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REVIEW ARTICLE

Monocyte-mediated drug delivery systems for the treatment of cardiovascular diseases

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Abstract Major advances have been achieved in understanding the mechanisms and risk factors leading to cardiovascular disorders and consequently developing new therapies. A strong inflammatory response occurs with a substantial recruitment of innate immunity cells in atherosclerosis, myocardial infarction, and restenosis. Monocytes and macrophages are key players in the healing process that ensues following injury. In the inflamed arterial wall, monocytes, and monocyte-derived macrophages have specific functions in the initiation and resolution of inflammation, principally through phagocytosis, and the release of inflammatory cytokines and reactive oxygen species. In this review, we will focus on delivery systems, mainly nanoparticles, for modulating circulating monocytes/monocyte-derived macrophages. We review the different strategies of depletion or modulation of circulating monocytes and monocyte subtypes, using polymeric nanoparticles and liposomes for the therapy of myocardial infarction and restenosis. We will further discuss the strategies of exploiting circulating monocytes for biological targeting of nanocarrier-based drug delivery systems for therapeutic and diagnostic applications.

Keywords Drug delivery systems · Liposomes · Polymeric nanoparticles · Monocytes · Monocyte subpopulations · Vascular injury

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Abbreviations

ALN-NPs	Alendronate nanoparticles
BPs	Bisphosphonates
CHD	Coronary heart disease
CM	Classical monocytes
CVD	Cardiovascular disease(s)
DDS	Drug delivery system(s)
DES	Drug eluting stent
EC	Endothelial cells
DM	Diabetes mellitus
EPR	Enhanced permeability and retention effect
Ga	Gallium
Gd	Gadolinium
IM	Intermediate monocytes
LipALN	Liposomal alendronate
LipCLOD	Liposomal clodronate
LipQDs	Liposomal quantum dots
MI	Myocardial infraction
MPS	Mononuclear phagocytic system
N_c	Number concentration
NCM	Non-classical monocytes
NPs	Nanoparticles
PCI	Percutaneous coronary intervention(s)
PEG	Polyethylene glycol
PLGA	Poly(d,l-lactide co-glycolide)
QDs	Quantum dots
QY	Quantum yield
siCCR2	siRNA sequence against CCR2
SMC	Smooth muscle cells

Cardiovascular disorders

Cardiovascular diseases (CVDs) are a global pandemic with more than 92 million Americans living with some

form of CVD and are the leading cause of death in North America [1]. Nevertheless, the prognosis of patients diagnosed with CVD has improved dramatically over the last decades. Major advances have been achieved in understanding mechanisms of CVD and the risk factors leading to it and in developing new therapies. This latter development is mainly attributed to the progress made in the field of small molecules therapeutics [2, 3] along with the advancement of percutaneous coronary interventions (PCIs) [4, 5]. Coronary artery disease (CAD), a prominent CVD manifestation, involves progressive accumulation of atherosclerotic plaque within the lumen of coronary arteries. This disease process may lead to partial or complete blockage of the blood vessel. Inflammation plays a major role in both chronic and acute CAD manifestations. Atherosclerosis, a chronic inflammatory disorder, involves both the innate and adaptive arms of the immune response mediating the initiation, progression, and ultimate thrombotic complications of atherosclerosis (see review in [6]). Inflammation also plays a major role in plaque vulnerability and occlusive blood clot formation, which leads to reduced blood perfusion to the myocardium resulting in acute myocardial infarction (MI).

The most prevalent non-surgical approach used for the revascularization of obstructed coronary arteries is PCI, consisting of balloon angioplasty (diagnostic and/or therapeutic) and stent deployment. However, the arterial wall injury, potentially exacerbated by stent deployment, causes neointimal tissue proliferation and re-narrowing of the arterial lumen termed restenosis. Stent deployment causes stretching of the artery, denudation of the endothelial layer, and compression of plaque, which often results in dissection of the tunica media and, occasionally, dissection of the adventitia. This damage could eventually result in in-stent restenosis [7–9]. To date, the most successful approach to restenosis is the use of drug-eluting stents (DESs; typically, polymer-coated stents) that deliver medication, such as anti-proliferating/inflammatory agents, directly to the site of vascular injury [10–14]. Although drug-eluting stents are in wide clinical use [12, 13, 15], several major problems associated with DESs still exist including late-stent thrombosis that frequently presents as MI, and the possible harm from a long-term implanted polymer with drugs [13]. Moreover, about a third of critical lesions cannot be stented, largely because they occur at branch sites or in small arteries. Hence, other methods for prevention of restenosis beyond the drug-eluting stent strategy are necessary.

Systemic pharmacological treatments have failed to prevent restenosis in humans, due to the inability to achieve the required dose at the site of injury without causing systemic side effects [2]. Various nanotechnology platforms are being investigated for the treatment of CVD in general, including liposomes, polymeric nanoparticles (NPs), dendrimers, micelles, and more [16–19]. The majority of nanotechnologies for treating restenosis or MI aim to specifically deliver therapeutic drug levels to the diseased site following systemic or local administration [20–24]. Another approach is based on modulating the systemic anti-inflammatory mechanism, e.g., inhibiting circulating monocytes [25, 26].

Here, we review the different strategies of depletion and modulation of circulating monocytes using particulate delivery systems for the therapy of restenosis and MI. The rational of innate-immunity intervention via monocyte modulation is more promising in an acute pathology (e.g., MI and restenosis) rather than in a chronic disease (e.g., atherosclerosis). We will further discuss the strategies of exploiting circulating monocytes for biological targeting of nanocarrier-based drug delivery systems (DDSs) for therapeutic and diagnostic applications.

