Liposome-based vaccines for immune modulation: from antigen selection to nanoparticle design

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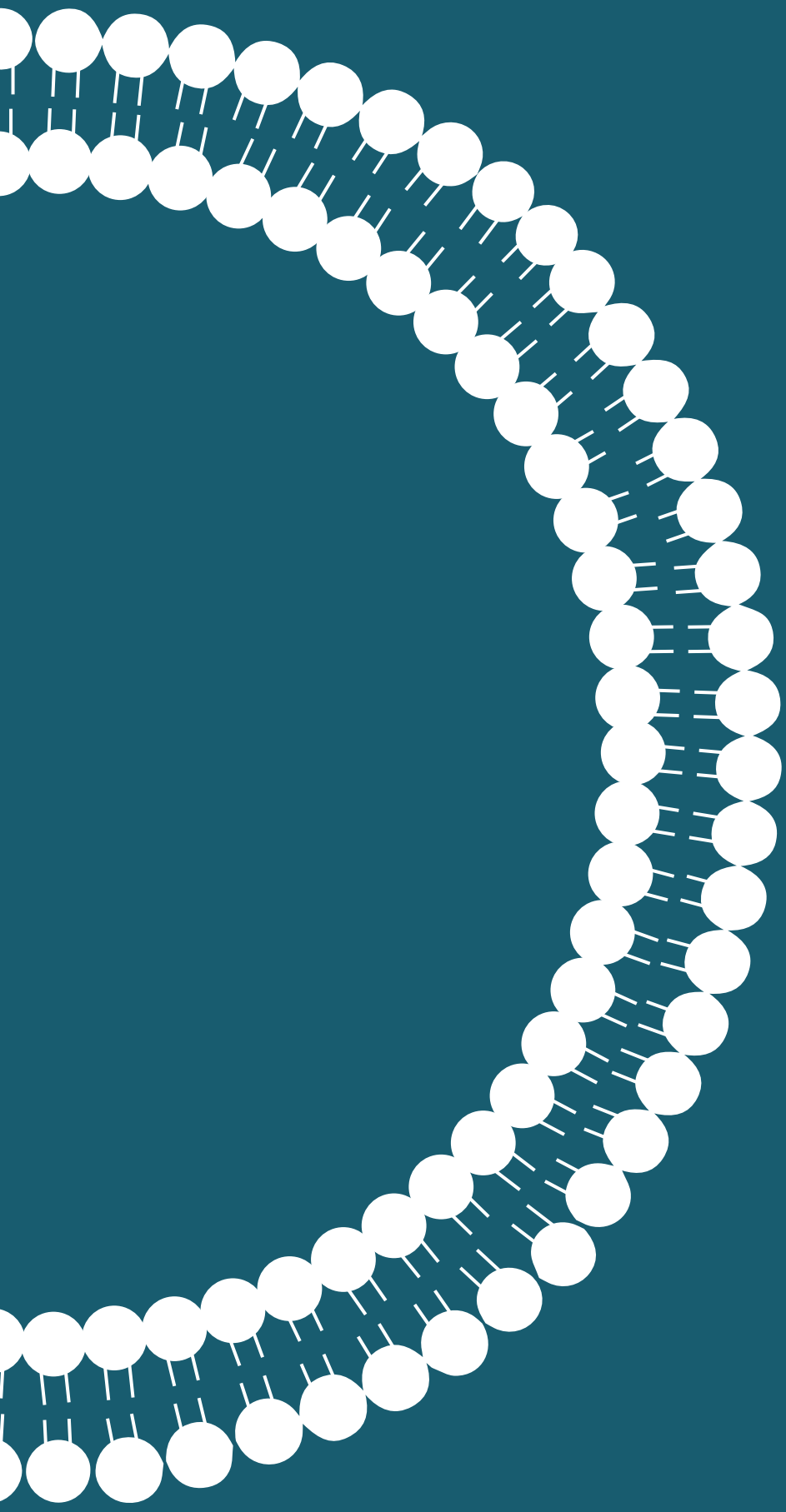

