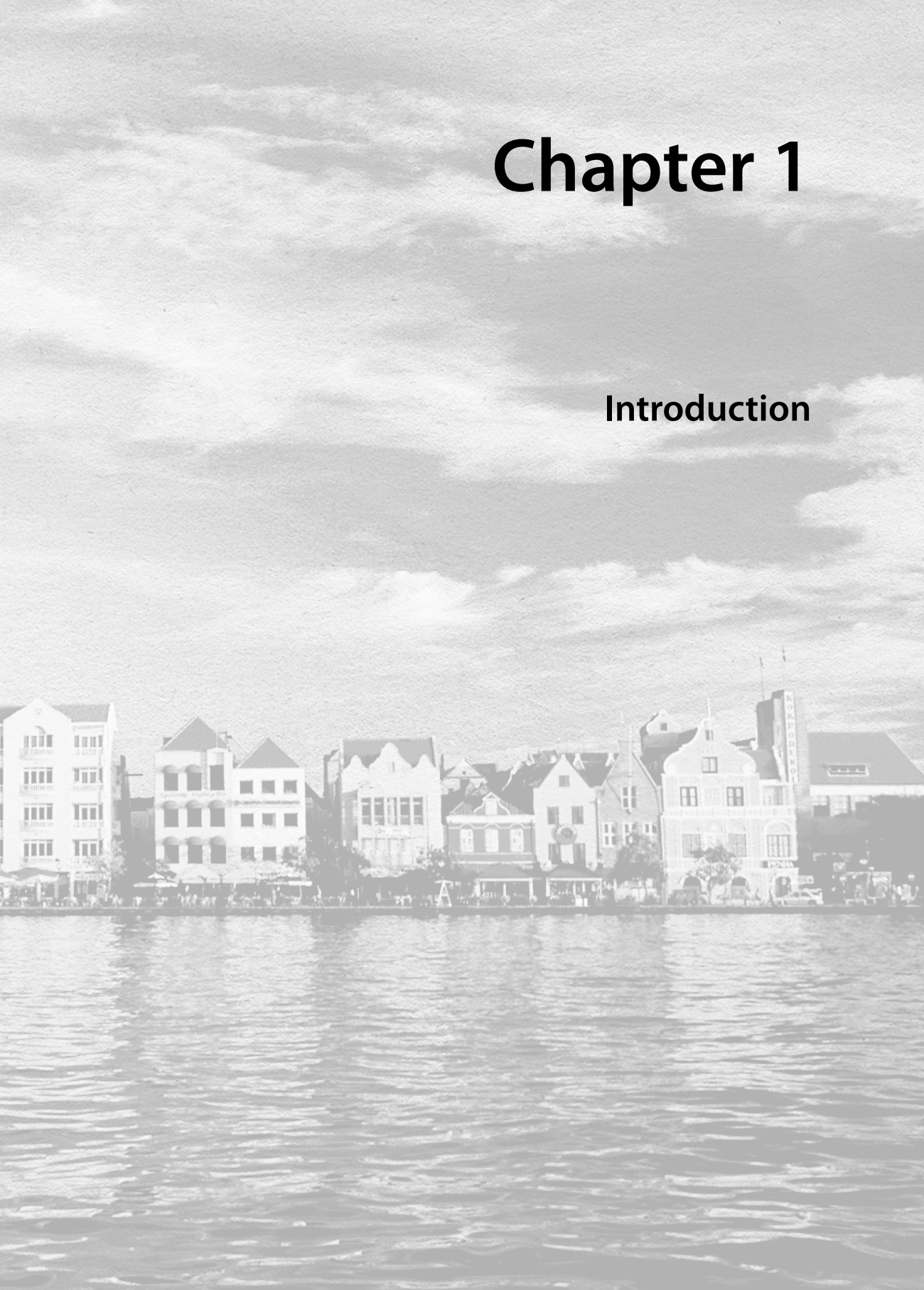
Chapter 1

Introduction



1. The immune system; basic mechanisms and function

The immune system constitutes a network of specialized bone marrow derived cells which detect, isolate and eradicate potential harmful microorganisms or malignant cells. It consists of two arms; the innate and the adaptive immune system which collectively protect the human body from pathogens [1,2]. Communication between cells of the immune system and other non-immune cells proceeds via cell-surface and secreted signaling molecules [3-5] produced in response to the detection of danger signals [5,6].

The innate immune system is activated after the detection of danger signals, for example an invading pathogen. The innate immune system comprises immune cells that can rapidly engage and elicit their effector functions forming the first line of defense. Innate immune cells are characterized by their antigen (Ag) non-specific effector functions, and lack of immunological memory. Innate immune cells recognize genetically conserved patterns expressed on non-self- and altered self-tissues [7-9].

Failure of the innate immune system to eliminate an invading pathogen leads to the activation of a more "tailor-made" immune defense mechanism; the adaptive immune system. Initial encounter of the adaptive immune system with a potentially harmful pathogen is characterized by a reaction time of 3 – 7 days. During this period, pathogen-specific lymphoid cells; B cells, $CD4^+$ and $CD8^+$ T cells are primed (activated). Upon priming, B and T cells vigorously multiply (proliferate) and exert their Ag-specific effector functions. The adaptive immune system can form immunological memory resulting in a rapid, within hours, secondary response which efficiently clears the pathogen upon reinfection with the same pathogen. For instance, the long lived protection against measles is based on the formation of immunological memory against the virus after original infection and clearance.

Some aspects of the immune system and vaccination will be briefly introduced in the following paragraphs which will facilitate the reader to interpret the research data described in this thesis.

2. First line of defense; the innate immune system and the detection of "danger"

Micro-organisms encode and express various molecular motifs, pathogen-associated molecular pattern (PAMP), crucial for their pathogenicity [2,10,11], such as DNA/RNA and/

or glycosylated proteins and lipids. The innate immune system has evolved to recognize these molecular motifs as danger signals and thus alarming for the presence of an invading pathogen.

