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## Engineering nanomaterials to address cell-mediated inflammation in atherosclerosis

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### Abstract

Atherosclerosis is an inflammatory disorder with a pathophysiology driven by both innate and adaptive immunity and a primary cause of cardiovascular disease (CVD) worldwide. Vascular inflammation and accumulation of foam cells and their products induce maturation of atheromas, or plaques, which can rupture by metalloprotease action, leading to ischemic stroke or myocardial infarction. Diverse immune cell populations participate in all stages of plaque maturation, many of which directly influence plaque stability and rupture via inflammatory mechanisms. Current clinical treatments for atherosclerosis focus on lowering serum levels of low-density lipoprotein (LDL) using therapeutics such as statins, administration of antithrombotic drugs, and surgical intervention. Strategies that address cell-mediated inflammation are lacking, and consequently have recently become an area of considerable research focus. Nanomaterials have emerged as highly advantageous tools for these studies, as they can be engineered to target specific inflammatory cell populations, deliver therapeutics of wide-ranging solubilities and enhance analytical methods that include imaging and proteomics. Furthermore, the highly phagocytic nature of antigen presenting cells (APCs), a diverse cell population central to the initiation of immune responses and inflammation, make them particularly amenable to targeting and modulation by nanoscale particulates. Nanomaterials have therefore become essential components of vaccine formulations and treatments for inflammation-driven pathologies like autoimmunity, and present novel opportunities for immunotherapeutic treatments of CVD. Here, we review recent progress in the design and use of nanomaterials for therapeutic assessment and treatment of atherosclerosis. We will focus on promising new approaches that utilize nanomaterials for cell-specific imaging, gene therapy and immunomodulation.

### INTRODUCTION

Atherosclerosis is an immunologically complex inflammatory condition within the intima of arterial vessels and a primary cause of cardiovascular disease (CVD). CVD continues to be the leading cause of death in the developed world and is responsible for over 17% of national health care expenditures alone in the United States. With 40% of the US population projected to experience some form of CVD by 2030, the total direct medical costs are expected to reach \$818 billion [1]. Numerous studies have implicated low density lipoprotein (LDL) as the instigator of atherogenesis, and a lowering of lifelong LDL levels by only 30% via diet [2, 3] or mutation [4, 5] can reduce the risk of an adverse cardiovascular event by 90%. In comparison, a similar 30% decrease in LDL levels by drugs such as statins, which have become a staple for therapeutic treatment of CVD [6], only reduce the risk of an event by 30%. The human body has long been estimated to only require

LDL cholesterol levels of approximately 25 mg/dl [7], while the mean value resulting from the high fat diets prevalent in North America and Europe is 136.2 mg/dl [8]. As a result, almost everyone over the age of twenty has subclinical atherosclerotic lesions [9]. Therapies must therefore account for the plaques that a lifetime of a Western diet has generated in the vessel walls of patients by addressing additional contributing factors, such as cell-mediated inflammation [10]. Atherosclerosis is primarily treated through surgery and/or a combination of therapeutic drugs such as platelet inhibitors, statins, antihypertensives and thrombolytics. Surgical intervention can involve stenting or artery bypass surgery. These current clinical strategies inadequately address the inflammatory component of atherosclerosis [11], and the targeting and modulation of inflammatory immune cells and their expressed factors may present a viable component of effective treatment regimens [12].

The activation state and function of inflammatory cells are strongly influenced by interactions with and factors released by a phagocytic cell population known as antigen presenting cells (APCs), which consists of diverse subsets of monocytes, macrophages, B cells, dendritic cells (DCs), and epithelial cells [13]. A primary role of these cells is to serve as sentinels that collect and process foreign and pathogenic molecules and particulates to generate appropriate immune responses, often stimulating controlled inflammation. Due to the highly phagocytic nature of APCs, they can be readily targeted by rationally designed nanoscale materials via multiple mechanisms. For example, the vast majority of intravenously injected nanomaterials are eventually cleared systemically by macrophages in the spleen and liver regardless of their intended *in vivo* targets, which was originally called the reticuloendothelial system and now referred to as the mononuclear phagocyte system (MPS) [14–16]. Nanomaterials have thus emerged as key components of delivery systems engineered to influence inflammation and immune responses, most notably vaccines and immunotherapies [17, 18]. Consequently, nanomaterials have found recent utility for targeting and modulating inflammatory cells that contribute to atherosclerosis. Encapsulation within nanomaterials can increase the pharmacokinetics of therapeutic drugs, improving their targeting to the lesions, and may reduce the need for surgery. Association with fluorophores, contrast agents and targeting ligands allow nanomaterials to serve as sensitive imaging tools to improve diagnosis of CVD and the efficacy of intervention. Furthermore, LDL is itself a nanoparticle composed of up to 1500 cholesterol esters and a single apolipoprotein B molecule, which maintains a diameter of  $21.4 \pm 1.3$  nm diameter and a height of  $12.1 \pm 1.1$  nm with strikingly low polydispersity [19].

The vast and ever-growing range of nanomaterials and nanostructures employed for therapeutic delivery are reviewed frequently [18, 20–22], and primarily consist of self-assembled aggregates formed from lipid or polymer amphiphiles (micelles, filomicelles and vesicles), metallic nanoparticles (mainly composed of Au, Ag, TiO<sub>2</sub>, ZnO, CuO, Fe<sub>2</sub>O<sub>3</sub>, and Fe<sub>3</sub>O<sub>4</sub>), solid core polymeric nanoparticles of which the most common are based on the clinically relevant component poly(lactic-co-glycolic acid) (PLGA), and finally high molecular weight polymers such as poly(ethylene glycol) (PEG) dendrimers and dextran. Here, we summarize nanomaterial-based therapeutic strategies aimed at investigating and addressing cell-mediated inflammation in atherosclerosis. Considerable progress has been achieved recently in the areas of imaging, gene therapy and immunotherapy for CVD, which will be the topics of focus in this review.