Chapter 1

Introduction and Thesis Outline

INNATE AND ADAPTIVE IMMUNITY

The immune system consists of cells and organs in charge of protecting the organism against external and internal threats. The immune system can be broadly divided into two arms, the innate and adaptive immunity. The innate immunity responds quickly but it is not specific, while the adaptive immune response takes more time to be mounted but it acts in a specific manner. The main link between innate and adaptive immune responses are antigen-presenting cells (APCs) such as dendritic cells (DCs). APCs can take up antigens, process them and present them on the cell surface bound to Major Histocompatibility Complex (MHC) molecules, called Human Leukocyte Antigen (HLA) in human. There are two major types of MHC molecules, MHC class I mostly presents antigens derived from proteins expressed within the cell, while MHC class II presents antigens that have been taken up from the extracellular environment. Antigens presented via MHC molecules can be recognized by cells from the adaptive immune system, such as T cells. T cells can recognize MHC-presented antigens via their T cell receptor (TCR). The vast diversity of TCRs allows the recognition of virtually any antigen that the immune system can encounter. When a T cell with a specific TCR recognizes their cognate antigen, it can undergo clonal expansion, giving rise to an antigen-specific T cell population that can react to that antigen throughout the body. When an antigen-specific T cell recognizes its target antigen it can, for example, directly kill the cell presenting that antigen or secrete molecules to potentiate the immune response. T cells can be broadly divided by the expression of two markers, cluster of differentiation 4 ($CD4^+$) and cluster of differentiation 8 ($CD8^+$), and therefore are called $CD4^+$ T cells and $CD8^+$ T cells. $CD4^+$ T cells, also known as helper T cells, recognize antigens presented by MHC class II molecules, while $CD8^+$ T cells, also known as cytotoxic T cells, can recognize antigens presented by MHC class I. An overactivation of the immune system in response to endogenous antigens is the root cause of autoimmune diseases such as multiple sclerosis or rheumatoid arthritis, and it also contributes to other diseases with high societal impact such as atherosclerosis, the main underlying cause of cardiovascular diseases (CVD).

ATHEROSCLEROSIS AS AN AUTOIMMUNE-LIKE DISEASE

Atherosclerosis is a chronic inflammatory disease of the arteries characterized by the accumulation of lipid-rich low-density lipoprotein (LDL) particles in the intima layer of the arteries. This deposition of LDL triggers an immune response that leads to the infiltration and accumulation of immune cells in the lesion site. The growth of the atherosclerotic lesions can narrow the arterial lumen, restricting blood flow towards certain parts of the body. Furthermore, the lesion can eventually rupture

generating a thrombus that can lead to myocardial infarction or stroke. The role of the immune system in the development of atherosclerosis has traditionally been underappreciated compared to the role of lipid metabolism, however several lines of evidence highlight the importance of immunity in both the initiation and progression of atherosclerosis[1-4]. One of the key players are T cells, with both $CD4^+$ and $CD8^+$ T cells found in atherosclerosis plaques of CVD patients[5, 6].

