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Nanoengineering Apolipoprotein A1-Based Immunotherapeutics

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In the slipstream of targeting the adaptive immune system, innate immunotherapy strategies are being developed. In this context, technologies based on natural carrier vehicles that inherently interact with the innate immune system, are increasingly being considered. Immunoregulatory nanotherapeutics based on natural apolipoprotein A1 (apoA1) are discussed here. This protein is a helical, amphipathic macromolecule and the main constituent of high-density lipoprotein. In that capacity, apoA1 interacts specifically with innate immune cells, such as monocytes and macrophages, to collect and transport lipophilic molecules throughout the body. Exactly these unique features make apoA1 a compelling elementary constituent of biocompatible self-assembled nanotherapeutics. Such apoA1-based nanotherapeutics (A1-nanotherapeutics) can be engineered and functionalized to induce or mitigate an innate immune response or to orchestrate an adaptive immune response through antigen delivery to dendritic cells. The authors first discuss apoA1's properties and how these can be exploited to generate libraries of A1-nanotherapeutics using advanced manufacturing approaches such as microfluidics or continuous flow methods. Using high-throughput in vitro screening methods and in vivo imaging to identify promising formulations are then recommend. Finally, three distinct immunotherapy strategies are proposed to effectively treat a variety of diseases—including cancer, infection, and cardiovascular disease—and promote allograft survival in transplantation.

1. Introduction

Although the majority of immunotherapies currently being developed engage the adaptive immune system, targeting the innate immune system is a compelling strategy for treating diseases in which dysregulated inflammation plays a role. Due to the phagocytic properties of many innate immune cells, nanomedicine holds tremendous potential to become a key in immunomodulatory therapy. When designing these nanoimmunotherapeutics, nature can provide a blueprint. In the human body, lipids—such as triglycerides and cholesterol esters—are transported by nanostructures called lipoproteins, mainly composed of phospholipids and apolipoproteins. Owing to their natural origin, lipoproteins are a unique template for designing next-generation nanotherapeutics: they are biocompatible and can cross biological barriers. Here we focus on high-density lipoprotein (HDL), the main protein component of which is apolipoprotein A1 (apoA1). HDL transports lipophilic molecules, originating from innate immune cells, among others, to the liver. It can

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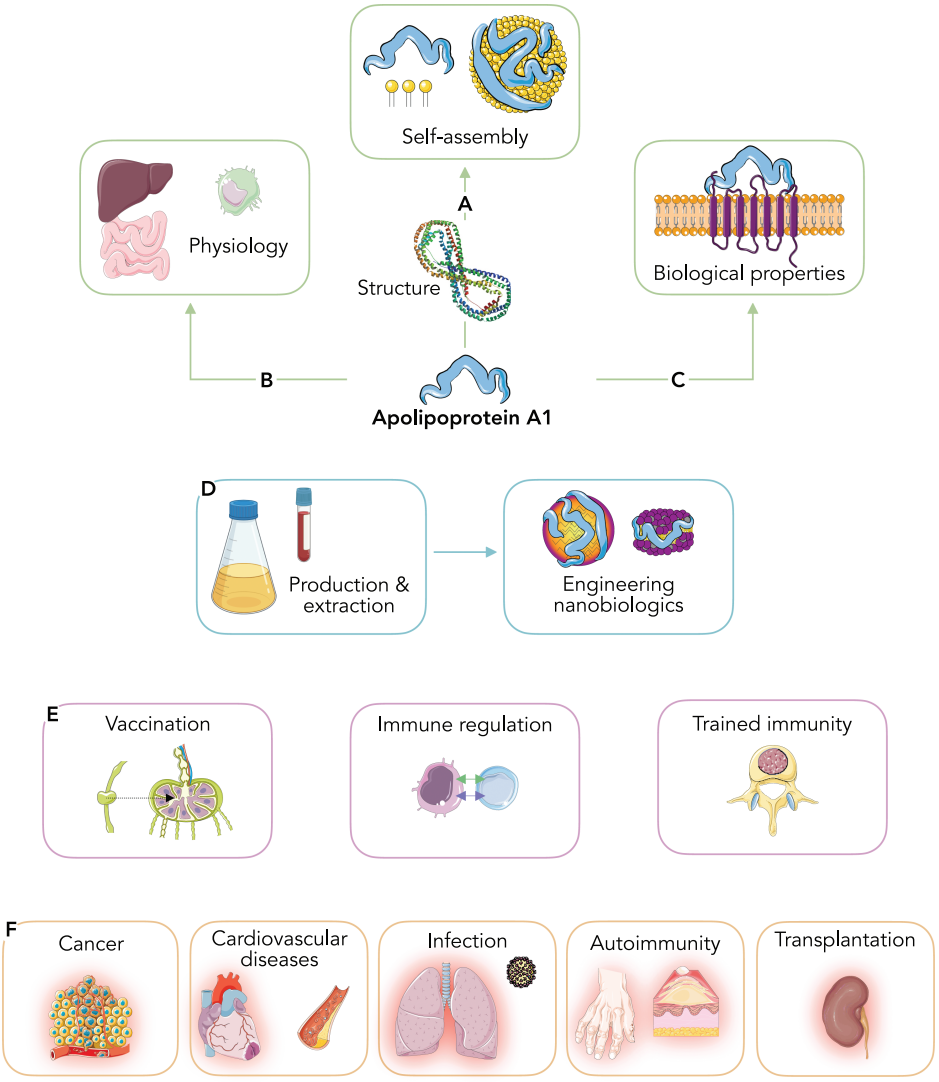

Figure 1. Overview of the chemical, physiological, and biological properties of apolipoprotein A1 (apoA1), and its function as an engineerable therapeutic platform to treat diseases. A–C) Properties and function of apoA1. D) Production and engineering of A1-nanomaterials. E) A1-nanotherapeutics as platform for immunotherapy. F) Treating diseases using A1-nanotherapeutics.

do so because of the binding of apoA1 to receptors on its target cells. These targeting, binding, and transportation capacities make apoA1 a foundational building block for immunoregulatory nanotherapeutics (A1-nanotherapeutics). Due to the endogenous character of apoA1-based nanomaterials (A1-nanomaterials), they have the ability to integrate in the natural pool of plasma lipoproteins. This means that they are very well tolerated and biocompatible, which allows for high dosing without adverse effects. In addition, their affinity for receptors expressed on the surface of myeloid cells promotes the targeting of these nanotherapeutics to the innate immune cell compartment.

While conventional nanomedicines are designed to evade the innate immune system, novel A1-nanotherapeutics deliberately engage myeloid cells to induce or mitigate a specific immune response. Because of these features, A1-nanotherapeutics can be applied to treat a great variety of diseases. Here, we will provide an overview of A1-nanomaterials' characteristics, engineering, and potential for treating immune-mediated diseases (**Figure 1**).

2. Physicochemical Properties of ApoA1

ApoA1 is a 28 kDa protein made up of several amphipathic α-helical repeats. These helices comprise 11 or 22 amino acids and are often separated by a proline (**Figure 2**A). One face of each helix is enriched in hydrophilic amino acids, whereas the opposite face is enriched in hydrophobic amino acids. The hydrophobic area interacts with phospholipid acyl chains in HDL and the hydrophilic amino acids interact with the aqueous environment.[1] The two faces of the apoA1 helices are separated by positively charged amino acids, such as lysine and arginine, which are proposed to interact with negatively charged phospholipid head groups, thereby anchoring apoA1 into HDL particles.[2]