Recognition of PAMP proceeds via several intra- and extracellular genetically conserved pathogen recognition receptors (PRRs). An important group of PRRs are the Toll-like receptors (TLRs). Ligation of TLRs results in intracellular signaling cascades and ultimately, cell activation and expression of cell-surface co-stimulatory molecules, chemokines and cytokines that signal to other cells in their environment, initiating inflammation [4,12,13].

Phagocytic myeloid cells form a major subset of the innate immune system, they are distributed throughout the body and participate in the surveillance of (non-)lymphoid tissues for the presence of invading microbiological threats. Phagocytic cells continuously engulf, process and "analyze" the content for possible PAMPs. The majority of phagocytic cells are formed by monocytes and macrophages (MΦ). The latter are also referred to as "scavenger cells and have an important role in the clearance of cellular debris, apoptotic bodies and pathogens from the body [14]. MΦ are specially equipped for this purpose as they efficiently translocate engulfed Ag into intracellular degradation compartments.

Monocytes are subdivided into two subsets based on their functional properties, the first subset, the "patrolling monocytes" have a special role in tissue repair and wound healing. The second subset are the so called "inflammatory monocytes" which produce tumor-necrosis factor and inducible nitric oxide synthase during infections [15]. Moncytes are also thought to contribute to an immune response by differentiating into macrophage- or dendritic cell (DC) like effector cells [15,16]. The final differentiation of monocytes is largely dependent on the type of danger signal detected.

Dendritic cells form a small percentage of the phagocytic cell population, 5–15%, but DCs are arguably the most important cell type of the innate immune arm as they link the innate and adaptive immune response. DCs and their functions will be described in more detail in paragraph 2.1.

2.1 Dendritic cells; linking innate and adaptive immunity

DCs function as the gate-keepers of the immune system [17-19]. DCs are strategically located throughout the body at sites where contact with "non-self" material is the most frequent, such as the skin and mucosal lining of the lungs and the gut. DCs use their extensive

arsenal of PRRs to detect invading pathogens and have a pivotal role during the onset and control of immune responses (Figure 1.1).

TLRs contain transmembrane signaling motifs and their ligands triggers intracellular signaling cascades which regulates among others NF-KB gene-transcription, important for cell activation. Ultimately, these signaling cascades result in the transformation of DCs into fully competent Ag presentation cells (APC), a process termed "DC maturation" (Figure 1.1). DC maturation is characterized by efficient processing of internalized exogenous Ag

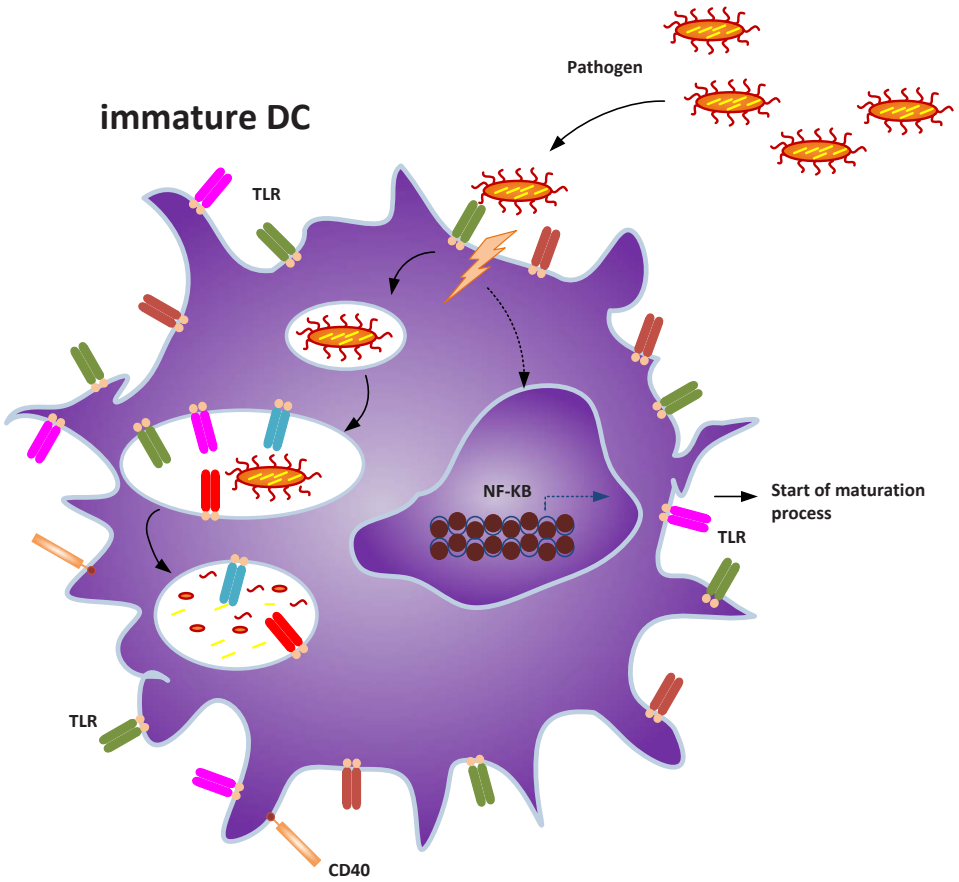


Figure 1.1 Immature DC encounters a pathogen and becomes activated.
Invading pathogens express molecular patterns which are recognized by DC via their TLRs expressed on the cell surface or inside intracellular compartments. TLR triggering activates intracellular signaling pathways which culminate in the NF-KB transcription and the initiation of DC maturation. The engulfed pathogens are translocated inside intracellular compartments, phagosomes, where they are killed and degraded.

and presentation in the context of major histocompatibility class (MHC) class I and class II molecules, increased expression of T co-stimulatory molecules and secretion of pro-inflammatory cytokines. These changes endow DC with the superior capacity to prime naïve and T cells (Figure 1.2).

