solid line), blood was drawn and blood derived lymphocytes were analyzed in an *ex vivo* DELFIA® EuTDA cytotoxicity assay. As graph 3A shows, specific killing of $5x10^4$ Vf10 target cells was generally low but well detectable after the boost. Given that the assay was performed directly ex vivo without any restimulation we decided to choose another read out for this challenging model. The same mice were sacrificed, without any further treatment, and at an age of 20 weeks. The prostatic tissue was excised and weight. As shown in figure 3B we could not detect any significant difference between TRAMP mice treated with empty MS (empty MS) or MS Lysate/CpG in combination with MS-polyI:C treated mice (MS-Lysate /CpG). A naïve C57BL/6 is given as a reference. The graph shows a composition of 5 individual mice per group, equally treated, starting at 10 weeks of age and ending at 20 weeks of age. One mouse of the MS-Lysate /CpG group had to be killed at an age of 16 weeks due to poor health conditions (zoonosis, reduced food uptake and lethargy).

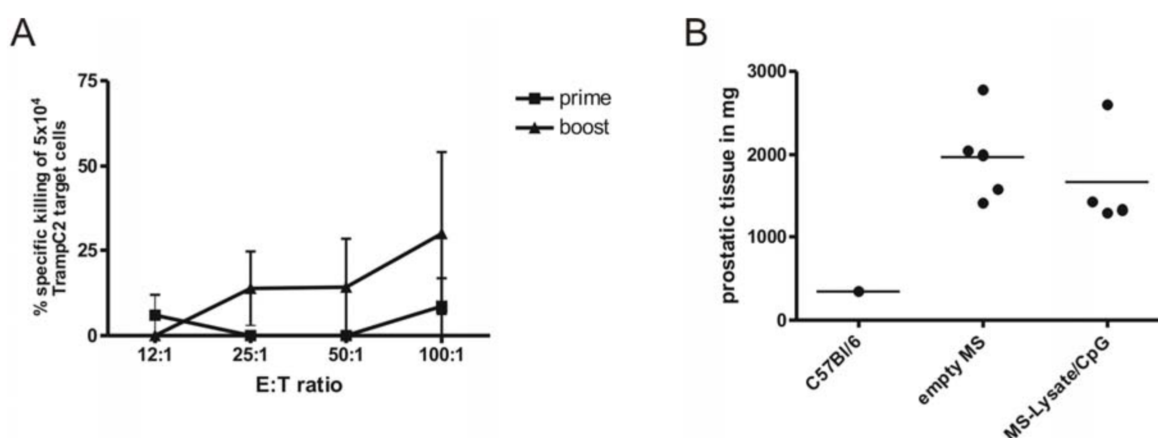

Figure 3: Anti tumor response after homologous prime-boost immunization with MS-Lysate/CpG and MS-polyI:C: A) TRAMP mice were immunized twice with 5 mg MS-polyI:C and 5 mg MS-Lysate/CpG in an interval of four weeks. Six days after each immunization blood lymphocytes were analyzed for ex vivo cytotoxicity in a DELFIA® EuTDA cytotoxicity assay. Values are shown for the primary immunization (squares) and for the booster immunization (triangles) at different effector to target ratios in % specific killing of $5x10^4$ TrampC2 target cells. The graph shows a single experiment using four independently treated TRAMP mice, starting therapy at the age of 10 weeks. B) Mice from experiment A) were sacrificed at an age of 20 weeks and prostatic tissue was excised (MS-Lysate/CpG). Prostatic tissue was defined as the *glandula vesiculosa*, the *glandula ampullaris*, the *pars anterior prostatae*, the *pars ventralis prostatae* as well as the *pars*

dorsalis prostatae. As a reference the prostatic tissue weight of a C57BL/6 at an age of 20 weeks is given as well in mg prostatic tissue weight. Similar to the mice in experiment A) a control group (n=5) was treated with empty MS twice, in an interval of four weeks, starting at the age of 10 weeks (empty MS).

A parallel experiment to proof the principle working-hypothesis, that using tumor lysate as an antigen in PLGA-MS is an appropriate tumor associated antigen in immunotherapy of prostate tumors, we inoculated C57BL/6 mice with $5x10^5$ EG-7 tumor cells, s.c. at the right flank. As soon as palpable tumors occurred we started treatment with either MS containing Ova/CpG (gray line; MS-Ova/CpG) or Lysate/CpG (black line; MS-EG-7Lysate/CpG) in combination with MS-polyI:C. A group of mice, treated with empty MS served as a control. Mice were killed if the tumors size exceeded a mean size of 15 mm in two orthogonal measurements. Figure 4 shows the % survival of a representative experiment. The experiment was conducted three times with similar results.

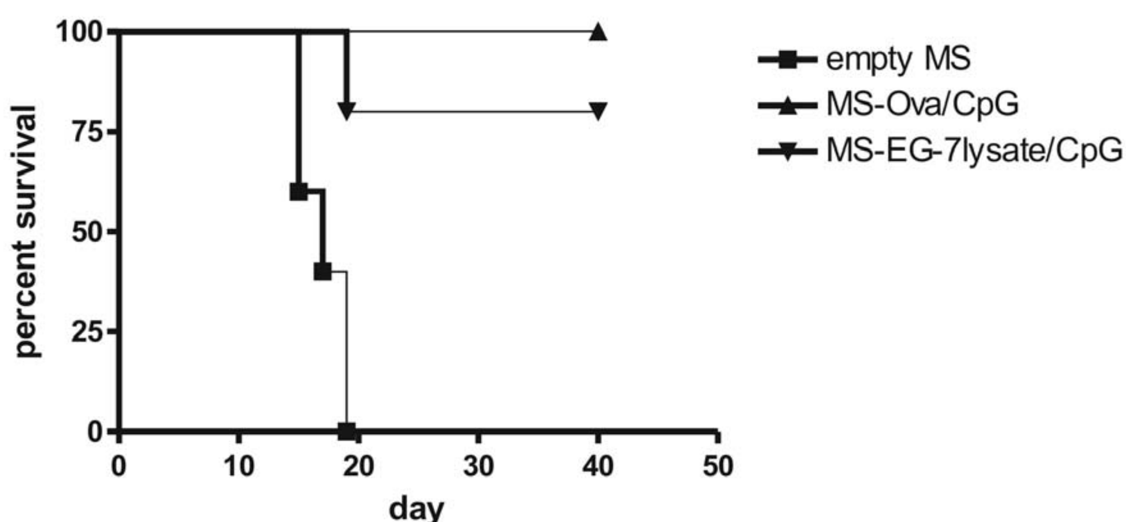

Figure 4: MS-Lysate/CpG mediated anti tumor response to solid, subcutaneous EG-7 tumors: C57BL/6 mice (n=5) were challenged with $5x10^5$ EG-7 cells s.c. at the right flank. As soon as palpable tumors occurred, mice were immunized with 5 mg MS-polyI:C and either 5 mg MS-EG-7Lysate/CpG (MS-Lysate/CpG) or 5 mg MS-Ova/CpG (MS-Ova/CpG). The control group was immunized with 5 mg empty MS. Mice were sacrificed when the mean tumor size, assed by two orthogonal measurements, exceeded 15 mm. Values are given in percent survival. The graph represents one out of three experiments with similar results.

Discussion

The availability of tumor-associated antigens (TAA) is a general problem in immunotherapy, regardless whether *ex vivo* pulsed DCs are used, if the approach is based on the transfer of autologous T cells or on *in vivo* loading of APCs. On the one hand, a tumor specific antigen is needed, on the other hand, the TAA needs to be available in substantial amounts. For peptides this is rather easy, since they can be synthetically synthesized in large amounts. The backside of the coin is that costs are immense and tumors might evolve to be resistant by down-regulating the expression of the protein from which the peptide stems (19, 20). Furthermore, peptides are restricted to certain MHC haplotypes, narrowing the pool of putative patients immensely. If the TAA is a whole protein, the production of large amounts gets challenging. The establishment of a protocol for recombinant production of desired proteins can take years or even longer and the problem of tumor evasion is also apparent in this approach. An advantage is that a protein encodes for a variety of putative epitopes, which might be helpful in clinics (21). At least the problem of costs and hand on time can be circumvented by tumor cells based vaccines (reviewed in (22)). An approach using lysate of melanoma tumors was pioneered in 1988 by Mitchell and colleagues (23). The tumor lysates were applied together with DETOX, a water-in-oil emulsion containing bacterial derived immunostimulatory adjuvants. The vaccine Melacine was launched by Corixa Corporation with about 10-20 % success in melanoma patients in first trials. Later studies revealed an influence of the HLA type of the patient (24) and confirmed the low but consistent efficacy in clinical phase I and II studies (25). Its carrier system is similar to IFA (26), a water in oil emulsion building a physical barrier and thereby hindering diffusion of the antigen and the adjuvants. IFA is still used in clinical trials, but will most likely never be approved for standard clinical use, due to considerable side effects.

In our approach we opsonize the tumor lysate to APCs by co-encapsulation in PLGA-MS together with the immunostimulatory adjuvants. This has been proven to be effective in vitro and in the mouse model. An improved uptake by APCs and thereby prolonged presentation in the lymphoid organs might improve the efficacy of the vaccine and even help to overcome the restraining effect of the HLA dependency. A prolonged Th cell activation and sustained supply of TAA loaded DCs to the lymphoid organs has been shown to effectively improve anti tumor

immunity in the TRAMP model (27, 28). Furthermore, it has been shown, that immunization with subdominant epitopes can help to overcome T cell tolerance (29). Since it is likely, that tumor lysate carries several epitopes we hypothesize that this might be an additional beneficial factor of our approach. We could show, that manufacturing of sterile PLGA-MS for non-contaminating injection is feasible and does not affect immunogenicity of encapsulated contents. Furthermore, we were able to show, that encapsulation of tumor lysates and co-administration with TLR ligands yields detectable cytotoxic T cell responses *ex vivo* and robust anti tumor immunity. In contrast to IFA, which is known for its severe side effects, the administration of PLGA-MS does not elicit any adverse effects. Thus our approach seems to be suitable and powerful enough for further studies in the TRAMP mouse. Moreover, the combination of the two encapsulated TLR ligands CpG-ODN and polyI:C and tumor lysate as a carrier of several TAAs is a potent alliance for the eradication of prostate tumors.