## 1. Cell-mediated inflammation in atherosclerosis

### 1.1 Inflammation in early lesions

The literature has demonstrated the participation of diverse inflammatory immune cell populations during all stages of atherosclerosis [23–26]. Several recent reviews delve into the cellular and biochemical details of this process that are beyond the scope of this review [27–29], and instead we intend to present the key concepts that have guided recent nanomaterials-based therapeutic strategies. The initial step in plaque formation is believed to involve dysfunctional endothelium, which permits infiltration and entrapment of LDL within the arterial intima [30]. LDL is retained via ionic bonds with proteoglycans in the extracellular matrix and subjected to oxidative modifications by enzymes and reactive oxygen species [31]. Continued irritative stimulations of the endothelium that include hypertension, dyslipidaemia, localized oxidation and inflammatory factors upregulate endothelial cell adhesion receptors to promote monocyte recruitment into the vessel walls [32, 33]. Here, monocytes encounter fatty deposits and are activated by cytokines, growth factors and modified lipid species, such as oxidized LDL (oxLDL), which drive their differentiation into lipid-laden macrophages, ie. foam cells, and inflammatory DC populations [34]. Initially, controlled inflammation resolution is maintained by molecules including interleukin-10 (IL-10), transforming growth factor- $\beta$  (TGF $\beta$ ), lipoxins, resolvins, protectins, maresins and prostaglandins [27, 35, 36]. This environment induces differentiation of monocytes into M2 macrophages that clear apoptotic debris and promote egression of inflammatory cells out of the plaque. Additionally, previously quiescent smooth muscle cells transition to a proliferative phenotype that expresses extracellular matrix components, generating a stabilizing fibrous cap composed primarily of fibrillar collagen that prevents plaque rupture [37, 38]. Thus early lesions are small, asymptomatic, and stable, demonstrating inflammation characteristic of wound healing and tissue regeneration.

### 1.2 Inflammation in late stage vulnerable plaques

Chronic intimal inflammation and hypercholesterolaemia can eventually lead to plaque instability, rupture and subsequent formation of a thrombus capable of occluding arteries and causing cardiac infarction. Hypercholesterolaemia promotes increased infiltration into lesions of Ly6C<sup>hi</sup> monocytes, which differentiate into macrophages that display an inflammatory phenotype similar to M1 macrophages (Fig. 1) [39]. Polarization towards M1 versus M2 macrophages within atheromas shifts the local inflammatory response from that of a healing/growth phenotype to that of a microbicidal/inhibitory capacity [40]. Although the accumulation of DCs within lesions has been shown to correlate with the stage of atherosclerosis, they have been found to be both atherogenic as well as atheroprotective, likely due to their heterogeneity [41–46]. Professional APCs, such as macrophages and DCs are highly phagocytic owing to their vast assortment of pattern recognition receptors (PRRs) that allow increased uptake of materials of diverse surface chemistries [47]. Although LDL uptake is regulated by a feedback system to prevent intracellular lipid overload, uptake of modified lipids including oxLDL by PRRs known as scavenger receptors can bypass this feedback to result in excessive accumulation of intracellular lipids [48, 49]. Accumulating foam cells release inflammatory cytokines including interleukin-1 $\beta$  (IL-1 $\beta$ ) and tumor necrosis factor (TNF), further recruiting inflammatory cell populations, such as T cells, mast

cells, and neutrophils. [23, 29, 50, 51]. The vasa vasorum serves as a key gateway for the entrance of migrating macrophages and leukocytes, converting the vessel adventitia into what has been characterized as an organized ectopic lymphoid tissue containing diverse immune cell populations [52–55]. These cytokines and recruited immune cells result in plaque instability via increased expression of reactive oxygen species and collagen-degrading metalloproteinases [28, 56, 57]. Furthermore, the progression of a secondary necrotic core composed of apoptotic cellular debris promotes the stimulation of APC intracellular PRRs toll-like receptors (TLRs) 7 and 9 by self-DNA complexes, inducing the release of pro-inflammatory  $T_H1$ -biased cytokines within atheromas [58]. Development of this necrotic core is a result of defective clearance of apoptotic cells as well as disrupted chemokine-mediated guidance cues that recruit and retain inflammatory cells within lesions [27, 56, 59]. Late stage plaques vulnerable to rupture therefore result from cell-mediated pro-inflammatory responses that promote dysregulated cellular chemotaxis, tissue damage and enzyme-induced weakening of the fibrous cap.

## 2. Design parameters for engineering nanomaterials that modulate inflammatory cells

### 2.1 Cellular interactions with nanomaterials

Evolutionarily driven interactions between APCs and bacterial, fungal and viral nano- and microstructures provides a powerful opportunity for rationally designed nanomaterials to influence inflammation-driven pathologies, such as atherosclerosis. Nanomaterials are broadly defined as any material with at least one external dimension that is less than 1000 nm [60], and the vast range of compounds that can be engineered within this size range presents immense opportunities to mimic the physiochemical interactions between APCs and pathogens for therapeutic applications. A diverse range of variables of course govern cellular interactions with materials that include surface chemistry, size, shape, elastic modulus and charge (Fig. 2) [17, 18, 61]. In a broader context, these different parameters ultimately determine two critical objectives: 1) specifying the biodistribution of nanomaterials so that they reach their intended cellular targets following administration and 2) controlling the release of transported payloads from nanomaterials to achieve the desired intracellular responses. With the plethora of new nanomaterials being developed, we find these two essential criteria to be often overlooked. We therefore discuss below the influence of nanomaterial properties on biodistribution and intracellular delivery.

