Vaccination and targeted therapy using liposomes : opportunities for treatment of atherosclerosis and cancer

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General Introduction

The immune system protects the body from infections by pathogens, such as viruses, bacteria and parasites, and sustains homeostasis. The immune system is broadly grouped into two branches: innate immunity and adaptive immunity. On the one hand, efficient elimination of pathogens requires fast recognition and clearance (phagocytosis), which is performed by immune cells of the innate immune system, such as macrophages and dendritic cells (DCs), which sense pathogens using pattern recognition receptors (PRRs)[1]. On the other hand, the adaptive immune system is involved with removing pathogens at later stages, using T and B lymphocytes which can recognize antigens. In this way, the adaptive immune system has a slower response time, but is more specific than the innate immune system and importantly has the ability to form immunological memory[2]. Antigen-presenting cells (APCs), such as DCs and macrophages, are essential for the interaction between the innate and adaptive immune system. This is due to these cells' ability to process pathogen-derived antigens after phagocytosis, and present parts of these antigens in the form of peptides on human leukocyte antigen (HLA) molecules, or major histocompatibility complex (MHC) class I or II molecules in the mouse. An antigenic peptide presented on such a molecule is then recognized by a T cell receptor (TCR), triggering an immune response; MHC-I and MHC-II activate $CD8^+$ and $CD4^+$ T cells, respectively[3]. $CD8^+$ T cells can kill target cells, such as cancer cells and cells that are infected[4]. Upon activation, they secrete interferon (IFN)-γ and tumor necrosis factor (TNF)-α, which are pro-inflammatory molecules that maintain the inflammatory environment[5,6]. These cytokines, in turn, can stimulate other immune cells, such as APCs and $CD4^+$ T cells[5]. $CD4^+$ T cells, also known as T helper (Th) cells, are important for their ability to positively or negatively regulate the magnitude of immune responses by interactions with B cells, $CD8^+$ T cells, and APCs. There are several types of $CD4^+$ T cell subsets that can arise from naïve $CD4^+$ T cells (Th0) depending on the inflammatory environment, including Th1, Th2, Th17 and regulatory T cells (Tregs) (Table 1)[7]. Th1 cells express the transcription factor T-bet and are induced by IFN-γ, interleukin (IL)-12 and IL-18[8,9]. Th1 cells, in turn, can secrete IFN-γ, TNF-α, and IL-2[7]. IL-2 is an important cytokine for T cell differentiation, as it can interact with all other $CD4^+$ T cell subtypes[10]. Th1 cells can also activate $CD8^+$ T cells and stimulate macrophages to become pro-inflammatory[11], generating a pro-inflammatory feedback loop. Th2 cells are activated by IL-4 and IL-33 and express the transcription factor Gata-3[12]. They produce IL-4, IL-5, and IL-13[11]. IL-4 induces B-cell class switching to immunoglobulin (Ig)E and suppresses Th1 responses[13]. IL-13 has similar effects to IL-4[14]. IL-5 supports B cell differentiation and enhances proliferation of eosinophils[15]. Th2 cells are thereby involved in allergic responses[12]. Th17 cells play an important role in auto-immune diseases and are vital for defense against extracellular bacterial and fungal infections[7]. They express RORγT and are induced by IL-6, IL-21, and transforming growth factor (TGF)-β. IL-6 is a pro-inflammatory cytokine[16], while TGF-β has inhibitory effects on immune cells, such as T cells, B cells, and macrophages[17]. IL-21 is implicated in several autoimmune diseases[18]. Th17 cells secrete IL-17, IL-21, IL-22, and IL-25[19]. IL17 and IL-25 are pro-inflammatory cytokines that act on many different cell types to stimulate the immune response[18]. IL-21 initiates an autoamplification loop for Th17 differentiation[18]. IL-22 is important for protection against infections[18]. Tregs are also induced by TGF-β, but in the absence of IL-6. They express the transcription factor FoxP3[20]. Tregs are important for the resolution of inflammation after infection, immune suppression, and immune homeostasis[21]. They

do so by producing the anti-inflammatory cytokines IL-10 and TGF-β[20]. Since both $CD8^+$ and $CD4^+$ T cells are important in the immune response, many diseases such as infection, allergy, cancer and autoimmune diseases involve an over- or under-active T cell response. In this thesis, we focus on two such diseases; atherosclerosis and cancer, which are the number 1 and 2 causes of death in the western world, respectively[22,23].

Table 1: Signature cytokines and function of selected $CD4^+$ T cell subsets.

$CD4^+$ T cell subset	Signature cytokines	Function
Th1	IFN-γ, TNF, IL-2	Fighting intracellular pathogens, cell-mediated inflammation, enhancing proinflammatory cytokine production
Th2	IL-4, IL-5, IL-13	Fighting helminth parasites, antibody-mediated inflammation
Th17	IL-17, IL-22, IL-21, IL-25	Fighting bacterial and fungal infections, recruitment of other immune cells, autoimmunity, enhancing proinflammatory cytokine production
Treg	IL-10, TGF-β	Suppressing other immune cells, maintaining immune tolerance, suppressing autoreactive T cells

Auto-immune diseases are examples of an over-active immune response, such as in a classic autoimmune disease like rheumatoid arthritis, but also in atherosclerosis[24-26]. Atherosclerosis is the predominant underlying pathology of cardiovascular disease which affects millions of people world-wide[22]. It is characterized by the accumulation of lipids in the form of low-density lipoprotein (LDL) in the subendothelial space in medium and large-sized arteries which leads to chronic inflammation[27]. This inflammation causes the modification of LDL to oxidized LDL (oxLDL), which attract immune cells such as monocytes[27]. Monocytes can differentiate into macrophages, and these, in turn, can phagocytose oxLDL, which leads to foam cell formation and plaque formation[28]. There are many different immune cells present in the atherosclerotic plaque, such as DCs, T and B lymphocytes[28,29]. While there is still debate about the specific role of T cells in atherosclerosis, it is generally accepted that Th1 cells progress the disease, while Tregs are protective[25,30]. Indeed, Tregs have abnormal activity in atherosclerosis[31]. Reestablishing immune homeostasis through induction of Tregs may, therefore, be an effective therapeutic approach[25].