References of chapter V:

1. Breslow, N., C.W. Chan, G. Dhom, R.A. Drury, L.M. Franks, B. Gellei, Y.S. Lee, S. Lundberg, B. Sparke, N.H. Sternby, and H. Tulinius. 1977. Latent carcinoma of prostate at autopsy in seven areas. The International Agency for Research on Cancer, Lyons, France. *Int J Cancer* 20:680-688.
2. Basler, M., and M. Groettrup. 2007. Advances in prostate cancer immunotherapies. *Drugs Aging* 24:197-221.
3. Greenberg, N.M., F. DeMayo, M.J. Finegold, D. Medina, W.D. Tilley, J.O. Aspinall, G.R. Cunha, A.A. Donjacour, R.J. Matusik, and J.M. Rosen. 1995. Prostate cancer in a transgenic mouse. *Proc Natl Acad Sci U S A* 92:3439-3443.
4. Shappell, S.B., G.V. Thomas, R.L. Roberts, R. Herbert, M.M. Ittmann, M.A. Rubin, P.A. Humphrey, J.P. Sundberg, N. Rozengurt, R. Barrios, J.M. Ward, and R.D. Cardiff. 2004. Prostate pathology of genetically engineered mice: definitions and classification. The consensus report from the Bar Harbor meeting of the Mouse Models of Human Cancer Consortium Prostate Pathology Committee. *Cancer Res* 64:2270-2305.
5. Wernert, N., E. Bieroff, and A. Hugel. 1997. Pathological aspects of prostate cancer and benign nodular hyperplasia. *Anticancer Res* 17:2907-2910.
6. Audran, R., K. Peter, J. Dannull, Y. Men, E. Scandella, M. Groettrup, B. Gander, and G. Corradin. 2003. Encapsulation of peptides in biodegradable microspheres prolongs their MHC class-I presentation by dendritic cells and macrophages in vitro. *Vaccine* 21:1250-1255.
7. Newman, K.D., P. Elamanchili, G.S. Kwon, and J. Samuel. 2002. Uptake of poly(D,L-lactic-co-glycolic acid) microspheres by antigen-presenting cells in vivo. *J Biomed Mater Res* 60:480-486.
8. Waeckerle-Men, Y., E.U. Allmen, B. Gander, E. Scandella, E. Schlosser, G. Schmidtke, H.P. Merkle, and M. Groettrup. 2006. Encapsulation of proteins and peptides into biodegradable poly(D,L-lactide-co-glycolide) microspheres prolongs and enhances antigen presentation by human dendritic cells. *Vaccine* 24:1847-1857.
9. Shen, Z., G. Reznikoff, G. Dranoff, and K.L. Rock. 1997. Cloned dendritic cells can present exogenous antigens on both MHC class I and class II molecules. *J Immunol* 158:2723-2730.
10. Manolova, V., A. Flace, M. Bauer, K. Schwarz, P. Saudan, and M.F. Bachmann. 2008. Nanoparticles target distinct dendritic cell populations according to their size. *Eur J Immunol* 38:1404-1413.
11. Heit, A., F. Schmitz, T. Haas, D.H. Busch, and H. Wagner. 2007. Antigen co-encapsulated with adjuvants efficiently drive protective T cell immunity. *Eur J Immunol* 37:2063-2074.
12. Schlosser, E., M. Mueller, S. Fischer, S. Basta, D.H. Busch, B. Gander, and M. Groettrup. 2008. TLR ligands and antigen need to be coencapsulated into the same biodegradable microsphere for the generation of potent cytotoxic T lymphocyte responses. *Vaccine* 26:1626-1637.
13. Solbrig, C.M., J.K. Saucier-Sawyer, V. Cody, W.M. Saltzman, and D.J. Hanlon. 2007. Polymer nanoparticles for immunotherapy from encapsulated tumor-associated antigens and whole tumor cells. *Mol Pharm* 4:47-57.

14. Hamdy, S., O. Molavi, Z. Ma, A. Haddadi, A. Alshamsan, Z. Gobti, S. Elhasi, J. Samuel, and A. Lavasanifar. 2008. Co-delivery of cancer-associated antigen and Toll-like receptor 4 ligand in PLGA nanoparticles induces potent CD8+ T cell-mediated anti-tumor immunity. *Vaccine* 26:5046-5057.
15. Goforth, R., A.K. Salem, X. Zhu, S. Miles, X.Q. Zhang, J.H. Lee, and A.D. Sandler. 2009. Immune stimulatory antigen loaded particles combined with depletion of regulatory T-cells induce potent tumor specific immunity in a mouse model of melanoma. *Cancer Immunol Immunother* 58:517-530.
16. Napolitani, G., A. Rinaldi, F. Bertoni, F. Sallusto, and A. Lanzavecchia. 2005. Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells. *Nat Immunol* 6:769-776.
17. Speiser, D.E., D. Lienard, N. Rufer, V. Rubio-Godoy, D. Rimoldi, F. Lejeune, A.M. Krieg, J.C. Cerottini, and P. Romero. 2005. Rapid and strong human CD8+ T cell responses to vaccination with peptide, IFA, and CpG oligodeoxynucleotide 7909. *J Clin Invest* 115:739-746.
18. Blomberg, K., R. Hautala, J. Lovgren, V.M. Mukkala, C. Lindqvist, and K. Akerman. 1996. Time-resolved fluorometric assay for natural killer activity using target cells labelled with a fluorescence enhancing ligand. *J Immunol Methods* 193:199-206.
19. Maeurer, M.J., S.M. Gollin, D. Martin, W. Swaney, J. Bryant, C. Castelli, P. Robbins, G. Parmiani, W.J. Storkus, and M.T. Lotze. 1996. Tumor escape from immune recognition: lethal recurrent melanoma in a patient associated with downregulation of the peptide transporter protein TAP-1 and loss of expression of the immunodominant MART-1/Melan-A antigen. *J Clin Invest* 98:1633-1641.
20. Slingluff, C.L., Jr., T.A. Colella, L. Thompson, D.D. Graham, J.C. Skipper, J. Caldwell, L. Brinckerhoff, D.J. Kittlesen, D.H. Deacon, C. Oei, N.L. Harthun, E.L. Huczko, D.F. Hunt, T.L. Darrow, and V.H. Engelhard. 2000. Melanomas with concordant loss of multiple melanocytic differentiation proteins: immune escape that may be overcome by targeting unique or undefined antigens. *Cancer Immunol Immunother* 48:661-672.
21. Mateo, L., J. Gardner, Q. Chen, C. Schmidt, M. Down, S.L. Elliott, S.J. Pye, H. Firat, F.A. Lemonnier, J. Cebon, and A. Suhrbier. 1999. An HLA-A2 polyepitope vaccine for melanoma immunotherapy. *J Immunol* 163:4058-4063.
22. Copier, J., S. Ward, and A. Dalgleish. 2007. Cell based cancer vaccines: regulatory and commercial development. *Vaccine* 25 Suppl 2:B35-46.
23. Mitchell, M.S., J. Kan-Mitchell, R.A. Kempf, W. Harel, H.Y. Shau, and S. Lind. 1988. Active specific immunotherapy for melanoma: phase I trial of allogeneic lysates and a novel adjuvant. *Cancer Res* 48:5883-5893.
24. Sosman, J.A., and V.K. Sondak. 2003. Melacine: an allogeneic melanoma tumor cell lysate vaccine. *Expert Rev Vaccines* 2:353-368.
25. Sondak, V.K., and J.A. Sosman. 2003. Results of clinical trials with an allogenic melanoma tumor cell lysate vaccine: Melacine. *Semin Cancer Biol* 13:409-415.
26. Freund, J. 1951. The effect of paraffin oil and mycobacteria on antibody formation and sensitization; a review. *Am J Clin Pathol* 21:645-656.
27. Shafer-Weaver, K.A., S.K. Watkins, M.J. Anderson, L.J. Draper, A. Malyguine, W.G. Alvord, N.M. Greenberg, and A.A. Hurwitz. 2009.

Immunity to murine prostatic tumors: continuous provision of T-cell help prevents CD8 T-cell tolerance and activates tumor-infiltrating dendritic cells. *Cancer Res* 69:6256-6264.

28. Degl'Innocenti, E., M. Grioni, A. Boni, A. Camporeale, M.T. Bertilaccio, M. Freschi, A. Monno, C. Arcelloni, N.M. Greenberg, and M. Bellone. 2005. Peripheral T cell tolerance occurs early during spontaneous prostate cancer development and can be rescued by dendritic cell immunization. *Eur J Immunol* 35:66-75.
29. Grossmann, M.E., T. Davila, and T. Celis. 2001. Avoiding tolerance against prostatic antigens with subdominant peptide epitopes. *J Immunother* 24:237-241.

Discussion

Discussion and outlook

Whether Ambrose Bierce was right when he said that “medicine is like a stone, flung down the Bowery to kill a dog on Broadway” and if he thought about immunotherapy is questionable. If so, the big challenge would be to get the stone flying over a distance of around 1 km from Bowery to Broadway. Figuratively spoken, PLGA-MS might be the tool to get the stone flying.

PLGA-MS have been shown to posses a variety of advantageous features for the loading of APCs *in vitro* and *in vivo*. Their ability to encapsulate virtually any antigen regardless of its size or origin, from peptides to proteins, from DNA to solid matters, and to release their otherwise protected contents slowly but constantly are the most interesting ones. It is not surprising, that numerous groups tried to study their potential to elicit immune responses, strong enough to cure tumor burden. Besides the fact that we could not detect an adverse effect of sterilization of PLGA-MS, we never experienced any negative side effects after the immunization with PLGA-MS. Other carrier systems, e.g., IFA, aluminum adjuvant or free CpG-ODN, in contrast, are known to commit for granulomas, erythemas (1, 2) or splenomegaly, a sign of toxicity (3). Apart from that, we could very convincingly show that PLGA-MS commit for a long lasting potential to induce proliferation of $CD4^+$ and $CD8^+$ T cells as well as effective and long lasting *in vivo* cytotoxicity. Given that IFA, the “gold standard” in immunization, and PLGA-MS yielded mostly similar results when being directly compared (chapter II), PLGA-MS compare favorably with IFA, providing a promising alternative.

After an immunization with PLGA-MS, we saw a rapidly decreasing CTL response after day 8. The percentage of IFN-γ^+ / $CD8^+$ splenocytes peaked at day 6, while being low on day 5 and on day 8 (data not shown). In general, an increased proliferation in combination with absence of IFN-γ at later stages is a characteristic of T cell anergy by functional exhaustion (4), probably due to antigen overload (5). This explanation goes in line with the earlier described depot effect of PLGA-MS, but is inconsistent with long lasting cytotoxic functionality shown in chapter II (figure 5).

Another interesting point of chapter II is that we found two approaches to further increase CTL responses upon PLGA-MS immunization. At first we could show that a TNF-α preconditioning of the site of injection increases the CTL response

significantly. This result goes in line with the original publication of Martin-Fontecha *et al.* in 2003 suggesting an improved DCs migration by the up regulation of CCL21 (6). Subsequently, we could show that homologous boosts with PLGA-MS significantly elevated levels of tetramer specific CD8+ lymphocytes population but not the IFNγ^{+} / CD8^{+} lymphocyte population of the spleen, 6 days after the booster immunization. The reason for this discrepancy could not be clarified, so far. It has been published for VLPs that homologous boost can effectively elevate the epitope specific T cell population (7), but the authors did not refer to the question, whether the IFNγ^{+} / CD8^{+} lymphocyte population is affected in this study as well. Nevertheless, heterologous boosting, using recombinant vaccinia virus, gave promising results in both read out systems and might be a candidate for clinical testing. The modified vaccinia Ankara (MVA) strain has been proven to be safe in clinical trials (8) and is commonly used as a vector for recombinant expression of TAAs in different systems (9-12).

Another, very versatile field of this thesis covers immunostimulatory adjuvants. In principle our approach makes use of TLR ligands, especially the TLR9 ligand CpG and the TLR3 ligand polyI:C. We found that the combination of CpG-ODN and polyI:C leads to increased CTL responses (data not shown), which was proposed by Napolitani *et al* in 2005 to be true for a variety of combinations (13). Up to now, we stick to our finding, that CpG-ODN have an increased impact, if they are in close proximity to the antigen (14) and used CpG-ODN in an co-encapsulated form with the antigen (15). This should be reconsidered if clinical trials are planned. Given that TLR9 is absent on human myeloid DCs (mDC) but only expressed on plasmacytoid DCs (pDC), it might be favorable to co-encapsulated polyI:C with the antigen. PDCs are able to cross-present endocytosed antigens (16) but are generally inferior with respect to T cell stimulation (17). In humans, TLR3 is known to be expressed on both, mDCs and pDCs, and it has been shown that polyI:C effectively activates mDCs and immature moDCs (18, 19). Moreover, polyI:C gave satisfying results when being co-encapsulated with ovalbumin in preliminary experiments (data not shown).