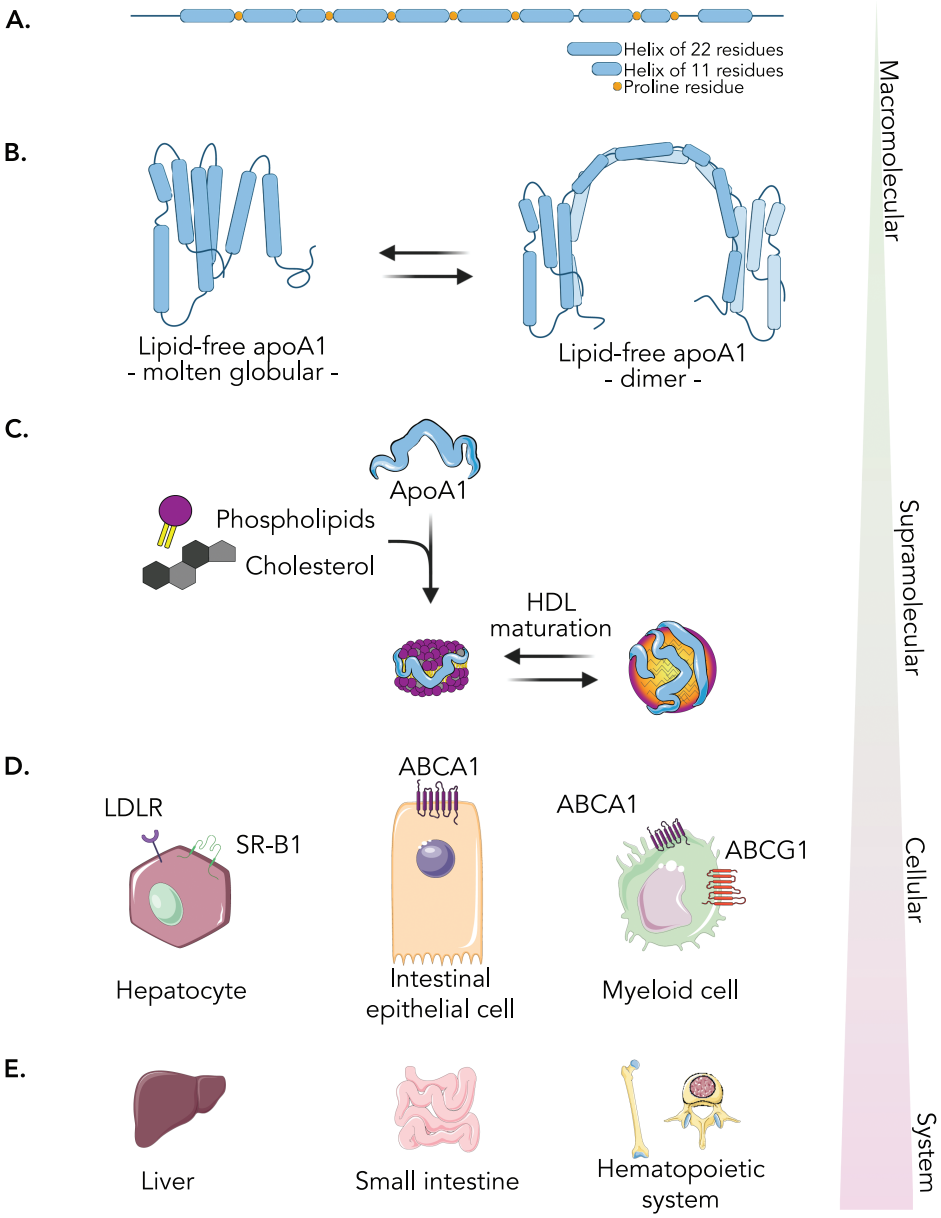

Figure 2. Natural properties of apoA1. A) Representation of apoA1's secondary structure. ApoA1 is composed of 12 alpha helices, often separated by a proline residue. B) Schematic representation of apoA1's tertiary and quaternary structure. Typically, apoA1 occurs as a molten globular monomer when not bound to lipids. When a dimer is formed, the conformation changes to facilitate lipid binding. C) ApoA1 combines with phospholipids and cholesterol to form nascent HDL. These discs mature to spherical HDL upon uptake of cholesterol and triglycerides. D) ApoA1 binds to several receptors on different cells. Examples include LDLR, SR-B1, and ABCA1 receptors. E) By interacting with these different receptors, apoA1 can affect multiple tissues and organs, such as the liver, small intestine, and hematopoietic tissue.

While the primary and secondary structures of lipid-free apoA1 have been extensively characterized, its tertiary structure remains more equivocal. The full-length lipid-free protein's crystal structure has not yet been resolved, likely due to its multiple conformations and tendency to aggregate.[3] However, two truncated structures that have been elucidated, 3R2P and 1AV1, represent apoA1 in dimeric or lipid-bound states, respectively.[4,5] Information about these crystal structures, together with data from studies using fluorescence resonance energy transfer, circular dichroism, chemical-crosslinking/mass spectrometry, and other techniques, indicate that lipid-free, monomeric apoA1 exists mostly in a globular, loosely folded state.[6–9] In such a state the α-helical regions remain intact, as the hydrophilic surface interacts with the environment while the hydrophobic regions face inwards.[10] It has been suggested that upon dimerization, helix 5 acts as a hinge region, causing subsequent helices to form a loop and fold away from the helix bundle at the N-terminus. In this looped conformation, apoA1 will form an antiparallel dimer with a second protein.[5] A schematic representation of this can be seen in Figure 2B.

Recently, Mei and Atkinson proposed that lipid binding to form HDL occurs when apoA1 is in a dimeric state. They suggest that when apoA1 is close to a lipid surface the C-terminal domain, especially helix 10, will bind the lipids. This anchors apoA1 into the lipid surface, filling the double loop of the dimer. Initiated by this, the N-terminal helical bundle opens and transforms apoA1's unstructured C-terminal domain into a helical conformation, facilitating the release of a nascent discoidal HDL in which apoA1 is wrapped around the lipids in a "double belt" arrangement.[3,5] Thorough reviews about apoA1's conformation in, and the formation of, nascent HDL have been published by Phillips and Gogonea.[11,12]

2.1. ApoA1 Mimetics

The properties of the apoA1 protein can be mimicked by short oligopeptides. These peptides are typically 16 to 38 amino acids long and resemble apoA1's amphipathic helical structure, but not necessarily its primary structure. Numerous mimetic peptides have been developed in the past decades. Most apoA1-mimetics associate with HDL particles similarly to the apoA1 protein. The resulting mimetic-containing HDL retains the ability to bind to macrophages and facilitate cholesterol efflux from these cells.[13] As mimetics are typically acquired by solid-phase peptide synthesis (SPPS) with high yield and purity, they are widely investigated as a potential therapeutic for atherosclerosis and inflammatory disorders.[14,15] One of the most studied mimetics is 18A, an 18 amino acid oligopeptide (DWLKAFYDKVAEKLKEAF). This peptide has been used in the formulation of nanodiscs and has been shown to associate with plasma HDL.[16] Acetylation of the N-terminal and amidation of the C-terminal of 18A resulted in the 2F peptide. This modified peptide showed increased lipid binding due to the changed charge on the peptide.[17,18] Another 18A variant is called 4F, referring to its four phenylalanine residues. This particular peptide has been synthesized in both all D-amino acids and all L-amino acids. Both variants of 4F are antiatherogenic and can dose-dependently increases preβ1 HDL levels in human plasma.[19] In addition to these mimetics, also longer peptides are being investigated, such as 37pA. This peptide consists of two 18A mimetics linked by a proline. This resembles the apoA1 protein in that the proline causes two individual amphipathic helical segments to form. As a result, 37pA possesses a greater lipid affinity and competes with apoA1 on the surface of HDL.[20]

3. Physiology and Biological Properties of ApoA1

The process of lipid transport and metabolism involves various lipoproteins including low-density lipoprotein (LDL), very low-density lipoprotein (VLDL), chylomicrons, and, most importantly, HDL.[21] The maturation phase of endogenous HDL influences the characteristics and composition underlying its heterogenicity. HDL can be divided into three sub-classes based on electrophoretic migration: α-migrating species, including the spherical classes of HDL2 and HDL3; β-migrating species such as discoidal HDL, lipid-poor apoA1, and free apoA1; and γ-migrating species.[22]

HDL consists of cholesterol, lipids, and apolipoproteins (Figure 2C). ApoA1 is one of the most important proteins in HDL and is primarily synthesized and secreted by the liver and small intestine as lipid-poor apoA1 and nascent phospholipid-rich cholesterol-poor HDL particles. During lipoprotein lipase-mediated intravascular triglyceride-rich lipoprotein lipolysis, cellular efflux and transfer of triglyceride-rich lipoprotein surface components add phospholipids and cholesterol to nascent HDL particles.[23] These bind to ATP-binding cassette transporter A1 (ABCA1), facilitating the addition of cholesterol and phospholipids to form pre-β-migrating discoidal HDL. The plasma enzyme lecithin cholesterol acyltransferase (LCAT) esterifies cholesterol to convert discoidal pre-β-migrating HDL into α-migrating HDL spheres. Subsequently, cholesteryl ester transfer through cholesteryl ester transfer protein (CETP) further modifies these spherical HDLs into other lipoproteins.[24] LCAT is crucial for HDL metabolism and, through its ability to convert free cholesterol into cholesterol ester (CE), fulfills an essential role in the transition from nascent to mature HDL. After esterification, cholesterol can be internalized, transforming nascent discoidal HDL into spherical HDL. Upon binding to ATP-binding cassette transporter G1 (ABCG1) and scavenger receptor-type B-I (SR-BI), HDL internalizes cholesterol, further matures, and increases in size. Triglycerides derived from LDL can also be transported to mature HDL in exchange for cholesterol, an interaction orchestrated by CETP (Figure 2D,E).

