The role of antigen cross-presentation in the vaccine-induced activation of cytotoxic T-lymphocytes

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: 05.04.2011

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“….it is […] hard to imagine that 'professional' antigen presentation by means of cross-presentation is not a chief part of priming T cell responses.”

Michael J. Bevan

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Summary

Antigen delivery systems that are efficient in inducing $CD8^+$ T-cell mediated immune responses are required for the development of novel vaccines against intracellular pathogens and cancer. Although many new approaches were already investigated in mice and also humans, low immunogenicity of tested vaccines is still a major challenge in the field.

Therefore it is important to study the molecular and cellular function of particular vaccines, in order to develop strategies that overcome this limitation. Beside “direct-presentation” of endogenous proteins, antigen “cross-presentation” by professional antigen presenting cells (APCs) is an essential pathway for the major histocompatibility complex (MHC) class I presentation of exogenous antigens. It becomes more and more evident that cross-presentation is not only involved in physiological responses to various pathogens and malignancies, but also mediates specific cytotoxicity in response to vaccines. This thesis aimed on analyzing the molecular and cellular requirements for antigen cross-presentation, especially in response to selected vaccines.

In ***chapter I*** we characterized the cell types involved in the cross-presentation of antigens that are encapsulated into a particulate biodegradable vaccine. These poly(lactic-co-glycolic) acid (PLGA) microspheres are approved as drug delivery system in humans and display promising properties to serve as therapeutic vaccine against cancer. In the murine system, we could show that cross-presentation of PLGA MS-encapsulated antigen can be performed by both dendritic cells (DCs) and macrophages. In contrast to the current idea that $CD8^+$ DCs are the important cell type in cross-presentation we provide evidence for a dominant role of $CD8^-$ DCs and macrophages.

In ***chapter II*** we investigated the intracellular fate of PLGA MS after phagocytosis by APCs. Based on the encapsulation of inorganic nanocrystals, we introduce a novel method to label PLGA MS for electron and fluorescent microscopy, as well as magnetic cell sorting. Using these tools, we were able to show that PLGA MS do not

enter the cytosol after uptake, but are stored in lysosomal vesicles for at least 72h. Since cross-presentation of encapsulated antigen already occurred at much earlier time points, our data have implications on the intracellular cross-presentation pathway.

In ***chapter III*** we studied the molecular requirements concerning antigen-stability on the efficiency of DNA vaccines and immune responses induced by recombinant vaccinia virus (VV). We could show that targeting antigens for rapid degradation increases direct-presentation *in vitro*, but in contrast inhibits *in vivo* responses to DNA vaccination and recombinant VV. The results shown here argue for a dominant role of cross-presentation in the systems analyzed.

In ***chapter IV*** we investigated the role of heat-shock proteins (HSPs) and other cellular factors on the cross-presentation of cell-associated antigen. We provide evidence that the stable full-length protein, but not antigenic peptides, is the source of antigen transfer to APCs. At the same time we exclude several HSPs as mediators for cross-presentation in this system. Finally we show biochemical strategies to find interaction partners of a viral antigen that can mediate the transfer to APCs.

Taken together the data accumulated in this thesis contribute to the development of novel strategies to enhance immune responses following vaccination.

Zusammenfassung

Für die Entwicklung von neuartigen Impfstoffen zum Schutz gegen intrazelluläre Pathogene und Krebs werden Antigen-Vektoren benötigt, die in der Lage sind ausgeprägte $CD8^+$ T-Zell-Antworten auszulösen. Obwohl es viele neue Lösungsansätze gibt, die bereits in der Maus und im Menschen erprobt werden, ist die geringe Wirkung vieler Impfstoffe noch immer eine große Herausforderung.

Aus diesem Grund ist es unerlässlich, die molekularen und zellulären Mechanismen des jeweiligen Impfstoffes zu untersuchen, um Strategien entwickeln zu können, die die Wirkung dieser Impfstoffe verbessern. Im Unterschied zur direkten Präsentation

von endogen synthetisierten Proteinen, stellt die Kreuz-Präsentation einen essentiellen Weg dar, durch den exogene Antigene auf Molekülen des Haupt-Histokompatibilitäts-Komplexes (MHC) der Klasse I präsentiert werden. Mehr und mehr stellt sich heraus, dass die Kreuz-Präsentation nicht nur an physiologischen Immunantworten gegen verschiedene Pathogene oder Krebs beteiligt ist, sondern auch die zytotoxischen Immunreaktionen auf verschiedenste Impfstoffe vermittelt. Ziel dieser Doktorarbeit war es, die molekularen und zellulären Voraussetzungen der Kreuz-Präsentation zu untersuchen. Dabei wurde der Fokus auf die Wirkmechanismen ausgewählter Impfstoffe gelegt.

Im ***ersten Kapitel*** wurden die Zelltypen charakterisiert, die bei der *in vivo* Kreuz-Präsentation von in bio-abbaubaren Mikrosphären (MS) verkapselten Antigenen eine Rolle spielen. Diese aus dem Material Poly(Laktat-co-Glykolat) Säure (PLGA) bestehenden MS sind als Trägersubstanz für Arzneistoffe zur Behandlung von Menschen zugelassen und erfüllen hervorragende Eigenschaften, die sie zusätzlich als therapeutischen Impfstoff gegen Krebs qualifizieren. Anhand von Studien in der Maus konnten wir zeigen, dass in Mikrosphären verkapselte Antigene sowohl von Dendritischen Zellen (DCs) als auch von Makrophagen kreuz-präsentiert werden können. Dabei spielten im Besonderen die $CD8^-$ DCs eine entscheidende Rolle.

Im ***zweiten Kapitel*** wurde untersucht, in welchen intrazellulären Kompartimenten PLGA MS nach der Aufnahme durch Phagozyten zu finden sind. Durch eine neuartige Markierungsmethode, die auf der Verkapselung von anorganischen Nanokristallen beruht, konnten PLGA MS für elektronen- und fluoreszenzmikroskopische Untersuchungen markieren werden. Mit dieser Technik konnte gezeigt werden, dass PLGA MS nach der Aufnahme durch Phagozyten in lysosomalen Vesikeln gespeichert werden und nicht ins Zytoplasma übergehen. Da eine Kreuz-Präsentation von verkapselten Antigenen jedoch bereits früher zu beobachten war, hat dieser Befund eine direkte Bedeutung für das mechanistische Verständnis der Kreuz-Präsentation von PLGA MS. Des Weiteren wurde durch Verkapselung von magnetischen FeO Nanokristallen die Möglichkeit einer PLGA MS-basierten magnetischen Zellsortierung demonstriert.

Das ***dritte Kapitel*** beschäftigte sich mit der Bedeutung der Antigen-Stabilität in Bezug auf den Erfolg von DNA Impfstoffen und rekombinanter Vaccinia Viren. Die Experimente haben gezeigt, dass kurzlebige Antigene *in vitro* zwar besser direkt präsentiert werden, *in vivo* die Antworten bei DNA Immunisierung und Infektion mit Vaccinia-Viren jedoch hemmen. Hier werden Immunantworten präferentiell durch langlebige Antigene ausgelöst. Die Ergebnisse weisen daher auf eine wichtige Rolle der Kreuz-Präsentation in diesen Systemen hin.

Im ***vierten Kapitel*** wurde die Rolle von Hitze-Schock-Proteinen (HSPs) und anderen zellulären Faktoren bei der Kreuz-Präsentation von zellassoziiertem Antigen untersucht. Die Ergebnisse zeigen, dass stabile, ungeschnittene Antigene, jedoch keine Peptide, für einen Transport zu APCs zur Verfügung stehen. Gleichzeitig konnten verschiedene HSPs als Mediatoren der Kreuz-Präsentation in diesem System ausgeschlossen werden. Am Ende des Kapitels werden Strategien aufgezeigt, mit denen künftig neue Antigen-Interaktionspartner gefunden werden können, die einen für die Kreuzpräsentation wichtigen Antigen-Transfer vermitteln können.

Zusammenfassend stellt diese Arbeit Ergebnisse bereit, die dazu genutzt werden können, neue Strategien für die Wirkungssteigerung von Impfstoffen zu entwickeln.

General introduction

Beside the discovery of antibiotics in the early days of the 20th century (Gosio 1893, Duchesne 1897, Fleming 1929), the development of vaccines against infectious diseases was probably the major breakthrough in the history of medicine. Initial attempts to vaccinate people against diseases like small pox date back to early approaches 200 B.C. in India and China. Simple inoculation of patients with living virus from different origins was used to trigger immunity; an approach that often had lethal consequences (Lombard et al. 2007). Again more than 2000 years had to pass until a novel understanding on the principles of vaccination led to a dramatic improvement of global health care. It was Edward Jenner, medical doctor at the end of the 18th century who systematical analyzed and discovered the basic principles of vaccination against small pox (Jenner 1801). This discovery not only had a direct impact on the prevention of small pox and other infectious diseases, but also set the cornerstone for a completely new scientific area: the field of immunology.

From the current point of view, Jenner's success was based on a very simple assumption. Prophylactic immunity against a lethal disease like small pox could be achieved by infecting patients with an attenuated form of the virus. He used the cow pox virus (vaccinia) that showed a rather mild progression in humans, to induce cross-protection against its close relative in humans. Even though the vaccination against small pox was extremely successful and soon spread around the globe, it needed another 150 years until scientists in the second half of the 20th century started to uncover the underlying immunological mechanisms.

Today, the basic components and principles of the immune system are well established. Owing to the continuous scientific success in the field of immunology, the track record of vaccination went on and today a large number of hazardous infectious diseases can be prevented by vaccination. However, there are still major challenges in field that need further research. Many vaccines require a functional cold chain from production to application or multiple injections due to low immunogenicity (Levine and Robins-Browne 2009). These requirements, together with financial aspects, impede the successful use of promising vaccines in developing countries, where they are needed the most. At the same time there are still infectious diseases, like malaria (Todryk and Hill 2007) or human

immunodeficiency virus (HIV) (McMichael et al. 2010) that still cannot be efficiently treated by prophylactic vaccination yet.

A different aspect of immunotherapy or immune modulation is the treatment of diseases that are not of infectious origin. Especially autoimmune disorders, allergies, and cancer are promising targets for immunological treatments, but the underlying principles are distinct from those involved in infectious diseases and still under intensive investigation (Ramshaw et al. 1997, Niederberger 2009, Hilkens et al. 2010, Luo et al. 2010, van den Broek et al. 2010). Nevertheless, there is a consensus that novel immunotherapies targeting non-infectious disease will strongly influence future health care systems.

Composition and organization of the immune system

The immune a system is remarkably adaptive defense system that has evolved in higher organisms to protect the host from invading pathogens and cancer. It consists of a non-hematopoietic component building the immunological organs and lymphatic vessels and a hematopoietic cellular compartment represented by immune cells and their effector molecules. All immune cells originate from the bone marrow that together with the thymus represents the primary lymphoid tissue of the organism (Murphy et al. 2008). The basic principle of the immune system is based on the discrimination between autologous tissues that should be protected and unknown structures that indicate pathogen invasion or development of malignancies. Based on the specificity of this antigen recognition, two distinct divisions of the immune system are described. Cells of *the innate immune system* express non-variable immune receptors, like those of the Toll-like receptor (TLR) family that are specific for common structures on bacteria or viruses (Kawai and Akira 2010). This pattern recognition leads to immediate defense mechanisms, including the release of pro-inflammatory cytokines, the clearance of infectious particles by phagocytes, and the production of antimicrobial proteins and peptides. Usually the great majority of pathogens that enter the organism are successfully eliminated by the innate immune response. However, there are situations where pathogens are able to circumvent this first line of defense. Due to evolutionary adaptations or massive infiltration the innate system can be overstrained and more specific mechanisms need to be activated.

This second line of defense is task of *the adaptive immune system*. Highly specific immune receptors recognize pathogen-derived antigens and activate lymphocytes to initiate a pathogen-specific immune response. This recognition and response to an individual pathogen strain focuses the resources of the adaptive immune system and is responsible for its superiority. The second advantage is the development of an immunological memory that protects the host from possible second infections with the same pathogen. Since the innate immune system is not variable, vaccinations always target the adaptive mechanisms. Independent of the actual target, the final goal of a usual vaccination protocol is the production of antibodies, activation of cytotoxic T-cells (CTLs), and the initiation of an immunological memory.

Protein degradation via the ubiquitin/ proteasome system

All proteins that are translated within a cell, according to their life time sooner or later become degraded, first into peptides and later into single amino acids (Lodish et al. 2004). This way a recycling of resources for the next generation of proteins is realized. Beside the protease activity in the endosomal compartment, the major cytosolic protease executing this protein degradation is the proteasome. It is a multimeric enzyme complex consisting of a core particle with protease activity, the 20S proteasome, and different regulatory subunits, including the 19S regulator PA700. This, together with the 20S core particle, forms the fully active 26S proteasome (Ferrell et al. 2000). The 20S proteasome is a barrel-shaped cylinder that is built of four stacked rings, each containing seven subunits. The outer two rings at the bottom and the top of the core particle mediate the interaction with regulatory particles and consist of subunits of the α-type. The two central rings are built of seven different β-subunits each, of which only three comprise proteolytic activity (β1, β2, and β5) (Groettrup et al. 2001c). The proteolysis is restricted to the central lumen of the cylinder, a mechanism that prevents unspecific access to the active sites and thus random degradation. The proteasome degrades proteins into peptides usually ranging from 3 to 25 amino acids in length (Groll and Clausen 2003). Afterwards recycling of amino acids is enabled by cytoplasmic amino peptidases.

Protein degradation via the proteasome is highly regulated since the half-life of enzymes or structural proteins is crucial for the function and the maintenance of cell

homeostasis or differentiation (Lodish et al. 2004). Degradation is restricted to proteins that are damaged, misfolded, or no longer needed and controlled via an ubiquitin conjugating system. Ubiquitin is a ubiquitously expressed 8.5kDa protein that can be covalently linked to target proteins via an isopeptide linkage. Proteins conjugated with a polyubiquitin chain interact with the 19S regulator of the 26S proteasome, which leads to the degradation of a labeled proteins (Hershko and Ciechanover 1998). After interaction with the 19S regulator polyubiquitin is cleaved off the target protein and recycled into monomers for the labeling of new targets. The attachment of ubiquitin to target proteins requires three essential steps. Initially it has to be activated by an ubiquitin activation enzyme (E1) that uses ATP to form an energy-rich thioester between itself and the C-terminus of ubiquitin. In a second step the activated ubiquitin is transferred to an ubiquitin conjugating enzyme (E2) that interacts with substrate specific ubiquitin ligases (E3). The E3 enzyme binds specifically to target proteins and brings them to close proximity with the activated ubiquitin, bound to the E2. In a last step ubiquitin is attached to the target protein either directly from the E2 (Joazeiro and Weissman 2000) or via the E3 (Huang et al. 1999). The interaction of E2 and E3, however, is responsible for the specificity of the ubiquitin conjugating system, since each individual protein requires a unique combination of E2 and E3 enzymes for its ubiquitinylation. Labeling of proteins with a single ubiquitin is not sufficient for proteasomal targeting. This is only realized after the formation of polyubiquitin chains, which usually have to form by K48-linkage of further ubiquitin monomers to the proximal ubiquitin of a conjugate (Li and Ye 2008). In some cases the formation of long polyubiquitin chains requires the presence of an additional assembly factor, called E4 (Hoppe 2005). The polyubiquitin chains that are generated this way are the signal for proteasomal degradation of proteins.

However, the degradation of proteins via the proteasome is not only essential to maintain and regulate the protein homeostasis of a cell, but has also important immunological function in term of immune surveillance.

Antigen presentation on MHC class I

Protein degradation via the proteasome produces a peptide spectrum of the entire proteome of a cell at a certain state that reflects its physiological condition. One

fraction of peptides that is generated this way is not recycled into single amino acids, but presented on the cell surface in association with molecules of the major histocompatibility complex (MHC) class I. This surface exposure of protein fragments is called antigen presentation and is the central element for responses of the adaptive immune system. MHC/peptide complexes on the cell surface are recognized by T-cell receptors (TCRs) that are specific for an individual MHC/peptide combination. With very few exceptions, all cells of the body present peptides on MHC class I that are derived from endogenously expressed proteins. This way the immune system can screen cells for expression of pathogen-derived or mutated proteins. MHC class I molecules bind preferentially peptides of 8 to 9 amino acids in length, which contain certain anchor residues that can vary between different MHC class I alleles (Rammensee et al. 1999). However, there is a general requirement for hydrophobic residues at the C-terminus of peptides in mice, whereas in humans MHC class I can bind peptides with both hydrophobic and basic C-termini. After generation in the cytosol, peptides have to be transported into the lumen of the endoplasmic reticulum (ER), where loading of MHC class I molecules occurs. Peptides are transported into the ER via an ABC transporter associated with antigen processing (TAP). Interestingly, the TAP co-evolved with the antigen presentation machinery to have similar preferences for anchor residues like the MHC class I molecules. This way it is achieved that potential MHC ligands are efficiently introduced into the ER. The loading of peptides on MHC molecules in the ER is mediated by numerous chaperon proteins. Whereas empty MHC molecules are retained within the ER, loaded complexes take the secretory pathway via Golgi to be presented on the cell surface (Schoenhals et al. 1999).

It has been shown that the peptides generated for antigen presentation are not only derived from degradation of intact proteins. Many immunodominant epitopes from viral origin are derived from extremely long-lived proteins. Nevertheless epitopes of these proteins are efficiently presented on MHC class I. An explanation for this phenomenon was summarized in the DRiPs-hypothesis by Yewdell and colleagues (Yewdell et al. 1996). According to this, DRiPs are “defective ribosomal products” that are generated by any cell during the process of protein translation. These improperly translated or misfolded polypeptides never reach their native state, but are rapidly targeted to ubiquitinylation and proteasomal degradation. It was shown that a large

fraction of MHC ligands are produced by the degradation of DRiPs (Schubert et al. 2000). This way it can be realized that not the half-time of a protein, but its actual production determines the amount of antigen presentation.

In the course of an infection specific changes in the MHC class I presentation machinery lead to an increase in antigen presentation. Induced by the pro-inflammatory cytokines TNF-α and IFN-γ the three constitutive proteolytic β-subunits of the proteasome are exchanged by their inducible counterparts β1i (LMP2), β2i (MECL-1), and β5i (LMP7). They are incorporated into the proteasome during neosynthesis to form so called immunoproteasomes. This inducible form of the proteasome shows an altered cleavage pattern and produces significantly higher amounts of peptides with basic or hydrophobic C-termini. This correlates with the required anchor residues for TAP and MHC class I and leads to an increased antigen presentation (Groettrup et al. 2001a, Goldberg et al. 2002). The different peptide spectrum that is presented during infections is also an important instrument to prevent autoimmunity, which often targets antigens that are produced by the constitutive proteasome. An additional proteasome subunit called β5t has been found in cortical thymic epithelial cells and has an important function in the selection process of antigen specific T-cells (Murata et al. 2007).

Antigens that are presented on MHC class I molecules are recognized by $CD8^+$ cytotoxic T-cells. Once activated, these cells recognize intracellular pathogens and malignancies and kill target cells by various effector mechanisms, including the release of cytotoxic granules (granzyme B) and the formation of molecular pores that destroy target cell homeostasis (Chavez-Galan et al. 2009).

Generation of antigen specific T-cells

Like all other cells of the immune system, T-cells originate from the bone marrow. However, in contrast to B-cells, they leave the bone marrow as immature cells and migrate to the thymus at an early stage during their development. Here they further mature into antigen specific T-cells. Due to the almost infinite number of possible MHC/peptide combinations, T-cells have to express an extremely large number of different TCRs. In contrast to cells of the innate immune system, which share

receptors of the same specificity, each individual T-cell expresses a different TCR. In cause of an infection, T-cells with a TCR specific for an infectious agent are activated (primed) and clonally expanded. This way the immune system has the flexibility to comprise a broad TCR repertoire, but is still able to produce high numbers of effector cells with the same specific affinity, if needed. In contrast to the large number olfactory receptors that can differentiate between 10000th of flavors, the TCRs are not germ line encoded, but are generated via a system of somatic recombination. This process called “VDJ-recombination” is based on the random combination of gene segments that together build the TCR (Spicuglia et al. 2006).

The random generation of TCRs requires a selection process that ensures two essential requirements. The receptor has to recognize peptides that are presented on “self” MHC molecules (MHC restriction) (Zinkernagel and Doherty 1974). This is realized in a first step of positive selection. Only T-cells that are able to receive a signal via their TCR are rescued from programmed cell-death. Furthermore, the TCR is not allowed to recognize “self” peptides presented on MHC with high affinity. This is realized in a second selection step, the negative selection, which is mediated by specialized cells that present “self”-peptides. The recognition of one of these “self” peptides is the sentence of death for a T-cell (Sebzda et al. 1999).

All remaining naïve T-cells that leave the thymus after selection are therefore specific for recognizing foreign peptides presented on “self” MHC molecules. This process of T-cell selection is the basis for the mechanisms of central tolerance. Nevertheless, this tolerance is not complete and is further improved by several other mechanisms, including peripheral tolerance, anergy, immunological ignorance and the presence of regulatory T-cells that under normal circumstances prevent autoimmunity (Thomas 2010).

Antigen presenting cells and the priming of T-cells

T-cells that survive the selection procedures in the thymus are released to the periphery. Such cells never encountered the antigen of their specificity and are therefore called “naïve”. These naïve T-cells are not able to fulfill their effector functions yet, but require an activation that can only be performed by professional

antigen presenting cells (APCs) and is called T-cell “priming”. This priming on the one hand requires the presentation of specific MHC-peptide complexes and on the other hand the surface expression of certain co-stimulatory molecules, which are exclusively exposed on matured APCs (CD80, CD86) (Basta and Alatery 2007). Only after being primed the naïve T-cells get activated, undergo a phase of proliferation, and are then able to fulfill their effector functions. This mechanism of T-cell activation requires some days and is the reason for the delayed start of the adaptive immune response.

Antigen presentation on MHC class II and the activation of B-cells

Antigen presentation on MHC class II is important for the activation of $CD4^+$ T-cells and can only be performed by APCs, namely dendritic cells (DCs), macrophages (MΦs) and B-cells. Peptides that are presented on MHC class II are exclusively provided by exogenous antigen (proteins or peptides from the extracellular milieu). Therefore, APCs take up antigens from the extracellular space by processes including endocytosis, phagocytosis, pinocytosis, and macropinocytosis. In lysosomal compartments with low pH values the exogenous antigens are degraded by specialized proteases (Cathepsins). For loading of peptides, the lysosomes fuse with secretory vesicles containing the MHC class II molecules and the peptides are attached to the binding pocket of these proteins (Jutras and Desjardins 2005). Afterwards the complexes are exposed to the cell surface and are accessible for the second class of T-cells, the $CD4^+$ T-helper-cells. They have important regulatory functions, for example the licensing of antibody production or isotype-switching in B-cells (Wan 2010).

Antigen cross-presentation

In 1976 Michael Bevan and colleagues published an experiment that changed the view on MHC class I presentation dramatically. Mice immunized with murine cells expressing a different MHC haplotype were nevertheless able to elicit an immune response against minor antigens that were specific for the donor cells. These responses could not be explained by the existing dogma of MHC class I antigen presentation. The hypothesis that MHC class I peptides are exclusively derived from

proteins synthesized within a cell had to be reconsidered. As mentioned above, the initial priming of $CD8^+$ T-cells is mediated by specialized APCs. Since the immune responses according to the MHC restriction had to be initiated by APCs of the recipient mice, Bevan's experiment provided evidence for a third antigen presentation pathway, which he termed "cross-presentation" (XP). In contrast to direct-presentation, XP is based on the uptake of exogenous antigens that are degraded into peptides and presented in association with MHC class I molecules.

The initial priming of $CD8^+$ T-cells following XP was called "cross-priming". It explains not only the immune responses after allogenic transplantation, but also many other physiologic immune reactions, including responses against tissue specific viruses, cancer, and the development of autoimmune diseases (Amigorena and Savina 2010). Also the induction of peripheral tolerance can be mediated via XP, a mechanism called "cross-tolerance" (Heath and Carbone 2001a). No matter if it is T-cell activation or induction of tolerance, XP is essential to mediate and control immune responses against tissue-specific antigens that cannot be presented by APCs via direct MHC class I presentation (Carbone and Bevan 1990, Rock 1996, Chen et al. 2004).

Cross-priming in diseases

Viruses show distinct strategies to evade from immune recognition by $CD8^+$ T-cell responses. Since direct-priming is an issue in T-cell activation, some viruses, including hepatitis B virus (HBV), polio virus, and Epstein-Barr virus (EBV), avoid the direct infection of APCs (Kurts et al. 2010a). This specific infection pattern has the consequence that $CD8^+$ T-cell responses against such viruses cannot be induced via direct antigen presentation. From the evolutionary point of view XP is therefore on the one hand an adaptation to cope with tissue specific viruses and on the other hand the reason for the relatively low number of viral infections that do not target APCs (Kurts et al. 2010a). The second and much more powerful evasion strategy of viruses is the expression of molecules (evasins) that inhibit components of the MHC class I loading machinery in APCs (Cunningham et al. 2010). Prominent examples for such strategies come from the family of herpes viruses (Reddehase 2002). The murine cytomegalovirus (MCMV) expresses evasins that lead to a failure of inducing

$CD8^+$ T-cell responses by infected APCs (Andrews et al. 2001). The fact however, that $CD8^+$ T-cell responses against MCMV can be found in the infected animal, indicate that non-infected APCs can induce anti-MCMV responses via cross-priming (Holtappels et al. 2004). XP is also responsible for the induction of immune responses against other viruses that express evasins, like Epstein-Barr virus (EBV) and herpes simplex virus (HSV-1) (Bickham et al. 2003).

Viral infections can generally trigger XP via a TLR-mediated type I IFN responses that leads to maturation of APCs and to enhanced XP (Le Bon et al. 2003, Schulz et al. 2005). The importance of this cell activation becomes evident when analyzing the persistence of viruses that can interfere with the IFN production. Examples for such an indirect inhibition of XP are known from hepatitis B and C virus (HBV, HCV) (Rehermann and Nascimbeni 2005, Hosel et al. 2009).

XP has also been shown to be an essential component of the immune defense against certain bacterial infections. Especially responses against intracellular bacteria, like the well studied model pathogen *Listeria monocytogenes,* depend on cross-priming. *Listeria* gets access to the cytoplasm of the host cells after infection. This leads to the induction of apoptosis in infected cells and the uptake of apoptotic cell debris by APCs that are able to cross-present pathogen-related peptides for the activation of $CD8^+$ T-cells (Jung et al. 2002). Interesting, with regard to the nature of the cross-presented antigen, is the fact that protein synthesis is not required in this case, which indicates that peptides of long-lived proteins are presented (Datta et al. 2006). A second bacterial system that was intensively studied is infection with *Mycobacteria*. During their infection cycle *Mycobacteria* persist in phagosomal vesicles of cells without translocation to the cytosol. Also in this case it was evident that XP of apoptotic material, originating from bacteria-bearing cells, by non-infected DCs was required for the induction of efficient immune responses (Schaible et al. 2003). Similar to viral antigens, also in case of infections with intracellular bacteria a migration of cross-priming DCs into the lymph nodes is essential for $CD8^+$ T-cell priming (Winau et al. 2006).

Furthermore, XP can be an important pathway for immune responses against non-infectious diseases, like cancer and autoimmune disorders. Tumor-derived antigens

are in most cases tissue-specific autoantigens or neoantigens originating from mutated proteins (Kurts et al. 2010a). In any case, these antigens are not present in antigen presenting APCs and can therefore not induce T-cell activation via direct-priming. Interestingly, it was shown that tumor-derived antigens can be efficiently taken up by DCs and are cross-presented in local tumor draining lymph nodes (Marzo et al. 1999, Hildner et al. 2008). However, at the same time tumor rejection does not occur because of inefficient $CD8^+$ T-cell activation. Several studies indicated that not the XP of tumor-derived antigens is the limiting factor in this system. Generally tumors lack pathogen-associated molecular patterns (PAMPs) that are required for efficient APC activation and maturation (Lyman et al. 2004). This might be one of the reasons why tumor antigens often only induce weak CTL responses (Stumbles et al. 2004) or even tolerance (Cuenca et al. 2003, Ney et al. 2009). Additionally, the mechanisms of central tolerance that lead to T-cells selection in the thymus reduce the number of tissue-specific T-cells available. Tumor therapy has therefore the challenge to reverse this immune balance by breaking tolerance for the activation of powerful CTL responses.

The opposite circumstances lead to the development of autoimmune diseases. Here the mechanisms of tolerance are already broken and immune responses against autologous tissues are induced. Autoimmune disorders that are based on the activity of $CD8^+$ T-cells, like diabetes type I (McDevitt and Unanue 2008) or multiple sclerosis (Goverman 2009), are still not completely understood. Although the induction of central tolerance in the thymus is quite efficient, there are nevertheless T-cells escaping that have auto-reactive potential. These cells can partially be controlled via APCs that cross-present tissue-derived antigen without further co-stimulation. This peripheral mechanism that inhibits auto-reactive $CD8^+$ T-cells is called cross-tolerance (Kurts et al. 1997). However, the balance between cross-tolerance and cross-priming can be modified by the antigen dose (Redmond and Sherman 2005) and also by the presence of additional infections that might trigger the maturation of APCs (Carbone et al. 1998). This can promote cross-priming instead of cross-tolerance and initiation of the disease (Kurts et al. 1998, Zehn and Bevan 2006). A second possible explanation for the induction of autoimmune diseases is the direct-presentation or XP of bacterial or viral antigens that mimic tissue-specific autoantigens. This mechanism is especially fatal since PAMPs are involved that can

lead to APC maturation and efficient CTL activation. However, neither for diabetes nor multiple sclerosis a correlation with triggering pathogens could be definitively shown (Benoist and Mathis 2001).

Cross-presenting cells

In contrast to direct-presentation and similar to MHC class II presentation, XP can only be performed by specialized cell types that originate from the bone marrow (Huang et al. 1994, Sigal et al. 1999, Sigal and Rock 2000). Macrophages were the initial cell type that was found to be capable of XP (Kovacsovics-Bankowski et al. 1993), but studies showing XP by DCs (Shen et al. 1997), B-cells (Ke and Kapp 1996, Heit et al. 2004), endothelial cells (Limmer et al. 2000) and even neutrophil granulocytes (Potter and Harding 2001, Tvinnereim et al. 2004) followed. Today, the impact of different APC types on XP is still not completely elucidated. Some cells that cross-present *in vitro* seem to be dispensable *in vivo*. Others that can stimulate CTLs *in vitro* seem to induce cross-tolerance *in vivo*. However, for the understanding of cross-priming *in vivo* it is essential to elucidate which APC types are involved.

Today it is believed that DCs are the major cross-presenting cells *in vivo*. Several lines of evidence led to this assumption. Initially it was shown that primary DCs isolated from lymph nodes of mice injected with the model antigen ovalbumin (OVA) were shown to present OVA-derived peptides to CTL cultures *in vitro* (Grant and Rock 1992). This experiment provided strong evidence that DCs are able to acquire exogenous antigen *in vivo* and to cross-present antigenic peptides in context of MHC class I. Vice versa, it was shown that primary DCs loaded with exogenous antigen *in vitro* were able to elicit robust immune responses if re-injected into mice (Pozzi et al. 2005). However, the strongest evidence in favor of a dominant role of DCs came from experiments in which DCs were depleted *in vivo* (Jung et al. 2002). Jung and colleagues generated a transgenic mouse (CD11c-DTR) that expresses the diphtheria toxin receptor under control of the CD11c promoter; the promoter for the linage marker of DCs. Administration of the toxin led to the specific *in vivo* depletion of DCs and was therefore a powerful tool to study DC function. In their experiments Jung *et al.* could show that XP of cell-associated antigen and class I responses against intracellular pathogens were dramatically reduced in mice lacking $CD11c^+$

cells. This finding, however, did not finally conclude the ongoing debate on cross-priming cell types. DCs themselves are a heterogeneous cell population consisting of various populations that originate from both, lymphoid and myeloid precursors (Banchereau et al. 2000).

The two major DCs subtypes in the spleen, the $CD8^+$ $CD11b^-$ $CD11c^+$ DCs and the $CD8^-$ $CD11b^+$ $CD11c^+$ DCs, are in the focus of APC candidates for *in vivo* XP (Guermonprez et al. 2002). Over the last years, strong evidence emerged that attributes a major role to the $CD8^+$ DCs subpopulation. This was true for studies performing experiments using cell-associated (den Haan et al. 2000, Belz et al. 2002b, den Haan and Bevan 2002) or soluble antigen (Pooley et al. 2001), as well as for infections with different viruses (Allan et al. 2003, Smith et al. 2003, Belz et al. 2004a) and intracellular bacteria (Belz et al. 2005). In contrast to this, the contribution of $CD8^-$ DCs is restricted to only few examples, like the XP of antigenic immune complexes (den Haan and Bevan 2002). The fact that all known DC subpopulations are able to present exogenous antigens on MHC class II, but only the $CD8^+$ DCs on class I, led to the assumption that $CD8^+$ DCs must show some special adaptations that increase their potential to cross-present (Schnorrer et al. 2006). According to what is known on DC function, peripheral DCs in the tissues capture antigens via infection or uptake. Then they migrate into the draining lymph nodes after maturation via inflammatory responses and initiate $CD8^+$ and $CD4^+$ T-cell responses. $CD8^+$ DCs however are thought to be lymph node resident (Carbone et al. 2004, Villadangos and Heath 2005). Therefore the questions occurred about how lymphoid organ resident $CD8^+$ DCs are able to efficiently cross-present antigens that originate from peripheral infections. There are two possible scenarios that might explain this experimental finding. Either migrating DCs change surface antigens after entering the lymph nodes and by this acquiring the $CD8^+$ phenotype. The second possibility would predict that peripheral antigens are transferred from migratory DCs to $CD8^+$ resident DCs, which then in turn would present the antigens to $CD8^+$ T-cells. Indeed, there are several publications that favor the second possibility, while excluding the first (Belz et al. 2004b, Allan et al. 2006).

Despite of the strong evidence for the dominant role of DCs, also other cell types were shown to cross-present antigens *in vivo*. Macrophages are able to engulf

antigens in the periphery and can migrate to draining lymph nodes after maturation (Shi and Rock 2002, Pozzi et al. 2005). Furthermore they are able to cross-present antigens *in vivo* and stimulate CTL responses (Grant and Rock 1992, Pozzi et al. 2005). Even though the experiments with the CD11c-DTR transgenic mice provided strong evidence for the dominant role of DCs, detailed analysis of the cell types depleted revealed that the depletion of DCs was not as specific as initially thought (Probst et al. 2005). Especially metallophilic marginal zone macrophages that only recently where discovered to have a strong impact on XP of certain antigens, were also depleted in the CD11c-DTR mouse (Backer et al. 2010). This and other findings point out that macrophages indeed might be more important for *in vivo* cross-presentation than currently described in the literature (Shen and Rock 2006). Up to now, the cross-priming APCs *in vivo* are not definitely identified.

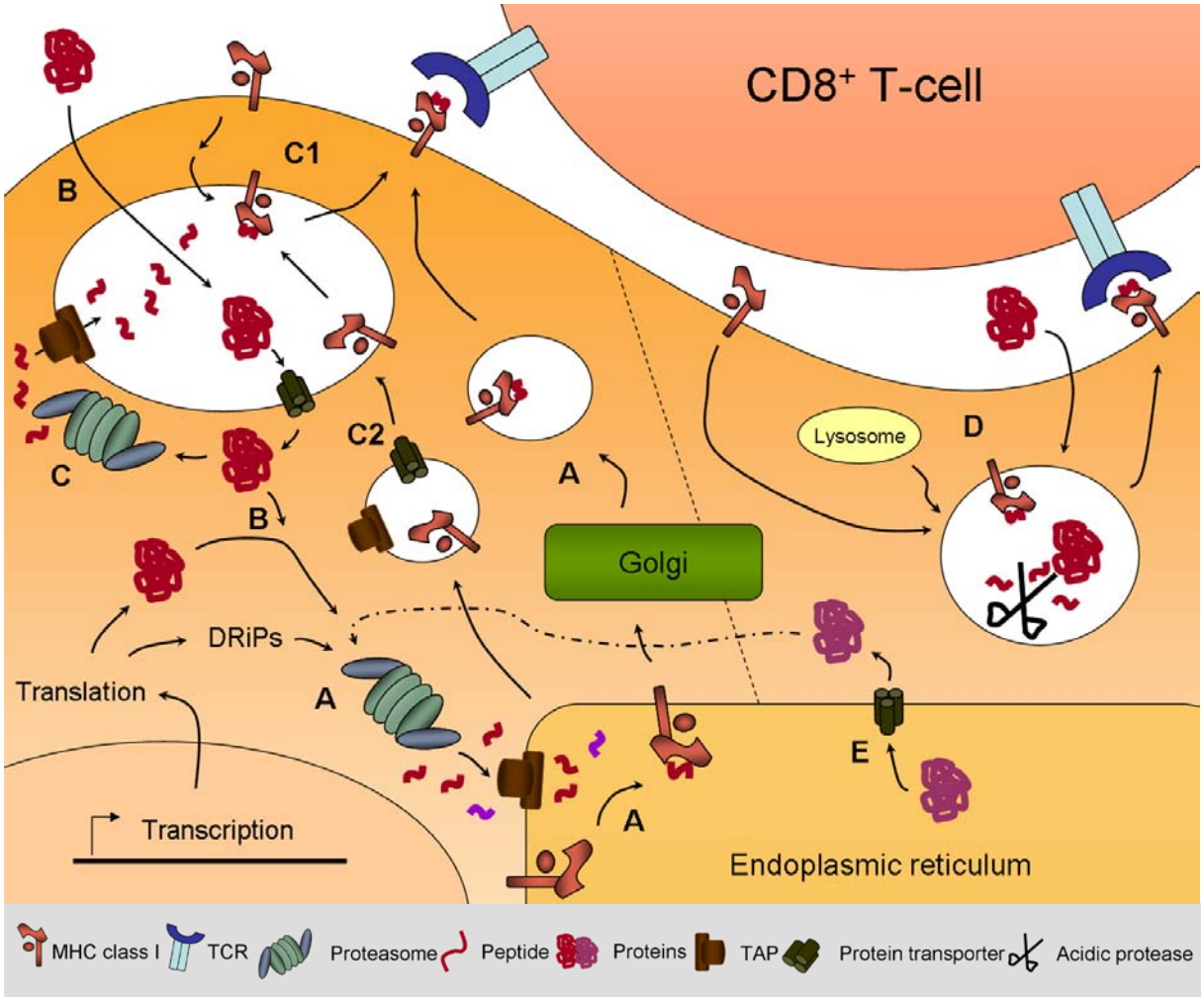
As mentioned earlier, there are other cell types that were shown to cross-present antigens *in vitro*. In a recent study on DNA vaccination using gene gun immunization, B-cells were found to cross-present antigens derived from keratinocytes. Although there were less efficient than DCs, B-cells contributed significantly to the overall cross-priming. These results were confirmed by using µMT knock-out mice that lack B lymphocytes (Cho et al. 2001, Heit et al. 2004, Hon et al. 2005). However, the contribution of B-cells on XP could be rather indirect. Specific antibodies produced by B-cell can complex antigens that in turn can be internalized by DCs via Fcγ receptors. Such a cross-priming of antigen complexes is quite efficient and does not require T-cell help. Therefore a lack of B-cells can indirectly lead to reduction in XP (den Haan and Bevan 2002, Schuurhuis et al. 2002, Schuurhuis et al. 2006).

Liver sinusoidal endothelial cells (LSECs) were shown to engulf exogenous antigen originating from neighboring live or dead cells and to present related peptides in the context of MHC class I molecules (Limmer et al. 2000, Bagai et al. 2005). However, LSECs express only low levels of co-stimulatory molecules (Katz et al. 2004, Tokita et al. 2006) and antigen presentation does not lead to T-cell priming. What was observed, is a rather immune suppressive function that can be categorized into the term cross-tolerance (Limmer et al. 2000, Limmer et al. 2005). Other cell types that were shown to cross-present under certain conditions *in vitro*, do not seem to have any significant impact on XP *in vivo* (Potter and Harding 2001).

Cross-presentation pathways

For XP, exogenous antigen is taken up by APCs, degraded into peptides, and presented on the cell surface in association with MHC class I molecules. However, this rather simple definition of XP is only the framework of intracellular mechanisms that are still not completely elucidated. Studies during the last decade have revealed that there is not one single intracellular pathway accounting for all XP events observed. In contrast, it seems there are multiple pathways that include different molecular mechanism depending on the cross-presenting cell type and the nature of antigens. XP can include mechanisms of the two classical antigen presentation pathways in combination with the machinery of non cross-presenting phagocytes (Amigorena and Savina 2010). This fact makes it difficult to develop a valid hypothesis on the principles of XP. Nevertheless, the molecular pathways leading to XP can be categorized in two principal routes. After uptake of antigens into endosomal compartments via endocytotic mechanisms, an initial step that is common for all antigens cross-presented, the pathways disperse. One being dependent on the activity of the proteasome and the TAP transporter, called the “cytosolic pathway” and a second being independent of these components, called the “vacuolar pathway” (Kovacsovics-Bankowski and Rock 1995, Moron et al. 2003, Rock and Shen 2005, Monu and Trombetta 2007). A summary of the most prominent cross-presentation pathways is visualized in *Scheme 1*.

Since the proteasome is a cytoplasmatic protease, its involvement in XP predicts the transition of antigens from the phagosome/endosome to the cytoplasm. Rock and colleagues were indeed the first to show that antigens located in endosomes after phagocytotic uptake can translocate to the cytosol (Kovacsovics-Bankowski and Rock 1995). They found that the ribosomal inhibitor protein gelonin, attached to beads, was able to inhibit protein translation in the cytosol after phagocytosis. At the same time they found increased cross-presentation of bead-associated antigens. These findings for the first time led to the assumption of a “phagosome-to-cytosol pathway”, an idea which is still represented by the first pathway described above. A similar experiment was performed by Lin *et al.* (Lin et al. 2008b). Using the fact that cytoplasmatic cytochrome C (Cyt C) induces apoptosis via the pro-apoptotic protein Apaf-1, they injected Cyt C into mice and analyzed the survival of different DC



Scheme 1: Intracellular pathways of "direct- and "cross-presentation". Endogenous antigens are transcribed in the nucleus and translated into proteins or defective ribosomal products (DRiPs) before they enter the direct-presentation pathway (A). Peptides are generated by proteasomal cleavage and transported into ER via TAP. Here loading of MHC molecules with peptides occurs and complexes take the secretory pathway via Golgi to the cell surface. Three routes are shown for the "cytosolic pathway" of cross-presentation: (B) Protein antigens enter endosomes by endocytosis and escape into the cytosol before acidification and formation of lysosomes. From here pathways divide. Cytoplasmatic antigens either enter the direct-presentation pathway (B) or are degraded by proteasomes in close proximity to the endosomes (C). Peptides generated this way can be re-imported into the endosomes via TAP. In the following, endosomal peptides can bind to recycling MHC class I molecules (C1). Protein and peptide transporters as well as empty MHC molecules as alternative for class I loading can be directly recruited to the endosome from the ER (C2). (D) "Vacuolar pathway" of cross-presentation. Antigen is internalised into endosomes that fuse with lysosomes. Acidification activates acidic phosphatases that degrade proteins into peptides. MHC class I loading is realized by recycling of empty MHC molecules from the cell surface. In all cases MHC/peptide complexes are delivered to the cell surface to interact with T-cell receptors of $CD8^+$ T-cells.

subtypes. Interestingly, they found that only $CD8^+$ DCs were depleted via Cyt C induced apoptosis. This result provided evidence that endocytosed Cyt C in $CD8^+$ DCs could translocate to the cytosol to fulfill its pro-apoptotic activity. Apart from these two examples there were other reports on the transition of antigens that all

together provide strong evidence that cytosolic transition of antigens exists (Norbury et al. 1995, Norbury et al. 1997, Rodriguez et al. 1999). The second definition of the “cytosolic pathway” is the dependence on the TAP transporter. Experiments using APCs of TAP-deficient mice or specific TAP inhibitors, like the viral peptide ICP47 (Rajcani et al. 2004), revealed a strong inhibition of XP for some antigens. These findings led to the assumption that antigens, which exit the endosomal compartment, are degraded via the proteasome and peptides enter the direct-presentation pathway via ER and Golgi (Scheme 1B). This converging of cross- and direct-presentation pathway was also supported by experiments showing that Golgi inhibitors can prevent XP for certain antigens (Huang et al. 1996, Raghavan et al. 2008).

A second possible scenario was hypothesized based on experiments that indicated a fusion of ER membranes with endosomes. When researchers initially thought about a possible mechanism how proteins could exit endosomes to enter the “cytosolic pathway”, they compared the situation with a retrograde protein transport in membranes of the ER. Luminal ER-resident proteins are normally degraded in the cytosol via the proteasome. The machinery responsible for this protein relocation is summarized with the term “ER-associated degradation (ERAD) (Raasi and Wolf 2007). The idea that ERAD indeed could account for the translocation of exogenous antigens from endosomes was initially supported by papers showing the presence or ER-membrane components within phagosomal membranes (Vembar and Brodsky 2008). Shortly after the proposed involvement of ERAD in XP, the group of P. Cresswell and colleagues performed experiments in which they could show that blocking of ERAD-associated proteins inhibited the conversion of endosomal proteins into the cytosol (Ackerman et al. 2006).