Restenosis and MI—inflammation-associated pathologies

Inflammation, a multi-factorial process that combines both cellular and non-cellular components, plays a pivotal role in the initiation, progression, and eventually resolution of many pathologies including autoimmune disorders [27], malignancies [28], and CVD [29, 30]. Following PCI, an inflammatory response is triggered in the injured arterial segment as a result of injury and denuding of the endothelial layer by the balloon catheter [8, 29, 31]. Immediately after endothelial denudation, platelets start to adhere to the injured arterial segment [32]. This platelet deposition mediates leukocyte (mainly neutrophils and monocytes) rolling, adhesion, and finally infiltration into the arterial wall by expressing p-selectin [33, 34]. The damaged endothelial cells are activated and secrete pro-inflammatory mediators such as VCAM-1, MCP-1, and p-selectin which also promote leukocyte recruitment [35]. It has been shown that in stented arteries, the first wave of leukocyte infiltration mainly consists of neutrophils, followed by a second wave of monocytes that lasts for a longer time [29, 36]. The number of infiltrating monocyte-derived macrophages (also termed not fully differentiated monocytes) has been shown to greatly differ with time after stenting in several animal models [37]. At the first week after stenting, nearly 40% of neointimal cells are macrophages. Their number decreases to 7% and then to 1%, 11 and 19 days after stenting, respectively. In addition, the number of macrophages in the artery correlates with smooth muscle cell (SMC) proliferation in the neointima (30, 8, and 1%, respectively) [37]. Thus, infiltration of monocytes/macrophages is closely associated with early SMC proliferation, which leads to neointimal formation and restenosis. It should be noted that monocyte infiltration into a balloon-injured artery is less pronounced than in a stented artery

probably due to the less severe injury [37, 38]. In the inflamed arterial wall, monocytes and monocyte-derived macrophages have specific functions in the initiation and resolution of inflammation, principally through phagocytosis, and the release of inflammatory cytokines and reactive oxygen species [39].

In MI, as in restenosis, a strong inflammatory response occurs with a substantial recruitment of innate immunity cells. Monocytes and macrophages are key players in the healing process that ensues MI. Myocardial infiltration by monocytes facilitates the scavenging of apoptotic neutrophils and necrotic cardiomyocytes and modulates cardiac remodeling. The first wave of leukocytes arriving to the injured myocardial tissue after MI is characterized with early and massive infiltration of neutrophils, with a postponed monocyte ingress commencing after 1 day [40, 41]. Monocyte-derived macrophages remained in high and relatively stable numbers at the first week, dropping to baseline levels on day 16. In contrast, tissue-resident macrophages remain in relatively low numbers from day 1 to day 7, indicating the profound role of blood/spleen monocytes in MI progression.

Monocyte subtypes

Recent research assigned monocytes into functionally distinguished subclasses demonstrating considerable heterogeneity with respect to their phenotype and function [39, 42]. Three subpopulations of monocytes are classified in humans as (i) classical monocytes (CM; $CD14^{++}CD16^{-}$), (ii) intermediate monocyte (IM; $CD14^{++}$ $CD16^{+}$), and (iii) non-classical monocyte (NCM; $CD14^{++}CD16^{++}$), each with different functions [42, 43]. Murine monocyte subtypes are characterized by the differential expression of Ly-6C: Ly-$6C^{high}$ (CM), Ly-$6C^{int}$ (IM), and Ly-$6C^{low}$ (NCM) [44]. In rats, two monocyte subsets have been described based on CD43 expression, $CD43^{high}$ monocyte (NCM) and $CD43^{low}$ monocytes (CM) [39, 42, 45, 46]. In MI, monocytes are recruited to the myocardium in two phases [40, 41, 47]. CM (i.e., "pro-inflammatory") are recruited to the injured myocardium during the first 3–4 days and facilitate the scavenging of apoptotic neutrophils and necrotic cardiomyocytes and the digestion of damaged tissue. NCM (i.e., "anti-inflammatory"; also termed patrolling monocytes) are recruited during the second phase, 5–7 days after MI (Fig. 1) [40]. A positive correlation between the level of circulating NCM, 12 days after stent implantation or MI, and in-stent late gain loss has been demonstrated [47, 48].

The role of monocyte subsets has been demonstrated in a murine injury model, induced by a subcutaneous sponge implantation. Bi-phasic monocytic response is observed; CM are the first monocyte subset recruited to the injured site, which are then slowly transitioned into repair macrophages [49]. CM express high levels of the chemokine receptor CCR2, important for monocyte localization in the injured tissue. The role of CCR2 in atherosclerotic plaque vulnerability was demonstrated in ApoE-deficient mice fed on high-fat diet. Adoptive transfer of $CCR2^{+/+}$Ly-$6C^{high}$ monocytes (CM) derived from $ApoE^{-/-}CCR2^{+/+}$ mice to $ApoE^{-/-}$ mice significantly increased the vulnerability of atherosclerotic fibrous caps and the expression of MCP-1 in the arterial plaque [50]. In contrast, when the same number of leukocytes, originating from the peritoneal cavity of $ApoE^{-/-}CCR2^{-/-}$ mice (NCM), is transferred to $ApoE^{-/-}$ mice, the vulnerability of the atherosclerotic fibrous caps and the expression of the chemokine MCP-1 are unaffected. These observations revealed the role of CM and CCR2/MCP-1 signaling in the pathology of plaque rupture and acute MI.

Monocytes as a target for CVD therapy

Based on the tremendous progress in understanding the role of inflammation in the development of cardiovascular complications following PCIs in general, and of monocytes/monocyte-derived macrophages in particular, several therapeutic approaches have emerged. These therapeutic measures are based on the rationale of intercepting the inflammatory cascade at the cellular-mediated inflammation level. One of the most profound advantages of particulate DDS, at least from a pharmaceutical perspective, is altering the biodistribution of the encapsulated drug in the body. Similar to Dr. Paul Erlich's search for the "magic bullet" a century ago, by changing the trajectory of the free drug, the therapeutic effect could be increased alongside with reduced toxicity. Since then, several strategies have been proposed with the objective of turning Erlich's idea into reality, including passive accumulation governed by long-circulatory nanocarriers (i.e., enhanced permeability and retention effect; EPR) [51–53], active ligand-receptor targeting [54], and active cellular mediated targeting (e.g., neutrophils, stem cells, and monocytes) [55].

Gladue et al. were one of the first to demonstrate the idea of delivering a drug to the inflamed tissue by exploiting the monocyte/macrophage massive infiltration [56]. It has been shown that the antibiotic drug azithromycin, even though found in soluble state, has the ability to accumulate in phagocytic cells, probably by active membrane carrier system. Consequently, an effective delivery of high azithromycin concentrations to the inflamed tissues has been achieved. In a similar manner, other drugs of interest, which do not intrinsically exhibit uptake ability, can effectively be internalized by phagocytic cells following encapsulation in a specially designed particulate delivery system.