Nevertheless, a second TLR ligand stimulating the MyD88 independent pathway is known to cause striking benefits *in vivo*, referring to CTL responses and also T-reg inhibition in mice (20). New B type CpG-ODN sequences, e.g., CpG 2006, have

been published to activate human pDCs effectively (21). Another advantage of these CpG-ODN is their ability to activate B cells (22), probably a crucial point in studies using unmethylated CpG-ODNs in combination with IFA. A possibility to exploit this feature of CpG-ODN could be the adsorption or coupling to PLGA-MS as it has recently been demonstrated by Fischer *et al.* (23) or the single encapsulation of CpG-ODNs in PLGA-MS.

Chapters III and IV deal with CD1d ligands. Ligands, binding the MHC class I like molecule CD1d, are potent activators of NKT cells and therefore interesting immune modulators. For PLGA-MS we could show, that the Th1/Th2 bias is rather balanced (chapter II; figure 1 and figure 3). Depending on the read out which was used, we saw either a slight Th2 shift (chapter II; figure 1) or a Th1 bias (chapter II; figure 3). An enticing idea was to modulate the system by the usage of a CD1d ligand that effectively drives the Th bias towards Th1. Additionally, the activation of the innate immune system represented by NKT cells would probably contribute to anti tumor responses (24) and have positive effects on the down regulation of the ongoing immune response (25, 26). In chapter III we investigated α-GalCer and α-C-GalCer for positive properties. Unfortunately, the hopes raised by Frank and colleagues (27, 28) faded again. On the one hand, we could not reproduce the published cytokine pattern of α-C-GalCer or its superior anti tumor reactivity, on the other hand neither α-GalCer nor α-C-GalCer gave any additional benefit when used in vaccination studies together with PLGA-MS. It is known that activation of NKT cells leads to improved activation of DCs and thereby enhanced cross-presentation of phagocytosed antigens (29). The absence of a co-stimulatory signal, normally provided by activated DCs, can lead to anergy of both, T cells and NKT cells. Since free α-GalCer can also be presented by B cells (30), which lack co-stimulatory molecules, efficient targeting to DCs is important. This can be achieved by encapsulation in or conjugation to microparticles (31). In our experiments, co-application of both CD1d ligands in a soluble form had even adverse effects regarding intracellular production of IFN-γ by $CD8^+$ lymphocytes in the spleen on day six after immunization, whereas co-application of encapsulated CD1d ligand showed no effect (chapter III; figure 4). The reasons for this remain unclear. The NMR-spectra for α-C-GalCer, which was synthesized in our laboratory by Mirko Zierke, under the supervision of Prof. Richard Schmidt, put the

chemical identity of our substance beyond doubt. Neither the experiments referring to the activation of DCs, the activation of NKT cells nor the tumor challenge experiments revealed any significant improvement. Only the serum cytokine pattern was slightly improved by α-C-GalCer (chapter III; figure 1), but not to the extent published previously. It can be hypothesized, that α-C-GalCer shows only marginally reduced binding properties compared to α-GalCer and therefore acts as a “super-antigen”. This would in turn lead to a less defined cytokine pattern and the released Th1 and Th2 cytokines would have opposing, and in turn neutralizing effects.

As a side project we investigated an α-C-diPheGalCer, a synthetic N-acyl analogue α-C-GalCer. Interesting about α-C-diPheGalCer is that it showed a cytokine pattern which was the opposite of what we were searching for. α-C-diPheGalCer induced after i.v. administration high amounts of IL-4 but very low amounts of IFN-γ (chapter IV, figure 2). We tested α-C-diPheGalCer in several models of autoimmunity and found α-C-diPheGalCer to be very effective, even in single dose administrations. Furthermore, there were no detectable signs of toxicity *in vitro* (chapter IV; figure 7). We could show that α-C-diPheGalCer lead to an efficient down regulation of the transcription factor T-bet on mRNA level (chapter IV; figure 3) and thus counteracted Th1 mediated autoimmunity. So far we did not prove that the Th2 polarization is NKT cell dependent, but large amounts of early cytokines are most likely produced by NKT cells. Experiments using $CD1d^{-/-}$ mice or $J\alpha18^{-/-}$ mice should conclusively clarify this issue. Another topic that needs to be investigated in the future is the efficacy of α-C-diPheGalCer after repeated injections. This might lead to a synergistic effect based on NKT cell anergy, which is characterized by the preservation of IL-4 production and the loss of IFN-γ secretion (32). Whether a depot effect by encapsulation in PLGA-MS is needed or not depends on the disease model. At least we could show that it is an option, even though, it still has to be optimized.
Another interesting aspect of α-C-diPheGalCer is, that co-injection in a soluble form together with PLGA-MS, significantly increases the CTL response (chapter IV; figure 8). In chapter III we have seen adverse effects for soluble α-GalCer and α-C-GalCer negatively correlating with the amount of IFN-γ apparent in the serum (chapter III, figure 4). A possible explanation for that might be a negative feedback

loop. Minami et al. showed in 2005, that DCs effectively trigger a Th1 cytokine polarization if they were pretreated with Th2 cytokines like IL-4 (33). In 2000, it could be demonstrated by Hochrein *et al.* for various DC subtypes, that treatment with IL-4 leads to the secretion of IL-12p70, the major Th1-inducing cytokine and in parallel down regulated its agonist IL12p40 (34). In our model the induction of early IL-4 by α-C-diPheGalCer activated NKT cells would lead to sustained production of IL-12p70 by DCs in the lymph nodes. The DCs, which engulfed PLGA-MS in the periphery and subsequently migrated to the lymph nodes would then be preconditioned by IL-4 and favor Th1 polarization of Th cells in the lymphoid organs. The situation would be contrary for α-GalCer and α-C-GalCer since both induce higher serum IFN-γ levels.

Chapter V explores the possibility of the encapsulation of tumor cell lysate. The fact that *ex vivo* responses could be detected and tumor experiments using EG-7 lysate proved, that in principle the encapsulation of lysate is effective, raised hopes for the future of this project. On the other hand the initial experiment in TRAMP mice (chapter V; figure 3B) were sobering. Admittedly, neither the group size of n = 4, nor the read out were sophisticated enough for such a challenging model. An alternative would be a computed tomography scan for malignant tumor mass. Nevertheless, we could induce a CTL response against tumor cells and even succeeded with a homologous boost (chapter V; figure 3A), strengthening the data obtained in chapter II (figure 2C).

In general, PLGA-MS represent an excellent carrier system for antigens and immunostimulatory adjuvants for immunotherapy of tumors. Our approach was proven to be at least equivalent to IFA, in some cases, e.g., tumor therapy, even better. Considering that IFA is apparently the most successful carrier system available and that PLGA-MS are flexible enough to cope with the exchange of immunostimulatory adjuvants for clinical trials, PLGA-MS are a very promising delivery device. An approach using autologous tumor tissue for lysates, which are co-encapsulated with suitable TLR ligands, seems to be feasible and passed the proof of principle. General features of the spray-drying technique, e.g., short manufacturing times and the ease of up-scaling, but also of the microparticles,

e.g., the possibility of sterilization by γ-irradiation, make PLGA-MS even more interesting for clinical trials.

Returning to the beginning of this chapter, PLGA-MS could get the stone flying, let’s get a dog …

References of discussion and outlook:

1. Miller, L.H., A. Saul, and S. Mahanty. 2005. Revisiting Freund's incomplete adjuvant for vaccines in the developing world. *Trends Parasitol* 21:412-414.
2. Gupta, R.K., J. Alroy, M.J. Alonso, R. Langer, and G.R. Siber. 1997. Chronic local tissue reactions, long-term immunogenicity and immunologic priming of mice and guinea pigs to tetanus toxoid encapsulated in biodegradable polymer microspheres composed of poly lactide-co-glycolide polymers. *Vaccine* 15:1716-1723.
3. Storni, T., C. Ruedl, K. Schwarz, R.A. Schwendener, W.A. Renner, and M.F. Bachmann. 2004. Nonmethylated CG motifs packaged into virus-like particles induce protective cytotoxic T cell responses in the absence of systemic side effects. *J Immunol* 172:1777-1785.
4. Wherry, E.J., J.N. Blattman, K. Murali-Krishna, R. van der Most, and R. Ahmed. 2003. Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. *J Virol* 77:4911-4927.
5. den Boer, A.T., G.J. van Mierlo, M.F. Fransen, C.J. Melief, R. Offringa, and R.E. Toes. 2004. The tumoricidal activity of memory CD8+ T cells is hampered by persistent systemic antigen, but full functional capacity is regained in an antigen-free environment. *J Immunol* 172:6074-6079.
6. MartIn-Fontecha, A., S. Sebastiani, U.E. Hopken, M. Uguccioni, M. Lipp, A. Lanzavecchia, and F. Sallusto. 2003. Regulation of dendritic cell migration to the draining lymph node: impact on T lymphocyte traffic and priming. *J Exp Med* 198:615-621.
7. Schwarz, K., E. Meijerink, D.E. Speiser, A.C. Tissot, I. Cielens, R. Renhof, A. Dishlers, P. Pumpens, and M.F. Bachmann. 2005. Efficient homologous prime-boost strategies for T cell vaccination based on virus-like particles. *Eur J Immunol* 35:816-821.
8. Harrer, E., M. Bauerle, B. Ferstl, P. Chaplin, B. Petzold, L. Mateo, A. Handley, M. Tzatzaris, J. Vollmar, S. Bergmann, M. Rittmaier, K. Eismann, S. Muller, J.R. Kalden, B. Spriewald, D. Willbold, and T. Harrer. 2005. Therapeutic vaccination of HIV-1-infected patients on HAART with a recombinant HIV-1 nef-expressing MVA: safety, immunogenicity and influence on viral load during treatment interruption. *Antivir Ther* 10:285-300.
9. Dreicer, R., W.M. Stadler, F.R. Ahmann, T. Whiteside, N. Bizouarne, B. Acres, J.M. Limacher, P. Squiban, and A. Pantuck. 2009. MVA-MUC1-IL2 vaccine immunotherapy (TG4010) improves PSA doubling time in patients with prostate cancer with biochemical failure. *Invest New Drugs* 27:379-386.
10. Gudmundsdotter, L., C. Nilsson, A. Brave, B. Hejdeman, P. Earl, B. Moss, M. Robb, J. Cox, N. Michael, M. Marovich, G. Biberfeld, E. Sandstrom, and B. Wahren. 2009. Recombinant Modified Vaccinia Ankara (MVA) effectively boosts DNA-primed HIV-specific immune responses in humans despite pre-existing vaccinia immunity. *Vaccine* 27:4468-4474.
11. McShane, H., A.A. Pathan, C.R. Sander, S.M. Keating, S.C. Gilbert, K. Huygen, H.A. Fletcher, and A.V. Hill. 2004. Recombinant modified vaccinia virus Ankara expressing antigen 85A boosts BCG-primed and naturally acquired antimycobacterial immunity in humans. *Nat Med* 10:1240-1244.

