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Targeting drug delivery in the vascular system: Focus on endothelium

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ABSTRACT

The bloodstream is the main transporting pathway for drug delivery systems (DDS) from the site of administration to the intended site of action. In many cases, components of the vascular system represent therapeutic targets. Endothelial cells, which line the luminal surface of the vasculature, play a tripartite role of the key target, barrier, or victim of nanomedicines in the bloodstream. Circulating DDS may accumulate in the vascular areas of interest and in off-target areas via mechanisms bypassing specific molecular recognition, but using ligands of specific vascular determinant molecules enables a degree of precision, efficacy, and specificity of delivery unattainable by non-affinity DDS. Three decades of research efforts have focused on specific vascular targeting, which have yielded a multitude of DDS, many of which are currently undergoing a translational phase of development for biomedical applications, including interventions in the cardiovascular, pulmonary, and central nervous systems, regulation of endothelial functions, host defense, and permeation of vascular barriers. We discuss the design of endothelial-targeted nanocarriers, factors underlying their interactions with cells and tissues, and describe examples of their investigational use in models of acute vascular inflammation with an eye on translational challenges.

Contents

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1. Introduction: Vascular endothelium and drug delivery

Circulation of blood through the vascular system represents the main mechanism for distribution of pharmacological formulations administered via intravascular, intramuscular, and other types of injections, and delivered via oral, pulmonary, nasal, sublingual, and other routes. Blood flow transports drugs and drug delivery systems (DDS). The rapidly growing roster of DDS devised to improve pharmacokinetics (PK), tissue distribution, and effects of drugs includes molecular carriers (e.g., antibodies, peptides, sugars, polyethylene glycol (PEG)), multimolecular carriers (e.g., liposomes and other nanocarriers), cells, and fragments derived from cells.

The endothelium exists as a lining surrounding the lumen of all blood vessels – arteries, arterioles, capillaries, venules, and veins separating blood from tissues. The degree of restriction of access to extravascular sites depends on the permeability characteristics of endothelial cells typical of a given vascular area under conditions of a specific pathology, as well as PK and other features of circulating agents, such as size. Generally, endothelium less effectively restricts diffusion to tissues of small drugs vs. large DDS. Yet, even large, micron-sized, long-circulating DDS having favorable PK enter some extravascular sites normally (e.g. hepatic sinuses) or in pathology (e.g. sites of edema and hemorrhage). Furthermore, endothelium, which represents an enormously extended surface area accessible to blood (3000–6000 m^2, Fig. 1) [1], is the key target for therapeutic interventions in many conditions.

Endothelial cells are the primary component of the endothelial lining of the circulatory systems (blood and lymphatic), heart (chambers and valves), and cavities in the central nervous system (brain ventricles). These highly specialized cells function in concert with other components of the barrier to form the endothelium. The *tunica intima* consists of endothelial cells and a basal membrane, which is the only component of the endothelium in capillaries. However, in larger vessels (e.g. veins and arteries), the *tunica intima* is surrounded by a layer known as the *tunica media*, which consists of smooth muscle cells, which regulate features such as vascular compliance. In different portions of the vasculature, the *tunica media* has different compositions. Finally, the *tunica adventitia* is a layer that consists of microvasculature within the vessel wall, such as the *vasa vasorum* [2].

The phenotype of endothelial cells is different within organs and larger vessels. For example, in tissues such as liver and spleen, the endothelium has large openings (hundreds of nanometers – microns in size) permitting movement of larger objects out of the bloodstream. Another specialized endothelium is that found in the glomerulus, where fenestrae are found that permit small solutes to be filtered into the renal tubules, while excluding proteins and cells larger than 5–10 nm in diameter [3]. A feature of endothelial cells within the lung and heart is the presence of a large number of caveolae ("little caves"), which seem to play a role in transendothelial transport.

In certain tissues, such as the brain, the endothelium forms an integral part of a very tight barrier, evolved to restrict passage of all but necessary substances, and, as such, lacks features such as fenestrae [4]. Results of recent studies indicate that, contrary to the previous conventional notion that the CNS vasculature lacks caveolae, the arterioles in the brain do contain abundant caveolae [5]. It is important to keep in mind, however, that caveolae have multiple functions, which likely vary in specific vascular areas, organs and endothelial phenotypes. These functions, in addition to the transport into and across the endothelial monolayer, include sensing of hydrodynamic and other physical forces, regulation of numerous signaling pathways. Thus, caveolae in the CNS arterioles seem to exert sensor rather than transport functions, consistent with the notion of restrictive transport across the BBB.

The endothelium is central in supporting the transport functions of the circulatory system, such as: tissue delivery of nutrients/oxygen, waste removal, and immune surveillance. It also controls vascular permeability, adhesiveness, contractility and angiogenesis, blood clotting

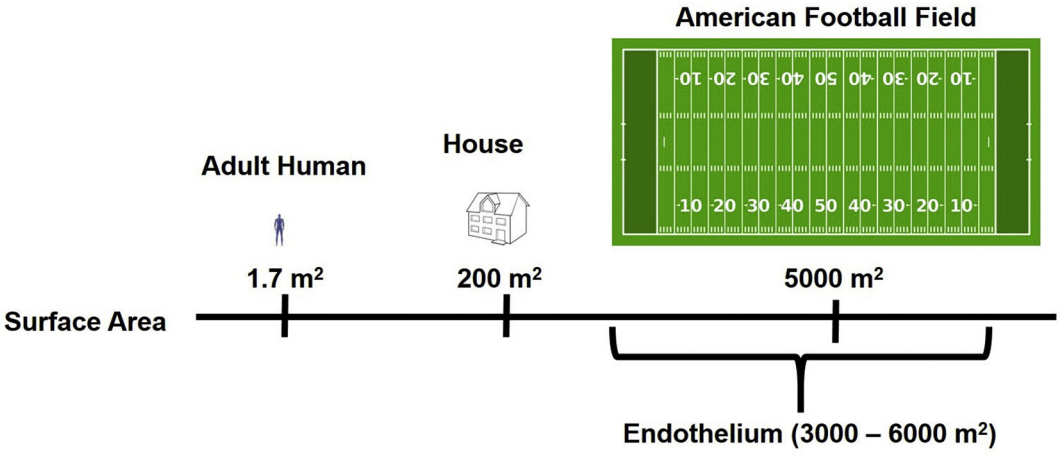

Fig. 1. The endothelium provides an extremely large surface area for drug delivery.

and fluidity, and blood/tissue exchanges [6]. As the endothelium is the critical interface between the bloodstream and extravascular sites in tissues, it serves as a key site for pharmacological interventions in inflammation, sepsis, acute respiratory distress syndrome (ARDS), blood clotting disorders, ischemia-reperfusion (I/R), hypertension, atherosclerosis, restenosis, diabetes, arthritis, tumor growth, and many others. Targeted drug delivery to endothelial cells has great potential in improving clinical outcomes in these severe pathologies [7–9].

2. Non-affinity-based targeting

Two distinct strategies to accumulate DDS in certain parts of the circulatory system employ either non-affinity- or affinity-based targeting (Table 1).

DDS may accumulate both in sites of interest and in off-target counterparts via binding or retention mediated by mechanisms with variable degrees of specificity. DDS endowed with specific affinity bind to components selectively or preferentially expressed in the target cell, tissue, or organ. This is essentially "active" (affinity-based) targeting, the subject of this paper. Conjugation to such affinity ligands using chemical or recombinant techniques yields DDS with targeting characteristics determined by the features of the binding epitope (surface density, accessibility, etc.), ligand (affinity, specificity, etc.), DDS (size, rigidity, etc.) and configuration of the conjugate (ligand density, spatial freedom, etc.). Alternative approaches bypass the need for cognizant molecular recognition of specific target determinants. For example, large particles (diameter > 10–30 μm) will lodge in the first microvascular bed encountered following injection (capillaries have a diameter of just a few μm). Mechanical retention also underlies uptake of nanoparticles aggregating in blood or forming large complexes with blood components after injection.

Some DDS have a relatively promiscuous affinity to a wide spectra of tissue counterparts. Cationic DDS bind to the glycocalyx and other anionic components of the plasma membrane of both endothelial and blood cells. DDS that are surface-decorated with Annexin V bind to membranes of apoptotic cells via exposed phosphatidylserine. While these approaches do have some specific affinity to components of the tissue, they have relatively little selectivity, hence we define these as non-affinity targeting strategies [10,11].