Monocytes and macrophages, professional phagocytic cells, extensively internalize many particulate moieties, such as apoptotic cells, bacteria, viruses, and micro- or nanoparticles [57]. It has been further demonstrated by us and others that

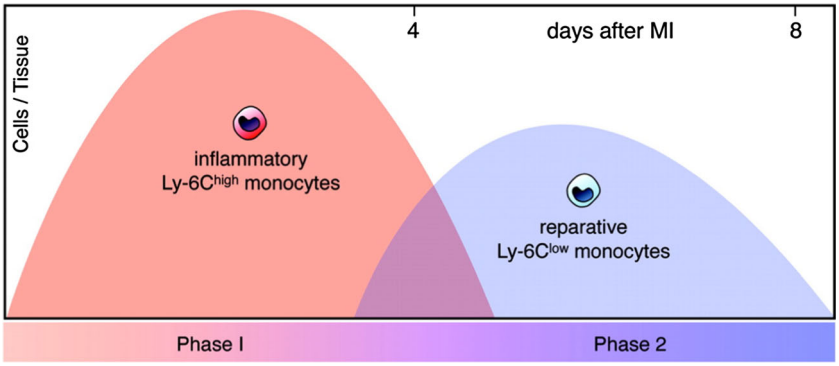

Fig. 1 The biphasic monocyte subpopulation response after myocardial infarction in the mouse. Adapted from reference [41]: Nahrendorf, M., Pittet, M. J., & Swirski, F. K. (2010). Monocytes: protagonists of infarct inflammation and repair. Circulation, 121(22), 2437–2445. https://doi.org/10.1161/CIRCULATIONAHA.109.916346. (MI—myocardial infarction)

internalization of NPs can be significantly enhanced by modifying their physicochemical properties, such as hydrodynamic size, surface-charge and shape, and in the case of liposomes, membrane fluidity [58, 59]. In contrast to particles with a hydrodynamic size of < 80 nm which tend to escape the mononuclear phagocytic system (MPS), particles larger than 100 nm are most likely to be eliminated from the circulation exclusively by monocyte/macrophage internalization [60]. It should be noted that particles larger than 0.5 μm may accumulate in the lungs and cause thrombosis. Of importance, particles under ~ 250 nm can be readily filter-sterilized. Hence, the optimal mean hydrodynamic diameter for achieving maximal monocyte/macrophage uptake is in the range of 80–250 nm. Surface charge also possesses great influence on NP uptake, whereas negatively and more potently, positively charged NPs are preferentially internalized into phagocytic cells through adsorptive endocytosis, in comparison to neutrally charged NPs [59, 61]. When a liposomal delivery system is used, the fluidity of the lipid membrane has been shown to negatively correlate with binding of serum opsonins and consequently with the degree of uptake, in vitro as well as in vivo [62, 63]. In order to increase membrane rigidity, a certain portion of cholesterol is inserted in the lipidic membrane, typically 50% of lipid molar ratio. Interestingly, it has been shown that cholesterol content in the liposomal bilayer influences the uptake extent in different phagocytic cells. Moghimi et al. showed that Kupffer cells internalize cholesterol-poor liposomes to a better extent in comparison to cholesterol-rich liposomes, and splenic resident phagocytes uptake preferentially cholesterol-rich liposomes in the presence of serum proteins [62]. The spatial geometry of the NPs is an additional important parameter affecting cellular internalization. It has been demonstrated that spherical NPs are internalized to a better extent by numerous cell lines in comparison to non-spherical NPs [58]. An additional, and frequently ignored, parameter which possesses a great impact on the extent of nanocarrier internalization by phagocytic cells is the number concentration (N_c) of the particulate delivery system, i.e., the number of vesicles administered rather than the concentration of the drug cargo [64]. It was shown that when increasing cellular uptake is desirable, the concentration of the encapsulated drug in each vesicle should be increased rather than the number of vesicles [65]. In contrast, when escape from the MPS is desired, administration of a relatively high N_c of particles/vesicles is advised in order to achieve, at least temporarily, saturation of phagocytes' internalization capacity.

Formulating a tailor-made delivery system, which takes into consideration all the above-mentioned properties, would allow for the drug cargo to be effectively accumulated at the targeted phagocytic cell. In that manner, a complete inhibition of monocyte recruitment or altering specific monocyte subtype, modulation of macrophages at the inflammation site can be achieved. In addition, particulate DDS effectively taken up by monocytes could also be used for diagnostic and therapeutic applications of the injured tissue, if the cargo is not toxic for the courier [65–67]. Beyond the scope of this review are some publications concerning the imaging of inflamed arteries by labeling tissue macrophages [68, 69] and their modulation for MI therapy by liposomes [70]. Although not discussed in the above-mentioned studies, it is reasonable to assume that such a DDS, which possesses the key parameters for promoting phagocytosis, will have a certain degree of monocyte-mediated accumulation.

Monocytes modulation by NPs

Nanotechnology offers new promising strategies for the treatment of various diseases [71]. NPs can be fabricated to perform more than one task simultaneously and can have a number of roles, such as acting as a therapeutic agent, a drug delivery vehicle, and/or as an imaging agent [72]. Many platforms of nanosized delivery systems have been proposed for the treatment of CVD, such as micelles, dendrimers, metallic and semiconductor nanocrystals, liposomes, and polymeric NPs [16, 19]. In view of the major role of inflammation in the pathogenesis of CVD, a sustained effort has been made to characterize the specific contributors and pathways and to identify non-invasive markers that will enable better targeting of NPs for treating CVD. Understanding the role of innate immune system in general, and the role of monocytes in CVD pathology in particular, enabled us and others to use them as a platform for treatment [26, 73–75].

Most of the publications on monocyte-mediated therapeutics focus on liposomes and polymeric NPs (Table 1).

Non-discriminative depletion of monocytes

Several publications describe the effect of partial and transient depletion of circulating monocytes/monocyte-derived macrophages on the inhibition of inflammation and promotion of tissue healing. Monocyte inhibition was demonstrated in inflammatory-related disorders such as CVD, endometriosis, and malignancies [26, 82–85]. The direct outcome of circulating monocyte depletion is the marked reduction of monocyte infiltration to the inflamed tissue, and consequently, the number of monocyte-derived macrophages at the site is decreased. Monocyte/macrophage depletion has been achieved by systemic injection of specially designed nanocarriers, mainly liposomes, polymeric NPs, and complexes, which exhibit increased uptake capability. The nanocarriers contain an active substance that inhibits monocytes such as a bisphosphonate (BP) [26, 61] or statins [50, 75]. It has been shown that systemic depletion of monocytes decreases the level of monocyte-derived macrophages in the artery, resulting in the attenuation of neointimal formation [25, 26, 76, 77].