The role played by the different T cell subsets in atherosclerosis is, to this day, subject of intensive research. In the case of $CD8^+$ T cells some evidence points towards pro-atherogenic role[7] while other studies suggest an atheroprotective role[8, 9]. The different subpopulations of $CD4^+$ T cells are also known to have different functions in the development of atherosclerosis lesions. T helper 1 (Th1) $CD4^+$ T cells are pro-inflammatory T cells that exert their function by releasing pro-inflammatory cytokines such as IFNγ that contributes to the activation of macrophages and further increases the inflammatory response[10, 11]. The pro-atherogenic or anti-atherogenic role of T helper 2 (Th2) cells is still controversial. The main cytokine produced by Th2 cells is IL-4 which can counteract the effect of atherogenic Th1 responses[12] however other experiments in mice have shown that the depletion of IL-4 reduced atherosclerosis plaque formation[13]. On the other hand, Th2 cells also produce IL-10, IL-5 or IL-13 and these cytokines have shown to be anti-atherogenic[14-16]. The other major subset of $CD4^+$ T cells is T helper 17 (Th17) cells, seems to have different roles in human and mouse atherosclerosis. These cells mainly produce IL-17, and while studies in mouse models of atherosclerosis have shown evidence of both pro-atherogenic and anti-atherogenic functions of Th17 cells, clinical evidence from patients shows that increased levels this T cell subset and IL-17 in circulation are linked to unstable angina and acute myocardial infarction[17]. Finally, another key population of $CD4^+$ T cells are T regulatory cells (Tregs). Tregs mostly secrete the anti-inflammatory cytokines IL-10 and TGFβ and increased levels of Tregs are atheroprotective in both mice and human[18, 19]. Tregs can be identified in mice by the expression of the transcription factor forehead box protein P3 (FoxP3), although in human FoxP3 alone is not a reliable marker of Tregs since it is also expressed by other T cells subsets upon activation[20]. Recent evidence suggests that Tregs can evolve from an anti-inflammatory phenotype towards a Th1/Th17 phenotype as atherosclerosis develops[21].

Most studies that try to identify the role of different T cell subsets in atherosclerosis do not take into account the antigen-specificity of the response and that might lead to apparently contradictory functions of the same T cell population. T cells specific for an atherosclerosis-relevant antigen could potentially have different effect on atherosclerosis compared to T cells targeting other antigens. T cells exert their function upon recognition of their target antigen, therefore the antigen-specificity of

T cell responses is key to unveil the role of each T cell population in atherosclerosis. Despite intensive research, the driving antigens of the immune responses in atherosclerosis are not fully elucidated. Recent single-cell TCR sequencing data showed presence of clonally expanded $CD4^+$ T cells in the plaque, suggesting antigen-specific T cell activation. Furthermore, these expanded $CD4^+$ T cells also expressed markers of recent T cell activation such as CD69, indicating that T cell activation occurs in the plaque. These data also showed an important overlap in the gene expression of T cells in plaques and T cells isolated from the synovial fluid of patients with psoriatic arthritis, a known autoimmune disease[22]. These results provide evidence of the long suspected autoimmune component of atherosclerosis. The potential target antigens for autoimmunity in atherosclerosis include heat-shock proteins (HSP) [23, 24] and β2-Glycoprotein I (β2GPI) [25], however the main suspect is ApolipoproteinB-100 (ApoB100), the main protein in LDL particles. The presence of antibodies and T cells against ApoB100 has been shown in both mouse models of atherosclerosis and in patients samples[26, 27]. These new insights into the role of autoimmunity in atherosclerosis could inform the development of new therapeutic approaches for the treatment of CVDs.

NEW THERAPEUTIC APPROACHES IN ATHEROSCLEROSIS

Since atherosclerosis has been traditionally considered a disease related to lipid metabolism, and more specifically to dysregulated LDL levels, the main therapeutic approach has targeted the high levels of LDL in patients. Lipid-lowering drugs, such as statins or proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors, are very effective at reducing LDL levels and the risk of cardiovascular disease[28, 29], however they do not target the important inflammatory component of atherosclerosis. In fact, patients undergoing intensive lipid-lowering therapy still present significant risk of suffering a cardiovascular event[30]. New therapeutic interventions that target the immune response in atherosclerosis could be the next frontier to significantly reduce the incidence of cardiovascular diseases. The Canakinumab Anti-Inflammatory Thrombosis Outcome Study (CANTOS) clinical trial showed that targeting inflammation is effective at reducing the risk of cardiovascular events in patients using standard lipid-lowering therapies but with elevated inflammatory markers[31]. This trial, however, also showed that the chronic use of therapies that dampen the overall immune response of patients leads to higher risk of fatal infections that can overshadow the benefits. The induction of antigen-specific immune tolerance mediated by Tregs could harness the advantages of anti-inflammatory therapies while avoiding the unwanted side effects of systemic immune suppression.