Mature HDL consists of a hydrophobic core containing triglycerides and CE encapsulated by a hydrophilic shell of apoA1 transporting the payload to hepatocytes in the liver, that metabolize the hydrophobic payload via SR-B1 mediated processes.[25,26] HDL's anti-inflammatory and antioxidative properties include neutralizing inflammatory agents such as LPS released from Gram-negative bacteria, flagellin, peptidoglycan or DNA unmethylated 5'-C-phosphate-G-3' (CpG) motifs, or even whole bacteria, thereby preventing systemic inflammation.[27] HDL is also involved in intercellular communication by facilitating the transportation of signaling proteins, lipids, and endogenous microRNAs to target cells.[28]

In addition to hepatocytes and intestinal epithelial cells, myeloid cells including monocytes and macrophages, and hematopoietic stem and multipotential progenitor cells (HSPCs) residing in the bone marrow express high levels of ABCA1 and ABCG1.[29–32] It has been shown that cholesterol efflux mechanisms controlled via these receptors affect HSPC proliferation indicating an important role for HDL in these processes.[29–32] Moreover, HDL suppresses myeloid cell proliferation, and the number of circulating monocytes and leukocytes, suggesting an important role in the development of atherosclerosis.[31]

HDL plays a large role in cardiovascular disease, a general term for a spectrum of heart and vascular dysfunctions.[33] Many of these dysfunctions are driven by imbalanced lipid metabolism and inflammation, which lead to excessive cholesterol and lipid deposition in, and immune cell recruitment to, the arterial vessel wall.[34] This process, called atherosclerosis, is associated with serious complications, including myocardial infarction, stroke, and kidney failure.[34] In atherosclerotic lesions, HDL extracts and transports cholesterol from foam cells—lipid-laden macrophages—via reverse cholesterol transport. Importantly, the risk of developing cardiovascular disease is inversely

correlated with HDL-cholesterol (HDL-c) blood levels.[35,36] The identification of $apoA1_{Milano}$, a natural apoA1 mutant, was an important finding in cardiovascular research.[37] $ApoA1_{Milano}$ contains a cysteine instead of an arginine at position 173, which facilitates the formation of $apoA1_{Milano}$-$apoA1_{Milano}$ dimer. This dimer accounts for approximately 70% of the $apoA1_{Milano}$ pool, which dramatically changes HDL blood values. The $apoA1_{Milano}$ carriers have very low levels of HDL-c, yet do not suffer from cardiovascular disorders.[38]

Research on HDL spurred the development of apoA1-based treatment aiming to clear cholesterol from the atherosclerotic vasculature.[39] Studies in cholesterol-fed rabbits demonstrated that weekly infusions of HDL or apoA1, isolated from the blood of normolipidemic rabbits, can effectively inhibit lesion development[40,41] and regress established fatty streaks in rabbit aortas.[42] Transgenic mice overexpressing human apoA1, by twofold more than normal, were significantly protected from atherosclerosis.[43–45] In healthy human subjects and different patient groups, apoA1/phospholipid complexes, also called reconstituted HDL (rHDL) or HDL mimetics, raised pre-β HDL levels, induced anti-inflammatory and antithrombotic effects, and improved endothelial function.[46–50] Further, researchers focused on fine-tuning rHDL composition to achieve optimal affinity to cholesterol, in vivo formulation stability, pharmacokinetics, and biodistribution.[51–54] The resulting HDL mimetics were predominately discoidal in shape and contained natural phospholipids, such as soybean phosphatidylserine, 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC), and/or sphingomyelin; some were negatively charged.[55]

Wild-type apoA1, isolated from human plasma, was used to produce the protein-lipid complex with soybean phosphatidylcholine (SRC-rHDL/CSL-111).[56] Four weekly infusions in patients with acute coronary syndrome (ACS) did not lead to plaque volume regression but displayed good safety and tolerability in both patients with stable atherosclerotic disease[57] and those who experienced acute myocardial infarction.[58,59]

Following the development of CSL-111, another HDL mimetic termed CSL-112 was formulated. This plasma-derived apoA1 has shown to rapidly increase the capacity of serum to efflux cholesterol in patients with stable atherosclerotic disease in a phase 2a trial.[57] Currently it is undergoing phase 3 clinical evaluation, where it is administered to patients who recently suffered an acute myocardial infarction in four weekly infusions. The aim of this study is to determine whether enhanced cholesterol efflux induced by CSL-112 treatment can reduce the rate of recurrent major cardiovascular events.[58]

CER-001 is another HDL mimetic, which entered phase 2 clinical trials and remains under clinical evaluation. It contains recombinant human apoA1, expressed in mammalian Chinese hamster ovary cells, egg sphingomyelin, and dipalmitolyphosphatidyl glycerol, mixed in a 97:3 mol% ratio. The first randomized multi-center trial showed no significant effects of 10-week CER-001 therapy in patients with ACS.[60] Further data analysis revealed significant plaque regression in patients with high coronary plaque burden.[61] In the following trial, CER-001 infusions did not regress coronary atherosclerosis in statin-treated patients with ACS and high plaque burden.[62] In response to these equivocal results, Zheng et al.[63] studied the uptake efficacy of zirconium-89 (^{89}Zr)-labeled CER-001 in carotid atherosclerotic plaques in eight patients. Despite significantly increased PET signal in carotid arteries at 24h and 48h after infusion, CER-001 did not improve either plaque area or inflammatory burden in patients with genetically determined low HDL levels.[64]

The Medicine Company pursued clinical translation of $apoA1_{Milano}$-based therapy. In the first randomized trial, the recombinant protein was formulated in a complex with POPC at 1:1 weight ratio. This HDL_{Milano} mimetic, named ETC-216, induced significant regression of coronary atherosclerosis in patients with ACS after 5 weekly doses,[65] but clinical development was halted during the third clinical trial after a serious adverse reaction[66] caused by a small quantity of residual host (microbial) cell proteins (HCP).[67] The manufacturing process was modified by selectively deleting the genes encoding the contaminating proteins and the recombinant $apoA1_{Milano}$ was reintroduced as MDCO-216, which has not caused immunostimulation.[66] However, $apoA1_{Milano}$-containing HDL mimetic (MDCO-216) did not benefit statin-treated ACS patients.[68]

In summary, although clinical trials found no evidence that exogenously administered HDL can ameliorate atherosclerotic disease burden,[60] they did uncover something critical: ApoA1 can be safely administered to humans at doses up to 45 mg kg^{-1}.[69] This finding paves the way for the development and clinical translation of A1-nanotherapeutics, which will require much (at least two orders of magnitude) lower apoA1 dosing.