### 2.2 Biodistribution

Modulation of inflammation for the purpose of altering and reversing the progression of atherosclerosis can occur through two different, and not mutually exclusive, biodistribution paradigms. First, systemic inflammation can be altered by targeting the high concentrations of immune cells residing within secondary lymphoid organs, mainly the lymph nodes and the spleen. Second, nanomaterials can be targeted to either the plaques themselves or to immune cells that are eventually destined to traffic to the plaques, such as  $LyC6^{hi}$  monocytes. In both cases, subcutaneous administration is likely to deliver the nanomaterials first to lymph nodes [62]. Intravenous administration can permit targeting of atheroma, but

would result in clearance of the vast majority of nanomaterials by the mononuclear phagocyte system (MPS) [63]. Clearance via MPS can occur within the blood and interstitial space [63], or through filtration and phagocytosis within the spleen, liver [64], and kidneys [65, 66]. Surface modification of nanomaterials by PEGylation is a common practice to reduce such clearance [67], via the ability of dense poly(ethylene glycol) layers to resist protein adsorption [68], but regardless of the surface modification, the vast majority of intravenously injected nanomaterials are still cleared by the MPS [14]. In the case of delivery to plaque sites, nanomaterials will need to transit across the endothelial lining of the blood vessel to access the arterial intima (Fig. 1). As plaques often possess dysfunctional endothelium, some nanomaterials may passively enter into the arterial intima directly, without transcytosis through endothelial cells. Additionally, in more advanced plaques, significant hypoxic conditions results in the expansion of microvessels (known in healthy contexts as vasa vasorum) into the plaque. This angiogenesis results in poorly structured vessels, which can also allow for the leakage of nanomaterials into the atherosclerotic plaques [69]. Beyond passive targeting, nanomaterials can also be surface functionalized to target receptors specific to dysfunctional endothelium, thus increasing their opportunities for diffusion into the plaque, uptake by phagocytic cells, or transcytosis by endothelial cells [70–72].

### 2.3 Mechanism of cellular uptake and intracellular delivery

In order to enter into cells, nanomaterials can be engineered to exploit several cellular mechanisms available for the uptake of extracellular materials. Detailed descriptions of these processes can be found in numerous recent reviews [73–75], and here we summarize several mechanisms that have been used for the targeting of proatherogenic inflammatory cells. Macropinocytosis, wherein cells constitutively engulf extracellular fluids, can allow internalization of nanomaterials in a non-targeted fashion [76]. Caveolar-mediated, clathrin-mediated, and alternative endocytosis pathways appear to have size restrictions of approximately 100 nm or less, and often require receptor recognition to allow for internalization [77, 78]. Phagocytosis is the most size-permissive, capable of internalizing particles as large as or larger than the cell, though it also requires receptor recognition [79]. While all cells are capable of endocytosis, antigen presenting cells are particularly adept at macropinocytosis and phagocytosis [80]. Nanomaterial surface charge can further enhance these membrane interactions and improve uptake. Due to the negative charge of cell membranes, positively charged nanomaterials can be attracted to, adhere to, or transit through the plasma membrane [81]. This benefit is to be weighed against the apparent cytotoxicity of cationic nanomaterials, polymers, and lipids [82]. Still, surface conjugation of nanomaterials with targeting moieties remains the most specific and promising route. Nanomaterials can be coated with whole antibodies, but smaller ligands such as individual Fab domains or single domain nanobodies have increasingly become more prevalent [46, 83–85]. A recent study using poly(lactic-co-glycolic acid) (PLGA) nanomaterials compared different monoclonal antibodies, each specific for a different DC surface marker, and all targeting moieties were found to improve T cell responses elicited by PLGA nanoparticles [86].

### 3. Nanomaterials to enhance the imaging of atherosclerosis

#### 3.1 Overview of imaging modalities utilizing nanomaterials

The clinical diagnosis and staging of atherosclerosis currently relies on indirect markers of disease, some of which only become apparent or detectable when mid-to-late plaques are prevalent. As evidence grows that early intervention could be a promising way to combat the disease, it is increasingly important that detection and staging methods are developed for the purpose of accurately assessing disease state [87]. There are a number of imaging technologies currently used clinically, namely: positron emission tomography (PET), x-ray computed tomography (CT), near-infrared fluorescence (NIRF), and magnetic resonance imaging (MRI). Due to their ability to achieve targeted delivery of often low solubility fluorophores and contrast agents, nanomaterials have recently found extensive use for the enhancement of NIRF and MR imaging.

NIRF imaging is of growing interest within the medical and research communities. Optical imaging typically fails *in vivo* due to poor tissue penetrance at visible wavelengths and high autofluorescence of tissues, with NIRF sidestepping both issues due to the properties of near-infrared wavelengths. NIRF is a high sensitivity technique, with relatively poor resolution. A variety of different nanomaterial-based delivery systems have been developed for NIRF, including fluorochrome-labelled PEG, dextran, silica and other polymeric nanomaterials [88, 89] as well as surface-stabilized quantum dots [90, 91]. Lobatto et al. recently correlated MRI images of vascular permeability in atherosclerotic lesions with the uptake of liposomal nanoparticles conjugated to the NIRF dye Cy7 both *in vivo* and *ex vivo* [69]. This important study demonstrated that microvessels within the vasa vasorum could permit nanomaterial accumulation within atheromas. The decreased toxicity and stable signal from PEGylated quantum dots have also found utility for imaging of microvessels. The functional microcirculation deep in mouse hind limb skeletal muscle was successfully imaged by laser scanning multiphoton microscopy and fluorescent 655-nm 5000-MW methoxy-PEGylated quantum dots [92].

MRI provides high resolution imaging of vessels and tissues, but has relatively low sensitivity compared to PET and NIRF. Magnetic nanomaterials are commonly used to visualize tissues and organs and typically have a magnetic core with a hydrophilic surface coating [93]. The magnetic core is usually composed of magnetite ( $\text{Fe}_3\text{O}_4$ ) and maghemite ( $\gamma\text{-Fe}_2\text{O}_3$ ) with a general formula of  $(\text{FeO})_{1-n}(\text{Fe}_2\text{O}_3)_n$  [94], and often solubilized with hydrophilic polymers [95]. Additionally, conjugation or loading of nanomaterials with chelated gadolinium ions (Gd) can enhance contrast during MRI [96]. The *in vivo* stability of magnetic NP has been improved by different methods, including dense packing and size-shape design [97]. The ability of ultra-small superparamagnetic iron oxide particles (USPIO)-enhanced MRI was shown to differentiate benign, inflammatory from malignant lesions [98]. Spatiotemporal mapping of SPIO nanoparticles by MRI QSM provided a reliable, rapid, non-invasive method for identifying organ-specific inflammation [99]. USPIO-enhanced MRI is feasible for *in vivo* assessment of vascularity and macrophage content in atherosclerotic carotid plaques, determining an association of these potential imaging biomarkers with plaque vulnerability [100]. Superparamagnetic iron oxide



nanoparticles (SPIONs) as contrast agents for MRI of inflammatory processes are useful for the *in vivo* MRI detection of macrophage infiltration [101].