Several approaches are in development for the induction of antigen-specific immune tolerance for the treatment of autoimmune diseases, such as the administration of glycosylated autoantigens or the delivery of the target antigens using tolerogenic nanoparticles[32, 33]. Nanoparticles can be used to deliver antigens to antigen-presenting cells and therefore induce an antigen-specific immune response (Figure 1). In the case of vaccines to treat inflammatory and/or autoimmune diseases, nanoparticles can be used to co-deliver a target auto-antigen and a tolerogenic adjuvant. Furthermore, certain nanoparticles such as liposomes are versatile systems that can drive the immune response towards a proinflammatory or an anti-inflammatory response depending on their physicochemical characteristics[34]. Liposomes are nanometric delivery systems consisting of a phospholipid bilayer enclosing an aqueous core. The phospholipid composition determines physicochemical properties of the nanoparticle such as surface charge and rigidity but also the set of proteins that will interact with the nanoparticle in a biological fluid, also known as protein corona[35]. In turn, the physicochemical properties and the protein corona will determine the immune response elicited by the nanoparticles. For example, cationic liposomes are known to induce potent pro-inflammatory responses that can target and eliminate tumour cells[36]. On the other hand, highly rigid anionic liposomes are known to have anti-inflammatory properties and are able to induce antigen-specific immune tolerance mediated by Tregs[37].

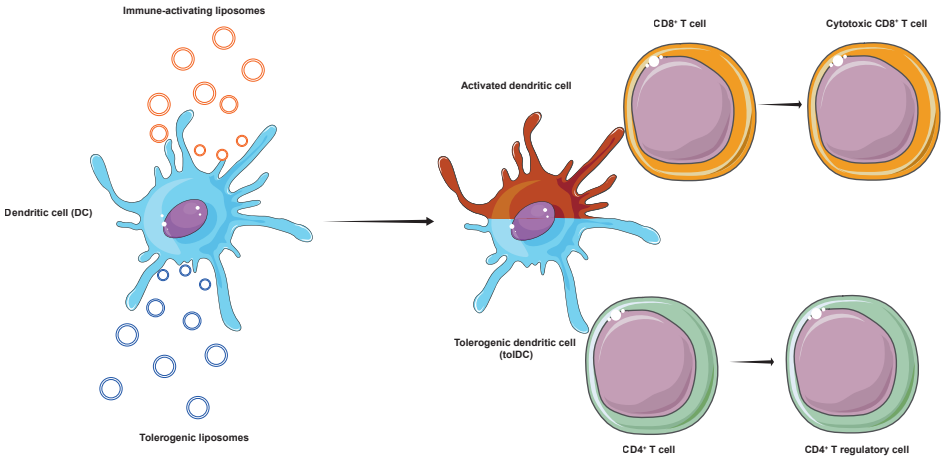

Figure 1. Overview of induction of pro-inflammatory or tolerogenic responses by dendritic cells (DCs) primed by different type of liposomes. The delivery of antigens to the DCs using immune-activating liposomes, such as cationic liposomes, leads to the generation of an antigen-specific cytotoxic CD8+ T cell response, useful to fight viral infections. The delivery of antigens to DCs using tolerogenic anionic liposomes leads to the generation of CD4+ T regulatory cell responses, useful to temper down immunity in the context of autoimmune diseases.

The preparation of highly rigid liposomes requires the use of phospholipids with high transition temperature, such as 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and 1,2-distearoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DSPG). These two phospholipids have a transition temperature of 55°C therefore the preparation of liposomes containing these lipids requires working at temperatures above 55°C. At a lab-scale, the most common methods to prepare these formulations is the lipid film hydration method followed by extrusion[38]. This method consists of the generation of a suspension of multilamellar vesicles by hydration of a dry lipid film. This suspension is subsequently forced through filters with increasingly smaller pore sizes to obtain a monodisperse formulation with the desired particle size, usually in the range of 100-200 nm. This preparation method presents several disadvantages such batch-to-batch variability and poor scalability due to the need of extrusion at high pressure and temperature, therefore new preparation methods are needed to further develop these formulations beyond pre-clinical testing. Microfluidic systems for the preparation of nanoparticles have been extensively studied in the last few years and this has led to the development of commercial microfluidic-based platforms for nanoparticle production[39]. Microfluidic systems allow the production of nanoparticle formulations in a continuous manner therefore one of the advantages of these systems is the scale-up potential[40].

