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Targeting vascular inflammation through emerging methods and drug carriers



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ABSTRACT

Acute inflammation is a common dangerous component of pathogenesis of many prevalent conditions with high morbidity and mortality including sepsis, thrombosis, acute respiratory distress syndrome (ARDS), COVID-19, myocardial and cerebral ischemia-reperfusion, infection, and trauma. Inflammatory changes of the vasculature and blood mediate the course and outcome of the pathology in the tissue site of insult, remote organs and systemically. Endothelial cells lining the luminal surface of the vasculature play the key regulatory functions in the body, distinct under normal vs. pathological conditions. In theory, pharmacological interventions in the endothelial cells might enable therapeutic correction of the overzealous damaging pro-inflammatory and pro-thrombotic changes in the vasculature. However, current agents and drug delivery systems (DDS) have inadequate pharmacokinetics and lack the spatiotemporal precision of vascular delivery in the context of acute inflammation. To attain this level of precision, many groups design DDS targeted to specific endothelial surface determinants. These DDS are able to provide specificity for desired tissues, organs, cells, and sub-cellular compartments needed for a particular intervention. We provide a brief overview of endothelial determinants, design of DDS targeted to these molecules, their performance in experimental models with focus on animal studies and appraisal of emerging new approaches. Particular attention is paid to challenges and perspectives of targeted therapeutics and nanomedicine for advanced management of acute inflammation.

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1. Introduction

The primary interface between blood and tissues is the monolayer of vascular endothelial cells lining the lumen of all blood vessels. This layer of cells provides an enormous surface area in contact with blood (3000–6000 m²), a scale approached in the human body only by the total surface area of erythrocytes (~3500 m²). Under basal conditions, endothelial cells provide many critical functions, including transport of nutrients & oxygen, immune surveillance, serving as a barrier, regulation of coagulation, among many others [1]. This privileged location in the body, set of important regulatory functions, and massive surface area underlies the observation that the endothelium is a critical site for pathological changes and, as such, pharmacological interventions in many inflammatory conditions.

Despite a complete lack of distributional barriers between the bloodstream and endothelial cells, achieving pharmacologically-relevant concentrations of small molecule and protein therapeutics within these cells remains a significant challenge. In part, this is due to the lack of any specific affinity for drug binding to endothelial cells. Furthermore, in some organs, first of all the brain, endothelial cells express efflux transporters (e.g. p-glycoprotein, breast cancer resistance protein) that actively pump drugs out of endothelial cells and back into the bloodstream [2]. This lack of natural affinity of drugs for endothelium provides a pharmacokinetic (PK) impetus to develop targeted drug delivery strategies able to concentrate drugs in these critically important cells.

Optimal delivery of therapeutics to protect against and treat vascular inflammation relies on unraveling a highly interdependent web of processes affecting drug disposition and action. In this review, we describe how drug delivery system (DDS) properties and pathological changes in the vasculature combine to impact PK and biodistribution of targeted DDS. With these factors in mind, we discuss the impact of drug targeting on treatment of acute

inflammation, as well as how therapeutic interventions may cause or exacerbate existing vascular inflammation.

2. Vascular inflammation

Endothelium is formed by a single cell layer that lines all blood vessels and is in direct contact with blood. The shape of endothelial cells varies between different vessel types, with a square shape in capillaries (~30–50 × 30–50 μm²) and an elongated shape in arteries (~10–20 × 40–50 μm²) [3]. In most organs, endothelial cells form a continuous cellular monolayer with tight but dynamic intercellular junctions. But in liver and spleen, the monolayer is discontinuous, resulting in gaps of a few hundred nm to a few microns that allow migration of large macromolecules and blood cells from blood to these organs [4–6]. Endothelial cells serve as a semi-permeable barrier and regulate the blood flow, exchanges between bloodstream and surrounding tissues, and quiescence of circulating leukocytes [7]. During pathological challenges, endothelial cells are activated and lead to alteration of vascular function and tone. These phenotypic changes in endothelial cells can be leveraged in the development and design of strategies to target therapeutics to the site of injury.

2.1. Blood flow

Under normal circumstances, quiescent endothelial cells form a non-adhesive surface with negatively charged glycocalyx that repels blood cellular elements and presents a highly thromboresistant surface by elaboration of mediators that prevent fibrin accumulation, including thrombomodulin, endothelial protein C receptor, tissue plasminogen activator, heparin-like substances, as well as anti-platelet mediators including ADP-decomposing enzyme CD39 and NO-producing enzyme NOS [8]. In sites of vascular damage, alterations in the coagulation and hemostatic systems,

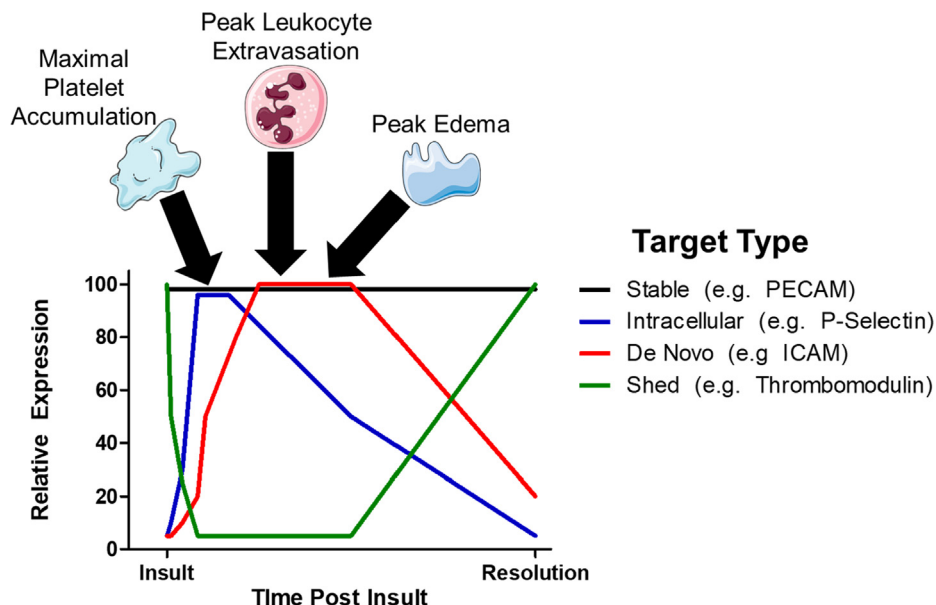


Fig. 1. Surface exposure of endothelial target molecules in inflammatory pathologies. Acute inflammation is a dynamic process. Changes in expression of endothelial surface proteins drive pathophysiological changes in the injured tissue. These changes in physiology can be harnessed to attain high levels of drug delivery in the target tissue.

Table 1
Comparison of main distribution mechanisms for nanoparticles.

Distribution Mechanism	Advantages	Disadvantages
Convection	Minimal distribution to healthy tissues (except RES) Delivery to tissues with inflamed/irregular vasculature can be achieved (e.g. tumor)	Highly inefficient process for pharmaceutical nanoparticles
Non-Specific Uptake	Nanoparticle properties can be engineered to either favor or avoid these pathways	Major route of elimination for nanoparticles Competes with mechanisms directing nanoparticles to target tissues
Affinity Targeting	Can achieve selective delivery to injured tissues, cells, sub-cellular compartments	Potential for delivery to undesired tissues expressing target antigen

resulting from inflammatory factors, increased cytokine levels, etc. causes endothelial cells to switch to a pro-thrombotic phenotype [9,10]. Pro-coagulant molecules are also exposed, such as: tissue factor (TF), phosphatidylserine (PS), P-selectin, and von Willebrand factor. Additionally, some proteins regulating coagulation

Table 2
Readouts of Endothelial Function and Inflammatory Status in Acute Pathologies.

Readout	Rodent	Human
Endothelial Function/Status	Extravasated proteins (e.g. albumin), dextran, and/or leukocytes Tissue Homogenate: CAM expression Intact Organ: Wet/Dry ratio	Plasma levels of biomarkers of endothelial injury [279]
Inflammatory Status	Tissue Homogenate: Pro-inflammatory cytokine levels (mRNA/protein), WBC infiltration	Plasma levels of pro-inflammatory cytokines Changes in WBC counts and coagulation parameters

Note: Unless otherwise noted, all tests used clinically could presumably be applied in rodent models.

(e.g. thrombomodulin) are shed from the cell surface and/or inactivated.

2.2. Sensory, signaling and regulatory functions

As endothelial cells represent a critical interface in the body, they are integral in signaling between components of blood and the surrounding tissues through their myriad of cell-surface receptors, including many that are used for drug targeting (see discussion below). In addition to sensing physical forces from flow shear stress, endothelial cells also transmit chemical signals, including oxygen and carbon dioxide, nutrients, vasoactive agents, transporting molecules (e.g., lipoprotein and albumin), pathological mediators (e.g., cytokines, proteases, reactive oxygen species (ROS)), and other natural or injected compounds [3].

2.3. Transport and exchange

Beyond signaling, endothelial cells also control transport of molecules, nutrients, and cells between blood and parenchyma via transcellular and intercellular mechanisms [11]. Under pathological conditions, pathways are activated that open gaps between adjacent endothelial cells and results in leakage of plasma proteins. In response to endothelial dysfunction, migration of leukocytes into tissues is also enhanced via upregulation of surface expression of cell adhesion molecules and integrins.