DDS may accumulate in some vascular areas via interactions with components of blood having natural affinity to these sites. For example, uptake of lipid nanoparticles (LNP) by hepatocytes occurs via a fortuitous interaction with apolipoprotein E (apoE), delivering LNP to the corresponding apoE receptors [12]. Opsonization, i.e., interaction of DDS with complement and IgG, favors clearance by cells comprising the reticuloendothelial system (RES) [13]. Further, reversible binding to blood cells (RBC, WBC) favors delivery of nanoparticles and other DDS to some vascular areas, organs, and pathological sites [14–16]. Understanding of regulation of these fortuitous hitchhiking mechanisms may enable one to employ this type of drug delivery in a more cognizant and controlled fashion.

Due to unpredictable scenarios of retention, delivery, and uptake of DDS in the vasculature, several groups have introduced and are currently in pursuit of methodologies that utilize in vivo screening of libraries of carrier formulations [17–19]. The outcomes, dependent on the screening approach (e.g. detection of biological activity of cargo or directly tracing a labeled component of the DDS), may yield exciting formulation variants displaying unusual tropisms to either normal or pathologically-altered organs. These discovery-driven approaches yield intriguing and often enigmatic results.

However, the DDS discussed above afford relatively limited, if any, control over addressing cargoes to specific vascular areas, cells, or cellular compartments of interest. DDS relying on size- and charge-mediated retention in the lumen of the first pass vascular areas are unlikely to enable control over cellular distribution and uptake vs. release of cargoes [20]. Engaging unknown components of the target might lead to unintended side effects. The utility of DDS evolving from discovery-driven, fortuitous, and non-specific methods for vascular drug delivery is hanging on the results of the ensuing efforts to identify target molecules and cells, as well as pharmacokinetics, biodistribution, toxicity, mechanism of delivery, and other parameters that may or may not permit emerging carriers to achieve their envisioned clinical applications.

3. Use of specific affinity ligands for targeting

Alternatively, to achieve efficient, selective targeting, one can employ ligands of surface determinants specific to or enriched in the area of interest [6,21–25]. Conjugation of DDS to affinity ligands using chemical or recombinant techniques is a common approach used to achieve specific targeting. Immunostaining, flow cytometry, Western blotting, PCR, phage display, functional genomics, and proteomics are useful approaches used to detect proteins enriched in the tissues of interest. Many moieties (e.g., antibody-derived, natural ligands, peptides, etc.) provide mechanisms for target recognition and have been used as ligands for vascular DDS [26–30].

The selection criteria of target determinants for drug delivery include: A) sufficient selectivity, specificity, and surface density of target epitopes in the desired site of action; B) accessibility of target epitopes to the intended carrier; C) engagement of target by the carrier must not induce any adverse effects; D) the target must provide the appropriate sub-cellular addressing of the carrier; and, E) the target should be useful in providing the appropriate kinetics of onset and duration and magnitude of effect to achieve the desired therapeutic outcomes.

3.1. Approaches for target identification

The hypothesis-driven approach focuses on target molecules meeting the criteria of utility in a given pathology, based on their location

Table 1
Comparison of targeting characteristics between passive vs. active approaches. Passive targeting may be reached by mechanical entrapment or non-specific features of DDS surface, while active delivery may be systemic (ubiquitous target expression) or local (tissue-specific target enrichment).

	Non-Affinity		Affinity	
	Carrier size or aggregation	Carrier surface features	Systemic Delivery	Site-Specific
Mechanism	Mechanical uptake in microvasculature down-stream injection (first pass)	Charge, Glycocalyx, ECM, PS, integrins	Binding to EC surface determinant epitopes	Using ligands of locally enriched epitopes or/and first pass
Addressing to vascular areas and EC types	Pre-capillary arterioles	Limited if any	Pan-endothelial or first pass in an organ	Diverse
Sub-cellular addressing	None	None or non-specific internalization	Cell surface and interior	As in systemic
Specificity	Low	Low	Variable to very high	High to very high

Abbreviations used in table: ECM: extracellular matrix, PS: phosphatidylserine, EC: endothelial cell, DDS: drug delivery systems.

in the body, cell surface density, specificity for the pathologically-altered region, function, membrane dynamics (clustering, internalization and shedding), and so on. This knowledge provides investigators with a rationale for choosing the target, targeting ligand, and configuration of the DDS.

The first two published reports of vascular targeting provide examples of the two primary approaches for target selection – hypothesis- and discovery-driven. Kennel, Huang, and co-workers obtained a monoclonal antibody from mice following immunization with rat lung homogenate that provided pulmonary targeting of immunoliposomes after IV injection [31,32]. Further research identified the target as thrombomodulin (TM), an endothelial transmembrane glycoprotein that converts thrombin [33] to exert anti-inflammatory and anti-thrombotic functions [34]. In clinical scenarios, meddling with TM is likely to be dangerous and produce severe adverse effects [35,36].

Meanwhile, Sergei Danilov obtained monoclonal antibodies to ACE (angiotensin-converting enzyme, an endothelial transmembrane glycoprotein that cleaves Ang I into a vasoconstricting and pro-inflammatory peptide Ang II) to test the hypothesis that ACE can be used for endothelial targeting. Indeed, anti-ACE antibodies accumulated in lungs after IV injection. Targeting to ACE, reported by Danilov and co-workers concomitantly with the Kennel and Huang paper, enabled delivery of drugs, carriers, imaging probes, and gene therapies to the pulmonary endothelium of multiple animal species, ranging from mice to humans, consistent with the high expression of ACE in the pulmonary vasculature [37–47].

It was observed that interaction of some anti-ACE antibodies with ACE lead to its shedding from the cell surface [47,48]. Extensive clinical use of ACE inhibitors implies that this effect may be tolerable or possibly beneficial in both hypertension and inflammation, and related conditions [21,49,50]. Anti-ACE mAbs were used for gene delivery in treatment of spontaneous [51] and hypoxic pulmonary hypertension [52]. In translational studies, no obvious adverse effects were observed following injection of anti-ACE in animals and humans [44,47].

3.2. Target determinants for vascular targeting

Three decades of research efforts pursuing both hypothesis- and discovery-driven approaches have yielded the roster of endothelial surface molecules that might serve as targets for drug delivery. These surface determinants include enzymes, cell adhesion molecules (CAM), integrins, receptors, transporting molecules, and other molecules, may localize to different regions of the endothelial cell membrane and to different areas of the vasculature (Table 2).

Ideal target determinants would be selectively expressed and accessible in the target site. However, there is no specific conventionally accepted gradation of the surface density of cellular epitopes, for many reasons including rather relative character of the results provided by many methods (FACS, immunostaining, ELISA) and mostly enigmatic clustering of the epitopes. It seems reasonable to categorize epitope density based on the B_{max} (maximal number of binding sites per cell determined using directly radiolabeled ligands), as high (10^5 and above), intermediate (10^3–10^5 sites per cell) and low (less than few thousands per cell).

3.2.1. Constitutive vs. inducible determinants

Under pathological conditions, endothelial cells undergo many changes that could impact targeting. Some constitutive surface molecules, such as TM, are shed from the endothelium in pathologies. For example, following acute lung injury, delivery of anti-TM mAbs to the lung was reduced by 50% [53]. This observation is in agreement with the notion that TM is shed by pulmonary endothelial cells following an inflammatory insult [54]. Another classical endothelial target, ACE, is also shed by the endothelium in pathologies [55]. This process has been shown to be mediated by several agents, including pro-inflammatory cytokines and oxidants, resulting in reduced uptake of ACE-targeted agents [56,57]. In thoracic imaging studies, it was revealed that sarcoidosis led to reduced pulmonary uptake of anti-ACE in patients [44]. Animal studies have revealed a similar reduction in lung targeting of anti-ACE in several models, including pulmonary edema, ischemia-reperfusion, and endotoxemia [57–59].