At the site of inflammation, DCs internalize pathogen-derived material present in the extracellular environment. In parallel, PAMPs are recognized as danger signals and initiate DC maturation. Mature DCs express the lymph node homing chemokine receptor, CCR7, permitting migration from the infection site towards the lymph nodes (LNs). In the LN the mature DC encounter T cells [20].

Stimulation of T cells by DCs is the first step in the activation of the adaptive immune system. In summary, DCs dictate the breadth and potency of an immune response via their capacity to activate the adaptive immune arm when the innate immune arm is incapable of clearing the disease causing entity. DCs play a critical role in balancing an ensuing response; a weak immune-response leaves the body vulnerable to the pathogen but an excessive immunological response can result in epitope spreading [21-25] which might cause damage to healthy tissues of the host [26,27]. Systemic Lupus Erythematosus (SLE) is a well-known disease resulting from excessive stimulation of auto-reactive T cells by DC presenting self-Ag derived from apoptotic cells [28].

3. The adaptive immunity; "acquired, antigen specific" effector functions

Adaptive immune responses can be sub-divided into a humoral response, carried out by B cells and type 2 $CD4^+$ T cells, and cellular response, carried out by type 1 $CD4^+$ and $CD8^+$ T cells. In contrast to immune cells of the innate immune system, B and T cells are characterized by their Ag-specific effector functions.

The nearly unlimited different Ag-specificities and different degree of affinity of the T-cell receptors (TCRs) T cells are a product of the enormous diversity in possible V(D)J gene-rearrangements at the chromosomal loci encoding the TCR [29]. T cells undergo positive and negative selection in the thymus. In the first round, T cells are screened which can successfully recognize self MHC class I molecules; positive selection. T cells failing to recognize MHC class I molecules are deleted. In a second screening, T cells are selected based on the affinity of their TCR for its specific epitope presented in MHC class I molecules.

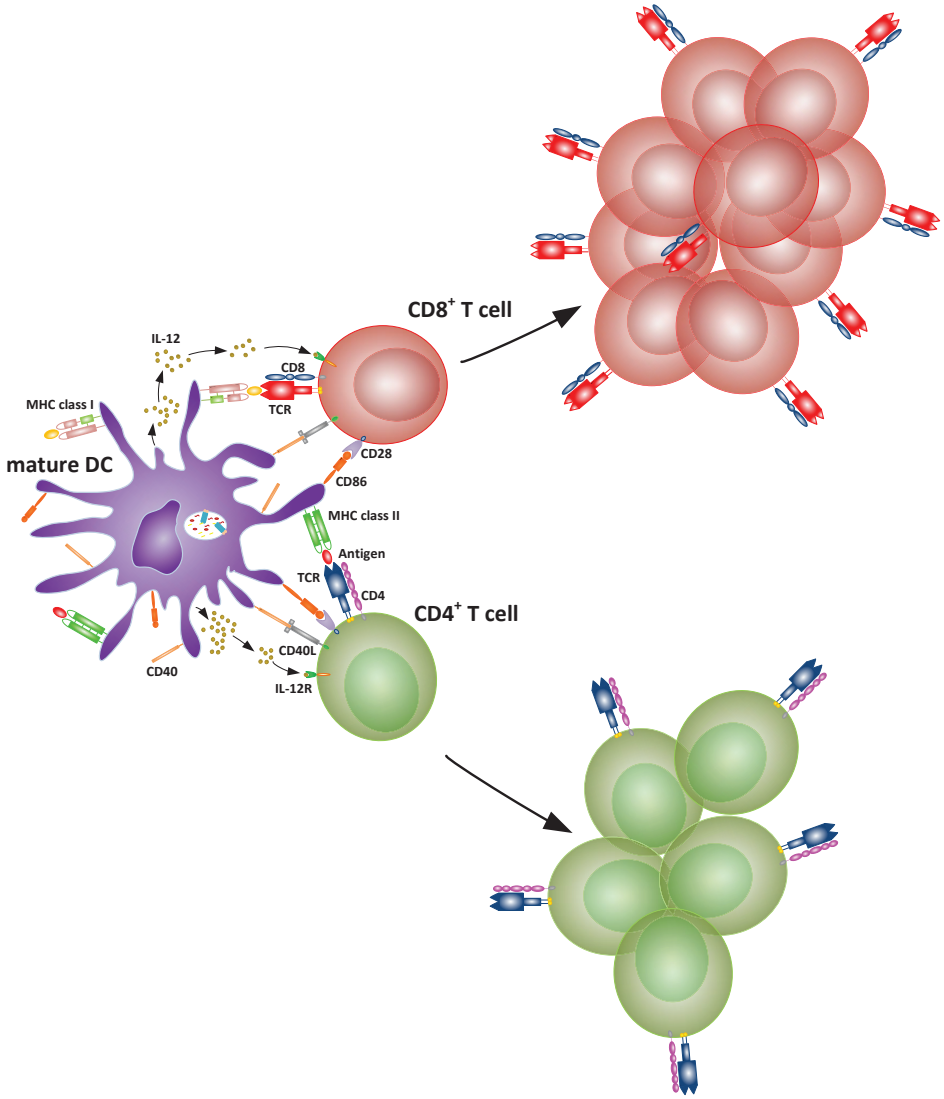


Figure 1.2 Mature DC prime and activate naïve T cells.

DC maturation leads to the up regulation of co-stimulatory molecules and production of cytokines important for an efficient activation of T cells. Mature DC acquires potent antigen processing and presentation capacity. Pathogen or vaccine specific epitopes are processed and presented in the context of MHC class I ($CD8^+$ T cells) or II molecules ($CD4^+$ T cells). The expression of the co-stimulatory molecules CD40, CD80 and CD86 on DC facilitates T cell activation and proliferation via the ligation of T cell expressed molecules, CD154 and CD28. In addition, IL-12 production by DC programs T cells to acquire a type 1 pro-inflammatory phenotype, characterized by high IL-2, IFN-γ and TNF-α production.

In this process, T cells showing a supra-threshold affinity TCR are deleted; negative selection. Negative selection is important for "central tolerance" and functions to prevent the release of high-affinity self-reactive T cells from the thymus into the periphery where they can cause autoimmunity [30-32].