2.4. Host defense and inflammation

Endothelial cells tightly regulate cellular and humoral mechanisms of both innate and adaptive immune defense. The membrane-bound complement regulatory proteins, membrane cofactor protein and CD59 are constitutively expressed on healthy endothelial cells, to prevent undesired activation of complement and provide protection against complement-mediated injury [12]. The endothelium is also a key player in maintaining homeostasis of IgG, through the salvage pathway mediated by the neonatal Fc receptor (FcRn) [13–15].

The interaction with defense cells is one of the biggest immune roles of endothelial cells. Increased production of ROS in the

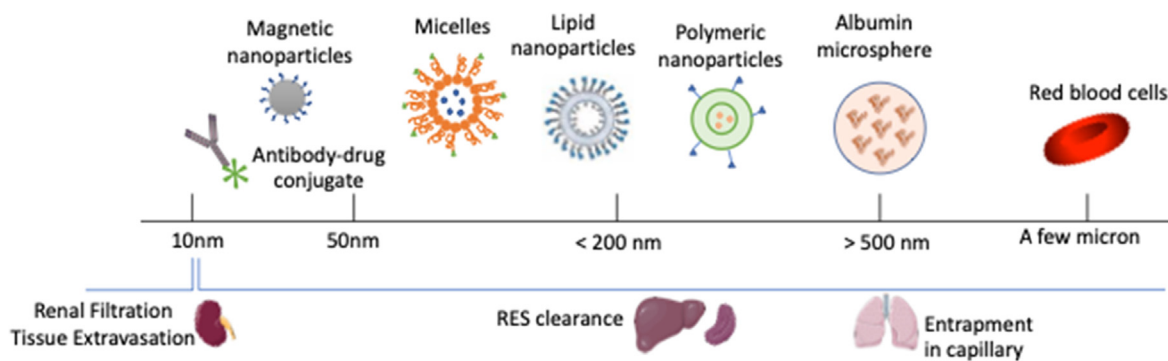


Fig. 2. Nanocarriers for vascular delivery. Typically, the nanocarriers size ranges from 10 nm to less than 1 μm . Antibody-drug conjugates and magnetic nanoparticles for imaging are below 50 nm. Micelles, lipid nanoparticles (liposome and solid lipid nanoparticles) and polymeric nanoparticles often yield a few hundred nanometers to a few microns. Particles smaller than 10 nm tend to be cleared by kidney from circulation or extravasate into tissue. Particles larger than 20 nm but smaller than 200 nm predominantly undergo reticuloendothelial system (RES) clearance, while above 500 nm particles tends to be trapped in the microvasculature.

vascular wall has been implicated in many pathologies, including hypercholesterolemia [16], myocardial infarction [17], lung/brain ischemia/reperfusion [18,19], hypertension [20], and organ transplantation [21], impaired the bioavailability of nitric oxide, which contributes to the vasoconstriction, blood flow disturbance, and platelet activation. Such activation of endothelium promotes vascular cell proliferation and inflammation, and release of cytokines and chemokines that activate and attract leukocytes to transmigrate from blood to tissue via exposure and upregulation of cell adhesion molecules (CAMs). The recruitment and activation of leukocytes in turn aggravates inflammation [22].

Pathological activation and damage of endothelial cells have been implicated in the pathogenesis of ischemic-reperfusion injury, local and systemic inflammation, acute respiratory distress syndrome (ARDS), sepsis, thrombosis, tumor growth, atherosclerosis, hypertension, restenosis, arthritis and many others [7,23–26]. These maladies involve, to varying degrees, abnormality of endothelial regulation, such as clotting, edema, abnormal blood flow, local metabolism, oxidative stress, inflammation, drug processing, and more [3,27–29]. Due to the strategic location and numerous functions in those maladies, the endothelium represents a key therapeutic target site [1,30–32].

This review primarily focuses on *acute* pathological conditions. When considering drug delivery to treat acute inflammation, many of the pathological changes described above have been utilized either to achieve selective drug delivery to sites of injury and to select pathways for therapeutic interventions (e.g. detoxification of ROS or blockade of pro-inflammatory signaling). Inflammatory pathologies are a dynamic process with changes in local expression of potential target epitopes and cell populations, among others (Fig. 1). In Table 2, we summarize readouts of vascular inflammation that can be measured in both preclinical (rodent) models and in patients. The potential impact of vascular inflammation on targeted drug delivery is highlighted below.

3. Pharmacokinetics of drug delivery systems

Due to the diverse array of DDS classes and properties, it is unsurprising that PK and biodistribution (BD) parameters can vary greatly. While the purpose of this review is not to provide an exhaustive description of DDS PK [33], a brief overview of typical mechanisms controlling the disposition of nanocarriers is provided. Following systemic administration, there are multiple competing processes that control blood and tissue exposure to DDS that can be modulated by engineering nanocarrier properties (see discussion below).

Tissue distribution of DDS can be controlled by several processes, including: 1) transport by bulk fluid flow (convection)

directly from the bloodstream to the interstitium in tissues where inter-endothelial pores are large enough to permit transport [34], 2) recognition and uptake by phagocytic cells with direct accessibility to the bloodstream (e.g. hepatic Kupffer cells), and 3) interactions with specific molecular targets conferred by affinity ligand conjugation (Table 1). The balance of these processes dictates relative distribution to desired vs. undesired sites.

However, distribution is in direct competition with elimination from the body, which is typically via either renal filtration of very small DDS (<10 nm) or via uptake by cells of the reticuloendothelial system (RES), which also affects distribution to tissues such as liver and spleen. As described below, engineering of DDS properties, such as size, charge, and flexibility, can provide some degree of control over PK/BD properties and is a viable strategy for enhancing targeted drug delivery.

4. Drug delivery system properties

Many classes of DDS have been utilized for targeted delivery to sites of vascular injury and inflammation, including antibody- and polymer-drug conjugates [35,36], liposomes [37], polymeric nanoparticles [38], nanoparticles, magnetic nanoparticles [39], solid lipid nanoparticles [40], and dendrimers [41]. In Fig. 2, we summarize typical size ranges and clearance pathways for selected nanoparticles.

4.1. Size

One of the most important and extensively studied parameters affecting nanoparticle behavior is size. Titanium dioxide, iron oxide, and gold particles often yield particles up to 50 nm [42–44]. Polymeric nanoparticles, liposomes, and core-shell micelles normally are hundreds of nanometers [45,46]. And lipid nanoparticles, silica nanoparticles have diameters from a hundred nanometers to a few microns [47,48]. It is well-established that nanoparticle size is a key factor affecting the route and efficiency of elimination from the body. Particles smaller than 10 nm tend to be cleared via renal filtration [49] and tissue extravasation [50]. Particles larger than 10–20 nm predominantly undergo RES clearance by phagocytes in organs such as liver and spleen [51]. Even larger particles (diameter > 500 nm) are likely to mechanically lodge in the first microvascular bed after injection, where capillaries only have a diameter of a few microns [52]. Generally, spherical particles with a diameter of 50–300 nm stay in the circulation longer because they are large enough to avoid kidney and liver sequestration, but small enough to escape splenic filtration [53].

Additionally, particle size may mediate cellular internalization pathways. For instance, small particles with a diameter of 60–100 nm tend to enter the cells through caveolae-mediated pathways [54,55]. Particles with the sizes ranging from 10 to 300 nm are often endocytosed through clathrin-mediated mechanisms [56,57], while larger particles are engulfed via micropinocytosis. Of course, particles can be driven to specific endocytic pathways via molecular targeting to specific cell surface receptors.

Furthermore, particle size can affect targeting to the vascular wall. Several groups have studied the role of size on margination to the vessel wall [58–61]. For example, Lee et al compared vascular distribution of spherical particles, sizing from 10 to 1000 nm [62], and Charoenphol et al elucidated the effect of particle size along with hemodynamics, vessel size, and blood rheology on adhesion to the vessel wall [59]. Both studies reported that larger particles preferentially accumulate to the vessel walls in a size-dependent manner.

4.2. Shape

In many cases, particle shape also affects circulation and cellular interactions. Gratton et al showed that rods are prone to show highest cellular uptake, followed by spheres, cylinders, and cubes when all the particles are above 100 nm [63]. Other studies also demonstrated lengthened circulation half-life and reduced liver accumulation of disk-shaped particles, when compared to spherical particles [64]. Flow-responsive filamentous polymeric micelles that align with the blood flow circulated up to 10 times longer than their spherical counterparts [65,66]. In addition to changes in non-target-dependent PK, changes in nanoparticle shape are also able to impact interactions with cell-surface targets. For example, studies of the effect of distinct shapes on vessel wall targeting, demonstrated that non-spherical particles underwent enhanced margination and adhesion to vessel wall vs. their spherical counterparts [60,67]. Direct comparisons between rod-shaped and spherical nanoparticles showed that under flow, rod-shaped particles were able to bind better to target-expressing cells and had improved delivery to target tissues [68–70]. Taken together, these results suggest that selection of an optimal nanoparticle shape may have dual benefits for drug delivery, both by reducing clearance by the immune system and by enhancing interactions with target cells.