12. Goonetilleke, N.P., H. McShane, C.M. Hannan, R.J. Anderson, R.H. Brookes, and A.V. Hill. 2003. Enhanced immunogenicity and protective efficacy against Mycobacterium tuberculosis of bacille Calmette-Guerin vaccine using mucosal administration and boosting with a recombinant modified vaccinia virus Ankara. *J Immunol* 171:1602-1609.
13. Napolitani, G., A. Rinaldi, F. Bertoni, F. Sallusto, and A. Lanzavecchia. 2005. Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells. *Nat Immunol* 6:769-776.
14. Blander, J.M., and R. Medzhitov. 2006. Toll-dependent selection of microbial antigens for presentation by dendritic cells. *Nature* 440:808-812.
15. Schlosser, E., M. Mueller, S. Fischer, S. Basta, D.H. Busch, B. Gander, and M. Groettrup. 2008. TLR ligands and antigen need to be coencapsulated into the same biodegradable microsphere for the generation of potent cytotoxic T lymphocyte responses. *Vaccine* 26:1626-1637.
16. Hoeffel, G., A.C. Ripoche, D. Matheoud, M. Nascimbeni, N. Escriou, P. Lebon, F. Heshmati, J.G. Guillet, M. Gannage, S. Caillat-Zucman, N. Casartelli, O. Schwartz, H. De la Salle, D. Hanau, A. Hosmalin, and C. Maranon. 2007. Antigen crosspresentation by human plasmacytoid dendritic cells. *Immunity* 27:481-492.
17. Kadowaki, N., S. Antonenko, S. Ho, M.C. Rissoan, V. Soumelis, S.A. Porcelli, L.L. Lanier, and Y.J. Liu. 2001. Distinct cytokine profiles of neonatal natural killer T cells after expansion with subsets of dendritic cells. *J Exp Med* 193:1221-1226.
18. Cella, M., M. Salio, Y. Sakakibara, H. Langen, I. Julkunen, and A. Lanzavecchia. 1999. Maturation, activation, and protection of dendritic cells induced by double-stranded RNA. *J Exp Med* 189:821-829.
19. Verdijk, R.M., T. Mutis, B. Esendam, J. Kamp, C.J. Melief, A. Brand, and E. Goulmy. 1999. Polyriboinosinic polyribocytidylic acid (poly(I:C)) induces stable maturation of functionally active human dendritic cells. *J Immunol* 163:57-61.
20. Warger, T., P. Osterloh, G. Rechtsteiner, M. Fassbender, V. Heib, B. Schmid, E. Schmitt, H. Schild, and M.P. Radsak. 2006. Synergistic activation of dendritic cells by combined Toll-like receptor ligation induces superior CTL responses in vivo. *Blood* 108:544-550.
21. Hartmann, G., G.J. Weiner, and A.M. Krieg. 1999. CpG DNA: a potent signal for growth, activation, and maturation of human dendritic cells. *Proc Natl Acad Sci U S A* 96:9305-9310.
22. Hartmann, G., R.D. Weeratna, Z.K. Ballas, P. Payette, S. Blackwell, I. Suparto, W.L. Rasmussen, M. Waldschmidt, D. Sajuthi, R.H. Purcell, H.L. Davis, and A.M. Krieg. 2000. Delineation of a CpG phosphorothioate oligodeoxynucleotide for activating primate immune responses in vitro and in vivo. *J Immunol* 164:1617-1624.
23. Fischer, S., E. Schlosser, M. Mueller, N. Csaba, H.P. Merkle, M. Groettrup, and B. Gander. 2009. Concomitant delivery of a CTL-restricted peptide antigen and CpG ODN by PLGA microparticles induces cellular immune response. *J Drug Target* 17:652-661.
24. Motohashi, S., K. Nagato, N. Kunii, H. Yamamoto, K. Yamasaki, K. Okita, H. Hanaoka, N. Shimizu, M. Suzuki, I. Yoshino, M. Taniguchi, T. Fujisawa, and T. Nakayama. 2009. A phase I-II study of alpha-galactosylceramide-pulsed IL-2/GM-CSF-cultured peripheral blood mononuclear cells in

patients with advanced and recurrent non-small cell lung cancer. *J Immunol* 182:2492-2501.
25. Roy, S., P.F. Barnes, A. Garg, S. Wu, D. Cosman, and R. Vankayalapati. 2008. NK cells lyse T regulatory cells that expand in response to an intracellular pathogen. *J Immunol* 180:1729-1736.
26. Shimizu, K., and S. Fujii. 2009. DC therapy induces long-term NK reactivity to tumors via host DC. *Eur J Immunol* 39:457-468.
27. Fujii, S., K. Shimizu, H. Hemmi, M. Fukui, A.J. Bonito, G. Chen, R.W. Franck, M. Tsuji, and R.M. Steinman. 2006. Glycolipid alpha-C-galactosylceramide is a distinct inducer of dendritic cell function during innate and adaptive immune responses of mice. *Proc Natl Acad Sci U S A* 103:11252-11257.
28. Schmieg, J., G. Yang, R.W. Franck, and M. Tsuji. 2003. Superior protection against malaria and melanoma metastases by a C-glycoside analogue of the natural killer T cell ligand alpha-Galactosylceramide. *J Exp Med* 198:1631-1641.
29. Fujii, S., K. Shimizu, C. Smith, L. Bonifaz, and R.M. Steinman. 2003. Activation of natural killer T cells by alpha-galactosylceramide rapidly induces the full maturation of dendritic cells in vivo and thereby acts as an adjuvant for combined CD4 and CD8 T cell immunity to a coadministered protein. *J Exp Med* 198:267-279.
30. Fujii, S., K. Shimizu, M. Kronenberg, and R.M. Steinman. 2002. Prolonged IFN-gamma-producing NKT response induced with alpha-galactosylceramide-loaded DCs. *Nat Immunol* 3:867-874.
31. Thapa, P., G. Zhang, C. Xia, A. Gelbard, W.W. Overwijk, C. Liu, P. Hwu, D.Z. Chang, A. Courtney, J.K. Sastry, P.G. Wang, C. Li, and D. Zhou. 2009. Nanoparticle formulated alpha-galactosylceramide activates NKT cells without inducing anergy. *Vaccine*.
32. Parekh, V.V., M.T. Wilson, D. Olivares-Villagomez, A.K. Singh, L. Wu, C.R. Wang, S. Joyce, and L. Van Kaer. 2005. Glycolipid antigen induces long-term natural killer T cell anergy in mice. *J Clin Invest* 115:2572-2583.
33. Minami, K., Y. Yanagawa, K. Iwabuchi, N. Shinohara, T. Harabayashi, K. Nonomura, and K. Onoe. 2005. Negative feedback regulation of T helper type 1 (Th1)/Th2 cytokine balance via dendritic cell and natural killer T cell interactions. *Blood* 106:1685-1693.
34. Hochrein, H., M. O'Keeffe, T. Luft, S. Vandenabeele, R.J. Grumont, E. Maraskovsky, and K. Shortman. 2000. Interleukin (IL)-4 is a major regulatory cytokine governing bioactive IL-12 production by mouse and human dendritic cells. *J Exp Med* 192:823-833.

Appendix

I References

Accapezzato, D., *et al.* (2005). "Chloroquine enhances human CD8+ T cell responses against soluble antigens in vivo." J Exp Med 202(6): 817-28.
Akira, S. (2003). "Mammalian Toll-like receptors." Curr Opin Immunol 15(1): 5-11.
Akira, S. (2006). "TLR signaling." Curr Top Microbiol Immunol 311: 1-16.
Amato, R.J., *et al.* (2009). "Phase 2 study of granulocyte-macrophage colony-stimulating factor plus thalidomide in patients with hormone-naive adenocarcinoma of the prostate." Urol Oncol 27(1): 8-13.
Ansell, S.M., *et al.* (2009). "Phase I study of ipilimumab, an anti-CTLA-4 monoclonal antibody, in patients with relapsed and refractory B-cell non-Hodgkin lymphoma." Clin Cancer Res 15(20): 6446-53.
Audran, R., *et al.* (2003). "Encapsulation of peptides in biodegradable microspheres prolongs their MHC class-I presentation by dendritic cells and macrophages in vitro." Vaccine 21(11-12): 1250-5.
Aurisicchio, L., *et al.* (2009). "Treatment of mammary carcinomas in HER-2 transgenic mice through combination of genetic vaccine and an agonist of Toll-like receptor 9." Clin Cancer Res 15(5): 1575-84.
Badiee, A., *et al.* (2007). "Enhanced delivery of immunoliposomes to human dendritic cells by targeting the multilectin receptor DEC-205." Vaccine 25(25): 4757-66.
Banchereau, J. and R.M. Steinman (1998). "Dendritic cells and the control of immunity." Nature 392(6673): 245-52.
Barber, D.L., *et al.* (2003). "Cutting edge: rapid in vivo killing by memory CD8 T cells." J Immunol 171(1): 27-31.
Barral, D.C. and M.B. Brenner (2007). "CD1 antigen presentation: how it works." Nat Rev Immunol 7(12): 929-41.
Basler, M. and M. Groettrup (2007). "Advances in prostate cancer immunotherapies." Drugs Aging 24(3): 197-221.
Beebe, G.W., *et al.* (1972). "Long-term mortality follow-up of Army recruits who received adjuvant influenza virus vaccine in 1951-1953." Am J Epidemiol 95(4): 337-46.
Behar, S.M., *et al.* (1999). "Diverse TCRs recognize murine CD1." J Immunol 162(1): 161-7.
Bendelac, A., *et al.* (2007). "The biology of NKT cells." Annu Rev Immunol 25: 297-336.
Berinstein, N.L. (2007). "Enhancing cancer vaccines with immunomodulators." Vaccine 25 Suppl 2: B72-88.
Billiau, A. and P. Matthys (2001). "Modes of action of Freund's adjuvants in experimental models of autoimmune diseases." J Leukoc Biol 70(6): 849-60.
Blander, J.M. and R. Medzhitov (2004). "Regulation of phagosome maturation by signals from toll-like receptors." Science 304(5673): 1014-8.
Blander, J.M. and R. Medzhitov (2006). "Toll-dependent selection of microbial antigens for presentation by dendritic cells." Nature 440(7085): 808-12.
Blattman, J.N. and P.D. Greenberg (2004). "Cancer immunotherapy: a treatment for the masses." Science 305(5681): 200-5.
Blomberg, K., *et al.* (1996). "Time-resolved fluorometric assay for natural killer activity using target cells labelled with a fluorescence enhancing ligand." J Immunol Methods 193(2): 199-206.

Bonifaz, L.*, et al.* (2002). "Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral CD8+ T cell tolerance." J Exp Med 196(12): 1627-38.

Borg, N.A.*, et al.* (2007). "CD1d-lipid-antigen recognition by the semi-invariant NKT T-cell receptor." Nature 448(7149): 44-9.

Bourquin, C.*, et al.* (2007). "Immunostimulatory RNA oligonucleotides trigger an antigen-specific cytotoxic T-cell and IgG2a response." Blood 109(7): 2953-60.

Breslow, N.*, et al.* (1977). "Latent carcinoma of prostate at autopsy in seven areas. The International Agency for Research on Cancer, Lyons, France." Int J Cancer 20(5): 680-8.

Brutkiewicz, R.R. (2006). "CD1d ligands: the good, the bad, and the ugly." J Immunol 177(2): 769-75.