Besides the production of tolerogenic liposomal formulations in large-scale, the translation of results from *in vitro* efficacy studies to *in vivo* and from animal models to human remain a challenge. The translation from *in vitro* to *in vivo* can be hindered by the very different biological environment in cell culture medium versus the mouse biological fluids. The difference in protein composition in these different environments can lead to the formation of significantly different protein coronas, that can in turn lead to different biological effects[41]. On the other hand, the translation of tolerogenic formulations from animal models, such as mice, to human is particularly challenging due to key differences in dendritic cells and Tregs in both organisms, therefore the use of human *in vitro* and *ex vivo* systems during pre-clinical testing of tolerogenic nanoparticle formulations is key.

LIPOSOMES AS IMMUNE POTENTIATORS IN PROPHYLACTIC VACCINES

In contrast to anionic liposomes, cationic liposomes are known for their immune activating properties, therefore these lipid nanoparticles can be used as prophylactic vaccines against infectious diseases. The induction of local anti-viral immune responses in the airways through vaccination is key for protection against respiratory viruses, such as SARS-CoV2 or influenza, however this has proven to

be difficult to achieve using subunit vaccines, the safest and easiest to manufacture type of vaccines available nowadays[42]. The use of cationic liposomes to co-deliver antigens and adjuvants such as cyclic-dimeric guanosine monophosphate (c-di-GMP) has been studied before as a therapeutic anti-cancer vaccine, and it has shown to induce potent antigen-specific $CD8^+$ T cell responses[43]. Furthermore, the cationic surface charge of these liposomes makes them suitable for intranasal delivery due to the favourable electrostatic interaction with the airways mucosa[44]. The combination of immune activating properties and the mucosal delivery of these formulations would make them an ideal subunit vaccine to induce local immune responses in the airways.

This thesis aims to advance the field of nanoparticle-based tolerogenic vaccines for the treatment of atherosclerosis. We examined three key aspects of these formulations including the elucidation of target antigens for these vaccines and the study of liposome-based and polymer-lipid hybrids for antigen delivery. Additionally, we developed a microfluidic method for the preparation of tolerogenic liposomal formulations, which allows for efficient encapsulation of peptide antigens and vitaminD3. Furthermore, we explore the use of liposomes to modulate the immune response towards activation and induction of protective anti-viral immunity.

THESIS OUTLINE

In **chapter 2**, we review the state of development of tolerogenic vaccines to treat cardiovascular disease. We summarized the lessons learned on the development of tolerogenic therapies for other inflammatory and autoimmune diseases such as rheumatoid arthritis, type 1 diabetes, or multiple sclerosis, and analysed the pre-clinical data available on the use of peptide-based vaccines to halt the development of atherosclerosis. Furthermore, we highlight the main challenges in the clinical translation of this therapeutic approach, such as the selection of the appropriate antigens, formulations, and target patient population for clinical trials. In **chapter 3**, we identify possible candidates for tolerogenic vaccines against atherosclerosis. To that end we used immunopeptidomics to identify antigens presented by HLA molecules in human atherosclerosis plaques from patients. We selected 20 peptides derived from ApoB100 that presented a high binding affinity for a wide range of HLA types. We observed that at least 25% of atherosclerosis patients present significant $CD4^+$ T cell responses against these ApoB100 peptides and that the level of T cell responses against these antigens correlated to the vulnerability of atherosclerosis plaques. These findings make the identified ApoB100 peptides potential targets for tolerogenic vaccines against atherosclerosis. In **chapter 4**, we explore the tolerogenic capacity of previously reported DSPG liposomes in human