4.3. Charge

In addition to nanocarrier size and shape, surface charge is also an important parameter that determines the nanocarrier's fate *in vivo*. As the cell membrane possesses a slight negative charge,

positively charged nanocarriers are taken up at a faster rate due to electrostatically favorable interactions [71]. Surface charge also affects corona composition, which is made up of multiple serum proteins [72]. Protein corona formation enhances recognition by phagocytes and accelerates the clearance of nanocarriers from the bloodstream [73,74]. In order to prolong the retention time of carriers in blood circulation and increase their chance to reach their target site, surface conjugation with polyethylene glycol (PEG) can neutralize the surface charge, preclude carriers from interacting with blood components, and thereby increase particle blood circulation half-life via avoidance of RES clearance [75].

4.4. Hydrophobicity

Hydrophobic interactions are one of the strongest non-covalent interactions in biological systems. They play a crucial role in various biological processes, such as biomolecule adsorption [76–78], cell membrane interaction and adhesion [77,79], cellular uptake [77], immune response [80], and hemolytic effects [81]. Jasinski et al conjugated fluorophores of different hydrophobicities to RNA nanoparticles and reported higher accumulation of the nanoparticles in vital organs in mice with stronger hydrophobicity [82]. In addition, hydrophobicity is closely related to immune response, which affects the fate of the nanoparticles. Moyano et al engineered gold nanoparticles with different degree of hydrophobic functional groups and reported increasing hydrophobicity elicits increased immune response [80].

4.5. Ligand density

While specific endothelial target determinants are discussed below, ligand density on the nanocarriers also affects greatly PK/BD and cellular interactions. Studies have shown that increasing nanoparticle avidity via increasing ligand number per nanocarrier resulted in enhanced binding affinity to endothelial cells and tissue uptake efficacy [83,84]. Additionally, introduction of spacers, such as PEG, is commonly used when conjugating ligand proteins to the nanocarriers. It decreases the crowding effect and steric hindrance, and can enhance binding to target proteins [85,86]. On the other hand, the targeting specificity of the nanocarriers depends on the ratio of ligand number and size (surface density). For example, increasing the size of antibody-drug conjugates from 40 nm to 300 nm led to improved pulmonary vascular targeting. However, further increasing the size reduced the specificity of targeting due to non-specific pulmonary uptake [87]. Such “sweet spots” of ligand density for nanocarrier and cell interaction likely varies between organs, cell types, and pathological conditions.

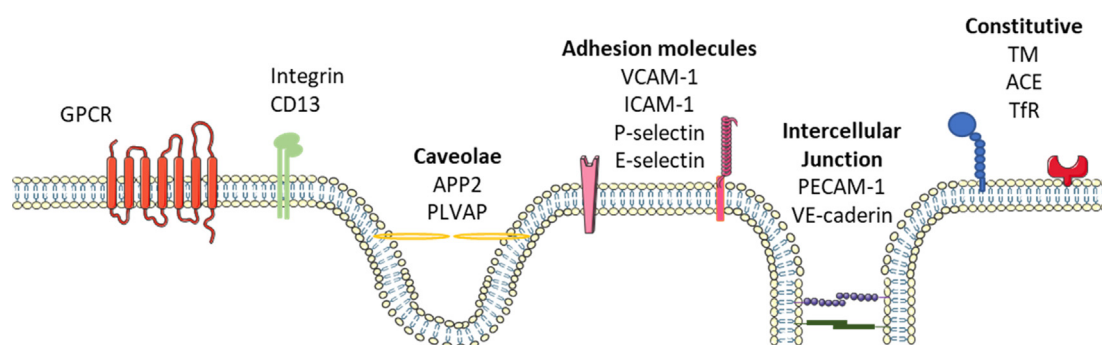


Fig. 3. Endothelial determinants for targeted drug delivery. GPCR: G protein-coupled receptors. Integrin and CD13: angiogenesis and tumor-related receptors. APP2 and PLVAP: caveolae-associated protein aminopeptidase P2 and plasmalemma vesicle associated protein, located in caveolae. VCAM-1, ICAM-1, P-selectin and E-selectin: adhesion molecules (CAMs). PECAM-1 and VE-cadherin: located at intercellular junction. TM, ACE and Tfr: thrombomodulin, angiotensin converting enzyme and transferrin receptor, constitutively expressed on endothelial cells.

Table 3
Expression of endothelial targets under pathological conditions for vascular drug delivery.

	Target Protein	Function	Location	Endocytosis Pathway
Stable	PECAM/CD31	Intercellular signaling Regulation of leukocyte migration	Pan-endothelial Intercellular borders	CAM-mediated
	TfR/CD71	Cellular uptake of iron	Ubiquitous Cell surface	Transcytosis Clathrin-mediated
Decreased	ACE/CD143	Conversion of angiotensin I to angiotensin II Inactivates bradykinin [123]	Pulmonary microvasculature Apical surface	Clathrin-mediated
	VE-cadherin/CD144	Control vascular permeability and leukocyte extravasation	Endothelial Intercellular junctions [280]	Clathrin-mediated
	Thrombomodulin/ CD141	Anti-inflammatory Anti-thrombotic	Endothelial Apical surface	Multiple
Increased	ICAM-1/CD54	Signaling adhesion Migration of leukocytes	Cell surface	CAM-mediated
	VCAM-1/CD106	Signaling adhesion and migration of leukocytes	Apical surface	Clathrin-mediated
	E-selectin/CD62E	Mediate leukocyte recruitment and blood clotting	Microvascular Apical surface	Clathrin-mediated
	P-selectin/CD62P	Mediate leukocyte recruitment and blood clotting	Microvascular Weibel-Plalade bodies	Unclear
	APN/CD13 (increased but shedded)	Digestion of peptides	Apical surface of angiogenetic vasculature	Clathrin-mediated Caveolar [281]
	Integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_5\beta_1$ CD44	Signaling, adhesion receptor and angiogenesis [282]	Cell surface	Clathrin-mediated
Unclear	APP2/XPNPEP2 PLVAP/PV1	Ectoenzyme, peptidase Microvascular permeability, structure and signaling	Caveolae Caveolae and fenestrae in lungs, heart and kidneys	Caveolar Caveolar
	TEM1/CD248	Tumor angiogenesis	Tumor endothelial cells	Unclear

4.6. Flexibility

Carrier flexibility is another key, and often underappreciated, factor that can modulate targeting. As the diameter of the blood vessel ranges from a few μm to mm, large (μm sized) rigid particles and aggregates formed by small particles tend to become entrapped in the microvasculature, while particles of similar size but with flexibility in shape can squeeze through the capillaries to pass through the microcirculation and prolong the circulation [53]. Merkel et al modulated the modulus of hydrogel particles by varying the cross-linking extent, and reported extended circulation of the particles with low-modulus and similar size as red blood cells (a few μm in diameter) [88]. Our group reported that flexible lysozyme-dextran nanogels with the diameters of 150 and 300 nm could access to plasmalemma vesicle associated protein (PLVAP), a marker found in caveolae, while rigid polystyrene particles could not [89]. Additionally, cross-linking of the flexible nanogels to increase their rigidity reduced targeting to caveolar epitopes, further highlighting the role of particle flexibility [90].

5. Target determinants

Numerous molecules localized on the surface of the endothelial cells can serve as targets for therapeutic delivery. Target determinants facilitate recognition of cells and enhance drug-loaded nanocarrier endocytosis by cells [91,92]. It helps them to bypass and evade the transporters responsible for efflux of cytotoxic drugs once released into the cytoplasm [58,93,94]. However, the nature of a 'good' molecular target is one that provides preferential delivery to desired sites, while minimizing drug uptake in tissues where adverse events are likely. Many of the strategies used to favor tissue distribution to desired sites were summarized above (**Drug Delivery System Properties**). In this section, we focus on the nature of specific molecular targets that have been used to promote preferential delivery to vascular beds of interest. Those molecules have been identified and characterized via selective proteomic mapping [95], *in vivo* phage display [96], or tracing ligand molecules, and include enzymes, receptors, integrins, transporting

molecules, and cell adhesion molecules (CAM), among others (Fig. 3). Expression of these markers may be pan-endothelial or selective to specific vascular beds, types of vessels, or endothelial activation status. Pathology may cause an increase, decrease, or no change in expression of target epitopes (Table 3), which contributes to selection of an optimal delivery strategy for generalized conditions (e.g. sepsis and disseminated intravascular coagulation) or local inflammation.

5.1. Stable expression

Platelet cell adhesion molecule-1 (PECAM-1, CD31) is expressed to different degrees on most leukocyte subtypes and platelets, yet at orders of magnitude higher density at the junctions between adjacent endothelial cells at the stable level of $0.2\text{--}2 \times 10^6$ copies per cell [97,98], even for multiple days after an acute inflammatory insult [99,100]. While *in vitro* cell culture studies have shown that PECAM expression is reduced following inflammation with no effects on leukocyte transmigration [101], *in vivo* expression seems to be stable following inflammation. Tissue-specific expression of PECAM has been correlated with endothelial surface area in organs [102]. As the pulmonary vasculature represents $\sim 25\%$ of the endothelial surface in the body and receives more than 50% of the combined arterial and venous cardiac output, delivery to PECAM leads to significant uptake in the lungs, which makes PECAM an important target for treatment of acute lung injury, pulmonary embolism, inflammation, and others [103–106].