Liposomes

Liposomes are spherical lipid vesicles with a bilayer membrane structure composed of natural or synthetic amphiphilic lipid molecules. The enormous versatility of the physical parameters of liposomes affords great potential for constructing tailor-made vehicles for a wide range of applications [86]. In addition, the unique structure of the liposomes can serve to encapsulate hydrophilic or hydrophobic agents, in aqueous core or lipidic membrane, respectively. Liposomes can be classified into three groups, conventional (classical) liposomes, which are short-circulating and are rapidly cleared by the MPS; long-circulating liposomes, including chemically modified liposomes such as with polyethylene glycol (PEG); and immunoliposomes, targeted liposomes with surface-attached ligands capable of recognizing and binding to cells of interest. A major breakthrough in liposomal drug delivery that occurred with the development of phospholipids grafted with PEG chains of molecular weight from 1 to 5 kDa [87, 88]. This provides a "cloud" of hydrophilic chains at the particle surface, which repels plasma proteins. These sterically stabilized ("stealth") liposomes are capable of evading the MPS resulting in a substantially prolonged circulation time of about 20 h in rodents and up to 45 h in humans. In contrast, conventional liposomes typically present a short circulating half-life ranging from minutes to a few hours in rodent models [65, 89]. Liposomes are considered to be one of the most potent DDSs by protecting the drug from degradation, modulating its pharmacokinetic properties, and generating a relatively high drug concentration in the tissue of interest [86, 90–93]. Thus, not surprisingly, liposomes are the most extensively tested nano-DDS in basic and clinical medicine with FDA approval.

The use of liposomes as a delivery system for BPs was first introduced by van Rooijen, who used liposomes to deliver clodronate into phagocytic cells in vivo [94]. This approach, termed the liposome-mediated macrophage "suicide" technique, has been used extensively in animals to eliminate blood monocytes and macrophages from different compartments of the body [95]. BPs are anti-resorptive drugs utilized clinically in bone-related disorders that involve excessive bone resorption, including osteoporosis, metastatic bone disease, and Paget's disease [96, 97]. Upon administration to humans or animals, BPs accumulate mainly in the bone tissue, with the remainder cleared rapidly from the circulation into the urine. The mechanism of action of BPs on osteoclasts is intracellular following phagocytosis of bone-adsorbed BPs [98]. BPs have poor membrane permeability in their free form. Negatively charged liposomes encapsulating BPs efficiently are characterized by enhanced intracellular internalization of the drugs by phagocytic cells, resulting in cells death (Fig. 2). It should be highlighted that targeting circulating monocytes requires the opposite approach of current nanomedicine technology since charged (rather than neutral) and not ultra-small NPs are required for effective phagocytosis, termed "biologic targeting." Thus, a cell-specific delivery system of BPs that is capable of depleting monocytes and macrophages effectively prevents restenosis in several models of animals injured by balloon or stent angioplasty, including rats and hypercholesterolemic rabbits (now in an ongoing clinical trial, see under the "Clinical studies" section) [25, 26, 76, 77]. Of advantage is the unique monocyte-targeting mechanism of action which completely spares endothelial cells (ECs) and SMC, allowing normal tissue repair. To verify the selective effect of liposomes on phagocytic cells, liposomal BPs were examined in SMC, EC, neutrophils, T cells, and hepatocyte cultures, with no inhibitory effect observed [59]. Reduction in neointimal hyperplasia and restenosis has been demonstrated in several animal models of vascular injury, following partial systemic inactivation and transient depletion of monocytes and macrophages by liposomal BPs (pamidronate, clodronate, or alendronate) [25, 76, 77]. There were no side effects since the depletion is both transient and partial, and monocyte levels return to basal levels after 5–7 days [76]. Liver and spleen macrophage numbers were also reduced to some extent by liposomal BPs 6 days after treatment [25], but the effective dose for preventing restenosis was not associated with any toxicity. It has been further demonstrated that the number of monocytes 24 and 48 h after balloon injury or stenting was significantly lower in liposomal-alendronate (LipALN) and

Table 1 Examples of monocyte-based –DDS in CVD therapy

Mode of action	Nano-carrier	Cargo agent	Experimental model	Outcome	Refs.
Non-discriminative depletion	Negatively charged liposomes	Alendronate Clodronate Simvastatin	Restenosis (rat, rabbit)	Depletion of monocytes and prevention of neointima formation	[25, 26, 75–77]
	PLGA-NPs	Alendronate Pitavastatin	Restenosis (rat, rabbit) Atherosclerosis in ApoE$^{-/-}$ mice	Depletion of monocytes and prevention of neointima formation Increased plaque stabilization	[50, 78]
	Albumin-NPs	Alendronate	Restenosis (rat)	Prevention of neointima formation	[79]
	Complex-NPs	Alendronic acid/Ga Alendronic acid/Gd	Restenosis (rat, rabbit)	Depletion of monocytes and prevention neointima formation	[80]
Discriminative depletion	Lipid-NPs	siCCR2	Atherosclerosis in ApoE$^{-/-}$ mice MI in mice	Reduction in CM recruitment and increased plaque stabilization	[74]
	PLGA-NPs	Pioglitazone inhibition of MCP-1	Atherosclerosis in ApoE$^{-/-}$ mice	Reduction in CM recruitment and increased plaque stabilization	[50, 81]
Targeting	Positively charged liposomes	Quantum-dots	Carotid injury (rat)	Fluorescent imaging of the injured artery	[65]
	PLGA-NPs	FITC	Atherosclerosis in ApoE$^{-/-}$ mice	Fluorescent imaging of the atherosclerotic plaque	[50]

liposomal-clodronate (LipCLOD)-treated animals. Moreover, reduction in the number of monocyte-derived macrophages at the injured arterial segment was observed in liposomal BP-treated rabbits 3 and 6 days after injury [25]. Thus, a systemic treatment manifested as a targeted, local one in the injured artery. Although LipALNs do not affect SMC directly, as can be seen in in vitro studies, the anti-proliferating effect on SMC observed in vivo is indirectly mediated via depletion of monocytes [59, 99].

The healing process following MI is different from other injuries since the cells of the myocardium do not regenerate. The rapid death of cardiac myocytes following an ischemic event signals the innate immune system to recruit neutrophils within 24 h and monocytes/macrophages shortly thereafter. Nahrendorf et al. injected LipCLOD immediately or 3 days after MI to assess the effect of monocytes on cardiac healing [40, 41, 100]. Monocytes were recruited to the injured myocardium in two phases. Ly-6C^{high} monocytes accumulate via CCR2 and dominate at the site of injury during the first 3 days (phase I), and Ly-6C^{low} monocytes accumulate preferentially via CX_3CR1 between 4 and 7 days after infarction (phase II). LipCLOD injection during phase 1 prevents healing and increases mice mortality. In contrast, LipCLOD injected 3 days after MI inhibits the reparative processes such as angiogenesis and extracellular matrix deposition [101–103].