Carbone, F.R. and M.J. Bevan (1990). "Class I-restricted processing and presentation of exogenous cell-associated antigen in vivo." J Exp Med 171(2): 377-87.

Carroll, R.G. and C.H. June (2007). "Programming the next generation of dendritic cells." Mol Ther 15(5): 846-8.

Cella, M.*, et al.* (1999). "Maturation, activation, and protection of dendritic cells induced by double-stranded RNA." J Exp Med 189(5): 821-9.

Celluzzi, C.M.*, et al.* (1996). "Peptide-pulsed dendritic cells induce antigen-specific CTL-mediated protective tumor immunity." J Exp Med 183(1): 283-7.

Cerundolo, V.*, et al.* (2004). "Dendritic cells: a journey from laboratory to clinic." Nat Immunol 5(1): 7-10.

Chang, Y.J.*, et al.* (2007). "Potent immune-modulating and anticancer effects of NKT cell stimulatory glycolipids." Proc Natl Acad Sci U S A 104(25): 10299-304.

Chen, Y.G.*, et al.* (2005). "Activated NKT cells inhibit autoimmune diabetes through tolerogenic recruitment of dendritic cells to pancreatic lymph nodes." J Immunol 174(3): 1196-204.

Chiba, A.*, et al.* (2004). "Suppression of collagen-induced arthritis by natural killer T cell activation with OCH, a sphingosine-truncated analog of alpha-galactosylceramide." Arthritis Rheum 50(1): 305-13.

Cho, H.C.*, et al.* (2008). "Cancer immunotherapeutic effects of novel CpG ODN in murine tumor model." Int Immunopharmacol 8(10): 1401-7.

Chu, R.S.*, et al.* (1997). "CpG oligodeoxynucleotides act as adjuvants that switch on T helper 1 (Th1) immunity." J Exp Med 186(10): 1623-31.

Copier, J.*, et al.* (2007). "Cell based cancer vaccines: regulatory and commercial development." Vaccine 25 Suppl 2: B35-46.

Coppieters, K.*, et al.* (2007). "A single early activation of invariant NK T cells confers long-term protection against collagen-induced arthritis in a ligand-specific manner." J Immunol 179(4): 2300-9.

Coquet, J.M.*, et al.* (2007). "IL-21 is produced by NKT cells and modulates NKT cell activation and cytokine production." J Immunol 178(5): 2827-34.

Degl'Innocenti, E.*, et al.* (2005). "Peripheral T cell tolerance occurs early during spontaneous prostate cancer development and can be rescued by dendritic cell immunization." Eur J Immunol 35(1): 66-75.

Delamarre, L.*, et al.* (2003). "Presentation of exogenous antigens on major histocompatibility complex (MHC) class I and MHC class II molecules is

differentially regulated during dendritic cell maturation." J Exp Med 198(1): 111-22.

den Boer, A.T., *et al.* (2004). "The tumoricidal activity of memory CD8+ T cells is hampered by persistent systemic antigen, but full functional capacity is regained in an antigen-free environment." J Immunol 172(10): 6074-9.

Dillman, R.O., *et al.* (2009). "Phase II trial of dendritic cells loaded with antigens from self-renewing, proliferating autologous tumor cells as patient-specific antitumor vaccines in patients with metastatic melanoma: final report." Cancer Biother Radiopharm 24(3): 311-9.

Doxsee, C.L., *et al.* (2003). "The immune response modifier and Toll-like receptor 7 agonist S-27609 selectively induces IL-12 and TNF-alpha production in CD11c+CD11b+CD8- dendritic cells." J Immunol 171(3): 1156-63.

Dreicer, R., *et al.* (2009). "MVA-MUC1-IL2 vaccine immunotherapy (TG4010) improves PSA doubling time in patients with prostate cancer with biochemical failure." Invest New Drugs 27(4): 379-86.

Dudziak, D., *et al.* (2007). "Differential antigen processing by dendritic cell subsets in vivo." Science 315(5808): 107-11.

Fais, F., *et al.* (2004). "CD1d is expressed on B-chronic lymphocytic leukemia cells and mediates alpha-galactosylceramide presentation to natural killer T lymphocytes." Int J Cancer 109(3): 402-11.

Fiedler, T., *et al.* (2002). "Regulation of CD1d expression by murine tumor cells: escape from immunosurveillance or alternate target molecules?" Int J Cancer 98(3): 389-97.

Figueroa-Perez, S. and R.R. Schmidt (2000). "Total synthesis of alpha-galactosyl cerebroside." Carbohydr Res 328(2): 95-102.

Fischer, S., *et al.* (2009). "Concomitant delivery of a CTL-restricted peptide antigen and CpG ODN by PLGA microparticles induces cellular immune response." J Drug Target 17(8): 652-61.

Fontana, R., *et al.* (2009). "Peripheral blood lymphocytes genetically modified to express the self/tumor antigen MAGE-A3 induce antitumor immune responses in cancer patients." Blood 113(8): 1651-60.

Forster, R., *et al.* (1999). "CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs." Cell 99(1): 23-33.

Freund, J. (1951). "The effect of paraffin oil and mycobacteria on antibody formation and sensitization; a review." Am J Clin Pathol 21(7): 645-56.

Fuji, N., *et al.* (2000). "Antitumor effect of alpha-galactosylceramide (KRN7000) on spontaneous hepatic metastases requires endogenous interleukin 12 in the liver." Clin Cancer Res 6(8): 3380-7.

Fujii, S., *et al.* (2004). "The linkage of innate to adaptive immunity via maturing dendritic cells in vivo requires CD40 ligation in addition to antigen presentation and CD80/86 costimulation." J Exp Med 199(12): 1607-18.

Fujii, S., *et al.* (2006). "Glycolipid alpha-C-galactosylceramide is a distinct inducer of dendritic cell function during innate and adaptive immune responses of mice." Proc Natl Acad Sci U S A 103(30): 11252-7.

Fujii, S., *et al.* (2002). "Prolonged IFN-gamma-producing NKT response induced with alpha-galactosylceramide-loaded DCs." Nat Immunol 3(9): 867-74.

Fujii, S., *et al.* (2003). "Activation of natural killer T cells by alpha-galactosylceramide rapidly induces the full maturation of dendritic cells in vivo and thereby acts as an adjuvant for combined CD4 and CD8 T cell immunity to a coadministered protein." J Exp Med 198(2): 267-79.

Fukushima, S., *et al.* (2009). "Multiple antigen-targeted immunotherapy with alpha-galactosylceramide-loaded and genetically engineered dendritic cells derived from embryonic stem cells." J Immunother 32(3): 219-31.

Gander, B. (2005). "Trends in particulate antigen and DNA delivery systems for vaccines." Adv Drug Deliv Rev 57(3): 321-3.

Godfrey, D.I. and M. Kronenberg (2004). "Going both ways: immune regulation via CD1d-dependent NKT cells." J Clin Invest 114(10): 1379-88.

Goforth, R., *et al.* (2009). "Immune stimulatory antigen loaded particles combined with depletion of regulatory T-cells induce potent tumor specific immunity in a mouse model of melanoma." Cancer Immunol Immunother 58(4): 517-30.

Gonzalez-Aseguinolaza, G., *et al.* (2002). "Natural killer T cell ligand alpha-galactosylceramide enhances protective immunity induced by malaria vaccines." J Exp Med 195(5): 617-24.

Goonetilleke, N.P., *et al.* (2003). "Enhanced immunogenicity and protective efficacy against Mycobacterium tuberculosis of bacille Calmette-Guerin vaccine using mucosal administration and boosting with a recombinant modified vaccinia virus Ankara." J Immunol 171(3): 1602-9.

Gorbachev, A.V. and R.L. Fairchild (2001). "Regulatory role of CD4+ T cells during the development of contact hypersensitivity responses." Immunol Res 24(1): 69-77.

Grajewski, R.S., *et al.* (2008). "Activation of invariant NKT cells ameliorates experimental ocular autoimmunity by a mechanism involving innate IFN-gamma production and dampening of the adaptive Th1 and Th17 responses." J Immunol 181(7): 4791-7.

Greenberg, N.M., *et al.* (1995). "Prostate cancer in a transgenic mouse." Proc Natl Acad Sci U S A 92(8): 3439-43.

Grossmann, M.E., *et al.* (2001). "Avoiding tolerance against prostatic antigens with subdominant peptide epitopes." J Immunother 24(3): 237-41.

Gudmundsdotter, L., *et al.* (2009). "Recombinant Modified Vaccinia Ankara (MVA) effectively boosts DNA-primed HIV-specific immune responses in humans despite pre-existing vaccinia immunity." Vaccine 27(33): 4468-74.

Guermonprez, P. and S. Amigorena (2005). "Pathways for antigen cross presentation." Springer Semin Immunopathol 26(3): 257-71.

Guillonneau, C., *et al.* (2009). "Combined NKT cell activation and influenza virus vaccination boosts memory CTL generation and protective immunity." Proc Natl Acad Sci U S A 106(9): 3330-5.

Gumperz, J.E., *et al.* (2002). "Functionally distinct subsets of CD1d-restricted natural killer T cells revealed by CD1d tetramer staining." J Exp Med 195(5): 625-36.

Gunn, M.D., *et al.* (1999). "Mice lacking expression of secondary lymphoid organ chemokine have defects in lymphocyte homing and dendritic cell localization." J Exp Med 189(3): 451-60.

Gupta, R.K., *et al.* (1997). "Chronic local tissue reactions, long-term immunogenicity and immunologic priming of mice and guinea pigs to tetanus toxoid encapsulated in biodegradable polymer microspheres composed of poly lactide-co-glycolide polymers." Vaccine 15(16): 1716-23.

Habjanec, L., *et al.* (2008). "Immunomodulatory activity of novel adjuvant formulations based on Montanide ISA oil-based adjuvants and peptidoglycan monomer." Int Immunopharmacol 8(5): 717-24.

Hamdy, S., *et al.* (2007). "Enhanced antigen-specific primary CD4+ and CD8+ responses by codelivery of ovalbumin and toll-like receptor ligand

monophosphoryl lipid A in poly(D,L-lactic-co-glycolic acid) nanoparticles." J Biomed Mater Res A 81(3): 652-62.

Hamdy, S.*, et al.* (2008). "Co-delivery of cancer-associated antigen and Toll-like receptor 4 ligand in PLGA nanoparticles induces potent CD8+ T cell-mediated anti-tumor immunity." Vaccine 26(39): 5046-57.

Harrer, E.*, et al.* (2005). "Therapeutic vaccination of HIV-1-infected patients on HAART with a recombinant HIV-1 nef-expressing MVA: safety, immunogenicity and influence on viral load during treatment interruption." Antivir Ther 10(2): 285-300.

Hartmann, G.*, et al.* (2000). "Delineation of a CpG phosphorothioate oligodeoxynucleotide for activating primate immune responses in vitro and in vivo." J Immunol 164(3): 1617-24.

Hartmann, G.*, et al.* (1999). "CpG DNA: a potent signal for growth, activation, and maturation of human dendritic cells." Proc Natl Acad Sci U S A 96(16): 9305-10.

Heath, W.R. and F.R. Carbone (2001). "Cross-presentation in viral immunity and self-tolerance." Nat Rev Immunol 1(2): 126-34.

Heit, A.*, et al.* (2007). "Antigen co-encapsulated with adjuvants efficiently drive protective T cell immunity." Eur J Immunol 37(8): 2063-74.