The lung is not the only organ receiving DDS targeted to PECAM and ICAM (see below). These determinants provide an opportunity to deliver drugs to diverse vascular areas using intra-arterial route, which enables to bypass the first pass phenomenon and thereby enrich local uptake downstream the injection site. For example, intracoronary injection of DDS targeted to PECAM and ICAM offers augmented uptake in the heart, whereas infusion into mesenteric, hepatic and cerebral arteries, respectively, provide orders of magnitude elevation of the uptake and effect of the drugs in these organs [53,107–110]. While these routes of administration could be technically challenging, there is clinical precedence for inserting

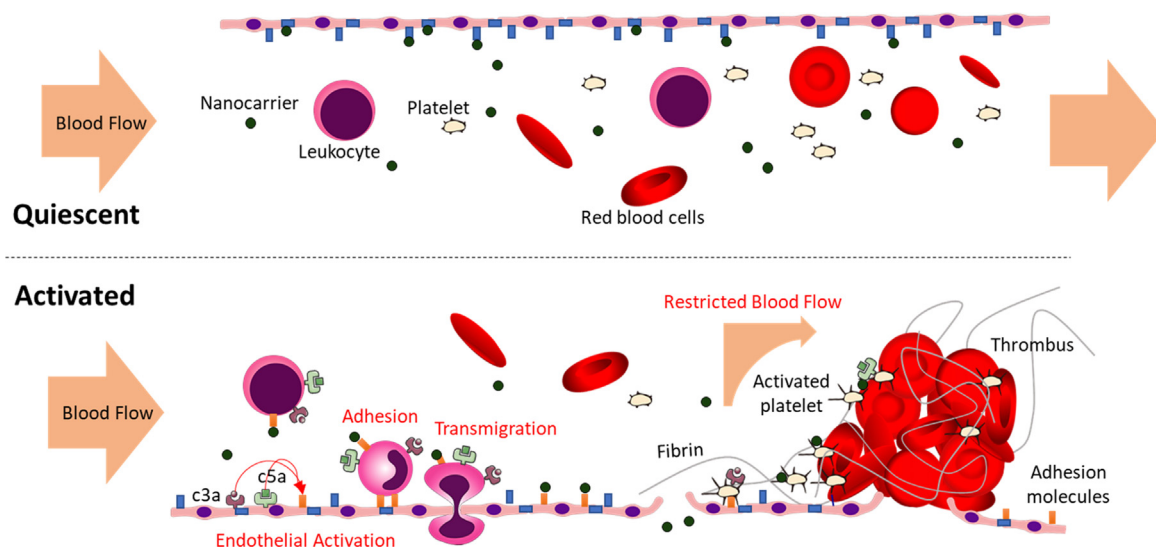


Fig. 4. Resting endothelial cells do not interact with leukocytes and platelets. Nanocarriers targeting constitutively expressed molecules (e.g. PECAM-1, ACE) can be delivered to the endothelium. Under inflammatory conditions, endothelial cells are activated and overexpress CAMs (VCAM-1, ICAM-1, P-selectin and E-selectin). Elevated activated complement components facilitate the upregulation of CAMs and enhancement of leukocytes activation and adhesion to endothelial cells and extravasation. Activated endothelial cells also open up the intercellular junctions, which promote vascular leakage. Activated platelets by inflammatory cues and activated complement components contribute to inflammatory cascades and induce aggregation. While the upregulation of epitopes on activated leukocytes, endothelial cells and platelets increase the targeting site of the nanocarriers, the thrombus formation restricts blood flow and accessibility of nanoparticles to the downstream endothelium.

catheters into these vessels. For example, removal of clots causing ischemic stroke can be performed using mechanical thrombectomy [111], which requires insertion of a catheter into vessels feeding the brain. Injection of targeted DDS using the same catheter following clot retrieval would be a feasible strategy to achieve high local delivery. Similarly, infusions into the hepatic [112] and coronary [113] arteries have been tested clinically for local delivery of therapeutics, demonstrating clinical potential for these routes of administration.

Transferrin receptor (TfR, CD71) is stably expressed on endothelial cells in a subset of organs, providing more tissue-specific cargo delivery. Multiple groups have reported enhanced brain delivery using TfR targeted nanocarriers [114–116]. However, TfR is not specific for endothelium and expressed heavily in hepatic cells. As result, DDS targeted to this determinant accumulate predominantly in this organ [117].

5.2. Target determinants decreased in pathological conditions

Pathological conditions often impact endothelial behavior, which may include suppression or shedding of surface molecule expression. For example, thrombomodulin (TM, CD141), a constitutively expressed surface molecule under basal conditions, regulates thrombosis and inflammation. Pulmonary and vascular pathologies suppress TM expression or activity [118]. Under acute conditions requiring oxygen ventilation, oxidative damage to the lungs causes reduction of delivery of anti-TM monoclonal antibodies to the lungs by 50% [119]. During infection, disseminated intravascular coagulation, inflammation, and sepsis, and as recently unveiled, in complications of COVID-19 an extracellular fragment of TM was cleaved from the apical surface, leading to enhanced microthrombosis and leukocyte adhesion [120,121]. Another constitutively expressed endothelial surface molecule is angiotensin-converting enzyme (ACE, CD143), which is also shed following pathological challenges. ACE is a metalloprotease that converts angiotensin I into angiotensin II, and indirectly regulates blood pressure. After exposure to inflammatory cues or oxidants, decreased tissue ACE activity, reduced uptake of ACE-targeted agents [122], and increased serum ACE

activity have been reported [123,124]. Animal studies and human studies have shown reduced lung targeting of anti-ACE in pulmonary edema, lung ischemia–reperfusion and sarcoidosis [1,125,126].

5.3. Target determinants increased in pathological conditions

In contrast to molecules such as TM and ACE, certain endothelial surface markers are upregulated by abnormal blood flow, oxidative stress and inflammation. Due to their enhanced exposure on activated endothelium, these molecules represent attractive targets to achieve selective drug delivery or imaging of an injured site. For example, under basal conditions, intercellular adhesion molecule-1 (ICAM-1, CD54) is expressed at the level of $0.2\text{--}1 \times 10^5$ copies per cell, but is upregulated to $0.5\text{--}3 \times 10^5$ copies per cell on activated endothelium [127]. Similarly, expression of inducible vascular cell adhesion molecule-1 (VCAM-1, CD106) also elevates within several hours [98]. Conjugation with antibodies of these molecules facilitates drug delivery to pathologically-altered endothelium in *in vitro* and in animal models of inflammation [107,128–130].

CAMs are involved in endothelial signaling and internalization of nanoparticles. Endocytosis via CAM-mediated pathway, which is triggered by redistribution, cross-linking, and clustering of CAMs following multivalent binding of nanocarriers to endothelial cells, induces rapid uptake of 85–90% of bound carriers within minutes [131]. Anti-VCAM conjugation not only allows the drug carriers to bind to activated endothelial cells, but also further activates endocytosis, which can enhance the *in vivo* imaging signals [132,133].

Selectins facilitate leukocyte and platelet adherence and activation. Resting endothelial cells sequester P-selectin (CD62P) within Weibel-Palade bodies and also suppress transcription of E-selectins. The activation of endothelial cells leads to exocytosis of Weibel-Palade bodies, exposing P-selectin on the luminal surface and inducing synthesis of E-selectin (CD62E) [7]. Anti-P-selectin antibodies target not only activated endothelium, but also activated platelets [134]. Targeting to these epitopes can provide excellent specificity in detecting and diagnosing activated

endothelium via radioisotope tracing [135] or ultrasound imaging [136].

It is worth noting that the expression of the endothelial surface molecules may alter over time under pathological conditions (Fig. 1). In acute inflammatory animal models, surface expression of selectins peaks within 2–6 hrs following inflammatory stimuli, and they disappear from luminal surface over time [137,138]. Therefore, the duration of the therapeutic effects may be restricted and thorough design of targeted treatment is essential based on knowledge of the kinetics of pathological changes.

6. Target accessibility

Pathological changes to the tissue not only can impact the absolute amount of target present, but also can modulate how accessible surface expressed target is to circulating nanocarriers (Fig. 4). This interplay between expression and accessibility can impact observed targeting to desired sites. Below, we summarize a few key physiological processes that can change in pathology and affect target accessibility.

6.1. Blood flow

In vivo, the behavior and activation status of endothelial cells can be affected by perturbations in blood flow. Cellular adaption to flow includes expression/suppression of surface molecules, endocytosis rate, signaling, etc. [139–141]. As such, changes in hemodynamics can alter processing of nanoparticles by endothelial cells. Due to different shear stresses at the vessel wall, endocytosis of ICAM-1 targeted nanocarriers appears to be superior in capillaries vs. arterioles/venules under physiological flow conditions [140]. Under pathological conditions, the accessibility of endothelial targets can be greatly affected due to masking (e.g. by adherent blood elements) or shedding as discussed above, or by changes in blood flow.

For example, reperfusion after ischemic insults such as ischemic stroke, myocardial infarction, organ transplantation, etc. [142–144] alters shear stress and can influence nanocarrier–endothelial cell interactions. The downstream cascade of inflammation caused by ischemia–reperfusion injury leads to upregulation of CAMs, which can facilitate binding of targeted nanocarriers [145–147]. Edema is a common consequence of inflammatory insults, resulting in excessive accumulation of interstitial fluid due to vascular leak [148]. This can result in turbulent or decreased blood flow. It has been observed that PECAM-targeted nanocarriers strongly accumulated in healthy lungs, but were shunted away from inflamed regions due to hypoxic vasoconstriction, which is a feature unique to the pulmonary circulation. On the other hand, targeting to locally upregulated ICAM in the inflamed lungs significantly enhanced the local delivery of nanocarriers, even in the presence of reduced blood flow [149].