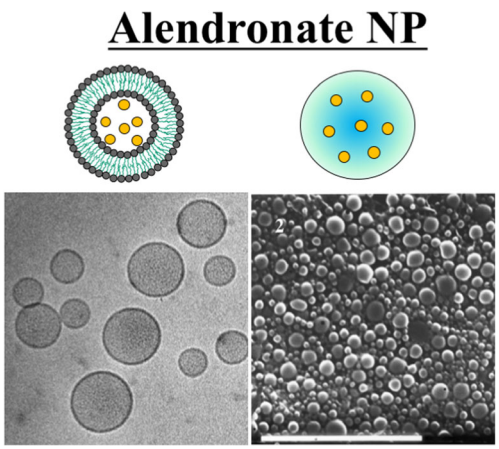

Fig. 2 Nanoparticles containing alendronate used to deplete monocytes in vivo (see text). Schematic description and transmission/scanning electron microscopy photomicrographs of alendronate encapsulated in negatively charged liposome (LipALN) (left) and PLGA-NPs (right) of ca. 120 and 210 nm, respectively

Several recent studies have demonstrated that massive monocyte infiltration to the infarcted tissue strongly correlates with improved wound healing and better prognosis [104, 105]. In another inflammatory model, promotion of wound repair in mice was achieved by local injection of macrophages [106].

Statins are extensively used in medical practice and have been shown to improve survival in patients with CVD [107, 108]. In the early 1990s, experimental studies have suggested that statins might reduce restenosis after balloon angioplasty [109]. Several preclinical studies have demonstrated that multiple systemic administrations of statins before and after surgery attenuate neointimal formation [110, 111]. Furthermore, accumulating clinical evidence apparently suggests favorable effects of statins on restenosis following stent deployment. However, all clinical studies, with a single exception [112], report a lack of significant effect from statins in preventing restenosis after PCI [113, 114]. Statins, like nitrogen-containing bisphosphonates, are inhibitors of the

mevalonate pathway blocking the prenylation of small GTPases [115]. These proteins regulate a variety of cell processes important for monocyte/macrophage function, including cell morphology, membrane ruffling, and trafficking of endosomes. In vitro and in vivo findings indicate that statins, in addition to their lipid-lowering effects, possess certain anti-inflammatory properties such as inhibition of pro-inflammatory cytokine production (e.g., TNF-α and IL-1β), C-reactive protein, cellular adhesion molecules (e.g., ICAM-1, P-selectin), and chemoattractant molecules (MCP-1) [116, 117]. Similar to alendronate [59], statins were shown to induce macrophage/monocyte apoptosis [118]. In the same line of the liposomal BP approach, Afergan et al. investigated the ability of simvastatin-laden liposomes, given IV, to deplete circulating monocytes and subsequently reduce neointimal formation [75]. Afergan et al. demonstrated that systemic administration of simvastatin encapsulated in liposomes significantly reduces neointimal formation following carotid injury in rats. In contrast, no reduction in neointimal formation was observed in rats treated with free simvastatin [75]. It should be noted that the approach of liposomal simvastatin treatment is fundamentally different than systemic administration of the free drug in solution, since the latter is not specific to a certain cell or organ. In addition, similarly to liposomal BPs, liposomal simvastatin toxicity was found to be limited to phagocytic cells, whereas no toxic effect was observed in SMC and EC. Importantly, peripheral blood monocytes returned to baseline levels 7 days following liposomal-simvastatin administration.

Polymeric NPs and complexes

Polymeric NPs are spherical objects, ranging from tens to hundreds of nanometers in size, and consisting mainly of biodegradable and biocompatible polymers, such as polylactide, poly(d,l-lactide *co*-glycolide) (PLGA) or natural polymers (e.g., albumin). Polymeric NPs have been used to incorporate a verity of therapeutic agents of both hydrophilic and hydrophobic nature [89]. Even though only the albumin-paclitaxel complex (Abraxane) has been approved for IV treatment, polymeric NPs have attracted a great deal of attention as potential candidates for drug and non-viral gene delivery vectors. This is because polymeric NPs can protect the cargo drug from degradation; overcome the absorption barrier of the cell membrane; escape the MPS under in vivo conditions (thus increasing the circulation time); and as opposed to liposomes, provides a sustained release of the drug. It has been demonstrated that following cell internalization into the cell by endocytosis, PLGA-based NPs can escape the endo-lysosomal compartment and slowly release the encapsulated payload in the cytoplasm [119].

Several polymeric NP- and complex-based carriers for the delivery of potent inhibitory agents to monocytes have caught special attention in the past several years [73, 81]. Choen-Sela et al. evaluated the anti-restenotic effect of a PLGA nanoparticulate formulation containing alendronate (ALN-NPs; Fig. 2) [78]. The formulated NPs, which presented negatively charged surface potential and a size of ~ 200 nm, exhibited a significant internalization and cytotoxic effect in monocyte/macrophage cell-line. Following SC administration of ALN-NPs to hyper-cholesterolemic rabbits, immediately after arterial injury, resulted in a significant attenuation of the neointimal formation [78]. It should be emphasized that the sustained release feature of polymeric NPs is not required in order to achieve effective monocyte depletion, and the rapid release of the drug inside the monocyte is of advantage for effectively inhibiting monocytes. Thus, the delivery system should protect its cargo until being phagocytized by circulating monocytes.

In a similar approach, PLGA-based NPs encapsulating pitavastatin (a potent statin that effectively inhibit the HMG-CoA reductase activity in rodents) has been investigated as a potential treatment for plaque destabilization and rupture in atherosclerosis [50]. It has been shown that the NPs were effectively and promptly taken up by circulating monocytes following IV administration to $ApoE^{-/-}$ mice. Pitavastatin NPs reduced the extent of monocytic fraction in total leukocytes 7 days after treatment. The depletion of circulating monocytes resulted in reduced monocyte-derived macrophages in the atherosclerotic plaque. However, the NPs also exhibited a certain extent of internalization into naïve cells (SMC and EC), which is of concern for side effects. Following recurrent weekly IV administration of pitavastatin NPs, a reduction in plaque destabilization and rupture was documented, and it was suggested to be mediated through the depletion of circulating monocytes.