### 3.2 Dual modality imaging

While the above-mentioned imaging techniques have been successfully used alone, they are often combined for multimodal imaging, which leverages the differences in sensitivity and resolution between several distinct imaging modalities. Common combinations include PET-CT, PET-MRI, and MRI-NIRF, though other combinations have proven useful [102, 103]. These combined imaging techniques work best if both sets of imaging probes/contrast agents have similar pharmacodynamic properties, a task made easier through conjugation to or incorporation in nanomaterials [104, 105]. Cellular fluorescence and MRI of vascular inflammation was achieved with a multi-functional high-relaxivity platform composed of iron-cobalt (FeCo) nanoparticles with Cy5.5-conjugated graphitic-carbon (GC) shells. [106]. These FeCo/GC nanoparticles were found to accumulate in inflammatory vascular macrophages *in vivo*. Vascular cell adhesion molecule 1 (VCAM-1) is up-regulated in numerous inflammatory processes, including early atherosclerosis. To localize and quantify VCAM-1 expression, VCAM-1 targeted liquid perfluorocarbon nanobeacons with <sup>19</sup>F fluorine in their cores were designed for both MR spectroscopy and imaging [107]. Additionally tobacco mosaic virus nanoparticles engineered to target VCAM-1 were labeled with both Cy5 and chelated Gd ions for dual MRI/NIRF imaging of atherosclerotic plaques [96]. Hybrid PET/MRI of inflammatory leukocytes in murine atherosclerotic plaques was achieved using 13 nm dextran nanoparticles labeled with zirconium-89 and NIRF fluorochrome VT680 [108]. Modulation of macrophage recruitment with RNAi-delivering nanoparticles verified that this technique could assess atherosclerotic inflammation.

## 4. Nanomaterials for gene therapy

### 4.1 Nucleic acid delivery via nanomaterials

Significant therapeutic potential exists in the controlled delivery of nucleic acids to modulate the expression of inflammatory mediators, either through transient modulation or permanent gene therapy. In both cases, the delivery of nucleic acids to target cells is required. Furthermore, the use of nucleic acids in translational medicine has a number of barriers that must be overcome. Therapeutically-relevant nucleic acids are readily degraded in the extracellular space by nucleases [109], and show poor tissue penetrance. They are difficult to functionalize with targeting moieties, and are poorly endocytosed by cells [110]. Once endocytosed, nucleic acids are sequestered and degraded within the endosome and lysosome, and effective therapeutic use would require endosomal escape and translocation into either the cytosol or nucleus. All of these difficulties can be resolved through the use of nanocarriers - viral, liposomal, polymeric, or otherwise. Nucleic acids are typically negatively charged due to their phosphate backbone, and thus many of the most effective nanocarriers are composed of cationic lipids or polymers. The benefits of nanomaterial-based delivery of nucleic acids are espoused in a number of reviews and include protection from nucleases, targeted delivery, and enhanced cytosolic availability [111–114].

## 4.2 Gene therapies targeting monocytes and macrophages

There has been considerable success in targeting nanomaterials carrying siRNA to monocytes and macrophages. siRNA targeting of mitogen-activated protein kinase kinase kinase 4 (Map4k4) resulted in reduced TNF- $\alpha$  and IL-1 $\beta$  secretion from macrophages systemically. This feat was all the more impressive as the nucleic acids were delivered orally, packaged within glucan nanoparticles [115]. Hyaluronin-stabilized liposome-based nanoparticles were targeted to leukocytes via surface-conjugated monoclonal antibodies against  $\beta$ 7 integrin for the delivery of cyclin D1 siRNA. These cells were blocked from proliferation, and agonist failed to result in increased secretion of type 1 cytokines, such as IL-2 and TNF- $\alpha$  [116]. Therapeutic regression of plaques can be improved by monocyte recruitment, which can shift the balance of lesion resident macrophages in favor of the non-inflammatory M2 phenotype. A seminal paper by Leuschner et al. demonstrated the systemic silencing of CCR2 within monocytes using siRNA-loaded nanoparticles, which resulted in a reduction of atherosclerotic lesion size [117]. Although prophylactically effective at limiting the progression of atherosclerosis, systemic silencing of CCR2 disrupts normal monocyte function and can potentially limit plaque regression during treatment for CVD [56].

Several nanomaterial formulations have taken advantage of the inherent phagocytic activity of macrophages for the *in vivo* delivery of microRNA mimics (miRNA or miR) and anti-miRNA (antagomir or antimir). Systemic delivery of liposomally encapsulated miRNA-181b mimics protected ApoE $^{-/-}$  mice from atherosclerosis [118]. miRNA-155 is notable in that it has clinical relevance in both cancer and atherosclerosis. Antagomir introduced into atherosclerotic mice via tail vein injection resulted in a decrease in plaque size [119]. Antagomir has yet to be encapsulated within nanocarriers that are targeted to specific cells within atherosclerotic plaques, though PLGA-encapsulated antagomir was tested as a lymphoma therapy [120]. That miR-155 is an important modulator in a number of cell types is further evidence that antagomir treatment should be carefully targeted to relevant cells to avoid off-target effects following systemic administration. miRNA-146a, another frequently discussed miR, reduces macrophage activation when delivered *in vivo* within liposomes [121]. Pluronic encapsulation and systemic introduction of antagomir to miRNA-342-5p suppresses atherosclerosis by reducing the number of macrophages and smooth muscle cells in plaques [122].