4. Producing and Engineering ApoA1

ApoA1 can be obtained from several sources (**Figure 3**A). Commercially available apoA1 is often acquired by isolating HDL from blood plasma through density ultracentrifugation and subsequent delipidation using organic solvents. This leads to precipitation of apoA1, which then needs to be renatured using denaturing agents, such as urea or guanidine hydrochloride solutions, followed by dialysis against the desired buffer.[70] Typically, this process results in purities of >90%. However, since apoA1 is extracted from blood, pathogens or other contaminants may be present. In addition, the large amounts of organic solvents needed to extract apoA1 might introduce protein changes. The method is also expensive and allows for very limited engineerability of apoA1.[71]

Recombinant expression is often used as an alternative. ApoA1 has been recombinantly expressed in several systems, most commonly in *Escherichia coli* and *Pichia storis*. These expression systems enable engineerability, as well as fast, affordable, and scalable apoA1 production.[72,73] Baculovirus-insect systems, Chinese hamster ovary cells, and human embryonic kidney cells have also been used to produce apoA1.[74]

Obtaining apoA1 mimetics is done using SPPS rather than recombinant expression.[75] As these peptides are typically under 40 amino acids in size, their synthesis usually results in high yield and purity. This process is scalable and sidesteps issues such as endotoxin presence, slow cell growth, and extensive purification protocols. Additionally, SPPS allows for very specific and selective engineering of apoA1 mimetics, which opens possibilities for oral delivery of these peptides, as well as increased stability in vivo.[76] Other than these advantages of apoA1 mimetics, it is also more cost-effective than the extraction of apoA1 proteins from plasma.[77]

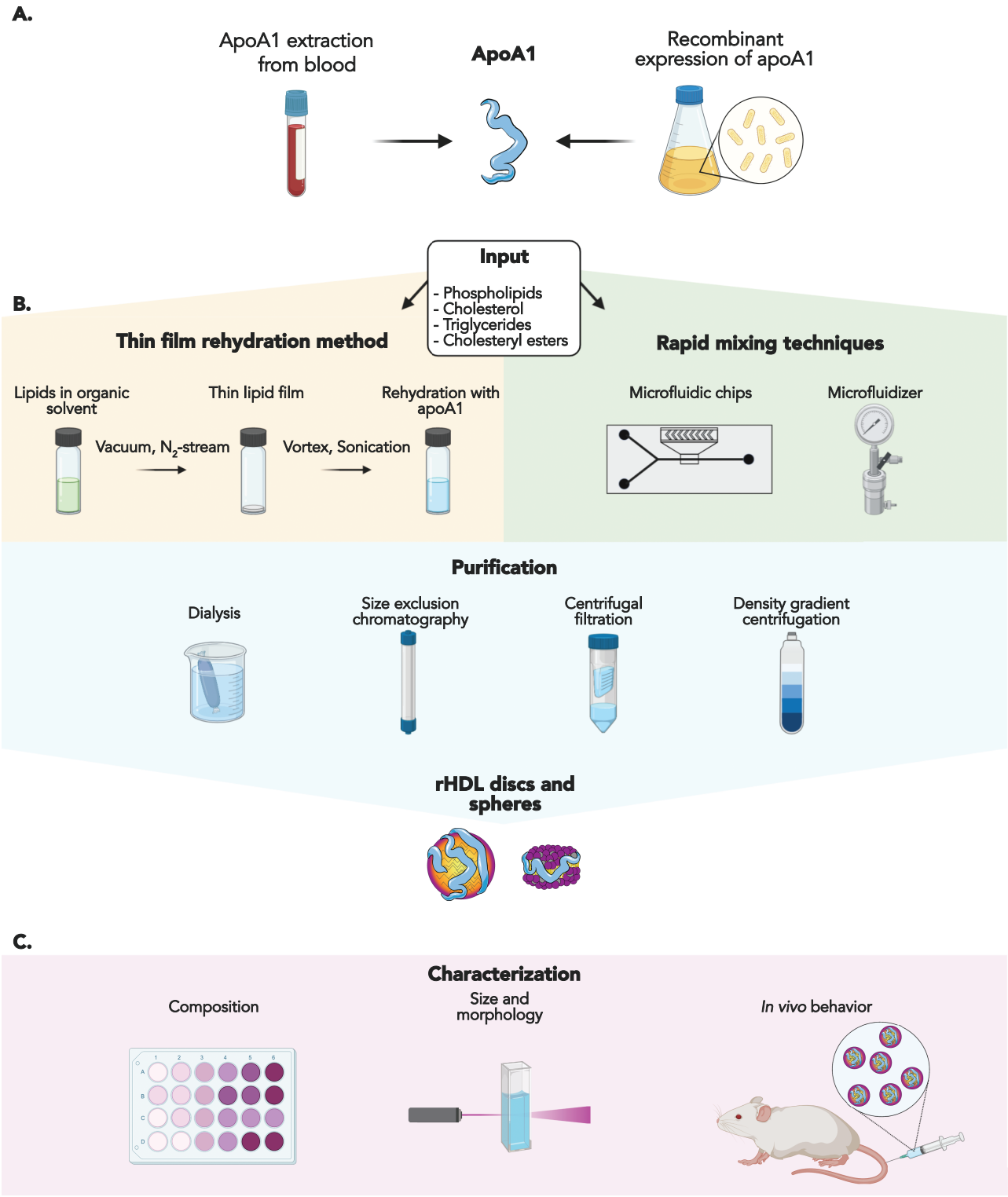

Figure 3. Engineering apoA1-based nanomaterials. A) ApoA1 is typically obtained by either extraction from blood samples or recombinant expression. B) ApoA1-based nanomaterials can be formulated using several techniques. The formulated materials subsequently need to be purified, which is usually done using size-based methods. C) A1-nanomaterials can be characterized three ways: composition, size and morphology, and in vivo behavior.

5. Engineering A1-Nanomaterials

ApoA1, together with phospholipids, can readily self-assemble into A1-nanomaterials. Because A1-nanomaterials offer a high degree of engineerability, individual components can be modulated to tune biological behavior. Additionally, small molecules or functionalized moieties can be incorporated to yield therapeutic effects.

A1-nanomaterial composition always involves apoA1, either as a full-length protein or a mimetic, to structurally stabilize the nanoparticle. Together with cholesterol and bilayer-forming phospholipids such as POPC or dimyristoylphosphatidylcholine, apoA1 self-assembles into small phospholipid bilayer discs. Spherical nanomaterials can be formed by adding hydrophobic compounds such as CE or triglycerides. These compounds will orient themselves to form a hydrophobic oily core, forcing the morphological change from discoidal to spherical. Phospholipids assemble into a monolayer at the interface between the hydrophobic core and the aqueous environment. Due to its amphipathic properties, apoA1 will integrate into the lipid monolayer and provide structural stability.

Traditionally, lipid mixtures are dissolved in organic solvents that are evaporated under a nitrogen stream or in vacuo. The resultant lipid film is subsequently rehydrated with aqueous buffer to form bilayer phospholipid vesicles. These vesicles can be broken down by adding apoA1 at 37 °C, aided by sonication of the protein-lipid suspension. Another approach combines these two steps by rehydrating the lipid film with aqueous buffer containing apoA1. After formulating, unincorporated components can be removed via dialysis or centrifugal filtration and monodisperse preparations can be isolated by gel chromatography or density gradient ultracentrifugation (Figure 3B). Although these techniques work well in an academic setting, they are difficult to scale for clinical applications. Therefore, microfluidic and high pressure homogenization[78] approaches have been developed to generate A1-nanomaterials in a continious fashion, thereby enabling large-scale production (Figure 3C).