In an attempt to decrease the degree of neointimal formation in the rat model of restenosis, mithramycin-laden polymeric NPs were formulated [120]. Mithramycin, an anti-tumor drug which selectively inhibits SMC proliferation in a dose-dependent manner, has been further shown to significantly inhibit circulating monocytes when encapsulated in a particulate delivery system [120]. However, following systemic administration of mithramycin NPs, no reduction in restenosis was observed possibly due to a short depletion period.

A polymeric particulate system, based on the natural polymer, albumin, for the encapsulation of BPs was introduced as a potential therapy for neointimal formation [79]. It has been shown that alendronate-albumin NPs exhibit a significant inhibitory effect on monocytes and a significant attenuation of restenosis in rats.

Another relatively new class of NPs is nanocomplexes, developed by Epstein and colleagues for the therapy of restenosis [80]. The nanocomplexes are composed of the negatively charged alendronic acid and a positively charged metallic counter ion, gallium (Ga), or gadolinium (Gd). The

self-assembly of the nanosuspensions is characterized by an optimal size and surface charge enabling effective uptake by monocytes. In addition, it has been shown that liposomal Ga inhibits macrophages in vitro [121], and that Gd promotes inhibition of phagocytosis in liver macrophages [122] as well as induce apoptosis of macrophages in vitro [123]. Following incubation with monocyte cell line, a substantial and synergistic inhibitory effect was discovered. Moreover, an effective uptake of the nanocomplexes by monocytes in vivo and subsequently a transient depletion has been demonstrated, 24 and 48 h after IV administration. Finally, the therapeutic effect of alendronate-Ga and alendronate-Gd nanocrystals was evaluated in the rat model of restenosis. It was found that systemic administration of alendronate-Ga complex at the time of carotid injury significantly inhibited neointimal formation. Moreover, the inhibition of restenosis by alendronate-Ga complex was found to be similar to the inhibition of restenosis following LipALN administration [77]. The alendronate-Gd complex also showed inhibition of restenosis, though insignificant [80].

Discriminative depletion of monocytes

Alternatively, to the non-discriminative depletion of circulating monocytes, several attempts to deplete only the pro-inflammatory monocytes (CM), while sparing the anti-inflammatory subtype (NCM), have been reported [74, 81]. Systemic administration of siRNA against messenger (mRNA) encoding for CCR2 (siCCR2) loaded in lipoidic NPs shows a substantial reduction in CM resulting in beneficent outcomes in several inflammatory-related pathologies (e.g., MI, atherosclerosis, colon cancer, pancreatic allograft rejection in type 1 diabetes) [74]. It has been shown that siCCR2-NPs accumulate in splenic phagocytic cells following IV administration, mainly in CM. Moreover, CM extracted from the spleen after treatment displays minimal levels of CCR2 mRNA and low expression of CCR2. The effect of siCCR2-NPs was also demonstrated by reduced CM migration. Treatment with NPs, containing clinically feasible doses of siCCR2, was administered daily for 3 days before MI or twice a week for 3 weeks in the atherosclerosis model. In both models, a substantial reduction in pro-inflammatory monocyte recruitment along with attenuation of disease progression were observed.

We examined the effect of timing on monocyte inhibition by LipALN in a rat model of MI (data not published). LipALN (10 mg/kg) injected IV 0, 2, and 4 days after MI had no effect on cardiac function in comparison to saline-treated rats. Our results are in accord with the study of van Amerongen et al. demonstrating that LipCLOD administered IV, 4 h before and 1, 3, and 6 days after MI, had a harmful effect to the cardiac tissue in comparison to control animals [101]. That is, CM infiltration of the myocardium during the early days (phase 1) after MI is essential for healing. The timing of monocyte depletion in restenosis is just before or after injury, and due to unknown specific mechanistic difference(s), only in MI the timing of treatment is critical.

Our group has recently demonstrated the significance of monocyte subpopulations in restenosis (published in this issue; Fig. 3) [46]. In the carotid artery injury model, both low- and high-dose LipALN (3 and 10 mg/kg, respectively) inhibit restenosis, while only the high-dose LipALN depletes monocytes. In contrast, LipCLOD, at an equivalent potency as low-dose LipALN, significantly reduced monocyte levels but had no effect on restenosis inhibition. The main finding is the correlation found between monocyte subclasses and restenosis inhibition. NCM levels were found higher in LipALN-treated rats but lower in LipCLOD-treated rats, 24 h after injury and treatment.

Nakashiro et al. further evaluated the approach of monocyte/macrophage subtype modulation by examining the effect of pioglitazone encapsulated in polymeric NPs on monocytes [81]. Pioglitazone, a peroxisome proliferator-activated receptor-γ (PPARγ) agonist with anti-diabetic activity, has a great impact on monocyte and macrophage polarity shifting them into the anti-inflammatory subtype [124]. Pioglitazone was shown to have atheroprotective

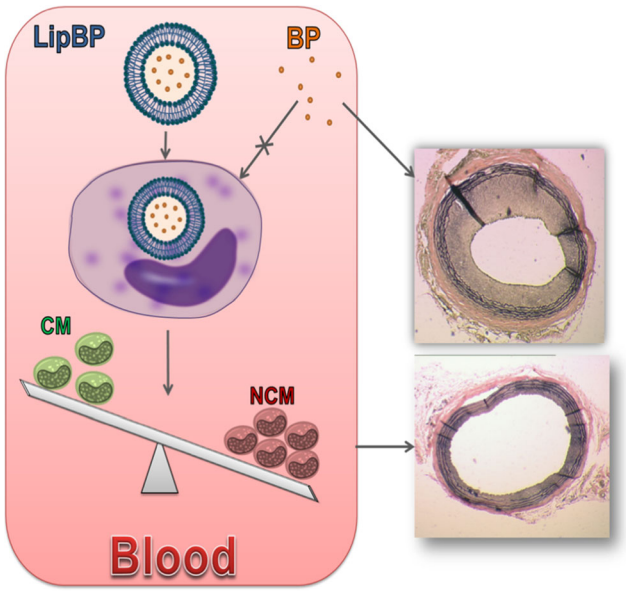

Fig. 3 Liposomes encapsulating bisphosphonates (LipBPs), but not free BPs, suppress neointima formation following vascular injury by partial and transient depletion of circulating monocytes and modulation of CM/NCM ratio. Elevation in NCM levels, 24 h after vascular injury is correlated with restenosis inhibition following LipBP administration. Adapted from reference [46]: E. Grad, K. Zolotarevsky, H.D. Danenberg, M.M. Nordling-David, D. Gutman, G. Golomb, The role of monocyte subpopulations in vascular injury following partial and transient depletion, Drug Deliv Transl Res (2017)

effects in diabetic patients [125]. Pioglitazone NPs given intravenously to ApoE$^{-/-}$ mice significantly decreased pro-inflammatory monocytes with a moderate increase of the anti-inflammatory subpopulation [81]. Moreover, pioglitazone NP treatment resulted in increased anti-inflammatory markers (arginase-1 and IL-10) expression along with a reduction in pro-inflammatory markers (IL-6, MMP-9, and extracellular MMP inducer). Finally, treatment with pioglitazone NPs has been shown to reduce the number of fibrous caps in atherosclerotic lesions in mice. It was suggested that the mechanism of action of pioglitazone NPs is modulation of monocyte subtypes resulting in alteration of CM/NCM proportion which consequently leads to elevated numbers of anti-inflammatory monocyte-derived macrophages in the atherosclerotic plaques, while the total number of macrophages remains unchanged.