Acute vascular inflammation upregulates adhesion molecule expression on the endothelial cells, exposes pro-coagulant molecules, promoting release of pro-inflammatory cytokines, enhances

platelet accumulation, sheds regulatory proteins, and eventually contributes to clot formation [150,151]. Thrombosis is an extreme case where blood flow is completely blocked due to clot formation. The reduction of nutrient and oxygen transport causes endothelial dysfunction and upregulates the expression of CAMs as a potential target for nanocarriers [152]. However, reduced flow near the clot may partially or completely shunt fluid transport of the nanocarrier away from the desired site [153]. Therefore, the interaction between nanocarrier and target may be primarily based on diffusion when local blood flow is reduced.

6.2. Impact of non-endothelial cells

Due to the direct contact between endothelial cells and blood, endothelial cells are able to interact with a myriad of cell types, including red blood cells, leukocytes and platelets. Endothelium-targeted nanocarriers, once entering the blood stream, not only adhere to endothelial surface molecules as designed, but may also interact with other cells types, both in target dependent or independent manners, which can modulate their interactions with endothelial cells.

Intracellular sequestration (P-selectin, chemokines) within Weibel-Palade bodies [154,155] and low basal expression (CAMs, E-selectin, integrins) of leukoattractants by endothelial cells minimize endothelial-leukocyte interactions under normal conditions. Due to the natural capacity of leukocytes to recognize and phagocytose particles, nanocarriers may be internalized by circulating immune cells. This can be minimized by increasing the ‘stealthiness’ of nanocarriers via techniques such as PEGylation, which decreases recognition of nanocarriers by leukocytes and increases their circulation time [75]. Following an inflammatory insult, increased local blood flow augments leukocyte delivery to the injured site; activation of endothelial cells promotes CAM expression and further recruitment of leukocytes; opening of intercellular junctions promotes localized leakage of plasma protein-rich fluid and extravasation of cells and other blood components [7]. All of these processes enhance the opportunity for targeted nanocarriers to interact with activated endothelial cells. However, competition with leukocytes for target binding may lead to reduced drug delivery. In the presence of leukocytes, anti-ICAM nanocarrier adhesion to endothelial cells significantly declined [156,157]. It is worth noting that transmigration of leukocytes to the subendothelial space happens within minutes [158]. Prolonging particle circulation time or optimizing ligand density on the particle surface could mitigate competition by leukocytes and enhance endothelial adhesion [156]. Additionally, expression of ICAM on the leukocyte surface is inducible upon inflammatory cues [159], which serves as a potential target for anti-ICAM nanocarriers. Those leukocytes endocytose targeted nanocarriers and respond to the recruitment by activated endothelial cells, potentially facilitating the delivery of the targeted nanocarriers to the injured area.

Platelets are another important mediator of hemostasis and thrombosis, which do not interact with endothelial cells under physiological conditions, but firmly adhere to the vessel wall upon

Table 4
Activation of endothelial cells by complement components.

	C1q	C5a	C3a	Terminal C complex
Upregulation of adhesion molecule expression	ELAM-1 ICAM-1 VCAM-1 [284]	E-selectin ICAM-1 VCAM-1 [285] P-selectin [286] vWF [287]	ICAM-1 VCAM-1 [288]	ELAM-1 VCAM-1 P-selectin E-selectin ICAM-1 [289,290]
Enhancement of leukocyte mobilization		Leukocyte adhesion to endothelial cells [286] and extravasation [291]	Leukocytes adhesion to endothelial cells [288]{Wu, 2016 #396}	Leukocytes extravasation [291]

activation and exposure of thrombogenic sub-endothelial layers [160]. Activated platelets release pro-inflammatory cytokines and chemokines, enabling platelets to recruit leukocytes to the site of inflammation. In an animal model of ischemic stroke, platelets roll and adhere to the cerebral vascular endothelium in a P-selectin dependent manner [161]. After activation, P-selectin translocates from α -granules of platelets to the cell membrane, hence can also serve as a local target for P-selectin binding agents [136,162].

Cross-talk between endothelial cells, leukocytes, and platelets is multi-directional and forms several positive feedback loops. Activation of endothelial cells recruits and activates leukocytes and platelets; leukocyte-derived molecules promote platelet-mediated fibrin deposition; activated platelets stimulate leukocytes to release chromatin that induces platelet aggregation on endothelium [163]. The upregulated adhesion molecules on the activated cells represent excellent targets for nanocarriers. However, the interactions between cells and nanocarriers are also affected by binding competition, disappearance/shedding of the target during pathological processes, endocytosis and so on. Therefore, the identification of cellular uptake after *in vivo* delivery of the targeted nanocarriers is a key characterization of delivery efficacy.

6.3. Role of complement proteins

Complement plays an essential role in many inflammatory cascades, which occurs shortly after insult and contributes significantly to the pathogenesis of stroke, myocardial infarction (MI), lung injuries, among other diseases [164–168]. The vascular endothelium is exposed to activated complement components and also expresses several complement proteins constitutively or induced by various cytokines [169,170]. Under physiological conditions, complement deposition on the endothelium is controlled by regulator proteins that are exposed in the plasmalemma and secreted by endothelial cells [171]. When excessive complements depositing on the endothelium due to pathological challenges, they can activate endothelial cells, increase vascular permeability, stimulate procoagulant pathways and exacerbate the inflammatory processes [172]. Table 4 summarizes the endothelial cell function changes after exposure to complement components. The upregulation of multiple CAMs represents potential targets for the therapeutic nanocarriers.

7. Pharmacologic strategies for treatment of vascular inflammation

Targeted drug delivery to the inflamed vasculature has been described for numerous conditions; however, here we focus on a select subset of acute conditions – ARDS/acute lung injury (ALI), ischemic stroke, and cardiac ischemia–reperfusion (MI). For simplicity, we will highlight only systemically delivered drug delivery strategies targeted to vascular epitopes.

DDS platform enables targeted delivery of variety of therapeutic modalities to the intended sites with different release mechanisms. For example, plasminogen activators or thrombomodulin, which directly exert their functions on the lumen surface, can be delivered via conjugation to antibody fragments to target non-internalizing endothelial epitopes [108,173,174]. Nucleic acids can be delivered into cytosol of endothelial cells to enable mediation of gene expression for therapeutic purposes [128,175]. DDS carrying small molecule drugs can target to endothelial cells and locally release drugs that directly or indirectly alters endothelial dysfunction [176,177]. Select examples of these delivery strategies are summarized below in a target-by-target manner.

7.1. PECAM-1

Due to its pan-endothelial expression, PECAM represents a potential target for drug delivery to multiple vascular beds. Antibody-mediated blockade of PECAM-mediated leukocyte migration has been explored as a therapeutic strategy for several decades [178,179]. For example, injection of anti-PECAM (Fab)₂ fragments was able to improve outcomes in a rat model of cardiac ischemia–reperfusion by reducing infarct size and cardiac MPO activity [180]. It was suggested that the effects of this direct blockade were due to inhibition of neutrophil transmigration into the cardiac tissue, in agreement with studies using anti-PECAM polyclonal antibodies in a feline model of myocardial ischemia–reperfusion [181]. Antibody blockade of PECAM was also shown to reduce infarct size and neutrophil infiltration in a mouse model of ischemic stroke [182].

Conjugation of antioxidant enzymes (e.g. superoxide dismutase (SOD), catalase) to anti-PECAM monoclonal antibody (mAb) has been extensively described for prophylaxis in various models of ARDS. In a model of oxidative stress induced by injection of anti-TM mAb/glucose oxidase conjugates, PECAM-targeted catalase was shown to improve outcomes of lung pathology (histological staining, endothelial barrier function) [183]. On the other hand, SOD was shown to protect against lipopolysaccharide (LPS)-induced lung inflammation, either alone [184] or in combination with nitric oxide donors [185]. These conjugates reduced markers of vascular inflammation in lung homogenates. Targeting to PECAM has also been used to reconstitute TM function in the pulmonary vasculature following acute inflammation. Delivery of TM alone improved endothelial barrier function and reduced markers of endothelial activation and WBC infiltration in LPS-induced lung injury models [118], and these results were further improved by co-delivering TM's natural partner, Endothelial Protein C Receptor (EPCR), to a distinct PECAM epitope [186].

In a mouse model of thromboembolic stroke, PECAM-targeted urokinase-like plasminogen activator (uPA) was able to not only dissolve fibrin microemboli, but also improve endothelial barrier function as measured by Evans blue extravasation [108]. In this condition, delivery of uPA to other vascular beds could help to prevent distal organ damage following stroke, particularly in the pulmonary vasculature. It has been reported that ~1% of acute ischemic stroke patients suffer a pulmonary embolism secondary to the initial injury [187] and, while not directly studied, it is conceivable that PECAM-targeted plasminogen activators could protect against this severe complication due to their high delivery to the pulmonary vasculature.