The presence of ER-membranes in phagosomes and the dependence of XP on components of ERAD changed the idea that peptides of exogenous antigens would have to necessarily enter the direct-presentation pathway. Interestingly, early after endocytosis of exogenous proteins, MHC/peptide complexes of the respective proteins were discovered in phagosomes (Guermonprez et al. 2003, Houde et al. 2003) and also endosomes (Burgdorf et al. 2007). These findings led to the hypothesis that peptide loading for XP could occur in endosomal compartments instead of the ER (Scheme 1C). This idea was supported by the discovery of TAP

transporters in the endosomal membrane (Guermonprez et al. 2003, Houde et al. 2003, Saveanu et al. 2009). It was even shown that the recruitment of TAP from the ER to endosomes is mediated via TLR ligands, indicating the importance of this pathway during infections (Burgdorf et al. 2007). However, although there is plenty of evidence indicating a delivery of proteins from the ER to the endosomal compartment, is has to be kept in mind that the methods showing this are either based on subcellular fractionation of ER and endosomes, which is extremely difficult, or on fluorescence microscopy with limited resolution. Hence, despite of the strong evidence a final detection of TAP recruitment to endosomes by high resolution electron microscopy is still missing (Amigorena and Savina 2010).

An alternative hypothesis to an escape of phagocytosed proteins from the endosomal compartment was brought up by the finding that a defect in lipid body metabolism led to reduced XP (Bougneres et al. 2009). Lipid bodies are cytosolic compartments that are surrounded by a membrane monolayer. During lipid metabolism these vesicles tend to fuse with phagosomal membranes, a process that generates regions of membrane instability and thereby allows the release of phagosomal content into the cytoplasm (Ploegh 2007).

The “vacuolar pathway” is the second route to process and present exogenous antigens on MHC class I (Scheme 1D). As shown for some bacterial, but also other antigens, XP can be independent of proteasomal inhibition, but sensitive to Inhibitors of lysosomal proteases, the cathepsins (Shen et al. 2004, Palmowski et al. 2006). The vacuolar pathway has great similarity with the MHC class II presentation pathway. Endocytosed antigens are stored in endosomes, which convert into lysosomes by acquisition of proton ATPases that lower the luminal pH. This drop in pH activates the acidic proteases of the lysosome and leads to the degradation of internalized antigens, which are then presented in the context of MHC class I. Loading of peptides following the vacuolar pathway is thought to occur either after internalization of MHC molecules from the cell membrane via a recycling mechanism. The vacuolar pathway is not only proteasome independent, but also insensitive to an inhibition of TAP and Golgi. Both pathways, the “cytosolic”- and the “vacuolar pathway” are still under intensive research, because major mechanistic details are still to be elucidated, especially *in vivo* (Amigorena and Savina 2010).

The nature of cross-presented antigens

What pathway a distinct antigen will utilize during XP is still largely unknown and hard to predict. However, the “nature” of antigen, namely its physiological state and context are thought to be pivotal for preferences in processing pathways and to a large extent also for the efficiency of XP (Amigorena and Savina 2010). Different antigenic material can be a source of cross-presented antigen. Most relevant under physiological conditions is however the XP of cell-associated proteins originating either from intracellular pathogens, especially viruses, or altered self-antigens during the development of cancer. However, independent of the antigen source there is a debate to what extent full-length proteins and smaller degradation products contribute to the overall cross-priming (Srivastava and Amato 2001, Rock and Shen 2005).

Three experimental reports provide evidence that full-length proteins are the major physiologic source of cell-associated antigen being cross-presented. The first finding described that the antigenicity of a donor cell lysate, when injected *in vivo,* correlated with the subcellular localization of the full-length protein. In case the antigenic protein was targeted to the cell surface, membrane preparations were able to elicit immune responses, whereas isolated cytoplasm was not (Shen and Rock 2004). The second line of evidence was provided by publications showing that the antigenicity of a donor cell lysate correlates with the stability of the antigenic protein (Norbury et al. 2004, Wolkers et al. 2004, Basta et al. 2005). The efficiency of XP was shown to increase with rising antigen stability. The third evidence that shows the importance of full-length protein was the fact that proteasomal inhibition in antigen donor cells does not inhibit XP, but in contrast is able to enhance its efficiency (Norbury et al. 2004, Basta et al. 2005). At the same time the expression of a mini-gene encoding the antigenic peptide in donor cells was sufficient for direct-presentation but not XP (Serna et al. 2003).

Despite the strong evidence for full-length proteins as antigens for XP, there were other independent investigations showing that also degradation products, especially peptides, can be efficiently cross-presented when associated with the chaperon family of heat-shock proteins (HSPs). HSPs isolated from tumor tissues were able to

elicit immune responses in mice and led to tumor regression of related cancers (Udono and Srivastava 1993). After this early publication, several other studies have shown similar potential of HSPs and indicate that the induced immune response is mediated by antigenic peptides bound to the surface of HSP molecules (Srivastava et al. 1994, Suto and Srivastava 1995, Blachere et al. 1997, Singh-Jasuja et al. 2000b, Berwin et al. 2002b, Kurotaki et al. 2007). According to this, cell lysates lose their antigenicity after depletion of major HSPs (Binder and Srivastava 2005a). Finally, the knock-out of heat-shock factor-1 (HSF-1), a major transcription factor of HSPs, led to reduced XP capacity in mice (Zheng and Li 2004).

The two experimental lines describing the XP of cell-associated antigen both are well documented, but are at the same time highly controversial. However, this discrepancy may in part be due to different experimental approaches and antigens used. Nevertheless, more research is required to evaluate the default mechanisms of XP, depending on the antigenic source and the nature of the antigen.

Model antigens in immunological research

Many experimental systems in immunological research utilize model antigens that are extremely well characterized in terms of protein stability, embedded antigenic epitopes, and other immune relevant parameters. Although this habit partially impedes the direct transfer of laboratory findings into the clinics, it has great advantages because of laboratory methods that are available. The quantification of antigen presentation on either MHC class I or II for example is an important issue to evaluate the efficiency of different antigen processing pathways, to study viral function, and to elucidate the underlying immunological mechanisms of vaccination. Levels of antigen presentation can be compared between wild type cells and others that originate from KO animals or that were treated with inhibitory molecules. This way, the contribution of cellular proteins, cell populations, or signaling pathways to antigen presentation can be estimated. The quantification of MHC/peptide complexes on the cell surface, however, is a methodical challenge. The TCR of MHC/peptide-specific T-cells is the natural ligand of antigen presenting cells. The fact that TCR-mediated T-cell activation correlates with the amount of MHC/peptide complexes presented is the basis for read-out systems that are able to quantify antigen

presentation. Intracellular cytokine staining for IFN-γ or IL-2 is a standard method to measure $CD8^+$ T-cell activation in responses to MHC class I presentation, a technique that requires the generation of specific T-cell lines to recognize an antigen of choice. This is only one example to explain why immunological research often sticks to a specific antigen, once a read-out system has been established.

One model antigen that accompanied immunologists since many years is the well characterized 45kDa chicken egg white protein ovalbumin (OVA) that is non-toxic, non-infectious, and can be cost efficiently produced in large amounts (Nisbet et al. 1981). During the last decades numerous OVA-specific laboratory tools were established that allow the investigation of complex immunological questions. A prominent and very helpful tool are for example OVA-specific T-cell hybridomas that allow quantification of MHC presentation (Karttunen et al. 1992a). These hybridoma cells were used in numerous studies to investigate direct- and XP (Berwin et al. 2001, Schliehe et al. 2011). Also commercially available OVA-specific MHC tetramers, an alternative method to quantify MHC presentation, or OVA-specific transgenic T-cell mouse models contribute to popularity of OVA as model antigen (Hogquist et al. 1994). Nevertheless, OVA represents everything but a physiological antigen and the relevance of results observed are often a concern of discussion among immunologist. Therefore, tools for the investigation of more physiological antigens were established as well, but do not yet reach the possibilities availably for the OVA system.

Another antigen that has been studied intensively is the nucleoprotein (NP) of the lymphocytic choriomeningitis virus (LCMV), a negative strand RNA-virus that belongs to the family *Arenaviridae* (Rowe et al. 1970, Oldstone 2002). First described in the mid 1930^{th} by C. Armstrong and colleagues (Amstrong and Lillie 1934, Traub 1935), LCMV became a well studied model system to investigate anti-viral immune responses. The infection of cells by LCMV is mediated via the cellular receptor α-dystroglycan (Cao et al. 1998), which leads to an uptake of virions into vesicular structures. LCMV is a non-cytopathic virus and the viral particles emerge by budding from the host cell (Modrow et al. 2003). The general structure of LCMV that can provoke lymphocytic choriomeningitis in mice, can be divided into the viral envelope and the nucleocapsid containing genetic information (Dalton et al. 1968). Beside

others, the most important proteins in terms of immune responses against LCMV are the glycoproteins GP1 (40-46kDa) and GP2 (35kDa) (Riviere et al. 1985, Borrow 1997) and the nucleoprotein (NP, 62kDa) that stabilizes the viral genome.

The characteristics of an ongoing LCMV infection is determined by multiple factors including route of infection, viral load, and genetic background of mice (Buchmeier et al. 1980). The injection of sublethal doses of LCMV leads to a rapid viral replication in mice, the induction of strong cytotoxic T-cell responses, and a final clearance of the virus with long-lasting immunological memory (Moskophidis et al. 1987). In C57BL/6 mice the immune response to LCMV is dominated by different epitopes (Kotturi et al. 2007). Two of them, the GP_{33-41} (H2-D^b) and GP_{34-41} (H2-K^b) are derived from the glycoprotein, whereas a third is derived from the nucleoprotein $NP_{396-404}$ (H2-D^b) (van der Most et al. 1996). Beside the immune response to these dominant epitopes there are numerous subdominant epitopes, including $GP_{276-286}$ and GP_{92-101} (H2-D^b), as well as $GP_{118-125}$ (H2-K^b) (van der Most et al. 1996, Kotturi et al. 2007, Masopust et al. 2007). A different situation was observed for BALB/c mice. Here, the NP-derived epitope $NP_{118-126}$ (H2-L^d) is dominating the CTL responses (van der Most et al. 1996, Gallimore et al. 1998).

Many groundbreaking immunological findings concerning direct- and cross-presentation were based on studies using the long-lived LCMV NP as model antigen (Rodriguez et al. 1997, Basta et al. 2005). Due to the strong immunological memory induced by LCMV, NP-specific T-cell lines can easily be raised from spleens of infected memory mice and by this providing an excellent readout system to study presentation of LCMV-derived epitopes. Furthermore, NP-specific hybridoma cell lines were developed and antibodies produced for the detection of proteins via flow cytometry or immunoprecipitation (Schwarz et al. 2000a, Schwarz et al. 2000b, Khan et al. 2001b). NP-expressing cell lines were used to investigate the cross-presentation of cell-associated antigen (Basta et al. 2005). Even the mechanisms of autoimmune diseases, like diabetes, were investigated with transgenic mouse models, based on the expression of LCMV-derived proteins in insulin producing cells of the pancreatic islets (von Herrath et al. 1994).

Novel approaches in immunotherapy

Treatment of diseases that do not primarily target the syndrome itself, but try to modulate the immune system to stimulate self-healing of the organism, are summarized under the term “immunotherapy”. This modulation can comprise the initiation, enhancement or suppression of an immune response against disease related antigens (Waldmann 2003).

Classical vaccination is one of the oldest forms of immune modulation with the aim to induce protective immunity against a certain infection. Disease-related antigens are delivered to the organism in form of attenuated pathogens or purified components mixed with adjuvant molecules (Waldmann 2003). For many infectious diseases, including measles, mumps, or rubella, this strategy was extremely successful and provided efficient vaccination protocols (Knuf et al. 2008). However, classical vaccines often induce B-cell-dominated immune responses, which are favorable to neutralize viral particles or opsonize extracellular pathogens, but can be a limitation for approaches where $CD8^+$ T-cells responses are required. This is the case for the induction of immune responses against autologous tissues for immunotherapy of cancer. There is a demand for novel vaccines that are able to elicit effective $CD8^+$ T-cell responses even in situations, where self-tolerance has to be broken (Storkus and Falo 2007). The use of patient-derived cell that are manipulated *in vitro* and re-injected *in vivo* is an example for alternative methods (Basler and Groettrup 2007a). Classical vaccines are also not able to actively decrease immune responses, as a major goal for immunotherapies against auto-immune diseases or allergies. Here, uncontrolled and dangerous immune reactions have to be down-regulated or, ideally, stopped (Miller et al. 2007).

Ideas to conduct immune responses to special requirements often utilize immune regulatory molecules, called immune modulators. This term also includes the adjuvants used for the classical vaccination against infectious diseases, but can be extended to other immune active molecules, such as interferons, chemokines, or other cell differentiation signal molecules (GM-CSF, G-CSF) (Berinstein 2007). However, immune modulators alone were not able to overcome the limitations of classical vaccination. This approach requires the development of novel antigen

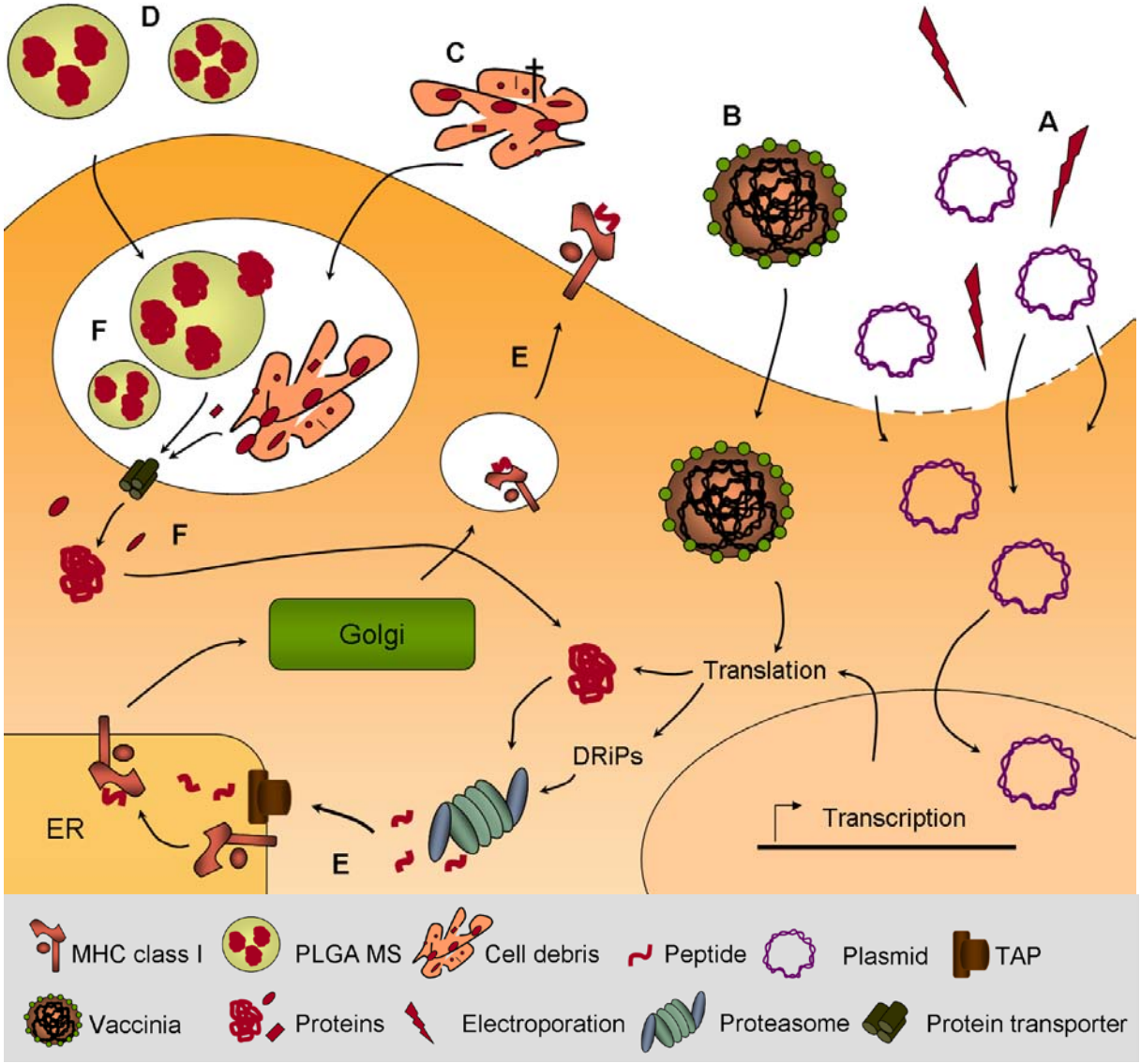
delivery systems that provide the immunological triggers with regard to the respective purpose.

The last part of this *introduction* will concentrate on three promising candidate vaccines that are currently under investigation to be applied for the induction of $CD8^+$ T-cell responses against intracellular pathogens and cancer (*Scheme* 2).

A: Biodegradable poly(lactic-co-glycolic) acid microspheres

The induction of $CD8^+$ T-cell-mediated immune responses can be achieved by delivery of exogenous antigens to cross-priming APCs. In case of immunotherapy against cancer, antigens of malignant tissues can be identified and either purified from *ex vivo* samples or recombinant expression systems. However, it was shown that soluble antigens are generally cross-presented very inefficiently and can induce tolerance, if administered in high doses (Heath and Carbone 2001b). Therefore, antigen coupling to particles in the low nanometer to micrometer range is an efficient strategy to deliver proteins for phagocytotic uptake (Jain 2000).

Biodegradable poly(lactic-co-glycolic) acid microspheres (PLGA MS) comprise ideal properties for such an antigen deliver to APCs. They are spherical particles ranging from 0.5 to 5µm in diameter and are entirely biocompatible (Jain 2000). After uptake they degrade into lactic acid and glycolic acid, which are metabolites of the citric acid cycle (Brady et al. 1973). Antigens can be encapsulated into PLGA MS in a process called “spray drying” (Gander 2005), a method that allows the co-encapsulation of antigens with adjuvant molecules (Heit et al. 2007, Schlosser et al. 2008a). After injection into mice and uptake by APCs, PLGA MS slowly hydrolyze and constantly release the encapsulated content for several weeks (Audran et al. 2003b, Waeckerle-Men et al. 2006). This depot effect and the possibility of co-encapsulating adjuvants, like the TLR9 ligand CpG, make PLGA MS a powerful tool for the induction of immune responses against cancer (Waeckerle-Men et al. 2006, Schlosser et al. 2008a, Mueller et al. 2011). Interesting, due to the controlled release, booster injection were not required in these cases. In addition to the usage of purified antigens, successful immunizations were also performed with encapsulated tumor lysate, which shows the



Scheme 2: MHC class I antigen presentation following DNA vaccination, vaccinia virus infection, and injection of PLGA microspheres in antigen presenting cells (APCs). (A) Electroporation of plasmid DNA in DNA vaccination leads to cellular uptake of genetic information into the cell. Plasmids are transcribed in the nucleus and translated into proteins or defective ribosomal products (DRiPs) in the cytoplasm. These are degraded by the proteasome and enter the "direct-presentation" pathway via ER and Golgi to the cell surface, where MHC/peptide complexes are presented (E). (B) Infection of APCs with vaccinia virus leads to the entry of viral particles into the cytoplasm were transcription and translation of viral proteins occur. Viral proteins then enter the direct-presentation pathway (E). Also non-APCs can be transfected by DNA or the infection with vaccinia virus. In this case exogenous antigens have to cross-presented by APCs in order to activate T-cells. (C) Cross-presentation of cellular debris from transfected or infected non-APCs. Cell debris is endocytosed and antigens escape to the cytosol to enter the direct-presentation pathway (F). (D) Phagocytotic uptake of PLGA microspheres. Encapsulated proteins are released in the endo/lysosomal compartment and escape from lysosomal degradation by translocation to the cytosol, proteins can enter the direct-presentation pathway (F).

potential of PLGA MS for individualized tumor therapy (Solbrig et al. 2007).

Although PLGA MS are used as a drug delivery system in the clinics for years, they are not approved as a tool for human immunotherapy yet. However, there are strong pre-clinical data showing the successful use of PLGA MS as protective and also therapeutic anti-cancer vaccine in animal models (Mueller et al. 2011). Interestingly, the cell types required for cross-priming of PLGA MS-encapsulated antigens as well as the intracellular pathways of XP were not elucidated completely and were therefore task of this thesis.

B: DNA vaccination

DNA vaccines are an alternative tool to PLGA MS in inducing $CD8^+$ T-cell responses for the defense against infectious diseases and the treatment of cancer (Fioretti et al. 2010). Vaccination protocols are based on the injection of naked plasmid DNA that encode for specific disease-associated antigens. Over the last decades it was shown that DNA vaccines have advantages in comparison to conventional antigen delivery systems. In contrast to these, DNA vaccination does not require the purification of antigens, but utilizes the protein translation machinery of recipient to produce recombinant proteins. After administration, the plasmid DNA is taken up by cells localized around the injection side (Donnelly et al. 2000). Following translation of antigens, degraded peptides are presented on MHC class I by either direct-presentation (transfection of APCs) or XP (transfection of tissue-specific cells) to prime specific CTLs (Scheme 2A). Additionally, DNA vaccines were also shown to induce humoral immune responses, which require peptide presentation on MHC class II and the activation of B-cells (Liu 2003, Raska et al. 2004, Raska et al. 2005).

Some of the advantages of DNA vaccines are their relatively cheap production and the independence of a functional cold chain (Giese 1998, Gurunathan et al. 2000). This makes them an ideal tool for vaccination approaches in developing countries, where the infrastructure does not allow the use of conventional vaccines. However, compared to classical vaccination, the overall immunogenicity of DNA vaccines is rather low. Injection of pure naked DNA into the muscle only leads to weak expression of the antigen and also to low CTL responses (Belakova et al. 2007).

Therefore, an increased immune-stimulatory potential of DNA vaccines is the primary goal of current research (Rice et al. 2008). The inefficiency is in part due to the low transfection rate of target cells by. Extracellular DNA is rapidly degraded by nucleases or cleared by phagocytes what makes efficient transfection an important issue. Current strategies to overcome this problem are either the physical delivery of plasmid DNA into the target cells or local manipulations at the injection side that can enhance cellular uptake (Luo and Saltzman 2000). Some of these approaches are electroporation, sonoporation, magnetofection, delivery via gene gun (DNA attached to gold particles), or tattooing (O'Hagan and Valiante 2003). In recent years, more and more evidence came up, showing that electroporation might be the most efficient tool to enhance immunogenicity of DNA vaccines (Wang et al. 2004). At least two effects are responsible for such enhanced immune responses after electroporation. On the one hand, the electric impulses that are delivered around the side of injection directly increase the uptake of plasmid DNA into the target cells, which leads to enhanced transfection efficiency and higher antigen expression. On the other hand the electric intrusion leads to a local inflammatory response via the release of pro-inflammatory cytokines (Chiarella et al. 2008). This way, the increased antigen production is combined with an inflammatory environment that can recruit and mature APCs and increase priming of immune effectors (Signori et al. 2010).

Apart from its delivery, also the DNA construct itself can be optimized, either by modification of the antigen or by co-expression of adjuvant molecules. Plasmids were optimized by including multiple expression cassettes for the simultaneous expression of antigens and adjuvant molecules, specialized promoter sequences, or nuclear targeting motives (Rinaldi et al. 2008). As an example, the introduction of a viral promoter from cytomegalovirus (CMV) upstream of the antigen sequence allowed antigen over-expression in a wide range of tissues and is therefore a commonly method to enhance expression rates (Montgomery et al. 1994). Also modification of the antigen sequence by codon optimization (Nagata et al. 1999, Deml et al. 2001) or addition of CpG motives (TLR-9 ligand) led to increased immunogenicity (Kojima et al. 2002, Coban et al. 2005). With the help of fusion proteins, intracellular localization, as well as antigen stability can be modified. Although the role for antigen stability during DNA vaccination is not completely elucidated yet, there were attempts to increase immunogenicity by linear fusion of antigens to ubiquitin (Rodriguez et al.

1997). If these findings can be independently reproduced, other protein fusions that alter the half-life of an antigen might also be efficient in enhancing immunogenicity of DNA vaccines. One candidate for such an approach is the ubiquitin-like modifier HLA-F associated transcript 10 (Fat10) (Liu et al. 1999). It is the only ubiquitin-like modifier so far identified that is, like ubiquitin, able to target proteins for proteasomal degradation (Hipp et al. 2005). Since the location of genes in the HLA regions usually correlate with a role in immunological function, the question emerged whether Fat10 could serve as molecular tool to target antigens for proteasomal degradation and antigen presentation (Fan et al. 1996). Indeed, it was shown that Fat10, which is not expressed in normal tissues, can be up-regulated by pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) (Raasi et al. 1999). This and the fact that Fat10 is constitutively expressed in organs of the immune system like spleen, lymph nodes, gut, and especially the thymus strengthens its possible role in immune responses (Lukasiak et al. 2008). Different publications suggest FAT10 to be involved in apoptosis (Raasi et al. 2001, Ross et al. 2006), formation of aggresomes (Kalveram et al. 2008), development of malignancies (Lee et al. 2003, Ren et al. 2006), or cell cycle regulation (Liu et al. 1999). However, the biological function of FAT10 remains to be further investigated. In this thesis, we used Fat10 NP fusion proteins, to study the role of Fat10 in antigen processing.

Taken together, DNA vaccines are an emerging field in immunotherapy and represent an alternative approach to protein-based delivery systems, like PLGA MS. Especially the development of novel strategies to enhance the intrinsic immunogenicity of DNA preparations is an important issue in immunotherapy and was therefore part of this thesis.

C: Recombinant vaccinia virus

In contrast to DNA vaccinations with low intrinsic immunogenicity, approached based on viral expression systems show efficient target cell transfection and maturation of APCs. The initiation of $CD8^+$ as well as $CD4^+$ T-cell responses and the induction of an immunological memory makes them promising tools for effective treatment of various diseases (Liu 2010). A group of viral candidate vaccines that belong into this category are recombinant vaccinia viruses (VV) (Scheme 2B).

VV belongs to the family of *Poxviridae* and are commonly known because of their use as vaccine against the related smallpox virus in humans. Even though cowpox virus was used as first vaccine against smallpox by Jenner, vaccinia is not the causing agent of cowpox, but a close relative possibly originating from infected horses (Huygelen 1996). Infection of humans with VV during a smallpox vaccination usually leads to a local infection, including the development of a pock and scar formation after wound healing. Vaccinia is a cytopathic virus that contains a linear double stranded DNA genome of roughly 170kbp, encoding for about 200 different proteins (Goebel et al. 1990) that stabilize both the viral genome and membranes (Cyrklaff et al. 2005, Heuser 2005). It can infect numerous cell types including dendritic cells and the entry of the virus into host cells is realized by protein-mediated fusion of the viral membrane with the host membrane (Senkevich et al. 2004). After infection and viral replication, the great majority of infectious particles remains in the host cells and is only released after cell lysis (Moss 2006). Interestingly, immune responses after infection with VV induce both the inert and the adaptive immune system. Pro-inflammatory cytokines at the beginning of an infection activate phagocytes, the complement system, and also NK-cells. Elimination of the virus, however, is realized by cells of the adaptive immune system. $CD8^+$ cytotoxic T-cells, as well as $CD4^+$ T_H-cell and antibody responses lead to an efficient clearance of the virus around one week after infection (Xu et al. 2004). The immunodominant MHC class I epitope in H-2^b mice is $B8R_{20\text{-}27}$, which is responsible for around 25% of the $CD8^+$ T-cells response (Moutaftsi et al. 2006). However, more than 45 different vaccinia epitopes were characterized that contribute to the overall response (Tscharke et al. 2005, Moutaftsi et al. 2006).

After the eradication of smallpox, vaccinia became less important as a vaccine, but was re-discovered as a promising vector for recombinant protein expression in mammalian cells. Recombinant VV are generated by relatively simple manipulations and up to 25kbp of foreign DNA can be integrated into the viral genome without changing the infectivity. This, together with the low infection selectivity, makes recombinant VV an excellent tool for the introduction of recombinant genes into a cell of interest (Guo and Bartlett 2004). However, it was the immunological research, in which recombinant VV had the most prominent impact. Basic research on the

activation of T- and B-cells was performed using vaccinia (Kennedy et al. 2009). At the same time recombinant VV have the potential to become a powerful vaccine in terms of cancer and other infectious diseases (Drake 2010). The introduction of antigens from malignant tissues or certain pathogens into the genome of the virus leads to the expression of the protein in host cells. This way the immunogenicity of vaccinia is utilized to induce immune responses and immunological memory against a recombinant antigen (Moss 2011).

Similar to DNA vaccines, the molecular mechanism involved in the induction of immune responses following VV infection are not completely understood. Especially the induction of $CD8^+$ T-cells responses is discussed controversially and was therefore further investigated in this thesis.

Aim of the thesis

The aim of this thesis can be summarized by three lines of experimental approaches:

In *chapters I and II* we aimed at understanding the underlying cellular mechanisms that are involved in the cross-presentation of PLGA MS-encapsulated antigen. Especially, we wanted to understand to what extend individual population of APCs contribute to the initiation of MS-specific immune responses *in vivo*. Therefore the role of DCs and MΦs, as well as two different subclasses of DCs, the $CD8^+$ and the $CD8^-$ DCs should be analyzed. In parallel we wanted to investigate, whether PLGA MS enter the cytoplasm after endocytosis or if they are stored in lysosomal vesicles. This question should be followed by electron- and confocal fluorescence microscopy.

In *chapter III* we planned to elucidate the role of antigen stability for the activation of immune responses after DNA vaccination and infection with recombinant VV. By comparing the presentation of short-lived versus long-lived antigens, we tended to find evidence for a major contribution of either direct- or cross-presentation, since both pathways have distinct antigen preferences.

Last but not least, in *chapter IV* we wanted to provide further mechanistic insides into the cross-presentation of cell-associated antigen, as it occurs after transfection of

DNA vaccines or infection with VV (Scheme 2C). A special focus was supposed to be set on the role of heat-shock proteins and other cellular factors that can enhance cross-presentation of cell-associated proteins, compared to purified antigens.

All together this thesis was supposed to provide novel insides into the cross-presentation of promising future vaccine candidates that can be utilized for the generation of improved vaccination protocols.

Chapter I

CD8$^-$ dendritic cells and macrophages cross-present PLGA particle-encapsulated antigen *in vivo*

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In revision in the *Journal of Immunology*

Abstract

The analysis of cell types involved in cross-priming of particulate antigen is essential to understand and improve immunotherapies using microparticles. Here we show that murine splenic dendritic cells (DCs) as well as macrophages (MΦs) are able to efficiently endocytose poly(lactic-*co*-glycolic) acid (PLGA) microspheres (MS) and to cross-present encapsulated antigens in the context of MHC class I molecules *in vitro*. In contrast to DCs, no significant class II restricted presentation by MΦs could be detected. A comparison of purified $CD8^+$ and $CD8^-$ DCs indicated that both DC subtypes are able to present OVA-derived epitopes on MHC class I and II *in vitro*. To determine the contribution of DCs and MΦs to cross-priming of PLGA MS *in vivo*, DCs were depleted in transgenic CD11c-DTR mice and MΦs were depleted by clodronate liposomes in wild type mice before immunising mice with OVA-encapsulated microspheres. Our results show, that the depletion of DCs or MΦs alone only led to minor differences in the OVA-specific immune responses. However, simultaneous depletion of DCs and MΦs caused a strong reduction of primed effector cells, indicating a redundancy of both cell populations for priming of particulate antigens. Finally, we analysed MS trafficking to draining lymph nodes after subcutaneous injection. It was evident that fluorescent MS accumulated within draining lymph nodes over time. Further analysis of MS-positive lymphatic cells revealed that mainly $CD8^-$ DCs and MΦs contained PLGA MS. The results presented in this work strongly suggest that *in vivo* cross-priming of PLGA MS-encapsulated antigen is performed by $CD8^-$ DCs and MΦs.

Introduction

Successful immunotherapy against cancer requires the induction of robust cytolytic T-lymphocyte (CTL) responses in combination with the appropriate immune environment and T-cell help (van der Most et al. 2006). We and others could recently show that injection of antigens encapsulated into biodegradable poly(lactic-*co*-glycolic) acid (PLGA) microspheres (MS) provoke strong CTL responses in mice (Waeckerle-Men et al. 2006) that protected from vaccinia virus infection (Schlosser et al. 2008a), tumor challenge, and lead to regression of already established tumors in therapeutic settings (Heit et al. 2007). The initial priming of CTLs requires MHC class I presentation by professional antigen presenting cells (APCs) that have the unique ability to provide co-stimulation via CD80 and CD86 after maturation (Mellman and Steinman 2001a). In contrast to “direct-presentation” of protein fragments synthesized within the cell, MHC class I presentation of exogenous antigen is based on a second pathway that was first described by M. Bevan in 1976 (Bevan 1976b) and is referred to as cross-presentation (Heath and Carbone 1999, Shen and Rock 2006). MS-encapsulated proteins represent one type of particulate antigen that has to be taken up and cross-presented by APCs in order to initially activate effector CTLs, a mechanism known as cross-priming (Bevan 1976b, Heath and Carbone 1999, Shen and Rock 2006). After phagocytosis, PLGA MS release encapsulated proteins over a long period of time (Tamber et al. 2005, Waeckerle-Men and Groettrup 2005), which enter the cross-presentation machinery. At least three different pathways have been described how antigenic peptides derived from exogenous antigens can be presented on MHC class I, depending on the nature of antigen and other factors (Shen and Rock 2006, Basta and Alatery 2007).

We and others could show that MS-encapsulated proteins are cross-presented in a proteasome and transporter associated with antigen processing (TAP) dependent way, which was sensitive to brefeldin A and independent of lysosomal acidification (Men et al. 1999, Shen et al. 2006). These data suggest an endosomal/lysosomal escape of encapsulated proteins that enter the direct-presentation pathway. This proposed mechanism for cross-presentation of MS-encapsulated antigen is referred

to as the phagosome-to-cytosol pathway (Kovacsovics-Bankowski and Rock 1995, Rodriguez et al. 1999).

Similar to other vaccines that are based on particulate antigen, it is still not completely understood, which APC subtypes are essential for cross-priming of MS-encapsulated antigen *in vivo*. The most prominent bone marrow-derived phagocytes able to provide co-stimulation are dendritic cells (DCs) and macrophages (MΦs), of which the DCs were long time thought to be the only cells type able to induce naïve T-cells *in vivo* (Pozzi et al. 2005). The pool of DCs can be further divided into several subpopulations. A distinction is drawn between conventional DCs (expression of $CD11c^{high}$) and plasmacytoid DCs (expression of $CD11c^{low}$ and B220) (Segura and Villadangos 2009). $CD11c^{high}$ DCs are further subdivided into resident DCs that develop and reside within lymphoid tissues and migratory DCs that differentiate in the periphery and constantly shuttle antigen into the lymphoid organs (Liu et al. 2007, Villadangos and Schnorrer 2007). $Gr1^{high}$ monocytes are able to actively enter inflamed tissue from peripheral blood and to differentiate into MHC class II^{high} $CD11c^{low}$ DCs (Randolph et al. 1999). Initiated by bacterial/viral infections, injection of adjuvants or release of pro-inflammatory cytokines, chemokines like CCL20 and CCL2 are produced by epithelial cells of the inflamed tissue. This finally leads to the recruitment of pro-inflammatory monocytes to the site of infection and to their differentiation into DCs. This mechanism is mediated via the chemokine receptors CCR2 and CCR6 expressed on $Gr1^{+}$ monocytes (Proudfoot 2002, Le Borgne et al. 2006). CCR2 was also shown to mediate homeostatic and inflammatory release of $Gr1^{high}$ monocytes from the bone marrow (Engel et al. 2008). Different DC species can have an impact on cross-priming depending on the nature and source of antigen (Lin et al. 2008a, Segura and Villadangos 2009). However, there is a consensus that the two populations of DCs found in lymphoid organs ($CD8^{+}$/$DEC205^{+}$ and $CD8^{-}$/$CD11b^{+}$), and of these especially the $CD8^{+}$ subtype, are essential for cross-priming of many antigens investigated (den Haan et al. 2000, Pooley et al. 2001, Allan et al. 2003, Belz et al. 2005, Schnorrer et al. 2006, Shortman and Heath 2010). $CD8^{-}$ DCs were published to have an impact on cross-priming of particulate antigen (den Haan and Bevan 2002, Moron et al. 2002). In contrast to DCs, the classification of MΦs ($CD11c^{-}$/$CD11b^{+}$/$F4/80^{+}$ in murine spleens) is less established and their general

impact on cross-priming is still restricted to few examples (Pozzi et al. 2005, Tacke et al. 2006). However, MΦs are the second type of APCs in the periphery, that internalize exogenous antigen and up-regulate the co-stimulatory molecules CD80/86 after being activated (Pozzi et al. 2005) (Figure 1A).

A major progress in showing a pivotal role of DCs for *in vivo* cross-priming was achieved by the generation of a transgenic CD11c-DTR mouse (Jung et al. 2002, Bar-On and Jung 2010). In those mice, the primate diphtheria toxin (DT) receptor is expressed under control of the CD11c promoter and consequently expressed exclusively on DCs. Injection of DT leads to *in vivo* depletion of DCs (Jung et al. 2002, Probst et al. 2005) and is therefore a powerful tool to investigate the role of DCs for the early immune response. The CD11c-DTR mouse was used to show the importance of DCs for cross-priming various model antigens (Jung et al. 2002, Probst and van den Broek 2005, Kassim et al. 2006). A different strategy was developed to study the *in vivo* impact of MΦs, which is based on clodronate liposome injection (Van Rooijen and Sanders 1994). Liposomes are taken up by phagocytes and accumulation of clodronate leads to MΦ-specific apoptosis (Van Rooijen and Sanders 1994, van Rooijen et al. 1996). Although, this way of MΦ depletion has been successfully applied for years, there are hardly any publications directly addressing the questions of cross-priming.

In this study we aimed to elucidate which populations of murine APCs are responsible for cross-priming of CTLs after vaccination with PLGA MS. Therefore we isolated $CD8^+$ and $CD8^-$ DCs as well as $CD11b^+$ MΦs and analysed their capacity to present MS-encapsulated antigen on MHC class I and II *in vitro*. While both DCs subpopulations were able to present epitopes derived from MS-encapsulated OVA on class I and II, isolated MΦs exclusively presented OVA-derived peptides on MHC class I. CD11c-DTR mice and clodronate liposome treatment were used to study the role of DCs and MΦs on MS cross-priming *in vivo*. We found that DCs and MΦs act in a redundant way, indicating that both APCs are able to cross-present MS-derived antigen *in vivo*. Additionally, we analysed MS-trafficking to the draining lymph nodes and show that $CD8^-$ DCs and MΦs are the major populations that are found in association this MS.

Materials and Methods

Mice, cells and media

C57BL/6 (H-2^b) mice were originally obtained from Charles River Laboratories and further bred in the animal facilities of the University of Konstanz. Transgenic CD11c-DTR and CCR$2^{-/-}$ mice were on the C57BL/6 background and a kind gift from C. Kurts (Institute for Molecular Medicine and Experimental Immunology (IMMEI) Bonn, Germany). CCR$6^{-/-}$ mice were of C57BL/6 background and kindly provided by U. Panzer (Universitätsklinikum Hamburg, Germany). All animals were kept under pathogen-free conditions in accordance with the rules of the veterinarian authority of Regierungspräsidium Freiburg and sacrificed at 6-12 weeks of age.

All cell culture media were obtained from Gibco, Invitrogen. Murine splenocytes were cultured in RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin/streptomycin (P/S). The B3Z $CD8^+$ T-cell hybridoma cell line, specific for the SIINFEKL ($Ova_{257-264}/K^b$) peptide of ovalbumin was a kind gift from N. Shastri (University of California, Berkeley, USA) and cultured in $IMDM^+$ +10% FCS + P/S (Karttunen et al. 1992a). The $CD4^+$ T-cell hybridoma DOBW specific for the class II epitope ISQAVHAAHAEINEAGR ($OVA_{323-339}$) was cultured in $D\text{-}MEM^+$ supplemented with 10% FCS, 100 U/ml P/S, 1 mM sodium pyruvate, 10 mM HEPES, 0.5 mM β-mercapto-ethanol and was kindly contributed by C.V. Harding (University School of Medicine, St. Louis, USA) (Harding et al. 1991).

Preparation of poly(lactic-co-glycolic) acid microspheres

MS were prepared from PLGA resomer RG502H (Boehringer Ingelheim, Germany). Ovalbumin and CpG were microencapsulated by spray drying as described elsewhere (Waeckerle-Men et al. 2005b, Schlosser et al. 2008a). Briefly, 50 mg ovalbumin (Grade V, Sigma) and 5 mg CpG oligonucleotide (1826, Microsynth, Balgach, Switzerland) were dissolved in 0.5 ml aqueous medium (aqueous phase) and mixed with 16 ml 5% PLGA in dichloromethane (organic phase). The two phases were emulsified by ultrasonication (Hielscher, UP200 H, Ampl. 40%) for 10 s on ice. The dispersion was spray-dried (Mini Spray-Dryer B-290, Büchi, CH-Flawil) at a flow rate of 2 ml/min and inlet/outlet temperatures of 40/38 °C. MS were harvested using

0.05% Synperonic® F68 (Serva, Germany), collected on cellulose acetate membrane filter and dried under reduced pressure (20 mbar) for 18 h. Immediately before use, MS were dispersed in phosphate buffered saline (PBS) or media by ultrasonication for 1 min. The covalent fluorescein labeling of resomer RG502H was adapted from (Weissenboeck et al. 2004, Yin et al. 2007). Briefly, resomer was activated by mixing 6 ml of each, 5% RG502H, 0.4% N-hydroxysuccinimide (NHS; Sigma-Aldrich, Switzerland) and 0.51% N, N´-dicyclohexylcarbodiimide (DCC; Sigma-Aldrich, Switzerland), dissolved in dichloromethane and inverting for 2 h at RT. Afterwards, 6 ml of 0,15% fluorescein cadaverine (Invitrogen, USA) in dichloromethane and 88 µl pyridine (Sigma-Aldrich, Germany) were added and further incubated overnight. Labelled polymer was washed using 5 mM HCl, precipitated by methanol, lysophilised and stored at 4°C until use. For the generation of fluorescein-labelled microspheres 35% labelled resomer was used according to the protocol described above.

CdSe/CdS/ZnS quantum dots were prepared in a one pot hot injection synthesis as described elsewhere (Mekis et al. 2003a, Talapin et al. 2004b), using a TOP/TOPO/HDA stabilizing mixture. MS labelled with fluorescent quantum dots (emission wavelength 583 nm, QD583) were generated by adding QD583 into the dichloromethane phase of the spray drying process described above. Ovalbumin and CpG were co-encapsulated as for non-fluorescent microspheres.

Preparation of splenocytes for cell sorting

5-10 mice per experiment were sacrificed and spleens collected in medium containing 2mg/ml collagenase D (ROCHE, Mannheim, Germany). The same medium was injected into the spleens before homogenization and incubation at 37°C for 30 min. The cell suspension was filtered and pre-purified by gradient centrifugation (Ficoll-Paque^{TM}PLUS; Amersham Biosciences, Germany).

DCs and MΦs were magnetically isolated by using commercially available micro beads (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's protocol. Briefly, single cell suspensions were incubated with CD11c beads for 15 min at 4°C, washed and loaded onto magnetic columns. The flow

through was collected and sorted for $CD11c^-/CD11b^+$ MΦs using CD11b micro beads. Positively sorted $CD11c^+$ and $CD11b^+$ cells were eluted, washed with PBS, and stored on ice until used for experiments.

$CD8^+$ and $CD8^-$ DCs were purified from the $CD11c^+$ elution of magnetically isolated splenocytes, using fluorescent activated cell sorting (FACS) (BD FACSAria™ II). The cells were labeled with FITC anti-CD11c and TriColor anti-CD8 before sorting. $CD11c^+/CD8^+$ and $CD11c^+/CD8^-$ cells were collected. The purity of all sorted cell types was analyzed by flow cytometry using FITC-coupled anti-CD11c, FITC-coupled anti-mouse CD11b, and TriColor-coupled anti-CD8. All antibodies were purchased from BD Pharmingen™.

Detection of antigen presentation by LacZ T-cell hybridoma assay

Antigen cross-presentation of $Ova_{257-264}$ was detected using the CD8 T-cell hybridoma cell line B3Z that expresses β-galactosidase under control of the interleukin-2 (Il-2) promoter (Karttunen et al. 1992a). $1x10^5$ hybridoma cells were incubated with $2x10^5$ APCs per well of a 96-well plate. Cells were co-incubated with 25 µg/well of either microspheres containing ovalbumin (MS OVA) or empty MS as control (MS empty). After 18 h of incubation at 37°C 7% CO_2 plates were washed with PBS and 100 µl of LacZ-buffer [0.13% NP40, 9 mM $MgCl_2$, 0.15 mM chlorophenolred-β-D-galactopyranoside (CPRG) (Roche, Germany) in PBS] was added for up to 4 h at 37°C. Absorbance was measured at 570/620nm using a Spectro Fluor Plus spectrometer (Tecan, Germany). The experiments using the $OVA_{323-339}$-specific class II hybridoma cell line DOBW were performed as described above for B3Z. After incubating the cells for 18 h, IL-2 secretion was measured from cell supernatants to quantify class II presentation by using the BD OptEIA™ mouse IL-2 ELISA kit (BD Biosciences, USA). Absorbance was detected at 450/570 nm.