Another approach to reduce the migration and infiltration of inflammatory monocytes to the injured tissue is by inhibition of MPC-1 expression in the tissue. It was shown that systemic gene therapy with plasmid laden NPs, which encodes a deletion mutant of MCP-1, attenuates the development and progression of atherosclerosis in ApoE-deficient mice [50].

Clinical studies

Recently, a phase IIa, randomized prospective, multicenter, double-blind clinical trial has been reported [126]. The objective of the study was to assess the safety and efficacy of a single IV bolus of LipALN in the treatment of de novo stenotic lesions in native coronary arteries in a population undergoing PCI with balloon pre-dilatation and implantation of a bare-metal stent. Underlying diseases of diabetes mellitus (DM) and acute coronary syndromes were considered in patient's outcome, in view of their different inflammatory state as evidenced by circulating monocyte levels. In-stent late loss was the primary endpoint follow-up at 6 months. The trial resulted in insignificant difference between the treatment and placebo groups in the average late lumen loss. The mere borderline significance of restenosis inhibition obtained in humans is most probably due to the markedly low dose used (a single and total dose of 0.01 mg per patient; $\sim 7 \times 10^4$ times lower than the dose used in rats). Nevertheless, sub-group analysis revealed a significant reduction in late lumen loss of 30% in the "inflammatory group," a group characterized by a baseline monocyte count higher than the basal median value (all enrolled patients were with no signs of active infection). This differential response suggests the potential for the personalized medicine approach. A phase IIb clinical trial of a single IV administration of liposomal alendronate is currently ongoing [127]. The primary objective of this study is to assess the safety and the efficacy of the liposomal formulation at the time of PCI and stenting in reducing restenosis in patients with DM (i.e., pro-inflammatory patients, having above-average inflammatory state with baseline monocyte count higher than the basal median value).

Pitavastatin-laden PLGA-NPs are currently being tested in a phase II clinical trial for the treatment of chronic critical limb ischemia, a peripheral arterial disease with pathophysiological characteristics similar to atherosclerosis [128]. The aims of this study are to evaluate the safety and efficacy of pitavastatin NPs and to identify the appropriate dosage regimen. Treatment with repeated intramuscular injections for 5 days is being examined in patients with chronic critical limb ischemia, which is difficult to vascularize. Results of the study have not yet been published.

Monocytes as transporters to inflammatory sites

The profound role of monocytes in inflammation and their unique phagocytosis capability makes them appealing for selective and active targeting approaches. Indeed, over the last several years, the use of monocytes for the specific transport of nanodelivery system to inflamed tissues has been demonstrated in various disease models. This includes the central nervous system [67], infections [129], and cancer [130]. However, key questions such as the portion of the nanoparticulate system taken up by monocytes, the number of monocytes as well as the number of cargo-containing monocytes infiltrating the target site, and the degree of un-degraded cargo drug released at the target site are yet inadequately answered.

One technique to address monocyte exploitation for drug delivery in CVD is by adoptive transfer of pre-extracted circulatory monocytes [131]. Following ex vivo internalization of the drug, the re-administered modified monocytes would deliver the therapeutic agent to the site of vascular disease as long as the cargo is not toxic to the cell carrier. In a recent patent, monocytes were proposed as a vehicle for therapeutic moieties in several CVDs [132]. It is claimed that monocytes, extracted from rats that underwent a carotid artery denudation using a balloon catheter, efficiently infiltrate the neointima of the injured artery following IV re-administration. It has been further claimed that extracted monocytes transfected ex vivo with a luciferase gene preserved their infiltration capability to the injured artery following re-administration. Rats which underwent a carotid injury were injected with monocytes transfected ex vivo to overexpress adiponectin [132]. Adiponectin, an endogenic hormone, promotes an anti-inflammatory phenotype in monocyte and macrophages. Morphometric analysis of injured arteries of rats treated with manipulated monocytes displayed a significant reduction in the formation of restenosis, in comparison to injured non-treated animals. Although this approach seems relatively facile, it is restricted by the amount of the drug internalized to the extracted cells and by the number of monocytes that can be administered.