In addition to delivery of biotherapeutics using protein conjugates and fusion proteins, targeting to PECAM has also been used to direct drug-encapsulating nanoparticles to the endothelium. Nanoparticles provide the flexibility to deliver a wide array of small molecules and/or biotherapeutics to intracellular compartments. Our group has described the use of pluronic nanocarriers that encapsulate either SOD or catalase and used them to prevent LPS-induced lung injury. Delivery of catalase improved endothelial barrier function, while SOD reduced expression of markers of endothelial activation and cytokines in tissue homogenate [188]. Small molecule drugs that have been delivered in this manner include liposomal EUK-134 (SOD/catalase mimetic) [105] and MJ33 [104], which both improved endothelial barrier function and reduced expression of inducible cell adhesion molecules in LPS-induced lung injury. Additionally, PECAM-directed lipid nanoparticles (LNP) and polymeric nanoparticles are able to deliver functionally active mRNA and plasmid DNA to the pulmonary vasculature, respectively [103,189]. Beyond the use of PECAM targeting in therapeutic strategies, iron oxide microparticles directed

towards PECAM have been tested as a probe to image vascular remodeling following ischemic stroke in mice [190].

7.2. ICAM-1

Targeting to ICAM provides an opportunity for enhanced delivery under inflammatory conditions, due to its inducible expression following injury. Direct blockade of ICAM using monoclonal antibodies has shown efficacy in models of acute lung injury or infection via reduction of neutrophil migration in several preclinical species, including mice [191,192], rats [193], and rabbits [194]. Blockade of ICAM advanced particularly far in the development pipeline for treatment of ischemic stroke, with favorable therapeutic outcomes in preclinical models [195–198]. However, this strategy ultimately failed in clinical studies due to lack of efficacy in ischemic stroke patients [199,200].

Beyond simple receptor blockade, affinity ligands directed towards ICAM have been used to deliver therapeutic cargoes to treat vascular inflammation. Similar to work described for PECAM targeting, delivery of SOD to ICAM via antibody conjugates provided significant protection against LPS-induced lung injury, as measured by expression of markers of endothelial activation [201]. Multiple groups have used ICAM as a target epitope for delivery of the corticosteroid dexamethasone, using nanostructured lipid carriers (NLC) and lysozyme-dextran nanogels in models of LPS-induced injury. These strategies were able to reduce expression of pro-inflammatory cytokines in Bronchoalveolar lavage fluid (BALF) and neutrophil diapedesis [202] and reduce expression of inducible CAMs in tissue homogenate [176]. Other classes of drugs that have been delivered to ICAM using nanoparticles to treat ALI include I κ B kinase-2 inhibitors, HMG-CoA reductase inhibitors, immunosuppressants, antibiotics, and therapeutic genes [203–206]. While the specific outcomes measured differed across studies, these ICAM-targeted strategies typically improved endothelial barrier function, reduced pro-inflammatory markers in BALF, and/or improved tissue architecture as measured by histology.

The strong first-pass extraction by the pulmonary vasculature provides a significant barrier to brain drug delivery. Nonetheless, there are several reports utilizing ICAM for delivery of enzymes to the brain for treatment of lysosomal storage disorders [207–211]. Our group has recently demonstrated that by injecting ICAM-targeted nanoparticles via the carotid artery, the pulmonary first pass effect is avoided and significant improvements in brain targeting can be achieved. Notably, brain targeting was further enhanced by acute inflammation, suggesting the potential utility of this approach in conditions such as stroke [107].

7.3. VCAM-1

VCAM is a highly inducible target under inflammatory conditions that has low basal expression in non-lymphoid tissues. This provides an advantage of specificity to the site of injury, allowing drug concentration in activated/inflamed endothelium. Blockade of VCAM has been tested as a therapeutic strategy in animal models of pancreatitis-induced lung injury [212] and ischemic stroke [213], with mixed results. However, due to the highly specific expression of VCAM at sites of inflammation, there has been great interest in using it as a target for delivery of therapeutics and imaging probes [214–217]. VCAM-targeted, dexamethasone-loaded SAINT-O-somes were tested in a mouse model of endotoxemia and were shown to promote drug accumulation at sites of inflammation; however, limited therapeutic effects were observed [218]. Using iron oxide microparticles, the dynamics of accessible VCAM expression have been studied in mouse models of stroke, with sustained expression for several days in the penumbra [219]. We have

recently described the utility of VCAM targeting in a model of TNF- α -induced acute neurovascular inflammation. Under these conditions, mAb and nanoparticles targeted to VCAM specifically accumulated in the inflamed region of the brain. Delivery of a therapeutic mRNA encoding thrombomodulin was able to completely reverse injury-induced brain edema, suggesting the utility of targeting to VCAM for treatment of neurovascular inflammation [128].

7.4. Selectins

Selectins represent potential selective targets for sites of inflammation, particularly at early time points following the initial insult due to the lack of need for *de novo* synthesis to increase surface expression (storage in Weibel-Palade bodies). Direct blockade of the endothelial selectin (E-selectin) using antibody fragments was shown to reduce neutrophil accumulation in alveoli following intratracheal administration of LPS [220]. Nanoparticle delivery to E-selectin has been reported in models of ALI, with enhanced pulmonary delivery being observed; however, no therapeutic studies have been reported to date [221,222]. Blockade of P-selectin (expressed on activated endothelium and platelets) has shown mixed effects in the treatment of acute lung injury, with beneficial effects observed in mice [192], but no therapeutic effects in sheep [223]. Additionally, pre-treatment with P-selectin antibodies was able to protect rats and gerbils from damage induced by ischemic stroke; however [224–227]. Delivery of vascular endothelial growth factor (VEGF) encapsulated in liposomes to P-selectin was tested in a model of rat coronary artery ligation and was shown to concentrate within the injured region of the heart, improve overall cardiac function, and promote vascularization of the tissue (measured by histology) [228–230].

7.5. Integrins

Similar to selectins, integrins are often upregulated in inflammatory conditions and represent a potentially promising class of targets to obtain specific delivery. The 'classical' targeting ligand used for integrins is the RGD peptide motif first identified as the cell attachment site of fibronectin [231]. Nimbolide-loaded liposomes coated with iRGD peptide were shown to inhibit LPS-induced cytokine storm in mice via reduction of oxidative stress and cytokine storm [232]. Additionally, it was shown that targeting to $\alpha_v\beta_3$ integrin via [c(RGDyK)] peptide was able to deliver curcumin-loaded exosomes to ischemic regions of the brain. This delivery strategy reduced expression of pro-inflammatory cytokines and pro-apoptotic gene expression in the ischemic region following stroke [233].

7.6. Transferrin receptor (TfR)

For over 30 years, TfR has been considered the gold standard for antibody-based targeting of the blood-brain barrier [234–236], with drug delivery strategies attempted for a myriad of pathologies via this targeting pathway. Similar to PECAM, TfR has stable expression and no selectivity for injured regions of the tissue vs. relatively normal regions; however, multiple groups have targeted therapeutics to TfR as a treatment strategy for acute neurovascular inflammation. One strategy utilized a direct conjugate between a TfR-binding aptamer and an NF- κ B decoy in order to treat neurovascular complications of endotoxemia. This construct was shown to function via reducing phosphorylated p65 expression and inhibited expression of inducible CAMs in brain homogenates [237]. Specifically, in acute ischemic stroke, selenium nanoparticles were targeted to TfR, which were able to improve stroke-related outcomes (infarct size, neuronal function, pro-apoptotic

gene expression) as well as improve blood–brain barrier function and reduce brain edema [238].

7.7. PLVAP

Delivery to PLVAP provides unique opportunities to access the caveolar endocytosis pathways, providing access to unique subcellular compartments and transcytotic pathways relative to other endocytic pathways [239]. Caveolar delivery of SOD was able to prevent expression of inducible CAMs in lung tissue and systemic expression of pro-inflammatory cytokines in a LPS-driven model of lung injury [240]. Recently, it was shown that PLVAP-targeted LNPs were able to provide significant mRNA delivery and protein expression in the lungs, opening the door to potential caveolae-targeted gene delivery strategies [241].

7.8. DDS bypassing affinity ligands

Moving beyond antibody/peptide-based targeting, there has been recent interest in targeted drug delivery that can be achieved without coupling of affinity ligands. It was shown that albumin-based nanoparticles were avidly taken up by pulmonary intravascular neutrophils following intraperitoneal LPS treatment, purportedly via Fc γ R interactions. By loading piceatannol into these particles, the investigators were able to reduce pulmonary neutrophil infiltration and myeloperoxidase (MPO) activity [242]. We have recently identified that nanoparticles containing agglutinated proteins were able to specifically target intravascular neutrophils in mouse models of ALL. One such particle, drug-free liposomes coated with DBCO-modified IgG, not only accumulated strongly in the inflamed lungs, but also was able to provide a significant reversal of LPS-induced protein and leukocyte accumulation in alveoli [243].

An alternative strategy is to use natural physiologic homing mechanisms for targeted drug delivery by either coating nanoparticles with cell membranes or loading drugs in extracellular vesicles. Platelet-derived extracellular vesicles loaded with an I κ B kinase-2 inhibitor were able to bind to the activated vascular walls, reduce reactive oxygen species production, improve endothelial barrier function, and reduce pro-inflammatory cytokine levels in the tissue, demonstrating the potential of using cell-derived particles for drug delivery in ALI [244]. Neutrophil-derived vesicles were loaded with Resolvin D2 (induces nitric oxide generation, prevents neutrophil infiltration) and were shown to interact with endothelial adhesion molecules following transient middle cerebral artery occlusion (MCAO). These vesicles reduced neutrophil infiltration, reduced expression of pro-inflammatory cytokines (TNF- α , IL-6, IL-1 β), and ultimately reduced infarct size in mice [245]. Platelet-mimetic extracellular vesicles were shown to accumulate in cardiac endothelial cells within the ischemic region of the tissue and that they promoted angiogenesis and improved cardiac function [246].