B cell receptors (BCRs) are membrane-bound immunoglobulins which recognize conformational epitopes which can be derived from various Ags, such as protein, polysaccharides, lipids and nucleic acids. BCRs are produced in process similar to TCRs, based on the V(D)J rearrangements [21,33]. Every B cell will express on its cell-surface BCRs with a single Ag-specificity. Ligation of BCRs initiates B cell maturation into plasma cells which secrete high amounts of soluble BCRs; antibodies [34]. Somatic hypermutation (SHM); a process whereby the total antibody avidity to a specific Ag is increased by "affinity maturation" of the genes encoding the Ag-specific BCR and by selection of higher-affinity B cell clones, regulates the efficiency of B cell responses. The B cells with the high(er) affinity BCR will out-compete the low(er) affinity B cells for the specific Ag resulting in apoptosis of the "weak" B cells. The net result is the induction of an Ag-specific high avidity antibody response through the activation of the selected high affinity B cells [21]. Secreted antibodies have two distinct functions 1) bind specifically to pathogens or their toxins, neutralizing the pathogen and inhibit their capacity to infect cells and 2) recruitment off- and signaling to other immune cells to target, engulf and kill the invading pathogen after binding by the antibody; antibody dependent cell cytotoxicity (ADCC).

TCRs, in contrast to BCRs, are expressed only as membrane-bound molecules. TCR-triggering via its specific epitope stimulates intracellular signaling cascades leading to T cell activation. TCR differ from BCR in an important way: TCR recognizes linear epitopes of proteins, lipids or glycolipids; short (peptide) fragments derived from pathogen-associated- or tumor associated Ag in the context of classical MHC [35].

3.1 Immune activation to self or non-self Ag by signals of danger

The danger-model proposed by Matzinger et al. [36-38] is an alternative theory to the original self-non self-theory set forward by Burnet et al who stated that an immune response can be explicitly be mounted to only "foreign" in other words, non-self-entities. In contrast, Matzinger's theory proposes that the immune system is also possible to against self-entities as long as there is a sense of "danger" present. Both theories offer plausible explanations for the activation of the immune system, however, both theories obviously have some

limitations [39]. Most cancers express self-Ag; in cancer patients the cancerous cells perhaps fail to trigger an adequate immune response because the (pre-)malignant lesions fail to present an imminent and acute sense of danger to the body. Considering Matzinger's danger theory from the viewpoint of vaccinology, it does offer an important basis for the use of adjuvants to enhance potency and efficacy cancer vaccines. Namely, adjuvants based on synthetic, well-defined small-molecular compounds mimicking PAMPs [22,24,25]. The addition of an adjuvant, will cause an acute sense of "danger" as the immune system will be tricked that a harmful pathogen is present in the body and thus prime (or boost) a cancer vaccine-specific immune response.

Cancer immunotherapy based on vaccination will be discussed in more detail in the following paragraphs.

4. Ag uptake, processing and presentation to T cells by DC

DC and other myeloid cells, for example MΦ, are very efficient phagocytic cells and possess multiple endocytic mechanisms allowing internalization of vast amounts of exogenous materials for intracellular processing. DC and MΦ have several shared characteristics [40,41] However DC differ from MΦ as they mainly contain intracellular compartments dedicated for Ag-processing [42] and Ag-storage [43] but less well equipped for Ag-degradation [43,44]. MΦ on the contrary contain mainly intracellular compartments specialized for Ag-degradation.

Internalized Ag is cleaved, trimmed and processed in a controlled manner by various proteases present inside endo-lysosomes and the cytosol [45-48]. DCs are specialized Ag presentation in MHC class II molecules, which are recognized by $CD4^+$ T cells (Figure 1.3), and MHC class I molecules, which are recognized by $CD8^+$ T cells [49]. MHC class I Ag presentation of exogenous material is known as Ag cross-presentation [46] (Figure 1.4).

Classically, exogenous materials were postulated to be processed only into MHC class II molecules whereas MHC class I molecules existed to present solely endogenous, self, produced proteins. These two processing pathways were described to function fully independent of each other. However it is now known that MHC class I and II Ag presentation consist of overlapping processing pathways [44,46,49]. MHC class I Ag cross-presentation is a crucial pathway by which the immune system can detect and respond to bacterial, viral and parasitic infections that exclusively invade non-hematopoietic cells or reside in extracellular environments. Notably, MHC class I Ag cross-presentation is the primary mechanism how