There are multiple strategies to control the physicochemical properties of A1-nanomaterials. First, composition is essential, as the apoA1-to-phospholipid ratio significantly influences the nanomaterial size. For example, a 1:100 (mol/mol) apoA1-to-phospholipid ratio will yield discs approximately 10 nm in diameter whereas a 1:420 ratio will result in a diameter of 36 nm.[79] Building upon this concept, differently composed, sized and shaped A1-nanomaterials can be produced by varying the phospholipid composition, amount of cholesterol, and presence of hydrophobic filler molecules.[80] Complementary to composition, formulation parameters such as temperature or microfluidic flow rates can be adjusted to further tune the A1-nanomaterial's physicochemical properties.[71]

Concurrent with the ability to precisely shape A1-nanomaterials, the possibility arises to introduce a therapeutically active compound. The most straightforward strategy to achieve this is to integrate small hydrophobic molecules. These compounds can easily be accommodated in the hydrophobic core and therefore be mixed in with the organic phase at desired quantities.[81,82] To modulate A1-nanomaterial's in vivo behavior, hybrid materials with a polymer or triglyceride core have been developed as drug delivery systems with controllable sizes.[83,84] Similar to small molecules, inorganic materials such as iron oxide, gold particles, or quantum dot nanocrystals can be incorporated for medical imaging purposes like optical imaging,[85,86] magnetic resonance imaging (MRI),[85] positron emission tomography (PET),[87] and computed tomography (CT).[86] For these types of nanomaterials, continuous flow production techniques are preferred, as lipid film hydration methods often induce inorganic particle aggregation. The A1-nanomaterials can also be surface functionalized with exogenous amphiphiles that integrate in the lipid corona. In general, small quantities (<2.5%) of active compounds will not affect A1-nanomaterial morphology, structural stability, or biological character.[88] Other strategies include directly conjugating therapeutic agents to phospholipids,[89,90] cholesterol,[91,92] or apoA1.[90]

After producing A1-nanomaterials, it is important to characterize their physicochemical properties for quality control. Here we present a selection of the techniques available for this. We characterize the A1-nanomaterials based on three different parameters: composition, size and morphology, and in vivo behavior (Figure 3C).

First, the composition needs to be determined to gain insights into materials lost during formation. For standard components such as cholesterol, phospholipids, apoA1, or triglycerides, various commercial colorimetric assays can determine material concentrations. Examples include the Smith assay for proteins and the Rouser assay for phospholipids. Alternatively, high-performance liquid chromatography (HPLC) combined with mass spectrometry (MS) can quantify specific components with great accuracy. For HPLC the nanomaterials need to be solubilized with water-miscible organic solvents, typically acetonitrile. Similarly, small molecule incorporation can be evaluated by extraction with organic solvents followed by HPLC analysis. To determine the incorporation of inorganics, such as iron oxide or gold particles, inductively coupled plasma mass spectrometry (ICP-MS) can be used.

Second, size, morphology, and size distribution are important parameters and are typically determined by complementary techniques.[93] Many tools are available including, but not limited to, size exclusion chromatography (SEC), dynamic light scattering (DLS), nanoparticle tracking analysis (NTA), transmission electron microscopy (TEM), and cryogenic TEM. DLS is a widely used technique that derives a hydrodynamic diameter from particle motion. It also provides a polydispersity index (PDI) that indicates sample heterogeneity. In tandem with DLS, SEC or NTA can be used to gain more precise insights in distinct subpopulations. However, the golden standard to evaluate nanomaterial size and morphology remains TEM or cryo-TEM.

Lastly, characterizing the in vivo behavior of A1-nanomaterials is essential for biomedical purposes. In general, nanomaterial size, charge, and stability are the main features that influence pharmacokinetics and biodistribution. Pharmacokinetics describes the material's movement in vivo, and biodistribution denotes spatial information about where the material accumulates. An important pharmacokinetic parameter is blood half-life, which can be determined by regularly taking blood samples and evaluating if the administered nanotherapeutic can still be detected. Hence, the material must be detectable in a complex media such as blood. If the material does not have an inherent

measurable feature, specific components can be labeled with fluorescent or radioactive probes. In addition, radioactive labels such as ^{89}Zr allow in vivo visualization by PET imaging,[87,88] reporting on both pharmacokinetics and biodistribution. Depending on the technique applied, biodistribution can be determined at different spatiotemporal resolutions. On a systems level, PET/CT and MRI can be used non-invasively. Alternatively, ex vivo measuring of organ bioluminescence, fluorescence,[81] or gamma-counting[94] yields quantitative biodistribution information. To evaluate biodistribution on a cellular level, flow cytometry[94] or cytometry by time of flight (CyTOF) are typically employed.

One of our key studies investigated the relationship between rHDL composition and its in vivo performance. We designed a library of 15 rHDL variants and compared their cholesterol efflux capacity, blood half-life, and atherosclerosis targeting selectivity.[84] POPC and triglyceride-containing nanoformulations achieved the highest rank, while PLGA-modified rHDL showed the most unfavorable properties. Some of our earlier work explored the in vivo behavior of rHDL, particularly its affinity for myeloid cells. By incorporating fluorescent dyes, we showed that rHDL preferentially targets macrophages and/or monocytes in the blood, atherosclerotic aorta, and immunologically relevant organs.[81,88,95] We also developed ^{89}Zr-rHDL for quantitative noninvasive guidance.[88] Using PET imaging, we showed three- to fourfold higher radioactivity concentrations in aortas of rabbits with atherosclerosis compared to healthy animals 5 d postadministration. In the porcine model, we observed increased radioactivity in atherosclerotic lesions two days after ^{89}Zr-rHDL injection. The same labeling strategy demonstrated TRAF6i-rHDL efficacy and safety in mice and non-human primates.[95] In a recent study, we designed rHDL with a perfluoro-crown ether payload (^{19}F-rHDL), an ^{19}F-MRI nanotracer for imaging systemic immune cell involvement in ischemic heart disease.[96] Atherosclerotic mice with myocardial infarction that received ^{19}F-rHDL displayed rapid myeloid cell egression from immune organs, including the spleen and bone marrow, by in vivo ^{19}F-rHDL MRI. The released pro-inflammatory myeloid cells accumulated in atherosclerotic plaques and at the myocardial infarct site. The aforementioned labeling approaches represent valuable tools to study rHDL's targeting efficacy, organ biodistribution, and complex systemic interactions with myeloid cells.

6. Immunotherapy with A1-Nanomaterials

As described in the previous section, important characteristics of A1-nanomaterials are their biocompatibility and favored affinity for innate immune cells, in particular myeloid cells. By exploiting these intrinsic properties, the engineering of A1-nanomaterials can find use in the activation of T-cells which is based on a cascade of events including antigen presentation (signal 1), co-stimulation (signal 2) and soluble cytokine secretion (signal 3) (**Figure 4**A). In other words, A1-nanomaterials can be applied as immunotherapy in the following areas: 1) vaccination, 2) costimulation / checkpoint inhibition, and 3) trained immunity.

6.1. Vaccination

A1-nanomaterials can serve as the foundation of vaccines (Figure 4B).[89] Vaccination focuses on signal 1 of the T-cell activation cascade by delivering an antigen to antigen-presenting cells (APCs). The antigen is taken up by APCs, which process it into small peptides. Depending on the pathway, these small peptides are then displayed on the APC surface by either MHC-II or MHC-I, also referred to as cross-presentation.[97] While the main goal of vaccination is the initiation of antigen presentation by APCs, nanotherapeutics might also enhance the induction of signal 2 and 3. Nanoparticles have been extensively investigated as antigen carriers, since they intrinsically enhance antigen uptake. Additionally, incorporating antigens into nanomaterials can prolong their presentation on APCs. While these nanoparticles depend on innate immune cells' phagocytic properties, A1-nanomaterials can be used as a carrier to actively target antigens to lymphoid organs.

Applying nanomaterials functionalized with adjuvants can facilitate the co-delivery of antigen and adjuvant to APCs. This has been demonstrated by Kuai et al. who showed that synthetic HDL nanodiscs can be formulated and loaded with monophosphoryl Lipid A (MPLA), a Toll-like receptor 4 agonist, and CpG motif, a TLR9 agonist.[98] They found that these synthetic HDL nanodiscs, consisting of phospholipids and apoA1 mimetic peptides (22A), could function as a neoantigen vaccination platform.[89] Carrying cholesterol-conjugated modified CpG as adjuvant as well as tumor (neo)antigens, these synthetic HDL nanodiscs improve antigen and adjuvant co-delivery to lymphoid organs as well as prolong antigen presentation by dendritic cells. Moreover, incorporating neo-epitopes induces the generation of a broad spectrum of T-cells acting synergistically with immune checkpoint inhibitors anti-PD-1 and anti-CTLA-4 to eradicate tumor cells. Thus far, this approach has been employed in multiple tumor models including glioma,[99] colon adenoma, and melanoma.[89] However, A1-nanotherapeutics as a vaccine platform can be applied to a broader range of diseases including various cancer types and infectious diseases caused by parasites, fungi, bacteria, and viruses.