## 4.3 Gene therapies targeting vascular endothelium and vascular smooth muscle cells

The vascular endothelium provides a number of signals critical for the initiation and propagation of atherosclerotic inflammation. Dysfunctional endothelium is an early marker for atherosclerosis [123], and the endothelium both expresses TLRs [124] and secretes inflammatory cytokines [125]. As such, modulation of these signals could be an effective option for the treatment of CVD. Liposomes have been targeted to the vascular endothelium using conjugated antibodies specific for VCAM-1, and successfully delivered siRNA against VE-cadherin to activated endothelial cells *in vivo* [126]. Most reports of miR and antagomir delivery to endothelial cells involve *in vitro* experiments or systemic application of non-encapsulated nucleic acids. For example, *in vitro* application of miR-221 to human umbilical vein endothelial cells resulted in inhibition of expression of AdipoR1. This resulted in



increased activity of NF- $\kappa$ B and an increased inflammatory response, while decreasing endothelial nitric oxide production [127]. The delivery of antagomir to miR-92a via retroorbital injection in atherosclerotic mice resulted in reduced endothelial inflammation and reduced plaque size [128]. In human atherosclerosis, smooth muscle cells (SMCs) in the arterial intima are some of the first cells to develop into foam cells [129]. Additionally, SMCs play a considerable role in the remodeling of the extracellular matrix in the developing atherosclerotic plaque, which can result in plaque destabilization and rupture. [130]. To combat plaque destabilization, matrix metalloproteinase-2 siRNA was encapsulated in polymeric nanostructures and was found to inhibit SMC migration *in vitro*, suggesting the potential to reduce atherosclerosis and restenosis [131].

## 5. Nanomaterials for immunotherapy against atherosclerosis

### 5.1 Cellular targets for immunotherapy

The critical roles of both innate and adaptive immunity during inflammation suggest immunotherapy as a potential component of effective treatment regimens for CVD [132, 133]. Immunotherapies have primarily focused on manipulating APCs with an emphasis on DCs, which have been shown to influence the maturation and homeostasis of atheromas [41–43]. Furthermore, DCs have evolved to interact with viruses and can thus be targeted and manipulated by nanomaterials that mimic viral structures and functions [134–137]. Notably, the specific functions of DCs depend on their subset, as some are proatherogenic [43] and others atheroprotective [42]. Plasmacytoid DCs (pDCs) have a particularly dichotomous role, serving as the primary source of proatherogenic type I interferons (IFN) [138–141] while also playing key roles in the activation of regulatory T cells (Tregs) that stabilize plaques and prevent rupture [142, 143]. Inflammation induced by pDCs is an essential first step during immune responses to viral infection in both humans and mice, and a link between viral infection and atherosclerosis has long been suspected [144–146]. Interestingly, both viral- and plaque-derived factors stimulate TLRs within pDCs [58] and promote atherosclerosis through mechanisms suspected to involve natural killer T (NKT) cells [23, 147, 148]. Influences of bacterial infection have also been implicated, and *mycobacterium bovis* BCG vaccination was recently found to promote an atheroprotective immunoregulatory profile in atherosclerotic mice via stimulation of pDCs [149]. These findings support targeting the immune cell component of atherosclerosis, via either vaccination or induction of tolerance, to be a viable strategy [150]. Relatively few attempts have been made to engineer nanomaterial delivery systems to enhance atheroprotective vaccination or immunotherapies, but recent work suggests that such strategies are rapidly gaining interest.

### 5.2 Vaccination against heart disease

Vaccines for the treatment of CVD have mainly focused on injections of antigens within oxLDL, primarily oxidized phospholipids and peptide fragments of apoB-100 [151]. With up to a 70% decrease in atherosclerosis being reported in some studies, vaccination has proven to be a promising approach. Although not completely understood, the mechanism behind the atheroprotective effect of vaccination against oxLDL has been strongly linked to antibody generation [152]. As in standard vaccination, packaging of the antigen and

adjuvant is critical, making nanomaterials a versatile platform for delivery. For example, chitosan nanoparticles served as carriers for plasmids during DNA vaccination against cholesteryl ester transfer protein (CETP) and were found to attenuate atherosclerosis in rabbits [153]. Another vaccine, HB-ATV-8, targets CETP as well, though it utilizes a synthetic peptide derived from CETP as the antigen, and is packaged within a lipid formulation [154, 155]. This nanoparticle vaccine was shown to be effective in preclinical trials, and is entering phase 1 development. Despite the demonstrated atheroprotective effects of parenteral immunization with native LDL or LDL-derived peptides and the established benefit of using nanoparticle vehicles during immunization, few other groups have reported nanomaterial-based vaccines for CVD.

### 5.3 Immunomodulation of inflammatory cells in atherosclerosis

Nanomaterial-based immunomodulation of inflammatory cell populations during CVD has primarily been achieved by enhancing the delivery of statins. In addition to hindering cholesterol biosynthesis and Rho-associated kinase (ROCKs) activity, statins also decrease cell-mediated inflammation by inhibiting 3-hydroxy-3-methylglutaryl-coenzyme-A (HMG-CoA) reductase [156, 157]. Recent findings have demonstrated the benefits of the controlled delivery of statins via nanomaterials, which permit better targeting of plaque-resident inflammatory cells as well as minimizes toxic side-effects in the liver [158, 159]. Duivenvoorden et al. developed recombinant HDL nanoparticles loaded with simvastatin that both decreased the number of plaque-resident macrophages as well their expression of genes related to inflammation [158]. Depending on the dosage, these particles were found to inhibit progression of plaque inflammation as well as decrease inflammation in advanced atheromas. But statin delivery is only the tip of the ice berg for nanomaterials in the area of immunomodulation. As evidenced by their stimulation of immune cells during vaccination against infectious diseases, cancer immunotherapy and the induction of tolerance, nanomaterials can be engineered to target and modulate a variety of different immune cell populations. Such tools may have great potential for the treatment of CVD, but have yet to be adequately explored.

## 6 Future directions and conclusions

Recent advances in nanotechnology and immunology now permit the rational design of targeted nanomaterials for the therapeutic and diagnostic probing and modulation of key inflammatory cell populations contributing to CVD (Table 1). Multi-disciplinary approaches that incorporate nanomaterials into the investigation of basic biochemical and cellular mechanisms contributing to atherosclerosis may reveal novel therapeutic targets. Atherosclerotic inflammation is influenced by cytokines expressed systemically by APCs located in distal organs such as the spleen as well as by factors released locally within lesions by monocyte-derived macrophages and foam cells. Neither of these inflammatory sources is sufficiently addressed by the current clinical approaches to the treatment of CVD.