Disappearance of endothelial determinants in pathologies occurs via multiple mechanisms. They include activation on the cell surface specific proteases and peptidases that cleave off transmembrane glycoproteins such as ACE and TM [48,60,61]. In addition, enzymes cleaving chondroitin sulfate, heparan sulfate, and other sugar moieties from ACE, TM, and many other endothelial surface glycoproteins shed these components of glycocalyx, which unmasks new binding sites on normally hidden epitopes. Reactive oxygen species, proteases, and other highly aggressive entities, including hypochlorous acid (bleach), released by activated leukocytes modify endothelial membrane components directly and via damage to endothelial cells themselves [62–64].

Stable constitutive molecules, such as Platelet-Endothelial Cell Adhesion Molecule (PECAM), can be used for prophylactic and/or therapeutic delivery [39,49]. Inflammation enhances Intercellular Adhesion Molecule 1 (ICAM-1) level in many cell types, including endothelia [65,66]. Pathological endothelia commonly express inducible markers including Aminopeptidase N (APN), Tumor Endothelial Marker 1 (TEM-1), Vascular Cell Adhesion Molecule 1 (VCAM-1), and *E*- and P-selectins [25,67]. These determinants are preferable for diagnostic imaging and therapeutic interventions due to their specificity for pathologically-altered endothelium [6,23,68–70]. Selectins exposed by pathological endothelium facilitate adhesion of WBC and platelets [71]. The kinetics of upregulation of surface epitopes are dependent on the mechanism of increased surface exposure. For example, P-selectin is mobilized from intracellular stores within 10–30 min [72]. However, *E*-selectin [73] and VCAM-1 [74] surface expression requires hours to increase due to the requirement for de novo protein synthesis (Fig. 2).

In massive hemorrhage, sepsis, severe sterile tissue injury, such as reperfusion or cytokine release syndrome, and other pathological conditions, endothelial cells cease their quiescent phenotype. Instead of maintaining blood fluidity and confinement to the vascular space, pathological endothelium aggravates thrombosis and inflammation. It promotes blood clotting via exposure of phosphatidylserine and exteriorization of von Willebrand Factor and P-selectin [75]. Activated endothelial cells release chemoattractants and cytokines luring migrating host defense cells and the luminal surface becomes pro-adhesive to leukocytes and blood components and the monolayer loses the barrier function as endothelial cells contract and the VE-cadherin "zipper" opens up [76,77]. These changes, concomitant with loss of anti-thrombotic and anti-inflammatory mechanisms, including TM/APC, CD39, and nitric oxide (converted into dangerous peroxinitrite $ONOO^-$ by elevated influx of reactive oxygen species) ignite and propagate the vicious cycle of acute vascular damage, multi-organ failure, and demise [78–80]. Rapidly emerging pathological and clinical data implicate this transformation in the pathogenesis of COVID-19, in particular contributing to severe morbidity and mortality ensuing from the pulmonary microvascular injury [81–83].

Beyond the endothelium, activated platelets also serve as a target for P-selectin binding agents [84]. In general, both selectins (e.g., P-selectin, *E*-selectin) and molecules such as VCAM-1 have a low and transient surface expression on endothelium. Because of this, targeting to these epitopes could provide excellent specificity in diagnostic imaging using radioisotopes [68] or ultrasound contrasts [84,85] to visualize pathologically activated endothelium. In general, these molecules are more closely associated with activated endothelia, in tissues such as the skin [86]. VCAM-1 is expressed relatively selectively in the inflamed cerebrovascular endothelium and VCAM-targeted DDS selectively and effectively target drugs, including mRNA, to the blood-brain barrier and normalize its pathological alterations in mouse models [87] (Fig. 3).

Hydrodynamic conditions may also modulate endothelial processing of targeted carriers [88,89]. It was observed that nanoparticles

Table 2
Target determinants for drug delivery to the endothelium.

Protein	Status, organ & vessel type	Cellular localization	Function(s)	Surface density	Internalization pathway	Changes in inflammation
ACE/CD143	Constitutive Pulmonary microvasculature	Apical surface	Peptidase, converts Ang I into Ang II, cleaves bradykinin	Intermediate	Yes. Clathrin endocytosis?	Suppressed
PECAM/CD31	Constitutive Systemic Pan-endothelial	Intercellular borders	Signaling Adhesion & migration of WBC	Very high (up to 10^6/cell)	Not free ligands. DDS induce CAM-endocytosis	Stable
ICAM-1/CD54	Constitutive Lungs	Lipid rafts & tetraspanin microdomains on cell surface	As above Immunologic synapse formation. Ligand of LFA-1, MAC-1	High	Similar to above	Elevated in inflammation
VCAM-1/CD106	Inducible CNS, skin, kidney	Apical surface	As above	Low	Clathrin endocytosis	Highly elevated in inflammation
E-selectin/ CD62E	Strictly inducible Microvasculature	Apical surface	Signaling. WBC rolling, blood clotting	Intermediate	Clathrin endocytosis	
P-selectin/ CD62P	Strictly inducible Microvasculature	Weibel-Palade bodies of EC, alpha-granules of platelets	Blood clotting, binding platelets, signaling, WBC rolling	As above	Unclear	De-encrypted & Inducible in inflammation & clotting
APP2/ XPNPEP2	Constitutive Lung, heart, kidneys Alveolar & cardiac microvasculature	Caveolae	Peptidase Decays substance P Transcytosis	High	Constitutive caveolar endocytosis	Unclear
PLVAP/PV1	As above	Caveolae and fenestrae	Structure aperture; signaling	As above and spleen & kidney	Constitutive caveolar endocytosis	
TfR/CD71	Ubiquitously distributed	Cell surface	Cellular uptake of iron into specialized endosomes	Regulated by cellular iron	Receptor-mediated endocytosis; transcytosis regulator	Stable
APN/CD13 [358]	Vasculature upon angiogenesis in tissue remodeling and tumors	Apical surface	Peptidase of Ang III and IV, peptides, chemokines	Unclear	Clathrin- and/or caveola-dependent [358]	Increased, shedded
Integrins [359,360] $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_5\beta_1$	Enriched in tumor vessels and other types of angiogenesis	Cell surface	Adhesion receptors for extracellular ligands; signaling; angiogenesis.	Variable	Type-specific (i.e. caveolin-dependent for $\alpha_5\beta_1$, etc.) [361]	Activated
VE-cadherin/ CD144 [362]	Constitutive, exclusively endothelial	Intercellular junctions	Cohesion and organization of the intercellular junctions; signaling; vascular permeability regulation	High	Clathrin-mediated endocytosis (p120-catenin- or phosphorylation-induced)	Decreased, causing high vessel permeability
TEM1/CD248 [363]	Constitutive in stromal fibroblasts. Expressed in tumor endothelial cells, but not in normal endothelium	Cell surface	Tumor angiogenesis	Variable	Unclear	Unclear
GP90/CD44 [364]	Most epithelial and lymphoid tissues. Avesicular zone of pulmonary endothelium [99]	Cell surface	Cell adhesion and migration; immune response; tumor angiogenesis. Hyalorunan receptor	Variable	Non-classical CD44-mediated endocytosis of hyalorunan [365]	Increased

Abbreviations used in table: ACE: angiotensin-converting enzyme, Ang I: angiotensin I, Ang II, angiotensin II, PECAM: platelet-endothelial cell adhesion molecule, WBC: white blood cell, DDS: drug delivery system, CAM: cell adhesion molecule, ICAM-1: intercellular adhesion molecule 1, LFA-1: lymphocyte function-associated antigen 1, MAC-1: macrophage-1 antigen, VCAM-1: vascular cell adhesion molecule 1, CNS: central nervous system, EC: endothelial cell, APP2/XPNPEP2: aminopeptidase P, PLVAP: plasmalemma vesicle-associated protein, TfR: transferrin receptor, APN: aminopeptidase N, *VE*-cadherin: vascular endothelial cadherin, TEM1: tumor endothelial marker 1.

targeted to either ICAM or PECAM had reduced uptake by endothelial cells following flow adaptation [90,91], and their uptake in endothelium in vivo appears to be inferior in arterioles vs. capillaries [91]. In contrast, acute increses in shear stress stimulates endocytosis of PECAM-targeted nanocarriers [90]. Activation of endothelial cells using cytokines (e.g. TNF-α) leads to enhanced internalization of ICAM-1-targeted nanoparticles, compared to naïve, quiescent cells [91].