6.2. Immune Regulation

Immune checkpoint proteins, including both co-stimulatory and co-inhibitory molecules, are master regulators of the immune response. They allow cognate interactions not only between T-cells and APCs, but also between innate and adaptive immune cells and endothelial cells, vascular smooth muscle cells, platelets, epithelial cells, and cancer cells.[95] A1-nanomaterials can be utilized to deliver molecules that regulate signal 2 of the immune response (Figure 4C). Here, immune tolerance can be achieved by inhibiting costimulatory molecules, while suppressing co-inhibitory molecules and immune checkpoints increases immune activation.

One such costimulatory interaction occurs between CD40 and its ligand (CD40L). TRAF6 is key to propagating CD40's signaling cascade into macrophages. Lameijer et al. developed an A1-nanotherapeutic, named TRAF6i-HDL, that incorporated a small molecule inhibitor of TRAF6 (TRAF6i).[95] TRAF6i-HDL inhibits TRAF6 binding to CD40, thereby decreasing monocyte recruitment.

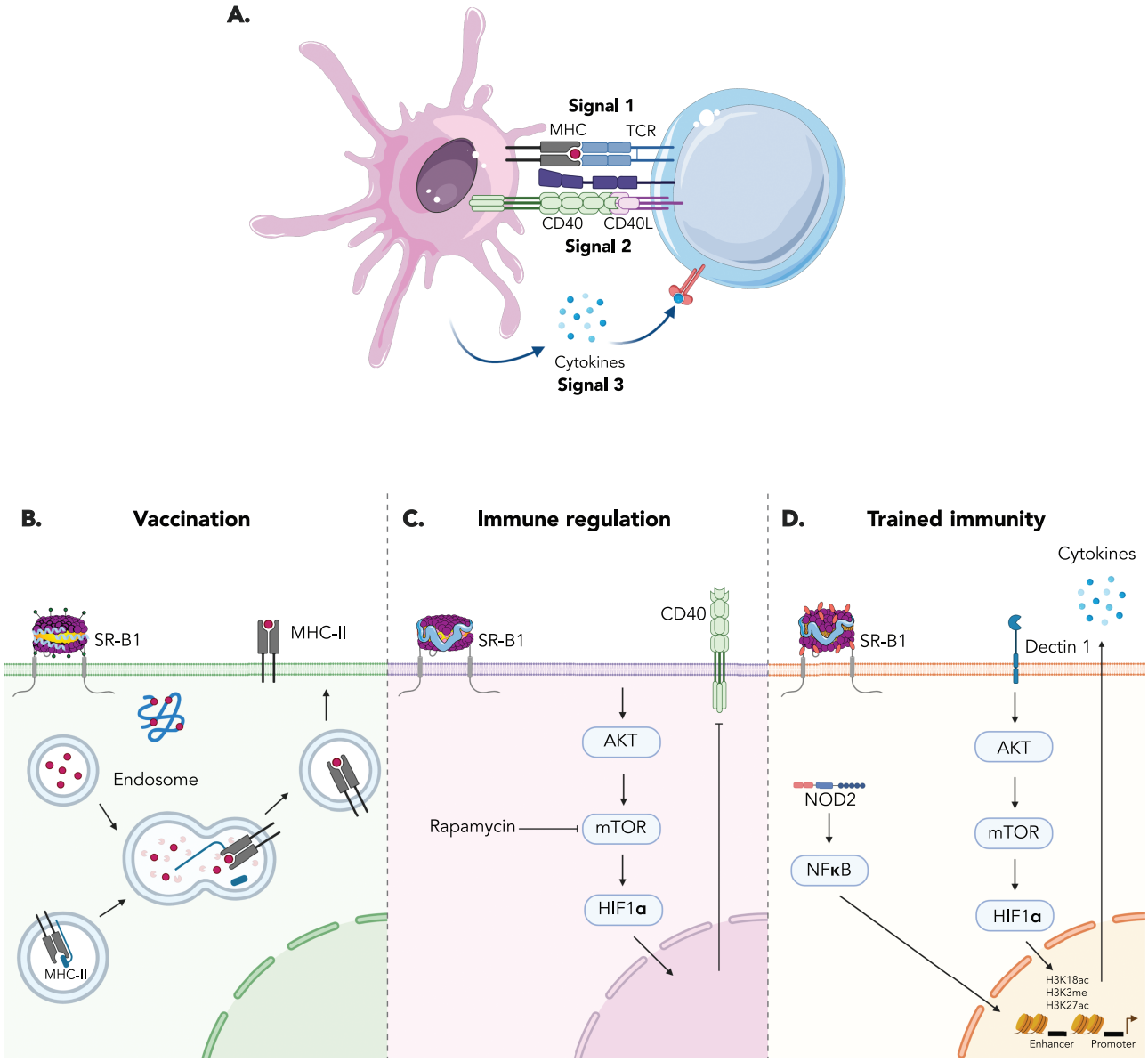

Figure 4. Exploiting A1-nanotherapeutics as platform for immunotherapy. A) APCs can activate T-cells via MHC protein complexes-facilitated antigen presentations (signal 1), co-stimulation (signal 2), and cytokines (signal 3). B) ApoA1 mimetic discs harboring antigens and adjuvants can serve as vaccination strategy to provide signal 1. C) Regulating immune responses via signal 2 by incorporating small molecules such as rapamycin. D) Inducing trained immunity using NOD2 agonist containing A1-nanotherapeutics, which primarily modifies the cytokine profile (signal 3).

Another immune checkpoint interaction is that between programmed cell death protein 1 (PD-1) and its ligand PD-L1. This immune checkpoint is of particular interest in several cancers. PD-1 plays an important role in promoting tolerance against endogenous cells by suppressing inflammatory T-cells. Cancer cells expressing PD-L1 on their membrane protect the tumor from the immune system. Several monoclonal antibodies developed to target the PD-1-PD-L1 interaction have effectively reduced tumor size.[100] However, treatment with a single antibody type is not sufficient to treat cancer in most patients, necessitating a cocktail of these co-inhibitory blockades. Although some nanoparticles have been developed to deliver these anti-cancer drugs,[101] A1-nanotherapeutics are not yet among them. While current treatments target tumor cells, A1-nanotherapeutics could be used to engage the immune cells that display PD-1.

Besides directing immune interactions between T-cells and tumor cells, checkpoints also regulate innate immune cells. For example, CD47 functions as a "do not eat me" signal, preventing phagocytic cells, including macrophages, from eradicating CD47-expressing cells. CD47 binding to signal regulatory protein alpha (SIRPα) located on the membrane of macrophages prevents phagocytosis by activating tyrosine phosphatase and prohibiting the accumulation of myosin at the phagocytic synapse site.[102] This mechanism helps malignant cells evade the innate immune system. Several therapies using monoclonal antibodies targeting CD47 have been developed to disrupt CD47-SIRPα

interaction.[103] A major side effect of these therapies is the development of anemia as a consequence of accelerated erythrocyte clearance due to the expression of CD47 on these cells.[104] A1-nanotherapeutics could specifically target phagocytic cells, avoiding side effects by reducing treatment doses. By making use of the phagocytic capacity of myeloid cells, A1-nanotherapeutics can bypass these effects by delivering therapeutic molecules that inhibit SIRPα expression. This approach might reduce treatment doses and side effects associated with CD47 antibody therapy.

6.3. Trained Immunity

Research in the past decade has discovered that cell populations of the innate immune system display long-term changes in their functional program through metabolic and epigenetic rewiring. Such reprogramming causes these cells to be either hyperresponsive or hyporesponsive, resulting in an altered immune response to secondary stimuli. This de facto innate immune memory, which has been termed "trained immunity," provides a powerful targeting framework to regulate the delicate balance of immune homeostasis, priming, training, and tolerance.

Unlike immunological memory rooted in the adaptive immune system, functional reprograming underlying trained immunity has been proven to last for at least three months to a year and is based on epigenetic reprogramming of transcription pathways instead of gene recombination.[105] Innate immune memory also differs from adaptive immunity in specificity and cell population. While adaptive immunity is antigen specific and mediated by B- and T-lymphocytes, trained immunity is characterized by a non-specific response and is localized in HSPCs residing in the bone marrow. These HSPCs have long lifespans with the ability to self-renew and pass on their altered epigenetic profile to daughter cells that enter the circulation and migrate to peripheral organs, where they deploy their trained effector function. A1-nanomaterials' ability to specifically target myeloid cells entails a powerful platform for trained immunity-regulating agents (Figure 4D).