A critical weakness of current therapies for atherosclerosis is cell-mediated inflammation [11], suggesting immunotherapy as a promising new approach to treatment [150]. As discussed above, nanomaterial-enhanced vaccination and immunotherapy are up-and-

coming untapped areas in need of further development. Such strategies will require new platforms engineered to target plaque-resident inflammatory cells, and several groups have recently developed nanomaterials for improved targeting of macrophages within lesions. Dextran nanoparticles were found to target macrophages within lesions because of the higher prevalence and activity of phagocytic cells within plaques and sites of inflammation [108, 160]. Synthetic HDL nanoparticles composed of PLGA and cholesteryl oleate with a triphenylphosphonium cation-decorated phospholipid bilayer coat were investigated by Marrache et al., and these HDL mimics were found to both target macrophage mitochondria and decrease lipid levels [161]. Sanchez-Gaytan et al. synthesized hybrid lipoprotein/PLGA nanoparticles capable of both drug delivery to and imaging of plaque-resident monocytes [162]. A smart nanosystem was recently shown by Oh et al. to be capable of photothermal ablation of inflammatory macrophages in high-risk plaques [163].

A majority of current research focuses on the major inflammatory players in atheromas: endothelial cells, macrophages/monocytes, dendritic cells, and smooth muscle cells. Consequently, nanomaterials designed to target the diversity of other immune populations contributing to plaque progression and instability are lacking and should be an objective of future strategies. Neutrophils and T cells are thought to play an important role in the secretion of factors within the atherosclerotic microenvironment. Neutrophils, which largely promote atherogenesis and plaque destabilization, aid in the activation of macrophages within the atheroma through the cellular release of neutrophil extracellular traps (NETs) [164]. Nanoparticles have been engineered to target neutrophils, including drug-laden albumin-polystyrene nanoparticles, which were able to reduce the adhesion of neutrophils to endothelium *in vivo*. [165] This result has implications for the treatment of CVD, as reduced numbers of recruited neutrophils could result in decreased macrophage activation and lower downstream numbers of recruited inflammatory cells, such as T<sub>H</sub>17 cells. T<sub>H</sub>17 cells, along with T<sub>H</sub>1 cells, are known to also contribute to atherogenesis through the secretion of pro-inflammatory cytokines. Nanoparticles can be targeted to T cells using surface-conjugated antibodies, such as PLGA nanoparticles with anti-CD4 antibodies. These nanoparticles, loaded with LIF, were found to shift CD4<sup>+</sup> T cells toward T<sub>Reg</sub> development and away from T<sub>H</sub>17 development *in vitro* and *in vivo* following *ex vivo* pretreatment of T cells [166]. Another potential source of atherosclerotic inflammation is NKT cell activation due to heightened type I interferon (IFN) levels [23, 147, 148]. TLR9-induced type I IFN, IL-12, and IL-18 expression from pDCs is essential for NK cell function during inflammation [167, 168] and may activate and recruit invariant NKT (iNKT) cells [23, 169–171], which are proatherogenic immune cell population within lesions [23]. Although the biology of NKT cells is poorly characterized and their role in the progression of atherosclerosis remains controversial, they may emerge as promising targets for future immunotherapies against CVD.

In addition to using imaging modalities to assess plaque progression and vulnerability, future approaches will utilize nanomaterials to enhance other techniques for early detection. For example, proteomics has shown promise for the identification of molecular indicators of disease, and nanomaterials can enhance purification and isolation of these markers from tissues and fluids. Stubiger et al. recently examined superparamagnetic Fe<sub>3</sub>O<sub>4</sub> nanoparticles of diverse surface chemistries that facilitate sample preparation for detection of oxidized

phospholipids using MALDI-MS [172]. Nanoparticles with dimethylamine-co-epichlorhydrine-co-ethylenediamine functionalized surfaces allowed quantification of increased levels of oxidized phospholipids in the plasma of ApoE<sup>-/-</sup> mice fed high fat diets.

Current clinical treatments for atherosclerosis focus on lowering serum levels of low-density lipoprotein (LDL) using therapeutics such as statins, administration of antithrombotic drugs, and surgical intervention. As with many complex pathologies, a single treatment strategy is not sufficient, and CVD remains the leading cause of death worldwide. With the aid of nanomaterials, novel approaches have recently emerged that permit targeting, imaging and manipulation of inflammatory cells within atherosclerotic lesions. There are early reports of clinical success in the use of nanoparticles for applications related to atherosclerosis. Work using silica-gold nanoparticles administered via an on-artery patch and plasmonic photothermal therapy has been demonstrated to reduce plaque burden in a 180-individual clinical trial [173]. This trial also utilized an alternative delivery mechanism in the form of magnetic microbubbles that were targeted to lesions. Pre-clinical work on the intravenous delivery of proresolving peptide Ac2-26 attached to PLGA-PEG nanoparticles has been promising, showing an increase in atheroprotective properties and a decrease in oxidative stress and necrosis in LDLR<sup>-/-</sup> mice [174]. Successful future therapies will likely combine the aforementioned clinically tested strategies with new approaches that focus on controlling cell-mediated inflammation. Rationally engineered nanomaterials have already demonstrated efficacy as platforms for cancer immunotherapy and vaccination against infectious diseases, and similar strategies may provide new opportunities for the treatment of CVD.