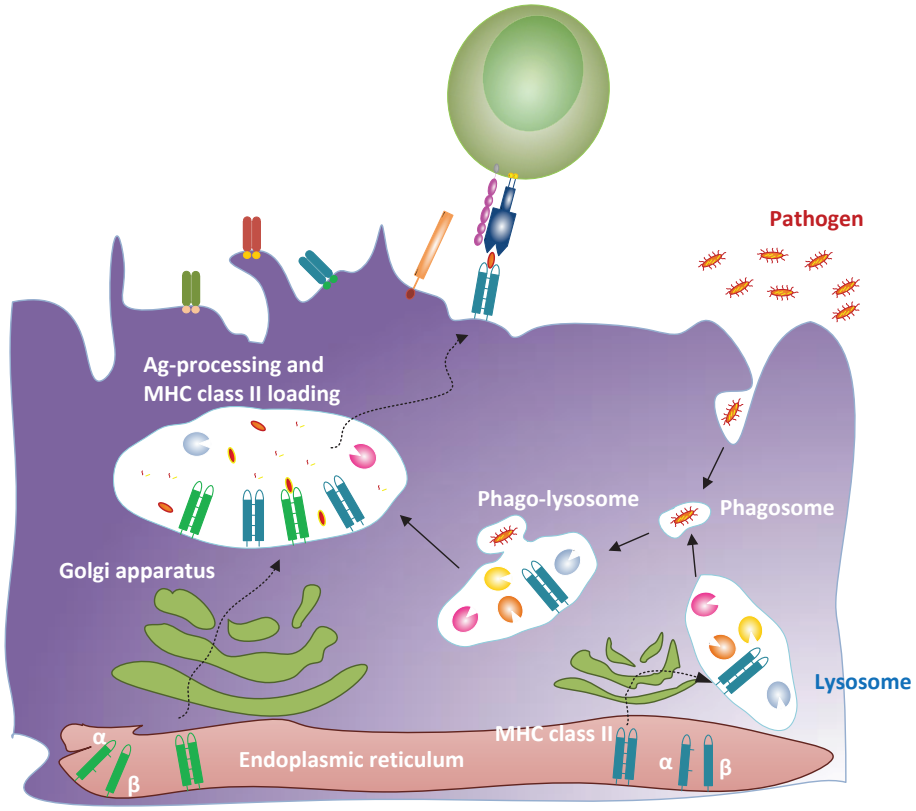


Figure 1.3 MHC class II processing and presentation of Ag by DC.
Exogenous antigens are internalized by DC inside phagosomes, or alternatively endosomes. Lysosomes inside DC, which are acidic intracellular compartments containing pH-sensitive proteases, so called cathepsins, fuse with phagosomes or endosomes. This fusion is also referred to as endo- or phagosomal maturation. This process is characterized by pH drop inside these compartments, thereby activating the cathepsins. The Ag content is degraded of into smaller peptide strands 12–20 aminoacid, epitopes. MHC class II molecules are assembled inside the endoplasmatic reticulum (ER) and translocated via the Colgi apparatus into MHC class II loading compartments. In these compartments, the epitopes are loaded on MHC class II molecules and transported to the cell-surface where they are recognized by $CD4^+$ T cells.

$CD8^+$ T cells are primed against tumor-associated Ags which are otherwise only presented on malignant cells [50-52].

DCs are the principal APC endowed with the capacity to cross-present Ag into MHC class I molecules. Depending on the type of Ag, DCs use phagocytosis, pinocytosis, fluid-

phase endocytosis and receptor-mediated endocytosis for Ag-internalization. It has been suggested that the mechanism of Ag-internalization dictates how the Ag is processed and presented by DC on MHC class I and II molecules [53].

4.1 Ag-processing by DC; mechanisms of MHC class I Ag cross-presentation

Several MHC class I Ag cross-presentation processing pathways have been reported [52,54,55]. For simplicity these pathways can be grouped in two principal pathways, commonly referred to as the *classical/cytosolic* (Figure 1.4) and an *endosomal/vacuolar* pathway.

Ag routed via the classical pathway is processed through similar mechanisms as endogenous self-protein Ag, mediated mainly by the proteasome, located in the cytosol. This suggests that internalized exogenous material must access the cytosol from the endosomes, become ubiquitinated and transferred to the proteasome system. The mechanisms involving the translocation of an Ag into the cytosol remains a matter of debate and extensive studies and is most likely influenced by the type of Ag. Proteasome-cleaved peptides are then transported into the Endoplasmic Reticulum (ER) by the transporter associated with antigen processing (TAP) for loading on newly formed MHC class I molecules (Figure 1.4). The majority of MHC class I epitopes are loaded on MHC class I molecules inside the ER. However, there is no firm evidence that peptide loading on MHC class I molecules occurs exclusively in the ER. Therefore, the cytosolic pathway of MHC class I Ag cross-presentation refers primarily to the intracellular location where exogenous Ag is processed, the cytosol, without taking into account the compartment where the loading of MHC class I molecules occurs.

The "endosomal/vacuolar" pathway is generally independent of proteasome activity and TAP-mediated transfer of cleaved peptides into the ER. However, Ag-processing through the endosomal/vacuolar pathway, is sensitive to endo-lysosomal proteases, such as cathepsins [48], and dependent on the pH-environment inside endo-lysosomes. The key factor distinguishing the two cross-presentation pathways is thus whether the internalized exogenous material is translocated from the endolysomes to the cytosol for processing or not [46].

5. Cancer

Cancer is the collective name given to more than 100 neoplastic diseases, which are characterized by uncontrolled growth of malignant cells, their subsequent metastasis and invasion of healthy tissues impairing their normal functioning. The development of cancer