Heit, A.*, et al.* (2005). "Protective CD8 T cell immunity triggered by CpG-protein conjugates competes with the efficacy of live vaccines." J Immunol 174(7): 4373-80.

Hermans, I.F.*, et al.* (2007). "Dendritic cell function can be modulated through cooperative actions of TLR ligands and invariant NKT cells." J Immunol 178(5): 2721-9.

Hermans, I.F.*, et al.* (2003). "NKT cells enhance CD4+ and CD8+ T cell responses to soluble antigen in vivo through direct interaction with dendritic cells." J Immunol 171(10): 5140-7.

Herrero-Vanrell, R.*, et al.* (2000). "Biodegradable PLGA microspheres loaded with ganciclovir for intraocular administration. Encapsulation technique, in vitro release profiles, and sterilization process." Pharm Res 17(10): 1323-8.

Hochrein, H.*, et al.* (2000). "Interleukin (IL)-4 is a major regulatory cytokine governing bioactive IL-12 production by mouse and human dendritic cells." J Exp Med 192(6): 823-33.

Hoeffel, G.*, et al.* (2007). "Antigen crosspresentation by human plasmacytoid dendritic cells." Immunity 27(3): 481-92.

Hsu, F.J.*, et al.* (1996). "Vaccination of patients with B-cell lymphoma using autologous antigen-pulsed dendritic cells." Nat Med 2(1): 52-8.

Huang, A.Y.*, et al.* (1994). "Role of bone marrow-derived cells in presenting MHC class I-restricted tumor antigens." Science 264(5161): 961-5.

Iezzi, G.*, et al.* (1998). "The duration of antigenic stimulation determines the fate of naive and effector T cells." Immunity 8(1): 89-95.

Im, J.S.*, et al.* (2006). "Expression of CD1d molecules by human schwann cells and potential interactions with immunoregulatory invariant NK T cells." J Immunol 177(8): 5226-35.

Ioannou, X.P.*, et al.* (2002). "CpG-containing oligodeoxynucleotides, in combination with conventional adjuvants, enhance the magnitude and change the bias of the immune responses to a herpesvirus glycoprotein." Vaccine 21(1-2): 127-37.

Ishikawa, A., *et al.* (2005). "A phase I study of alpha-galactosylceramide (KRN7000)-pulsed dendritic cells in patients with advanced and recurrent non-small cell lung cancer." Clin Cancer Res 11(5): 1910-7.

Jahng, A., *et al.* (2004). "Prevention of autoimmunity by targeting a distinct, noninvariant CD1d-reactive T cell population reactive to sulfatide." J Exp Med 199(7): 947-57.

Jahng, A.W., *et al.* (2001). "Activation of natural killer T cells potentiates or prevents experimental autoimmune encephalomyelitis." J Exp Med 194(12): 1789-99.

Jain, R.A. (2000). "The manufacturing techniques of various drug loaded biodegradable poly(lactide-co-glycolide) (PLGA) devices." Biomaterials 21(23): 2475-90.

Janeway, C.A., Jr. (1989). "Approaching the asymptote? Evolution and revolution in immunology." Cold Spring Harb Symp Quant Biol 54 Pt 1: 1-13.

Jegerlehner, A., *et al.* (2007). "TLR9 signaling in B cells determines class switch recombination to IgG2a." J Immunol 178(4): 2415-20.

Jiang, S., *et al.* (2005). "Activated CD1d-restricted natural killer T cells secrete IL-2: innate help for CD4+CD25+ regulatory T cells?" Eur J Immunol 35(4): 1193-200.

Jiang, W., *et al.* (2005). "Biodegradable poly(lactic-co-glycolic acid) microparticles for injectable delivery of vaccine antigens." Adv Drug Deliv Rev 57(3): 391-410.

Johansen, P., *et al.* (2000). "Revisiting PLA/PLGA microspheres: an analysis of their potential in parenteral vaccination." Eur J Pharm Biopharm 50(1): 129-46.

Johnston, D. and J.C. Bystryn (2006). "Topical imiquimod is a potent adjuvant to a weakly-immunogenic protein prototype vaccine." Vaccine 24(11): 1958-65.

June, C.H. (2007). "Adoptive T cell therapy for cancer in the clinic." J Clin Invest 117(6): 1466-76.

Kadowaki, N., *et al.* (2001). "Distinct cytokine profiles of neonatal natural killer T cells after expansion with subsets of dendritic cells." J Exp Med 193(10): 1221-6.

Kaparakis, M., *et al.* (2007). "Mammalian NLR proteins; discriminating foe from friend." Immunol Cell Biol 85(6): 495-502.

Kawano, T., *et al.* (1997). "CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides." Science 278(5343): 1626-9.

Kedl, R.M., *et al.* (2003). "Epitope dominance, competition and T cell affinity maturation." Curr Opin Immunol 15(1): 120-7.

Kim, H.Y., *et al.* (2005). "NKT cells promote antibody-induced joint inflammation by suppressing transforming growth factor beta1 production." J Exp Med 201(1): 41-7.

Kim, S., *et al.* (2009). "Vaccination with recombinant adenoviruses and dendritic cells expressing prostate-specific antigens is effective in eliciting CTL and suppresses tumor growth in the experimental prostate cancer." Prostate 69(9): 938-48.

Knutson, K.L., *et al.* (2006). "IL-2 immunotoxin therapy modulates tumor-associated regulatory T cells and leads to lasting immune-mediated rejection of breast cancers in neu-transgenic mice." J Immunol 177(1): 84-91.

Kobayashi, E., *et al.* (1995). "KRN7000, a novel immunomodulator, and its antitumor activities." Oncol Res 7(10-11): 529-34.

Koch, M., *et al.* (2005). "The crystal structure of human CD1d with and without alpha-galactosylceramide." Nat Immunol 6(8): 819-26.

Kopecky-Bromberg, S.A., *et al.* (2009). "Alpha-C-galactosylceramide as an adjuvant for a live attenuated influenza virus vaccine." Vaccine 27(28): 3766-74.

Krishnamachari, Y. and A.K. Salem (2009). "Innovative strategies for co-delivering antigens and CpG oligonucleotides." Adv Drug Deliv Rev 61(3): 205-17.

Kronenberg, M. (2005). "Toward an understanding of NKT cell biology: progress and paradoxes." Annu Rev Immunol 23: 877-900.

Kronig, H., *et al.* (2009). "Allorestricted T lymphocytes with a high avidity T-cell receptor towards NY-ESO-1 have potent anti-tumor activity." Int J Cancer 125(3): 649-55.

Kubach, J., *et al.* (2005). "Dendritic cells: sentinels of immunity and tolerance." Int J Hematol 81(3): 197-203.

Lanzavecchia, A. (1996). "Mechanisms of antigen uptake for presentation." Curr Opin Immunol 8(3): 348-54.

Leite-de-Moraes, M.C., *et al.* (2002). "Ligand-activated natural killer T lymphocytes promptly produce IL-3 and GM-CSF in vivo: relevance to peripheral myeloid recruitment." Eur J Immunol 32(7): 1897-904.

Liang, P.H., *et al.* (2008). "Quantitative microarray analysis of intact glycolipid-CD1d interaction and correlation with cell-based cytokine production." J Am Chem Soc 130(37): 12348-54.

Liu, K., *et al.* (2005). "Innate NKT lymphocytes confer superior adaptive immunity via tumor-capturing dendritic cells." J Exp Med 202(11): 1507-16.

Liu, Y.J. (2001). "Dendritic cell subsets and lineages, and their functions in innate and adaptive immunity." Cell 106(3): 259-62.

Lu, S. (2009). "Heterologous prime-boost vaccination." Curr Opin Immunol 21(3): 346-51.

Ludewig, B., *et al.* (2001). "Rapid peptide turnover and inefficient presentation of exogenous antigen critically limit the activation of self-reactive CTL by dendritic cells." J Immunol 166(6): 3678-87.

Maeurer, M.J., *et al.* (1996). "Tumor escape from immune recognition: lethal recurrent melanoma in a patient associated with downregulation of the peptide transporter protein TAP-1 and loss of expression of the immunodominant MART-1/Melan-A antigen." J Clin Invest 98(7): 1633-41.

Malyala, P., *et al.* (2009). "Enhancing the therapeutic efficacy of CpG oligonucleotides using biodegradable microparticles." Adv Drug Deliv Rev 61(3): 218-25.

Mann, J.F., *et al.* (2009). "Lipid vesicle size of an oral influenza vaccine delivery vehicle influences the Th1/Th2 bias in the immune response and protection against infection." Vaccine 27(27): 3643-9.

Manolova, V., *et al.* (2008). "Nanoparticles target distinct dendritic cell populations according to their size." Eur J Immunol 38(5): 1404-13.

MartIn-Fontecha, A., *et al.* (2003). "Regulation of dendritic cell migration to the draining lymph node: impact on T lymphocyte traffic and priming." J Exp Med 198(4): 615-21.

Mateo, L., *et al.* (1999). "An HLA-A2 polyepitope vaccine for melanoma immunotherapy." J Immunol 163(7): 4058-63.

Matsuda, J.L.*, et al.* (2008). "CD1d-restricted iNKT cells, the 'Swiss-Army knife' of the immune system." Curr Opin Immunol 20(3): 358-68.

Matsumoto, M. and T. Seya (2008). "TLR3: interferon induction by double-stranded RNA including poly(I:C)." Adv Drug Deliv Rev 60(7): 805-12.

McBride, S.*, et al.* (2006). "Cell-associated double-stranded RNA enhances antitumor activity through the production of type I IFN." J Immunol 177(9): 6122-8.

McCarthy, C.*, et al.* (2007). "The length of lipids bound to human CD1d molecules modulates the affinity of NKT cell TCR and the threshold of NKT cell activation." J Exp Med 204(5): 1131-44.

McShane, H.*, et al.* (2004). "Recombinant modified vaccinia virus Ankara expressing antigen 85A boosts BCG-primed and naturally acquired antimycobacterial immunity in humans." Nat Med 10(11): 1240-4.

Mellman, I. and R.M. Steinman (2001). "Dendritic cells: specialized and regulated antigen processing machines." Cell 106(3): 255-8.

Men, Y.*, et al.* (1999). "MHC class I- and class II-restricted processing and presentation of microencapsulated antigens." Vaccine 17(9-10): 1047-56.

Men, Y.*, et al.* (1996). "Induction of sustained and elevated immune responses to weakly immunogenic synthetic malarial peptides by encapsulation in biodegradable polymer microspheres." Vaccine 14(15): 1442-50.

Men, Y.*, et al.* (1995). "A single administration of tetanus toxoid in biodegradable microspheres elicits T cell and antibody responses similar or superior to those obtained with aluminum hydroxide." Vaccine 13(7): 683-9.

Miconnet, I.*, et al.* (2002). "CpG are efficient adjuvants for specific CTL induction against tumor antigen-derived peptide." J Immunol 168(3): 1212-8.

Miller, L.H.*, et al.* (2005). "Revisiting Freund's incomplete adjuvant for vaccines in the developing world." Trends Parasitol 21(9): 412-4.

Minami, K.*, et al.* (2005). "Negative feedback regulation of T helper type 1 (Th1)/Th2 cytokine balance via dendritic cell and natural killer T cell interactions." Blood 106(5): 1685-93.

Mitchell, M.S.*, et al.* (1988). "Active specific immunotherapy for melanoma: phase I trial of allogeneic lysates and a novel adjuvant." Cancer Res 48(20): 5883-93.