In vivo cytotoxicity assay

Mice were immunized subcutaneously (*s.c.)* by injection of 5 mg MS containing OVA and CpG in 200 µl PBS at the base of the tail as described earlier (Schlosser et al. 2008a). On day 4 or 5 after injection (as indicated) splenocytes from naïve donor mice were prepared and incubated for 1 h at 37°C in the presence or absence of

10^{-6} M SIINFEKL peptide. After intensive washing with PBS, cells were labeled with two intensities of carboxyfluorescein succinimidyl ester (CFSE) for 10 min at 37°C, using 0.75 µM ($CFSE^{low}$) for control and 7.5 µM ($CFSE^{high}$) for peptide pulsed cells. Labeled cells were washed, resuspended and mixed at a ratio of 1:1 in PBS and $2x10^7$ labeled splenocytes in 200 µl were injected *intravenously* (*i.v.).* After 16-18 h mice were sacrificed, spleen cell suspensions were prepared, and the ratio of CFSE labelled cells analyzed by flow cytometry. For the detection of cytotoxic activity in the CD11c-DTR mice, donor cells were additionally stained with CellVue® Clared (Polysciences, USA) to avoid overlapping of the CFSE signal with transgenic DCs expressing GFP. This protocol and the calculation of specific cytotoxicity were adapted from (Dunbar et al. 2007). Specific cytotoxicity = (1 – [ratio naive /ratio immune]) x 100, and ratio = $\%CSFE^{low}$/ $\%CSFE^{high}$.

Depletion of dendritic cells and macrophages

For the depletion of DCs, transgenic CD11c-DTR mice were used. These mice express the primate diphtheria toxin receptor under control of the CD11c promoter and treatment with diphtheria toxin (DT; Sigma, Germany) leads to depletion of $CD11c^+$ cells (Jung et al. 2002). The *in vitro* depletion of DCs after magnetic sorting was performed by adding 1 µg/ml DT to the cells during hybridoma assay. Splenocytes of C57BL/6 mice were used as controls. *In vivo* depletion of DCs was carried out 18 h prior to MS challenge by *i.v.* injection of 100 ng DT per mouse (Jung et al. 2002). Injection of DT into C57BL/6 mice served as control.

Clodronate liposomes (CL) to deplete MΦs *in vivo* were prepared as described elsewhere (Van Rooijen and Sanders 1994). Clodronate for liposome production was a kind gift from Roche Diagnostics GmbH (Mannheim, Germany). CL or control liposomes containing PBS (PBS-L) were administered 18 h prior to the MS treatment. To deplete MΦs in the draining lymphatics as well as in the blood system and spleen, 200 µl of liposome solution was injected twice per mouse; *i.v.* and at the base of the tail. Successful depletion was monitored by staining of splenocytes with FITC anti-F4/80 antibodies, followed by flow cytometry.

Analysis of microsphere trafficking to the draining lymph nodes

5 mg OVA/CpG microspheres, labelled with QD583 (left hind leg) and non-fluorescent OVA/CpG microspheres as control (right hind leg), were injected into the footpads of the same animal. After 1 to 5 days post injection *lymphonodi popliteus* were taken and grated to single cells suspensions. Samples were stained with FITC anti-CD11c, APC anti-CD11b in order to separate DCs and MΦs and PacificBlue anti-CD8 to distinguish between $CD8^+$ and $CD8^-$ DCs. Antibodies were obtained from BD Bioscience. Staining was performed for 20 min on ice before samples were fixed with 4% paraformaldehyde, washed with PBS and analysed by flow cytometry (BD FACSAria™ II). Fluorescent quantum dots were detected in the PE channel. Autofluorescent cells were excluded using the PE-Cy5 channel. Kinetics of MS trafficking was performed by using 4 mice per group. For acquisition the lymph nodes from two mice were pooled.

Results

Uptake of fluorescein-labeled microspheres by primary murine splenocytes

The uptake of MS-encapsulated antigen by APCs is the first essential step in the cascade of antigen processing pathways, finally leading to successful priming of immune effectors. In order to investigate what cell types are able to internalize microspheres we incubated primary splenocytes from C57BL/6 mice with PLGA particles covalently labeled with fluorescein cadaverin. These fluorescent MS allowed the characterisation of phagocytotic cells by performing co-stainings for various marker molecules (Fig. 1B). As expected, fluorescein-labeled MS were almost exclusively taken up by MHC class II positive cells, representing the pool of professional APCs. Further analysis showed that both DCs ($CD11c^+$) and macrophages ($CD11c^-$/ $CD11b^+$) actively internalize MS. Only low numbers of $CD11c^-$ $CD8^+$ or $CD11c^-$ $CD4^+$ cells internalized MS, indicating that there is no uptake by T-cells. To ensure that fluorescein signals were due to active uptake rather than extracellular attachment of labeled MS to the cell surface, we performed the same experiments at 4°C where no significant uptake could be detected (data not shown).

DCs and MΦs cross-present microsphere-encapsulated OVA in vitro

After confirming that DCs and MΦs were capable to actively take up MS, we performed a study to analyse the capacity of cross- and class II presentation. First, we compared MΦs and DCs magnetically isolated via the general DC-marker CD11c (Fig. 1A). MΦs were obtained from the CD11c-negative flow throw by positive sorting for CD11b. Purity of isolated APC populations in elution fractions was between 70 and 85 % (Fig. 2A+B). After sorting, $CD11b^+$ cells were analyzed for the number of remaining $CD11c^+$ DCs; only a small number of $CD11c^{low}$ and no residual $CD11c^{high}$ cells could be detected (Fig. 2B).

In order to investigate the intensity of antigen presentation, isolated DCs and MΦs were incubated with MS containing ovalbumin (MS OVA) or empty MS (MS empty). Cross-presentation was evaluated by measuring activation of the SIINFEKL-

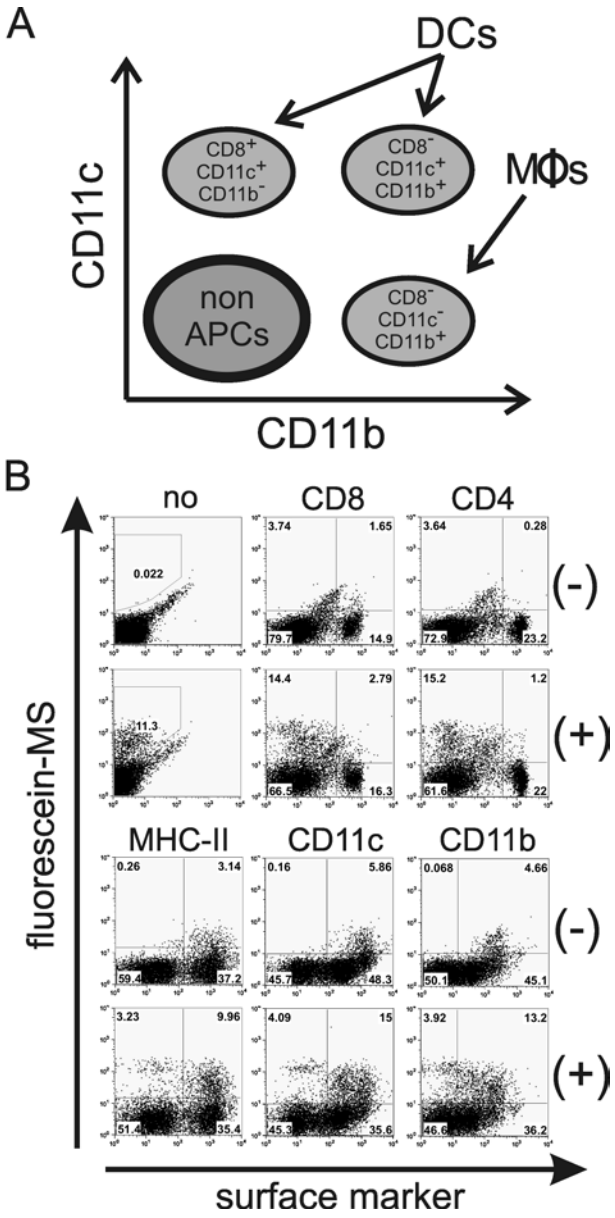

Figure 1: Uptake of PLGA-microspheres (MS) by primary murine splenocytes. A, Professional antigen presenting cells (APCs) in the murine spleen can be subdivided into two populations of dendritic cells (DCs) and macrophages (MΦs) according to the expression pattern of indicated marker molecules. B, Spleens from naïve C57BL/6 mice were taken, and isolated splenocytes were purified by Ficoll gradient centrifugation. Splenocytes were cultured in the presence of control (-) or fluorescein-labelled MS (+) for 1h, followed by flow cytometric analysis of MS uptake. Co-stainings with indicated surface markers were performed to detect MS uptake by specific cell populations. The graph showing CD11b was pre-gated on $CD11c^-$ events. Fluorescein-MS signals were detected in the FITC channel. Images show representative results of two independent experiments.

specific CD8 T-cell hybridoma line B3Z. Both cell types analyzed were clearly able to cross-present OVA derived SIINFEKL on MHC class I (Fig. 2C). Cells from the same purification were used to study class II presentation via activation of the CD4 T-cell

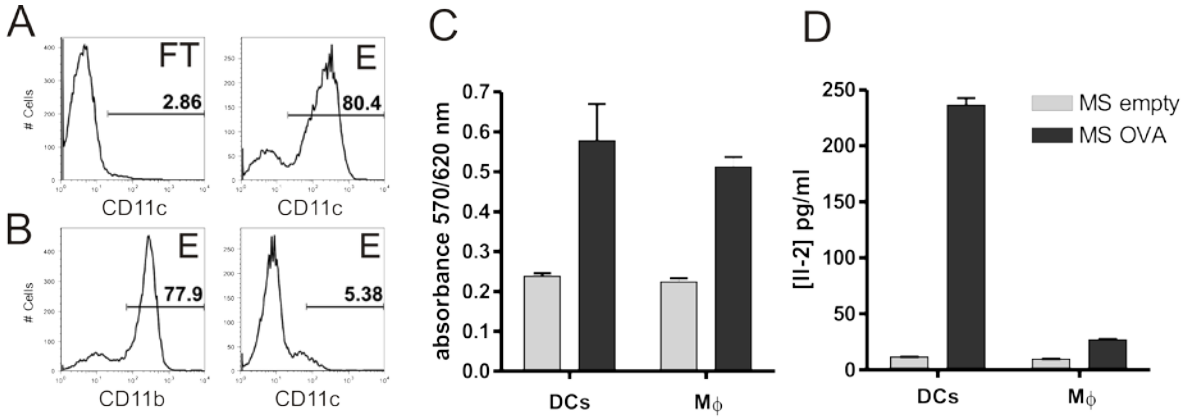

Figure 2: Cross-presentation and MHC class II restricted presentation of microsphere- (MS) encapsulated ovalbumin (OVA) by dendritic cells (DCs) and macrophages (MΦs) *in vitro.* $CD11c^+$ DCs and $CD11b^+$ MΦs were magnetically purified from spleens of C57BL/6 mice. The purities of $CD11c^+$ DCs (A) and $CD11c^-CD11b^+$ MΦs (B) after magnetic sorting in either elution (E) or flow through (FT) was determined by staining for CD11c and CD11b, followed by flow cytometry. Representative purifications are shown. C, Cross-presentation by DCs and MΦs. APCs were cultured in the presence of empty (MS empty) or OVA containing (MS OVA) MS for 18 h by incubation with the OVA-specific CD8 T-cell hybridoma B3Z. Activation of B3Z cells was detected in a colorimetric LacZ assay (absorbance 570/620 nm). D, MHC class II restricted presentation by DCs and MΦs. APCs were cultured in the presence of empty (MS empty) or OVA containing (MS OVA) microspheres with DOBW hybridoma cells. After 18 h culture supernatants were analysed for IL-2 production by ELISA. Shown results are representative of at least 3 independent experiments.

hybridoma line DOBW. In contrast to their ability to cross-present, MΦs were not able to induce IL-2 secretion by class II restricted DOBW cells (Fig. 2D). $CD11c^+$ DCs however present the OVA derived class II epitope ISQAVHAAHAEINEAGR leading to a robust secretion of IL-2 by DOBW hybridoma cells.

Depletion of DCs confirms cross-presentation activity of MΦs in vitro

To exclude that cross-presentation activity of the $CD11b^+$ fraction was due to small numbers of contaminating DCs we used splenocytes isolated from CD11c-DTR mice. This system allows the specific depletion of $CD11c^+$ cells by addition of diphtheria toxin (DT) during culture of cells *in vitro* (Jung et al. 2002). MΦs are $CD11c^-$ and therefore protected from DT treatment. Magnetic cell sorting was performed to purify MΦs and cross-presentation was studied in the presence or absence of DT (Fig. 3). As expected no effect of DT on control cells isolated from C57BL/6 could be detected. Both the $CD11c^+$ and $CD11b^+$ cell fractions were able to cross-present microsphere encapsulated OVA, as shown above (Fig. 2C). In contrast to this, no

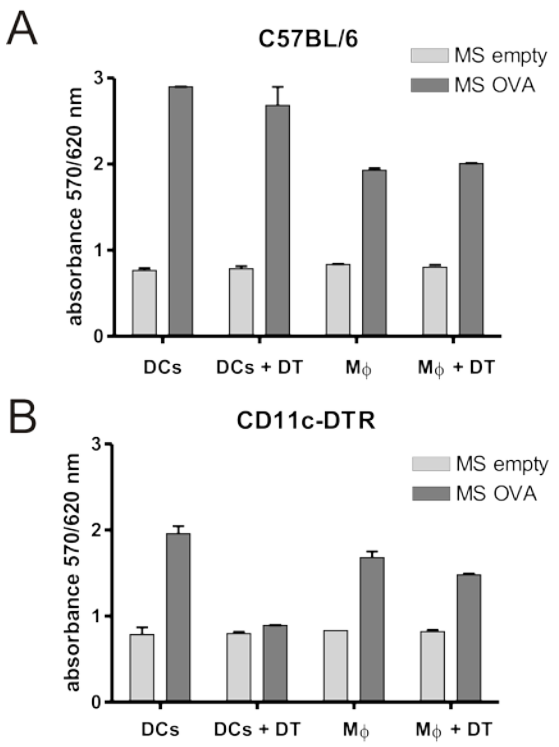

Figure 3: *In vitro* treatment of purified dendritic cells (DCs) and macrophages (MΦs) with diphtheria toxin (DT) confirms cross-presentation by MΦs. Splenocytes from either C57BL/6 (A) or transgenic CD11c-DTR mice (B) were magnetically sorted for $CD11c^+$ dendritic cells (DCs) and $CD11b^+$ MΦs. APCs were incubated with empty (MS empty) or ovalbumin (OVA) containing microspheres (MS OVA), followed by co-culture with OVA-specific B3Z hybridoma cells. Cross-activation of B3Z cells was determined using a colorimetric LacZ assay (absorbance 570/620 nm), either in the presence (+DT) or absence of DT.

cross-presentation of DCs purified from CD11c-DTR mice could be observed in the presence of DT. However, $CD11b^+$ cells isolated from CD11c-DTR mice were able to cross-present even in the presence of DT (Fig 3B). This experiment clearly showed that MΦs indeed cross-present MS-encapsulated antigen *in vitro* and that this activity was not due to contaminating DCs.

$CD8^+$ and $CD8^-$ DCs present microsphere-encapsulated antigen on MHC I and II

Two important populations of murine splenic DCs can be distinguished by their expression of the CD8 surface marker (Fig. 1A). We were interested to study possible differences in the capacity of $CD8^+$ and $CD8^-$ DCs to cross- and class II present MS-associated antigen. Using a combination of magnetic CD11c sorting (as shown in Fig. 2) and subsequent purification by FACS (staining for CD11c and CD8) we obtained highly pure DC subpopulations (Fig. 4A). Antigen presentation was

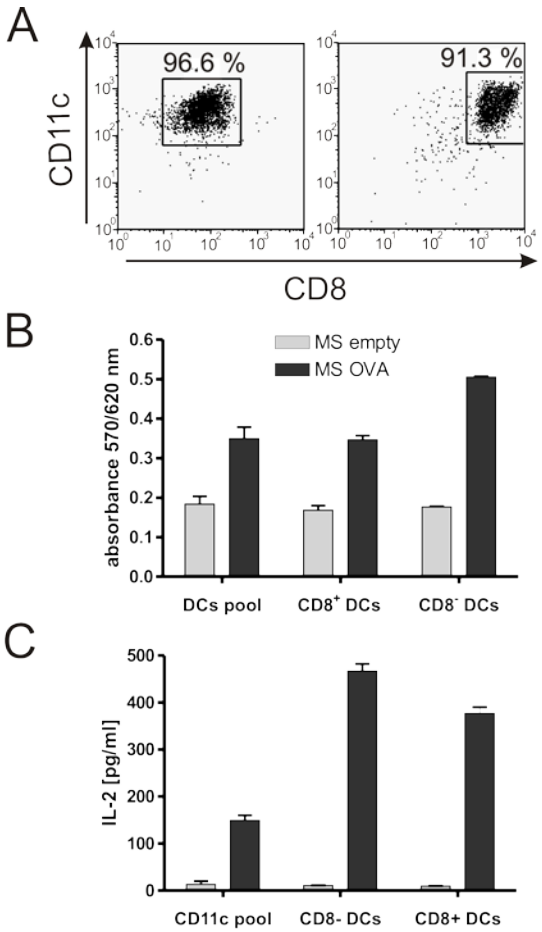

Figure 4: Cross-presentation and MHC class II restricted presentation of microsphere- (MS) encapsulated ovalbumin (OVA) by $CD8^+$ and $CD8^-$ dendritic cells (DCs) *in vitro*. $CD11c^+$ DCs were magnetically pre-sorted, stained for CD8 expression and further subdivided into $CD8^+$ and $CD8^-$ DCs by fluorescence activated cell sorting (FACS). A, Representative purity analysis of $CD8^+$ (right) and $CD8^-$ DCs (left) after cell sorting. B, Cross-presentation by $CD8^+$ and $CD8^-$ DCs. APCs were co-cultured with empty (MS empty) or OVA containing microspheres (MS OVA) and OVA-specific B3Z hybridoma activation monitored in a colorimetric LacZ assay (absorbance 570/620 nm). C, MHC class II restricted presentation by $CD8^+$ and $CD8^-$ DCs. APCs were cultured in the presence of MS and DOBW hybridoma cells. After 18 h the culture supernatants were analysed for their concentration of IL-2 by ELISA. Shown results are representatives of 3 independent experiments.

assayed by co-incubating isolated cells with OVA-containing MS and the respective hybridoma cell line B3Z (class I) or DOBW (class II). In both cases $CD8^+$ and $CD8^-$ DCs were able to present OVA derived epitopes in a similar manner (Fig 4B, C). However, $CD8^-$ DCs showed insignificantly, but reproducibly elevated levels of class II presentation (Fig. 4B).

Recruitment of pro-inflammatory DCs from monocyte-derived blood precursors to the sites of inoculation is not required for efficient cross-presentation of microsphere-encapsulated OVA

Beside Langerhans cells (LCs), dermal DCs, and tissue resident MΦs, there is another APC population with phagocytotic activity at the site of infection that has the ability to prime T-cells. Recent publications have shown that CCR6-dependent recruitment of monocyte-derived DCs to inflamed tissues is essential for cross-presentation of soluble antigen after mucosal or skin immunizations (Le Borgne et al. 2006). At the same time, CCR2- and CCR6-mediated signaling was shown to mediate the recruitment and accumulation of immature DCs in response to particulate antigen (Osterholzer et al. 2005). In order to analyze the impact of monocyte-derived pro-inflammatory DCs on the T-cell priming after MS immunization, we compared the specific CTL responses after MS injection in C57BL/6 and CCR2 or CCR6 knock-out mice (Fig. 5). Our results clearly indicate that active DC recruitment via CCR2 or CCR6 is not required for the cross-presentation of MS-encapsulated OVA. Knock-out mice for both cytokine receptors showed similar SIINFEKL-specific cytotoxicity *in vivo*, compared to the wild type control. We conclude from these findings that tissue-resident APCs rather than infiltrating pro-inflammatory DCs are responsible for the initiation of CTL responses after MS vaccination.

$CD8^-$ DCs and MΦs are the major cell types that associate with MS-derived antigen in vivo

After showing that $CD8^+$ and $CD8^-$ DCs as well as MΦs were able to cross-present MS-derived antigen *in vitro* and depletion of DCs and MΦs led to a strong reduction of cross-presentation *in vivo*, we were interested to analyze the role of APC subpopulations at the site of T-cell priming. Therefore we injected mice with MS-OVA/CpG (control, right hind leg) and fluorescent MS-OVA/CpG/QD583 (MS QD583, left hind leg) into the footpad and measured MS trafficking into the draining lymph nodes. Starting already on day 1 post injection we were able to detect accumulation of fluorescent MS in “left” (+) but not “control” (right, (-)) lymph nodes (Fig. 7A). This accumulation constantly increased up to day 5 post injection. To elucidate whether MS were associated with lymphatic cells or “free”, we distinguished two populations

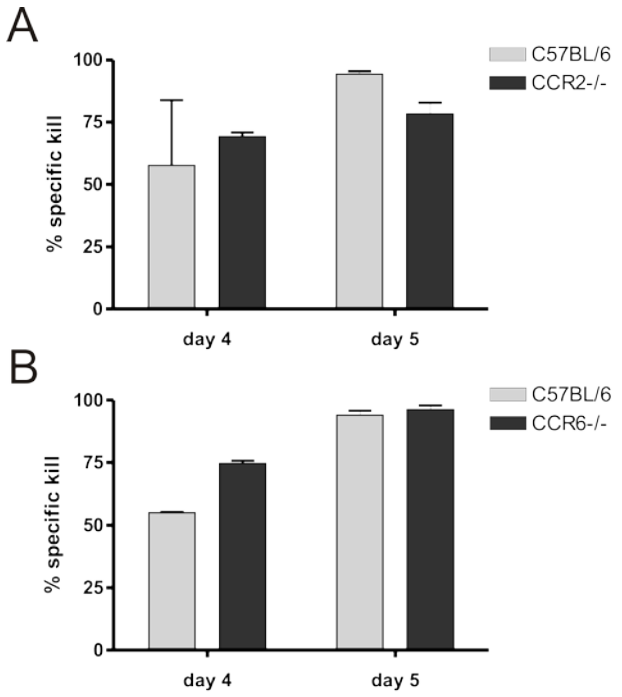

Figure 5: The role of C-C chemokine receptors (CCR) 2 and 6 for cross-priming of microsphere-(MS) encapsulated ovalbumin (OVA). Naïve C57BL/6 mice and CCR2$^{-/-}$ (A) or CCR6$^{-/-}$ knock-out mice (B) were immunised with 5 mg MS-OVA/CpG at the base of the tail. On days 4 and 5 *in vivo* cytotoxicity assays were performed by *i.v.* injection of peptide pulsed/unpulsed CFSE-labelled splenocytes. After 18 h mice were sacrificed and cytotoxicity was evaluated by FACS analysis and calculation of specific cytolysis was performed as described in the materials and methods section. The results are representative for 2 independent experiments with 2-3 mice per group.

by flow cytometry according to their scattering pattern (Fig. 7B). Interestingly, we could detect a substantial amount of “free” MS that might have entered the lymph nodes by draining lymph flow. However, significantly more MS were associated with lymphatic cells. In order to characterize individual cell populations that were positive for fluorescent MS, we distinguished CD8$^+$/CD8$^-$ DCs and MΦs (Fig. 7C+D). Analyzing the individual APC populations we found mainly CD8$^-$ DCs and MΦs to be positive for fluorescent MS. Only very low numbers of MS-containing CD8$^+$ DCs could be detected.

DCs and MΦs cross-prime microsphere-encapsulated antigen in vivo

After showing the general capability of MΦs to perform the cross-presentation of MS-encapsulated antigen *in vitro*, we were interested in confirming these data *in vivo*. To selectively deplete APCs in the living animal, we used two model systems to either

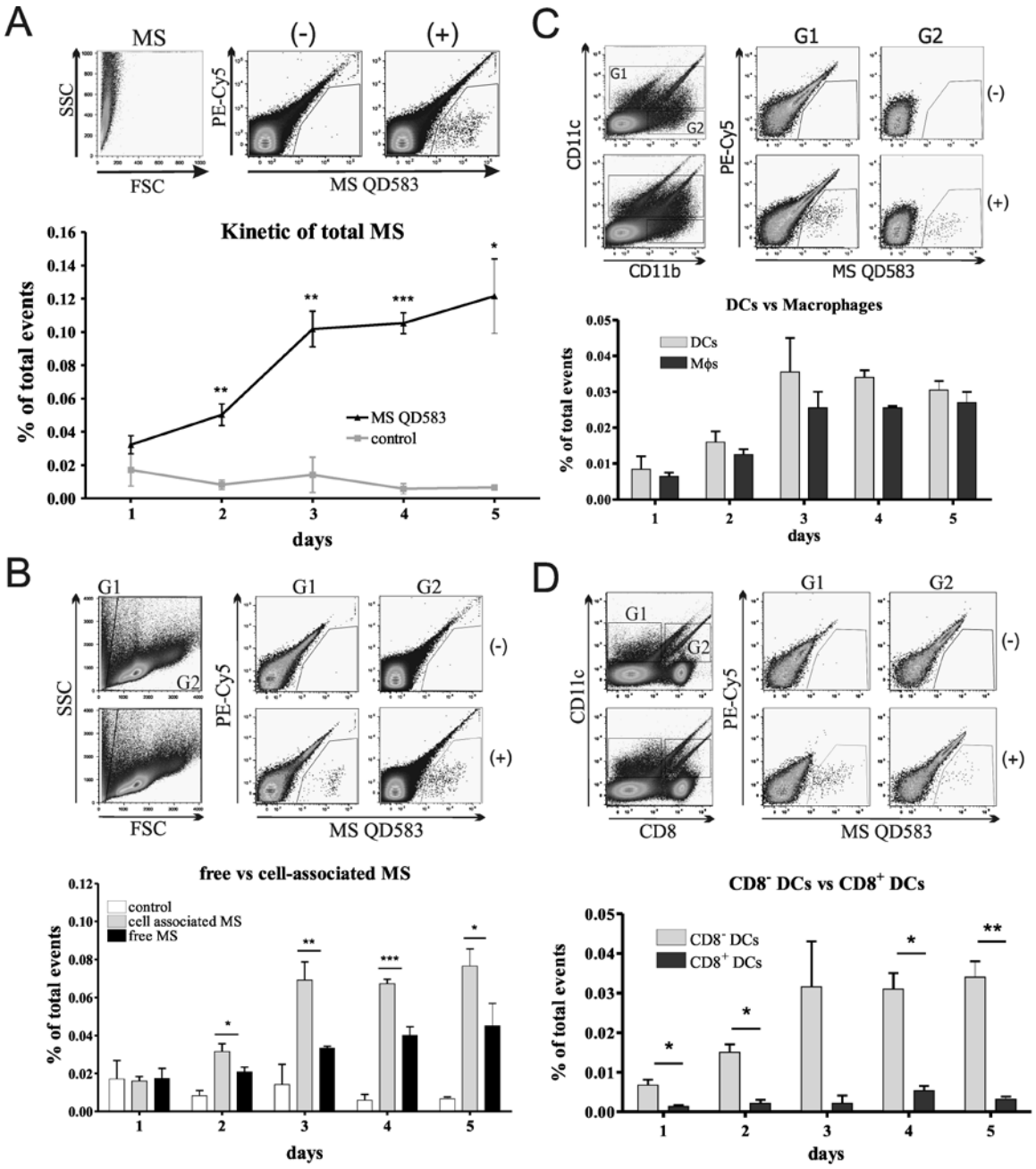

Figure 7: Cell type-specific uptake of fluorescent microspheres (MS) in the lymph node. Mice were injected with MS containing OVA/CpG into the right (-) and fluorescent OVA/CpG/QD583 into the left (+, MS QD583) hind footpad of the same animal. Total events were separated by scattering pattern into "free MS" and "cell-associated" events (B, G1 and G2). Autofluorescent signals were excluded from MS^+ cells by gating on MS $QD583^+/PE\text{-}Cy5^-$ events. Lymph nodes were prepared at indicated time points after injection of MS and percentage of MS $QD583^+$ events were evaluated by flowcytometry. A, Graph shows MS detected in right (-) versus left (+) lymph nodes. The upper left image shows the scattering pattern of MS alone. B, Graph shows ratio of "free" (G1) versus "cell associated" (G2) MS in left lymph nodes (+), or right lymph nodes as control (-). C, Comparison of MS uptake by dendritic cells (DCs) and macrophages (MΦs) in left lymph nodes (+) at indicated time points after MS injection. "Cell-associated" events were gated and total numbers of $CD11c^+$ cells (G1) were compared with $CD11b^+$ MΦs (G2). D, Comparison of MS uptake by $CD8^+$ and $CD8^-$ DCs in left lymph nodes (+). "Cells-associated" events were gated and MS uptake by $CD8^-$ (G1) and $CD8^+$ (G2) DCs was compared. All graphs show representative results of three independent experiments with 4 mice each. On top of each graph representative flow cytometry images for left (+) or control (-) lymph nodes are shown. Significance was calculated by unpaired student's t-test. Significance values are: * $p \leq 0.05$, ** $p \leq 0.005$, *** $p \leq 0.0005$.

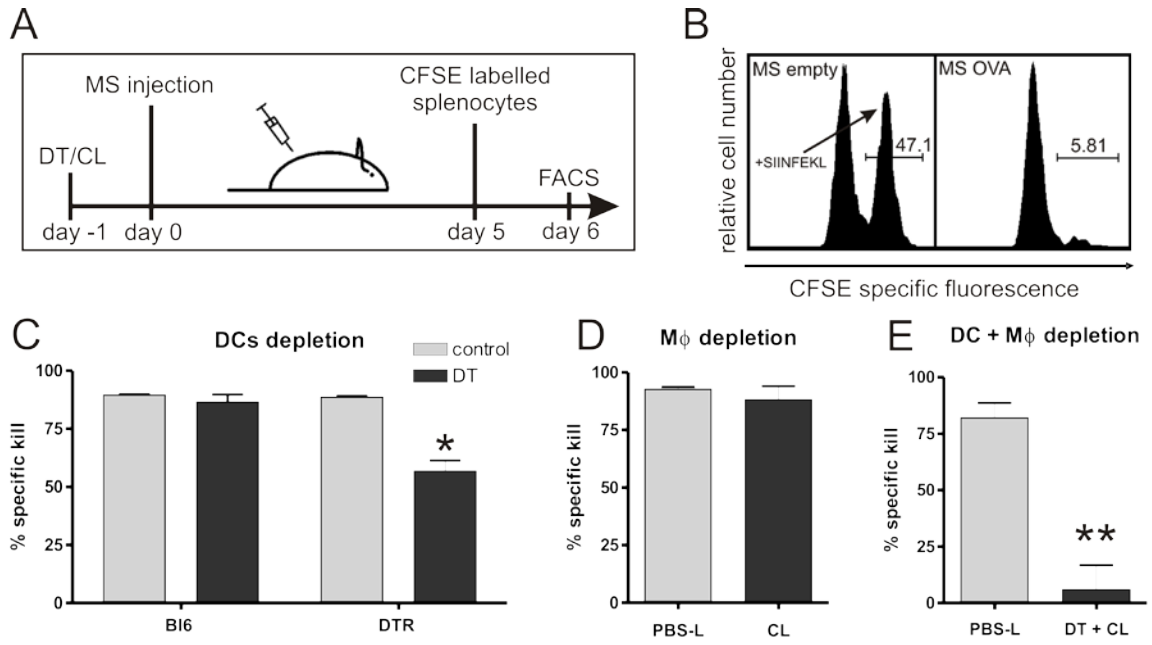

Figure 6: Impact of dendritic cell (DCs) and macrophage (MΦs) depletion on cross-priming of microsphere- (MS) encapsulated ovalbumin (OVA) *in vivo*. Naïve C57BL/6 (D) or transgenic CD11c-DTR mice (C+E) were immunised with 5 mg MS-OVA/CpG at the base of the tail. 18 h prior to MS injection, C57BL/6 and/or transgenic mice (Bl6 and DTR) were left untreated (control) or were injected with either 100 ng diphtheria toxin (DT) (DC depletion, *i.p.*), 2 x 200 µl control (PBS-L) or clodronate liposomes (CL) (MΦ depletion, *i.v.* and base of the tail) or a combination of both, as indicated. On day 5 after MS injection, *in vivo* cytotoxicity assays were performed by *i.v.* injection of peptide pulsed/unpulsed CFSE-labelled splenocytes. After 18 h mice were sacrificed and the cytotoxicity was evaluated by FACS analysis. The calculation of specific cytotoxicity was performed as describe in the materials and methods section. A, Schematic overview of the depletion experiments. B, Example to illustrate detection of the OVA-specific immune response using the *in vivo* cytotoxicity assay in MS OVA immunized C57BL/6 mice on day 5 post immunization. C, Impact of DT treatment on MS cross-priming in C57BL/6 and transgenic CD11c-DTR mice. D, Impact of MΦ depletion on MS cross-priming in C57BL/6 mice. E, Cross-priming activity after depletion of both DCs and MΦs in CD11c-DTR mice. Shown results represent 3 independent experiments with 2-4 mice per group.

deplete DCs, MΦs or both. DC depletion using CD11c-DTR mice, as demonstrated *in vitro* (Fig. 3), can also be applied *in vivo* (Fig. 6A). After DT injection, mice lack $CD11c^+$ DCs for 2-3 days, before cells start to repopulate (Jung et al. 2002, Probst et al. 2005). To avoid the influence of recovering DCs we chose a system where we could study the effect of DC depletion on the very early immune response. Using the *in vivo* cytotoxicity assay, strong SIINFEKL restricted cytotoxicity could be observed as early as day 5 after immunization of untreated mice (Fig. 6B). Therefore we used day 5 after vaccination as time for analysis, which reflected priming in the absence of DCs. Interestingly, we were not able to see strong differences in SIINFEKL-specific killing between CD11c-DTR mice either treated with DT or not (Fig. 6C). The

experiments revealed only a small but significant reduction of about 20% specific cytotoxicity. From this experiment we conclude that $CD11c^+$ DCs depleted in the transgenic animals are not the only APCs cross-priming MS restricted responses. To evaluate the role of MΦs *in vivo*, we used clodronate liposomes (CL) to deplete MΦs in C57BL/6 mice. Liposomes are taken up by MΦs and clodronate accumulates within the cells, leading to MΦ-specific apoptosis. Control animals were treated with liposomes containing PBS only. Neither on day 4 (data not shown) nor on day 5 significant differences in OVA-specific cytotoxicity could be detected between mice treated with CL or PBS-liposomes (PBS-L) (Fig. 6D). These data illustrate that MΦs are not essential for cross-priming of MS-encapsulated antigen *in vivo*. To test if they have any impact on cross-priming at all, we performed a similar experiment in CD11c-DTR mice. Transgenic animals treated with DT and CL lack both $CD11c^{high}$ DCs and MΦs (Fig. 6E). Control mice were treated with PBS-L only. The results of this experiment indicate that MΦs indeed have an impact on cross-priming in the situation when no DCs are available. In contrast to mice lacking single APC populations, the depletion of DCs and MΦs reduced OVA-specific cytotoxicity to much lower levels than seen for any of the two treatments alone. From these experiments we conclude that DCs and MΦs have redundant functions concerning the cross-presentation of MS-encapsulated antigens.

Discussion

The development of therapeutic vaccines against cancer is a central challenge of immunology. A key requirement to achieve tumor regression is the induction of a robust CTL response. In most cases this is tightly linked to breaking tolerance for the malignant tissue, which calls for powerful immunisation strategies. Vaccines based on particulate antigens show high potency in inducing antigen-specific responses, in contrast to soluble antigens, which often induce ignorance (Storni et al. 2005). For PLGA MS, but also for other particulate antigen like virus like particles, liposomes, ISCOMs or non-degradable antigen-coupled beads, very little is known about the antigen presentation capacities and the *in vivo* relevance of different APC subtypes. However, a careful analysis of cell types involved in T-cell priming is an essential requirement for the optimization, comparison and choice of a suitable antigen system for a certain vaccination approach. During the last years, we and others published PLGA MS to be a promising tool for the induction of anti-tumor responses by combining all advantages necessary for successful clinical outcome. Ideal properties for phagocytotic uptake by APCs, the possibility of co-encapsulating antigenic material with T_H-I polarising adjuvants, immunization independent of MHC haplotype and the depot-effect with protracted antigen release qualify MS as antigen delivery devices for immunotherapy (Tamber et al. 2005, Waeckerle-Men et al. 2006, Schlosser et al. 2008a).

Previous studies highlighted the potency of MS vaccination in induction of T_H-I dominated immune responses (Men et al. 1999, Waeckerle-Men et al. 2006, Heit et al. 2007, Schlosser et al. 2008a). In this report, we aimed at identifying the different APC types involved in priming of T-cells after MS injection. Therefore we analyzed two DC subtypes and MΦs purified from murine spleens for their capacity to present MS-encapsulated OVA on MHC class I and II. Although it has been shown earlier that antigen uptake by APCs does not necessarily correlate with its cross-presentation (Schnorrer et al. 2006), uptake of MS is the first requirement for antigen presentation in this system. Primary splenocytes were incubated with fluorescent MS and phagocytotic cells types were identified via the expression of different surface markers. Cells containing MS were MHC class II positive and co-expressed CD11c,

CD11b or F4/80, the lineage markers of DCs and MΦs. These results confirmed that MS indeed are efficiently taken up by various phagocytes. To directly address the question of antigen presentation, we purified DCs and MΦs to compare their ability to present antigen-derived peptides to specific T-cell hybridomas. Our results indicate that not only phagocytosis, but also cross-presentation is very similar for isolated DCs and MΦs. Although this is the first report on cross-presentation of MS-encapsulated antigens by primary MΦs, our data go in line with other *in vitro* studies showing cross-presentation by MΦs for several antigens (Kovacsovics-Bankowski et al. 1993, Houde et al. 2003, Basta et al. 2005). Interestingly, our experiments analyzing presentation on MHC class II, indicated that MΦs in contrast to DCs are unable to stimulate the class II restricted hybridoma DOBW. This lack of class II presentation was not due to low expression of MHC-II (data not shown). One likely explanation for this inability of MΦs was recently published and deals with differences in the enzymatic machinery between DCs and MΦs. According to this, DCs are specialized for antigen presentation by preventing maximal lysosomal acidification and thereby inhibiting proteolytic destruction of antigens (Lennon-Dumenil et al. 2002, Delamarre et al. 2005). Amigorena and colleagues postulated a mechanism, in which nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase II is recruited to the early phagosomes of DCs, which increases the pH values by constantly producing low levels of reactive oxygen species (Savina et al. 2006). This mechanism prevented antigen degradation by cathepsins in DCs but not in MΦs, explaining why DCs are the exclusive cell type for cross-presentation of many antigens. In the experiments shown in this report DCs and MΦs were both able to cross-present MS-encapsulated OVA. We hypothesize that the lack of class II presentation by MΦs is a consequence of epitope destruction by acidic proteases, which are inhibited in DCs due to the mechanism described above.

The fact that cross-presentation is not impaired in MΦs favours the idea of an early endosome/lysosome to cytosol transition of encapsulated antigens. This indicates again that cross-presentation after MS uptake is mainly dependent on proteins entering the direct-presentation pathway. In contrast to this, class-II presentation is dependent on lysosomal degradation and loading of MHC-II complexes within organelles of the endosomal pathway (Watts et al. 2003). A second way to explain the lack of class II presentation by MΦs could be differences in the intracellular fate

of MS between DCs and MΦs, due to alterations in endosomal acidification. It was reported that PLGA particles can change surface charge from anionic to cationic due to a pH drop in secondary endosomes. This might result in a MS-membrane interaction, leading to an escape of the entire particle into the cytosol (Panyam et al. 2002a). This escape would definitely prohibit peptide loading in an MHC class II loading compartment. At the same time cross-presentation would be permitted because of immediate access to the direct-presentation machinery. We are currently studying the intracellular fate of MS in order to evaluate these possibilities (see *chapter II*).

To confirm the ability of MΦs to cross-present MS *in vitro*, we used splenocytes from CD11c-DTR mice and treated isolated APCs with DT. As expected, cross-presentation of DCs but not MΦs was sensitive to DT, excluding impurities in the MΦ preparation. The fact that DT only depletes $CD11c^{high}$ cells, but not $CD11c^{low}$ plasmacytoid DCs (Jung et al. 2002), also excludes a role of $CD11c^{low}$ DCs for cross-presentation in our *in vitro* experiments. The next step was to further subdivide the DCs population into the two major populations found in lymphoid organs, $CD8^+$ and $CD8^-$ DCs. High purity of isolated cell fractions allowed the conclusion that both DC isoforms were able to present MS-encapsulated OVA on class I and II. Differences in cross-presentation, which were reported for soluble, cell-associated or viral antigens (den Haan et al. 2000, Allan et al. 2003, Belz et al. 2005) were not observed for MS-encapsulated antigen. Probably due to rapid cytosolic transition of antigens and independence of lysosomal degradation, phagocytosis and cross-presentation are directly correlated in this case.

The final goal of our work was to elucidate the impact of individual APC populations on cross-priming after injection of MS *in vivo*. First, we wanted to evaluate the impact of tissue resident APCs versus pro-inflammatory DCs that differentiate *de novo* from peripheral blood monocytes. The recruitment of phagocytotic APCs to the site of injection might be a requirement for successful T-cell induction. The chemokine receptors CCR2 and CCR6 were reported to trigger the infiltration of monocytes into the inflamed tissue and their differentiation into pro-inflammatory DCs. These are able to take up antigens and migrate to the sites of T-cell activation, mediated via a chemokine gradient of the CCR-ligands CCL2 and CCL20. We used CCR2 and

CCR6 knock-out mice to study the role of monocyte-derived DCs. Our experiments clearly indicated that neither CCR2 nor CCR6 deficiency has an influence on the strength of the CTL response. Reports from literature showing an essential impact of CCR signalling for cross-presentation of soluble antigen (Le Borgne et al. 2006) or the accumulation of immature DCs in response to particulate antigen (Osterholzer et al. 2005) seem not to be required for microsphere-based vaccination. The CCR knock-out experiments have also implications on the role of Langerhans cells (LCs). Beside the dermal DCs, LCs represent the second resident DC-type within the skin and are therefore potential candidates for MS uptake and T-cell priming. In the murine system these cells express the chemokine receptors CCR2 and CCR6 (Dieu et al. 1998, Caux et al. 2000, Merad et al. 2002), which might be important for the recruitment of LCs already established in the tissue to the sites of inflammation. However, our CCR knock-out experiments argue against the requirement of such a mechanism in our system, which goes in line with the recent finding that CpG-mediated immune responses does not require monocyte recruitment (Le Borgne et al. 2006).

The *s.c.* injection of immunized mice probably leads to cross-priming of CTLs in the draining lymph nodes. By subcutaneous injection of fluorescent MS into mice, we could show that labeled particles accumulate in lymph nodes over time. This is in accordance with a recent study showing that DCs loaded with PLGA MS have a comparable capacity to migrate along a CCL19/21 chemokine gradient, compared to unloaded DCs (Waeckerle-Men et al. 2004). The CCR7 dependent chemotaxis principally showed that MS-loaded DCs have the ability to home into draining lymph nodes after phagocytosis. Although there is a study showing that large particles like MS (Ø 0.5 – 5 µm) are not able to freely diffuse to the draining lymph nodes, but require cellular transport especially by DCs (Manolova et al. 2008), we found a substantial amount of “free” MS within our lymph node preparations. Since the size of MS is very heterogeneous, it might well be that particles smaller than 0.5 µm occur, which might have the ability to reach the lymph node exclusively by draining lymph flow. On the other hand, “free” MS in our lymph node preparations could originate from cells dying within the lymph node after having actively shuttled MS. This mechanism could allow secondary uptake by lymph node resident APCs. However, a secondary antigen uptake in lymphoid organs was shown to depend on $CD8^+$ DCs

(Allan et al. 2003). Very interestingly, we could show here that mainly $CD8^-$ DCs and MΦs, but not $CD8^+$ DCs were positive for fluorescent particles within the lymph nodes. These results are consistent with a very similar experiment performed with FITC-labelled latex beads (Shi and Rock 2002). Beads accumulated in draining lymph nodes after *s.c.* injection and only ~ 50% were associated to $CD11c^+$ cells. Although not show, the authors suggested that the other 50% of fluorescent cells had to be MΦs. In our experiments we extend this finding to PLGA MS and add more information by showing that fluorescent $CD11c^-$ cells indeed expressed the MΦ marker CD11b. Even though $CD8^+$ DCs were able to cross-present MS-derived antigen *in vitro*, they probably have a very low impact on *in vivo* cross-priming, simply due to the fact that they to not co-localize with the antigen. MΦs were shown to have the capacity to take up antigens in the periphery and to stimulate naïve T-cells in draining lymph nodes. Nevertheless, we can not exclude that fluorescent signals in MΦs are entirely dependent on uptake of "free" MS entering the lymph node by draining lymph flow. In any case, MΦs at the sites of T-cell priming are positive for MS which makes it very likely that they play a role in antigen presentation.

To further examine the role of DCs and MΦs *in vivo*, we used cell depletion strategies. CD11c-DTR mice were used in a number of studies showing the exclusive role of DCs in cross- and direct-presentation (Jung et al. 2002, Probst and van den Broek 2005, Kassim et al. 2006). Nevertheless, the DTR model has some limitations. First of all, the kinetics of DC depletion has to be taken into account. DCs start to recover as early as 2 to 3 days after DT treatment (Jung et al. 2002, Probst et al. 2005), making it difficult to analyze the immune response of a vaccine with long term antigen release like MS. To be confident that cross-priming of CTLs occurred during the short window of DC depletion, we analyzed the immune response already on day 4 and 5 after immunization. These are the earliest time points where specific CTL activity could be detected *in vivo* after immunization this MS. Intracellular cytokine staining for interferon-γ in $CD8^+$ T-cells from isolated spleens peaks only on day 6 after immunization (Schlosser et al. 2008a). Second, DT treatment of CD11c-DTR mice also leads to unspecific depletion of some other cell types (Probst et al. 2005). Using the DTR mouse, we could show that cross-priming of OVA-specific CTLs is significantly reduced by DC depletion, but only to a small extent. Around 80% of the total CTL activity detected in C57BL/6 mice remained even in the absence of DCs.