3.2.2. Cellular localization of ligands and ligand-coated DDS

Endothelial surface determinants localize to diverse domains of the plasmalemma. Proteins such as PECAM and VE-cadherin function to maintain the endothelial barrier, and, as such, are often found at inter-endothelial junctions [92,93]. On the other hand, VCAM-1 and ICAM-1, which function in leukocyte adhesion and migration, are largely found on the apical surface of the membrane within specific micro-domains [94–96], or 'rafts' [97]. Within the pulmonary endothelium, GP85 is found on the luminal surface within an organelle-free portion of the cell [98,99].

Various forms of endocytosis (e.g. clathrin, etc.) are used by endothelial cells to internalize ligands bound to selectins [100–102]. This process allows entry of a variety of *E*-selectin targeted agents, such as liposomes [103], drugs [103,104], and nucleic acids [105]. However, not all endothelial surface epitopes are internalized as efficiently. Studies revealed that anti-PECAM mAbs are not internalized and that anti-ICAM-1 mAbs are internalized, but are subsequently recycled back to the cell surface [106]. This feature of CAMs is desirable for therapeutics that require prolonged exposure in the vessel lumen, such as anti-thrombotics [107]. It should be noted that this process can be modulated by the avidity of the drug carrier, as multivalent protein conjugates and nanoparticles targeted to PECAM and ICAM-1 are able to enter endothelial cells via an inducible CAM-mediated endocytosis mechanism [108]. By tuning the valency of a DDS, sub-cellular delivery of CAM-targeted agents can be controlled, to a certain extent.

Accessibility of particles to specific endothelial epitopes is dependent on the localization of the epitope within the membrane and vascular conditions [109]. Epitopes that may not be suitable for affinity

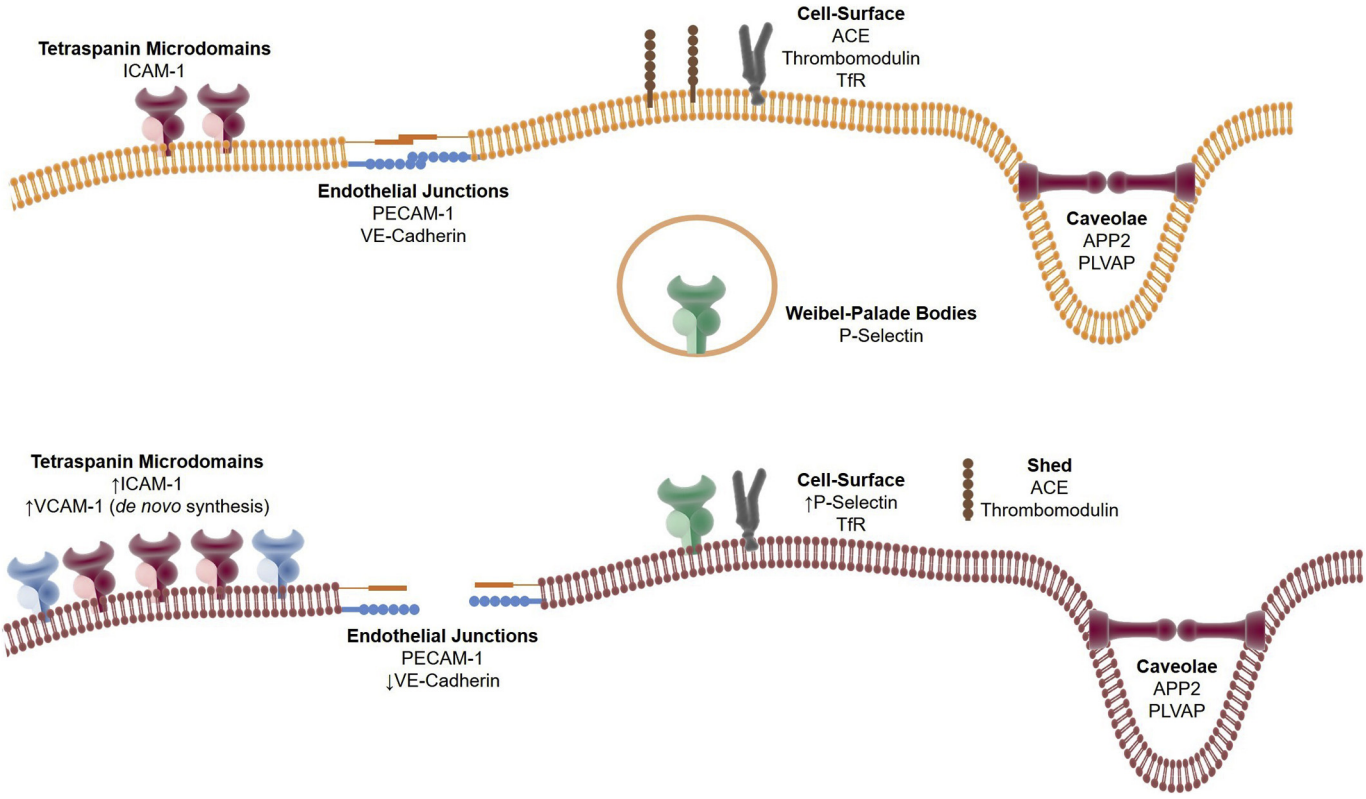

Fig. 2. Inflammation affects surface expression and cellular localization of endothelial targets. Upper panel: Receptor expression on quiescent endothelium. Certain proteins are localized to specific cell surface microdomains, such as tetraspanin-rich (ICAM-1), cell-cell junctions (PECAM-1, VE-Cadherin), and caveolae (APP2, PLVAP), while others may be found throughout the cell surface (ACE, thrombomodulin, TfR). Lower panel: Receptor expression, localization, and accessibility change upon endothelial activation. ICAM-1 and VCAM-1 expression in the tetraspanin domains is increased, largely via de novo synthesis. Accessibility to endothelial junction proteins PECAM-1 and VE-Cadherin may change due to reduced cell-cell interactions. P-selectin is upregulated on the membrane through mobilization of intracellular stores found in Weibel-Palade Bodies. Other proteins such as ACE and thrombomodulin are lost from the cell surface through a shedding mechanism.

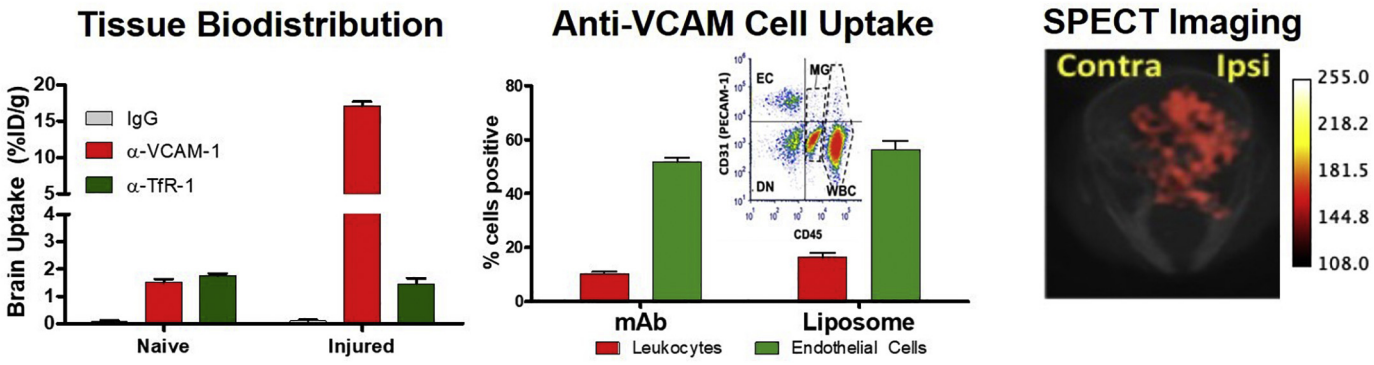

Fig. 3. Targeting to VCAM-1 enables selective delivery to the inflamed cerebral vasculature. Left panel: Absolute uptake of anti-VCAM-1 mAb in the brain of mice injured via an intrastriatal injection of TNF-α exceeds that of the 'gold standard' for brain delivery, Transferrin Receptor (TfR), by an order of magnitude. Middle panel: Flow cytometry on brain homogenates reveals that over 50% of endothelial cells are positive for VCAM-targeted agents (either mAb or liposome) following IV injection. Following IV injection of fluorescently labeled mAbs and liposomes under the same conditions as in the left panel, brains were disaggregated and stained to determine mAb/liposome association with leukocytes and endothelial cells. Inset: Typical dot plot showing cell types identified via this approach. Right panel: SPECT imaging of VCAM-targeted liposomes labeled with ^{111}In demonstrates selective uptake in the injured hemisphere of the brain. Units in the scale bar are presented as arbitrary intensity units. Figures adapted from [87]. Colors are consistent across panels.