Miyamoto, K.*, et al.* (2001). "A synthetic glycolipid prevents autoimmune encephalomyelitis by inducing Th2 bias of natural killer T cells." Nature 413(6855): 531-4.

Mizuno, M.*, et al.* (2004). "Synthetic glycolipid OCH prevents insulitis and diabetes in NOD mice." J Autoimmun 23(4): 293-300.

Montanari, L.*, et al.* (2003). "Poly(lactide-co-glycolide) microspheres containing bupivacaine: comparison between gamma and beta irradiation effects." J Control Release 90(3): 281-90.

Morita, M.*, et al.* (1995). "Structure-activity relationship of alpha-galactosylceramides against B16-bearing mice." J Med Chem 38(12): 2176-87.

Moss, R.B.*, et al.* (2000). "In vitro immune function after vaccination with an inactivated, gp120-depleted HIV-1 antigen with immunostimulatory oligodeoxynucleotides." Vaccine 18(11-12): 1081-7.

Motohashi, S.*, et al.* (2009). "A phase I-II study of alpha-galactosylceramide-pulsed IL-2/GM-CSF-cultured peripheral blood mononuclear cells in patients with advanced and recurrent non-small cell lung cancer." J Immunol 182(4): 2492-501.

Mumprecht, S., *et al.* (2009). "Programmed death 1 signaling on chronic myeloid leukemia-specific T cells results in T-cell exhaustion and disease progression." Blood 114(8): 1528-36.
Mutwiri, G.K., *et al.* (2004). "Strategies for enhancing the immunostimulatory effects of CpG oligodeoxynucleotides." J Control Release 97(1): 1-17.
Nakano, H., *et al.* (2009). "Blood-derived inflammatory dendritic cells in lymph nodes stimulate acute T helper type 1 immune responses." Nat Immunol 10(4): 394-402.
Napolitani, G., *et al.* (2005). "Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells." Nat Immunol 6(8): 769-76.
Naumov, Y.N., *et al.* (2001). "Activation of CD1d-restricted T cells protects NOD mice from developing diabetes by regulating dendritic cell subsets." Proc Natl Acad Sci U S A 98(24): 13838-43.
Navabi, H., *et al.* (2009). "A clinical grade poly I:C-analogue (Ampligen) promotes optimal DC maturation and Th1-type T cell responses of healthy donors and cancer patients in vitro." Vaccine 27(1): 107-15.
Nelson, D., *et al.* (2000). "In vivo cross-presentation of a soluble protein antigen: kinetics, distribution, and generation of effector CTL recognizing dominant and subdominant epitopes." J Immunol 165(11): 6123-32.
Nestle, F.O. (2002). "Dendritic cell vaccination for the treatment of skin cancer." Recent Results Cancer Res 160: 165-9.
Nestle, F.O., *et al.* (1998). "Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells." Nat Med 4(3): 328-32.
Newman, K.D., *et al.* (2002). "Uptake of poly(D,L-lactic-co-glycolic acid) microspheres by antigen-presenting cells in vivo." J Biomed Mater Res 60(3): 480-6.
Numata, Y., *et al.* (2005). "Therapeutic effect of repeated natural killer T cell stimulation in mouse cholangitis complicated by colitis." Dig Dis Sci 50(10): 1844-51.
Obst, R., *et al.* (2007). "Sustained antigen presentation can promote an immunogenic T cell response, like dendritic cell activation." Proc Natl Acad Sci U S A 104(39): 15460-5.
Ohlschlager, P., *et al.* (2009). "Enhancement of immunogenicity of a therapeutic cervical cancer DNA-based vaccine by co-application of sequence-optimized genetic adjuvants." Int J Cancer 125(1): 189-98.
Pal, E., *et al.* (2001). "Costimulation-dependent modulation of experimental autoimmune encephalomyelitis by ligand stimulation of V alpha 14 NK T cells." J Immunol 166(1): 662-8.
Panyam, J., *et al.* (2002). "Rapid endo-lysosomal escape of poly(DL-lactide-co-glycolide) nanoparticles: implications for drug and gene delivery." Faseb J 16(10): 1217-26.
Parekh, V.V., *et al.* (2005). "Glycolipid antigen induces long-term natural killer T cell anergy in mice." J Clin Invest 115(9): 2572-83.
Peter, K., *et al.* (2001). "Induction of a cytotoxic T-cell response to HIV-1 proteins with short synthetic peptides and human compatible adjuvants." Vaccine 19(30): 4121-9.
Porcelli, S., *et al.* (1993). "Analysis of T cell antigen receptor (TCR) expression by human peripheral blood CD4-8- alpha/beta T cells demonstrates preferential use of several V beta genes and an invariant TCR alpha chain." J Exp Med 178(1): 1-16.

Probst, H.C., *et al.* (2005). "Resting dendritic cells induce peripheral CD8+ T cell tolerance through PD-1 and CTLA-4." Nat Immunol 6(3): 280-6.
Proudfoot, O., *et al.* (2007). "Receptor-mediated delivery of antigens to dendritic cells: anticancer applications." Mol Pharm 4(1): 58-72.
Qu, C., *et al.* (2009). "MHC class I/peptide transfer between dendritic cells overcomes poor cross-presentation by monocyte-derived APCs that engulf dying cells." J Immunol 182(6): 3650-9.
Ramirez, M.C. and L.J. Sigal (2002). "Macrophages and dendritic cells use the cytosolic pathway to rapidly cross-present antigen from live, vaccinia-infected cells." J Immunol 169(12): 6733-42.
Reddy, S.T., *et al.* (2006). "Targeting dendritic cells with biomaterials: developing the next generation of vaccines." Trends Immunol 27(12): 573-9.
Reis e Sousa, C. (2001). "Dendritic cells as sensors of infection." Immunity 14(5): 495-8.
Rock, K.L. (1996). "A new foreign policy: MHC class I molecules monitor the outside world." Immunol Today 17(3): 131-7.
Rock, K.L. and L. Shen (2005). "Cross-presentation: underlying mechanisms and role in immune surveillance." Immunol Rev 207: 166-83.
Ronet, C., *et al.* (2005). "NKT cells are critical for the initiation of an inflammatory bowel response against Toxoplasma gondii." J Immunol 175(2): 899-908.
Roy, S., *et al.* (2008). "NK cells lyse T regulatory cells that expand in response to an intracellular pathogen." J Immunol 180(3): 1729-36.
Sakuishi, K., *et al.* (2007). "Invariant NKT cells biased for IL-5 production act as crucial regulators of inflammation." J Immunol 179(6): 3452-62.
Sallusto, F., *et al.* (1998). "Rapid and coordinated switch in chemokine receptor expression during dendritic cell maturation." Eur J Immunol 28(9): 2760-9.
Schakel, K. (2009). "Dendritic cells--why can they help and hurt us." Exp Dermatol 18(3): 264-73.
Schlosser, E., *et al.* (2008). "TLR ligands and antigen need to be coencapsulated into the same biodegradable microsphere for the generation of potent cytotoxic T lymphocyte responses." Vaccine 26(13): 1626-37.
Schmieg, J., *et al.* "A multifactorial mechanism in the superior antimalarial activity of alpha-C-GalCer." J Biomed Biotechnol 2010: 283612.
Schmieg, J., *et al.* (2003). "Superior protection against malaria and melanoma metastases by a C-glycoside analogue of the natural killer T cell ligand alpha-Galactosylceramide." J Exp Med 198(11): 1631-41.
Schoenberger, S.P., *et al.* (1998). "T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions." Nature 393(6684): 480-3.
Schulz, O., *et al.* (2005). "Toll-like receptor 3 promotes cross-priming to virus-infected cells." Nature 433(7028): 887-92.
Schwarz, K., *et al.* (2005). "Efficient homologous prime-boost strategies for T cell vaccination based on virus-like particles." Eur J Immunol 35(3): 816-21.
Schwarz, K., *et al.* (2000). "Overexpression of the proteasome subunits LMP2, LMP7, and MECL-1, but not PA28 alpha/beta, enhances the presentation of an immunodominant lymphocytic choriomeningitis virus T cell epitope." J Immunol 165(2): 768-78.
Shafer-Weaver, K.A., *et al.* (2009). "Immunity to murine prostatic tumors: continuous provision of T-cell help prevents CD8 T-cell tolerance and activates tumor-infiltrating dendritic cells." Cancer Res 69(15): 6256-64.
Shappell, S.B., *et al.* (2004). "Prostate pathology of genetically engineered mice: definitions and classification. The consensus report from the Bar Harbor

meeting of the Mouse Models of Human Cancer Consortium Prostate Pathology Committee." Cancer Res 64(6): 2270-305.

Shen, Z., *et al.* (1997). "Cloned dendritic cells can present exogenous antigens on both MHC class I and class II molecules." J Immunol 158(6): 2723-30.

Shephard, E., *et al.* (2008). "A multigene HIV type 1 subtype C modified vaccinia Ankara (MVA) vaccine efficiently boosts immune responses to a DNA vaccine in mice." AIDS Res Hum Retroviruses 24(2): 207-17.

Shimizu, K. and S. Fujii (2009). "DC therapy induces long-term NK reactivity to tumors via host DC." Eur J Immunol 39(2): 457-68.

Silk, J.D., *et al.* (2004). "Utilizing the adjuvant properties of CD1d-dependent NK T cells in T cell-mediated immunotherapy." J Clin Invest 114(12): 1800-11.

Silk, J.D., *et al.* (2008). "Structural and functional aspects of lipid binding by CD1 molecules." Annu Rev Cell Dev Biol 24: 369-95.

Simpson-Abelson, M.R., *et al.* (2009). "IL-12 delivered intratumorally by multilamellar liposomes reactivates memory T cells in human tumor microenvironments." Clin Immunol 132(1): 71-82.

Singh, A.K., *et al.* (2001). "Natural killer T cell activation protects mice against experimental autoimmune encephalomyelitis." J Exp Med 194(12): 1801-11.

Singh, M., *et al.* (1997). "Immunogenicity and protection in small-animal models with controlled-release tetanus toxoid microparticles as a single-dose vaccine." Infect Immun 65(5): 1716-21.

Singh, N., *et al.* (1999). "Cutting edge: activation of NK T cells by CD1d and alpha-galactosylceramide directs conventional T cells to the acquisition of a Th2 phenotype." J Immunol 163(5): 2373-7.

Slingluff, C.L., Jr., *et al.* (2000). "Melanomas with concordant loss of multiple melanocytic differentiation proteins: immune escape that may be overcome by targeting unique or undefined antigens." Cancer Immunol Immunother 48(12): 661-72.

Solbrig, C.M., *et al.* (2007). "Polymer nanoparticles for immunotherapy from encapsulated tumor-associated antigens and whole tumor cells." Mol Pharm 4(1): 47-57.

Sondak, V.K. and J.A. Sosman (2003). "Results of clinical trials with an allogenic melanoma tumor cell lysate vaccine: Melacine." Semin Cancer Biol 13(6): 409-15.

Sosman, J.A. and V.K. Sondak (2003). "Melacine: an allogeneic melanoma tumor cell lysate vaccine." Expert Rev Vaccines 2(3): 353-68.

Sozzani, S., *et al.* (1998). "Differential regulation of chemokine receptors during dendritic cell maturation: a model for their trafficking properties." J Immunol 161(3): 1083-6.