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### LAY SUMMARY

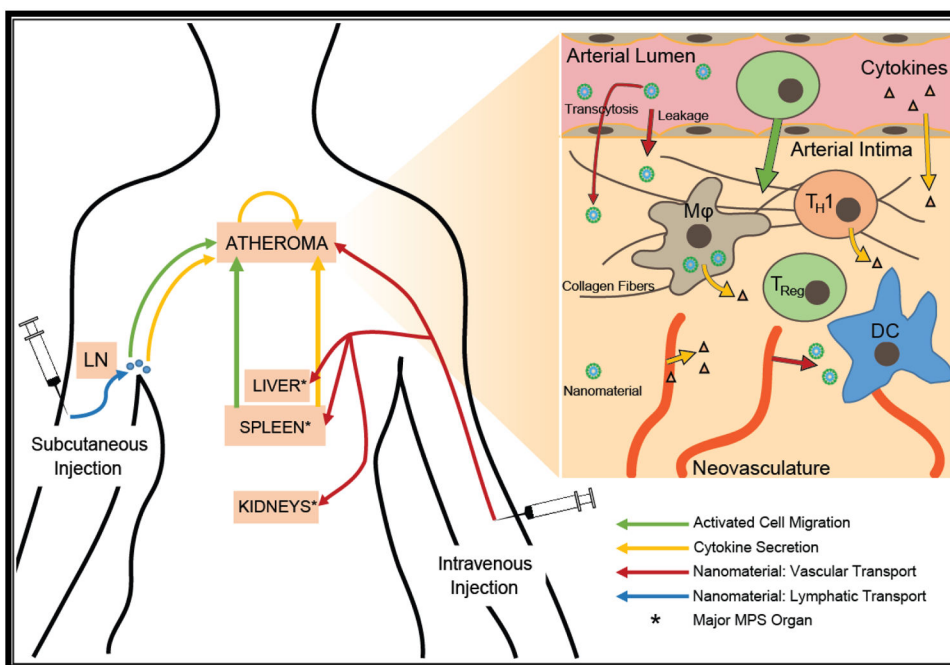
Cardiovascular disease (CVD) continues to be the leading cause of death in the developed world and is a considerable economic burden. The underlying mechanism of most CVD is atherosclerosis, an inflammatory condition characterized by cellularly complex plaque deposits within arterial vessel walls. Therapies that adequately address cell-mediated inflammation and diagnostic tools for early detection of vulnerable plaques are critical weaknesses of current clinical strategies for treating CVD, and nanomaterials have been engineered to uniquely address both of these needs. Here, we review recent progress in the use of nanomaterials designed to enhance imaging and therapeutic intervention of atherosclerotic inflammation.

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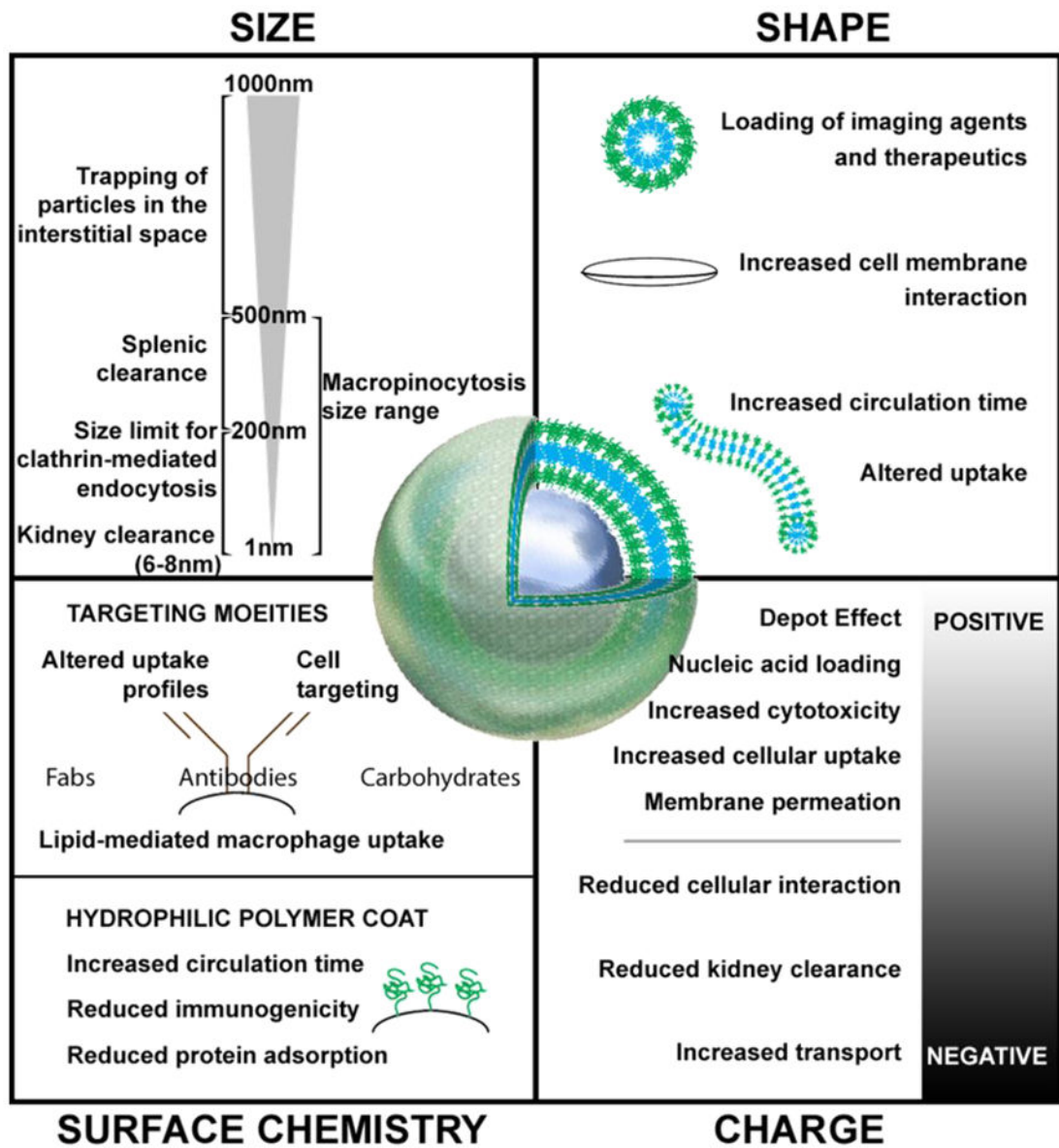
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**Figure 1. Comparison of subcutaneous and intravenous routes of administration for nanomaterial-based treatment of atherosclerotic lesions**

Choice in route of administration may alter nanomaterial biodistribution and the resulting immunomodulation. Subcutaneous injection is most likely to target nanomaterials to nearby lymph nodes, where they are internalized and processed by antigen presenting cells (APCs). These cells may then activate other immune cells, promoting their trafficking to the atheroma and/or secretion of cytokines that can elicit systemic pro- or anti-inflammatory responses. Intravenous injection is more likely to result in targeting of the atheroma directly for local imaging or modulation of inflammation, but the majority of nanomaterials may be lost to phagocytic cell populations within the major organs of the mononuclear phagocyte system (MPS): the liver, spleen, and kidneys. As a secondary lymphoid organ, the spleen is a major site of nanomaterial uptake and processing by APCs. These splenocytes may activate other immune cells and/or secrete cytokines systemically, both of which can alter atheroma development and progression remotely.