8. Challenges and opportunities

8.1. Effects of specific targeting to endothelium

When designing drug delivery strategies for treatment of acute vascular inflammation, the potential for significant, life-threatening side effects must be taken into account. In general, patients with severe inflammatory conditions (e.g. ARDS, stroke, MI) are much more fragile than healthy subjects or even patients with chronic inflammation. This fragility means that adverse events that appear to be relatively minor or even are absent in certain subjects may be much more severe in acute critical illness.

Additionally, under inflammatory conditions there are large changes in the local immune system, which could lead to changes in host defense response to drug delivery systems, potentially resulting in adverse events.

8.1.1. Epitope-specific targeting, dual targeting, CEPAL and target-specific effects

The first step in any targeted drug delivery strategy is selection of an appropriate target epitope. Ideally a target epitope will provide selective, high levels of drug uptake at the site of injury without causing adverse effects on the tissue. For example, targeting recombinant thrombomodulin fused with scFv of anti-ICAM provides more potent protective effects than PECAM-targeted counterpart, since ICAM is located in the endothelial membrane next to molecular partner of thrombomodulin, EPCR [247]. Furthermore, binding to distinct epitopes on the same target determinant may provide an additional control of the fate and effect of the DDS. For example, endothelial cells differently internalize and traffic intracellularly nanocarriers directed to different PECAM epitopes, and the rate of intracellular delivery via these epitopes differentially regulated by flow [248].

In this context, dual targeting of DDS directed to distinct epitopes providing optimal spatiotemporal anchoring of complementary agents is of interest. For example, dual targeting to two distinct epitopes on PECAM mutually enhances binding of antibodies, fragments and conjugates directed to both parts of PECAM molecule (Collaborative Enhancement of Paired Affinity Ligands, CEPAL). [186,249–251]. Moreover, recombinant thrombomodulin and its molecular partner EPCR fused with scFv fragments of two adjacent yet distinct epitopes of PECAM provide cumulative enhancement of both targeting and functional effect of these molecular cargoes [186].

These powerful interventions may exert effects not necessarily beneficial or even benign. In some cases, the matter of selecting the optimal epitope or target is the matter of benefit/risk ratio. Unintended consequences of vascular targeting are the key issue, taking into account that the level of tolerance of side effects in the patients with conditions discussed in this paper is much lower than in oncological conditions. For example, inhibition of certain endothelial surface determinants including peptidases ACE and APP ensuing from anchoring of DDS may lead to undesirable elevation of their substrates, vasoactive peptides including substance P and bradykinin (both are increasing endothelial permeability) [252,253].

More recently, our group compared the impact of targeting two different proteins involved in inter-endothelial junctions on endothelial barrier function. In this work, it was shown that targeting to PECAM did not have any adverse effects on the pulmonary endothelium, while targeting to VE-cadherin resulted in pulmonary edema and release of pro-inflammatory cytokines in otherwise healthy mice [254]. In another study, the effects of delivery of glucose oxidase to two PECAM vs. thrombomodulin was evaluated. Delivery to both targets resulted in neutrophil transmigration in the lung; however, targeting to thrombomodulin also resulted in severe pulmonary thrombosis, hypothesized to be due to blockade of the endogenous functions of thrombomodulin [255]. These results highlight that inappropriate selection of a target epitope can lead to substantial toxicities in the target organ, which may be further exacerbated under inflammatory conditions.

8.2. Systemic drug-induced vascular damage

For any drug in the systemic circulation, there is potential for significant interactions with the endothelial cells lining blood vessels. As these cells represent the initial barrier to drug distribution into tissues, it is all but guaranteed that drug molecules will

encounter endothelial cells and have the potential to elicit a biological response from these cells. In fact, capillary leak syndrome is a reported side effect in 62 clinical trials of investigational anti-cancer drugs [256]. Knowledge of the mechanisms of these effects provides a unique opportunity to defuse them by administering endothelial-targeted drugs designed to counteract adverse effects from systemically administered drugs. This represents a potential new frontier in the field of vascular-targeted drug delivery – using endothelial targeted drug delivery as a strategy to mitigate side effects associated with systemically administered drugs.

8.2.1. Nanoparticles

It is well-appreciated that administration of nanomaterials may result in unwanted effects (for a more focused review, readers are directed to [257]), an observation that has been brought to the forefront of public consciousness with widespread administration of nanoparticle-based COVID-19 vaccines [258]. Several engineerable nanoparticle properties are known to contribute to toxicities, including material, size, shape, surface chemistry, and elasticity [257]. Here, we focus on endothelial toxicities derived from nanoparticle administration and strategies to mitigate this response.

Interactions between nanoparticles and the complement system can lead to severe adverse effects, termed complement activation-related pseudo-allergy (CARPA) [259], which manifests as severe cardiopulmonary and hemodynamic effects and has been observed after administration of liposomal drugs [260,261] and possibly for mRNA/LNP vaccines [262]. As the name implies, these acute inflammatory effects are due to activation of the complement cascade following nanoparticle administration. In the inflammatory signaling that occurs during CARPA, many blood cells (leukocytes, platelets) are activated along with endothelial cells. This can result in consequences such as edema and thrombosis, ultimately resulting in severe cardiopulmonary symptoms [263]. This highlights the need to evaluate toxicity of nanoparticles both in the naïve state and in relevant models of pathology, as in situations where the immune system is 'primed', such as inflammation, it would be unsurprising if the propensity for immune-mediated toxicities (e.g. CARPA) is enhanced in these conditions.

Recently, it was identified that high doses of untargeted siRNA-encapsulating LNP accumulated within hepatic endothelial cells and resulted in endothelial activation (upregulation of VCAM, E-selectin, ICAM), cytokine release (IL-6, G-CSF, etc.), and hepatotoxicity. However, this was circumvented by specific targeting of particles to hepatocytes using GalNAc, thereby minimizing exposure of endothelial cells to nanoparticles [264]. In the case where drug delivery to endothelium is the root cause of toxicities but is not necessary for therapeutic effects, this strategy has merit in prevention of vascular inflammation.

8.2.2. T-cell immunotherapy

Both CAR-T cell therapy and bispecific T-cell engaging (BiTE) antibodies elicit adverse neurological events that are thought to be, in part, due to endothelial activation. In a study of patients treated with CD19-targeting CAR-T, neurological side effects were associated with increased markers of endothelial activation (capillary leak, enhanced blood–brain barrier (BBB) permeability, cytokine storm, diffuse intravascular coagulation) [265]. In fact, neurotoxicity severity was shown to be correlated with peak serum concentrations of cytokines that activate endothelial cells (IL-6, IFN- γ , TNF- α) and in patients with fatal neurotoxicity histology showed disrupted endothelium and microthrombi in brain vessels. The standard of care for treating CAR-T-induced cytokine release is administration of an IL-6R blocking mAb (e.g. tocilizumab) [266]; however, local blockade of endothelial activation in

the primary organ experiencing toxicities could have unexplored benefits relative to systemic cytokine blockade.

Similar to CAR-T therapy, there have been significant neurological adverse events reported following administration of BiTE. It has recently been shown that a potential mechanism of this is bridging of endothelial and T-cells by a CD19/CD3 engaging construct (blinatumomab), creating a pseudo-immune synapse on the surface of the BBB, which was postulated to be a potential first step in BiTE-induced neurotoxicities [267]. Further investigation revealed that systemic cytokine release and endothelial activation is not required for tumoricidal activity of BiTEs, suggesting that mitigation of cytokine release in susceptible organs (e.g. brain) is a potentially viable strategy for mitigating adverse events [268].

8.2.3. Immunotoxins

Immunotoxins combine the high specificity of mAb with extreme potency of certain toxins (e.g. ricin-like toxins, pseudomonas exotoxin, etc.). Dating back to some of the earliest clinical trials of immunotoxins, capillary leak syndrome has been a recurring adverse event for these constructs [256,269] and administration of corticosteroids was investigated as a potential strategy to mitigate these effects [270]. It has been proposed that this severe, adverse effect could be due to many pathways involving endothelial cells: including direct activation/damage due to pre-emptive toxin release, acute inflammation, and cytokine release [271]. While improved design of stable constructs may be able to pre-emptively inhibit some of these effects, targeted delivery of drugs designed to mitigate endothelial activation and inflammation may have potential in improving the safety profile of immunotoxins.

8.3. Barriers to clinical translation

To date, there are no vascular-targeted drug carriers being used clinically to treat acute inflammation. While direct blockade of epitopes upregulated in inflammation has been pursued in clinical trials (see discussion of anti-ICAM above), no strategy has provided therapeutic benefits in patient populations. The differences seen between preclinical species and patients could be due to a myriad of factors, including, but not limited to: animal models of disease not representing clinical pathophysiology, differences in target expression patterns and accessibility between species, and challenges in scale up/manufacturing of complex drug carriers from lab scale to commercial scale. As understanding of key determinants of PK, biodistribution, and efficacy continues to improve, it is anticipated that our ability to anticipate and predict the behavior of drug carriers in patients will be enhanced, resulting in a greater probability of successful clinical translation.