A1-nanomaterials can be applied to either promote or inhibit trained immunity.[106,107] Inducing trained immunity can help treat tumors protected from the patient's immune system by an immunosuppressive microenvironment, while inhibiting trained immunity is a relevant treatment modality in the context of cardiovascular diseases, autoimmune disorders, and organ transplantation.

7. A1-Nanotherapeutic Applications

The human body defends itself through the immune system. It orchestrates molecular and cellular immune responses that eliminate infections caused by pathogens (such as bacteria, viruses, fungi, and parasites) and also has an immune surveillance role in fighting tumor development.[108,109] In addition, the immune system helps maintain homeostasis through processes such as tissue development and repair.[110–112] Both host defense and healing must be very tightly yet subtly controlled. Different immune system components must be activated in the correct sequence, at the right time, in the proper place, and for the needed period of time. Because immune-mediated mechanisms are present in various aspects of tissue and organ homeostasis, they are implicated in pathologies as diverse as cancer and infections as well as in autoimmune diseases (multiple sclerosis, rheumatoid arthritis), inflammatory and metabolic disorders (atherosclerosis, diabetes), and even chronic neurodegenerative disorders (Alzheimer's disease, Parkinson's disease). As our understanding of the immune system and its role in health and disease grows, it is becoming increasingly evident that major diseases can potentially be much more effectively treated with innovative immune system-directed therapies. In this section, we discuss recent translational experimental studies in which A1-nanotherapeutics were successfully applied.

7.1. Cancer and Infection

Trained immunity, a functional state of primarily the myeloid cell compartment, increases non-specific resistance against infection. "Trained" myeloid cells are characterized by metabolic and epigenetic rewiring, making these cells hyperresponsive against a range of pathogen and damage associated molecular patterns. While the induction of trained immunity using the BCG vaccine was recently shown to be effective in providing elderly patients with enhanced non-specific protection against a range of infections, including respiratory infections,[113] we recently demonstrated—for the first time—the effectiveness of inducing trained immunity as an anti-cancer strategy. Priem et al. developed so-called nanobiologics that were surface-decorated with the NOD2 agonists muramyl dipeptide (MDP) or muramyl tripeptide (MTP). Through extensive screening, a "lead" trained immunity-inducing nanobiologic was selected and evaluated in comprehensive translational studies, involving a mouse melanoma model, state-of-the-art in vivo imaging, immune landscape profiling, combination immunotherapy, as well as safety and biodistribution work in mice and nonhuman primates. Upon intravenous administration, nanobiologics efficiently accumulated in bone marrow, exerting epigenomic and transcriptomic changes in myeloid progenitor cells, which led to a rebalancing of the tumor microenvironment. Depending on the regimen, the nanobiologic immunotherapy effectively reduced tumor growth and increased sensitivity to checkpoint inhibitor drugs in a melanoma mouse model that is refractory to immune checkpoint blockade (**Figure 5**A).[94] MDP and MTP are small biomolecules, derived from peptidoglycans found on the surface of mycobacteria. Due to this, the MDP/MTP-based nanobiologic therapy induces trained immunity via the same programs as BCG vaccination. Since BCG vaccination reduces sensitivity to respiratory tract infection,[115] nanobiologic immunotherapy may also be effective to increase resistance against infections.

As detailed above, A1-nanotherapeutics might also be used as a vaccination platform to treat cancer and fight and prevent infections. James Moon and his team developed an apoA1 nanodisc-based vaccination platform consisting of phospholipids and an apoA1 mimetic.[89] Accumulation of these A1-nanotherapeutics loaded with antigen peptides and adjuvants in the lymph nodes increases the codelivery of antigens and adjuvant to lymphoid organs and prolongs antigen presentation by dendritic cells compared to potent adjuvants including CpG and Montanite

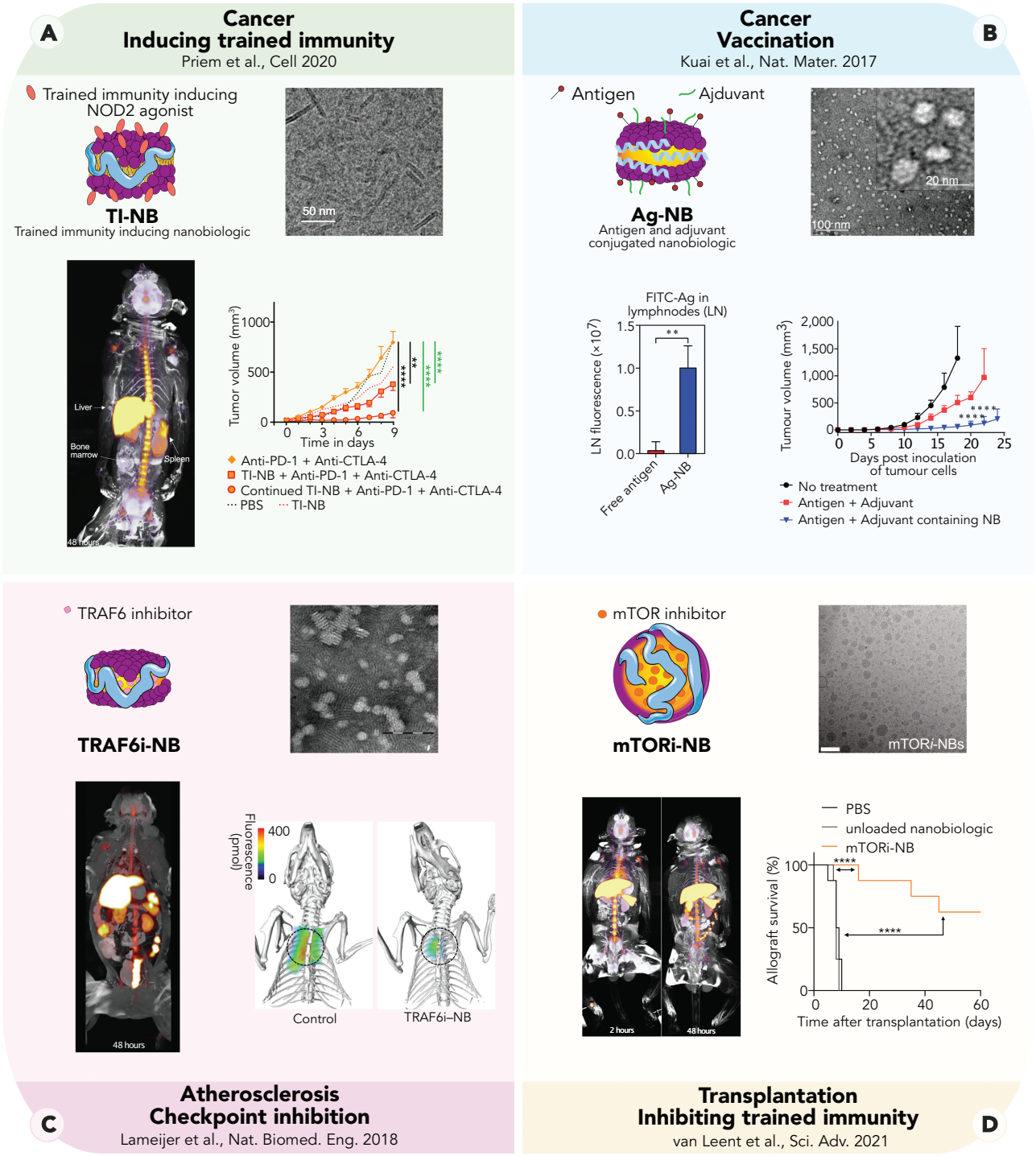

Figure 5. Treating diseases with A1-nanotherapeutics. A) Priem et al. demonstrate how A1-nanodiscs containing a trained immunity inducing NOD2 agonist can be employed to treat tumors. Adapted with permission.[94] Copyright 2020, Elsevier. B) Kuai et al. present a neo-antigen platform based on A1-nanomaterials. Antigen and adjuvant loaded nanodiscs formulated with apoA1 mimetic peptides are used to vaccinate against tumors. Adapted with permission.[89] Copyright 2016, Springer Nature. C) Lameijer et al. developed a A1-nanotherapeutic loaded with TRAF6i, a checkpoint inhibitor, to reduce atherosclerotic plaque inflammation. Adapted with permission.[95] Copyright 2018, Springer Nature. D) mTOR inhibitor containing A1-nanobiologics can promote allograft survival by inhibiting trained immunity. These therapeutics were also found to be safe in nonhuman primates, as demonstrated by van Leent et al. Adapted with permission.[115] Copyright 2021, The Authors, some rights reserved; exclusive licensee AAAS. Distributed under a Creative Commons Attribution NonCommercial License 4.0 (CC BY-NC).