in vitro and *ex vivo* skin model and we showed that the inclusion of vitaminD3 in the anionic liposomal formulation is required to induce T regulatory cells in human experimental models. In **chapter 5**, we develop a microfluidic method to produce tolerogenic liposomes containing DSPG in a continuous and scalable manner. Furthermore, we show that this microfluidics system can be used to encapsulate the tolerogenic adjuvant vitaminD3 and ApoB100-derived immunogenic peptides with high efficiency. In **chapter 6**, we explore the role of nanoparticle rigidity in the tolerogenic capacity of formulations by comparing DSPG-containing liposomes to solid PLGA nanoparticles covered with an anionic lipid bilayer. In **chapter 7**, we use cationic liposomes loaded with peptides derived from SARS-CoV2 or influenza and the adjuvant c-di-GMP, inducing strong antigen-specific T cell responses systemically but also locally in the lungs. The T cell responses induced by vaccination with the liposome formulation correlated with lower viral load in the lungs of vaccinated mice upon viral challenge. In **chapter 8**, we summarize and discuss the main findings of this thesis.

REFERENCES

1. Libby P. Inflammation in atherosclerosis. Nature. 2002;420(6917):868-74.
2. Tsiantoulas D, Diehl CJ, Witztum JL, Binder CJ. B cells and humoral immunity in atherosclerosis. Circ Res. 2014;114(11):1743-56.
3. Ley K, Miller YI, Hedrick CC. Monocyte and macrophage dynamics during atherogenesis. Arterioscler Thromb Vasc Biol. 2011;31(7):1506-16.
4. Paulsson G, Zhou X, Tornquist E, Hansson GK. Oligoclonal T cell expansions in atherosclerotic lesions of apolipoprotein E-deficient mice. Arterioscler Thromb Vasc Biol. 2000;20(1):10-7.
5. Jonasson L, Holm J, Skalli O, Bondjers G, Hansson GK. Regional accumulations of T cells, macrophages, and smooth muscle cells in the human atherosclerotic plaque. Arteriosclerosis. 1986;6(2):131-8.
6. Hansson GK, Holm J, Jonasson L. Detection of activated T lymphocytes in the human atherosclerotic plaque. Am J Pathol. 1989;135(1):169-75.
7. Kyaw T, Winship A, Tay C, Kanellakis P, Hosseini H, Cao A, et al. Cytotoxic and proinflammatory CD8+ T lymphocytes promote development of vulnerable atherosclerotic plaques in apoE-deficient mice. Circulation. 2013;127(9):1028-39.
8. Chyu KY, Zhao X, Dimayuga PC, Zhou J, Li X, Yano J, et al. CD8+ T cells mediate the athero-protective effect of immunization with an ApoB-100 peptide. PLoS One. 2012;7(2):e30780.
9. van Duijn J, Kritikou E, Benne N, van der Heijden T, van Puijvelde GH, Kroner MJ, et al. CD8+ T-cells contribute to lesion stabilization in advanced atherosclerosis by limiting macrophage content and CD4+ T-cell responses. Cardiovasc Res. 2019;115(4):729-38.
10. Chen J, Xiang X, Nie L, Guo X, Zhang F, Wen C, et al. The emerging role of Th1 cells in atherosclerosis and its implications for therapy. Front Immunol. 2022;13:1079668.
11. Voloshyna I, Littlefield MJ, Reiss AB. Atherosclerosis and interferon-gamma: new insights and therapeutic targets. Trends Cardiovasc Med. 2014;24(1):45-51.
12. Davenport P, Tipping PG. The role of interleukin-4 and interleukin-12 in the progression of atherosclerosis in apolipoprotein E-deficient mice. Am J Pathol. 2003;163(3):1117-25.
13. King VL, Szilvassy SJ, Daugherty A. Interleukin-4 deficiency decreases atherosclerotic lesion formation in a site-specific manner in female LDL receptor-/- mice. Arterioscler Thromb Vasc Biol. 2002;22(3):456-61.
14. Pinderski Oslund LJ, Hedrick CC, Olvera T, Hagenbaugh A, Territo M, Berliner JA, Fyfe AI. Interleukin-10 blocks atherosclerotic events in vitro and in vivo. Arterioscler Thromb Vasc Biol. 1999;19(12):2847-53.
15. Binder CJ, Hartvigsen K, Chang MK, Miller M, Broide D, Palinski W, et al. IL-5 links adaptive and natural immunity specific for epitopes of oxidized LDL and protects from atherosclerosis. J Clin Invest. 2004;114(3):427-37.
16. Cardilo-Reis L, Gruber S, Schreier SM, Drechsler M, Papac-Milicevic N, Weber C, et al. Interleukin-13 protects from atherosclerosis and modulates plaque composition by skewing the macrophage phenotype. EMBO Mol Med. 2012;4(10):1072-86.
17. Cheng X, Yu X, Ding YJ, Fu QQ, Xie JJ, Tang TT, et al. The Th17/Treg imbalance in patients with acute coronary syndrome. Clin Immunol. 2008;127(1):89-97.