These data show that cell types other than CD11c^{high} DCs, presumably macrophages, have to be responsible for residual CTL priming.

In contrast to our initial hypothesis we could not detect differences in cytotoxic activity after MΦ depletion between CL treated and untreated mice. This indicated that MΦs are not essential for immunizations using MS. Interestingly however, mice treated with DT and CL one day prior to the injection of MS showed a much stronger reduction of CTL priming and initiating a cytotoxic response compared to DT treatment alone. The mild phenotype of the single depletions and the strong reduction of immune activation after joint depletion of DCs and MΦs, allowed us to conclude that both cell types are able to cross-prime CTLs after MS injection. It seems that DCs and MΦs have redundant function concerning the immune activation. At the same time LCs are not depleted by DT treatment using the CD11c-DTR mouse (Bar-On and Jung 2010). Therefore the low response of CD11c-DTR mice treated with DT and CL also argues against an important role of LCs following MS injection.

The powerful CTL responses achieved by MS-based vaccination may in part be due to the redundancy of cell targeting. PLGA MS are specifically taken up by phagocytes and encapsulated antigen is cross-presented by a large number of primary APCs and cell lines (data not shown) and also *in vivo* cross-priming can be performed by DCs and MΦs. Our data challenge the popular idea of targeting antigen selectively to certain APCs for successful immunization. Promiscuous antigen delivery can be an advantage for the performance of future vaccines.

Acknowledgements

We thank Eva-Maria Boneberg and Michael Basler for help with cell sorting. Christian Kurts and Ulf Panzer are acknowledged for providing knock-out mice and Nilabh Shastri and Clifford V. Harding for contributing T-cell hybridomas.

Chapter II

Microencapsulation of inorganic nanocrystals into PLGA microsphere vaccines enables their intracellular localization in dendritic cells by electron and fluorescence microscopy

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Published in the *Journal of Controlled Release*

J. Control. Release (2011), doi:10.1019/j.conrel.2011.01.005

Abstract

Biodegradable poly-(D,L-lactide-*co*-glycolide) microspheres (PLGA MS) are approved as a drug delivery system in humans and represent a promising antigen delivery device for immunotherapy against cancer. Immune responses following PLGA MS vaccination require cross-presentation of encapsulated antigen by professional antigen presenting cells (APCs). While the potential of PLGA MS as vaccine formulations is well established, the intracellular pathway of cross-presentation following phagocytosis of PLGA MS is still under debate. A part of the controversy stems from the difficulty in unambiguously identifying PLGA MS within cells. Here we show a novel strategy for the efficient encapsulation of inorganic nanocrystals (NCs) into PLGA MS as a tool to study their intracellular localization. We microencapsulated NCs as an electron dense marker to study the intracellular localization of PLGA MS by transmission electron microscopy (TEM) and as fluorescent labels for confocal laser scanning microscopy. Using this method, we found PLGA MS to be rapidly taken up by dendritic cells and macrophages. Co-localization with the lysosomal marker LAMP1 showed a lysosomal storage of PLGA MS for over two days after uptake, long after the initiation of cross-presentation had occured. Our data argue against an escape of PLGA MS from the endosome as has previously been suggested as a mechanism to facilitate cross-presentation of PLGA MS encapsulated antigen.

Introduction

Poly(lactide-co-glycolide) microspheres (PLGA MS) are spherical, biodegradable polymer particles that can be loaded with a great variety of therapeutic molecules (Mathiowitz et al. 1997, Walter et al. 2001). They are approved by the US Food and Drug Administration (FDA) to be used as drug delivery system in humans. Recent studies highlight the promising role of PLGA MS as antigen delivery system for the induction of $CD8^+$ T-cell dependent immune responses against cancer (Waeckerle-Men et al. 2005a, Schlosser et al. 2008b). They show ideal properties for phagocytotic uptake by professional antigen presenting cells (APCs), like dendritic cells (DCs), and macrophages (MΦs), and offer the possibility to co-encapsulate antigenic material with immune stimulatory adjuvants (Schlosser et al. 2008b).

In this study, we combined the biocompatibility of PLGA MS with the exceptional properties of inorganic nanocrystals (NCs). NCs are colloidal particles, which exhibit a size in the low nanometer range. Corresponding NCs to a wide range of macroscopic inorganic materials can be prepared. Within the nanometer scale, their characteristic properties often differ dramatically from those of the corresponding bulk material. The particle size in turn is responsible for the exceptional change of magnetic, electronic, and optic properties of the NCs. Semiconducting NCs (quantum dots, QDs) show fluorescence at an emission wavelength determined by the intrinsic electronic band gap structure of the material and confinement effects depending on the particle size (Peng 2009). Parameters like high photostability, the narrow emission signal and small stokes shift of QDs provide an extraordinary potential for replacing organic dyes within the biological diagnostic (Gao et al. 2005). A further examples to illustrate the special properties of NCs are super-paramagnetic iron oxide nanoparticles (SPIONs). While the macroscopic iron oxide is ferromagnetic, its corresponding NCs show super-paramagnetic characteristics (Tromsdorf et al. 2007). Among others, the “hot injection” method is a one pot procedure for synthesizing colloidal, monodisperse, and crystalline nanoparticles (Doneg et al. 2005).

A common way to produce PLGA MS is the so called spray drying evaporation technique (Cal and Sollohub 2010). A homogenous emulsion, containing the PLGA

polymer precursor resomer (organic phase) and usually hydrophilic proteins or other therapeutics (aqueous phase), is sprayed into vacuum. During this process the solvent evaporates and PLGA polymer formation occurs, generating spherical microparticles. Depending on the resomer and the spray drying conditions, the particular properties of microparticles can vary (Walter et al. 2001). Using this technique, we introduce a straightforward protocol to efficiently encapsulate NCs of different materials into PLGA MS. This novel strategy allows the application of the diverse properties of NCs for investigating PLGA MS function and offers interesting options for the development of laboratory tools or novel strategies to fight cancers.

Three examples are shown to demonstrate the potential of NCs encapsulation. Although PLGA MS are used as a drug delivery system for years and are intensively studied as a tool to induce T-cell responses, the intracellular fate of microparticles after uptake by APCs is still poorly understood and controversially debated (Panyam et al. 2002b, de Jesus Gomes et al. 2006, Shen et al. 2006, Yoshida and Babensee 2006, Cartiera et al. 2009). Transmission electron microscopy (TEM) studies analysing intracellular distribution of PLGA MS were so far dependent on osmium tetroxide incorporation (Panyam et al. 2002b), colloidal gold labelling (Mathiowitz et al. 1997), or the abnegation of any electron dense marker (Walter et al. 2001, de Jesus Gomes et al. 2006, Gomes et al. 2006, Trombone et al. 2007). Here we show that lead sulfide (PbS) QDs serve as an excellent tool to label PLGA MS with an electron dense marker for replacing toxic osmium tetroxide or rather inefficient colloidal gold labelling. In a second approach we encapsulated fluorescent cadmium selenide (CdSe) QDs to demonstrate their ideal properties for bioimaging. Numerous studies performed microscopy analysis of PLGA MS labelled with fluorescent dyes like fluorescein isothiocyanate (FITC) or coumarin (Peyre et al. 2004, Yoshida and Babensee 2006, Trombone et al. 2007). However the high photo sensitivity of such dyes hampers to take high resolution images by confocal laser scanning microscopy (LSM), Z-stack analysis, or time course experiments requiring multiple excitation of the same object. Using CdSe QDs encapsulated PLGA MS, we were able to perform high resolution LSM images of PLGA MS after phagocytosis by APCs. Using the lysosomal marker LAMP1 we used LSM to show co-localisation with fluorescent PLGA MS, indicating the lysosomal storage of phagocytosed particles.

Cell sorting based on magnetic beads is a well established and commercially available method to purify individual cell populations from biological samples (Miltenyi et al. 1990, Grutzkau and Radbruch 2010). This technique is usually based on covalent labelling of magnetic beads with antibodies specific for a certain lineage marker. Antibody binding attaches magnetic beads to the cell surface and allows purification of the labelled cell type via a magnetic column. In a third experimental setting, we tried to combine the ideal properties of PLGA MS for phagocytosis with the super-paramagnetic characteristics of FeS nanoparticles, to generate magnetic PLGA MS for a novel cell sorting approach. Rapid internalisation of PLGA MS by phagocytes was exploited to deplete such cells from a heterogeneous cell suspension. Our results demonstrate that many powerful properties of NCs can be utilized for biomedical research by encapsulation into PLGA MS.

Materials and methods

Synthesis of quantum dots

Nanoparticles were synthesized by hot injection method. PbS-nanoparticles (PbS org) were prepared as described elsewhere (Nagel et al. 2007), using a TOP/OA stabilizing mixture. The synthesized PbS-nanoparticles were transferred into water by a ligand exchange (PbS aq.). To this aim, particles were incubated with an excess of hydroxyl amine for 30 min. The mixture was centrifuged and the precipitated particles were solved in water. CdSe/CdS/ZnS quantum dots were prepared in a one pot hot injection synthesis as described elsewhere (Mekis et al. 2003b, Talapin et al. 2004a), using a TOP/TOPO/HDA stabilizing mixture.

Poly(lactic-co-glycolic)acid microsphere preparation

PLGA MS were prepared from resomer RG502H (Boehringer Ingelheim, Germany) by spray drying as described elsewhere (Waeckerle-Men et al. 2005a, Schlosser et al. 2008b). Briefly, phosphate buffered saline only (MS-empty) or 50 mg ovalbumin (Grade V, Sigma) in phosphate buffered saline (MS-OVA) (aqueous phase) were emulsified with 5% PLGA in dichloromethane (organic phase) by ultrasonication (Hielscher, UP200 H, Ampl. 40%). The emulsion was spray-dried (Mini-Spray-Dryer B-290, Büchi) at a flow rate of 2 ml/min and inlet/outlet temperatures of 40/38°C. Immediately before use, MS were dispersed in media by ultrasonication. PbS nanoparticles were encapsulated by either adding particles to the aqueous (PbS aq.) or the organic phase (PbS org.) of the emulsion. The encapsulation of fluorescent cadmium selenide and magnetic iron oxide nanoparticles was achieved by adding particles to the organic phase of the emulsion.

Transmission electron microscopy (TEM)

Microspheres were embedded in a low-viscosity epoxy resin (Spurr) as described elsewhere (Spurr 1969). Slices of 100 nm were prepared and analyzed without further contrast agents. BMDCs were grown on petriPERM® dishes (Sigma-Aldrich). Cells were pulsed with PbS quantum dot-labeled PLGA MS for 1 h. For TEM preparation, cells were fixed after indicated times with 2% glutardialdehyde solution. Samples were washed in sodium cacodylate buffer and treated with 1% OsO_4. Cells

weredehydrated by increasing concentrations of ethanol. After reaching 100% ethanol, cells were embedded in Spurr. Slices of 80 nm thickness were prepared and samples further contrasted using lead citrate and uranyl acetate. Images were acquired at 80 kV using a Zeiss EM10-S electron microscope.

Cell lines and media

All cell culture media were purchased from Gibco, Invitrogen. The murine dendritic cell line DC2.4 (H-2^b) was a kind gift from K. Rock (University of Massachusetts Medical School Worcester, MA) and cultured in RPMI 1640, 10% FCS, 100 U/ml penicillin/ streptomycin (P/S). Bone marrow-derived dendritic cells (BMDCs) were prepared and maintained as described elsewhere (Schlosser et al. 2008b). Murine peritoneal macrophages (H-2^b) were cultured in RPMI 1640 supplemented with 10% FCS, 100 U/ml P/S. The $CD8^+$ T-cell hybridoma cell line B3Z, specific for the SIINFEKL ($Ova_{257-264}/K^b$) peptide of ovalbumin was a kind gift from N. Shastri (University of California, Berkeley, USA) and cultured in IMDM, 10% FCS, 100 U/ml P/S (Karttunen et al. 1992b). The murine fibroblast cell line B8 (H-2^d) (Groettrup et al. 1995a) was cultured in IMDM, 10% FCS, 100 U/ml P/S.

Preparation of primary cells

Bone marrow-derived dendritic cells (BMDCs) were prepared from naive C57BL/6 mice as previously described (Schlosser et al. 2008b). For microscopy BMDCs were differentiated on cover slips and used on day 6. Peritoneal macrophages (pMΦs) were prepared by *intra peritoneal* (*i.p.*) injection of 3% thioglycolate solution into C57BL/6 mice. After three days, peritoneal cells were washed out of the abdominal cavity. Cells were cultured for 2 days and adherent cells were used for microscopy as well as cross-presentation assays.

Confocal laser scanning microscopy (LSM)

Cells were cultured on cover slips and CdSe quantum dot-labelled microspheres were added on day 2. Unbound microspheres were removed by washing cells with phosphate buffered saline. Cells were fixed in 4% paraformaldehyde, treated with ammonium chloride solution (50 mM in phosphate buffered saline), and permeabilized in 0.2% triton X-100. Samples were blocked with 0.2% fish gelatine (G-7765, Sigma) in phosphate buffered saline. Anti-LAMP1 primary antibody

(553792, BD Pharmingen) was diluted 1:50 in 0.2% fish gelatine. Secondary Alexa488 conjugated anti-rat Ig (A11006, Invitrogen) was diluted 1:300 in phosphate buffered saline. Cells were embedded using Dapi-Fluoromount-G (SouthernBiotech). Samples were analyzed using an LSM510meta (Zeiss). Cadmium selenide specific fluorescence was exited by UV laser. Microscopy images were processed using the software AxioVs40 V4.7.2.0 (Zeiss).

Detection of antigen presentation by LacZ T-cell hybridoma assay

Presentation of $Ova_{257-264}$ was detected using the $CD8^+$ T-cell hybridoma cell line B3Z (Karttunen et al. 1992b). Cells were co-incubated with APCs and 25 µg/well of either microspheres containing ovalbumin (MS-OVA) or empty microspheres (MS-empty). After 18 h, LacZ-buffer [0.13% NP40, 9 mM $MgCl_2$, 0.15 mM chlorophenolred-β-D-galactopyranoside (CPRG) (Roche, Germany) in phosphate buffered saline] was added for 4 h at 37°C. Absorbance was measured at 570/620 nm using a SpectroFluorPlus spectrometer (Tecan).

Magnetic depletion of phagocytes by PLGA MS containing super-paramagnetic iron oxide nanoparticles (SPIONs)

PLGA MS containing SPIONs were prepared as described above. 50 µg/ml magnetic or empty PLGA MS were added to heterogeneous 50:50 cell suspensions of B8 (H-2^d) and DC2.4 (H-2^b) cells for 1 h. For magnetic separation of phagocytes, cells were applied to MACS columns (Miltenyi Biotec) and washed with phosphate buffered saline. Specific depletion of phagocytes was analysed by flow cytometry using FITC anti-mouse H-$2D^b$ and PE anti-mouse H-$2D^d$ (BD Biosciences). Specific depletion was calculated following this equation: Specific depletion =(1–[ratio non-sorted/ratio sorted])x100, and ratio =%H-$2D^{b+}$/%H-$2D^{d+}$.

Results

Efficient encapsulation of PbS quantum dots after addition of nanoparticles to the organic phase of the spray drying process

The encapsulation of QDs into PLGA MS can be achieved by two possible strategies. The PLGA emulsion used for spray drying consists of an organic and an aqueous phase. NCs to be encapsulated can therefore be added to either one of the two phases. In order to test both possibilities, we first tried to encapsulate NCs via the aqueous phase of the emulsion. Therefore, PbS QDs were hydrophilised by exchanging the stabilizing hydrophobic ligand against a hydrophilic one (PbS QDs (aq.)). Using these particles, we achieved encapsulation of electron dense material into PLGA MS (Fig. 1B,C). However the QD inclusions were not evenly distributed throughout the microspheres and only low numbers of particles showed QD incorporation. At the same time, PLGA MS that did contain QDs were found to contain large aggregates of nanoparticles. In a second experimental setting we used hydrophobic QDs (PbS QDs (org.)) to reach our aim. Indeed, addition of PbS QDs (org.) into the organic phase of the spray drying emulsion led to an improved distribution of NCs inclusions (Fig. 1E,F). PLGA MS were prepared for TEM analysis and were found to be entirely labelled with electron dense NCs. The distribution of QDs within single microspheres was smoother, compared to the preparation in aqueous solution (Fig. 1G).

A comparison of the two experimental strategies revealed that the addition of QDs into the organic phase of the spray drying emulsion led to the most efficient and even encapsulation of nanoparticles into the PLGA MS. Hence, this experimental setup was chosen for all further experiments.

Encapsulation of PbS quantum dots is a suitable methods to label PLGA microspheres with an electron dense marker for electron microscopy

Initially we started to encapsulate QDs into PLGA MS to have an electron dense marker that allows identification of microspheres after phagocytotic uptake by

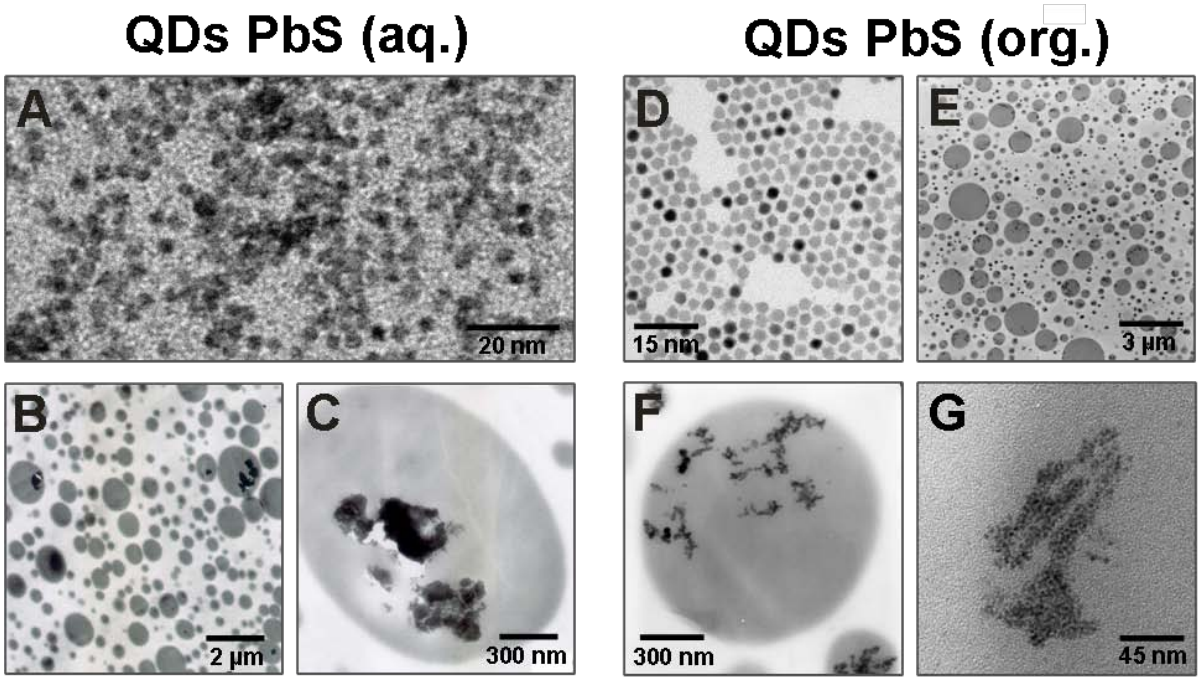

Figure 1: Transmission electron microscopy (TEM) images of PbS nanocrystals (NCs) before and after encapsulation into PLGA microspheres (PLGA MS). (A) Hydrophilic PbS NCs (QDs PbS (aq.)) directly after synthesis. (B,C) TEM images of PLGA MS preparations after including PbS (aq.) into the aqueous phase of the spray drying emulsion. (D) Hydrophobic PbS NCs (ODs PbS (org.)) directly after synthesis. (E,F) TEM images of PLGA MS preparations after including QDs PbS (org.) into the organic phase of the spray drying emulsion. (G) Image of encapsulated QDs PbS (org.) within a PLGA MS at higher magnification. Scale bars indicate the size of the images. PLGA MS images were performed from 50 nm slices. Figure panels show representative images.

immune cells. The labelling of PLGA MS with a defined electron dense material is essential to distinguish phagocytosed microparticles from subcellular structures that also occur in the absence of microspheres (Fig. 2, control). As shown in figure 1, we successfully labelled PLGA MS with electron dense PbS QDs and were therefore able to identify PLGA MS within the cytoplasm of bone marrow-derived dendritic cells (BMDCs), when analyzing TEM images of samples after phagocytosis. Interestingly, we found most microspheres present in larger vacuole-like organelles, each containing multiple particles. These structures were observed at 4 and 48 h after addition of labelled microspheres to BMDCs (Fig. 2; upper right, lower left).

As we were interested whether microspheres after phagocytotic uptake do enter the cytoplasm or stay in defined membrane-enclosed compartments, we had a closer look at cellular positions, where microspheres and cytoplasm are in close proximity (Fig. 2; lower middle, lower right). For some contact sides it was evident that

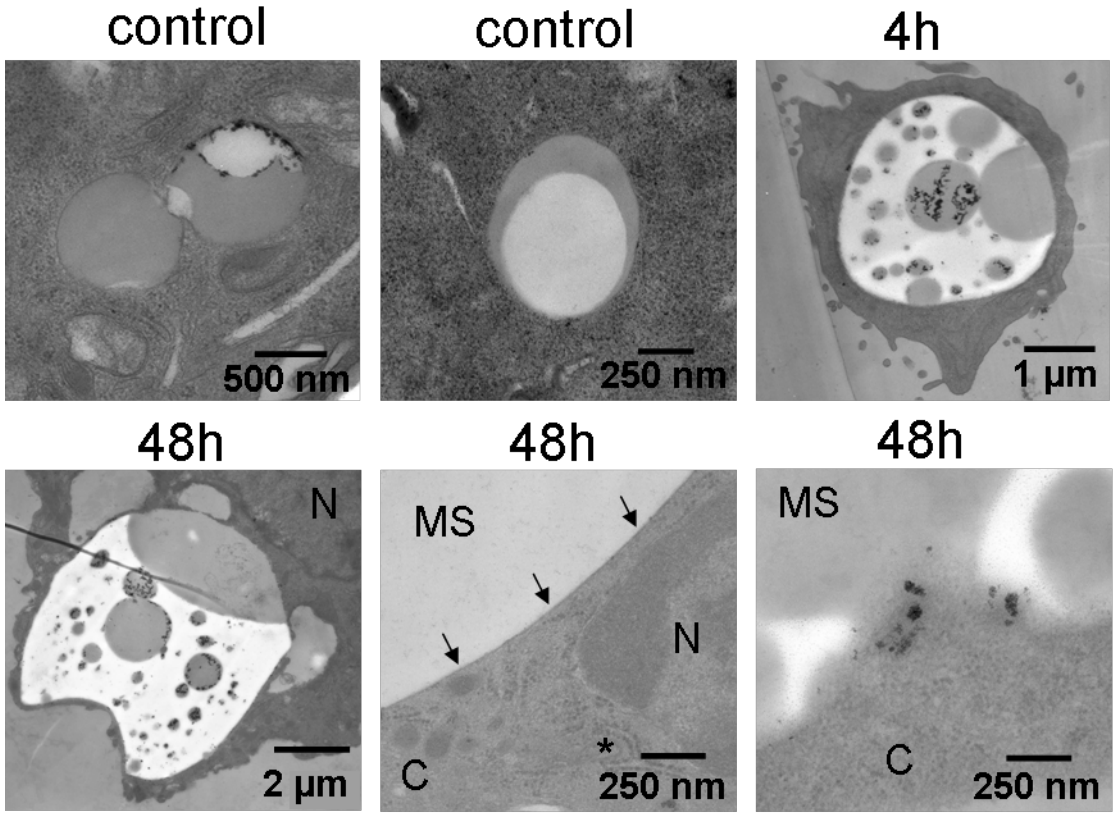

Figure 2: Transmission electron microscopy (TEM) images of PbS quantum dot-labelled PLGA microspheres after uptake by bone marrow derived dendritic cells (BMDCs). BMDCs were grown on petriPERM® dishes and microspheres were added or not (control). After 1h extracellular microspheres were washed off and BMDCs were further inoculated in medium for the indicated time points before preparation for TEM. The following abbreviations are used: MS, microsphere; N, nucleus; *, endoplasmatic reticulum with ribosomes; C, cytoplasm. Scale bars indicate the size of cellular structures. Arrows indicate enclosing membrane. Images were obtained from 50 nm slices. Figure shows representative images.

microspheres and cytoplasm were separated by a surrounding membrane. Other slices depicted microspheres that fused with the cytoplasm without a visible membrane. We observed microspheres that were surrounded by a membrane 48h after particle uptake. However, we wanted to confirm these results with an independent method. Therefore we performed uptake experiments with fluorescent PLGA MS, followed by confocal LSM.

Encapsulation of fluorescent CdSe QDs shows co-localization of PLGA MS with LAMP1$^+$ organelles in a dendritic cell line even 72 h after phagocytosis

After having established the encapsulation of PbS QDs into the PLGA MS preparations, we applied this procedure to NCs with other properties. Here we used CdSe QDs with an emission maximum of 583nm to study intracellular distribution of PLGA MS by confocal LSM. Encapsulation was performed by adding nanoparticles to the organic phase of the spray drying emulsion. Indeed, fluorescent CdSe QDs were efficiently encapsulated into the PLGA MS (TEM, data not shown).

Material which is engulfed by phagocytes usually ends up in the late endosomal/lysosomal compartment of the cell, where digestion is enabled by acidification (Honey and Rudensky 2003). In order to investigate whether microspheres are targeted to the lysosomal compartment, we chose LAMP1 as a marker molecule to label the membrane of lysosomes. At different time points after phagocytosis of fluorescent PLGA MS by the DCs line DC2.4, we looked for co-localisation with the lysosomal marker LAMP1 (Fig. 3). Shortly after addition of PLGA MS (10min) we observed attachment of particles to the outer membrane of the cells. Microspheres were not labeled by anti-LAMP1 staining (green). After 30min microspheres were already taken up by DC2.4 cells. However, no co-localization with LAMP1 could be observed at this early time point. Interestingly, as early as 2h after addition of microspheres, LAMP1$^+$ staining appeared around the microparticles, indicating the presence of a lysosomal membrane. This co-localization was getting even more prominent with time. At all later time points analysed, microspheres were surrounded by a distinct LAMP1$^+$ staining (24h and 48h, data not shown). From these data we conclude that the microspheres were efficiently taken up by DC2.4 cells and enter the lysosomal compartment as early as 2h after phagocytosis. Within the first 72h microspheres did not enter the cytoplasm, but localized in LAMP1$^+$ organelles.

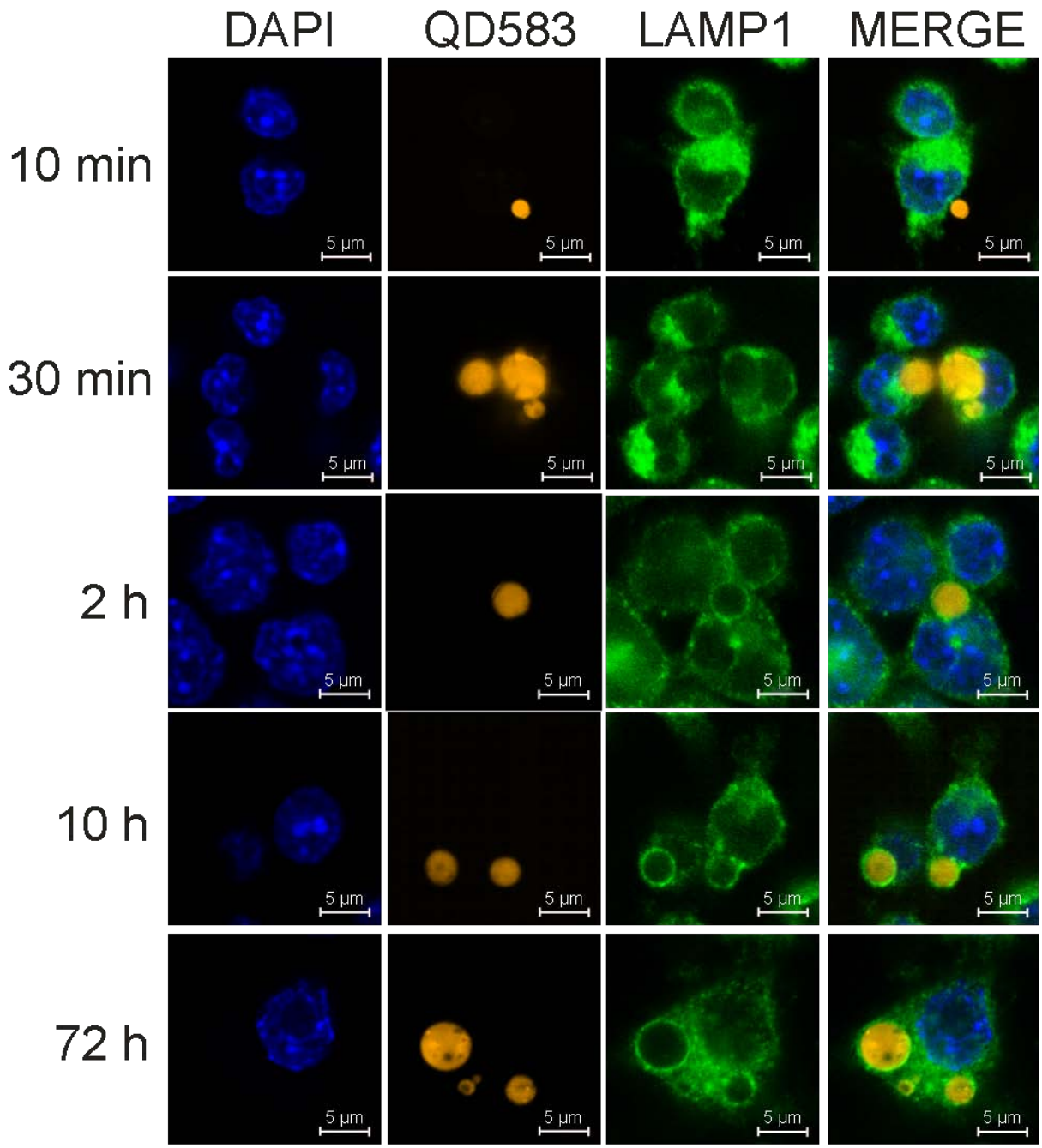

Figure 3: Confocal images showing the uptake of fluorescent microspheres by DC2.4 cells and co-localisation with the lysosomal marker LAMP1. DC2.4 cells were grown on cover slips and fluorescent CdSe quantum dot-labelled microspheres were added. After 1h (10 and 30 min for the first two time points) unbound microspheres were removed by washing with phosphate buffered saline and cells were further incubated in medium. At indicated time points cover slips were washed with phosphate buffered saline and cells fixed in 4% paraformaldehyde. Confocal laser scanning microscopy was performed after intracellular staining of cell nuclei (DAPI, blue) and the lysosomal marker LAMP1 (LAMP1, green). CdSe quantum dot-labelled microspheres were excited with a UV-laser (QD583, yellow). Images in the very right panel show merged channels (MERGE). Shown examples are representative images from the indicated time points after co-incubation.

Microspheres taken up by different primary antigen presenting cells localize in LAMP1$^+$ organelles even 48 h after phagocytosis

In order to extend the data observed for the cell line DC2.4, we prepared primary mouse DCs and macrophages, to further examine the intracellular fate of phagocytosed microparticles. Interestingly, also for these primary cells it was evident that both, at 4h and 48h after phagocytosis, microspheres were surrounded by a bright LAMP1$^+$ staining (Fig. 4), indicating the presence of a lysosomal membrane. For both cell types and time points we were not able to find significant numbers of microspheres that were not enclosed by bright LAMP1 staining. Hence, we conclude, that the lysosomal storage of PLGA MS within LAMP1$^+$ organelles is a general feature that is common in all APC lines analysed. This lysosomal storage has direct implications for the understanding of antigen presentation following microsphere based vaccination.

Efficient cross-presentation of PLGA MS-encapsulated ovalbumin by APCs despite of lysosomal storage of microparticles

The lysosomal storage of PLGA MS is a crucial parameter for the cross-presentation pathway. For the activation of CD8$^+$ T-cells by vaccination, it is critical that antigenic peptides are presented on MHC class I molecule (Kurts et al. 2010b). As discussed later, this cross-presentation of PLGA MS-encapsulated antigens was shown to be dependent on proteins entering the “direct-presentation pathway”. Our observation of a lysosomal storage of PLGA-particles argues that cross-presentation can only occur after translocation of proteins, but not entire particles, from the endo/lysosomal compartment into the cytosol. We analysed the intracellular fate of PLGA MS in three different cell types. Performing an *in vitro* assay, we wanted to demonstrate that cross-presentation of encapsulated ovalbumin can be observed in these cells, despite of lysosomal localization of PLGA particles. DC2.4 cells, BMDCs, and pMΦs were incubated with MS containing either ovalbumin (MS OVA) or left empty as control (MS empty). Additionally, $OVA_{257-264}$ specific CD8$^+$ T-cell hybridomas were added to detect cross-presentation of the SIINFEKL epitope on MHC class I molecules. As demonstrated for other APCs, all three cell types were able to

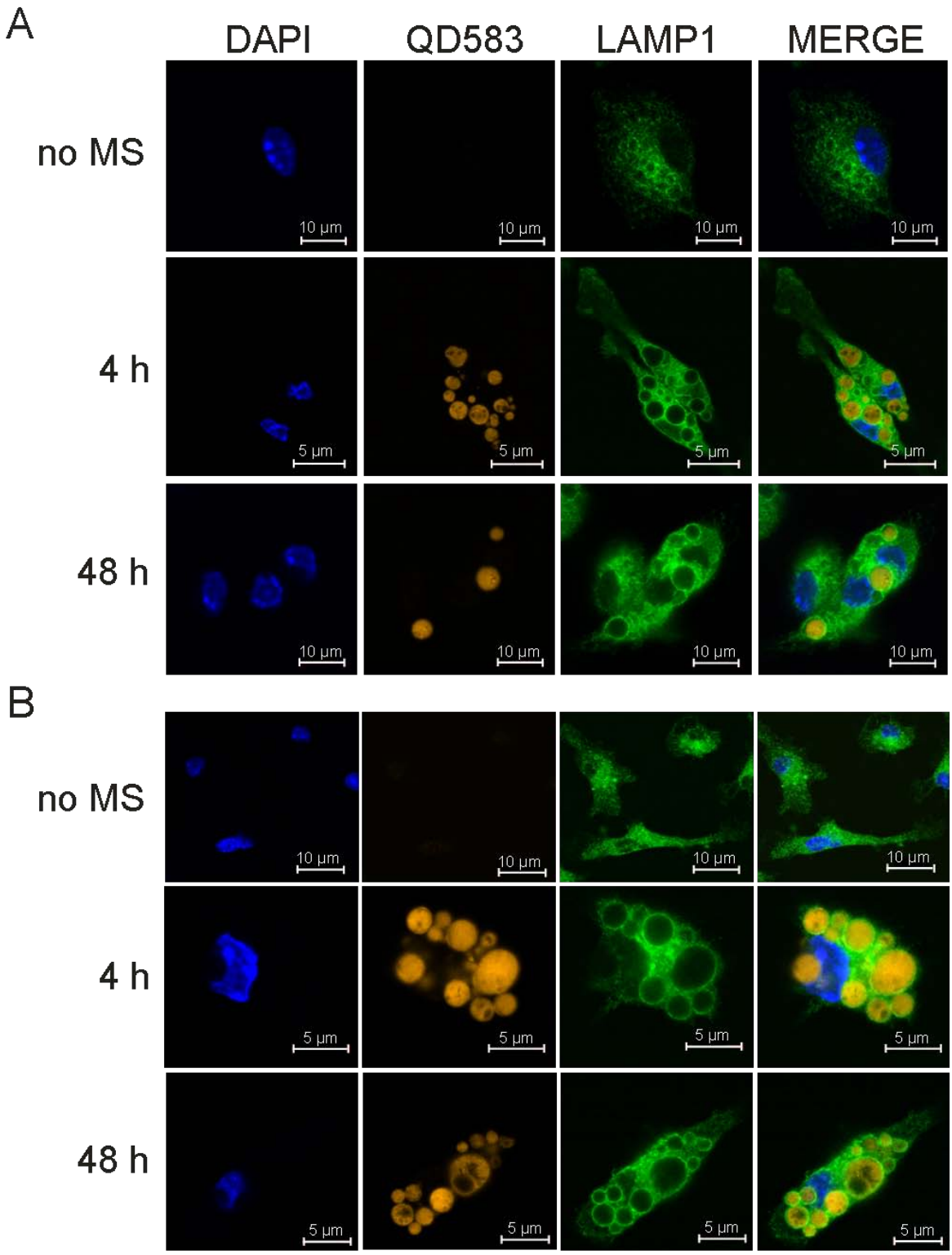

Figure 4: Confocal laser scanning microscopy (LSM) images of fluorescent CdSe quantum dot-labelled microspheres after uptake by primary peritoneal macrophages (A) and bone marrow derived dendritic cells (BMDCs) (B) and co-staining for the lysosomal marker LAMP1. Cells were grown on cover slips and fluorescent CdSe quantum dot-labelled microspheres were added for 1 h. Unbound microspheres were washed off with phosphate buffered saline and cells were further incubated in medium. At indicated time points cells were fixed with 4% paraformaldehyde and intracellular staining of cell nuclei (DAPI, blue) and the lysosomal marker LAMP1 (LAMP1, green) was performed. CdSe quantum dot-labelled microspheres were excited with the UV-laser (QD583, yellow). Images on the very right show merged channels (MERGE). Shown examples are representative results for the indicated time points.

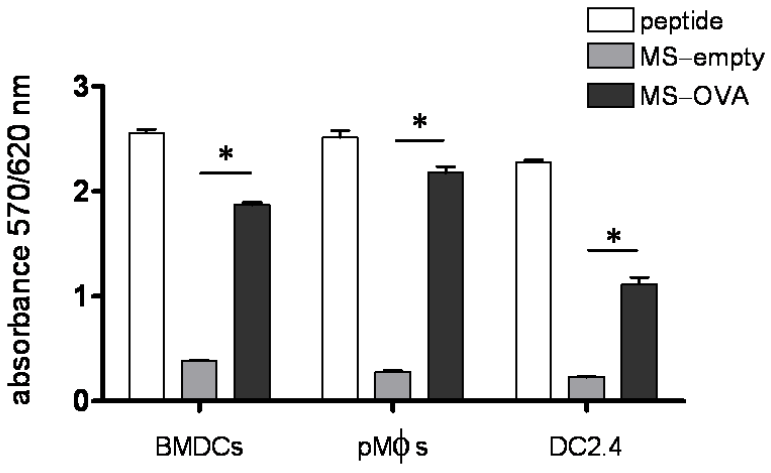

Figure 5: *In vitro* cross-presentation of PLGA MS-encapsulated ovalbumin by different antigen presenting cells 16 h after uptake. DC2.4 cells, bone marrow-derived dendritic cells (BMDCs), or peritoneal macrophages (pMΦs) were incubated with $OVA_{257-264}$-specific $CD8^+$ T-cell hybridomas B3Z, in the presence of either external $OVA_{257-264}$ peptide (peptide), empty microspheres (MS-empty) or microspheres containing OVA (MS-OVA). Activation of B3Z cells was detected in a colorimetric LacZ assay (absorbance at 570 nm with reference wave length of 620 nm). Significance was calculated by student's t-test from two independent experiments: (*)=$p<0.05$.

efficiently cross-present MS-encapsulated OVA after 16h (Fig. 5). From this finding we conclude that antigens encapsulated into PLGA MS are released from hydrolysing particles within the endo/lysosomal compartment and are then translocated into the cytoplasm.

Encapsulation of super-paramagnetic iron oxide nanoparticles (SPIONs) into PLGA MS for depletion of phagocytes from biological samples

PLGA MS in the micrometer range are efficiently taken up by phagocytes but not other cell types. This specific difference can be utilized to deplete phagocytes from biological samples by magnetic sorting using encapsulated SPIONs. In a straight forward proof of principle experiment we tried to deplete dendritic cells from a heterogeneous cell culture. The murine fibroblast cell lines B8 (BALB/c-derived, H-2^d) and the DC cell line DC2.4 (C57BL/6-derived, H-2^b) were cultured in a ~1:1 ratio. To illustrate the possibility to magnetically deplete DCs, PLGA MS containing SPIONs (MS-SPIONs) or empty microspheres (MS-empty) were added to the cell mixture for 1h. Afterwards cells were harvested and applied to a magnetic column. The two cells lines can be distinguished due to differences in the expression of the major histocompatibility complex (MHC) class I alleles (H-2^b and H-2^d). The flow through

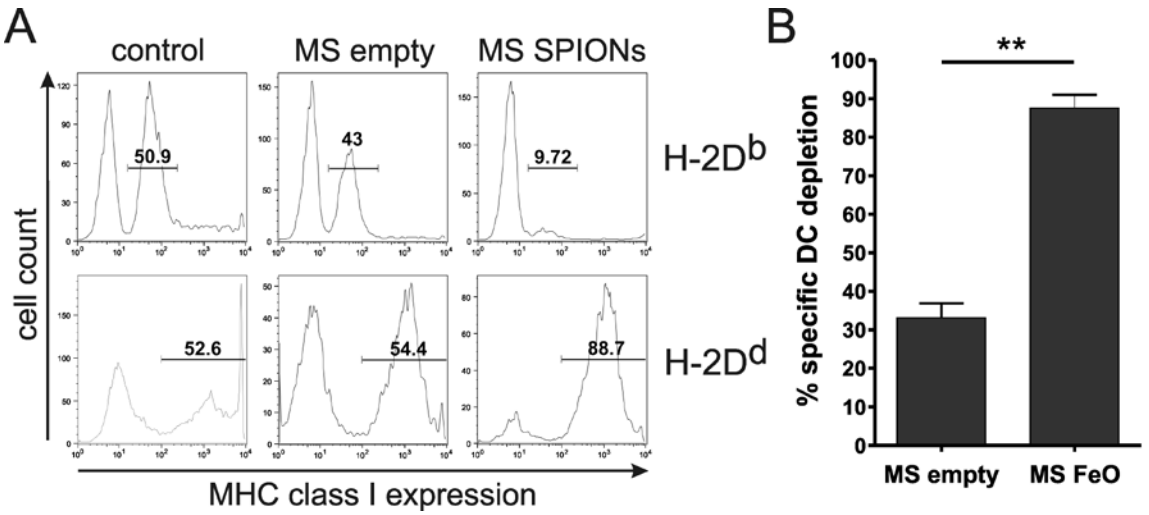

Figure 6: Transmission electron microscopy (TEM) images of PbS quantum dot-labelled PLGA microspheres after uptake by bone marrow derived dendritic cells (BMDCs). BMDCs were grown on petriPERM® dishes and microspheres were added or not (control). After 1 h extracellular microspheres were washed off and BMDCs were further inoculated in medium for the indicated time points before preparation for TEM. Following abbreviations are used: MS, microsphere; N, nucleus; *, endoplasmatic reticulum with ribosomes; C, cytoplasm. Scale bars indicate the size of cellular structures. Arrows indicate enclosing membrane. Images were obtained from 50 nm slices. Figure shows representative images.

was collected and analysed for the expression H-2 molecules (Fig. 6). We were able to magnetically deplete around 90% DC2.4 cells from the heterogeneous culture indicating the usefulness of this approach.

Discussion

Attempts to combine the properties of NCs with biocompatible polymer particles are still restricted to very few examples. In two recent publications, a method was introduced to externally label PLGA nanoparticles with NCs as potential biological probe for bioimaging and MRI spectroscopy (Cheng et al. 2008, Kuo 2009). The authors show homogenous distribution of NCs covering PLGA particle surface by TEM and are able to detect fluorescent PLGA nanoparticles by fluorescence microscopy. Other efforts illustrate the possibility to encapsulate NCs into PLGA particles using the nanoprecipitation method (Yang et al. 2006, Nehilla et al. 2008). In this report, we introduce a novel protocol to efficiently encapsulate NCs into PLGA MS using the spray drying method. Encapsulation by spray drying has a number of advantages. First, it does not modify the surface characteristics of PLGA particles, which might alter their characteristic biodistribution pattern. Second, generation of NC-encapsulated PLGA MS by spray drying does not require additional purification of particles from non-encapsulated NCs, as needed for nanoprecipitation (Nehilla et al. 2008). The encapsulation of NCs described here can be easily performed by any laboratory using the spray drying technique to produce PLGA MS, without any procedural adaptation.