9. Predictive tools

9.1. *In vitro* approaches

An ongoing challenge in drug development is successful implementation of *in vitro* assays that correlate with results obtained in the target patient population. While it is impossible to perfectly recapitulate the *in vivo* environment using *in vitro* approaches, development of methods that provide insights into what will happen when a therapeutic is administered is of great interest. An approach that has the potential to integrate multiple factors relating to drug disposition and effects in an *in vitro* platform is microfluidics-based assays. The effects of single-chain variable fragment (scFv) fused to thrombomodulin was tested in a microfluidics system where whole blood was flowed over endothelial cells. Both endothelial-targeted and RBC-targeted thrombomodulin were shown to reduce coagulation induced by TNF- α stimulation

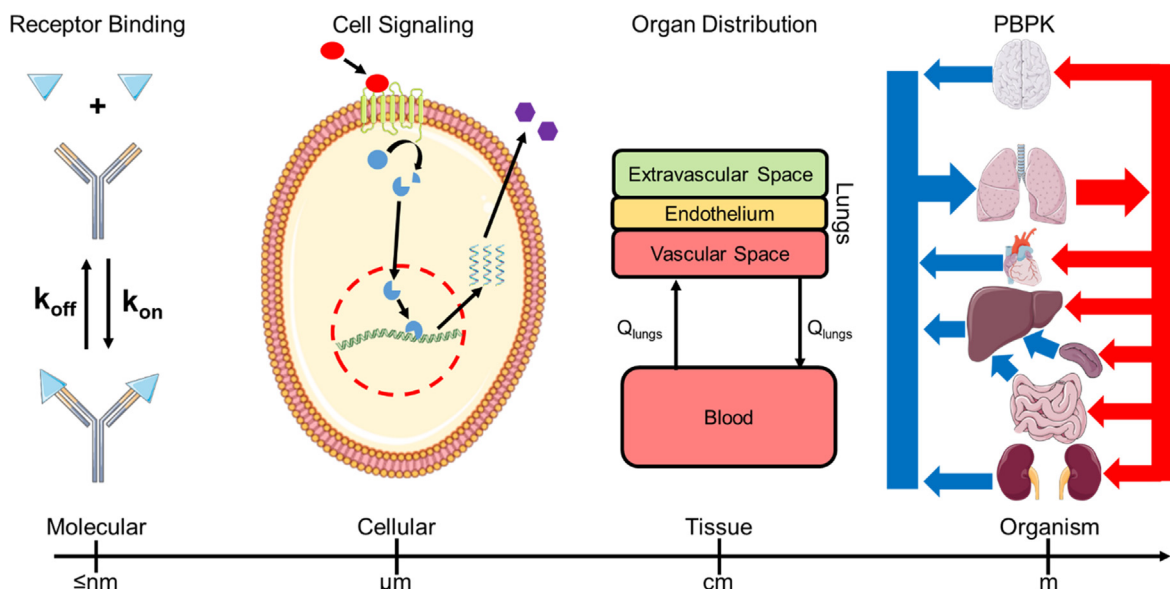


Fig. 5. Mathematical modeling of targeted drug delivery can be utilized across size scales spanning several orders of magnitude, ranging from binding interactions (nm scale) all the way to physiologically-based pharmacokinetics (PBPK) at the organism-wide scale (meters).

[129,272], providing motivation for further studies of these agents in animal models of vascular inflammation.

Development of *in vitro* assays that correspond to *in vivo* readouts of safety could allow investigators to defuse potential toxicities prior to administering a single dose to preclinical species. In a recent study, our group compared the effects of mAbs directed against two endothelial markers – PECAM and VE-Cadherin, as well as combinations of PECAM-binding mAbs – to identify potential adverse effects of this delivery strategy. *In vitro* experiments using mouse and human endothelial cells demonstrated that targeting to VE-Cadherin led to reduced endothelial barrier function, while PECAM targeting did not. These results correlated with *in vivo* experiments in mice, where VE-Cadherin targeting induced albumin extravasation in lung and induced expression of pro-inflammatory markers, while PECAM targeting was shown to be safe [254]. Other approaches have been used to probe potential endothelial toxicities of systemically administered therapeutics, including cytokine storm induced by CAR-T therapy [273] and induction of neurological toxicities due to T-cell engaging therapies promoting endothelial cell-T cell interactions [267]. In each of these studies, not only were potential mechanisms of toxicities investigations, but potential solutions to these undesired effects were also identified. Early identification of potential adverse effects of a drug delivery strategy provides opportunities to devise alternative, safe strategies or to develop approaches to mitigate these effects.

9.2. *In silico* approaches

Mathematical modeling is regularly implemented in the development of small molecule drugs and biologics in order to: a) identify relationships between drug exposure and response, b) characterize inter-individual variability in pharmacokinetics/pharmacodynamics (PK/PD), and c) identify safe and efficacious dose levels in the patient population. PK/PD models can span the range of purely empirical curve fitting (simple compartmental models) to intricate, predictive models that incorporate physiological determinants of drug disposition (physiologically-based pharmacokinetics). Use of mechanism-based models provides greater confidence in predictions that are made from these approaches;

however, there is a relative paucity of these models that have been developed for vascular targeted agents.

In silico modeling can be utilized to span across a wide range of spatial (molecular to whole-body level) and time (fractions of a second to years) scales, and selection of a model scale should be based on the desired outputs (Fig. 5). Systems biology and quantitative systems pharmacology (QSP) are two interrelated modeling disciplines that describe the kinetics of cellular signaling processes in response to a stimulus. QSP has been applied with increasing regularity in early drug discovery, in order to identify novel targets and drug combinations for a myriad of diseases. While this has not yet been applied to describe acute vascular inflammation, it is conceivable that these approaches could be used to identify dysregulated signaling pathways that contribute to adverse outcomes in vascular inflammation and to identify candidate therapeutic molecules for targeted drug delivery strategies.

Molecular modeling is an approach that can be used to probe 'nano-scale' interactions between targeted DDS and the target cells. By focusing on this scale, processes that 'macro-scale' models may be insensitive to can be probed to evaluate their impact on drug delivery. For example, this class of model has been applied to consider hydrodynamic forces on DDS and monovalent vs. multivalent engagement of DDS with endothelial cells [274–277]. By understanding the impact of these parameters on behavior of DDS on such a small spatial scale, it is conceivable that DDS properties could be engineered to identify the 'optimal' parameter space for delivery not only to the target tissue, but also to the desired cellular and subcellular compartments.

Finally, classical PK modeling is often used to describe disposition of therapeutics on the 'macro-scale', either in hypothetical compartments (mammillary models) or in physiologically-relevant spaces (physiologically-based pharmacokinetics (PBPK)). PBPK modeling provides the added advantage of being able to describe drug concentrations in tissues, as well as in the bloodstream. We have applied a semi-PBPK approach to describe the impact of acute lung inflammation induced by unilateral instillation of LPS on the sub-tissue level biodistribution of targeted nanoparticles. This model incorporated pathology-induced changes in lung physiology induced by the injury, such as altered target receptor expression, pulmonary edema, and hypoxic

vasoconstriction and was able to make *a priori* predictions of the impact of injury on ICAM-targeted, PECAM-targeted, and untargeted nanoparticle distribution to the injured region of the lung [149]. By quantifying the relative importance of pathophysiological changes in the lung tissue, we were able to understand mechanisms underlying the observation that targeting to ICAM concentrated nanoparticles to the greatest extent in the injured region, despite overall lung uptake being higher for PECAM targeting.

We have also developed a semi-PBPK model capable of describing the blood and lung PK of mAb, scFv, and mAb-thrombomodulin conjugates with affinity for ICAM and PECAM in mice. This model was able to accurately characterize differences in vascular targeting resulting from: a) monovalent vs. bivalent target binding and b) changes in clearance organs due to differences in molecular size and interactions with FcRn [278]. While this model has only been implemented to date in naïve animals, PBPK approaches allow for straightforward incorporation of physiological changes induced by injury or disease. This would allow for *in silico* investigations of uptake by target organs in models of injury and identification of efficacious dosing regimens. Additionally, due to their basis in physiology, PBPK models provide useful platforms for interspecies scaling, allowing for projection of the behavior of vascular targeted therapeutics in higher species.

10. Conclusions

Due to its unparalleled surface area, unique position as an interface between blood and tissues, and critical role in many pathologies, the vascular endothelium represents a key therapeutic target for drug delivery, particularly in acute inflammatory conditions. Over the past several decades, approaches for specific drug delivery to endothelial cells have been investigated in a myriad of pathologies and in several organs. These reports have not only demonstrated the therapeutic potential of endothelial drug delivery, but have also provided insights into how drug delivery system properties and pathology interface to affect outcomes. As these technologies approach clinical translation, it is critical that studies not only address questions of tissue-level delivery and efficacy, but also of cellular and sub-cellular addressing and adverse effects in translationally-relevant models of pathology. Development of highly predictive methodologies, both *in vitro* and *in silico* will provide additional insights into potential mechanisms of efficacy/toxicity and may guide design of clinical studies. Looking forward, drug delivery to the endothelium has potential not only to treat the inflammatory pathologies that have been the focus of decades of research (ARDS, stroke, myocardial infarction, etc.), but also may be useful as strategies to mitigate severe inflammatory phenotypes associated with next-generation therapies, such as immunotherapies used to treat cancer. Future directions of using emerging nanocarriers for vascular inflammation targeting must involve balancing between targeting efficacy and therapeutic potentials, and mitigating potential risks under pathological conditions.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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