**Figure 2.** Size, shape, charge, and surface chemistry are important variables to consider when designing nanomaterials for the modulation of inflammatory cells.

Table 1

Summary of nanomaterials designed to image or modulate atherosclerotic lesions.

Nanomaterial	Cells Targeted	Targeting Moiety	Cargo	In Vivo Therapeutic Effect	Reference
<b>Gene Therapy</b>					
POPC, SAINT-C8, Chol, DSPE-PEG2000, DSPE-PEG2000-Mal	Endothelial Cells	Anti-(E-selectin) and Anti-(VCAM-1) Antibodies	VE-cadherin siRNA	Untested. In vitro reduction of VE-cadherin expression in primary endothelial cells	121
$\beta$ 1,3-D-glucan, PEI	Macrophages	Dectin-1 mediated phagocytosis	TNF- $\alpha$ siRNA or Map4k4 siRNA	Cellular mRNA silencing and reduced TNF- $\alpha$ serum levels	110
Hyaluronan, DPPE, PC, Chol	Gut mononuclear leukocytes	anti-( $\beta$ 7 integrin) antibody	Cyclin D1 siRNA	Alleviated intestinal inflammation in DSS-induced colitis	111
Lipid C12-200, DSPC, Chol, PEG-DMG	Ly-6C <sup>high</sup> monocytes and macrophages	None	CCR2 siRNA	Reduction in myocardial infarct size, inflammatory atherosclerosis, tumor-associated macrophages. Prolongs islet graft survival	112
Lipofectamine 2000	Peripheral blood mononuclear cells	None	miR-181b mimics	Protection against atherosclerosis in ApoE <sup>-/-</sup> mice, inhibition of NF- $\kappa$ B signaling, reduced vascular inflammation	113
PLGA	pre-B-cell lymphoma cells	Penetratin	Anti-(miR-155) PNA	Diminished tumor growth	115
Lipofectamine 2000	Ly-6C <sup>high</sup> monocytes and macrophages	None	miR-146a mimics	Reduction in TNF- $\alpha$ mRNA levels, attenuated atherosclerosis	116
DA-PEI1.8	Smooth muscle cells	None	MMP-2 siRNA	Untested. Reduced MMP-2 expression in vitro in smooth muscle cells	125
<b>Imaging</b>					
Dextran-coated iron oxide	Monocytes and macrophages	Dextran, particle size	Fluorine-18, VT680 fluorochrome	PET-CT detection of monocytes and macrophages within the aneurysmatic wall	154
PEI, Core-shell fluorescent silica nanoparticles	Cos7 cells	None	CMV-luc plasmid DNA	Untested. Cytoplasmic escape of plasmid DNA and fluorescent nanoparticles	84
CuS, ZnS, TOPO, PEG-PE	MCAIV tumor cells	TAT	Quantum dot	Successful uptake of nanoparticles by MCAIV tumor cells	85
NaYF4 nanoparticles, polyglutamic dendrons	None: Vascular Mapping	None	None	Mapping of cortical vasculature down to 400 $\mu$ m below tissue surface	86
FeCo/Graphitic-Carbon, DSPE-PEG	Macrophages	None	Cy5.5	In situ and ex vivo fluorescence and in vivo MRI detection of	101

Nanomaterial	Cells Targeted	Targeting Moiety	Cargo	In Vivo Therapeutic Effect	Reference
Tobacco mosaic virus-based nanorods	None; VCAM-1 detection	VCAM-1 targeting peptide	Cy5, Gd(DOTA)	Ex vivo detection of plaques from ApoE <sup>-/-</sup> mouse aorta	91
CE, glycerine, lissamine rhodamine B sulphonyl, MPB-PEG-DSPE	None; VCAM-1 upregulation detector	VCAM-1 targeting peptide	Rhodamine	Successful detection of upregulated VCAM-1 expression in ApoE <sup>-/-</sup> mouse kidneys	102
Methoxy PEG5000	None; microvascular imaging	None	Quantum dots	In vivo imaging of microvascular units, LPS induction results in non-specific uptake by phagocytes in vivo	87
Epichlorohydrin-crosslinked dextran	Monocytes and macrophages	None	VT680, zirconium-89	In vivo PET/MRI detection of monocyte/macrophage infiltration of atherosclerotic plaques	103
SPIO nanoparticles	Macrophages	None	None	In vivo detection of macrophage infiltration in aortic walls	95
<b>Immunomodulatory</b>					
Chitosan	Microfold cells	None	CETP plasmid DNA	Slowing down the formation of atherosclerotic plaques	147
PC, lyso-C <sub>12</sub> PC, LPA	Antigen presenting cells	None	CETP (CHLLVDFLQSL) peptide	Reduced liver lipid-associated histopathology, reduced atherosclerosis	148, 149

POPC = 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; Chol = cholesterol; DSPE-PEG2000 = 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-(polyethylene glycol)-2000; DSPE-PEG2000-Mal = 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000]; PEI = polyethyleneimine; DPPE = 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine; PC = phosphatidylcholine; DSPC = Distearoylphosphatidylcholine; DA-PEI1.8 = deoxycholic acid - polyethyleneimine; TOPO = tri-n-octylphosphine oxide; PEG-PE = poly(ethylene glycol) phosphatidylethanolamine; SPIO = superparamagnetic iron oxide; DOGS-NTA = 1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] nickel salt; CE = perfluoro-15-crown-5-ether; MPB-PEG-DSPE = N-[(w-[4-(p-maleimidophenyl)butanoyl]amino)poly(ethylene glycol)2000]1,2-distearoyl-sn-glycero-3-phosphoethanolamine; lyso-C<sub>12</sub>PC = 1-lauroyl-2-hydroxy-sn-glycero-3-phosphocholine; LPA = 1-oleoyl-2-hydroxy-sn-glycero-3-phosphate; CETP = cholesteryl ester transfer protein