The results presented in this report are to our knowledge the first examples to illustrate the potential of PLGA-encapsulated NCs as a tool to study intracellular distribution of particles by TEM and confocal co-localization studies. Understanding the intracellular fate of particulate vaccines after uptake by APCs is critical for the development of successful immunotherapies. For the induction of protective cytotoxic T-cell responses against cancer or other tissue specific immune targets, it is critical that the antigen encapsulated into PLGA MS is targeted to the MHC class I presentation machinery of APCs. Only these specialised immune cells are able to initially activate naïve T-cells by providing co-stimulatory signals (Mellman and Steinman 2001b). Generally, there are two pathways known how antigen-derived peptides can be presented on MHC class I molecules. Endogenous protein that are synthesised within the cell are usually degraded via the ubiquitin/proteasome system into polypeptides that are loaded onto MHC I molecules within the endoplasmatic

reticulum (Groettrup et al. 2001b). From there MHC/peptide complexes are translocated to the cell surface to allow T-cell receptor signalling. This way of MHC class I presentation is referred to as the “direct-presentation pathway”. In contrast, proteins that are taken up by APCs from extracellular compartments primarily enter the endo/lysosomal pathway. However, peptides derived from such proteins can still be presented on MHC class I via a pathway termed “cross-presentation” (Kurts et al. 2010b). For the cross-presentation of antigens that are encapsulated into PLGA MS the intracellular localization of hydrolyzing particles is critical to understand the mode of action of this vaccine. There are several publications addressing the intracellular localization after administration of PLGA particles. However, the data are inconsistent and two hypotheses can be found. In one of the first studies which analysed the intracellular distribution of biodegradable particles the authors used colloidal gold labelling to visualize nanoparticles (40-120nm) by TEM (Mathiowitz et al. 1997). In this study particles were found to enter the cytoplasm and could additionally be detected in Golgi and secretory vesicles of liver and epithelium cell. This paper was followed by a number of other publications that independently showed the endo/lysosomal escape of PLGA particles after uptake by MΦs (Gomes et al. 2006), smooth muscle cells (Panyam et al. 2002b), and epithelial cells (Cartiera et al. 2009). Biochemical evidence for an endosomal escape was provided by studying DCs and B-cells (Shen et al. 2006). An endosomal escape would allow release of encapsulated proteins into the cytoplasm, where they could be targeted to the “direct-presentation” machinery.

However, a number of other publications using similar techniques were published, finding no evidence for cytosolic transition of PLGA particles. A study using DCs and MΦs showed the presence of a phagocytotic membrane after uptake of particles (Walter et al. 2001). Independent reports showed membrane-enclosed PLGA particles after uptake by peritoneal exudates cells (de Jesus Gomes et al. 2006) and peritoneal MΦs (Trombone et al. 2007). These findings would favour other mechanisms for the cross-presentation of encapsulated antigen. Nevertheless, there is a general consensus that either the encapsulated antigens or the entire PLGA particles have to enter the cytoplasm in order to achieve efficient cross-presentation (Audran et al. 2003a). The heterogeneous results observed for this important question, may be in part due to differences in cell-types used, protocols for PLGA

polymerization and size distribution of particles. However, also the lack of electron dense material in PLGA preparations for TEM and photobleaching of conventional dyes might be responsible for misinterpretations. To circumvent this, we established a protocol to label PLGA MS with NCs. Our TEM analyses not only showed the efficient encapsulation of NCs, but also indicate the presence of an enclosing membrane 48 h after phagocytotic uptake of PLGA particles. More evidence came from our experiments performed by confocal LSM. Fluorescent QDs were encapsulated into PLGA MS and allowed co-localisation of intracellular particles with the lysosomal marker Lamp1. It was evident that even after 48h PLGA MS were located inside of $Lamp1^{+}$ organelles. The kinetic of particle uptake confirmed earlier studies performed by TEM (de Jesus Gomes et al. 2006). Taken together, our data show the lysosomal storage of PLGA MS after phagocytosis (Fig. 2-4). This has direct implication for the cross-presentation of encapsulated antigen, since MHC class I presentation already occurs at much earlier time points (Fig. 5). We hypothesise that not the entire PLGA particles, but proteins released within the lysosomes do cross the lysosomal membrane to enter the direct MHC class I loading machinery.

In the third part, we showed that encapsulation of SPIONs into PLGA MS leads to magnetic particles that can be applied for magnetic cell sorting. Magnetic nanoparticles are studied as contrast agents for magnetic resonance imaging (MRI) in humans (Pankhurst et al. 2003). Small size of particles allows distribution with the blood stream throughout the organism. SPIONs itself are not toxic and can be applied *in vivo* (Hergt et al. 2006a). To specifically label malignant tissues there were attempts to couple SPIONs to tumor-specific antibodies (Yang et al. 2007). This approach would allow exact determination of tumor size and distribution by MRI. Here we introduce the encapsulation of magnetic nanoparticles into PLGA MS using the spray drying method. PLGA MS in the micrometer range are specifically endocytosed by phagocytes only. Therefore, we were able to successfully use magnetic PLGA MS to clear a biological sample from phagocytic cells. This technology is specific for phagocytes but independent of antibody recognition. It might therefore be an easy and straightforward way to deplete phagocytes from a mixed suspension of cells.

Conclusions

Here we report on a method on how PLGA MS, successfully used as antigen carrier devices in vaccination, can be labeled either with electron dense PbS nanocrystals for intracellular localization of MS by electron microscopy or with CdSe nanocrystals for fluorescence microscopy. Microencapsulation of nanocrystals into PLGA MS by spray drying allowed us to localize PLGA MS within dendritic cells and macrophages at different time points after phagocytosis. We show that PLGA MS do not escape endosomes but remain in $LAMP1^+$ lysosomes up to three days after uptake although cross-presentation of microencapsulated antigen occurred much earlier. Microencapsulated nanocrystals are therefore instrumental for elucidating the cell biological pathways of the extraordinarily efficient cross-presentation afforded by PLGA MS. Furthermore, we show that ferromagnetic nanocrystals can be used for the efficient magnetic separation of phagocytic and non-phagocytic cells.

Acknowledgements

We thank Elisa May and the Bioimaging Center of Konstanz University for experimental support in fluorescence microscopy. We acknowledge the contribution of the DC2.4 cell line by Kenneth Rock and the B3Z hybridoma by Dr. Nilabh Shastri. This study was supported by a grant from Deutsche Krebshilfe (grant no. 107943 to M.G.). The authors declare that they have no conflict of interest.

Chapter III

Stable antigen is required for $CD8^+$ T-cell responses after DNA vaccination and infection with recombinant vaccinia virus *in vivo*

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Manuscript in preparation

for the *Journal of Virology*

Abstract

The efficient induction of cytotoxic T-cell (CTL) responses against infectious diseases and cancer is an ongoing challenge of today's immunology. The nature of an antigen and especially its half-life are critical parameters for the development and improvement of prophylactic vaccines and immunotherapies. Depending on the source of antigen and the infectious agent, priming of CTLs requires "direct-" and/or "cross-presentation" of antigenic peptides on major histocompartibility complex (MHC) class I molecules by professional antigen presenting cells (APCs). However, both pathways have preferences concerning the antigen stability. Whereas the direct-presentation pathway was shown to be efficient in presenting peptides derived from rapidly degraded proteins, cross-presentation is dependent on long-lived antigen species. Therefore, comparative immunizations with antigens of different half-life allow conclusions about the underlying mechanism of the respective vaccination.

In this report, we analysed the role of antigen stability on DNA vaccination and vaccinia virus infection using altered versions of the same antigen. The well studied nucleoprotein (NP) of lymphocytic choriomeningitis virus (LCMV) is a very long-lived viral model antigen that can be targeted for proteasomal degradation by N-terminal fusion to ubiquitin or, as we show here, with the ubiquitin-like modifier FAT10. This proteasomal targeting was used as a tool to study the impact of antigen stability under various experimental conditions. Whereas direct-presentation by cells either transfected with NP encoding plasmids or recombinant vaccinia viruses (VV) *in vitro* was enhanced in the presence of the short-lived form of the antigen, the opposite was observed *in vivo* after DNA vaccination and infection with recombinant VV. In this case, best induction of NP-specific $CD8^+$ T-cell responses was detected in the presence of the long-lived antigen. Our experiments provide evidence that targeting antigens for proteasomal degradation does not improve the immunogenicity of DNA vaccines and recombinant VVs. It is the long-lived antigen that is required for efficient class I restricted immune induction.

Introduction

Vaccination strategies for inducing $CD8^+$ T-cell responses against intracellular pathogens and cancer are based on the major histocompartibility complex (MHC) class I presentations of antigenic peptides by professional antigen presenting cells (APCs) (Ramirez and Sigal 2004, van den Broek et al. 2010). In these specialized immune cells, two classes of MHC I restricted antigen presentation pathways exist in parallel that can both be utilized for immunotherapy and vaccination. Proteins that are expressed within the APCs are usually degraded via the ubiquitin/proteasome system (Sorokin et al. 2009) and the generated peptides are translocated into the endoplasmic reticulum (ER) via the transporter associated with antigen processing (TAP). Peptides are then loaded on MHC class I molecules, before they take the secretory pathway to be presented on the cell surface. This pathway is referred to as the “direct-presentation” pathway (Hansen and Bouvier 2009). In contrast to this, APCs can also acquire exogenous antigens that are either derived from expression by non-professional cells, pathogens, or delivered as particulate vaccines. The mechanism facilitating this way of antigen presentation was discovered by M. Bevan in 1976 and was later termed “cross-presentation” (Bevan 1976a, Basta and Alatery 2007, Lin et al. 2008a). Since both, direct- and cross-presentation can lead to MHC class I presentation of antigenic peptides and the priming of naïve cytotoxic T-cells (CTLs), they are both interesting targets for vaccinations aiming at specific CTL induction. However, the two pathways favour different antigen properties, especially concerning the protein stability. Generation of peptides for direct-presentation was shown to strongly depend on the formation of defective ribosomal products (DRiPs) (Yewdell et al. 1996, Khan et al. 2001b, Yewdell et al. 2001, Pierre 2005). Co-translational misfolding and rapid proteasomal targeting leads to an increased peptide generation that can be loaded on MHC class I molecules. In contrast to this, there are studies indicating that cross-presentation depends on stable antigens that are not immediately targeted for proteasomal degradation (Basta et al. 2005, Donohue et al. 2006).

These biases for differences in antigen stability have to be taken into account when aiming at the induction of CTL responses by vaccination. However, for many

vaccines the relative contribution of direct- and cross-presentation was not elucidated yet. Therefore, it is hard to predict whether stable or unstable antigens are able to increase the immunogenicity of a particular vaccine.

DNA vaccination is a promising approach to induce MHC class I restricted immune responses (Choo et al. 2005, van den Berg et al. 2010, Liu 2011). Despite a relatively low intrinsic immunogenicity it has the advantage of combining the low-cost production with easy handling and the independence of a functional cold chain. These properties give DNA vaccines the excellent potential to be used especially in developing countries (Carvalho et al. 2010). In recent years DNA vaccines against various targets were investigated and published (Choo et al. 2005, Wu 2007, Alam and McNeel 2010). Optimization of target vectors, especially overcoming the low immunogenicity, is currently in the focus of interest (Leroux-Roels 2010). To induce $CD8^+$ T-cell responses after *intramuscular* (*i.m.*) injection of DNA constructs, encoded antigens can be directly presented by transfected APCs. If this does not occur, antigens expressed by muscle and fibroblast cells have to be cross-presented by professional cells (Ulmer and Otten 2000). Depending on the contribution of both possibilities modification of antigen stability could provide beneficial immunogenicity for a vaccination approach using DNA.

A similar situation is observed for viral vectors that are used for immunotherapy and vaccination. Genetically modified strains of vaccinia virus (VV), the effective protective vaccine against small pox, were developed as tools to induce immune responses against recombinant antigens (Jacobs et al. 2009, Moss 2011). Even though anti-VV responses are dominated by $CD4^+$ T-cell and B-cell responses, the broad spectrum of infected cell types offers interesting opportunities to also induce class I restricted responses via direct- and cross-presentation. Infection with VV *in vivo* also targets APCs, which could allow induction of VV-induced $CD8^+$ T-cell responses by direct-presentation, as recently suggested (Xu et al. 2010). Modification of antigenic stability can therefore possibly be used to enhance MHC class I restricted responses.

In this study we investigated the role of protein stability on MHC class I presentation after DNA vaccination and infection with VV. As model antigen we choose the

extremely long-lived nucleoprotein (NP) of the murine lymphocytic choriomeningitis virus (LCMV) (Zinkernagel 2002). LCMV is a frequently used model to study anti-viral immune responses. It belongs to the arena virus family and consists of two structural proteins, the NP and the glycoprotein (GP). Infections with LCMV induce strong NP- and GP-specific CTL responses in mice (Butz and Bevan 1998). The LCMV proteins were used as model antigens to study direct- and cross-presentation (Basler and Groettrup 2007b, Pavelic et al. 2009). Importantly, for the LCMV NP it was shown that cross- but not direct-presentation is dependent of the long-lived form of the antigen and independent of neosynthesis. Additionally, in this system defective ribosomal products (DRiPs) were published to be the major antigen source for direct-presentation (Basta et al. 2005).

Antigen stability and protein degradation in general are dependent on a highly regulated degradation machinery that maintains homeostasis and can lead to cellular differentiation, adaptation or division. Generally, proteins that are supposed to be degraded via the proteasome are conjugated to the 8kDa protein ubiquitin via a ubiquitin conjugating enzyme cascade (Groettrup et al. 2001c, Sorokin et al. 2009). This conjugation leads to proteasomal recognition of the substrate and to its degradation. Beside ubiquitin there is a family of proteins called ubiquitin-like modifier that can also be specifically conjugated to target proteins. However, from all ubiquitin-like modifiers only the F-locus associated transcript 10 (Fat10, 18kDa) is, like ubiquitin, able to target proteins for proteasomal degradation (Groettrup et al. 2008). In this study we tried to use ubiquitin-NP as well as Fat10-NP fusion proteins to shorten the half-life of the LCMV NP model antigen. This approach allowed us to investigate the role of antigen stability on immune induction after DNA vaccination and recombinant VV infection.

We show for the first time that N-terminal fusion of Fat10 to a viral antigen leads to a reduction in protein stability as reported for ubiquitin. Further, we provide evidence that protein stability is a critical parameter that can strongly influences the outcome of a specific immunization approach. Whereas direct-presentation after transfection or infection with recombinant VV of cell lines *in vitro* was increased when using short-lived NP-fusion proteins as antigens, the opposite case was observed for DNA vaccination and recombinant VV infection *in vivo*. Our data indicate that targeting an

antigen for proteasomal degradation must not necessarily improve a vaccination protocol, as proposed in the past (Rodriguez et al. 1997).

Materials and Methods

Mice, cells and media

C57BL/6 (H-2^b) and Balb/c (H-2^d) mice were originally obtained from Charles River Laboratories and further bred in the animal facilities of the University of Konstanz. BL/6 x Balb/c F1 mice were generated by crossing of C57BL/6 and Balb/c mice. All animals were kept under specific pathogen-free conditions in accordance with the rules of the veterinarian authority of Regierungspräsidium Freiburg and used for the experiments at 6-12 weeks of age.

Primary murine CTL-lines were cultured in RPMI 1640, 10% FCS, 20 U/ml interleukin-2, 50µM β-mercapto-ethanol, 50 µg/ml gentamycin. The Human embryonic kidney cell line HEK293 (Graham et al. 1977) was maintained in DMEM, 10% FCS, 100 U/ml penicillin/streptomycin (P/S). The murine fibroblast cell line B8-wt (H-2^d) (Groettrup et al. 1995b) was cultured in IMDM, 10% FCS, 100 U/ml P/S. B8-D^b are B8 cells, stably transfected with H2-D^b and were cultured in IMDM, 10% FCS, 100 U/ml P/S, 5 µg/ml puromycin (Basler et al. 2004). The human cell line 143B (TK^-) was maintained in MEM, 10% FCS, 100 U/ml P/S, 25 µg/ml BrdU (ATCC: CRL-8303). BSC-40 cells are kidney-derived epithelial cells from *Cercopithecus aethiops* and were cultured in MEM, 10% FCS, 100 U/ml P/S (ATCC: CRL-2761). Primary peritoneal macrophages were cultured in DMEM, 10% FCS, 100 U/ml P/S. All cell culture media and supplements were obtained from Gibco, Invitrogen.

Generation of NP constructs

The plasmids pCMV_NP and pCMV_ Ub-NP were kindly provided by L. Whitton (Scripps Research Institute, USA, CA) (Rodriguez et al. 1997). The plasmid pCMV_FAT10-NP encoding a N-terminal Fat10 fusion protein of the NP was generated as follows: mouse Fat10 was amplified by PCR from pBKCMV_HA-FAT10-GFP (kindly provided by G. Schmidke, University of Konstanz, Germany) generating a N-terminal XhoI and a C-terminal EcoRI restriction side using the primer pair: fwd 5´-TGG TAC CTC GAG ATG GCT TCT GTC CGC ACC-3´ and rev 5´-ATA CTA GAA TTC TGC CAC AGT GCA GTG TGT-3´ introducing a GG to VA mutation at the C-terminal end of the amino acid sequence of Fat10. According to the ubiquitin system this mutation protects Fat10 from being cleaved off the

substrate by putative de-Fat10ylating enzymes. NP was amplified by PCR from pCMV_NP using the primer pair: for 5`-TAT GAT GAA TTC ATG TCC TTG TCT AAG GAA GT-3´ and rev 5´-ATC CCC GCG GCC GCT TAG AGT GTC ACA ACA TT-3´ introducing an EcoRI and a NotI restriction side. Both fragments were digested with EcoRI and ligated before Fat10-VA-NP was amplified by PCR using the primers Fat10_for and the reversed primer NP_rew. The amplified Fat10-VA-NP construct was then introduced into the XhoI/NotI side of pCMV.

Pulse chase experiments

Analysis of protein stability of different NP constructs was performed by radioactive pulse chase experiments, which were adopted from (Schwarz et al. 2000c). Therefore, HEK293 cells were transfected with NP encoding pCMV constructs using Fugene 6 (Roche). After 18h cells were washed two times with PBS and then incubated in medium lacking methionine (RPMI-1640 medium modified, R5713, Sigma). After 1.5 h of starvation, radioactively labelled ^{35}S methionine was added to the cells in a concentration of 0.25 mCurie/ml. After 1 h of radioactive labelling ^{35}S methionine was removed, cells washed with PBS, and further incubated in cold medium. At indicated time points cells were washed with ice cold PBS and cell pellets were frozen at -20°C. Then pellets were resuspended in lysis buffer [50 mM Tris-HCl pH 8, 150 mM NaCl, 1 mM EDTA, 2% NP40, protease inhibitors, in H_2O] and cells were lysed for 30 min on ice. Afterwards cell debris was removed by centrifugation and radioactivity of the supernatant was quantified using a scintillation counter (Top Count NXT^{TM}, Packard). Detected values were used to equalise supernatants before NP-specific immunoprecipitation (IP). In a step of pre-clearance protein G beads (Sigma) were added to the cell supernatant for 1 h. Afterwards, beads were removed by centrifugation and fresh protein G beads were added in the presence of the NP specific antibody KL53 (Schwarz et al. 2000b). Samples were incubated on a rotator at 4°C. After 18 h beads were washed three times with ice cold NET-TON buffer [650 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl pH 8, 0.5% Triton X-100, 1 mg/ml ovalbumin (Sigma)] and NET-T buffer [150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl pH 8], respectively. Then beads were resuspended in SDS-sample buffer, heated for 5 min/95°C, and finally loaded on a 10% SDS-PAGE gel. After electrophoresis gel was dried and exposed to a radiosensitive photo plate. After 24 to

48h radioactive bands were visualized using a phosphoimager (Molecular Imager® FX, Bio-Rad).

Generation of specific CTL lines

Naive C57BL/6 or Balb/c mice at 6 to 8 weeks of age were intravenously (*i.v.*) infected with 200 pfu LCMV. Starting from 3 weeks after infection, memory mice were used for the generation of $NP_{396-404}$ and $NP_{118-126}$ specific CTL lines. Spleens from memory mice were homogenized and isolated splenocytes were further purified by Ficoll density gradient centrifugation (GE Healthcare). Splenocytes were further cultured in 6-well plates ($1.5*10^7$ cells/well) directly pulsed with 10^{-6} M NP396 or NP118 peptide. Medium was renewed every second day. On day 5 density Ficoll gradient centrifugation was repeated to remove dead cells. Remaining splenocytes were washed with PBS and further cultured in the presence of IL-2. Specific NP396 (H2-D^b) and NP118 (H2-L^d) CTL lines were used between day 7 and 9 of culture.

Preparation of primary peritoneal macrophages

Peritoneal macrophages (pMΦs) were prepared by *intraperitoneal* (*i.p.*) injection of 2 ml 3% thioglycolate solution into BL/6 x Balb/c F1 mice. After three days, peritoneal cells were washed out of the abdominal cavity using 10 ml of PBS. Cells were cultured for 2 days and adherent cells were used for transfection and antigen presentation assays.

In vitro antigen presentation assay

To determine the amount of direct-presentation after transfection of NP encoding plasmids cells were electroporated using the Amaxa technology (Lonza). B8, B8-D^b, or pMΦs were harvested and used in a concentration of $2*10^6$ cells per 100 µl. 1 µg plasmid DNA was used for individual transfections. Electroporation was performed according to the user's manual with the MEF2 kit and program A01 of the Amaxa device. 1 µg of plasmid encoding GFP was co-transfected as internal control to determine transfection efficiency in individual transfections. After transfection, cells were cultured in complete medium. After 18 h cells were harvested and the amount of GFP^+ cells was analysed by flow cytometry. To determine antigen presentation of NP-derived epitopes, transfected cells were titrated in 96-well plates starting with an initial concentration of $2*10^5$ cells (1/1) in 100 µl RPMI medium per well. All samples

were prepared in duplicates. $2*10^5$ NP-specific CTLs in 100 µl RPMI medium containing brefeldin A in a concentration of 20 µg/ml (2x) were added to each of the wells. After 4 h incubation at 37°C/5% CO_2 activation of CTLs was determined by intracellular cytokine staining and flow cytometry. Error bars were calculated from duplicates.

Intracellular cytokine staining (ICS)

ICS was performed to detect intracellular accumulation of IFN-γ as a measure of CTL activation. Samples were centrifuged and 50 µl TriColor anti-mouse-CD8a 1:150 in PBS (Invitrogen) was added to each well on ice and incubated for 15 min in the dark. After washing with ice cold PBS, 50 µl of 3% para-formaldehyde (Acros Organics) solution was added for 5 min at room temperature. Afterwards, samples were again washed with PBS. Staining for IFN-γ was performed by adding 50-µl FITC anti-mouse-IFN-γ (kind gift from M. Basler, University of Konstanz) 1:1000 in PBS, 1% Saponin. The staining was incubated over night at 4°C. Before acquisition by flow cytometry (FACScan, BD), samples were washed, resuspended in PBS and kept on ice in the dark.

DNA immunization

NP expressing pCMV plasmids for DNA vaccination were generated in *E. coli* TOP10 F´ and purified using a plasmid purification kit (Midi plasmid Kit, Sigma). Plasmid concentration was determined by a spectrophotometer (NanoVue, GE Healthcare) and adjusted to 2 µg/µl. 100 µg plasmid DNA in 50 µl were injected and electroporated into each of the two hind legs of C57BL/6 mice *intramuscular* (*i.m.*) using an *in vivo* electroporation device (kindly provided by Ichor Medical Systems, San Diego, Ca). DNA immunization was repeated two times after 14 and 28 days. On day 7 after the last boost, mice were sacrificed and splenocytes prepared. The NP396-specific immune response was quantified by ICS.

Generation of recombinant vaccinia virus

Recombinant vaccinia virus (rVV) expressing the LCMV NP was kindly provided by R. Zinkernagel (Schulz et al. 1989). rVV expressing ubiquitin-NP (Ub-NP) and Fat10-NP fusion proteins were generated as follows: NP sequences were introduced into

SalI/NotI restriction site of the vaccinia transfer plasmid pSC11-S-B-A-K-N (kindly provided by Bernhard Moss, NIAID NIH, USA, WA), generating the transfer plasmids pSC11-S-B-A-K-N_Ub-NP and pSC11-S-B-A-K-N_Fat10-VA-NP. Both constructs were amplified by PCR from pCMV-Ub-NP/pCMV-Fat10-VA-NP using the primer pair: for 5´- TAT GAT GTC GAC ACT CTA GAG GAT CCG GTA C-3´ and rev 5´- ATC CCC CCA TGG TTA GAG TGT CAC AAC ATT-3´, introducing restriction sites for 5´SalI and 3´NotI. Generation of rVV was adapted from (Talavera and Rodrigues 1991). Briefly, sub-confluent 143B (TK^-) cells were infected with wild type VV strain Wyeth (kindly provided by M. van den Broeck, ETH, Zürich) at a multiplicity of infection (MOI) of 0.1. After 2 h unbound virus was removed by addition of fresh medium. Afterwards vaccinia transfer plasmids were transfected into TK^- cells using Fugene 6 (Sigma). After 48 h medium was removed and cells were embedded in 1% Agarose in MEM containing 10% FCS, 25 µg/ml BrdU, 300 µg/ml X-Gal (Sigma). Plaques that appeared blue within 6 h of time were picked and resuspended in 500 µl MEM, 2% FCS. Isolated viral samples were frozen 3 times at -70°C and then used to re-infect sub-confluent TK^- cells. Purification of plaques was repeated until in three following re-infections only blue plaques could be detected. After the last isolation rVV were used to infect BCS-40 cells to generate large amounts of virus. The viral titer of rVV was determined and aliquots frozen at -70°C.

Virus titer determination

The titer of rVV infected BSC-40 cell lysates was analyzed after 3 freeze and thaw cycles of cell suspensions. One day prior to the experiment BSC-40 cells were plated in 24-well plates to reach confluence the next day. Lysates of rVV infected BCS-40 cells were titrated to the 24-wells in 1:10 dilutions, starting with an initial dilution of 1:1000. In case of *ex vivo* determination of viral titer in ovaries of infected mice, ovaries were taken on day 4 after infection and physically disrupted by dounce homogenization in MEM, 10% FCS. Suspensions were frozen and thawed 6 times. Titrated amounts of lysate were added to 24-wells of BSC-40 cells starting with pure lysate. After infection BCS-40 cell were cultured in MEM, 10% FCS, 100 U/ml P/S and incubated for 24 to 48 at 37°C, 5% CO_2. Afterwards medium was removed and plaques visualized by addition of 0.5 % crystal violet solution for 1 h. Then plates were washed in a water bath and plaques were counted to calculate the number of plaque forming units (pfu) in the stock lysate.

Determination of immune responses in vaccinia virus infected mice

For analysis of immune responses in mice during VV infection C57BL/6 mice were injected with $2*10^6$ pfu wild type or recombinant VV *i.p.*. Non injected mice served as control. On day 7 after infection mice were sacrificed and splenocytes prepared and re-stimulated with 10^{-6} M of $B8R_{20-27}$, $NP_{396-404}$, or $NP_{118-126}$ peptide, in the presences of 10 µg/ml brefeldin A for 5 h. Afterwards activation of CTLs was quantified by ICS and flow cytometry.

Results

N-terminal fusion of the LCMV nucleoprotein with ubiquitin or the ubiquitin-like modifier FAT10 decreases stability of the long-lived protein

The half-life of a protein is critical for its physiological function. At the same time, peptides for MHC class I presentation are derived from proteins that are degraded

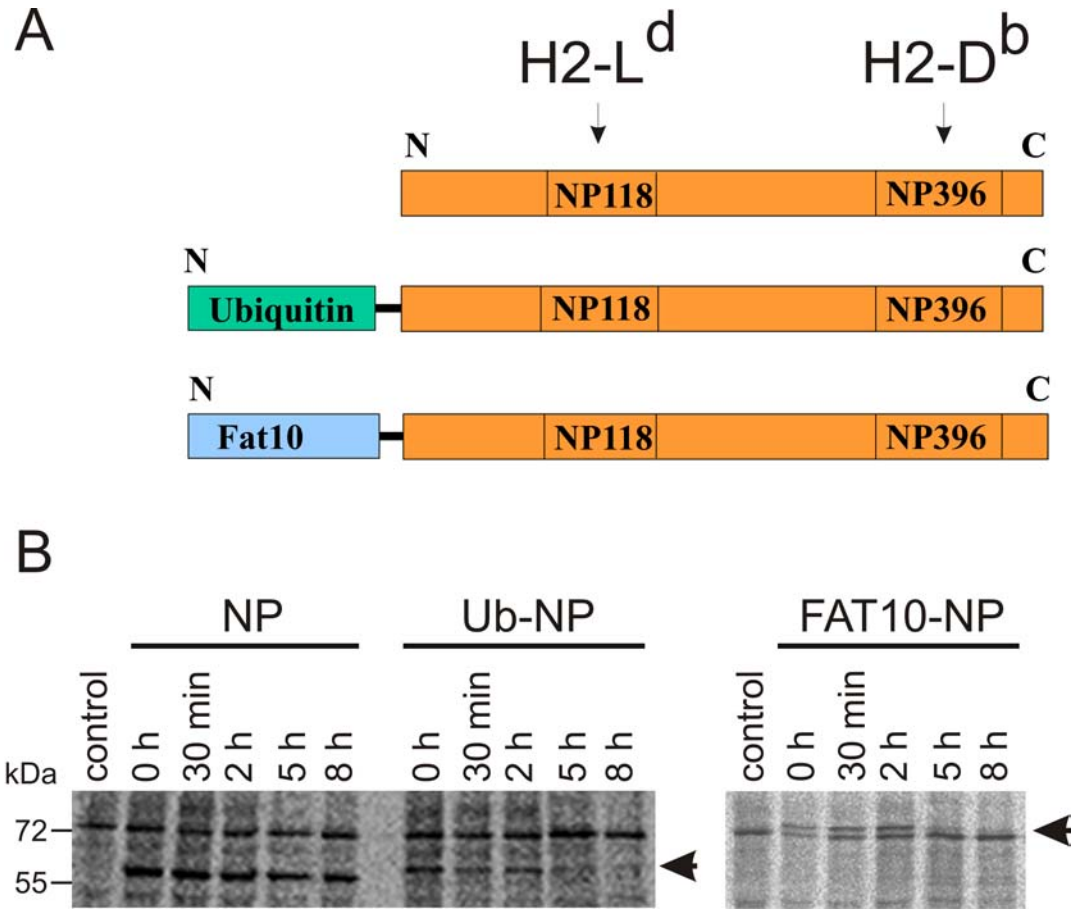

Figure 1: pCMV constructs used for the analysis of antigen presentation after transfection and DNA immunisation. A, The lymphocytic choriomeningitis virus (LCMV) nucleoprotein (NP) (orange) was either left unmodified or was cloned in linear N-terminal fusion with either ubiquitin (Ub-NP) or FAT10 (FAT10-NP). Arrows indicate the two NP epitopes that were used in this study; the H2-L^d restricted $NP_{118-126}$ (NP118) and the H2-D^b restricted $NP_{396-404}$ (NP396). B, Pulse chase experiments to analyse the stability of different NP constructs. HEK293 cells were transfected with individual constructs and metabolically labelled with S^{35} methionine. After indicated time points cells were lysed and NP was immunoprecipitated with the anti-NP antibody KL53. Samples were then separated by SDS-PAGE, dried and imaged on a radio-imager. Arrows indicate the size of NP or indicated NP fusion proteins.

via the ubiquitin/ proteasome system. However, not only peptides of degraded mature proteins are thought to contribute to the overall MHC class I presentation, but also the degradation of DRiPs. Although both direct- and cross-presentation have been investigated intensively, a direct comparative study that analyses the impact of antigen stability for different vaccines was not performed yet.

Initially, we used three different constructs to analysed the role of antigen stability (Fig. 1A). The LCMV NP was used without any modification or as fusion protein of the NP with either ubiquitin or FAT10. To show the modified antigen stability of fusion proteins we performed radioactive pulse chase experiments after transfection of DNA

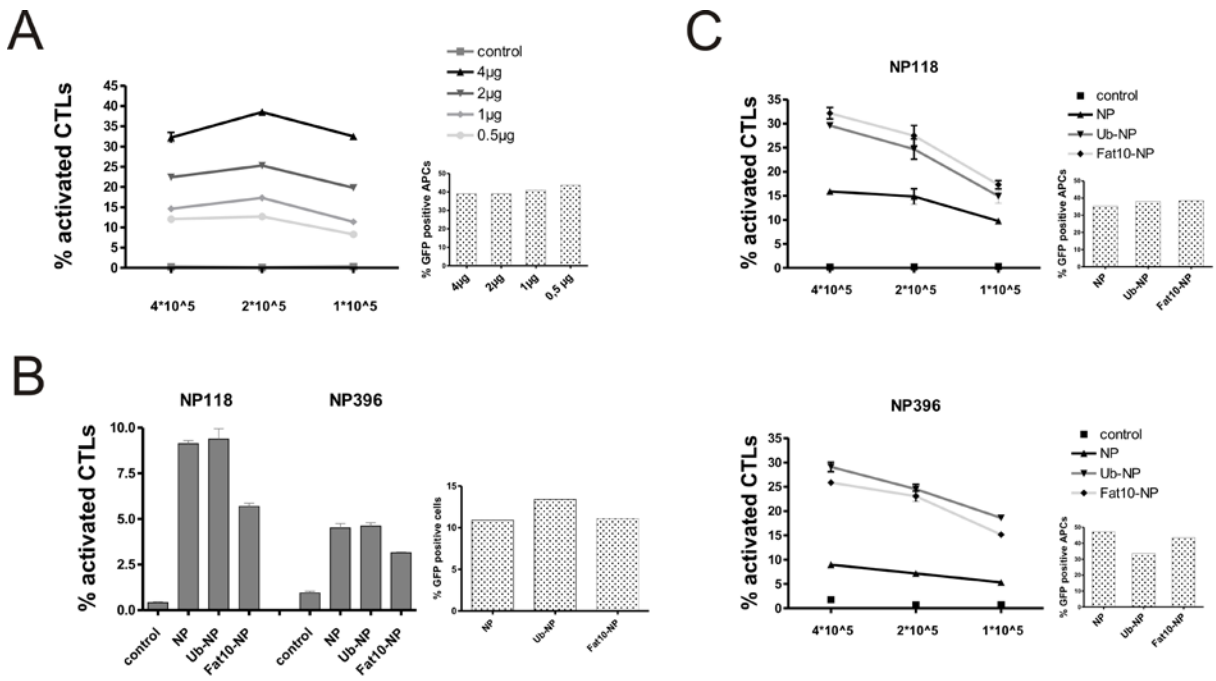

Figure 2: Direct-presentation after transfection of lymphocytic choriomeningitis virus (LCMV) nucleoprotein (NP) constructs *in vitro*. A, Titration of pCMV NP transfection. Indicated amounts of pCMV NP DNA was transfected into B8D^{b} cells and presentation of the epitope NP396 was analysed by NP396-specific cytotoxic T-cells (CTLs) after 18 h. Antigen presentation was measured by intracellular cytokine staining (ICS). For each condition a plasmid encoding for GFP was co-transfected to compare transfection efficiency. B, Thioglycolate induced peritoneal macrophages (pMΦs) from C57BL/6 x Balb/c F1 mice were transfected with 1 µg of pCMV encoding for the NP alone (NP) or NP fusions with ubiquitin (Ub-NP) or FAT10 (FAT10-NP). After 18 h CTLs specific for the NP epitopes NP118 and NP396 were added and antigen presentation measured via ICS. Co-transfection with GFP served as transfection control. C, B8 cells (H-2^{d}) and B8-D^{b} cells (H-2$^{d/b}$) were transfected with pCMV constructs (as described in B) and antigen presentation was analysed after 18 h by co-incubation with specific CTL lines and ICS. GFP co-transfection served as control. Numbers on the X axis of the graph indicate number of transfected cells. All experiments shown are representative results of at least two independent repeats.

constructs in HEK293 cells (Fig, 1B). While the NP alone was found to be stable during the time period analysed, fusion with ubiquitin reduced the half-life of the NP leading to almost complete degradation. Interestingly, also fusion with FAT10 led to a destabilisation of the NP, similar to what was observed for the ubiquitin fusion. This experiment not only validates the NP constructs to be adequate for a comparative study of antigen stability and its impact on antigen presentation, but also shows for the first time the FAT10-mediated degradation of a viral antigen. In the following experiments, presentation of two different epitopes was analysed. The H2-D^b restricted $NP_{396-404}$ and the H2-L^d restricted $NP_{118-126}$ (Fig. 1A).

Targeting the LCMV NP to proteasomal degradation increases direct-presentation in fibroblast cells but not macrophages

In a first experimental setting we analysed the impact of protein stability on direct antigen presentation after transfection of plasmid DNA. In order to detect a possible difference between individual NP constructs we had to ascertain our transfections would not lead to a saturation situation with maximal CTL activation. Therefore, we performed an experiment to titrate the amount of plasmid DNA transfected into the antigen presenting cells (Fig. 2A). As expected, we observed increasing antigen presentation with transfecting increasing amounts of NP-expressing plasmid. Although best CTL activation was observed at a concentration of 4 µg/6-well, we decided to use 1 µg plasmid DNA for all further transfections. This way we could exclude oversaturation of the system as a reason for insignificant difference between experiments performed with individual constructs.

We analysed the role of antigen stability for direct-presentation in thioglycolate induced pMΦs (Fig. 2B). C57BL/6 and Balb/c mice were crossed to breed H2-$D^{d/b}$ expressing F1 offspring. This way we could analyse the presentation of both the NP118 and the NP396 epitope in one experiment. pMΦs were transfected with pCMV plasmids encoding either the NP alone or unstable fusion protein. Interestingly, pMΦs presented equal amounts of peptides derived from the stable NP or the unstable Ub-NP. However, less peptides were presented in cells transfected with the construct encoding for FAT10-NP. This observation was found for both epitopes analysed and was not due to alterations in transfection efficiency (Fig. 2B,

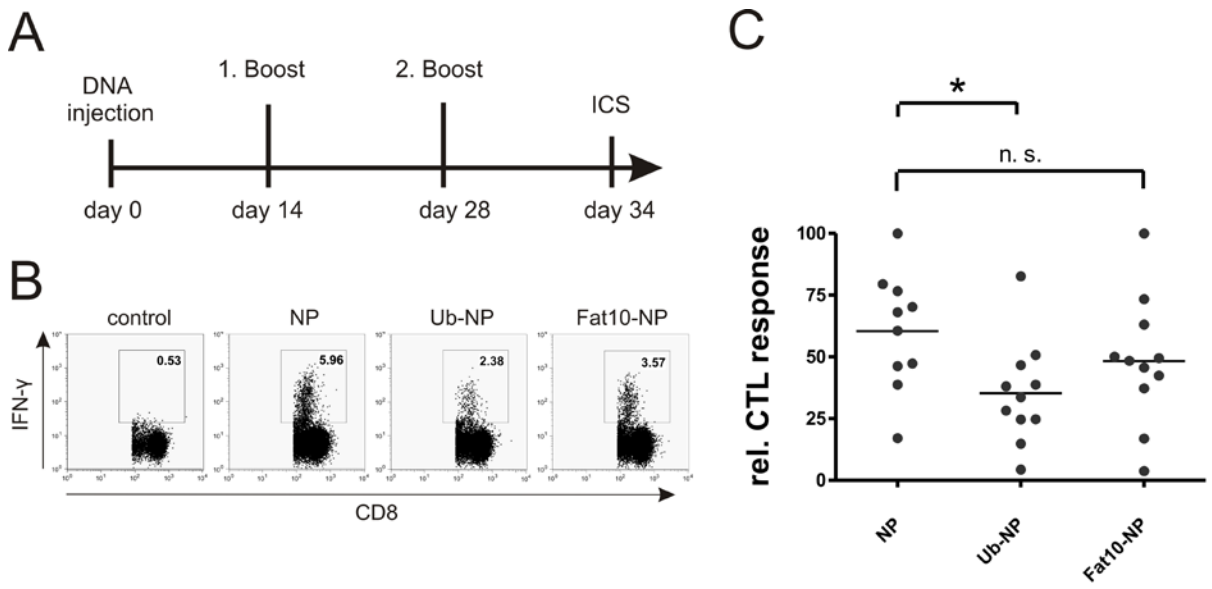

Figure 3: DNA immunization of C57BL/6 mice with constructs expressing either the long-lived lymphocytic choriomeningitis virus (LCMV) nucleoprotein (NP) or short-lived fusion proteins of the NP with either ubiquitin (Ub-NP) or FAT10 (FAT10-NP). A, Experimental setup. Mice were injected and electroporated *intramuscularly* with 100 µg DNA into the limbs on day 0. Homologous boosts were performed on days 14 and 28. On day 36 mice were sacrificed and splenocytes were re-stimulated with $NP_{396-404}$ peptide. A. After 4 h cells were fixed and labeled by intracellular cytokine staining (ICS) for IFN-γ. B+C, Results of DNA immunization. B, Representative ICS results for non-immunized mice (control) or mice immunized with DNA constructs as mentioned above. C, Combined results of 2 independent experiments (n=11). To compare results, the highest response of each individual experiment was set to 100%. All other values were calculated accordingly: rel. CTL response = (100 / $\%IFN\gamma^{+}_{MAX}$) x $\%IFN\gamma^{+}_{SAMPLE}$. Statistic analysis was performed by unpaired student's T-test. * → $p \leq 0,05$.

GFP control). We repeated the experiment in the mouse fibroblast cell line B8. Surprisingly, the results obtained in the B8-system were significantly different compared to those observed in the pMΦs (Fig. 2C). Whereas transfection of the stable form of the NP only led to a basal presentation of NP derived epitopes, fusion of the NP to ubiquitin or FAT10 was able to dramatically enhance direct-presentation by a factor of 2 to 4, depending on the epitope and the effector to target ratio. These experiments indicate that there might be mechanistic differences in the direct antigen presentation machinery of professional and non-professional antigen presenting cells.

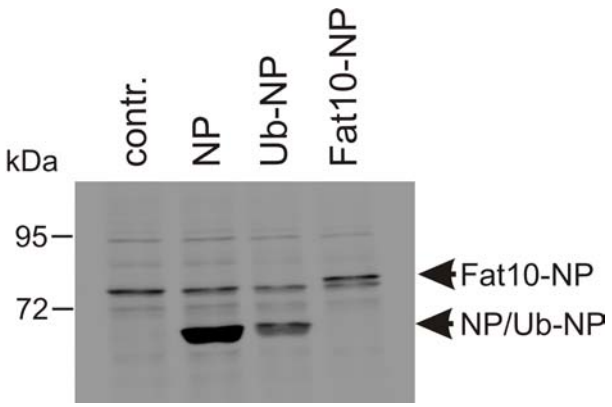

Figure 4: Analysis of recombinant protein expression by different recombinant vaccinia viruses (rVVs). HEK293 cells were left untreated as control (contr.) or infected with rVVs expressing either the wild type lymphocytic choriomeningitis virus nucleoprotein (NP) or fusion constructs of the NP with ubiquitin (Ub-NP) or Fat10 (Fat-NP). 3 h after infection cells were starved for 1h and then incubated with radioactive medium containing ^{35}S methionine for an additional hour. After lysis samples were incubated with NP specific beads for immunoprecipitation of the NP. The precipitate was separated by SDS-PAGE and analysed using a phosphoimager. Arrows indicate expected protein sizes.

DNA vaccination using a construct encoding the stable form of the LCMV NP is more efficient in inducing antigen specific-immune responses in vivo compared to rapidly degraded NP fusion proteins

In DNA vaccination, constructs encoding the antigen of interest are usually injected *intramuscularly*. Therefore, one can assume that large fractions of cells transfected during DNA vaccination are muscle cells and cells of the connective tissue. However, the initiation of immune responses can only be induced by professional APCs. Hence, either the number of APCs directly transfected during DNA vaccination must be large enough to stimulate an immune response or antigens have to be acquired exogenously and processed via cross-presentation.

To test whether targeting of an antigen to proteasomal degradation can be an advantage for the induction of immune responses via DNA vaccination we immunized mice with constructs expressing either the stable NP of fusion proteins of the NP and ubiquitin or FAT10. After two booster injections, we analysed the immune response in mice by intracellular cytokine staining. Interestingly, we found that the percentage of NP396 specific CTLs was significantly higher in mice immunized with a construct expressing the stable NP, compared to the fusion protein with ubiquitin (Fig. 3). Responses against the FAT10-NP fusion protein was also reduced compared to the stable NP, but differences were not significant. Therefore, our experiments clearly indicate that targeting a protein for rapid degradation is of no

benefit for DNA vaccination. In conrast, long-lived proteins are able to induce strongest immune responses. These findings have to be taken into account when designing new DNA vaccines.

Enhanced direct-presentation of LCMV NP-derived epitopes by peritoneal macrophages after infection with rVV expressing short-lived NP fusion proteins in vitro

In recent years, recombinant VV was introduced as a potential vaccine not only against small pox, but also as tool to initiate immune responses against various other immune targets (Basler and Groettrup 2007a). Since optimization of expression constructs is an important issue for successful vaccination strategies, we were interested to study the role of antigen stability on the immune response of a recombinant antigen expressed by vaccinia virus. Therefore we generated rVVs expressing the same constructs that we also used for the DNA vaccination above; the long-lived LCMV NP or short-lived fusion proteins of the NP with either ubiquitin or the ubiquitin-like modifier Fat10 (compare Fig. 1A). Initially, we wanted to confirm that recombinant proteins were indeed expressed in rVV infected cells. Therefore we infected HEK293 cells with different rVVs and analysed NP expression by NP-specific immunoprecipitation and SDS-PAGE after radioactive pulse with ^{35}S methionine (Fig. 4). While there was no NP-specific precipitate detected in the non-infected control, bands of the expected sizes were found for NP (62kDa), Ub-NP (70.5kDa), and Fat10-NP (80kDa).