targeting include those that are masked by the glycocalyx, located deep within cell-cell junctions, or are inside membrane invaginations [110]. Pathological changes in tissues can alter the accessibility of target epitopes. A feature of certain pathologies is the shedding of the endothelial glycocalyx, which may increase accessibility of certain target epitopes, such as ICAM-1 [111]. However, the endothelial surface may become coated with leukocytes and thrombi, which would reduce accessibility to target determinants [58]. Recent advances in techniques such as proteomics [112] in vivo phage display [113–115] have provided additional insights into accessibility of membrane targets. As carrier size increases, accessibility to epitopes becomes increasingly more important (e.g., accessibility is less of a concern for peptides than for immunoliposomes).

Proteins located within caveolae, such as Aminopeptidase P (APP) and Plasmalemma Vesicle Associated Protein-1 (PLVAP) [116], are accessible only to relatively small drug carriers. This is due to the nature

of the flask-shaped membrane invaginations that are characteristic of caveolae. It is generally thought that the caveolar mouth has a diameter of about 50 nm [117,118]. Functions of caveolae include endothelial trafficking and cellular signaling [119–124]. A key feature of caveolae that distinguishes them from other cholesterol-rich domains (e.g., lipid rafts) is the presence of the protein caveolin-1. Caveolar pathway(s) transport ligands including albumin [125] and chemically-modified albumin [126] across the endothelium [127]. In rats, mAbs directed against several caveolar antigens are able to efficiently target and transmigrate across the pulmonary vasculature, consistent with the large number of caveolae present in the pulmonary endothelium [6].

However, conjugation of APP and PLVAP antibodies with nanoparticles larger than 50-70 nm obliterates targeting, due to the presence of the caveolar neck [128]. It has been reported that caveolae are able to merge, forming "caveolosomes", which are capable of internalizing large particles. However, these experiments were perfomed in vitro using static cell culture systems, which may not be reflective of true in vivo endothelial physiology [116,129,130]. The spatial accessibility of caveolar targets to circulating nanocarriers is a hot topic. For example, flexible anti-PLVAP nanogels with a diameter of 150–300 nm enter caveolae and accumulate in the lungs, whereas their rigid nanoparticle counterparts do not [131].

Ferritin-based nanocarriers have been investigated as a DDS that can be used in a modular and stimuli-responsive manner [132]. Ferritin particles conjugated with anti-PLVAP accumulate in the lungs after intravenous injection and enter caveolae, since their size does not exceed 20 nm. Since the ferritin carriers are so small, conjugation of the ligand and enzymatic cargo to external and internal facets of ferritin nanocages, respectively, improves targeting [133]. Interestingly, ferritin nanocarriers targeted to ICAM also appear to have greater access to the endothelial surface in the pulmonary vasculature compared to larger carriers [134,135].

3.3. Intracellular endothelial delivery

A conventional approach to achieve intracellular delivery utilize affinity ligands capable of anchoring DDS to epitopes that are permissive of endocytosis. Diverse endothelial determinants have been implicated in internalization [50,136–138]. In general, identification and selection of affinity ligands is empirical in nature [139,140]. Approaches such as phage display facilitate selection of internalizable ligands [141–143]. In one example, phage display was used to select several VCAM-1-binding peptides [144]. Peptides that were rapidly endocytosed were used for molecular imaging of vascular inflammation [86,144]. Antibodies to APP, PLVAP or GP90 enter caveolae [6] and those to *E*-selectin or VCAM-1 [103,104] enter clathrin-mediated endocytosis [101,102].

In some cases, coating carriers with affinity ligands permits internalization to occur at least as efficiently as monovalent ligands, if not more efficiently. This may occur due to multivalent carriers eliciting strong cell signaling and actin rearrangement [145]. Both VCAM-1 antibodies [104,146] and VCAM-1 targeted DDS have been reported to enter endothelial clathrin-mediated endocytosis [86,144]. Anti-transferrin receptor (TfR) mAbs bound to TfR and free transferrin also enter endothelial cells via clathrin-mediated endocytosis [147,148]. However, coupling ligands to carriers may actually reduce cellular uptake due to their exceeding size limits of endocytosis. Caveolar and clathrin-mediated endocytosis have size limits of 50–70 [149] and 200–300 nm [70], respectively. Findings made using in vitro, static systems should be taken with a grain of salt, as they may use non-physiologically-relevant endocytic pathways that have not been confirmed in vivo thus far [127].

The uptake and trafficking of ICAM-targeted DDS vary. Endothelial cells are not effective in internalizing monovalent fusion proteins that do not initiate clustering of epitopes, whereas bivalent anti-ICAM and anti-ICAM conjugates recycle rapidly to the luminal surface, unlike multivalent nanoparticles targeted to ICAM, which traffic to the lysosomes [91,106,108,150–154]. Binding to different epitopes may also change the efficiency of entrance into cells [110]. Nanocarrier geometry may also affect uptake and subcellular trafficking [108,155]. For example, disk shaped particles targeted to ICAM-1 have a lower rate of endocytosis compared to spheres of a similar size. Once inside the cell, the rate of lysosomal trafficking is inversely related to size (e.g., small particles traffick more quickly) [155].

Silvia Muro, whose studies greatly advanced the field of endothelial intracellular delivery, devised a novel DDS platform for ICAM-1 targeted delivery of nanocarriers loaded with enzyme replacement therapies for lysosomal storage diseases (LSD) [153,156]. LSDs are morbid conditions caused by mutation of lysosomal enzymes [157]. In LSD, enzyme replacement therapy (ERT) is the standard of care. Clinically, this approach is applied by chronic injections of recombinant forms of the deficient lysosomal enzyme [158]. The systemically injected proteins are then internalized by cells in a manner dependent on mannosylation of proteins, via either mannose receptor and/or mannose-6-phosphate receptor [159]. Currently approved therapies for treatment of LSD include ERT, substrate reduction inhibitors, and small molecule chaperones [160–162]. Type B Niemann-Pick disease (NPD) is caused by deficiency/malfunction of the protein acid sphingomyelinase (ASM). Deficiency of ASM results in increased deposition of sphingomyelin and cholesterol enzymes [157].

In LSD, endothelial cells are often a key pharmacologic target [156], but endothelial delivery of ERT is not very effective [161,163]. It has been reported that endothelial cells have increased ICAM-1 expression in many LSD, which may provide a route for improved delivery of recombinant enzymes [164,165]. Delivery of ERT using ICAM-1-targeted nanoparticles has been shown to improve endothelial targeting and pharmacologic effects in several animal models of LSD [153,166,167]. In a mouse model of Pömpe disease, an ~600-fold improvement in lung delivery was observed for ICAM-1-targeted acid α-glucosidase (the enzyme deficient in this disease), relative to untargeted enzyme [168]. By adjusting geometric features of the carrier, delivery via ICAM-1 targeting can be optimized. Use of relatively small (100–200 nm), spherical carriers provided maximal delivery to the lysosomes as compared to particles with other features (e.g., discoidal, micron-sized/spherical) [169].

The Muro group has also identified that interactions between nanocarriers and endothelial ICAM-1 results in activation of enzymes responsible for sphingomyelin metabolism in the membrane and endocytosis of a wide range of sizes of targeted particles [170]. However, this pathway is dependent on sphingomyelinase expression and coating of particles with both anti-ICAM-1 and ASM permits internalization in NPD [166]. Additionally, it was shown that larger carriers (>200 nm) coated with ASM and anti-TfR were also internalized [171].

CAM-mediated endocytosis achieved via targeting to ICAM-1 has been shown to be more efficient than clathrin-mediated endocytosis via TfR targeting in delivery of enzymes to the lysosome [154]. However, dual targeting to ICAM-1 and TfR provided a different tissue distribution than either mono- or non-targeted carriers [171]. The utility and significance of these findings represent an area of active investigations [156].