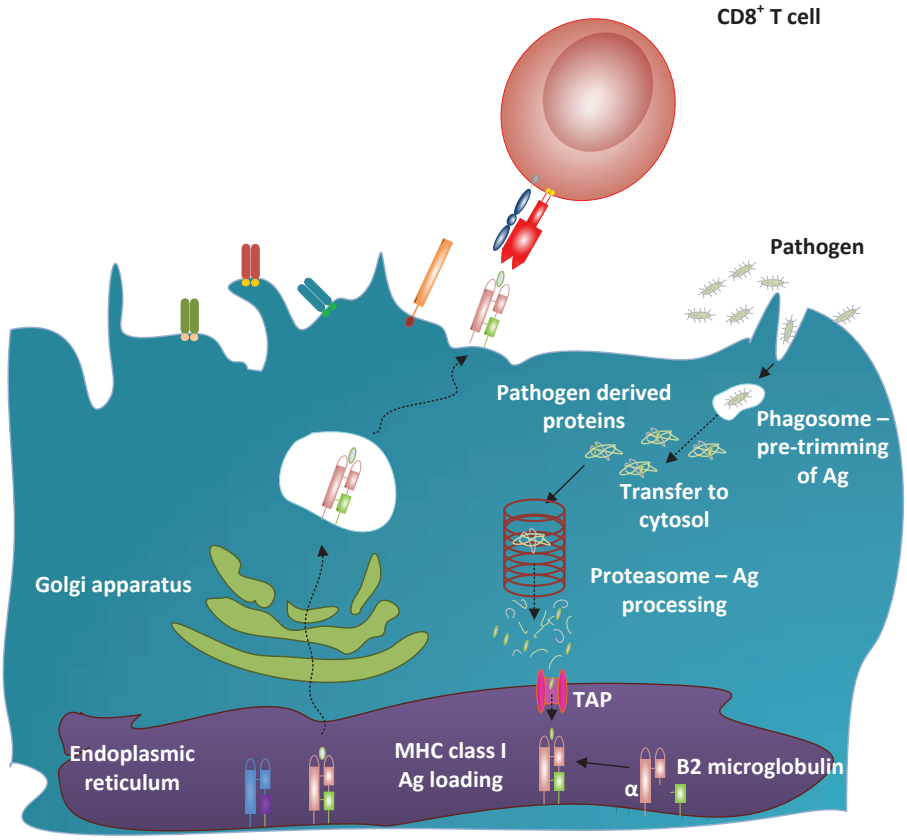


Figure 1.4 MHC class I Ag (cross-)presentation by DC.
Exogenous Ag engulfed by DC are present inside phagosomes or endosomes (Figure 1.3). The Ag content is translocated from these compartments into the cytosol by yet unknown mechanisms. Inside the cytosol, Ag-derived proteins are degraded by the cytosolic protease, the proteasome, into short 8–9 aminoacids peptide strands. The transporter associated with antigen processing (TAP) next transfers the peptide strands from the cytosol into the ER where MHC class molecules are assembled and loaded with their specific epitopes. The loaded MHC class I molecules are then transported via the Colgi apparatus to the cell surface where CD8+ T cells are able to triggered via the TCR.

is a multistep process originating from DNA mutations in oncogenes or tumor-suppressor genes and, importantly, failure to repair mutated damaged DNA sequences. Malignant transformation and DNA mutations can be caused by both exogenous and endogenous triggers; carcinogens [56-58]. Succeeding DNA mutations malignant cells acquire various

hallmarks of cancer; continuous proliferative signaling, insensitivity to growth suppressors, resistance of apoptosis, activation of replicative immortality, induction of angiogenesis, and activation of metastasis invasion of other organs [59,60]. Cancer can partially be designated an immunological disease, already at the initial stages of carcinogenesis, (pre-)malignant lesions and the immune system are involved in a two way battle. The immune system is able to recognize tumors as implied by 1) rejection of experimental tumors 2) increased carcinogenesis in immunodeficient animals and/or 3) increased incidence of some cancers in immunodeficient patients and in the elderly. A strong evidence for potent tumor-specific immunity is provided by studies on cancer patients with paraneoplastic syndrome. For example, oncoproteins of neural origin can in some cases of breast and ovarian cancer be expressed by the tumor. In healthy individuals these (onco)proteins are expressed only in immune-privileged sites, such as neurons. However, in these cancer patients, a strong $CD8^+$ T-cell response is generated which effectively controls tumor growth but also induces severe auto-immune neurological diseases. Thus, in cancer patients despite the induction of a tumor specific immune response, the tumor is not controlled and grows out; tumor escape. Mechanisms resulting in tumor escape are many. DNA mutations does not only modulate oncogenes and tumor suppressor genes but also facilitate carcinogenesis by driving tumor promoting inflammation [61,62], angiogenesis [63,64] and induction of local immune suppression via the attraction/induction of T regulatory cells [65,66] and myeloid derived suppressor cells (MDSCs) [67]. Several other factors have been attributed to the overall lack of a potent anti-tumor response in cancer patients [68-70] and combined these factors lead to a weak immunogenic tumor microenvironment allowing escape of the tumor from surveillance by the immune system.

5.1 Cancer; disease prevalence in the Netherlands

In the Netherlands, 100,600 new cases of cancer were diagnosed in 2011. This number is 4% higher than the previous year, 96,500. Skin cancer is the most common with 14,400 cases followed by breast cancer (14,100), colorectal cancer (13,300), lung & tracheal cancer (11,700) and 11,400 cases of prostate cancer. The stepest rise was seen with skin cancer with 1,500 new cases in 2011 compared to 2010. The expectation is that an annual increase of 3% in total cancer cases will be evident for the next ten years

Life expectancy of cancer patients has increased approximately with 3 years in the past decade. In general, the longer people live the higher their chances of being diagnosed

with cancer. Another factor contributing to the rise of cancer prevalence is the change in daily activities. For example, more women reported being a regular smoker, a habit which most likely is the cause the increase of lung cancer among women. Nowadays, the chance of getting cancer for women is 1 in 3, was 1 in 4, and for men 1 in 2, was 1 in 3. This clear increase is most evident in patients of 85 years and older. Unhealthy diets, alcohol consumption and lack of physical exercise have also been related to the increase in cancer.

In 2011, 43,139 persons in the Netherlands died from cancer or related complications. That is 42% of the the total cancer cases in the same year. Thus, it is very clear that better treatment modalities are required (Dutch Association of Comprehensive Cancer Centers).

6. Vaccines

The use of classical prophylactic vaccines dates back to the late 18th century when it was shown by Jenner and others that humans could be protected against small-pox by cross-immunity against cow-pox after inoculation with pus from cow-pox blisters. This important observation initiated the field of vaccine development. Although Jenner successfully induced immunity and protection in his patients, he was not aware of the entity causing this protection. Koch et al. then showed that infectious diseases are caused by pathogenic microorganisms, each one responsible for a particular disease. These findings led to the culture of artificially weakened strains of virulent pathogens by Pasteur, which were then used as vaccines against rabies and anthrax.

Immunostimulatory agents, adjuvants, were introduced in the 20th century by Gaston Ramon. "Adjuvant" is derived from the latin word adjuvare (translation "to help"). An adjuvant potentiates the working of a vaccine by hyperactivation of the immune system. The use of aluminum salts based adjuvants (alum) were one of the first to be applied in the modern era to boost immune responses elicited by prophylactic vaccines. Alum remained for decades the only clinically approved adjuvant for human use. Alum effectively enhances Type 2 (T_H2) humoral responses, prolongs antibody production and promotes the formation of memory B cells. Nowadays there are other clinically approved adjuvants based on water-in-oil (w/o) emulsions such as MF59^{TM} (Novartis) and the adjuvant systems (AS) marketed by Glaxo-Smith-Kline. These adjuvants are used primarily as agents to enhance the efficacy of prophylactic vaccines which is based on the induction of neutralizing antibodies.