In a next step, we wanted to investigate whether differences in antigen stability influence the direct-presentation of antigens after VV infection. Therefore, we prepared peritoneal macrophages (pMΦs) from Bl6 x Balb/c F1 mice (H2-$D^{d/b}$) and infected these cells with NP-expressing recombinant or wild type VV. To detect direct-presentation of NP-derived epitopes, we incubated infected pMΦs with NP-specific CTL-lines and detected activation by intracellular cytokine staining for IFN-γ (Fig. 5). Interestingly, and similar to our transfection experiments (Fig. 2C), we found the stable form of the NP to be presented with significantly less efficiency compared to the epitopes derived from short-lived NP fusion proteins. However, a direct comparison of the two NP fusion proteins Ub-NP and Fat10-NP reveals that although

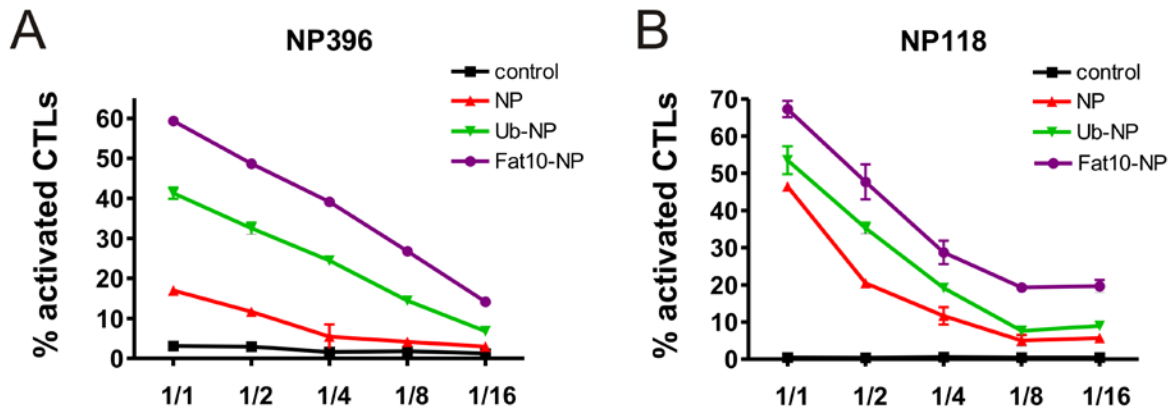

Figure 5: Direct-presentation of peritoneal macrophages (pMΦs) after infection with different recombinant vaccinia viruses (rVV) *in vitro*. pMΦs were prepared from Bl6 x Balb/c F1 mice as described above and infected with rVV expressing either the long-lived lymphocytic choriomeningitis virus nucleoprotein (NP), short-lived fusion proteins of the NP with either ubiquitin (Ub-NP) or FAT10 (FAT10-NP) or wild type vaccinia as control (control). After 3 h infected pMΦs were washed with PBS and indicated dilutions were incubated with a constant number of cytotoxic T-cell (CTL) lines specific for the NP epitopes NP396 ($H2\text{-}D^b$) (A) and NP118 ($H2\text{-}L^d$) (B). CTL activation was analysed by intracellular cytokine staining (ICS) for interferon-γ.

there was no obvious difference in antigen stability, epitopes derived from rVV expressing Fat10-NP were presented significantly better by a factor of around 50%. This observation was true for both epitopes analysed, the NP396 and the NP118. Antigen presentation and activation of CTLs was not saturated, since indicated differences were observed for all titrated ratios.

Stable antigen is more potent in inducing CTL responses in mice after infection with recombinant vaccinia virus in vivo

After analysing direct-presentation *in vitro*, we were interested to study the role of antigen stability after VV infection *in vivo*. In an initial experiment we compared the viral titers of wild type vaccinia and the different recombinant VV on day 4 after infection to ensure possible differences in immune induction were not due to alterations between individual virus clones (Fig. 6A). While no virus could be detected in ovaries of uninfected mice, comparable amounts of virus was found in wild type and recombinant VVs. No significant difference could be calculated between individual clones. These results indicate that there was no difference in viral proliferation and that possible differences in the immune response following VV infection cannot be due to altered virus amplification. $CD8^+$ T-cells responses against vaccinia are relatively low compared to other viruses, but dominated by the B8R

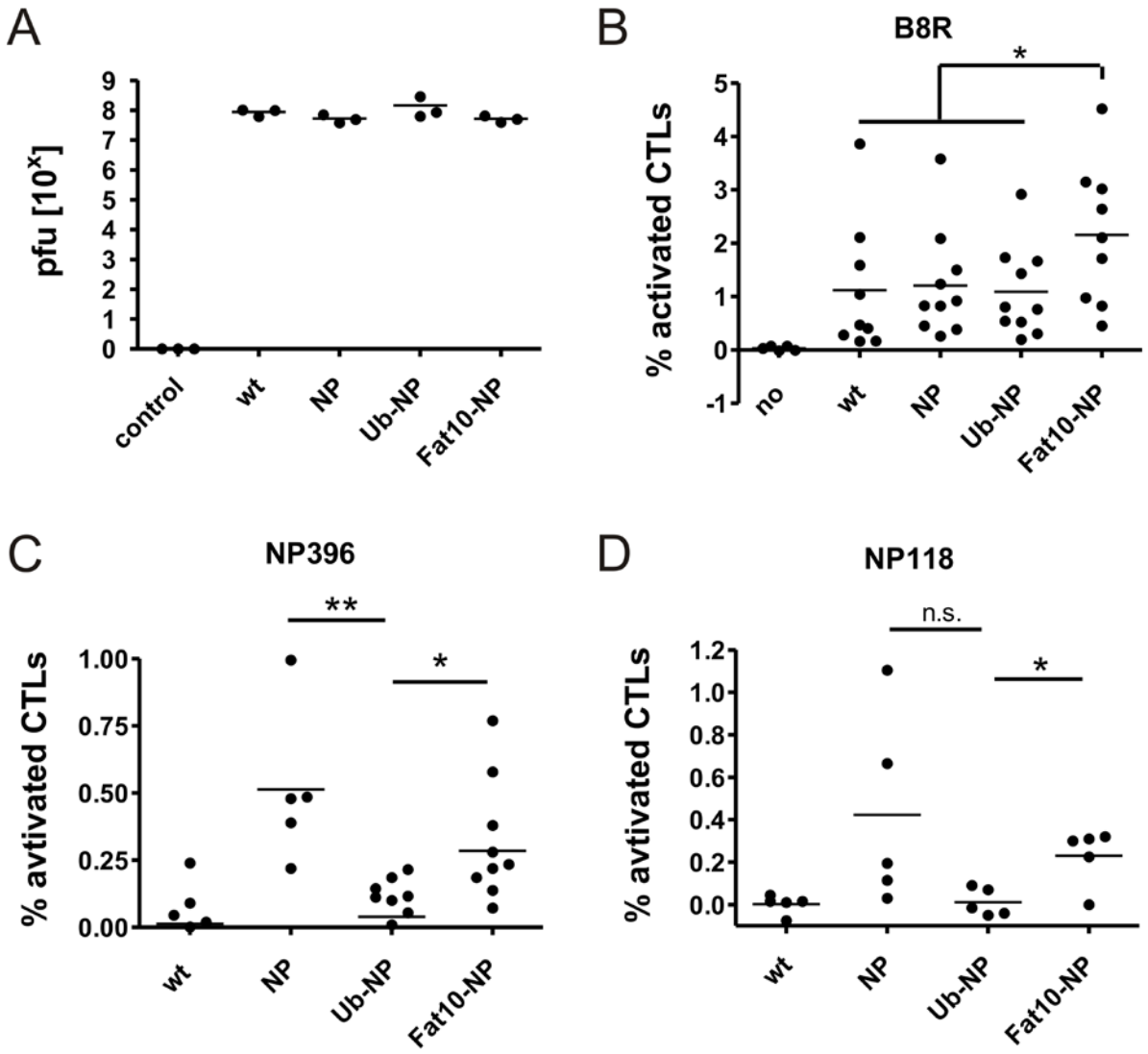

Figure 6: Analysis of cytotoxic T-lymphocyte (CTL) responses in mice immunised with different recombinant vaccinia viruses (rVV). A, Comparison of viral titers in ovaries of C57BL/6 mice on day 4 after infection with wild type vaccinia virus, rVV expressing the long-lived lymphocytic choriomeningitis virus (LCMV) nucleoprotein (NP), rVV expressing the short-lived fusion proteins of the NP with either ubiquitin (Ub-NP) or FAT10 (FAT10-NP), or with PBS as control (control). Viral titer was determined as described in the materials and methods section. Results are indicated in plaque forming units (pfu) per one pair of ovaries. B+C+D, Spleens of infected and control mice (no) were removed on day 7 after infection and splenocytes were re-stimulated *in vitro* with the VV epitope B8R (B) or the LCMV epitopes NP396 (C) or NP118 (D). After 5 h, CTL activation was analysed by intracellular cytokine staining (ICS) for interferon-γ (IFN-γ) and samples analysed by flow cytometry. Graphs show percent IFN-γ positive cells from all $CD8^+$ lymphocytes (% activated CTLs). Statistic analysis was performed by using an unpaired student's T-test: * → $p \leq 0,05$, ** → $p \leq 0,01$

protein-derived epitope $B8R_{20-27}$ in C57BL/6 mice. As additional control, we compared the B8R-specific immune responses in mice immunised with either wild type VV or different rVV clones (Fig. 6B). Since the only difference between individual viruses is the introduction of NP and NP derivates, the B8R-specific

response should be similar in mice infected with different viruses. In fact, there was no significant difference in the B8R-specific immune response on day 7 after infection with wild type VV and the recombinant VV clones NP and Ub-NP. Surprisingly however, we observed significantly higher numbers of B8R-specifc CTLs in mice immunized with the rVV expressing Fat10-NP. As shown above, this increase could not be due to differences in viral propagation.

At the same time we also analysed the activation of NP-specific $CD8^+$ T-cells (Fig. 5C+D). Interestingly, we observed highest induction of NP-specific CTLs in mice injected with the recombinant VV expressing the long-lived form of the NP. In contrast to this, there was hardly any response detectable in mice immunized with the virus expressing the short-lived Ub-NP. Since there were no difference in viral propagation and induction of B8R specific immune responses we conclude from these experiments that stable antigens are favoured in inducing $CD8^+$ T-cell responses after vaccinia infection. An intermediate phenotype was observed after infection with recombinant VV expressing Fat10-NP. CTL responses were significantly stronger compared to rVV Ub-NP, however did not reach the level of rVV NP. These results were observed after infection of C57BL/6 (NP396) as well as Balb/c mice (NP118). Our results indicated that recombinant vaccines based on vaccinia should preferentially express long-lived antigens in order to enhance $CD8^+$ T-cell based immune responses.

Discussion

The development of novel vaccines against infectious diseases and cancer is still a challenging field of biomedical research. Immunogenicity, biological safety and cost efficiency are key words often discussed in connection with the requirements of novel vaccines. However, especially biological safety and high immunogenicity are parameters, which are often mutually exclusive. In his report, we tended to study the molecular requirements on antigen stability for two kinds of vaccines that are promising candidates for future immunotherapy and vaccination.

DNA vaccines combine many advantages necessary for being used as a successful tool in clinical immunology (Belakova et al. 2007). However, low immunogenicity of DNA constructs hampers their contemporary application and occupies researchers seeking to enhance immune efficiency (Coban et al. 2008). Current approaches that intend to enhance DNA-mediated immune responses include various delivery systems (e.g. electroporation, gene gun), co-administration of cytokines, or other pro-inflammatory molecules (Kutzler and Weiner 2004). Also the application of adjuvants, like plasmid encoded CpG elements (Toll-like receptor 9 (TLR-9) ligand), was shown to enhance DNA-induced immune responses by various groups (Kojima et al. 2002, Coban et al. 2005). However this effect is recently questioned by studies using TLR-9 knock-out mice (Spies et al. 2003, Babiuk et al. 2004). Although $CD4^+$ T-cells responses can be induced by DNA vaccination, the majority of studies concentrate on the initiation of CTL responses, since $CD8^+$ T-cells are important to clear infected or malignant cells. There is an ongoing debate on the molecular mechanisms involved in the initial priming of $CD8^+$ T-cells after DNA vaccination. Of special interest are the cell types involved in antigen priming and whether direct- or cross-presentation accounts for antigen-specific responses. Initial studies in the 1990s have clearly demonstrated that priming of CTLs following DNA vaccination is dependent of bone-marrow derived cells (Huang et al. 1994, Corr et al. 1996, Doe et al. 1996). Similar to other immune responses, this finding was later specified by naming DCs as the major CTL priming cell type in DNA vaccination (Gurunathan et al. 2000). Due to the fact that the vast majority of antigen after *intramuscular* injection of DNA is produced by myocytes (Donnelly et al. 2000), it was questioned whether direct-presentation by the relatively low number of transfected APCs could account

for the overall CTL priming, as documented in some reports (Porgador et al. 1998, Akbari et al. 1999). For cross-presentation, antigens expressed by myocytes and other tissue-specific cells would have to be transferred to APCs that can internalize exogenous antigens and present peptides in the context of MHC class I. Indeed, such a transfer of antigens from myocytes to APCs was formally shown and associated with antigen cross-presentation (Fu et al. 1997). The understanding of the antigen presentation pathway after DNA immunization is important to further enhance its immunogenicity.

In the current study we investigated the role of antigen stability on the efficiency of DNA vaccination. By N-terminal fusion of the LCMV NP to either ubiquitin or Fat10 we were able to convert the long-lived nature of the NP into rapidly degraded derivates that resemble the viral protein in all parameters (e.g. epitopes, expression profile) except its half-life (Fig.1). Although this approach to alter antigen stability was already used before (Rodriguez et al. 1997), we are the first demonstrating Fat10 to target a viral antigen for rapid degradation. As introduced before, rapidly degraded proteins and DRiPs are the major sources of the direct MHC class I antigen presentation pathway (Yewdell et al. 1996, Schubert et al. 2000, Khan et al. 2001b, Yewdell et al. 2001, Pierre 2005). In accordance with these findings one would assume that proteasomal targeting of an antigen can lead to enhanced direct-presentation. Using the B8 mouse fibroblast cell line we could show that ubiquitin-NP and Fat10-NP fusion proteins are more efficiently presented after transfection *in vitro* compared to the construct expressing the wild type NP (Fig. 2). Even though these results fit to the DRiPs hypotheses, we did not observe increased NP-specific presentation after proteasomal targeting in pMΦs. The reasons for this are unclear. It might be that MHC class I processing is more efficient in pMΦs and therefore peptide loading could already be saturated after transfection of wild type NP. Also the maturation status could account for the differences observed (see below).

We were interested in observing the effects of antigen stability on DNA injection *in vivo*. The dependence of direct-presentation on DRiPs in combination with the finding that cross-presentation of the LCMV NP entirely depends on the stable form of the antigen (Basta et al. 2005, Donohue et al. 2006) allows an interpretation of our findings concerning the general mechanism of antigen presentation in DNA

vaccination. If direct-presentation by APCs would be the major mechanism, proteasomal targeting of the antigen should enhance CTLs production. Vice versa, the long-lived wild type protein should give best results, if a response is based on cross-presentation. We therefore immunized mice with either wild type NP or fusion proteins and analysed the $CD8^+$ T-cells specific immune response directly *ex vivo*. Interestingly, we found best results in mice that were immunized with the long-lived wild type NP. The responses after injection of ubiquitin constructs was significantly reduced, which is in contrast to an earlier study using the same constructs (Rodriguez et al. 1997). However, in this study the authors could only indirectly show the NP-specific immune responses by analysis of viral clearance after an LCMV infection that was performed weeks after immunization. This rather indirect method was required because low intrinsic immunogenicity of DNA constructs did not allow direct *ex vivo* analyses. We could circumvent this by using a novel electroporation devise as tool to enhance immunogenicity, as also demonstrated by other recent studies (Belakova et al. 2007). Thus, we were able to analyse CTL responses directly *ex vivo*, which makes our results more reliable and allows a different interpretation of the data. Cross-presentation of DNA encoded antigens has to be a major contribution to the overall immune response to a DNA vaccine. Otherwise it could not be explained why short-lived NP fusion proteins show enhanced antigen presentation *in vitro* but reduced responses *in vivo*. Our data are in accordance with different lines of evidences that indicate cross-presentation of DNA encoded antigens after DNA immunization. In a cellular transfer model it was shown that donor restricted immune responses were still initiated in mice, if APCs were injected as long as 21 days after DNA immunization (Doe et al. 1996). It is hard to imagine that after such a long time donor APCs could still be transfected to perform direct-presentation. It is therefore likely that antigens expressed by other cell types were acquired and immune responses induced via cross-presentation. Controversial data were published concerning the induction of apoptosis in transfected cells. While some studies are in favour of cross-presentation by showing enhanced CTL responses when co-administered pro-apoptotic proteins via the same DNA construct (Sasaki et al. 2001, Ferguson et al. 2002), others argue for direct-presentation because of increased CTL responses if co-administering anti-apoptotic proteins (Kim et al. 2003). One of the strongest findings that is in accordance with our data in favour of a role of cross-presentation comes from two studies, in which the authors could show efficient CTL

priming in mice that were immunized with DNA constructs expressing the antigen under a tissue-specific promoter that is not active in APCs (Loirat et al. 1999, Cho et al. 2001). If one assumes tight promoter regulation, these studies show that DNA mediated immune responses can be entirely independent of direct-presentation.

Since our experiments did not show entire inhibition of immune responses after targeting of the LCMV NP for rapid degradation we conclude from our experiments that cross- as well as direct-presentation contributes to the overall priming of CTL after DNA immunization. This idea fits well to recent studies showing a redundant function of direct- and cross-presentation in DNA vaccination (Prasad et al. 2001, Wolkers et al. 2001). Depending on the antigenic nature and the provided antigen dose both pathways seem to be possible (Heath et al. 2004).

A similar debate on the specific involvement of distinct antigen presentation pathways is also found for viral infections. Recombinant VVs are another promising vaccine that efficiently induces $CD8^+$ as well as $CD4^+$ T-cell responses in mice (Brave et al. 2007). Compared to DNA vaccines they have the advantage to show significantly higher transfection efficiency. This infection leads to the expression and presentation of recombinant antigens. Similar to the situation described above, it was not elucidated yet, to what extent direct- or cross-priming contribute to the initial T-cell activation. However, as shown for DNA vaccines, also the recombinant VV-induced immune responses were published to require the presence of bone marrow-derived APCs (Lenz et al. 2000).

We generated recombinant VV strains that either express the long-lived wild type form of the LCMV NP or short-lived fusion proteins of NP with ubiquitin or Fat10. According to what was expected, we found enhanced direct-presentation *in vitro*, if cells were infected with recombinant VV strains expressing the short-lived fusion proteins (Fig. 4). It was interesting to observe that infection of cells with recombinant VV expressing Fat10-NP resulted in highest CTL activation. However, although the viral replication was similar for the Fat10-NP expressing strain, we observed significantly higher responses to the vaccinia epitope B8R. Therefore the enhanced direct-presentation could be due to effects not related to the Fat10 construct. We are currently trying to elucidate this observation. In contrast to our DNA transfections we

did not detect any difference in direct antigen presentation between professional and non-professional cell types. Maturation of APCs in cause of VV infection and up-regulation of the MHC class I pathway might account for this difference. Interestingly, our *in vivo* studies indicated opposite preferences for CTL priming. The recombinant VV expressing the long-lived wild type form of the NP was found to be most potent in inducing CTLs. In contrast to this, the short-lived ubiquitin-NP failed to induce any response. These findings are especially striking since there was no difference in viral replication *in vivo* as well as responses to the VV epitope B8R (Fig. 6). Therefore, difference in NP-specific responses must be due to the antigenic nature of different NP variants.

Following the argumentation introduced above, our results clearly favor a dominant, if not exclusive, role of cross-presentation for MHC class restricted immune responses after VV infection. Interestingly, a very similar study was performed with equal results using a recombinant VV expressing either the long-lived tyrosinase or a short-lived ubiquitin fusion (Gasteiger et al. 2007). Also in this case the wild type protein was much more potent in inducing an immune responses compared to its short-lived derivate. In a different report, co-expression of inhibitory proteins of cytomegalovirus that block direct-presentation reduced responses to a recombinant antigen (Basta et al. 2002). Therefore, the authors argue that cross-presentation of viral antigens could be a mechanism to overcome viral evasion strategies in case direct-presentation is inhibited. Rapid degradation of antigen was also shown to inhibit CTL responses against other viruses, like semliki virus (Huckriede et al. 2004), vesicular stomatitis virus, sendai virus (Lizee et al. 2003), and others (Sigal et al. 1999). Another possible reason for a requirement of cross-presentation is provided by a recent study that shows reduced levels of co-stimulatory molecules in VV infected APCs (Gasteiger et al. 2007, Mueller et al. 2011). Co-stimulation, however, is required for efficient priming, which might therefore be dependent on non-infected cross-priming APCs. Along this line it was shown that VV-infected DCs do not respond as well to cytokines, which blocked migration (Humrich et al. 2007), affected cytoskeleton assembly, and cell contractility (Sanderson et al. 1998, Schepis et al. 2006). This mechanism could inhibit infected cells from migrating to draining lymph nodes where CTL priming occurs.

Our finding that increased antigen stability leads to better CTL priming can also be explained by the fact that VV-induced apoptosis and necrosis in infected cells (Engelmayer et al. 1999). Therefore, infected APCs could simply just not have enough time to reach the draining lymph nodes to directly present VV-derived epitopes. This scenario makes it likely that infected apoptotic as well as necrotic cells, either APCs or tissue specific cells, serve as antigen donors for cross-presentation by non-infected APCs. The evidence that apoptotic cells can indeed be a source of cross-presentation was provided by a study from the late 90s (Albert et al. 1998a). This process would require a long-lived antigen as also shown in other cross-presentation models with cellular antigen source (Norbury et al. 2004).

There are only very few studies that are in favour of direct-presentation as the dominant mechanism in VV infection. Surprisingly, proteasomal targeting of an HIV antigen was shown to increase CTL induction (Tobery and Siliciano 1997), which is in contrast to the previously mentioned studies and our results. Other reports show an importance of direct-presentation, which is however not exclusive (Basta et al. 2002, Norbury et al. 2002, Shen et al. 2002). We therefore conclude that antigen cross-presentation is an important mechanism to induce CTL responses after VV infection, a finding that goes in line for what was observed with other cytopathic viruses (Shen et al. 2004). The few exceptions that appear in the literature are most probably due to special properties of the antigen used and should not be overestimated compared to the increasing number of reports in favour of cross-presentation.

Finally, it has to be discussed to what extent Fat10 fusion to the LCMV NP alters its potential in antigen presentation and CTL priming. In our *in vitro* transfection experiments we could not detect any difference in direct-presentation comparing the two fusion proteins ubiquitin-NP and Fat10-NP. However, in our *in vivo* experiments using DNA vaccination as well as recombinant VV infection the Fat10-NP constructs showed an intermediate phenotype. In both systems the NP-specific responses of Fat10-NP was stronger compared to those of the constructs expressing ubiquitin-NP but, however, did not reach the level achieved with wild type NP. Differences in the half-life of ubiquitin-NP and Fat10-NP would have been an elegant explanation for the results observed. However, since there were no obvious differences detected in

the radioactive pulse chase experiments, other mechanisms have to explain the rescue of Fat10-NP-specific responses. One hypothetical speculation is the hypothesis, that peptides after Fat10 mediated degradation enter a partially different pathway compared to those occurring after modification with ubiquitin. It was shown in numerous publications that not only stable proteins, but also peptides in association to cellular factors like heat-shock proteins (HSPs) can be an efficient source of antigen for cross-presentation (Binder 2006). Here we speculate that peptides originating from Fat10 but not ubiquitin conjugates are targeted not only to the direct-presentation machinery but also to cellular factors like HSPs, establishing a pool of stable peptides that could represent an antigen source for cross-presentation. The inducible character of Fat10 would allow the stabilization of peptides during inflammation and by this means increase immune responses via cross-presentation. However, until now we have no evidence for such a pathway whose discovery could provide novel insights into the biology of Fat10 to further distinguish its function in comparison to ubiquitin.

The results presented in this report provide evidences that a stable antigen is required to achieve robust immune responses after DNA vaccination and infection with recombinant VV. A clinical trial studying an anti-malaria vaccine based on a modified VV (MVA) is already based on the expression of stable antigens (McConkey et al. 2003). Our results are in accordance with other studies showing the importance of cross-presentation after DNA and recombinant VV vaccination and should therefore be taken into account for the development of novel vaccination strategies that aim to improve immunogenicity.

Chapter IV

Cross-presentation of the LCMV nucleoprotein is dependent on the full-length antigen and mediated by heat-sensitive factors others than heat-shock proteins

Christopher Schliehe, Sameh Basta, and Marcus Groettrup

Manuscript in preparation

Abstract

Antigen cross-presentation by professional antigen presenting cells (APCs) is essential to induce and control central defence mechanisms of the adaptive immune system against tissue-specific antigens, like tumor and viral proteins or auto-antigens. In contrast to “direct-presentation” on major histocompatibility complex (MHC) class I, “cross-presentation” (XP) describes the uptake, processing, and MHC class I presentation of exogenous antigens. A favoured model to study anti-viral immune responses is the arenavirus lymphocytic choriomeningitis virus (LCMV) with its two structural proteins, the glycoprotein (GP) and the long-lived nucleoprotein (NP). By using LCMV NP-transfected cell lines as antigen donor cells (ADCs) in an *in vitro* cross-presentation assay, we show here that the soluble, mature form of the full-length NP is the antigenic source for cross-presentation in this system. Immune depletion of the NP from antigenic supernatants completely abrogated cross-presentation. Recently we could show the improvement of XP after heat-shock treatment of ADCs, which was sensitive to the specific Hsp90-family inhibitors. Here we used the heavy metal ions of nickel and cadmium to induce cellular stress response. Although the Hsp90-family seems to be essential, we could exclude their up-regulation as reasons for increased cross-presentation. Furthermore, we were not able to find evidences that heat-shock proteins (HSPs) directly interact with the NP to target receptor-mediated endocytosis by APCs. Neither the depletion of HSPs from antigenic supernatant, nor competition with purified Hsp90 was able to reduce cross-presentation. However, heat-inactivation of antigenic supernatants abolished cross-presentation, showing that heat-sensitive factors are essential for mediating the uptake of antigens via receptor mediated processes. CD91, a currently discussed HSP-receptor, was excluded as a major player in this system. Finally we show strategies to identify possible NP interaction partners that could promote cross-presentation by cell lysate fractionation via liquid chromatography, immune-precipitation and two-dimensional gel electrophoresis. Since the efficiency of cross-presentation is strongly dependent on the antigen transfer between ADCs and APCs, the results presented in this work have direct implications for the treatment of primary antigenic material that is used for vaccination in immunotherapy against cancer.

Introduction

Antigen presentation by professional antigen presenting cells (APCs), like dendritic cells (DCs) or macrophages (MΦs), is a central requirement for the induction of specific immune responses against foreign pathogens, cancer, and auto antigens. APCs that constantly sample antigens in the periphery get activated by pathogen-derived or "natural endogenous" adjuvants (Matzinger 2002, Medzhitov and Janeway 2002, Pulendran 2004, Rock et al. 2005), which induce migration to draining lymph nodes and up-regulation of the co-stimulatory molecules CD80 and CD86 (Mellman and Steinman 2001a). These co-stimulatory molecules are exclusively expressed by APCs and are, in combination with antigen presentation on MHC class I and II, essential for the initial activation of naive T-cells, a process referred to as priming. Beside the presentation of exogenous antigens on MHC class II for the activation of $CD4^+$ T_H-cells, there are two possible pathways of antigen presentation on MHC class I. Viral antigens or proteins derived from intracellular bacteria are expressed within the APCs and consequently presented via the "direct-presentation" pathway. According to this pathway, cytosolic proteins are degraded via the proteasome and generated peptides enter the endoplasmic reticulum (ER) via the transporter associated with antigen processing (TAP). Finally, the epitopes are presented on the cell surface in association with MHC class I molecules (Groettrup et al. 2001c). Antigens that are not synthesized within the APCs, like proteins of tissue-specific viruses or cancer-related antigens have to be endocytosed by APCs in order to become available for $CD8^+$ T-cell priming. This pathway for MHC class I presentation of exogenous antigens was discovered by M. Bevan in 1976 (Bevan 1976b) and called "cross-presentation" (XP). The initial activation of cytotoxic T-cell responses against tissue-specific antigens is therefore called "cross-priming" (Rock and Shen 2005, Rock 2006, Basta and Alatery 2007, Villadangos et al. 2007, Lin et al. 2008a). XP is the exclusive way to induce $CD8^+$ T-cell responses against tissue-resident viruses and cancer. Nowadays, the mechanisms and the molecular pathways are still not elucidated completely and are controversially debated.

One of the basic questions currently discussed, is the mechanism of antigen transfer from the infected or mutated tissue cell, the antigen donor cell (ADC), to the cross-

priming APCs. There is a variety of possibilities how antigen transfer can occur. DCs are able to actively endocytose cell-associated material from tissues undergoing necrosis or apoptosis. This way they clear the periphery and collect antigens for XP (Albert et al. 1998a, Fadok et al. 1998, Platt et al. 1998, Fadok et al. 2001, Kanduc et al. 2002, Pittoni and Valesini 2002, Fonteneau et al. 2003, Freigang et al. 2003). In contrast to responses against tissue-specific pathogens, which generally rely on the same mechanisms, anti-tumor responses have to be induced in the absence of bacterial or viral patterns. However, there is a general consensus that transfer of immunogenic antigens has to be facilitated by additional factors, which act either specifically as antigen shuttles or non-specifically as “natural endogenous adjuvants” leading to APC activation (Shi et al. 2003, Zeng et al. 2003, Hu et al. 2004, Rock et al. 2005). It was published that necrotic tumor cells or tumor cell lysates are able to induce the maturation of APC (Basu et al. 2000, Sauter et al. 2000, Somersan et al. 2001). This was due to molecules, like HSPs (Binder et al. 2000b, Sauter et al. 2000, Singh-Jasuja et al. 2000a, Kuppner et al. 2001, Flohe et al. 2003), fibronectins (Okamura et al. 2001), hyaluronic acid (Termeer et al. 2002), or uric acid (Shi et al. 2003, Hu et al. 2004), that were released into the medium. It was shown that different Toll-like receptors (TLRs) participate in HSP-signaling (Datta and Raz 2004), especially TLR-4 (Asea et al. 2000, Ohashi et al. 2000, Beutler et al. 2001, Vabulas et al. 2002). Another hint that TLRs might be involved in activating the XP machinery was the discovery, that MyD88, a molecule involved in the signal transduction pathway of most TLRs (excluding TLR-3), was required for XP *in vivo* (Palliser et al. 2004). In general, the important role of adjuvant molecules was further indicated by the finding that cell-associated antigen is much more efficiently cross-presented, compared to purified proteins (Binder and Srivastava 2005a). There are different groups of other receptors which were published to directly play a role in XP. Some of these are Fc receptors for IgG (Guermonprez et al. 2002) which lead to uptake of immune complexes (Regnault et al. 1999) or opsonized liposomes (Machy et al. 2000), the scavenger receptor family members, like SR-A (Berwin et al. 2003, Basta et al. 2005) or CD36 which was discussed in connection with the uptake of apoptotic material (Albert et al. 1998b).

A special role in mediating XP is assigned to the family of HSPs, in addition to their function as endogenous adjuvant. Molecular chaperones generally support

expression and folding of newly synthesized proteins, or prevent their aggregation during cellular stress (Hartl 1996, Feldman and Frydman 2000). HSPs, which were initially discovered in the fly *Drosophila melanogaster* (Ritossa 1962, Tissieres et al. 1974, Ritossa 1996), are evolutionarily highly conserved members of this chaperone family (Lindquist 1986), but have special importance in situations of cellular stress. Elevated temperatures, exposure to toxic substances, or osmotic shocks alter the expression pattern of HSPs in order to prevent protein damage (Williams and Morimoto 1990, Hansen et al. 1991, Hartl 1996, Jakob 1996, Basu and Srivastava 2003, Hfaiedh et al. 2005), buffering consequences of mutations (Rutherford and Lindquist 1998) and controlling apoptosis (Takayama et al. 2003). Similar to the heat-shock, treatments with heavy metal ions, as provided by nickel or cadmium salts, stimulate the anti-stress response of cells and finally lead, among other responses, to an up-regulation of heat-shock proteins (Lindquist 1986, Basu and Srivastava 2003). Many HSPs are expressed in a constitutive and a stress inducible isoform (Menoret and Bell 2000), such as the two isoforms of Hsp90, the inducible Hsp90α (Hsp86) and the constitutive Hsp90β (Hsp84), or Hsp70, the inducible Hsp70 and the constitutive Hsc70. HSP expression is regulated by stress sensitive transcription factors, like the heat-shock factor-I (HSF-I) (Zheng and Li 2004). The four major HSPs of mammalian cells are Hsp90, gp96, Hsp70 and calreticulin (Binder and Srivastava 2005b).

Numerous publications examined HSPs and their potential role in XP, especially for the process of antigen transfer from ADCs and specific uptake by APCs. First evidences for HSPs being involved in inducing CTL responses, came from experiments where HSPs purified from cancer cells were able to induce a tumor-specific immune response (Tamura et al. 1997). Recently, increasing numbers of studies postulated and tested purified HSPs from malignant tissues as potent anti-cancer vaccines, showing strong immune responses in mice (Stebbins et al. 1997, Neckers and Neckers 2002, Dai et al. 2003, Nicchitta 2003, Zeng et al. 2003, Binder 2006). HSP/antigen complexes, composed of Hsp70, Hsp90 or gp96, either purified from malignant tissues and infected or transfected cells were able to serve as antigen source for XP *in vitro* (Castellino et al. 2000, Basu et al. 2001, Berwin et al. 2002b) and *in vivo* (Suto and Srivastava 1995). Furthermore, it was shown that HSPs can interact with antigenic peptides as well as full proteins (Suto and Srivastava 1995,

Blachere et al. 1997, Singh-Jasuja et al. 2000b, Nicchitta 2003, Binder and Srivastava 2004, Gullo and Teoh 2004, Hickman-Miller and Hildebrand 2004, Shen and Rock 2004, Binder 2006, Binder et al. 2007) and bind on APCs in a receptor-dependent manner. This interaction was shown to lead to an antigen-specific uptake (Arnold-Schild et al. 1999, Wassenberg et al. 1999, Binder et al. 2000a, Singh-Jasuja et al. 2000b, Basu et al. 2001, Srivastava 2002, Binder et al. 2004). The *in vitro* loading of HSPs with antigenic peptides was able to induce specific immune responses *in vivo*, in contrast to peptide alone (Blachere et al. 1997, Ciupitu et al. 1998). Furthermore, also full-length proteins as possible source for XP may require the help of HSPs. It was shown, that fractions of lysed cells would only induce XP, if the full protein co-localized with HSPs in the same fractions (Shen and Rock 2004).

One important HSP-receptor that was published to mediate the binding and uptake of HSP-antigen complexes, is the surface molecule CD91 expressed on APCs (Basu et al. 2001). CD91 was shown to participate in the HSP-dependent XP of different antigens (Binder et al. 2000a, Basu et al. 2001, Berwin et al. 2002a, Binder and Srivastava 2004, Binder et al. 2004). Nevertheless, it is not clear, whether HSP-associated peptides or full proteins are the major source of antigen under physiological conditions.

Hsp90 plays a special role in the maturation of hormone receptors and protein kinases (Stebbins et al. 1997) as well as in the degradation of proteins by the proteasome (Imai et al. 2003). The functional Hsp90-chaperoning is dependent on ATP-hydrolysis (Obermann et al. 1998) and can be inhibited by reagents that block ATP binding (Whitesell et al. 1994, Stebbins et al. 1997). Hsp90 is thought to interact with several other proteins to fulfill its effector function (Jakob 1996). Interestingly, several publications show the involvement of Hsp90 in XP (Srivastava 1997, Neckers and Neckers 2002, Basu and Srivastava 2003, Gullo and Teoh 2004, Binder et al. 2007).

We could recently show that heat-shock treatment of ADCs expressing the LCMV NP, enhances the ability of APCs to cross-present this antigen *in vitro* (Basta et al. 2005). This effect was sensitive to the specific Hsp90-family inhibitors geldanamycin

and herbimycin, what suggests an important role of the Hsp90 family members for XP in this system.

In this report we wanted to further characterize the molecular requirements for the XP of the cell-associated LCMV NP. Therefore, we analyzed the role of HSPs on XP with a special focus on Hsp90. Interestingly, neither the depletion of different HSPs, nor competition with purified Hsp90 did reduce the amount of XP. In contrast, purified Hsp90 was able to strongly promote XP similar to the TLR-4 ligand LPS.

Furthermore, our experiments clearly indicated that not antigenic peptides but the entire native form of the full length NP is required for efficient XP in this system. Heat-inactivation of antigenic supernatants reduced XP to background levels, indicating that properly folded, soluble factors others than Hsp90 have to interact with surface receptors on the APCs in order to mediate the antigen uptake.

To find such factors we used an immune precipitation approach in combination with two dimensional gel electrophoresis and show that there are proteins specifically interacting with the LCMV NP. Taken together, the results presented in this work indicate that there are XP-promoting molecules others than HSPs, which target full-length antigens to uptake and presentation by APCs.

Materials and Methods

Mice, cells and media

C57BL/6 (H-2^b) and BALB/c (H-2^d) mice were originally obtained from Charles River Laboratories and further bred in the animal facility of the University of Konstanz. Animals were kept under specific pathogen-free conditions in accordance with the rules of the veterinarian authority of Regierungspräsidium Freiburg.

All cell culture media were obtained from Gibco, Invitrogen. Primary murine CTL-lines were cultured in RPMI 1640, 10% FCS, 20 U/ml interleukin-2, 50 µM β-mercapto-ethanol, 50 µg/ml gentamycin. Bone marrow-derived dendritic cells (BMDCs) were cultured in RPMI 1640 supplemented with 10% FCS, 10% GM-CSF supernatant, 0.1% β-mercapto ethanol, 100 U/ml penicillin/streptomycin (P/S). The Human embryonic kidney cell line HEK293 (Graham et al. 1977) and the murine fibroblast cell line B8-wt (H-2^d) (Groettrup et al. 1995b) were cultured in DMEM, 10% FCS, 100 U/ml P/S. HEK-NPA3 stably expressing the LCMV nucleoprotein (Basta et al. 2005) were cultured in DMEM, 10% FCS, 2.5 µg/ml puromycin, 100 U/ml P/S. B8-NP64 cells expressing the LCMV nucleoprotein under a tetracycline-inducible promoter (Khan et al. 2001b) were kept in DMEM, 10% FCS, 5 µg/ml puromycin, 400 µg/ml hygromycin, 500 ng/ml tetracycline. Removing tetracycline from the medium induces nucleoprotein expression. The murine macrophage cell line BMC (H-2^b) and the dendritic cell line DC2.4 (H-2^b) were a kind gift from K. Rock (University of Massachusetts Medical School Worcester, MA) and cultured in RPMI 1640, 10% FCS, 100 U/ml P/S.

Preparation of marrow-derived dendritic cells (BMDCs)

BMDCs were prepared from bone marrow of naive C57BL/6 mice at 6 to 8 weeks of age. Animals were sacrificed and the femurs and tibias removed and washed with PBS. The bone marrow was flushed out of the bone using a syringe filled with PBS and an injection needle. Red blood cells were lysed with 1.88 g/l ammonium chloride buffer. After lysis cells were filtered and cultured in BMDC medium in 6-well plates at a density of $2.5x10^6$ cells/well. Three days after preparation, additional medium

was provided. On day five the medium was completely renewed and adherent cells where used for the respective experiments on day 6.

Western blot analysis

Proteins were electrotransferred from SDS-PAGE gels to a nitrocellulose membrane by semi-dry blotting. After protein transfer, the membrane was blocked with PBS-T (PBS, 0.2 % Tween 20) containing 5 % milk powder. Following primary antibodies were used over night: anti-Hsp70 (W27, sc-24, mouse monoclonal IgG2a, Santa Cruz Biotechnology), anti-Hsc70 (B-6, sc-7298, mouse monoclonal IgG2a, Santa Cruz Biotechnology) anti-calreticulin (H-170, sc-11398, rabbit polyclonal IgG, Santa Cruz Biotechnology), anti-Hsp90α (C-20, sc-8262, goat polyclonal IgG, Santa Cruz Biotechnology), anti-Hsp90β (D-19, sc-1057, goat polyclonal IgG, Santa Cruz Biotechnology), anti-Hsp90α/β (H-114, sc-7947, rabbit polyclonal IgG, Santa Cruz Biotechnology), anti-HA (HA-7, mouse ascites fluid IgG1, Sigma), anti-LMP7 (Khan et al. 2001a), anti-MECL-1 (Groettrup et al. 1997). Secondary antibodies coupled to HRP were obtained from DAKO, Denmark. All antibodies were diluted in PBS-T. Blots were developed using SuperSignal® West Pico (Thermo Scientific). Chemiluminescence was immediately measured by using the Molecular Imager ChemiDoc XRS (Bio-Rad, Munich, Germany).

Generation of primary NP396 specific CTL lines

Naive C57BL/6 mice at 6 to 8 weeks of age were intravenously (*i.v.*) injected with 200 pfu LCMV. Starting from 3 weeks after infection, memory mice were used for the generation of NP396-specific CTL lines. Spleens from memory mice were taken and homogenized. Isolated splenocytes were further purified by Ficoll density gradient centrifugation (GE Healthcare) and washed with PBS. DC2.4 cells were pulsed with 10^{-6} M NP396 peptide and γ-irradiated with 10.000 rad using a γ-radiation machine (Siemens, Germany). Afterwards splenocytes were co-cultured in 6-well plates ($1.5*10^7$ cells/well) with irradiated peptide pulsed DC2.4 cells (10:1) in CTL-medium. On day 5 density gradient centrifugation was repeated to remove dead cells. Remaining splenocytes were washed with PBS and further cultured in the presence of IL-2. Specific NP396 CTL lines were used between day 7 and 9 of culture and specificity measured independently of the respective experiment (Fig. 1A).

In vitro cross-presentation assay

APCs (BMCs, BMDCs) were harvested by Trypsin/EDTA (Gibco, Invitrogen) and washed with PBS. The cells were cultured in 96-well plates at a concentration of $2x10^5$ cells/well (RPMI 1640, 10% FCS). ADCs (HEK-wt, HEK-NP, HEK-HANP, B8-wt or B8NP64) were used at a concentration of $2x10^6$ cells per ml for the respective treatments. Heat-shock was performed in a water bath at 45°C for 15 min. Combined lysis and UV light treatment (Ly/UV) was performed by freezing samples in liquid nitrogen and exposure to UV-light using an UV Stratalinker^{TM} 2400 (Stratagene, USA) for 8 min on ice. Denaturation of cell supernatants was performed for 5 min at 95°C. Independent of the respective treatment or controls, 100 µl of ADC suspensions or supernatants were added to the APCs. Chemicals or proteins were added directly into the co-culture in indicated concentration. Co-incubation was performed for 18 h overnight at 37°C. Then plates were centrifuged and medium was removed. Specific CTLs (day 7 to 10) were harvested and resuspended in RPMI-medium to a final concentration of $1x10^6$ cells/ml. Brefeldin A was added in a concentration of 10 µg/ml, to block secretion of cytokines. After gently inverting the suspension, 200 µl of CTL suspension was added to each sample of the 96-well plate. After 4 to 6 h at 37°C, 7% CO_2, IFN-γ positive CTLs were detected by intracellular cytokine staining (ICS).

Intracellular cytokine staining (ICS)

ICS was performed to detect intracellular accumulation of IFN-γ as a measure of CTLs activation. Samples were centrifuged and 50 µl TriColor anti-mouse-CD8a 1:150 in PBS (Invitrogen) was added on ice and incubated for 15 min in the dark. After washing with ice cold PBS, 50 µl of 3% para-formaldehyde (Acros Organics) solution was added for 5 min at room temperature. Afterwards, samples were again washed with PBS. Staining for IFN-γ was performed by adding 50 µl FITC anti-mouse-IFN-γ (kind gift from M. Basler, University of Konstanz) 1:100 in PBS, 1% saponin. The staining was incubated overnight at 4°C. Before acquisition by flow cytometry (FACSscan, BD), samples were washed, resuspended in PBS and kept on ice in the dark.

Intracellular detection of LCMV NP and Hsp90 via flow cytometry

ADCs were harvested and fixed with 37% formalin for 20 min at RT. Afterwards cells were washed with PBS and resuspended in 1% Triton X-100 for permeabilisation of cell membranes. After incubation for 20 min at RT cells were again washed with PBS and incubated overnight at 4°C in the presence of either the NP-specific B-cell hybridoma supernatant VL4 (hybridoma kindly provided by R. M. Zinkernagel, University Hospital Zurich) or PBS as control. The next day samples were washed with PBS twice and FITC anti-rat secondary antibody (BD) was added for one hour at RT. For the intracellular staining of Hsp90, PE anti-human Hsp90α/β (F-8, sc-13119 PE, Santa Cruz Biotechnology) was added 1:50 in PBS after cell lysis and incubated for 1 h at RT. After two washing steps with PBS samples were analyses by flow cytometry.

Assessment of cell death by flow cytometry

Apoptosis was detected by incubation of cells with FITC anti-phosphatidyl-serine (PS) antibodies for 20 min on ice (BioVision). Afterwards samples were washed with PBS and analyzed by flow cytometry or further stained for necrosis. Necrotic cell death was detected via flow cytometric analysis of propidium iodide (PI) incorporation. Cells were kept in PBS and PI was added in a concentration of 1 µg/ml one minute before acquisition by flow cytometry. The extent of apoptosis/necrosis was analyzed as percent PS/PI positive cells.