4. Interaction of DDS with endothelial targets: vascular targeting modulation

Nanocarriers can be decorated by ligands in many ways. Interplay between features of the carrier (e.g., size, shape, flexibility, etc.), the ligand (e.g., affinity, density, etc.), and the target epitope (e.g., accessibility, density, etc.) creates a complex matrix of parameters controlling interactions between a DDS and its target.

4.1. Dual targeting

Carriers co-coated by antibodies to both inducible and stable high-density molecules can, in theory, selectively bind to pathological cells

[172,173]. Dual P-selectin/ICAM-1 targeted particles carrying equimolar ratios of both ligands were reported to bind to the inflamed endothelium better than mono-targeted particles [172,174,175]. This dual targeting approach improved imaging in mouse models of inflammation using contrast enhancing particles targeted to either P-selectin and VCAM [176] or P-selectin and ICAM-1 [177]. Dual targeting of anti-ICAM/anti-TfR nanoparticles has shown that individual ligands could promote targeting to a specific tissue vasculature (ICAM-1: inflamed lungs, TfR: brain) [171].

4.2. Collaborative endothelial targeting

Dual therapeutic targeting to vascular lumen can be achieved by utilizing a recently discovered phenomenon where distinct monoclonal antibodies directed to adjacent epitopes in the extracellular region of PECAM stimulate binding of each other [178]. The effect was dubbed Collaborative Enhancement of Paired Affinity Ligands, or CEPAL, and is dissimilar to generally observed binding patterns of ligands to same target molecule inhibiting binding of each other [48,179,180]. This unusual finding can be explained by increased accessibility of an epitope to its ligand due to conformational changes in the PECAM molecule induced by binding of a paired "stimulatory" ligand [181]. In-depth investigation of mechanisms underlying this effect revealed several findings: CEPAL is independent of PECAM-PECAM homophilic interactions in the plasma membrane and can be observed with recombinant PECAM protein [181]. Given the known roles of PECAM-1 in promoting endothelial quiescence and in maintaining cell junction integrity [182], investigations of possible unintended effects of CEPAL on vascular function have shown only modest or insignificant effects. Interestingly, these effects were observed in response to antibodies to PECAM-1, whether given solo or in pairs [183].

CEPAL was applied to achieve enhanced effects of protein therapeutics using anti-PECAM scFv fusion proteins [178]. A key advantage of this dual targeting strategy was demonstrated by co-delivery of thrombomodulin (TM) and its natural endothelial binding partner, endothelial protein C receptor (EPCR) using paired anti-PECAM scFv fusions. *Co*-administered scFv/TM and scFv/EPCR partnered in activation of protein C (PC) and reduced markers of lung inflammation in a model of acute lung injury [184]. Interestingly, the CEPAL effect also increased delivery of PECAM-targeted nanocarriers, a finding that opens up a new avenue for the field of nanomedicine in optimizing and enhancing delivery of diverse therapeutic cargoes encapsulated in endothelial-targeted nanocarriers [185]. The CEPAL mechanism, occurring by exposure of partially occult epitopes on PECAM protein, may be a generalizable phenomenon and is likely to occur for other molecules and antibody pairs.

4.3. Ligand surface density

A key parameter controlling targeted nanocarrier delivery is the surface density of the affinity ligand. However, selection of the optimal density is not a straightforward process, as this value is dependent on features of the carrier, the affinity ligand, and the target epitope. In other words, selection of the maximal surface density may not be the optimal strategy for achieving the maximal selectivity and magnitude of drug delivery. For example, an intermediate ligand density has been shown to provide enhanced selectivity of targeting [186,187]. An excessive ligand density ("ligand overcrowding") impedes congruency of ligand and target molecules [186,188]. Selectivity of both delivery and imaging of vascular inflammation has been shown to be improved via reduction of surface density to an optimal value [128,189]. Recent studies have shown that ligand density can play a role not only in selectivity of delivery, but also in sub-cellular trafficking. Delivery of ICAM-1-targeted nanocarriers across the blood-brain barrier was demonstrated to be optimal at an intermediate ligand density, which balanced efficient uptake and detachment from target following transport [150].

4.4. Carrier geometry and flexibility

In addition to a carrier's affinity, its geometry and flexibility regulate pharmacokinetics, circulation, tissue permeation, and interactions with cells [190–194]. Carrier size is a factor in route of administration, target access, and pharmacokinetics. Carriers smaller than 20–30 nm can be administered via diverse routes, while larger carriers generally require vascular injection for systemic administration [193,195–198]. Once in circulation, carriers of small (<10 nm) diameter are prone to rapid renal clearance [199]. A size range of ~100–200 nm generally avoids filtration to the space of Disse in the liver and entrapment in the splenic sinusoids, giving longer circulation and more opportunities for target engagement [200]. For instance, it has been noted that larger ICAM- and PECAM-targeted particles have enhanced uptake in the RES, and a reduction of specificity of vascular delivery relative to untargeted carriers [155,201]. Indeed, at larger extremes of injectable particle size (>1–3 μm), both greater splenic retention and non-specific entrapment in lung capillaries is observed [202–204].

Larger carriers generally have lesser access to targets and adhered carriers may experience greater dragging force from blood, leading to target detachment [198,205,206]. For instance, larger carriers were shown to be incapable of targeting PLVAP, expressed in caveolae in lung endothelium [128]. However, in studies of targeting to PECAM, increasing carrier size from 40 nm to 300 nm increased specific adhesion in the lungs, implying that greater engagement with accessible endothelial targets may be achieved for larger carriers [201].

Nanocarrier shape, and interplay between shape and size, is also an important factor in targeting behavior [192,207]. Carriers that are non-spherical in nature may circulate longer than similarly sized spheres due to reduced recognition by host defense cells [109,208]. Discoid, ellipsoid, and filamentous carriers have prolonged circulation relative to spheres [155,209,210], in part due to reduced uptake by macrophages [211]. Rods and discoid particles avoid side effects associated with engagement with host defense cells in the lungs, unlike spherical carriers [210].

Eluding clearance from circulation and non-specific uptake may lead to non-spherical nanocarriers with more specific targeting to vascular endothelium. ICAM-targeted disks adhered to the pulmonary vascular endothelium more specifically than spherical counterparts [155]. Non-spherical and spherical particles have distinct propensity for margination in flow [212,213], modulating their affinity interactions under flow [214–216] and facilitating efficient non-spherical particle targeting to endothelial cells in the lungs and brain [217]. However, at an extreme of carrier elongation, worm-like filomicelles align with flow and avoid binding to vascular cells [218]. Filomicelles targeted to ICAM-1 had similar specificity of targeting as spheres, but a lower magnitude of uptake in the lung [209].

The above discussion of shape and size largely presumes rigid particle geometry, but many nanocarriers are capable of dynamic changes in shape and size. Methods for adjusting the flexibility of nanocarriers have been described for a variety of materials, with specific focus on lipid and polymeric vesicles [195,219–222]. Mechanical flexibility has a clear impact on systemic and target-specific behaviors of nanoparticles.

Generally, greater flexibility correlates to prolonged circulation of nanocarriers [195,218,219]. In part, prolonged circulation of soft nanoparticles may be attributable to reduced non-specific uptake in phagocytic cells [223,224]. In macrophages and other immune cells, harder particles are internalized more readily than soft particles [195,219,224,225]. A relationship between particle flexibility and ease of particle uptake may also be emerging in results with endothelial cells, where in vitro and in silico studies show passive and affinity-mediated nanoparticle uptake being impeded in softer particles [195,219,226]. Further, recent results

show that harder (GPa bulk modulus) non-targeted 200 nm spheres associated with and transported across brain endothelium in a microfluidic model to a greater degree than softer (MPa bulk modulus) spheres [227].