18. Ait-Oufella H, Salomon BL, Potteaux S, Robertson AK, Gourdy P, Zoll J, et al. Natural regulatory T cells control the development of atherosclerosis in mice. Nat Med. 2006;12(2):178-80.

19. George J, Schwartzenberg S, Medvedovsky D, Jonas M, Charach G, Afek A, Shamiss A. Regulatory T cells and IL-10 levels are reduced in patients with vulnerable coronary plaques. Atherosclerosis. 2012;222(2):519-23.

20. Roncarolo MG, Gregori S. Is FOXP3 a bona fide marker for human regulatory T cells? Eur J Immunol. 2008;38(4):925-7.

21. Wolf D, Gerhardt T, Winkels H, Michel NA, Pramod AB, Ghosheh Y, et al. Pathogenic Autoimmunity in Atherosclerosis Evolves From Initially Protective Apolipoprotein B(100)-Reactive CD4(+) T-Regulatory Cells. Circulation. 2020;142(13):1279-93.

22. Depuydt MAC, Schaftenaar FH, Prange KHM, Boltjes A, Hemme E, Delfos L, et al. Single-cell T cell receptor sequencing of paired human atherosclerotic plaques and blood reveals autoimmune-like features of expanded effector T cells. Nature Cardiovascular Research. 2023.

23. Wick G, Jakic B, Buszko M, Wick MC, Grundtman C. The role of heat shock proteins in atherosclerosis. Nat Rev Cardiol. 2014;11(9):516-29.

24. Almanzar G, Ollinger R, Leuenberger J, Onestingel E, Rantner B, Zehm S, et al. Autoreactive HSP60 epitope-specific T-cells in early human atherosclerotic lesions. J Autoimmun. 2012;39(4):441-50.

25. George J, Harats D, Gilburd B, Afek A, Levy Y, Schneiderman J, et al. Immunolocalization of beta2-glycoprotein I (apolipoprotein H) to human atherosclerotic plaques: potential implications for lesion progression. Circulation. 1999;99(17):2227-30.

26. Bjorkbacka H, Alm R, Persson M, Hedblad B, Nilsson J, Fredrikson GN. Low Levels of Apolipoprotein B-100 Autoantibodies Are Associated With Increased Risk of Coronary Events. Arterioscler Thromb Vasc Biol. 2016;36(4):765-71.

27. Marchini T, Hansen S, Wolf D. ApoB-Specific CD4(+) T Cells in Mouse and Human Atherosclerosis. Cells. 2021;10(2).

28. Cheung BM, Lauder IJ, Lau CP, Kumana CR. Meta-analysis of large randomized controlled trials to evaluate the impact of statins on cardiovascular outcomes. Br J Clin Pharmacol. 2004;57(5):640-51.