Spada, F.M., *et al.* (1998). "CD1d-restricted recognition of synthetic glycolipid antigens by human natural killer T cells." J Exp Med 188(8): 1529-34.

Sparwasser, T., *et al.* (2000). "Bacterial CpG-DNA activates dendritic cells in vivo: T helper cell-independent cytotoxic T cell responses to soluble proteins." Eur J Immunol 30(12): 3591-7.

Speiser, D.E., *et al.* (2005). "Rapid and strong human CD8+ T cell responses to vaccination with peptide, IFA, and CpG oligodeoxynucleotide 7909." J Clin Invest 115(3): 739-46.

Steinman, R.M. and Z.A. Cohn (1973). "Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution." J Exp Med 137(5): 1142-62.

Steinman, R.M., *et al.* (2003). "Tolerogenic dendritic cells." Annu Rev Immunol 21: 685-711.

Storni, T. and M.F. Bachmann (2004). "Loading of MHC class I and II presentation pathways by exogenous antigens: a quantitative in vivo comparison." J Immunol 172(10): 6129-35.

Storni, T., *et al.* (2004). "Nonmethylated CG motifs packaged into virus-like particles induce protective cytotoxic T cell responses in the absence of systemic side effects." J Immunol 172(3): 1777-85.

Susumu, S., *et al.* (2008). "Cross-presentation of NY-ESO-1 cytotoxic T lymphocyte epitope fused to human heat shock cognate protein 70 by dendritic cells." Cancer Sci 99(1): 107-12.

Taniguchi, M., *et al.* (2003). "The regulatory role of Valpha14 NKT cells in innate and acquired immune response." Annu Rev Immunol 21: 483-513.

Thapa, P., *et al.* (2009). "Nanoparticle formulated alpha-galactosylceramide activates NKT cells without inducing anergy." Vaccine.

Timmerman, J.M., *et al.* (2002). "Idiotype-pulsed dendritic cell vaccination for B-cell lymphoma: clinical and immune responses in 35 patients." Blood 99(5): 1517-26.

Toledo, H., *et al.* (2001). "A phase I clinical trial of a multi-epitope polypeptide TAB9 combined with Montanide ISA 720 adjuvant in non-HIV-1 infected human volunteers." Vaccine 19(30): 4328-36.

Ueno, Y., *et al.* (2005). "Single dose of OCH improves mucosal T helper type 1/T helper type 2 cytokine balance and prevents experimental colitis in the presence of valpha14 natural killer T cells in mice." Inflamm Bowel Dis 11(1): 35-41.

van der Vliet, H.J., *et al.* (2003). "Polarization of Valpha24+ Vbeta11+ natural killer T cells of healthy volunteers and cancer patients using alpha-galactosylceramide-loaded and environmentally instructed dendritic cells." Cancer Res 63(14): 4101-6.

Velmourougane, G., *et al.* (2009). "Synthesis and evaluation of an acyl-chain unsaturated analog of the Th2 biasing, immunostimulatory glycolipid, OCH." Bioorg Med Chem Lett 19(13): 3386-8.

Verdijk, R.M., *et al.* (1999). "Polyriboinosinic polyribocytidylic acid (poly(I:C)) induces stable maturation of functionally active human dendritic cells." J Immunol 163(1): 57-61.

Vremec, D., *et al.* (1992). "The surface phenotype of dendritic cells purified from mouse thymus and spleen: investigation of the CD8 expression by a subpopulation of dendritic cells." J Exp Med 176(1): 47-58.

Waeckerle-Men, Y., *et al.* (2006). "Encapsulation of proteins and peptides into biodegradable poly(D,L-lactide-co-glycolide) microspheres prolongs and enhances antigen presentation by human dendritic cells." Vaccine 24(11): 1847-57.

Waeckerle-Men, Y., *et al.* (2005). "Delivery of tumor antigens to dendritic cells using biodegradable microspheres." Methods Mol Med 109: 35-46.

Waeckerle-Men, Y. and M. Groettrup (2005). "PLGA microspheres for improved antigen delivery to dendritic cells as cellular vaccines." Adv Drug Deliv Rev 57(3): 475-82.

Waeckerle-Men, Y., *et al.* (2004). "Phenotype and functional analysis of human monocyte-derived dendritic cells loaded with biodegradable poly(lactide-co-glycolide) microspheres for immunotherapy." J Immunol Methods 287(1-2): 109-24.

Waeckerle-Men, Y.*, et al.* (2006). "Dendritic cell-based multi-epitope immunotherapy of hormone-refractory prostate carcinoma." Cancer Immunol Immunother.

Wagner, H. (2009). "The immunogenicity of CpG-antigen conjugates." Adv Drug Deliv Rev 61(3): 243-7.

Wagner, T.L.*, et al.* (1999). "Modulation of Th1 and Th2 cytokine production with the immune response modifiers, R-848 and imiquimod." Cell Immunol 191(1): 10-9.

Warger, T.*, et al.* (2006). "Synergistic activation of dendritic cells by combined Toll-like receptor ligation induces superior CTL responses in vivo." Blood 108(2): 544-50.

Weigmann, B.*, et al.* (1997). "Diminished contact hypersensitivity response in IL-4 deficient mice at a late phase of the elicitation reaction." Scand J Immunol 45(3): 308-14.

Wernert, N.*, et al.* (1997). "Pathological aspects of prostate cancer and benign nodular hyperplasia." Anticancer Res 17(4B): 2907-10.

Wherry, E.J.*, et al.* (2003). "Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment." J Virol 77(8): 4911-27.

Wilson, M.T.*, et al.* (2002). "Immunotherapy with ligands of natural killer T cells." Trends Mol Med 8(5): 225-31.

Wipf, P. and J.G. Pierce (2006). "Expedient synthesis of the alpha-C-glycoside analogue of the immunostimulant galactosylceramide (KRN7000)." Org Lett 8(15): 3375-8.

Wu, D.*, et al.* (2006). "Design of natural killer T cell activators: structure and function of a microbial glycosphingolipid bound to mouse CD1d." Proc Natl Acad Sci U S A 103(11): 3972-7.

Wu, L. and L. Van Kaer (2009). "Natural killer T cells and autoimmune disease." Curr Mol Med 9(1): 4-14.

Yamamura, T.*, et al.* (2007). "Understanding the behavior of invariant NKT cells in autoimmune diseases." J Neuroimmunol 191(1-2): 8-15.

Yanagihara, S.*, et al.* (1998). "EBI1/CCR7 is a new member of dendritic cell chemokine receptor that is up-regulated upon maturation." J Immunol 161(6): 3096-102.

Yarovinsky, F.*, et al.* (2006). "Toll-like receptor recognition regulates immunodominance in an antimicrobial CD4+ T cell response." Immunity 25(4): 655-64.

Yoshimoto, T.*, et al.* (1995). "Role of NK1.1+ T cells in a Th2 response and in immunoglobulin E production." Science 270(5243): 1845-7.

Yu, K.O.*, et al.* (2005). "Modulation of CD1d-restricted NKT cell responses by using N-acyl variants of alpha-galactosylceramides." Proc Natl Acad Sci U S A 102(9): 3383-8.

Zafir-Lavie, I.*, et al.* (2007). "Novel antibodies as anticancer agents." Oncogene 26(25): 3714-33.

Zhang, T. and D. Herlyn (2009). "Combination of active specific immunotherapy or adoptive antibody or lymphocyte immunotherapy with chemotherapy in the treatment of cancer." Cancer Immunol Immunother 58(4): 475-92.

II Record of achievement / Eigenabgrenzung

Chapter II: I performed all experiments except for figure 6; protective setting for MO-5 tumors and the initial experiment for figure 6; therapeutic setting for EG-7 tumors.

Chapter III: I established the assays and supervised the experiments for figure 1, 2 and 3. I performed experiments for figure 4 and figure 5.

Chapter IV: I performed all experiments except initial experiments for figure 2 and the synthesis of α-C-diPheGalCer.

Chapter V: I performed all experiments.

III List of publications

1. Schlosser E, **Mueller M**, Fischer S, Basta S, Busch DH, Gander B and Groettrup M. – (2008). – TLR ligands and antigen need to be coencapsulated into the same biodegradable microsphere for the generation of potent cytotoxic T lymphocyte responses. – *Vaccine* 26(13): 1626-1637.
2. Fischer S, Schlosser E, **Mueller M**, Csaba N, Merkle HP, Groettrup M, and Gander B. - (2009). - Concomitant delivery of a CTL-restricted peptide antigen and CpG ODN by PLGA microparticles to induce cellular response. - *J Drug Target* 17(8): 652-661.

Chapter II was submitted as a research article to the journal “Cancer Research” in March 2010

Chapter III was written for submission as a brief definitive report for the journal “Journal of Experimental Medicine”

Chapter IV was written to support a patent registration in collaboration with the University of Pittsburgh.

IV Acknowledgements

I owe my deepest gratitude to Prof. Marcus Groettrup, my advisor, my boss, but also a steady source of motivation and inspiration for me. He worked industriously and spared no effort to support every group member whenever and wherever he could. His perpetual energy and enthusiasm, the excellent working conditions he created, the fruitful meetings and the lively discussions engraved my picture of scientific work and conveyed a feeling of support through-out the last four years.

I was delighted to interact with Prof. Christof Hauck, who agreed being the second advisor for this thesis. I really appreciate the expeditious and competent help with “my” microspheres, which was provided by Prof. Bruno Gander. In addition to that he agreed being my third advisor. Thank you very much.

I am really grateful to Michael Basler. His expertise was always a landmark for me to orient my work. For me, he sets an example of a top researcher for his realism and his passion on research. Thanks for the supervision that supervisors don't have time for, the proofreading, our critic, and so much more.

After all those years, I've got quite a list of people, for which I would like to express thanks. Brigitte, for helping out with all the things a student needs. Those, who accompanied my time in the lab and who contributed in some or the other way to this thesis: Ulli (she has everything or knows at least where it might be), but also Tina, Gerardo and Bernd, who have been invaluable on the practical side of things. The people from TFA for their patience and flexibility. Katrin, Neha, Valerie, Andrea, Franzi, and Khalid for their help and for creating a pleasant and convivial atmosphere in the lab. Elmar, for being the labs sunshine, and Peter, for being a “real” immunologist. I am heartily thankful to Gunter, whose encouragement, guidance and control was particularly important for my scientific, but also my general life.
In particular, I would like to thank Christopher for his friendship and the discussions during the past four years. Stay gold! And I would like to thank Eva Schlosser, who was my supervisor “long time ago”. Thanks for all the ideas, the breaks and the “breakfasts”. That was a good time.

I would also like to thank Jennifer, Chiara, and Mirko “the gatherer” Zierke for teaching me what it means to be “the almighty supervisor”.

This thesis would not have been possible without Karin and “her” Jörg. With her I endured and survived the experience of studying biology. Her unending encouragement and support straitened me up when the going was tough.
I would like thank Annette and “her” Jörg for the gallons of coffee we had, for the cigarette breaks, the nights out, the discussions about science, the lab, or about life and about things one should do. Thanks for being my friends.

Lastly I want to thank my “pack”. I could not ask for more from my parents. Their constant support through-out my life, but especially when I encountered any kind of difficulties, made it easy following the paths, opening up for me. My brother Patrick, my role model, whom I followed probably unconsciously since I was able to “walk”. Finally, and most importantly: Schotte, my motivation, my pillow, my lighthouse, and my tower of strength. You are second to none!

… my friends, regardless where they are, and those I forgot ...