As opposed to auto-immune diseases, cancer is an example of a disease associated with a dysfunctional pro-inflammatory immune response. Cancer is a major health concern, with approximately 1.7 million diagnoses and an estimated 600,000 deaths in the United States in the year 2019[23]. Cancer comprises a group of diseases involving abnormal sustained cell proliferation, replication, and survival. Benign cancers are contained and will not spread to other parts of the body, while malignant tumors have the potential to metastasize[32]. In cancer, T cells can recognize tumor-associated antigens[33,34]. These antigens can be tumor-associated antigens which are epitopes that are expressed on healthy cells but over-expressed on tumor cells[35]. Another type of tumor antigens are neoantigens. These result from non-synonymous somatic mutations that encode new amino acid residues, leading to new peptides that can be presented

on the cell surface of tumor cells[36]. However, the tumor can escape immune detection and attack by inducing immunosuppression. The main challenge in cancer treatment is, therefore, to induce strong pro-inflammatory $CD8^+$ and $CD4^+$ T cell responses to overcome this immune escape[37].

The goal of immunotherapy is to manipulate $CD4^+$ and $CD8^+$ T cell responses to either become more active to fight diseases such as infections or cancer[38], or to suppress the immune system in the case of auto-immune diseases or chronic inflammatory diseases[39]. Strategies include cytokine therapy[40], cell-based therapies (DCs[41] or T cells[42]) or the use of immune active drugs[43]. However, often these are non-specific, labor-intensive and expensive to make, quickly degraded or metabolized in the body, and/or have side effects at therapeutic doses[44-49]. Nanoparticulate drug delivery systems can overcome the limitations of immunotherapy; they can be designed to protect the drugs/biologicals and (indirectly) target specific immune cells, which allows for therapeutic efficacy, reduces the required dose and minimizes side-effects[50]. Nanoparticles can be made of different materials, such as polymers[51], metals[52], lipids[53], proteins[54], or a combination of the above. The choice of material depends on multiple factors, such as the properties of the cargo, the desired release rate of the cargo (i.e. sustained, delayed, burst), and the desired pharmacokinetics and biodistribution. Some of these materials have intrinsic immune effects[55], which makes them very interesting for use in immunotherapy. For instance, particles displaying the polymer poly(maleic anhydride-alt-1-octadecene)[56] or the anionic phospholipid phosphatidylglycerol[57] on their surface can induce tolerance. Conversely, particles composed of cationic phospholipids[58] or the polymer PC7A[59] can have strong pro-inflammatory effects. Furthermore, the physicochemical properties of the particles such as size, shape, rigidity (as reviewed in **chapter 2**), and charge[60] can affect the immune response. For example, small nano-sized particles show higher uptake by APCs and stronger cellular Th1 responses than larger particles[61].

Here we focus on liposomes because they have been FDA-approved for certain therapies[62], they can encapsulate a range of compounds, and their properties can be specifically tuned to induce immunogenicity or tolerance[53]. Liposomes are particles consisting of one or more lipid bilayers encapsulating an aqueous core. Both the liposomal composition and the formulation process determine the physicochemical properties of liposomes[53]. When using liposomes as a vaccine delivery system, their physicochemical properties can be tuned to obtain the desired immune response[63]. In this thesis, we prepared immune-suppressing liposomes to treat atherosclerosis, and immune-activating liposomes to treat cancer. Apart from APC uptake, liposomes can be functionalized with targeting molecules to be retained by other cell types or tissues. This allows for the delivery of drug substances into specific cells which may otherwise not be reached when using non-delivery approaches[64]. We used this approach to target liposomes to atherosclerotic plaques to deliver a drug substance to reverse foam cell formation.

Thesis Outline

Since liposomes are very versatile drug delivery systems, the research presented here focuses on using liposomes in two different treatment strategies; vaccination and delivery of a small molecule, and in two different disease models; cancer and atherosclerosis. For each of these treatment strategies, the liposomal formulation was tailored to obtain the desired therapeutic effect. **Chapter 2** reviews some of the most important physicochemical properties (size, shape, and rigidity) that determine the immunological effects of liposomes in the body. In **chapter 3** we present a detailed study on the effect of liposomal rigidity, as measured by atomic force microscopy, on antigen-specific Treg responses for anionic liposomes. In **chapter 4**, we show that our optimized anionic liposomes can induce potent antigen-specific Treg responses, and can be used to delay atherosclerosis progression in a mouse model. **Chapter 5** also focuses on liposomal treatment of atherosclerosis, but here liposomes were prepared to target to foam cells in atherosclerotic plaques to deliver a small molecule. **Chapter 6** again centers around using liposomes in immunotherapy; we used cationic liposomes in combination with an adjuvant to treat cancer in mice. Finally, we summarize the overall findings in **chapter 7** and discuss perspectives of using liposomes for vaccination and targeted drug delivery.

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