Changes in the mechanical properties of nanoparticles have yielded changes in affinity-directed targeting behavior. For PEG hydrogel nanoparticles, softer nanoparticles outperformed similar rigid particles in targeting to ICAM in the lungs [195]. However, in vivo observations with soft nanoparticles must consider the impact of prolonged circulation times on the extent of engagement with target molecules. The softer PEG nanoparticles also maintained higher circulating concentrations, meaning the lung to blood localization ratio was largely unaffected by flexibility of the ICAM-targeted particles in this case. Controlling for this factor, a microfluidic study showed that soft disc nanoparticles outperform rigid counterpart discs in adhesion to a target surface under flow [216]. It is hypothesized that this result may reflect the capacity of soft particles for increased target engagement and for resistance to target disengagement via fluid forces on the nanoparticles.

A specific application for soft particles in endothelial targeting was noted in studies of nanoparticles targeted to PLVAP. As noted above, PLVAP localizes to caveolae in the lungs and targeting to caveolae is limited for particles larger than the caveolar neck (>50-100 nm) [128]. However, soft (~50 kPa) dextran nanogels of 150–300 nm diameter successfully targeted to PLVAP in the lungs (Fig. 4), where analogous rigid polystyrene particles, functionalized with the same antibody, did not [131]. Further, tuning the modulus of the dextran nanogels via crosslinking resulted in abrogation of PLVAP targeting in inverse proportion to Young's modulus of the crosslinked particles [228] (Fig. 4D). Therefore, engineering of nanoparticle softness may affect particle access to caveolae, representing an important route for internalization in endothelial cells and possibly for transcytosis.

5. Modulating vascular targeting via control of route and PK

5.1. Local infusion

Compared to other routes of administration, the intravenous route has a distinct advantage in pulmonary delivery, as the lungs receive the entire first pass of venous blood [229]. In addition to first pass benefits, delivery to the pulmonary endothelium is particularly attractive, as it comprises ¼ of the entire surface area of endothelium. Because of the high perfusion of lungs and large endothelial surface area, delivery to endothelial antigens (e.g. ICAM-1, PECAM) is pharmacokinetically favored in the pulmonary vasculature.

An infusion via vascular catheters in conduit vessels favors uptake in the microvasculature immediately downstream of the injection site. This principle was successfully employed for PECAM and ICAM-targeted DDS: local infusion enhanced uptake in the vasculature in the brain, mesentery, and heart in several species, including mice, rats, dogs, and pigs [229–231] (Fig. 5a). Further, infusion in the isolated organs bypasses numerous factors impeding targeting and boosts local delivery, providing mechanistic insights and potential utility of vascular targeting for improving grafts for organ transplantation.

5.2. RBC Hitchhiking

The intravascular route can be further manipulated by using red blood cells (RBC) as a transport vehicle for DDS that are both actively and passively targeted [232,233]. Nanocarrier DDS can massively increase the mass of drug delivered to the target organ, but are not without their inherent problems, such as short half-life due to rapid clearance by the reticuloendothelial system [234]. The liver, spleen, and intravascular leukocytes, aided by the complement cascade, all contribute to this rapid clearance.

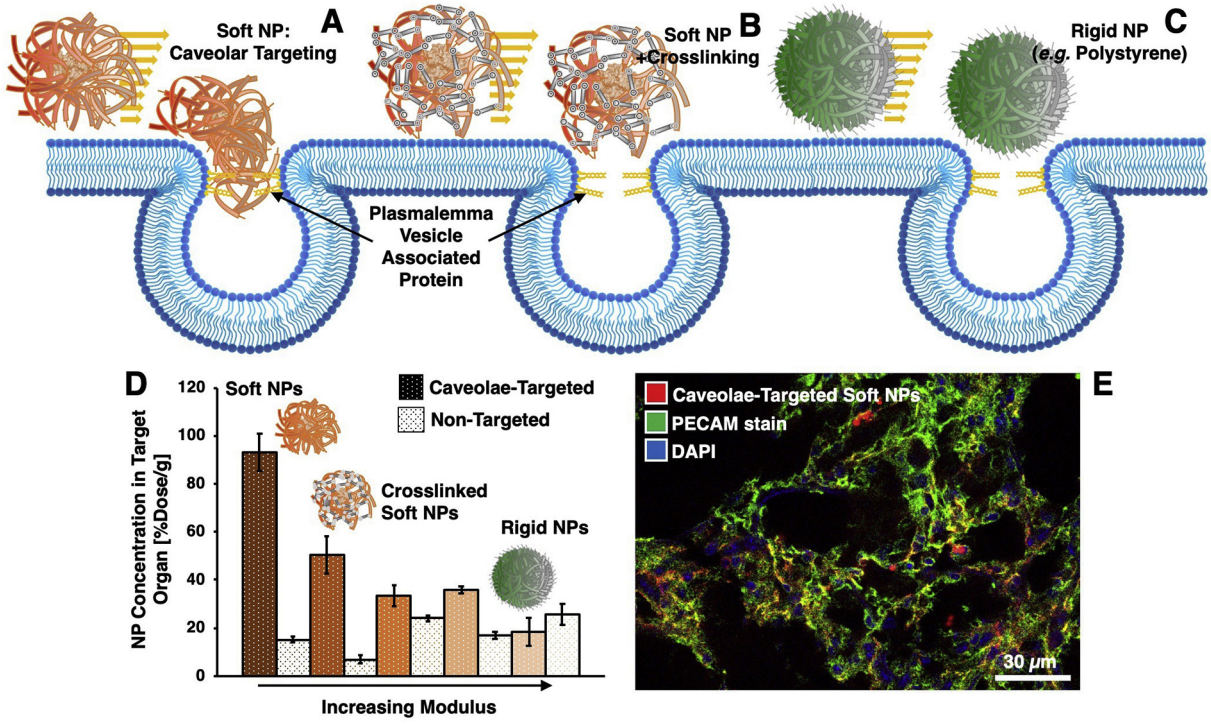

Fig. 4. Carrier flexibility modulates engagement of targeted carriers with caveolar plasmalemma vesicle associated protein (PLVAP) in lung endothelium. Carrier mechanical properties can affect how well carriers access targets (e.g. by soft particles "squeezing" into small spaces, as depicted in (a)). PLVAP is located in a sterically concealed position, the caveolar neck, in lung endothelial cells. Soft dextran particles (a) were able to target PLVAP more effectively than crosslinked dextran particles (b) or rigid polystyrene particles (c) of similar size. As evaluated in radioisotope tracing (d) and fluorescence (e) studies, PLVAP targeting efficacy in the lungs decreased as nanoparticle Young's modulus (as determined by AFM) increased (d). Adapted from [131,228].