Although tumors do stimulate humoral responses and the production of tumor-specific antibodies with cytotoxic effects [61,62,71], tumor cell killing is primarily achieved through the mechanisms of the cellular immune system, in other words T cells. For the purpose of tumor specific vaccination, therapies are required to boost not only the antibody response but more importantly the tumor specific T cell response.

7. Cancer immunotherapy

The natural capacity of the body`s own immune system to recognize and eradicate cancers allows the possibility for treatments which enhances anti-tumor effector mechanisms, cancer immunotherapy. The need for new treatments against cancer is direct consequence of the critical challenges imposed by conventional treatments against cancers, such as surgery, chemotherapy and radiotherapy. Their clinical efficacy is poor and causes significant adverse effects in treated individuals. There is a high requirement for a more personal, tumor-specific and efficacious cancer therapy with considerably lower treatment-related adverse effects.

Significant improvements in immunology have provided greater understanding of the interactions between malignant and immune cells. It is now well accepted that avoiding destruction by the immune system is a hallmark of cancer [60]. This knowledge also allows the development of novel strategies and medical interventions aiming to boost the immune system against a growing tumor. Several cancer immunotherapies have been successfully devised which are currently undergoing (pre-)clinical testing or have already been approved for standard 1st line therapy. These include the enhancement of B cell responses [72-75], antibody-based cancer immunotherapy [71,76-78], adoptive cell transfer of cytotoxic $CD8^+$ T cells [79-82], DC based vaccines [83-85], inhibitors of immune checkpoint blockade, such as the FDA approved anti-CTLA4 mAb, YERVOY® (Ipilumimab) [86-89] or cancer vaccines based on proteins [90,91], short peptides encoding minimal CTL epitopes [92-96] or the main focus of the tumor immunology group at LUMC, long peptide vaccines [97-102].

Vaccination against cancer represents a promising treatment modality and is based on the principle of activating or boosting specific T cell responses against a tumor-associated Ag (TAA). From the pharmaceutical point of view; vaccinations with (long) peptides offers the possibility of having an "off the shelf" product which can be manufactured in large numbers and under GMP-conditions. More and more TAA are being discovered and described [103-106] allowing the production of long peptide vaccines for these targets.

7.1 Cancer immunotherapy; therapeutic vaccines

Successful cancer immunotherapy requires a strong pro-inflammatory Type 1 (TH1) $CD4^+$ and $CD8^+$ T cell responses. Advances in molecular immunology have led to the development of a broad range of novel synthetic adjuvants that are currently being explored in clinical trials in combination with vaccines [60,107-109]. Adjuvants, such as synthetic TLR agonists mimic PAMPs expressed by pathogen resulting in immune activation of the immune system. The Ag-composition of vaccines themselves have also undergone considerable developments; from completely undefined material such as pus from cow-pox blisters, modern immunologists aim to vaccinate with precisely defined Ag, from DNA-sequences, protein or peptides, encoding or representing a specific pathogen-associated or tumor-associated Ag (TAA). Therapeutic cancer vaccines aim to successfully activate or boost an effective anti-tumor T cell immune response. DC hold the key to this process, thus the main objective of vaccination regimens against cancer should be the specific and efficient delivery of the vaccine, encoding a TAA, to DC.

7.2 Cancer Immunotherapy; soluble Ag vaccines – pros and cons

Historically, protein and/or peptides in their soluble, native, form were the first vaccine Ag candidates tested in pre-clinical experimental tumor models or in the clinic. These vaccines have led to promising observations of enhanced tumor-specific T cells responses [110-112]. Nevertheless, in most, if not all, clinical trials, soluble protein and peptide vaccines have failed to induce complete and durable responses in cancer patients despite increasing tumor immunity.

Regarding soluble protein vaccines, it's suggested that their capacity to boost the $CD8^+$ T-cell repertoire against a tumor to be rather poor [113-115]. Efficient anti-tumor immune responses require potent cytotoxic $CD8^+$ T cell responses to achieve the desired clinical benefit.

Synthetic short-peptide (SSP) vaccines, encoding minimal MHC class I molecule binding epitopes on the other hand considerably boost the $CD8^+$ T cell tumor immunity which translated into improved clinical responses. But vaccinations with SSP are associated with significant limitations on the long term [116-119]. SSP-vaccines do not directly stimulate $CD4^+$ T cell responses. It is well known that the co-activation or $CD4^+$ T cells is crucial in all aspects of $CD8^+$ T cell responses and plays an important role during the priming, effector and memory phase $CD8^+$ T cells [120-125]. Thus when SSP are used as vaccines, the ensuing $CD8^+$ T cell responses are short-lived [116] and of sub-optimal potency. Other restrictions

related to the use of SSP vaccines are the necessity for HLA-typing for each patient to be treated and tolerance induction due to SSP presentation by non-professional APC [116]. Another disadvantage of SSP is the short-lived *in vitro* Ag presentation in comparison to SLP [126] which, next to SSP loading on non-professional APC might underlie the vanishing $CD8^+$ T-cell responses observed *in vivo* post-vaccination [116].

The concept of synthetic long peptide (SLP) vaccines was introduced by Melief et al. [114,115,127], as way to improve the efficacy of peptide vaccines. The SLPs are overlapping synthetic peptides of 15–35 amino acids that 1) cover the entire sequence of the native protein TAA to which an immune response is targeted to, 2) SLP require DC-specific internalization and processing for optimal presentation in MHC class I and class II molecules and 3) do not require HLA-typing as ingestion by DC of overlapping strands of peptides allows epitope selection *in vivo* based on the patient's own HLA-profile 4) facilitates simultaneous priming of T-cells against multiple dominant and subdominant epitopes stimulating a broad T-cell response [114]. Therapeutic vaccinations with SLP encoding the E6 and E7 oncoproteins of high risk HPV16 successfully boosts $CD4^+$ and $CD8^+$ T-cell responses in pre-clinical murine models of cervical cancer and in patients with (pre-)malignant disease of the cervix and the vulva [128,129]. SLP vaccines have also been used against other types of cancers [119,130-132] and against other immunological diseases [133,134]. In a direct comparison, SLP vaccines were more efficient in inducing $CD8^+$ T-cell responses than protein vaccines [119] and lead to stronger and more effective Ag-specific immune responses.