29. Sabatine MS, Giugliano RP, Keech AC, Honarpour N, Wiviott SD, Murphy SA, et al. Evolocumab and Clinical Outcomes in Patients with Cardiovascular Disease. N Engl J Med. 2017;376(18):1713-22.

30. Murphy SA, Cannon CP, Wiviott SD, McCabe CH, Braunwald E. Reduction in recurrent cardiovascular events with intensive lipid-lowering statin therapy compared with moderate lipid-lowering statin therapy after acute coronary syndromes from the PROVE IT-TIMI 22 (Pravastatin or Atorvastatin Evaluation and Infection Therapy-Thrombolysis In Myocardial Infarction 22) trial. J Am Coll Cardiol. 2009;54(25):2358-62.

31. Ridker PM, Everett BM, Thuren T, MacFadyen JG, Chang WH, Ballantyne C, et al. Antiinflammatory Therapy with Canakinumab for Atherosclerotic Disease. N Engl J Med. 2017;377(12):1119-31.

32. Tremain AC, Wallace RP, Lorentz KM, Thornley TB, Antane JT, Raczy MR, et al. Synthetically glycosylated antigens for the antigen-specific suppression of established immune responses. Nat Biomed Eng. 2023.

33. Krienke C, Kolb L, Diken E, Streuber M, Kirchhoff S, Bukur T, et al. A noninflammatory mRNA vaccine for treatment of experimental autoimmune encephalomyelitis. Science. 2021;371(6525):145-53.

34. Benne N, van Duijn J, Kuiper J, Jiskoot W, Slutter B. Orchestrating immune responses: How size, shape and rigidity affect the immunogenicity of particulate vaccines. J Control Release. 2016;234:124-34.

35. Pattipeiluhu R, Crielaard S, Klein-Schiphorst I, Florea BI, Kros A, Campbell F. Unbiased Identification of the Liposome Protein Corona using Photoaffinity-based Chemoproteomics. ACS Cent Sci. 2020;6(4):535-45.

36. Varypataki EM, Benne N, Bouwstra J, Jiskoot W, Ossendorp F. Efficient Eradication of Established Tumors in Mice with Cationic Liposome-Based Synthetic Long-Peptide Vaccines. Cancer Immunol Res. 2017;5(3):222-33.

37. Benne N, van Duijn J, Lozano Vigario F, Leboux RJT, van Veelen P, Kuiper J, et al. Anionic 1,2-distearoyl-sn-glycero-3-phosphoglycerol (DSPG) liposomes induce antigen-specific regulatory T cells and prevent atherosclerosis in mice. J Control Release. 2018;291:135-46.

38. Zhang H. Thin-Film Hydration Followed by Extrusion Method for Liposome Preparation. Methods Mol Biol. 2017;1522:17-22.

39. Zhang H, Yang J, Sun R, Han S, Yang Z, Teng L. Microfluidics for nano-drug delivery systems: From fundamentals to industrialization. Acta Pharm Sin B. 2023;13(8):3277-99.

40. Jahn A, Stavis SM, Hong JS, Vreeland WN, DeVoe DL, Gaitan M. Microfluidic mixing and the formation of nanoscale lipid vesicles. ACS Nano. 2010;4(4):2077-87.

41. Lee SY, Son JG, Moon JH, Joh S, Lee TG. Comparative study on formation of protein coronas under three different serum origins. Biointerphases. 2020;15(6):061002.

42. Burchill MA, Tamburini BA, Pennock ND, White JT, Kurche JS, Kedl RM. T cell vaccinology: exploring the known unknowns. Vaccine. 2013;31(2):297-305.

43. Benne N. Vaccination and targeted therapy using liposomes: opportunities for treatment of atherosclerosis and cancer. The Netherlands: Leiden University; 2020.

44. Leal J, Smyth HDC, Ghosh D. Physicochemical properties of mucus and their impact on transmucosal drug delivery. Int J Pharm. 2017;532(1):555-72.