Generation of stable transfected HEK-HANP and HEK-SHHNP cell lines

The plasmid pcDNA3.1_HA-NP, encoding an N-terminal HA-tagged LCMV NP, was generated by amplification of the nucleoprotein from pCMV_NP (Rodriguez et al. 1997) via PCR, using following primers: fwd 5´-GGC GAA TTC GGT ACC TAT GTC TTT GTC TTT GTC CAA AGA A-3´ and rev 5´GAT TAC TCT AGA TTA GAG TGT CAC AAC ATT GGG-3´. The construct was then inserted into the Asp718/XbaI restriction sites of pcDNA3.1_HA-LacZ (G. Schmidke, University of Konstanz, Germany) by replacing the LacZ gene. The plasmid pcDNA3.1_SHH-NP, encoding an N-terminal Strep-His-HA-tag was generated by amplification of the HA-tagged nucleoprotein from pcDNA3.1_HA-NP via PCR, using following primers: fwd 5´-GAA GCC CTC GAG ATG GCC TAC CCC TAC GAC-3´ and rev 5´TCG CGG CTC GAG

TTA GAG TGT CAC AAC ATT-3´. The construct was then inserted into the Xho I restriction sites of pcDNA3.1_Srep-His-EGFP (G. Schmidke, University of Konstanz, Germany) by replacing the EGFP gene. The stable cell lines HEK-HANP and HEK-SHHNP were generated using Fugene 6 (Roche Diagnostics) and co-transfection of puromycin resistance plasmids. Positive clones were identified by intracellular NP staining and anti-HA western blot analysis.

Purification of Hsp90 from murine spleens

The purification of Hsp90 was performed in a 3-step protocol adopted from (Srivastava 1997). Briefly, HEK293 cells were grown, pellets collected for purification, and stored at -20°C. Cells were lysed for 20 min on ice by adding 4 volumes of 30 mM sodium bicarbonate pH 7.1 in the presence of 1 mM PMSF. After lysis, the cell suspension was centrifuged first at 2000g, 4°C, 10 min, and the supernatant was further purified by ultracentrifugation at 100.000g, 4°C, 90 min. Subsequently, ammonium sulfate was slowly added to a final concentration of 50%. Precipitated proteins were removed by centrifugation and the supernatant was further concentrated to 70% ammonium sulfate. Precipitated protein fractions containing Hsp90 were washed with 70% ammonium sulfate and then resuspended in 10 volumes of PBS containing 2 mM Mg^{2+} and Ca^{2+}. After further purification by centrifugation, supernatant was loaded on concanavalin A beads (0.5 ml/ml initial cell pellet) which were equilibrated with PBS containing 2 mM Mg^{2+} and Ca^{2+}. Unbound fraction was then dialyzed 3 times for 12 h against 100 volumes of [20mM Na_2PO_4, 250 mM NaCl, 1 mM PMSF, in water, pH 7.4] and finally loaded on a monoQ column which was equilibrated with the same buffer. Elution was performed by linear NaCl gradient from 0 to 600 mM. Fractions were collected and analyzed for the presence of Hsp90 by SDS-PAGE and western blot analysis. We were able to purify 150-300 µg Hsp90 per 1 g of initial wet weight.

Depletion of individual HSPs

For the depletion of individual HSPs, HEK-NPA3 cells were harvested, washed twice with PBS and finally resuspended at a density of $2*10^5$ cells per 250 µl. Cell suspensions were lysed by freezing in liquid nitrogen and were then treated with UV-light (Ly/UV), as described above. Cell debris was removed by centrifugation at 20.000 g for 10 min at 4°C. Afterwards, protease inhibitors were added in the

following concentrations: 5 µM aprotinin, 100 µM leupeptin, 50 µM pepstantin A, 1 mM PMSF. Protein A affinity beads (Sigma) were equilibrated with PBS and added to the cell supernatant. For depletion of HSPs the following antibodies were added: anti-Hsp86 (Ab-1, RB-119-PO, rabbit polyclonal, NeoMarkers), anti-Hsp84 (Ab-1, RB-118-P, rabbit polyclonal, NeoMarkers), anti-Hsp70 (820, Stressgen), anti-calreticulin (SPA600, Stressgen). The supernatant was incubated with antibodies and beads for 12 h at 4°C on a rotating incubator. Protein A beads were removed by centrifugation and immune precipitation was repeated twice for each sample. HSP depletion was monitored by western blot analysis.

Gel chromatographic fractionation of HEK-HANP cell supernatants

HEK-HANP cells were grown to 90% confluence and then harvested by Trypsin/EDTA (Gibco, Invitrogen). The pellet was resuspended in PBS to reach a concentration of $5*10^7$ cells/ml, frozen in liquid nitrogen and treated with UV light on ice after thawing. The cell debris was removed by centrifugation and the supernatant was filtered using a 0.2 µm sterile filter before loading the sample on a Sephadex 200 gel chromatography column (Amersham Biosciences). Sephadex 200 was equilibrated and run with PBS and 500 µl fractions were collected. Fractions were analysed by SDS-PAGE and western blot and used as antigenic source for XP in the *in vitro* assay.

Purification of the LCMV NP

The purification of NP was performed following a 1-step protocol. HEK-SHHNP cells were grown to about 95% confluence and harvested by Trypsin/EDTA (Gibgo, Invitrogen). Cells were lysed using hypotonic lysis buffer [20 mM Tris pH 7.8, 150 mM NaCl, 0.1% Triton X-100] and cell debris was removed by centrifugation and filtration (0.2 µm). Strep-tactin matrix was equilibrated with PBS and cell supernatants were loaded after dilution with 5 volumes of PBS. Unbound proteins were washed off the column with PBS and specific elution of Strep-tagged NP was performed with a commercial elution buffer containing d-desthiobiotin (Sigma). Fractions collected during loading, washing, and elution were analyzed by SDS-PAGE and western blot.

2-dimensional gel electrophoresis

HEK-HANP cells were grown to about 95% confluence and harvested by Trypsin/EDTA (Gibgo, Invitrogen). Pellets were resuspended in PBS, frozen in liquid nitrogen and treated with UV light on ice after thawing, as described above. Cell debris was removed by centrifugation and the supernatant was filtered using a 0.2 µm sterile filter before protease inhibitors were added (Complete mini, EDTA-free, Roche). HA-NP was precipitated overnight using anti-HA affinity beads (Sigma). On the next day the beads were washed 6 times with PBS to remove unbound proteins. In a first dimension, samples were separated by isoelectric focusing using Non-Equilibrium-pH-Gel-Electrophoresis (NEPHGE). Therefore, beads were resuspended in 80 µl of NEPHGE-Sample buffer [9,5 M urea, 2% NP40, 5% ampholine 3-10 (Serva), 5% mercaptoethanol, 0.3% SDS] in an electric shaker at 27°C over night. Next, the samples were loaded on the first dimension gel [6 M urea, 30% acrylamide/bisacrylamide, 2ml 10% NP40, 10% ampholine 3-10] and covered with 20µl of overlay solution [9 M urea, 2.5% ampholine 3-10]. Gels were run with 400 V for 4 h. Afterwards gel rods were prepared for SDS-PAGE separation by incubation 2 x 20 min in equilibration buffer [10% glycerol, 10% β-mercaptoethanol, 2.3% SDS, 90 mM Tris-HCl, pH 8.8]. For the second dimension, the gel was loaded on a SDS-PAGE gel and covered with agarose buffer [2% agarose, 10% glycerol, 0.5% SDS, 0.5 M Tris pH 6.8, 2% 2-mercaptoethanol, bromphenol-blue]. The second dimension was then run for 1100 V*h overnight. For detection of proteins after electrophoresis, silver stainings were performed in the absence of formaldehyde, as required for protein identification via mass spectrometry.

Results

Induction of necrotic cell death in antigen donor cells enhances cross-presentation of the LCMV nucleoprotein

The induction of CTL responses against cell-associated antigens is strongly influenced by the physiological condition of the ADCs. In a recent publication we have shown, that heat-shock treatment of ADCs enhanced the efficiency of various APC lines to cross-present the LCMV NP *in vitro (Basta et al. 2005)*. This enhancement was interpreted to be the result of HSP up-regulation during cellular stress. In this report, we could verify that Hsp90 was indeed up-regulated in ADC samples treated with heat-shock (Fig. 1B). Expression increased and reached a maximum 18 h after heat-shock treatment. Steady state levels were re-established after 24 h to 48 h (data not shown). Based on the hypothesis that increased expression of HSPs might lead to an induction of cross-presentation, we were interested to investigate other stress-inducing conditions. ADCs stably expressing the

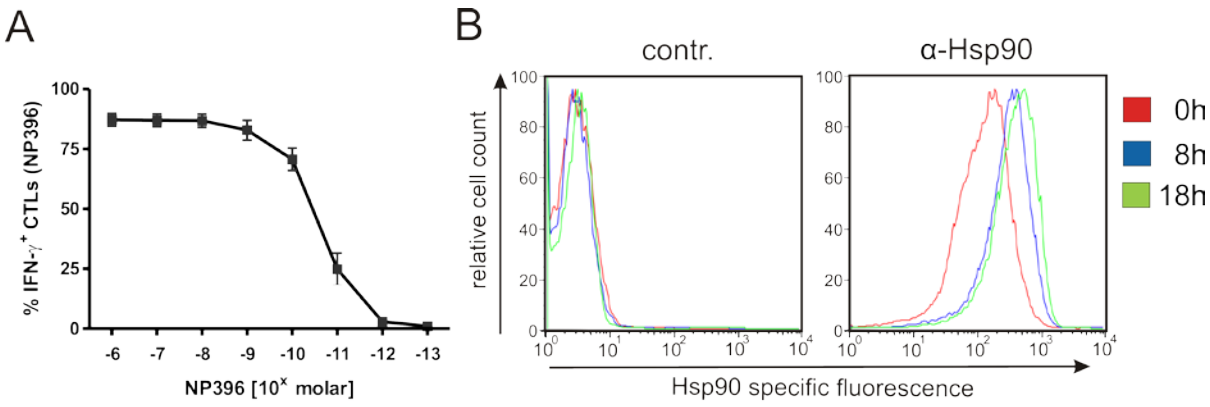

Figure 1: A, Titration of NP396-specific cytolytic T-cell lines (CTLs). Spleens of LCMV memory mice were homogenized and cells re-stimulated in the presence of DC2.4 loaded with NP396 peptide and interleukin-2. On days 7 to 9 cells were used for experiments and simultaneously tested for specificity and sensitivity towards DC2.4 cells loaded with indicated amounts of NP396 peptide for 4 h. Intracellular IFN-γ staining was performed to measure the percentage of activated CTLs. The graph shows the means of 6 individual CTL-lines. B, Up-regulation of intracellular Hsp90 in response to heat treatment. HEK293 cells were incubated at 45°C for 15 min. Intracellular Hsp90 expression was detected at indicated time points. Results are representative for two individual experiments.

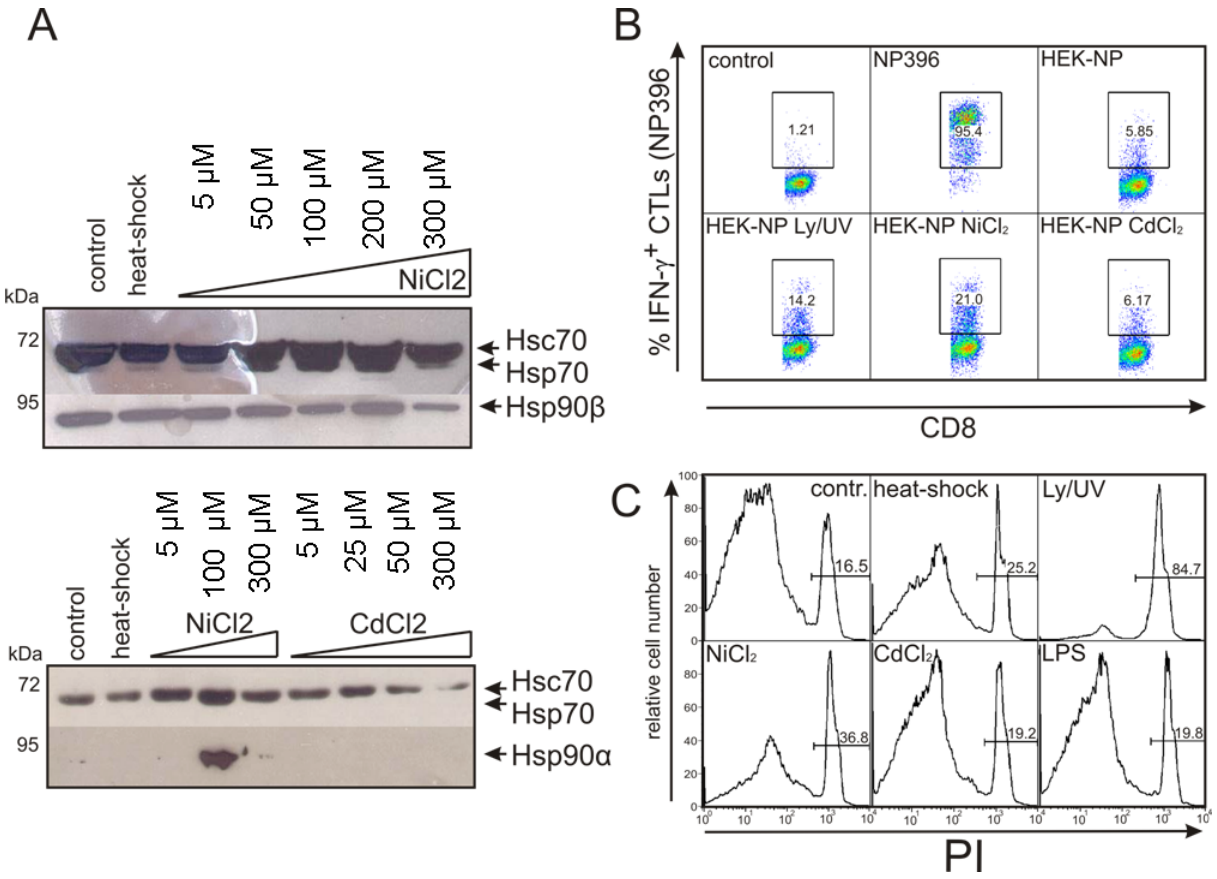

Figure 2: Induction of heat-shock proteins by nickel and cadmium salts and their effect on cross-presentation. A, HEK-NP were treated with either heat-shock, indicated concentrations of $NiCl_2$, $CdCl_2$ or left untreated as control. After 18 h cells were lysed and western blots performed for Hsc70, Hsp70 or Hsp90α. Equal loading was assured by western blot against the constitutive Hsp90β (as indicated or data not shown). B, HEK-NP cells were treated by either Ly/UV, 300 µM $NiCl_2$ or 5 µM $CdCl_2$, and co-cultured with BMC cells for 18 h. Afterwards NP396-specific cytotoxic T-lymphocytes were added to detect cross-presentation of the LCMV NP by intracellular IFN-γ staining. Co-incubation in the presence of external peptide served as positive control (NP396). C, Necrotic cell death of HEK-NP cells treated with either heat-shock, 300 mM $NiCl_2$, 5 mM $CdCl_2$ or left untreated as control (contr.). 18 h after treatment cells were harvested and propidium iodide was added 1 min before sample acquisition by flow cytometry. Shown results are representative for at least two independent experiments.

LCMV NP were incubated with increasing concentration of either $NiCl_2$ or $CdCl_2$ and induction of Hsp70 and Hsp90α was examined by western blot analysis (Fig 2A+B). Increasing concentration of $NiCl_2$ treatment led to an induction of Hsp70, much stronger than in the heat-shock or control sample, which only showed background expression (Fig 2A). Detection of constitutive Hsc70 and Hsp90β served as loading control. Also Hsp90α was up-regulated in samples treated with nickel ions, showing the highest expression at a $NiCl_2$ concentration of 200 µM. High nickel concentrations of 300 µM show reduced expression of constitutive HSPs indicating reduced viability. In contrast to our intracellular staining (Fig. 1B), we were not able to detect up-

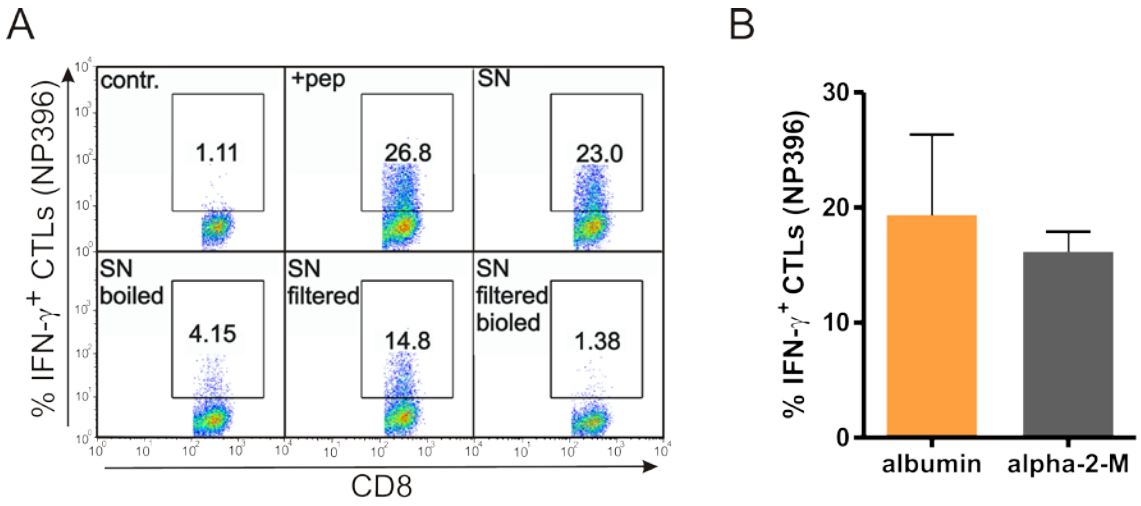

Figure 3: A, Cross-presentation of HEK-NP cell lysate supernatants (SN) before and after denaturation (boiled) or filtering through a 0.2 µm sterile filter (filtered). BMC cells were incubated in the presence of cell supernatants as indicated. After 18 h NP396-specific cytotoxic T-lymphocytes were added to detect cross-presentation of the LCMV NP by intracellular IFN-γ staining. Results are representative for 2 independent experiments. B, CD91 competition. BMCs or BMDCs (data not shown) were incubated with HEK-NP Ly/UV cell supernatant in the presence of either 1 mg/ml albumin or 1 mg/ml alpha-2-macoglobline (alpha-2-M). After 18 h NP396-specific cytotoxic T-lymphocytes were added to detect cross-presentation of the LCMV NP by intracellular IFN-γ staining. Graph shows an average of three independent repeats.

regulation of Hsp70 or Hsp90α in samples treated with heat-shock. Also treatments with $CdCl_2$ did not show increased HSP expression using this read-out. Nevertheless we were interested to study the effect of heavy metal ions on XP. NP-expressing ADC lines either without additional treatment, Ly/UV or in the presence of $NiCl_2$ or $CdCl_2$, were co-incubated with APCs and NP396-specific CTLs in order to detect XP (Fig. 2C). Nickel induced XP to levels higher than seen for the Ly/UV treatment. In contrast to this, samples treated with cadmium did not show altered responses. In order to evaluate the role of cell death in this setting we treated ADCs and analyzed the percent of necrotic cells by propidium iodide staining (Fig. 2D). Indeed, it was evident that nickel in contrast to cadmium treatment showed a higher proportion of necrotic cells. Also for the heat-shocked and especially Ly/UV treated samples elevated numbers of necrotic cells could be detected. Hence, the amount of XP in these experiments correlated with the induction of cell death, rather than up-regulation of HSPs. Although, these experiments do not support the notion that up-regulation of HSPs is the reason for increased XP, their general role as antigen shuttle cannot be excluded.

Denaturation of antigenic supernatants strongly interferes with cross-presentation

The transfer of antigens from ADCs to APCs is thought to be mediated by specific carrier proteins. Denaturation of antigenic cell fractions unfolds conformation of proteins, but does not harm primary epitope sequences of potential antigens. To evaluate whether correct folding is required for the cross-presentation of the LCMV NP, we compared the antigenic activity of HEK-NPA3 lysates before and after denaturation. In the same experiment we analyzed whether antigenic particles larger then 0.2 µm could be an essential source for XP. Interestingly, filtering the antigenic supernatant of HEK-NPA3 cells only led to a small reduction of MHC class I presentation. In contrast to this, denaturation reduced XP to background levels independently of filtration prior to the treatment (Fig. 3A). This experiment clearly indicates that receptor-ligand interactions, but not particulate cell debris, are required for the XP of the LCMV NP.

Antigen uptake by APCs is not mediated via CD91

Even though we did not find evidences for enhanced cross-presentation via up-regulation of HSPs, we have shown before that XP of the LCMV NP is sensitive to specific Hsp90-family inhibitors (Basta et al. 2005). Here we used α2-macroglobulin, one of the natural CD91 ligands (Binder et al. 2001), to compete with HSP binding. If CD91 is involved in uptake of antigenic HSP-complexes, the amount of XP should be reduced in the presence of α2-macroglobulin. However, we were not able to detect specific differences for NP XP in the presence of either albumin (contr.) or α2-macroglobulin (Fig. 3B). From this experiment we conclude that XP of the LCMV NP is independent of CD91 receptor binding.

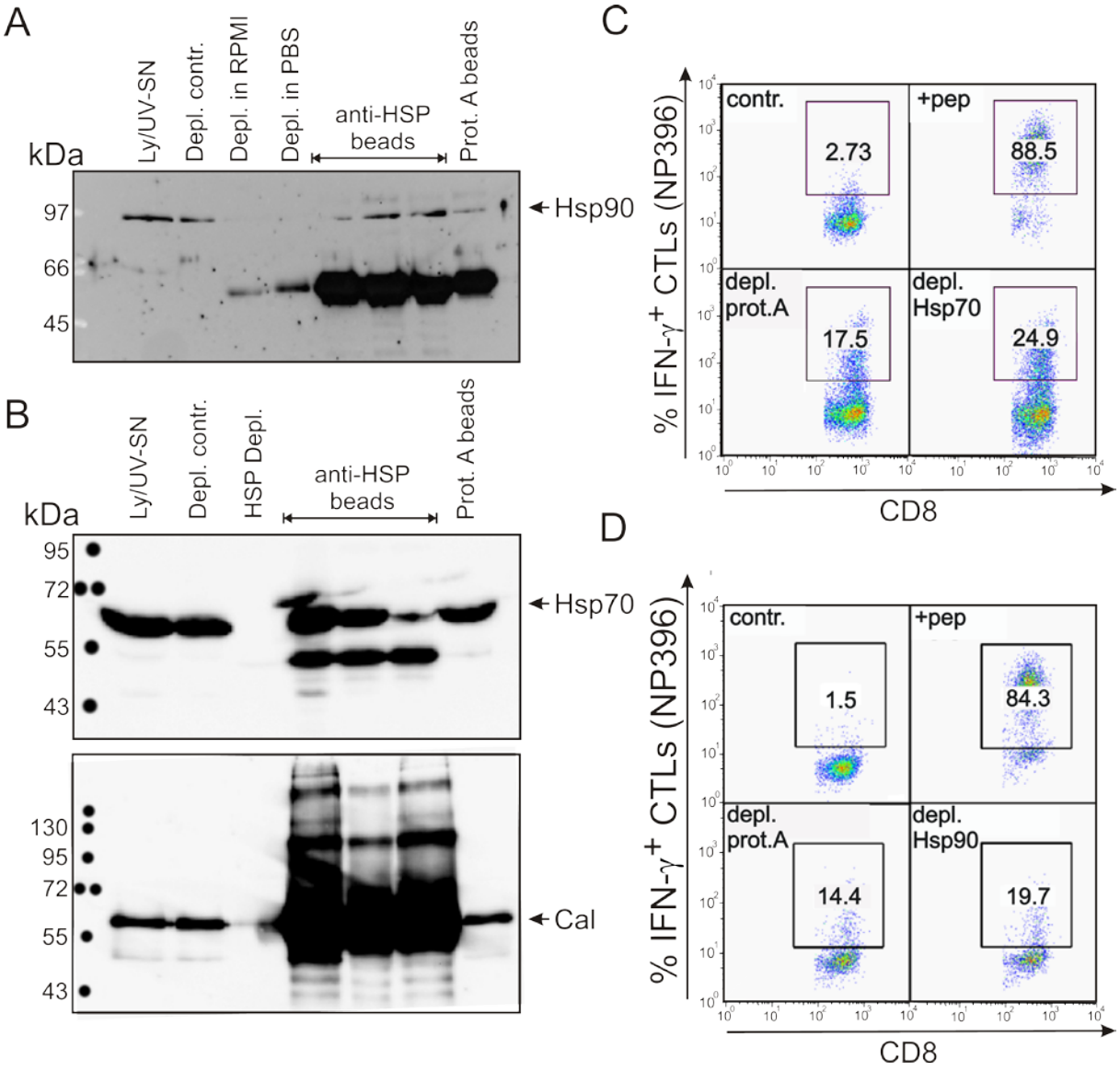

Figure 4: Depletion of heat-shock proteins (HSPs). HEK-NP cells were harvested and prepared in a concentration of $2*10^6$ cells/ml. Ly/UV treatment was performed, cell debris removed by centrifugation and protease inhibitor cocktail added to the lysate supernatant. A, Western blot analysis of Hsp90 depletion. Hsp90 was removed from cell lysates by three rounds of depletion in either medium or PBS. Incubation with protein A beads in the absence of anti-HSP antibodies served as control. B, Western blot analysis of Hsp70 and calreticulin depletion. HSPs were depleted using antibodies against Hsp70 and calreticulin. Lysates were performed in PBS. C+D, Analysis of cross-presentation efficiency in the presence or absence of Hsp70 or Hsp90. $2*10^5$ BMC cells (BMDCs, data not shown) per 96-well were incubated with 100 µl of either control (depl. Prot. A) or HSP-depleted HEK-NP cell supernatant (depl. 70 or depl. Hsp90). After 18 h NP396 specific cytolytic T-lymphocytes (CTLs) were added to detect cross-presentation of the LCMV-NP. Activation of CTLs was monitored by intracellular staining for IFN-γ and acquisition of samples by flow cytometry. Cross-presentation assays were performed twice with similar outcome.

Depletion of Hsp70 or Hsp90 from antigenic supernatants does not affect cross-presentation of the LCMV NP

In order to directly investigate the role of individual HSPs we developed depletion strategies for Hsp90, Hsp70, and calreticulin. Antigenic cell lysates from NP-expressing HEK-NPA3 cells were prepared and proteins were depleted by immunoprecipitation. Successful depletion was followed by western blot analysis (Fig. 4A+B). Equal loading was monitored by re-acquisition of blots against β-actin (data not shown). Interestingly neither the depletion of Hsp90 nor Hsp70 showed an effect on NP XP (Fig. 4C+D), what indicates that these proteins are not essential in mediating NP presentation and may be not directly involved in specific antigen shuttling.

Competition with purified Hsp90 increases XP efficiency similar to LPS

In order to unambiguously exclude HSPs as important players in our system, we tried to confirm our results in a third independent experiment. Assuming a potential role for Hsp90 in shuttling antigenic material to the APCs, cross-presentation should be inhibited by the addition of saturating amounts of purified Hsp90. Therefore, we established the purification of Hsp90 from naïve background in order to compete with Hsp90-antigen complexes for receptor binding. Hsp90 was isolated in a 3-step protocol with high purity. A representative chromatogram from the last purification step an analysis of fractions by SDS-PAGE and western blot are shown (Fig. 5A+B+C).

Unexpectedly, we observed a strong increase rather than a decrease in XP when co-incubating purified Hsp90 with NP-expressing cells in our assay (Fig. 5E). Since Hsp90 was shown to bind to TLRs, we decided to test if the increase in XP could be due to activation of APC. Therefore, we compared the results observed for Hsp90 with the TLR4 ligand LPS (Fig. 5F). These experiments show that LPS strongly induces XP similar to Hsp90.

Taken together, we hypothesize that HSPs in the LCMV system are not required as specific vehicles for antigen transfer to APC, but have an unspecific function in APC activation by providing a cellular danger signals.

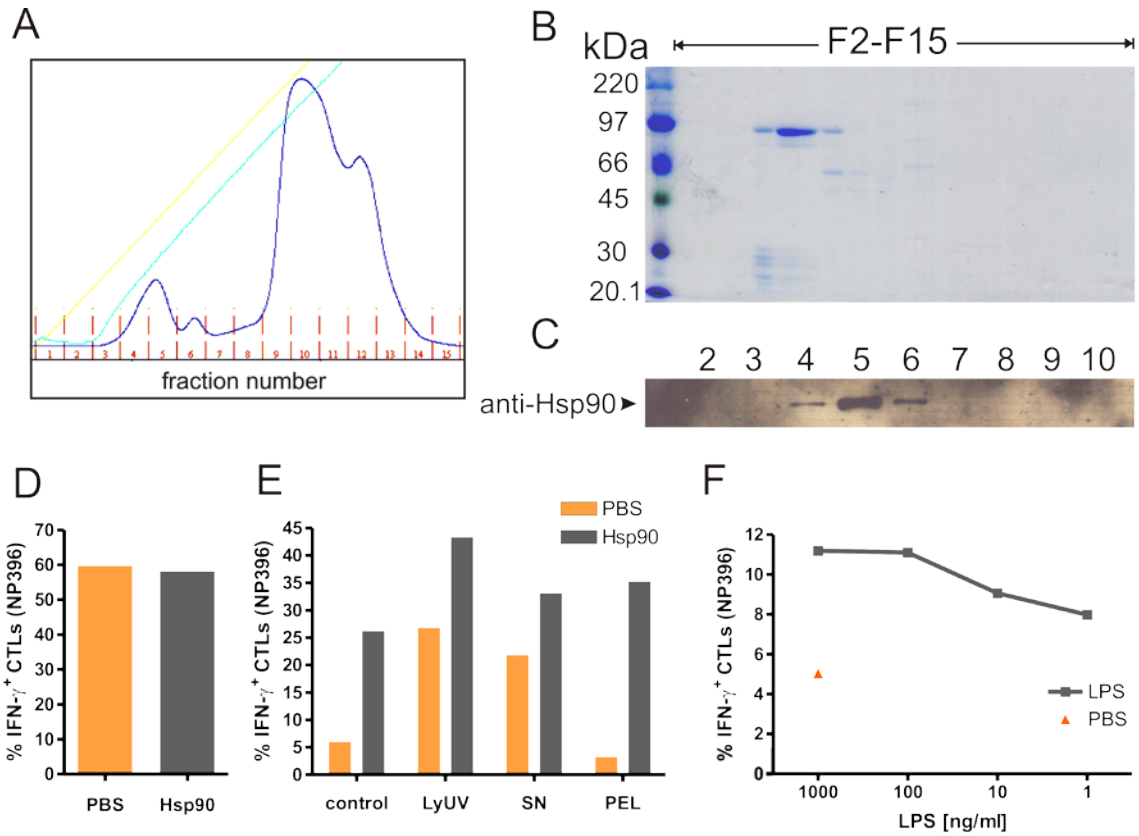

Figure 5: Purification of Hsp90 and its role in cross-presentation. A, Representative result of the FPLC anion exchange chromatography run as final step of the Hsp90 purification. Proteins were eluted by linear NaCl gradient. B, Coomassie staining of SDS-PAGE analysis of protein fractions after separation by FPLC. C, Western blot analysis of FPLC fractions 2-10. The membrane was developed with antibodies specific for Hsp90α/β. D, Effect of purified Hsp90 on direct-presentation after external peptide loading. $2*10^5$ BMCs were incubated in the presence (Hsp90) or absence (PBS) of Hsp90. After 18 h NP396 peptide was added at a concentration of 10^{-10} M and NP396-specific cytotoxic T-lymphocytes were used to measure antigen presentation. E, Effect of purified Hsp90 on cross-presentation of the LCMV NP. HEK-NP cells were harvested and used in a concentration of $2*10^6$ cells/ml. Cells were either left untreated (control) or were Ly/UV treated (Ly/UV). Additionally Ly/UV treated samples were separated by centrifugation into a soluble (SN) and pellet fraction (PEL). 100 µl of the respective solution were incubated with $2*10^5$ BMCs in the presence or absence of purified Hsp90. F, HEK-NP and BMC cells were co-incubated in the presence or absence of indicated concentrations of LPS. E+F, After 18 h NP396-specific cytotoxic T-lymphocytes were added to detect cross-presentation of the LCMV NP by intracellular IFN-γ staining and flow cytometry. Graphs are representative for at least two independent experiments with similar results.

Fractionation of ADC lysates to find cross-priming activity

The denaturation experiments shown above did indicate that cross-presentation of the LCMV NP is dependent on receptor-ligand interactions. In order to find interaction partners we decided to fractionate antigenic supernatants of HEK-NPA3 cells by gel filtration chromatography. Using this approach, it is possible to separate the full-length NP from possible degradation products with antigenic

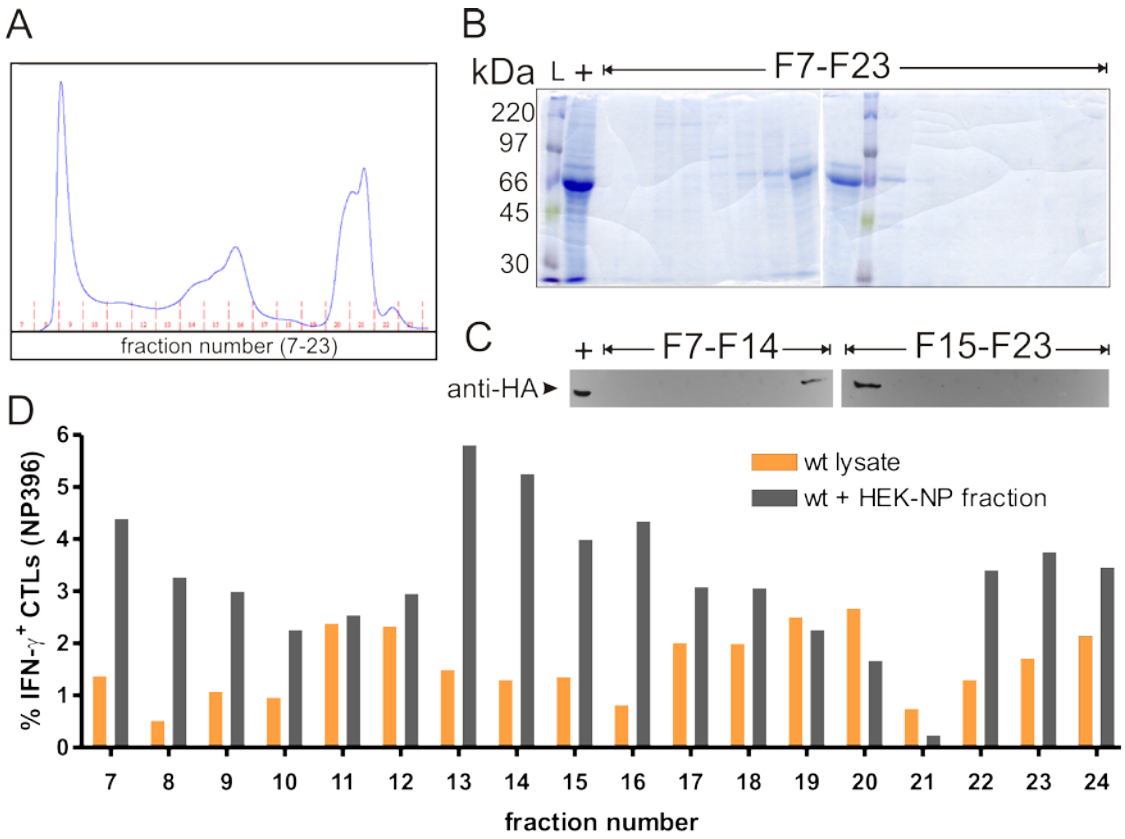

Figure 6: Analysis of antigenic properties of HEK-HANP lysates fractionated by FPLC. A, HEK-NP cells in a concentration of $8*10^6$ cells/ml were treated with Ly/UV and cell debris was removed by centrifugation. Supernatant was separated by FPLC using size exclusion column. Graph shows a representative chromatography run. B, Coomassie staining of protein fractions 7-23 separated by SDS-PAGE. C, Western blot analysis of chromatography fractions 7-23. Membranes were developed with anti-HA antibodies, specific for HA-tagged NP. D, $2*10^5$ BMCs per 96-well were incubated with 100 µl of the indicated FPLC fraction or PBS, in the presence HEK293 cell lysates. After 18 h NP396 specific cytotoxic T-lymphocytes were added in order to detect cross-presentation of the LCMV NP by intracellular IFN-γ staining and flow cytometry.

potential and larger complexes of NP bound to other proteins. Although we succeeded in separating the full-length NP from fractions containing larger protein-complexes or smaller protein-fragments (Fig. 6A+B), we were not able to detect any NP-specific signals by western blot analysis in the high molecular weight fractions of the purification (Fig. 6C). This suggests that the NP was not associated to other proteins in the native state. Nevertheless, when analyzing the chromatography fractions for the capacity to induce XP by APCs we observed that not only the two fractions, which actually contained the full-length NP, but also some high- and low-molecular weight fractions showed XP activity (Fig. 6D).

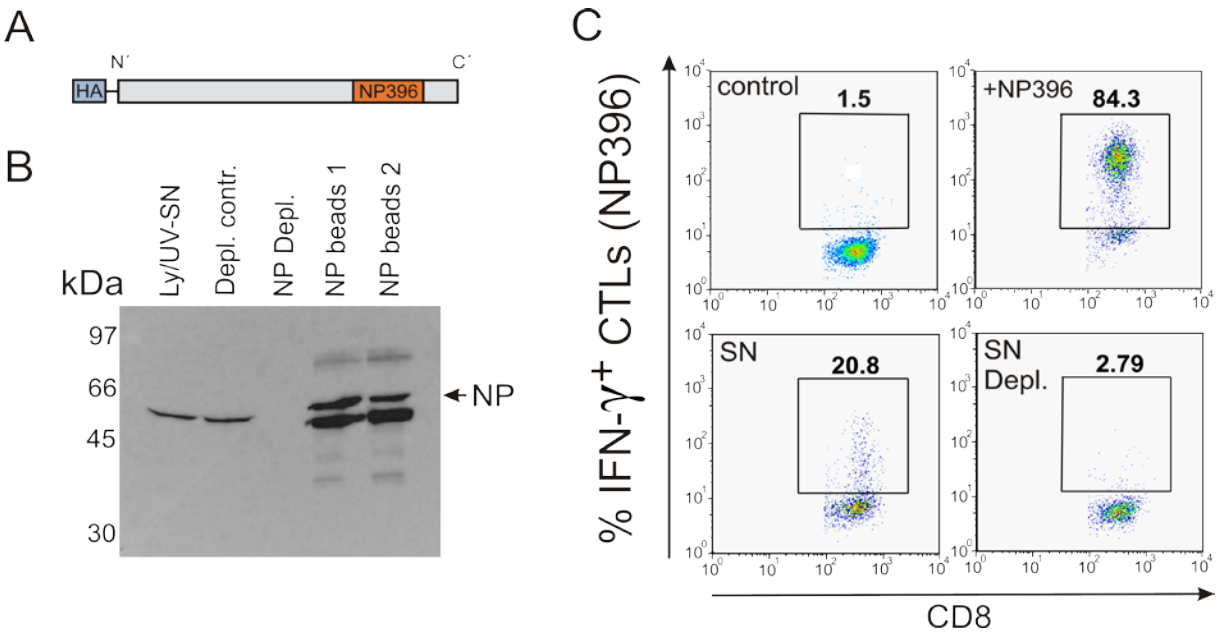

Figure 7: Depletion of full-length NP reduces cross-presentation to background levels. A, Schematic view of the HA-tagged version of the LCMV nucleoprotein (NP). B, Western blot analysis of NP depletion from antigenic supernatants. $2*10^6$ HEK-HANP cells/ml were treated with Ly/UV and cell debris was removed by centrifugation. Supernatant was incubated two times for 12 h with anti-HA beads in the presence of protease inhibitors. Afterwards the samples were analyzed for successful NP depletion (NP Depl.) by western blot analysis. Depletion with protein A beads served as control (Depl. contr.). Membrane was developed with anti-HA antibodies. C, Analysis of cross-presentation in the presence (SN) or absence (SN Depl.) of the LCMV NP. $2*10^5$ BMCs per 96-well were left untreated (contr.), treated with NP396 peptide or co-incubated with HEK-HANP cell supernatants as indicated. After 18 h NP396-specific cytotoxic T-lymphocytes were added to detect cross-presentation of the LCMV NP by intracellular IFN-γ staining and flow cytometry. Results shown are representative for two independent experiments.

Therefore, it remains possible that NP degradation products might be a source for cross-presentation, as well as larger complexes. Hence, this experiment was not able to resolve the question whether the full-length NP, degraded protein fragments, or NP peptides are the major antigen source of XP in this system.

Depletion of full-length NP prevents cross-presentation

In order to evaluate the antigenic role of peptides or larger degradation products versus the native antigen we established the depletion of the full-length NP from antigenic cell supernatants. Therefore, we generated a HEK293 cell line, expressing the NP with an N-terminal HA-tag (Fig. 7A) and established a successful depletion strategy. Lysates of HEK-HANP cells were made and absence of NP after depletion

was

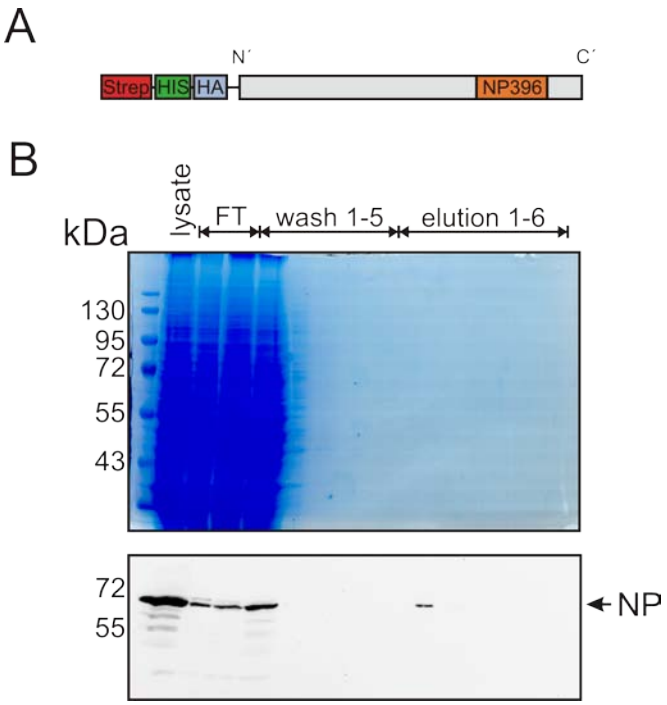

Figure 8: Purification of the LCMV nucleoprotein (NP). A, Triple-tagged version of the LCMV NP, expressing N-terminal Strep, 6 x histidin (HIS) and hemaglutinin (HA) tag. B, HEK293 cells were stably transfected with triple tagged NP and cell lysates were loaded on a streptactin column. Fractions from NP purification were collected and separated by SDS-PAGE (upper part). Same fractions were also analysed by western blot anti-HA (lower part).

was confirmed by western blot analysis (Fig. 7B). When antigenic lysates were added to APCs in a XP assay we observed a complete loss of CTL activation in case when the full-length NP was depleted from cell lysates (Fig. 7C). Since the epitope NP396 is located at the C-terminal end of the NP, this experiment unambiguously excludes antigenic peptides as source for XP. The entire XP activity is therefore mediated by the native form of the full-length NP.