(Figure 5B).[89] Furthermore, tumor growth was reduced via a comprehensive T-cell mediated immune response as a result of vaccination with multiple epitopes. Combination therapy of these A1-nanomatierials and immune checkpoint inhibitors showed potent tumor eradication in colon and melanoma tumor models. This platform could also be deployed in personalized medicine via the incorporation of tumor-specific mutant neo-epitopes targeting the tumor of a specific patient.

7.2. Inflammatory and Autoimmune Diseases

While trained immunity provides protection against infection and malignancies, exacerbated immune responses can drive autoimmune diseases and chronic inflammatory conditions, including atherosclerosis, an important underlying cause of cardiovascular disease. Central in immune cells' metabolic regulation is the mammalian target of rapamycin (mTOR). Van Leent, et al. developed nanobiologics inhibiting mTOR (mTORi-NB) and a downstream target known as S6 kinase-1 (S6K1i-NB). Both mTORi-NB and S6K1i-NB therapy effectively reduced arterial plaque inflammation in a mouse model of advanced atherosclerosis.[115] To better understand the mechanism behind this reduced inflammation, transcriptome modifications were investigated, leading to the identification of prosaposin as a mediator of anti-inflammatory effects and this protein's regulatory role in immunometabolism. The study exemplifies how A1-nanotherapeutics can be employed as not only treatments for various diseases but also tools to study immunometabolism and the mechanisms underlying trained immunity.

In addition to targeting mTOR and S6K1 in myeloid cells, we have applied a similar nanobiologic therapeutic approach to enhance deliver simvastatin (S-NB)[81] and an LXR agonist,[116] and a TRAF6 inhibitor (TRAF6i-NB)[95,117] to myeloid cells generally and plaque-associated macrophages specifically. In atherosclerotic Apoe$^{-/-}$ mice, a one-week S-NB regimen markedly decreased inflammation in advanced atherosclerotic plaques.[81] We also evaluated S-NB in large animal models of cardiovascular disease. Towards that goal, we scaled S-NB's production using microfluidizer-based high-pressure homogenization to allow studies in rabbits and pigs,[78] while we implemented a positron emission tomography/magnetic resonance imaging (PET/MRI) workflow to study inflammatory processes relevant to atherosclerosis, in live animals and non-invasively. In both species, we found significantly reduced atherosclerotic parameters in the S-NB-treated group. Similar to S-NB, TRAF6i-NB nanotherapy resulted in smaller lesion size and lower inflammatory burden in atherosclerotic mice (Figure 5C). Extensive transcriptomic and immune landscaping efforts disclosed this was due to monocytes' impaired migration capacity.[95,117] Importantly, TRAF6i-NB therapy was found to be safe in nonhuman primates.

7.3. Transplantation

Braza et al. showed that trained immunity-inhibiting nanobiologics can be applied to promote allograft acceptance.[118] The studied A1-nanotherapeutic, mTORi-HDL, contained the small molecule drug rapamycin. The target of rapamycin, mTOR, regulates cytokine production through trained immunity. Rapamycin treatment thus prevents epigenetic and metabolic modifications that underlie trained immunity.[106] In an experimental heart transplant mouse model, the mTORi-HDL therapeutic targeted cells in both the bone marrow and the heart allograft, further underlining A1-nanobiologics' preference for myeloid cells. Additionally, this therapeutic prolongs allograft survival, with less toxicity than oral rapamycin treatment. Short-term combination therapy using mTORi-HDL and TRAF6i-HDL resulted in long-term allograft survival, even achieving >70% survival 100 d post-transplantation. Similar results were also obtained in a study by van Leent and co-workers in which they demonstrate a modular approach for the development of A1-nanotherapeutics. In this study an A1-nanomaterial platform is shown in which a broad range of drugs can be incorporated, among which rapamycin.[119] In addition to demonstrating the immunomodulatory effect of the A1-nanomaterial platform both in vitro and in vivo, they also showed that this is a scalable platform which can be safely applied in nonhuman primates (Figure 5D).

8. Outlook

The twenty-first century is witnessing a convergence between the fields of nanomedicine and immunotherapy.[120] While the traditional nanomedicine paradigm has been efficient drug delivery by nanomaterials that were designed to evade premature clearance by phagocytic immune cells, for immunotherapeutic purposes the design prerequisites must be reconsidered. One of the key lessons learned—since the introduction of Doxil in 1995[121] and all way to the deployment of lipid nanoparticle technology for the mRNA COVID-19 vaccines[122]— is that the nanomaterials' molecular constituents should be as close to natural molecules as possible. In that context, the exploitation of fully natural nondelivered vehicles can be considered. Indeed, viral delivery systems are already employed for gene therapy,[123,124] while molecular components from viruses can be used to engineer nanomaterials.[125] Extracellular vesicles and exosomes are other natural platforms that gained a lot of traction, particularly for the delivery of RNA.[126]

While nanomaterials based on viruses or extracellular vesicles hold great potential, for effective innate immunotherapy myeloid cell engagement is a prerequisite. In this review, we focus on HDL's main protein constituent apoA1. ApoA1 is a natural protein that forms nanoaggregates with other biomolecules, in particular fatty molecules like phospholipids, cholesterol and triglycerides, but also microRNAs.[127] ApoA1's inherent affinity for myeloid cells makes A1-nanomaterials highly attractive for innate immune regulation and we showcase apoA1's utilization for developing myeloid cell-engaging nanotherapeutics.

Among A1-nanomaterials' different immunotherapeutic applications, vaccination through effective antigen presentation,[89] innate immune inhibition and stimulation,[94,119] as well as immune costimulation[95] and checkpoint blockade[118] are feasible and highly effective in preclinical studies. We expect these A1-nanotherapeutics' impact will primarily rely on effective clinical translation in the current twenty-twenties. This will rely on the implementation of large libraries containing A1-nanomaterials[84] and high throughput screening, in vitro and in mouse models. From these screens, "laws" on structure-function

relationships can be extracted through computational modeling methods, including machine learning.[128,129]

To close the clinical translation gap, large animal models are highly valuable. For example, Binderup et al. showed, in 2019, that producing and evaluating A1-nanotherapeutics is scalable from mice to larger animals, i.e., rabbits and porcine cardiovascular disease models.[78] In that context, non-invasive imaging can be integrated to quantitatively visualize a nanotherapeutic's in vivo behavior as well as to evaluate its therapeutic effects systemically.[130]

Translational efforts with injectable HDL mimetics by several companies did not uncover their desired atheroprotective function in cardiovascular patient trials.[60] These studies did demonstrate, however, that apoA1-/lipid- based nanomaterials can be safely administered to humans at high dose, much higher than will be required for A1-nanoimmunotherapeutics presented in this review, paving the clinical translation way.

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Competing Interests

W.J.M.M., and Z.A.F., are scientific co-founders of and have equity in Trained Therapeutix Discovery. W.J.M.M. and Z.A.F. have consulting agreements with Trained Therapeutix Discovery.

Keywords

apolipoprotein A1, immunoengineering, immunotherapy, nanomedicine, nanotherapeutics, trained immunity

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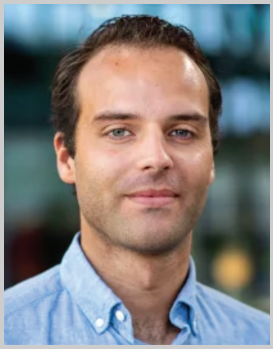

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