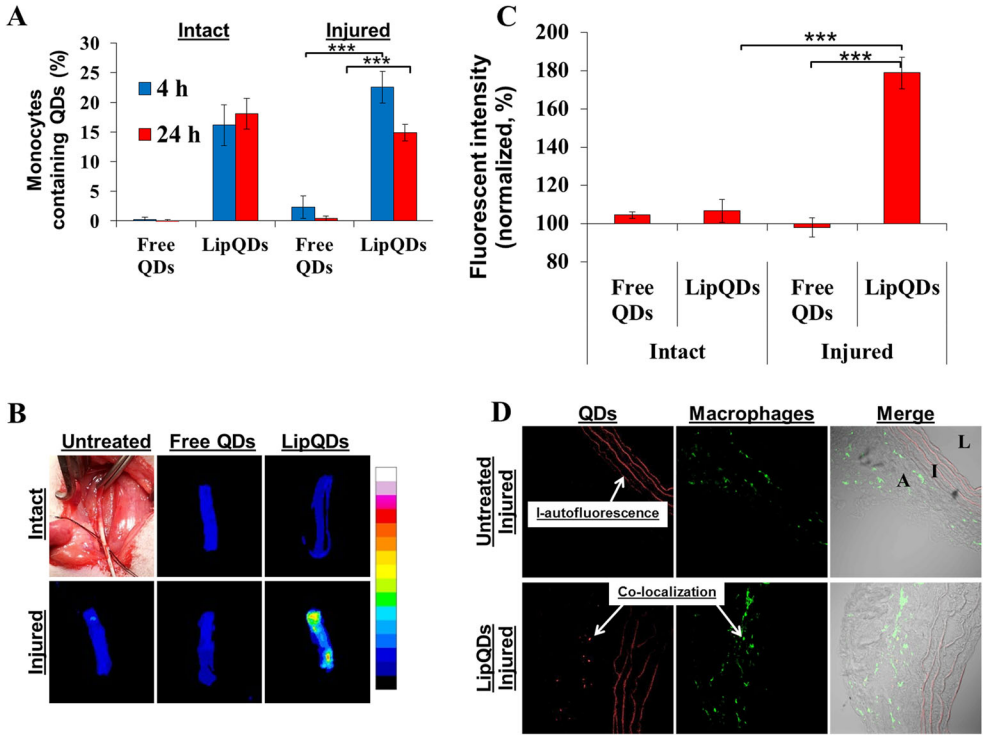

Fig. 4 Liposomal-QDs (LipQDs) accumulate in the injured carotid artery of rats (a model of arterial inflammation) 24 h post-treatment with LipQDs. **a** The phagocytosis of LipQDs by circulating monocytes 4 and 24 h after IV injection (200 nM, 2 mL) was determined by means of FACS analysis. Harvested blood monocytes were labeled with anti-CD68 antibody (FITC), and the percentage of QD-containing monocytes was assessed. Only LipQDs treatment administered to both injured and intact animals resulted in marked internalization by circulating monocytes. **b** The harvested injured carotid arteries (inset shows the surgical procedure) were scanned by means of a typhoon scanner, and the fluorescent intensities were quantified by means of the ImageJ software (**c**). Fluorescent intensities were normalized to untreated control and are presented as the mean ± SEM, $***P < 0.001$ (n = 4–6 animals in each group). Color scale bar: max = 14,815, min = 837. Note the high and selective QDs accumulation in the injured artery only following LipQDs treatment. **d** Co-localization of QDs and monocytes in injured arteries of rats 24 h post-treatment with LipQDs. The harvested injured arteries were blocked in OCT and sectioned. Representative confocal microscopy images were taken, and LipQD co-localization with tissue-infiltrated monocytes was assessed. Lumen (L), internal elastic lamina (I), and adventitia (A). QDs are shown in red, monocytes are shown in green (anti-ED1, FITC), and co-localization is shown in yellow. Adapted from reference [65]: G. Aizik, N. Waiskopf, M. Agbaria, Y. Levi-Kalisman, U. Banin, G. Golomb, Delivery of Liposomal Quantum Dots via Monocytes for Imaging of Inflamed Tissue, ACS Nano 11(3) (2017) 3038–3051

More importantly, the efficacy of this approach is questioned in view of the limited number of monocytes that can be homed to the site of inflammation. Biodistribution studies of monocytes following reimplementation, administered in different routes, shows that monocytes accumulate predominantly in the lungs at the first few hours, to a lesser extent in the liver and spleen, and only a minor fraction at the inflamed site. The cells accumulating in the lungs eventually depart and accumulate mostly in the liver and spleen [77, 133, 134].

It has been shown that following IV administration of negatively charged liposomes labeled with the fluorescent marker rhodamine, a significant fluorescent signal is detected only in the injured artery, whereas the parallel intact carotid artery did not show any signal [25]. The liposomal formulation is detected in circulatory monocytes for a very short time, suggesting that liposomes' accumulation in the artery is mediated via monocytes. Furthermore, the accumulation of the fluorescently labeled liposomes is annulled when the fluorescently labeled liposomes are co-administrated with liposomal BPs, which deplete monocytes. Thus, monocytes could serve as a carrier for DDS in inflammatory-related diseases.

Recently, we formulated positively charged liposomes loaded with quantum dots (LipQDs) for a stable fluorescent imaging of inflammation (Fig. 4) [65]. QDs, nanometer-sized fluorescent semiconductor crystals, are characterized with superior optical properties such as narrow emission band tunable by size through quantum confinement, wide excitation spectra, increased photostability, and high quantum yield (QY) [135, 136]. We hypothesized that by encapsulating QDs in positively charged liposomes, an increased accumulation of QDs in the inflamed arterial wall can be achieved, following monocyte infiltration. In addition, the lipidic vesicles would confer an increase optical and structural stability to the QDs in both serum proteins and acidic milieu characterizing blood circulation, inflammatory tissue, and cells' lysosomes. It has been demonstrated in the rat carotid injury model of restenosis that the "biological targeted" LipQDs formulation, which

displayed a remarkable and extremely stable encapsulation yield, endowed the QDs with superior qualities, in comparison to free QDs, in terms of higher QY and longer fluorescent decay life-time in quenching conditions of the blood and lysosomes. A substantial internalization of LipQDs by monocytes is achieved in both cell lines and animal models of vascular injury, with no toxicity. The QD delivery system has favorable biodistribution, characterized with sufficient accumulation and retention at the inflammatory site of carotid-injured rats. In contrast, QD accumulation is detected in neither injured arteries of rats treated with free QDs nor non-injured arteries treated with liposomal or free QDs, demonstrating the selectivity of the delivery system. Furthermore, histochemical analysis of carotid arteries has shown co-localization of QDs with macrophages only in rats that underwent injury and treated with LipQDs (Fig. 4).

Katsuki et al. demonstrated the use of polymeric NPs for the potential monocyte-based delivery of therapeutic agents in a mice atherosclerotic model [50]. PLGA NPs labeled with FITC were readily internalized into monocytes, which significantly accumulated in the atherosclerotic plaques.

It should be highlighted that despite the encouraging results in the field of monocyte-based delivery, in order to translate this strategy into clinical use, it is mandatory for the therapeutic agent to be stable in the monocyte harsh environment (e.g., endosomal and lysosomal compartments) and to be released from the monocytes at the target site.

Conclusions

Monocytes play a major role in the inflammatory response in CVD. Harnessing their professional phagocytic capability to internalize DDS seems promising for the therapy of various inflammatory-associated disorders. In this review, we summarized the strategies to modulate monocytes and exploiting them for "biologic-targeting." Depletion of circulating monocytes using DDS has been shown to have beneficial effects in animal models of CVD (restenosis and MI), and ongoing clinical trials would certainly shed more light in this evolving field. There is also extensive and promising research focusing on monocyte subtypes as another therapeutic target. Additionally, monocytes can be exploited diagnostically as carriers for delivering imaging agents to specific inflammation sites.

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Compliance with ethical standards

Conflict of interests EG and GA declare that they have no conflict of interest. GG has a financial stake in Biorest Ltd.

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