Purification of recombinant NP

To confirm the hypothesis that XP of the LCMV NP requires the help of additional factors that promote antigen uptake by APCs, we wanted to test the XP efficiency with purified NP. Therefore, we established purification protocols for the LCMV NP based on bacterial, yeast and eukaryotic expression system. Although NP was expressed, unfortunately purification failed using bacterial and yeast expression systems (data not shown). Thus, we decided to generate an NP-expressing HEK cell line, using a NP construct encoding for an additional strep/His-tag at the N-terminus of the protein. Using this cell line we were able to successfully purify the LCMV NP (Fig. 8A+B). However, since this system was not able to deliver the amounts of NP needed to study

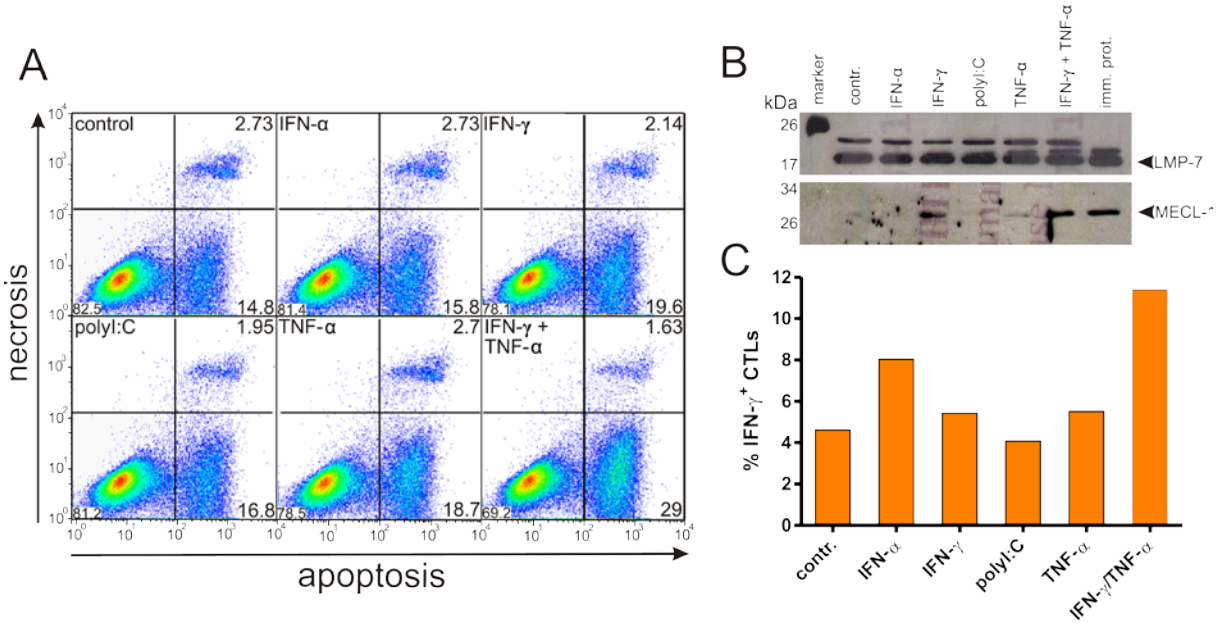

Figure 9: Induction of cross-presentation by pro-inflammatory cytokines. A, Flow cytometric cell death analysis of HEK-NP cells treated with indicated molecules. Necrotic cells death was detected by incorporation of propidium iodide (PI) whereas apoptotic cells were stained with antibodies against phosphatidyl-serine (Annexin V). B. Western blot analysis of cytokine and polyI:C treated HEK-NP cells for the expression of immune proteasome subunits LMP-7 and MECL-1. C, Cross-presentation of the LCMV NP by BMCs, co-incubated with HEK-NP cells either treated with indicated cytokines or untreated as control (contr.). After 18 h NP396-specific cytotoxic T-lymphocytes were added to detect cross-presentation of the LCMV NP by intracellular IFN-γ staining and flow cytometry.

the XP of purified antigen, we are currently optimizing our purification protocols in order to improve protein yield.

Simultaneous stimulation of ADCs with TNF-α and IFN-γ induced cross-presentation of the LCMV nucleoprotein

Here we tackled the question, whether a cytokine treatment of ADCs would alter the XP efficiency of expressed antigens. Therefore, we treated NP-expressing donor cells with various cytokines or adjuvant, before using them as antigen donor for the XP assay. As expected, we were able to induce the immune proteasome subunits LMP-7 and MECL-1 by treatment with IFN-γ, TNF-α or a combination of both, which had synergistic effects (Fig 9B). All other conditions tested showed no altered subunit expression compared to the control. Interestingly, combined treatment with TNF-α and IFN-γ led to a significantly stronger activation of CTLs (Fig. 9C) as well as the highest expression of immune proteasome subunits. In order to investigate possible reasons for increased cross-presentation after cytokine treatment, we analyzed the

amount of dying cells. As shown before (Fig. 2), cell death might be an important factor to target protein into the exogenous antigen presentation pathway. ADCs were treated with indicated factors and cell death was analyzed by flow cytometry (Fig. 9A). Although we could not find elevated numbers of necrotic cells in any of the conditions, there was a significant increase in the number of apoptotic cells in samples treated with the combination of TNF-α and IFN-γ.

Identification of interaction-partners of the LCMV NP by 2-dimensional gel electrophoresis

Using a biochemical approach, we performed a screen for physiological interaction partners of the LCMV NP. Interacting proteins could be interesting candidates for molecular factors other than HSPs that might mediate the transfer and uptake of the NP by APCs. Since the Ly/UV treatment of antigenic cell supernatants led to a strong increase of XP we decided to further analyze this issue. HEK293 and HEK-HANP cells were lysed and treated with UV-light. Supernatants were then incubated with HA-affinity beads in order to precipitate the NP and associated proteins. The protein precipitates were loaded on 2-dimensional gels and separated by isoelectric focusing and denaturing SDS-PAGE (Fig. 10). The gels were developed by silver staining and anti-HA western blot (Fig. 10). Noticeably, only a single spot could be detected on western blots from HEK-HANP lysates, which was absent in the control (Fig. 10C+D). This spot represented the full-length NP. These data suggest that UV-light treatment of cell supernatants does not lead to covalent cross-linking of the NP to other proteins, at least not in a concentration detectable by western blot analysis. However, gels stained with silver indicated that there are additional protein spots in the samples precipitated from HEK-HANP cell lysates. These spots represent proteins that were co-immunoprecipitated with the NP and had to be non-covalent interaction partners in the cell lysate. To identify these molecules, analysis of the dissected gel spots was performed by mass spectrometry. Unfortunately, the protein concentration within the collected samples was too low to identify individual proteins.

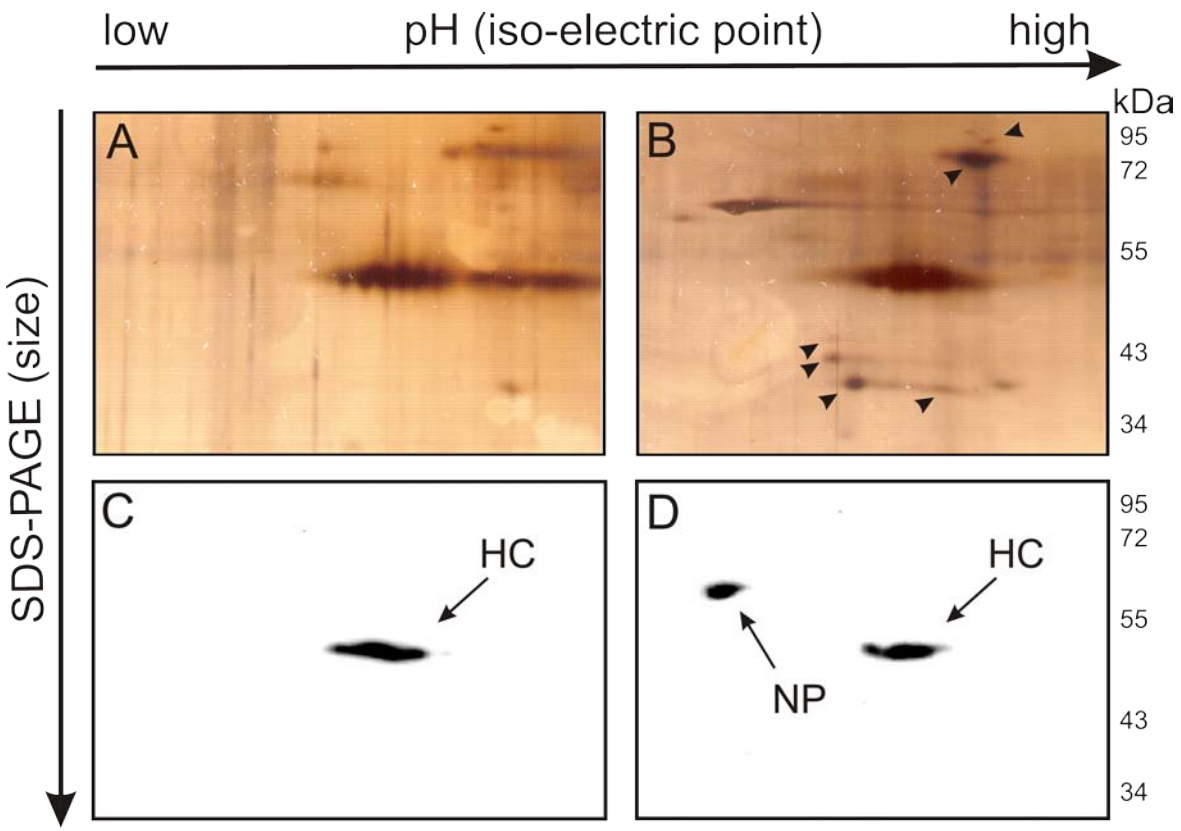

Figure 10: Two-dimensional analysis of LCMV nucleoprotein (NP) binding proteins as possible co-factors for mediating cross-presentation. HEK293 (A+C) or HEK-HANP (B+D) cells were harvested and treated with Ly/UV. Cell debris was removed by centrifugation and supernatants were incubated with anti-HA beads for 12h in the presence of protease inhibitors. Beads were then loaded on a two dimensional gel, separating precipitated proteins by iso-electric point in a first step and conventional SDS-PAGE in a second step. Gels were either stained by silver-staining (A+B) or blotted on a membrane an analysed by anti-HA western blot (C+D). Indicated spots were extracted from the gel and analyzed by mass spectrometry. Arrows in C+B indicate the nucleoprotein (NP), the heavy chain of the antibody used for immune precipitation (HC) and NP-binding proteins (in B).

Only the NP-specific spot was confirmed. Nevertheless, the presented data are the first evidences for NP-interacting proteins that could be promising candidates in inducing cross-presentation. Currently we try to scale up our experimental procedures in order to increase the final protein concentration to allow analysis via mass spectrometry.

Discussion

The investigation of how tissue specific, cell-associated antigens get access to antigen presentation by professional antigen-presenting cells is still not elucidated completely. Numerous efforts have been made in order to comprehend the induction of cytotoxic $CD8^+$ T-cell responses, which is are central requirement for the immune defence against intracellular pathogens and cancer. According to this, the more attention is being drawn to cross-priming of antigens expressed by tumors or tissue-specific viruses as an essential step to clear malignant or infected tissues.

For cancer, it was shown that patients treated with immunosuppressing drugs show higher incidences for malignancies (Haagsma et al. 2001, Adami et al. 2003, Serraino et al. 2007). Therefore, it could be assumed that malignant developments during an organism's life time are in most cases successfully rejected without the need of any therapy. This idea was summarised as the "cancer immunosurveillance hypothesis" (Dunn et al. 2002). Additional to the mechanisms of the inert immune system, tumor regression in many cases is dependent on cytotoxic T-cells primed against tumor-associated auto-antigen. How this T-cell priming works has been a mystery for a long time and is still not completely understood (Matzinger 2002). The successful cross-priming of CTL responses requires a well-defined mechanism of antigen recognition, transfer and uptake by APCs, and the cross-presentation on MHC class I molecules. Uncovering this pathway will have an strong impact on the development of future vaccines. It seems that malignancies actually established in the tissue evade from immune recognition by accumulating mutations (Khong and Restifo 2002). This could be possibly due to mutation of cancer-associated epitopes that in most cases of cancer lead to successful regression. Understanding why certain cancers can circumvent these defense mechanisms might be the key for effective immunotherapy. It is necessary to consider that cell-associated antigen are the most physiological source for immune responses primed via XP. Given the notion that the XP pathway evolved according to these requirements, the use of antigenic material in association with natural endogenous adjuvants from autologous tissues might, therefore, be the most promising strategy for the design of anti-cancer

vaccines. In this report we used the nucleoprotein from LCMV as a model antigen to study cross-presentation of cell-associated proteins. Similar to the requirement of anti-tumor responses we used a system that is depended on antigen transfer from donor cells to APCs, and XP in the absence of viral patterns. A special interest was assigned to cellular factors that on the one hand are able to promote APC maturation and on the other hand can specifically shuttle antigens to APCs for XP.

Our finding that heat-shock treatment of antigen donor cells induces XP of the LCMV NP and that this enhancement was sensitive to the activity of Hsp90 family chaperones (Basta et al. 2005), initiated a study to further characterize the role of HSPs in the LCMV system. Our results are consistent with other reports showing enhancement of XP by HSP-inducing treatments (Melcher et al. 1998, Feng et al. 2001). Elevated cell temperatures lead to a classical heat-shock response (Lindquist 1986) and HSP up-regulating serves as an elegant explanation to understand enhanced XP in heat-treated samples.

In figure 2 we tested whether a heat-independent induction of HSP expression would have the same enhancing effects on XP, like the actual heat-shock. We used heavy metal ions in concentrations that were shown to give highest HSP up-regulation (Basu and Srivastava 2003). Interestingly, cellular treatment with 300 µM nickel was able to strongly promote the cross-presenting capacity of APCs. In contrast to this, $CdCl_2$ did not alter the amount of XP compared to the untreated control. Although we could show that nickel ions induce HSP expression, concentrations of 300 µM showed decreasing amounts of HSPs in our system and also the constitutive proteins were reduced in these samples (Fig. 2A+B). Since high nickel concentrations are close to being toxic to cells, it is reasonable to explain the reduced protein expression by the induction of cell death. In contrast to nickel, cadmium was published to induce HSP expression already at a concentration of 5 µM, which is in the non-toxic range (Skreb and Fischer 1984, Steiner et al. 1998). However, 5 µM cadmium was not able to induce XP. Our experiments indicated that the induction of HSP expression might not be the critical event that leads to better XP. According to the literature, the difference in the molecular action of the two ions might explain the observed effects. Interestingly, nickel ions were reported to lead to APC maturation due to their properties to act as contact sensitizer (Basu and Srivastava 2003, Mizuashi et al.

2005). The XP promoting effect of nickel ions in our experiments could, therefore, be an unspecific adjuvant effect, independent of HSPs up-regulation. In addition, the induction of cells death could play a role for the effects observed with cellular stress. Similar to nickel, cadmium has been published to induce HSP-expression (Lindquist 1986, Williams and Morimoto 1990, Hiranuma et al. 1993), although we could not show this by western blot analysis in our settings (Fig. 2B). Strikingly, the cellular stress response to cadmium is more sensitive compared to nickel ions and HSP-expression is already induced at much lower concentrations (Skreb and Fischer 1984, Steiner et al. 1998). This results in a lower toxicity as a side effect of the cadmium treatment. When analysing the induction of necrotic cell death we found elevated numbers in samples treated with nickel but not with cadmium. Thus, necrotic cell death is a second possible explanation for the increased XP in the nickel-treated samples.

Another cell treatment that provides arguments against HSP-expression as reason for increased XP is the Ly/UV condition introduced above (Basta et al. 2005). XP of Ly/UV-treated material can not be explained by increased HSP-expression, since cells were lysed by shock-freezing and HSP levels were not altered. The release of cytoplasm from necrotic cells into the medium might stimulate XP via the action of endogenous adjuvants. Interestingly, only UV-light treatment of cell lysates activates the full cross-presenting activity (data not shown). This could be due to chemical cross-linking of molecules. Either NP-complexes with cellular factors that are required for XP are stabilized or the formation of particulate antigen is induced, which can be very efficiently endocytosed and presented by APCs (Storni et al. 2005). The experiments presented in figure 10 help to discriminate between these two possibilities.

If it is not the quantity of HSPs in treated cells that leads to an increase in XP, it might be their availability that matters. Especially the intracellular relocation by targeting HSPs to the cell surface was previously discussed in the literature. It was shown by different groups that artificial surface expression of HSPs let to specific immune responses against antigens expressed in modified ADCs (Zheng et al. 2001, Dai et al. 2003). Other studies mention the induction of immune responses against tumor tissues, expressing Hsp70 on the cell-surface (Multhoff et al. 1997). However, since

we were not able to find evidence for increased HSP surface expression, we further focused on antigenic cell supernatants and a role of the Hsp90 family (data not shown).

Beside the cytosolic Hsp90, another member of this family is the ER-resident gp96, which plays a crucial role in chaperoning and shuttling antigenic peptides (Arnold et al. 1995, Singh-Jasuja et al. 2000b, Srivastava and Amato 2001). However, for the LCMV NP system it was clearly shown that the long-lived and non-processed form of the protein is cross-presented, which is in line with our immune depletion experiments shown in the present report (Norbury et al. 2004, Basta et al. 2005). Due to the N-terminal HA-tag and the C-terminal location of the NP396 epitope, it is highly unlikely that peptide fragments depleted from the cell supernatant are cross-presented in this case (Fig. 7). This fact excludes defective ribosomal products (DRiPs) as antigen-source for XP in this system. Although there are reports that gp96 is able to bind full-length proteins, this option is restricted to molecules of the secretory pathway (Argon and Simen 1999). The NP is targeted to the cytoplasm of the cell (Oxenius et al. 1995) and it is therefore unlikely that it can directly interact with the ER-associated gp96.

The specific uptake of antigens for XP by APCs can be promoted by various receptor-ligand interactions. Among the known HSP-receptors, CD91 might be of special interest for the XP of the LCMV NP. It is not only a receptor of different HSPs, but was shown to have the highest affinity to Hsp90 (Basu et al. 2001). Therefore, we were studied the role of CD91 in our system. Alpha2-macroglobuline (α2-M), a physiological ligand for CD91 was used to evaluate the need for CD91 (Fig. 3). Although CD91 was shown to be essential for XP of gp96-peptide complexes (Binder et al. 2000a) or HSPs from bacterial background (Tobian et al. 2004) and other antigens, competition assays failed to inhibit XP of the NP. This clearly indicates that CD91-independent uptake mechanisms are involved in our experiments. Furthermore, this finding also supported the idea that not gp96 but Hsp90 might be involved in the XP of the NP, because CD91 was shown to be essential for the uptake of gp96-peptide complexes (Binder and Srivastava 2004). However, CD91-independent cross-presentation was reported as well (Berwin et al. 2002a). Experiments using the same LCMV NP system showed that fucoidin, an inhibitor of

SR-A family, had inhibitory effects on XP (Basta et al. 2005). Other publications directly show the involvement of SR-A in HSP-uptake by APCs (Berwin et al. 2003). Also the scavenger receptor LOX-1 was reported to be involved in clearing apoptotic cells by acting as HSP-binding receptor on APCs and targeting HSP-antigen complexes for cross-presentation (Delneste 2004). The CD91 competition experiments performed in this study are, therefore, in line with previous findings, showing that HSP-mediated XP can be CD91 independent.

Cytokines like TNF-α or IFN-γ have been shown to induce immune proteasome formation and thus alter the repertoire of peptides, used for direct-presentation (Groettrup et al. 2001a). If cross-presentation is an important evolutionary conserved mechanism throughout higher organisms, one could assume that cytokine treatment of peripheral tissue might also lead to intracellular modulations that prepare non-immune cells in being better antigen donors for cross-presentation. We treated ADC with various cytokines of adjuvants in order to investigate the effects of XP. Interestingly, we could show that a synergistic treatment with IFN-γ and TNF-α led to an increased XP (Fig. 8). This finding correlated with the induction of apoptotic cell death, which can explain increased antigen presentation. Hence, not only material released from necrotic but also apoptotic cells can serve as antigen donor for XP. This process might involve certain apoptosis receptors, which are discussed in the literature (Albert et al. 1998b, Platt et al. 1998). One molecule that was associated with XP of apoptotic cells is CD36. But although it was clearly shown that this receptor mediates phagocytotic uptake of antigens from apoptotic contexts (Fadok et al. 1998), it is controversial if this uptake leads to class-I processing and XP by APCs. Some studies promote this assumption (Albert et al. 1998b), but more and more evidences came up that show CD36 not to be required for XP of apoptotic cells (Belz et al. 2002a, Schulz et al. 2002). Other candidates, which were shown to be important for the clearance of apoptotic cells include SR-A (Suzuki et al. 1997), the integrin $\alpha_v\beta_3$ (Savill et al. 1993), CD14 (Devitt et al. 1998) or LOX-1 (Fadok et al. 2001). Beside these, various possible receptor molecules are mentioned in the literature, but their status for XP is unclear and can not be discussed here (Platt et al. 1998, Pittoni and Valesini 2002). However, the fact that apoptotic cells can be a source for cross-priming *in vivo* was clearly shown (Albert et al. 1998a). Our results fit well with other publications pointing at specific XP mechanisms based on the uptake

and processing of apoptotic material (Savill et al. 1993, Albert et al. 1998b, Devitt et al. 1998, Fadok et al. 1998, Rovere et al. 1999, Pittoni and Valesini 2002, Schulz and Reis e Sousa 2002).

Hence, depending on the physiological conditions of antigen donor cells different uptake mechanisms are possible and likely to occur in parallel. Nevertheless there are convincing evidences that receptor-mediated uptake mechanisms indeed play a major role. Although it was published that antigen uptake via receptor-independent pinocytosis or macropinocytosis can lead to XP (Norbury et al. 1995, Norbury et al. 1997, Binder and Srivastava 2005b), it is rather unlikely that these unspecific mechanisms are responsible for XP of the LCMV NP in the system used here. Other cell-associated antigens were shown to be 100.000 times more potent in inducing an immune response, compared to purified proteins (Binder and Srivastava 2005a). This fact cannot be explained by unspecific uptake mechanisms. Second, we could show in this report, that heat-denaturation of antigenic cell supernatants interferes with XP (Fig. 3). From these experiments we draw two conclusions. First, the antigenic activity could be conserved after removing cellular fragments and particles from the soluble fraction. This fact in combination with the low XP activity in the pellet-fraction leads to the hypothesis that soluble antigen is a major player in XP of the LCMV-NP. Second, the loss of XP after heat-inactivation indicated the involvement of cellular factors that are required in a native configuration. This is a clear indication for the involvement of receptor-mediated uptake mechanisms. The immune system is exposed to numerous antigens that need to be cross-presented for the induction of effector responses. Therefore, it is rather unlikely that each antigen itself has to specifically bind to an uptake receptor on APCs. More likely is a receptor interaction with evolutionary-conserved molecules that mediate the XP of an antigen. This consideration is supported by the fact that denatured antigen is still presented on MHC class-II, as used for the induction of antibody responses. Thus, we hypothesize that specific signals leading to class-I processing and presentation are delivered by heat-sensitive factors.

In order to elucidate in what form the antigenic NP is available in cell supernatants, we fractionated lysates by gel filtration chromatography (Fig. 6). Using this approach we found a rather distributed activity within the different fraction. Not only fractions

that contained the full-length NP were able to induce XP, but also high and low molecular weight fractions had considerable XP activity. However, this is clearly contradicting to our experiment where no residual XP activity was detected after depleting the full-length NP protein (Fig. 7). XP activity in the high molecular mass fractions might be due to the formation of larger complexes, but no NP could be detected in those fractions by western blotting. Hence, the fractionation experiment was not able to identify the source of antigen in this system.

Following the idea that Hsp90 might be responsible for the induction of NP XP we purified Hsp90 from non-transfected cells to compete with possible Hsp90-antigen complexes for receptor binding (Fig. 5). Interestingly, we detected increased XP instead of inhibition. From this experiment we concluded, that on the one hand Hsp90 does not shuttle antigenic NP to APCs in a receptor-dependent manner, but on the other hand leads to a strong increase in cross-presentation, probably due to rather unspecific adjuvant effects. Independent of the cell treatment prior to the experiment and the cellular fraction, Hsp90 was able to promote cross-presentation. This finding is in accordance with other reports, showing that Hsp90 can bind to TLR4, and by this means induce the activation of APCs. However, there is a debate in the field whether APC activation by HSP preparations can be due to endotoxin contaminations (Rock et al. 2005). Different reports could show that the full adjuvant-properties of some HSP-preparations could be due to LPS contaminations (Bausinger et al. 2002, Gao and Tsan 2003b). Although we cannot completely exclude possible LPS contaminations of our Hsp90 preparation, the purification from HEK293 cells makes it rather unlikely. However, the fact that exactly the same XP-enhancing effects were observed with direct LPS treatments, confirms on the one hand the high impact of adjuvants on XP, but also challenges a potential role of Hsp90 due to possible endotoxin contaminations. Both, heat inactivation and polymyxin B treatment have been common techniques to exclude endotoxin contaminations as adjuvant-components in HSP-preparations (Kol et al. 1999, Rock et al. 2005). However, these treatments are controversially discussed and were therefore not applied in this study. There are papers showing that low concentrations of LPS can be heat-sensitive and treatments with polymyxin B could not completely eliminate endotoxic properties from the solution (Bausinger et al. 2002, Gao and Tsan 2003b, a).

The depletion of HSPs from antigenic supernatants is a different approach that was used to show their involvement in XP for other systems (Binder and Srivastava 2005a). Therefore, we set up a similar approach and established the depletion of Hsp90, Hsp70 and calreticulin. Interestingly, we were not able to find significant reduction of XP in any of the cases tested. Depletion of Hsp90, the most likely candidate to mediate the XP of the LCMV NP, did not alter the efficiency of antigen presentation by APCs. Even though Binder *et al.* detected strong reduction of XP only after depletion of all four major HSPs, they already observed tendencies in the single depletions. This, together with the findings mentioned above, brings us to the conclusion that HSPs do not act as a specific antigen carriers to shuttle the NP in this system.

The LCMV NP expressed by antigen donor cells is a powerful tool to study the XP of cell-associated antigen. In this study we were able to show that the soluble, full length protein is the antigenic source for XP in this system. Although we could not see any specific effect of HSPs, they promote the XP pathway by providing activation/maturation signals to the APCs as part of the cocktail of endogenous adjuvants released from dying cells. Further characterization of proteins involved in the specific antigen transport and uptake mechanism will help to identify important factors for XP, apart from members of the HSP family. The presented data suggest that there are antigenic systems, which are entirely independent of peptides being transferred to the APCs. The nature of native antigens is a critical parameter that has to be taken into account in order to perform successful immunotherapy.

Final Discussion

The vision of early scientists to develop biological agents that clear the world from infectious diseases was until now only partially successful. The exceptional eradication of small pox in the second half of the 20th century is a prominent example to name the efficiency of vaccination programs, but however, not many more approaches of such an exceptional global success can be found. Even pathogens that we classify back into the dark days of middle ages, like pest and cholera, are not completely vanished but reappear, whenever situations of natural catastrophes or poverty do not allow maintenance of a certain level of hygiene (Kesteloot 2004). Therapies against other diseases that can be prevented by vaccination are extremely costly or require a functional cold chain from production to application. Therefore, these approaches are only applicable in developed countries. From the vaccination program against small pox we learn that random prophylactic vaccination is able to reduce the number of cases, but did not lead to the final success of small pox eradication. This was only possible after applying the “ring vaccination strategy” that was introduced by the WHO as a response to initial difficulties (Lau et al. 2005). The ring strategy was based on the vaccination of individuals that lived in the primary or secondary social environment of the person diseased. This way an ongoing epidemic was inhibited and a further expansion of the virus retarded. Taken this as a paradigm, it will not be possible to eradicate pathogens that persist in the environment. Cholera, typhus, and Ruhr are examples for diseases that emerge in situation of lacking hygiene of the drinking water or food and can only be suppressed locally (John et al. 2011). However, vaccination programs in these cases are an essential component of active emergency management, whenever they are available (Graves et al. 2010).

Different aspects are important for approaches that try to use vaccination against non-infectious diseases, like cancer. In this case, a prophylactic immunotherapy is only possible in rare cases, for example when malignant developments are based on viral or bacterial infections (Grce et al. 2010). Therefore, the focus of current research lies on the development of therapeutic vaccines that can be individualized to the respective patient.

In this thesis, the basic requirements of a successful immunotherapy were introduced and optimization strategies discussed concerning promising candidate vaccines. The

initial two chapters dealt with a cellular analysis of PLGA microsphere function. In a number of preclinical studies we and others provided evidence that the encapsulation of proteins into PLGA microspheres is a potent tool to deliver antigen to phagocytes. Mice injected with loaded microspheres were able to develop $CD8^+$ T-cell responses, which not only protected animals from viral challenges and tumor growth after transplantation of tumor cell lines, but were also sufficient to reject already established tumors in a therapeutic manner (Waeckerle-Men and Groettrup 2005). Compared to other vaccine preparations, including DNA vaccination and recombinant VV infection, PLGA MS have the great advantage to be applicable in individualized tumor therapy. Without the need of tumor-specific antigen identification, the encapsulation of complete tumor lysates into PLGA MS provides the possibility to induce anti-tumor responses, specific to the tumor of the respective patient (Solbrig et al. 2007). Taker together, the preclinical studies in mice qualify PLGA microspheres to take the next step of being applied in clinical trials soon. Since microspheres made of PLGA are already used as a delivery system for conventional drugs in humans, the accreditation to be utilized as human vaccine seems to be possible. However, the promising results observed in mice have to be confirmed in human studies to show corresponding effects on the human immune system. This is a crucial step for immune applications that were developed in the murine system. Although mice and humans are very similar in their organization of the immune system, there are details, which can be different and responsible for varying clinical outcome (Mestas and Hughes 2004). Although the induction of immune responses and the effects on tumor or viral challenge was studied intensively, the knowledge of what cell types are involved in microsphere-mediated responses is still very limited. A mechanistic understanding of microsphere function, however, is required to guide the project into a phase I clinical trial.

In *chapter I* we therefore analyzed the cell types involved in microsphere function. It was already known that CTL priming after microsphere injection is dependent on professional APCs, but the participation of individual cell types was not investigated. DCs were shown to be the most efficient APCs that can cross-present antigens *in vivo (Jung et al. 2002)* and we therefore assumed that also MS-encapsulated antigens would be cross-presented especially by DCs. From initial *in vitro* experiments using cell lines, we however knew that MS can be efficiently cross-

presented also by MΦs. This observation, together with the efficient endocytosis of MS by all kinds of phagocytes, was the basis for a comparative study to investigate the role of DCs and MΦs in MS function. Drawing two lines of evidence, we could show that both DCs and MΦs were able to cross-present MS-encapsulated antigens. Isolation of cellular fractions from murine spleens as well as *in vivo* depletion of individual cell population in the course of an MS vaccination indicated the contribution of both DCs and MΦs in CTL priming. Since the idea the MΦs can prime T-cells in response to MS injection is quite revolutionary we are currently performing experiments to further strengthen our conclusions. As already mentioned before, the depletion of DCs in transgenic CD11c-DTR mice has the disadvantage that it can only be performed transiently (Jung et al. 2002). Since DCs recover before finishing the experiment, there is a remaining possibility that T-cell priming detected in DC-depleted mice is due to recovering cells. To finally eliminate this insecurity, we will use CD11c-DTR → C57BL/6 Thy1.1 bone marrow chimeras. In those mice DCs originate from the donor and can be depleted with repetitive injection of diphtheria toxin, without side effects for the recipient mouse. This experiment will further define the role of MΦs in this system.

In parallel to the question of MΦ function, we were also interested to study the role of different DCs subpopulations. This analysis required a complex purification strategy that was only successful by combining magnetic- and fluorescence-activated cell sorting. Additionally, we performed experiments to investigate which cell types were associated with MS in the draining lymph nodes. This was performed by utilizing CdSe quantum dot labeled MS that were introduced in more detail in *chapter II*. Having this novel method, we could show that although both the $CD8^+$ and the $CD8^-$ DCs could cross-present *in vitro*, only $CD8^-$ DCs showed to be associated with the antigen in the draining lymph nodes. Since $CD8^+$ DCs, however, are currently thought to have the strongest impact on XP of many antigens, we decided to perform additional experiments to confirm these results with an alternative method. Only recently, the transcription factor BATF3 was introduced to drive development and differentiation of DCs (Hildner et al. 2008). Interestingly, although BATF3 is also expressed in $CD8^-$ DCs, mice lacking this transcription factor are depleted specifically from the $CD8^+$ DC subset. Using BAFT3 KO mice we will test our hypothesis that mainly $CD8^-$ DCs and MΦs are required for MS-mediated CTL priming.

In *chapter II* we introduced a novel method to label MS for electron and fluorescent microscopy. Similar to the question, which cell types are involved in cross-priming of MS-encapsulated antigen, the intracellular pathways required for this process were not completely elucidated. Due to the lack of adequate labeling methods, the question, whether MS enter the cytoplasm of phagocytes, as proposed by some authors (Panyam et al. 2002a, Gomes et al. 2006), could not be finally solved. Our approach to label MS with nanocrystals was therefore a promising method to elucidate further details of the intracellular distribution of MS. Electron microscopy was the technique of choice to answer the question if MS are stored in membrane-surrounded vesicles after phagocytosis. However, we could only answer this question by incorporation of electron dense nanocrystals into the PLGA particles. MS itself are not electron dense enough to be identified after internalization. This novel technique was able to avoid highly toxic osmium tetroxide incorporation, which is used as a contrasting agent in electron microscopy since many years (Luftig and McMillan 1981). The incorporation of nanocrystals also allowed us to incorporated fluorescent quantum dots to label MS for fluorescent microscopy. Quantum dots have exceptional properties concerning their fluorescence intensity and bleaching resistance. In contrast to initial experiments were FITC-labeling of MS was used, we could provide high resolution images, utilizing fluorescent quantum dots. Having these two novel methods we were able to provide evidence for a lysosomal storage of MS after uptake.

In a proof of principle experiment we introduced a third type of MS, in which we incorporated super-paramagnetic FeO nanocrystals. We could show, that these MS can be used to separate phagocytic from non-phagocytic cells. Additionally to what was already discussed above we want to mention that FeO nanoparticles also have implications on direct cancer treatments using high-energy magnetic resonance to induce heat-shock in tumor cells. Bases on strong magnetic fields, iron particles that are targeted to tumor cells (e.g. via attachment to antibodies) in the patient are heated to induce apoptosis in malignant tissues (Hergt et al. 2006b, Frimpong and Hilt 2010). Although this technique was already established in mice it faces some difficulties in humans. The resonance energies required for the elevation of cellular temperatures into the toxic range (~45°C) *in vivo*, are not approved to be used in

humans. The necessity of these high resonance energies after systemic application of iron particles is largely due to the fact that the number of particles per cell is too low (Hergt et al. 2006b). FeO nanoparticles encapsulated in PLGA-MS as performed in our study might circumvent this difficulty. PLGA-MS in the micrometer scale encapsulate large numbers of magnetic nanoparticles and deliver as a batch. This increases the number of FeO particles per cell and lower resonance energies could be sufficient for the induction of heat-induced cytotoxicity. This might allow the application of this promising method also in humans. PLGA-MS are efficiently delivered to draining lymph nodes. Therefore, FeO PLGA-MS would allow the treatment of lymphatic metastasis of any type of cancer. Other rare malignancies are based on hyperproliferation of phagocytes like DCs (Bothra et al. 2005). DCs tumors could therefore be treated using PLGA-MS as a delivery system for FeO nanoparticles that are able to generate heat-induced cytotoxicity.

Two other promising vaccines were investigated in *chapter III*. Plasmid DNA injection and infection with recombinant VV can induce CTL responses to an antigen of interest. Beside their successful use in mice there are already a number of clinical trials running that study the potential of these vaccines in humans (McConkey et al. 2003, Bodles-Brakhop et al. 2009).

As a comparison, both, DNA vaccines and recombinant VV viruses have their specific advantages in terms of application. While approaches based on viral delivery system generally have difficulties to enter the clinics because of biosafety, DNA vaccines are less hazardous, but often lack the immunogenicity required. At the same time it has to be kept in mind that viruses, also if used as a delivery system provoke anti-viral immune responses in the patient. This might lead to a protective immunity, which can suppress booster immunization or vaccinations for other antigens using the same vector (Basak et al. 2004, Bolhassani et al. 2011). This could actually be the most critical point also with regard to recombinant VV immunization. To overcome this limitation, a heterologous immunization strategy can be applied. Immune responses following DNA immunization can be improved by heterologous booster injection of recombinant VV (McConkey et al. 2003). Other disadvantages of viral delivery system are the higher production costs and the possible integration of viral genes into the genome of the host (Vergati et al. 2010).

However, despite these disadvantages of recombinant VV, there are a number of promising clinical trial running, which study their efficiency for malignant melanoma (Kim-Schulze and Kaufman 2009) and prostate cancer (Kaufman et al. 2002). Other viral vectors that were successfully used to induce immune responses are based on herpes simplex virus (Marconi et al. 2009), adenovirus and BCG (Hilleman 2000), or even bacterial delivery systems (Moreno et al. 2010). A different possibility is the delivery of DNA-based vaccines by non-living vectors like liposomes, nanospheres, MS (Bolhassani et al. 2011), or virus-like particles (Roy and Noad 2008).

However, a central question for the mechanistic understanding of DNA and recombinant VV vaccines is still not elucidated completely. The initial priming of $CD8^+$ T-cell responses can be performed by either direct- or cross-presentation and both pathways prefer different properties of an antigen. Knowing the impact of these mechanisms on the outcome of a vaccination protocol can therefore contribute to the enhancement of immunogenicity by optimizing the vaccine for either one of the two pathways. Consistent with previous results, we could show here that cross-presentation has a major contribution to anti-DNA and also VV responses (Doe et al. 1996, Loirat et al. 1999, Cho et al. 2001). Stable antigens were found to enhance $CD8^+$ T-cells responses compared to rapidly degraded derivates. From the evolutionary point of view this finding makes a lot of sense. Numerous pathogens try to evade from immune recognition by interfering with the direct-presentation machinery (Cunningham et al. 2010). Therefore, the cross-presentation of stable antigens in many cases is the only way to induce $CD8^+$ T-cells responses.

Interestingly there were also attempts to combine DNA vaccination with a PLGA MS based delivery system (Lin et al. 2010). This way PLGA MS encapsulation can protect DNA from degradation and leads to enhanced uptake by phagocytes. In general, the attachment of DNA to microparticles was shown to enhance vaccine efficiency (Doria-Rose and Haigwood 2003). However, since our results indicate that cross-presentation is the major mechanism of T-cell priming in DNA-based immunizations, it might be a disadvantage to only target DNA to phagocytes. A large number of transfected non-professional tissue cells are probably needed to produce enough antigens for cross-presentation. Independent of this objection, PLGA

MS/DNA preparations were shown to have toxic potentials, probable to DNA stabilizers that have to be co-encapsulated (Fu et al. 2000).

In *chapter III* we also discussed the possibility to transfer antigens via apoptotic or necrotic cells. DNA vaccines that co-express pro-apoptotic proteins were shown to increase immunogenicity (Sasaki et al. 2001, Ferguson et al. 2002). At the same time VV infected cell undergo apoptosis and secondary necrosis due to the viral infection. In both cased apoptotic/necrotic cells are efficiently cross-presented by non-transfected or non-infected APCs.

The mechanism by which such cell-associated antigens can be cross-presented by APCs was analyzed in *chapter IV*. Especially the role of HSPs and other cellular factors was investigated. In accordance to the results observed for DNA vaccines and recombinant VV, we found the long-lived LCMV NP to be source of antigen, when delivered in a cellular context. This further strengthens the hypothesis that dying cells can be the antigen source for cross-presentation in these systems. Viral infection and electroporation are also inducers of cellular stress, which leads to an up-regulation of HSPs and the initiation of an inflammatory response (Wallace et al. 2009). However, in *chapter IV* we excluded at least two of the major HSPs to be responsible for cross-presentation of the LCMV NP. The cellular factor that mediates cross-presentation in this system was not elucidated yet. We were able to exclude some cellular players that were discussed to have an impact of cross-presentation of cellular antigen and showed ways to identify novel molecules that might be involved.

In the course of this thesis we investigated different processes, which together are important for cross-presentation of antigens and cross-priming *in vivo*. Starting with the initial observation that mice can induce MHC class I restricted immune responses to an exogenous antigen, we tended to learn more about the cell types involved, the intracellular mechanisms of antigen delivery to MHC class I, the role of the antigenic nature, and finally the contribution of cellular factor that serve as adjuvants and mediators in the cross-presentation of cell-associated antigen. It is interesting to observe that the different antigen delivery system (PLGA-MS, DNA, recombinant VV, cell-associated antigen) all induce immune responses via very similar pathways.

There are “many roads to cross-presentation”, but the outcome is always MHC class I presentation (Groothuis and Neefjes 2005).

A complete understanding of the mechanism involved in XP is still a major challenge in the field of immunotherapy. However, only this will improve current vaccination protocols to induce efficient and long-lasting immune responses and immunological memory. The results presented in this thesis provide important contributions to this approach and might therefore help to further enhance the promising utilities of cross-presentation-based vaccines.

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Appendix

I. Abbreviations

α-2M	Alpha-2-macroglobuline
ADCs	Antigen donor cells
APCs	Antigen presenting cells
ATP	Adenosine-triphosphate
BATF	Basic leucine zipper transcription factor
BFA	Brefeldin A
BMDCs	Bone marrow-derived dendritic cells
CCL	CC chemokine
CCR	CC chemokine receptor
CD	Cluster of differentiation
CL	Clodronate liposomes
CPRG	Chlorophenolred-ß-D-galactopyranoside
CTLs	Cytotoxic T-lymphocytes
Cyt C	Cytochrome C
DNA	Deoxyribonucleic acid
DCs	Dendritic cells
DRiPs	Defective ribosomal products
DT	Diphtheria toxin
DTR	Diphtheria toxin receptor
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmatic reticulum
ERAD	Endoplasmatic reticulum-associated degradation
F1	First offspring generation
FACS	Fluorescence activated cell sorting
Fat10	HLF-F associated transcript 10
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
GFP	Green fluorescent protein
GP	Glycoprotein
HA	Hemagglutinin
HBV	Hepatitis B virus
HC	Heavy chain
HCV	Hepatitis C virus
HSF	Heat-shock factor
HSPs	Heat-shock proteins
HSV	Herpes simplex virus
ICS	Intracellular cytokine staining
IL	Interleukin

IFN	Interferon
kbp	Kilo base pairs
kDa	Kilo Dalton
KO	Knock-out
LAMP	Lysosomal-associated membrane protein
LCMV	Lymphocytic choriomeningitis virus
LCs	Langerhans cells
LPS	Lipopolysaccharide
LSECs	Liver sinusoidal epithelial cells
LSM	Fluorescent laser scanning microscopy
MCMV	Murine cytomegalovirus
MΦs	Macrophages
MHC	Major histocompartibility complex
MRI	Magnetic resonance imaging
MS	Microspheres
MVA	Modified virus Ankara
NADPH	Nicotinamide adenine dinucleotide phosphate
NC	Nano crystals
NP	Nucleoprotein
OVA	Ovalbumin
P/S	Penicillin/streptomycin
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PE	Phycoerythrin
pfu	Plaque forming unit
PLGA	Poly(lactic-co-glycolic)acid
pMΦs	Peritoneal macrophages
QDs	Quantum dots
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
SPIONs	Super-paramagnetic iron oxide nanoparticles
TAP	Transporter associated with antigen processing
TCR	T-cell receptor
TEM	Transmission electron microscopy
T_H	T_{Helper}-cells
TK	Thymidine kinase
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Ub	Ubiquitin
UV	Ultra-violet light
VDJ	variable-divers-joining
VV	Vaccinia virus
XP	Cross-presentation

II. Record of achievement / Eigenabgrenzung

Chapter I: I performed the results presented in figures 1, 2, 5, and 6. Experiments for Figures 3, 4, and 7 were performed by Michael Fehlings, Sabrina Engelhardt, and Chiara Redaelli, under my direct supervision. I wrote the entire manuscript.

Chapter II: I performed all experiments, except the images for figures 1A+B, which were taken by Constanze Schliehe. Electron microscopy images in figure 2 were taken by Joachim Hentschel. I wrote the entire manuscript.

Chapter III: I performed all experiments presented in this chapter and wrote the entire manuscript.

Chapter IV: I performed all experiments presented in this chapter and wrote the entire manuscript.

Acknowledgements

First of all I would like to thank Prof. Dr. Marcus Groettrup, who gave me the opportunity to perform this PhD thesis in his group. Thank you for the excellent and fruitful supervision of my thesis. Thank you for your constant interest in my project, for your motivation, and for giving me the space for my own ideas. I appreciate a lot the way you supported me, especially during the time when experimental difficulties required a reorientation of the project. Thank you for this great time.

I thank Prof. Dr. Thomas Brunner for his kind agreement to serve as reviewer of this thesis. Not less I would like to thank Dr. Martin Bachmann and Prof. Dr. Christof Hauck for having been members of my thesis committee.

I would like to thank Dr. Marc Müller, Dr. Michael Basler, Dr. Aleksandra Shypitsyna and Maxim Bothe for proofreading this thesis.

I thank the IRTG1331 for accepting me as a member and fellow of their graduate school. Especially, I appreciate a lot that I got the chance to organize the “First Inselsymposium on the roots of future biology” here in Konstanz, together with Dr. Jens Selige and other students of the graduate school.

I thank the Bioimaging Center of Konstanz University, especially Dr. Elisa Ferrando May for their great help with confocal microscopy and the pleasant working atmosphere. In line with this I would like to thank Dr. Joachim Hentschel for collaboration in electron microscopy. His knowledge and patience were essential components for succeeding in our project.

I would also like to thank Prof. Dr. Horst Weller and his group, especially Dr. Constanze Vaupel, for the fruitful collaboration and supply with inorganic nanocrystals.

Special thanks go to Dr. Michael Basler for constant immunological and technical support, fruitful discussions and help with mouse applications and animal work. I thank Ulrike Beck for constant support in the mouse house, in the lab, and for her

friendship during my theses. I thank Dr. Gunter Schmidke for support with radioactive work and cloning. Gerardo Alvarez I would like to thank for assistance in the lab, especially for introduction and help with electroporation of DNA vaccines. Last but not least, I would like to thank Dr. Marc Müller for his friendship and his great help in the microsphere projects.

I also thank my former diploma students Michael Fehlings and Sabrina Engelhardt for giving me the opportunity to test my supervising qualities and for much more.

I would like to thank the entire group of immunology Konstanz for the great working atmosphere. Especially Andrea for having a good time in sharing the room, Khalid for having so much fun in the lab, Tina for reminding me of the mountains and the infinity of travelling opportunities on a daily base, Elmar for his friendship in the lab and for training sessions, Annette for fruitful discussions and some comedy, Neha for hindi lessons, Stef for taking over my lab jobs during writing, and all the other members for making it fun to come to the lab every day.

Special thanks go to my family, especially to my parents. From the very beginning I felt furthered in whatever I did. Thank you for always believing in me, for your interest in what I am doing and for your patience. Thank you for being there for me!

Finally I would like to thank my girlfriend Amalie who I fortunately met at the beginning of my thesis. Thank you for your support and love!