The positive effect on the anti-tumor responses and resulting clinical benefits are well described for SLP vaccines. But still, soluble SLP vaccines carry some disadvantages especially related to the method of administration. Montanide-based water-in-oil (w/o) emulsions are mostly used to formulate SLP vaccines, but also protein vaccines, for administration to patients enrolled in clinical trials [133-138]. Montanide w/o emulsions function as an Ag-depot and triggers inflammation at the site of injection. However, the properties of Montanide which cause inflammation are poorly described. In addition, the use of w/o formulations cause significant local side effects in treated patients because of their non-biocompatible/non-biodegradable properties. Moreover, unpredictable Ag release rates and lack of long-term stability of the w/o emulsions limit pharmaceutical scalability [128,129,139].

Besides the disadvantages related to the delivery system, once released from the w/o emulsion based Ag-depot, SLPs are rapidly cleared via the kidney from the body [140,141] because of their typically small size of ≤ 5 kD. As a result, injected SLPs are inefficiently

target to- and internalized by DCs when administered s.c. or i.d. *in vivo*. Thus alternative methods to deliver SLP vaccines are highly required. Particulate vaccine carriers prepared from bio-degradable, biocompatible polymers offer a suitable substitute for Montanide or other w/o emulsions due to their relative ease of pharmaceutical formulation and immunological properties.

7.3 Cancer immunotherapy; particulate vaccine carriers based on PLGA-nanoparticles

To date, many particulate vaccine carriers have been successfully formulated from various types of biocompatible polymers [142-150]. These resulting "particulate vaccines" boosts Ag-specific humoral and cellular responses with higher efficiency compared to soluble vaccines. Their method of action is for a large part based on facilitated uptake of particulate Ag by APCs compared to soluble protein- and/or peptide Ag. From a cancer therapy perspective, one would desire to develop particulate carriers, carrying TAA that can efficiently target DC, either actively or passively, promote Ag processing and MHC class I and II presentation and finally generate of potent immune responses capable of tumor control [151-154].

Biodegradable particulate vaccine carriers prepared with the polymer Poly-(Lactic-co-Glycolic-acid) (PLGA) have yielded positive results as a carrier for various types of Ag, from DNA, proteins to peptides [152-158]. The use of PLGA-nanoparticles (PLGA-NP) offer some unique advantages over the administration of the soluble vaccine-Ag encoding TAA or the use of W/O based delivery vehicles; these include 1) PLGA is an FDA approved polymer 2) protection of the Ag cargo from premature degradation, 3) encapsulation of Ag in NP increases the total size of the vaccine and slows renal clearance, 4) enhanced uptake of the Ag by DC. 5) PLGA-NP makes it possible to accommodate both Ag and adjuvant in "one" particle to create a single immune activating "pathogen-like entity" and finally 6) PLGA-NP immunogenicity can be further modified by coupling of various ligands to- or surface coating of the NP to modulate the *in vivo* bio distribution and immune cell specific uptake of particles.

Owing to these favorable characteristics of PLGA-NP as vaccine delivery carriers and the crucial requirement to improve the immunogenicity of SLP-vaccines currently administered in Montanide a study was designed to assess several aspects of PLGA-NP as potential clinically applicable delivery vehicle/vaccine carrier for SLP-vaccines.

8. Scope of this thesis

Chapter 2 describes our studies exploring the mechanisms of long peptide-Ag-processing by DC. Understanding these mechanisms will allow further fine-tuning of SLP vaccines, with the goal to enhance their *in vivo* potency which may ultimately lead to improved treatment of cancer patients. We set out to enhance SLP-vaccine potency through the encapsulation in PLGA-NP.

In ***chapter 3*** we studied the feasibility to encapsulate SLP in PLGA-NP as a method to improve the immunogenicity of SLP. This study focused on the physical and formulation criteria necessary to successfully encapsulate SLP in PLGA-NP (PLGA-SLP). Subsequently, we studied the efficacy of cross-presentation by DC of PLGA-SLP in comparison to soluble SLP. We next studied the intracellular mechanisms used by DC to process PLGA-SLP in ***chapter 4*** and in addition describe the *in vivo* vaccine potency of PLGA-SLP in comparison to soluble SLP.

In ***chapter 5*** we report the application of PLGA-NP encapsulating protein Ag as a delivery vehicle to enhance DC-mediated stimulation of Ag-specific T cells *ex vivo* which could be used for adoptive T cell immunotherapy.

Because plain PLGA-particles have sub-optimal adjuvant properties *in vivo*, the optimization of PLGA-NP vaccines to achieve efficient anti-tumor responses is the topic of ***chapter 6*** in which nanoparticles and microparticles where studied in a head-to-head comparison in their capacity to activate B and T cell responses.

Chapter 7 continuous with the optimization of PLGA-NP vaccines where PLGA-NP vaccines were formulated co-encapsulating protein Ag and TLRL combined with active targeting of DC via CD40 molecules expressed on the cell-surface. In ***chapter 8*** a follow up study was performed to analyze different targeting strategies to enhance delivery of PLGA-NP encapsulated Ag to DC. For this purpose, PLGA-NP encapsulating TLRL and Ag were targeted to CD40 (a TNF-receptor family molecule), DEC-205 (a C-type lectin receptor) and CD11c (an integrin receptor).

Finally, in ***chapter 9*** we will discuss the most important findings described in this thesis and present a general overview. The contribution of the results to the further understanding of the immune system and the field of cancer vaccine development will put into context of known literature. Finally we will highlight the clinical relevancy of our findings and debate the future perspectives for particulate carriers as vehicles for SLP-vaccines.

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