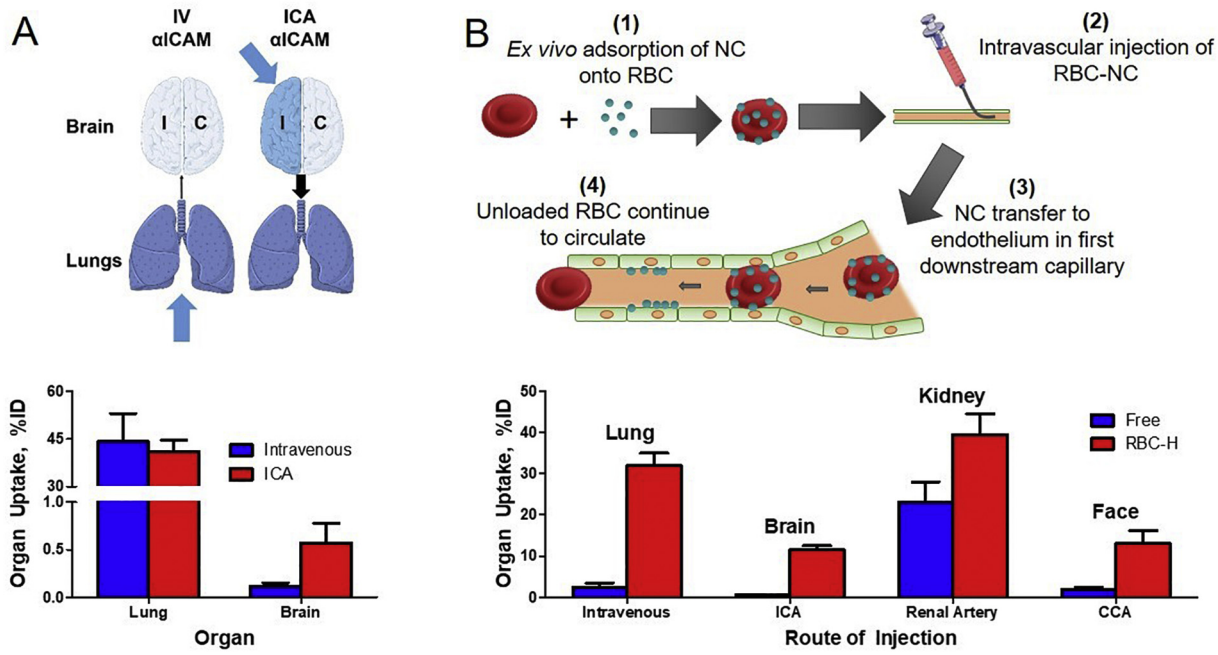

Fig. 5. Utility of the first-pass effect in tissue targeting. A: Intravenous (IV) infusion of ICAM-targeted nanocarriers (NC) results in a large portion of the dose being deposited in the lungs. On the other hand, infusion into the internal carotid artery bypasses the lung first pass and permits delivery to the brain. B: Ex vivo adsorption of untargeted NC onto RBC, termed RBC hitchhiking (RBC–H), allows transfer of NC to endothelium in the first capillary downstream of the injection site. This technology has been shown to increase delivery to several tissues by varying the injection site. Additional abbreviations used in figure: ICA (internal carotid artery), CCA (common carotid artery). Figures adapted from [231,232].

Red blood cell hitchhiking (RBC—H) was devised to evade this rapid clearance by the RES. Early studies used passive adsorption to non-covalently attach polystyrene nanoparticles onto RBCs and found that the half-life of RBC-adsorbed nanocarriers was longer than that of free nanoparticles. Additionally, the lung-to-clearing organ (liver & spleen) ratios were significantly improved using RBC-H [232]. This landmark work; however, was limited to rigid nanoparticles having no drug delivery utility, nonspecific attachment to RBC, and a circulation time of less than 24 h. Notably, increased delivery to the target tissue was achieved by injecting RBC-coupled nanoparticles immediately upstream of target organ, thus increasing first-pass delivery (Fig. 5b).

Upon careful loading that does not compromise RBC biocompatibility, RBC carrying surface-bound cargoes do not accumulate in any organ, including the reticuloendothelial system. However, the surface-bound cargoes that are loaded on RBC in a reversible fashion detach from RBC and transfer to the microvascular bed encountered by RBC. In the case of intravascular injections of RBC carrying such cargoes, the first major microvascular bed downstream injection site, i.e., first pass. In IV injection this is the pulmonary vasculature.

Additional studies incorporated biocompatible nanocarriers into RBC-H using liposomes, polymeric nanocarriers, and adeno-associated virus (AAV). The first pass effect was again amplified using RBC-H with 40-fold increase in lung targeting compared to free nanocarrier after intravenous injection. Importantly, this effect was also observed after intra-arterial injection via the carotid artery and measuring uptake in the immediate downstream organ, the brain, achieving brain uptake 143-fold than that of free nanocarriers [232]. Studies have shown therapeutic benefit using this technique in vivo in mouse models of acute pulmonary embolism and lung metastasis [232,233].

RBC-H has shown many advantages over intravenous delivery of free nanocarriers, but is not without room for improvement. The initial work focused largely on first-pass delivery to the immediate downstream organ, relying on the interaction of RBCs with the endothelium to encourage mechanical dissociation of nanoparticle from RBC vehicle. The promise of RBC-H as a drug delivery system that successfully evades the RES will likely need to incorporate better control over the RBC-nanoparticle association (and thus dissociation) and effect on the carrier RBC. One method to increase control of RBC-nanocarrier interaction is the use of targeted binding rather than passive adsorption. The interaction of RBC with RBC membrane targeting moieties has been characterized using drug-coupled fusion proteins. Specifically, anti-RBC-membrane scFv fused to thrombomodulin were evaluated in vitro in a microfluidic system. While some targeting to certain RBC surface epitopes caused pathologic increases in membrane rigidity (Band 3/Glycophorin A), targeting to others (RhCE) did not. Importantly, therapeutic efficacy of fusion-TM was retained when compared to soluble TM [235]. Future generations of RBC-H may utilize nanocarriers targeted to the RBC membrane to improve control of RBC-nanocarrier association, dissociation, and ultimately prolong time in circulation beyond the first pass effect.

5.3. Molecular engineering of targeting ligands

Many groups utilize antibody fragments (e.g. Fab, scFv, nanobody, etc.) as affinity ligands to achieve targeted drug delivery. Despite many advantages, such as ease of production in microbial systems and lack of Fc fragment-induced toxicities, these fragments are often inferior to full mAbs as isolated targeting ligands due to rapid blood clearance and poor stability of binding due to their monovalent nature. Additionally, commonly used amine-based conjugation strategies often inhibit the function of these small affinity ligands [236]. As such, there have been a myriad of protein engineering strategies proposed to improve the drug delivery properties of antibody fragments.

Improving circulation time of affinity ligands can be critical in conferring them with favorable biodistribution properties for therapeutic applications. This has been achieved in many ways, including fusion to long-circulating proteins (IgG and albumin) [237–240] or attachment of hydrophilic polymers (PEG) [241,242] to reduce renal elimination. Fusion to proteins such as albumin or albumin-binding molecules confers a long half-life, in part, due to interactions with the neonatal Fc receptor (FcRn), which serves as a salvage receptor preventing the intracellular catabolism of albumin [243] and IgG [244–246]. We have

recently reported that fusion of a VCAM-1-targeting nanobody to an albumin-binding nanobody leads to 25–40-fold improvements in uptake in target tissues over a 24 h period, relative to the anti-VCAM-1 nanobody alone [247].

To overcome challenges in coupling of therapeutic cargoes to small affinity ligands, site-specific conjugation strategies are becoming more prevalent. These typically involve building in motifs for conjugation near the C-terminus of the antibody fragment. Some of the more common strategies for site-specific conjugation of antibodies and fragments include Sortase A transpeptidation (recognizing the sequence LPXTG) [248] and conjugation to unpaired cysteines introduced into the molecule. These strategies have been successfully used to attach therapeutic cargoes and radiolabels to targeting ligands without adverse effects on their function [236,249–253]. In addition to engineering of antibody fragments, our group has recently reported a strategy for engineering full-length mAbs for site-specific modification by Sortase A via CRISPR/Cas9-mediated engineering of hybridoma cells [254,255]. The use of site-specific bioconjugation strategies permits oriented coupling of targeting ligands on the surface of nanoparticles, which has been demonstrated to improve their specificity for endothelial targets [252,256].

Nucleic acid aptamers are a recently developed class of affinity ligands that selectively bind to targets with high affinity via unique three-dimensional structures [257]. Aptamers are short, single-stranded DNA oligonucleotides that can be developed for a variety of target molecules using techniques analogous to phage display for antibody selection [258]. Aptamers directed against TfR have been used to deliver therapeutics both to [259] and across [260] the blood-brain barrier in animal models. This presents another potential approach for selective delivery to the endothelium.

6. Envisioned biomedical utility in acute vascular pathologies

Investigations in both animal models and in patients [6,19,41,44,45,69,138,261–263] have demonstrated affinity targeting to both normal and pathologically altered endothelium [40,264–266]. The delivery of enzyme replacement therapies to improve lysosome storage disease treatment was discussed above in the section on intracellular endothelial delivery. Here we provide a brief overview of a few areas of potential medical utility of this approach, with a focus on thrombosis, acute vascular inflammation, and ischemia (Fig. 6). Applications of endothelial targeting in select pathologies are summarized in Table 3.

Endothelial targeted DDS can deliver a great variety of anti-thrombotic agents, with different mechanisms for release and or activation at the intended site. They include plasminogen activators [230,267–269] and thrombomodulin [64,270–272] fused with scFv binding to surface endothelial epitopes and exert their activities in the lumen directly. Alternatively, DDS targeted to the same epitopes carrying encapsulated small drug molecules such as anti-inflammatory drugs (which have both direct and indirect anti-thrombotic effects) employ release of drugs from the cell-bound DDS either in blood or in endothelial cells [205,273]. Further, delivery of mRNA for TM (and likely other anti-thrombotic proteins) deliver their cargo into the cytosol of endothelial cells enabling synthesis and luminal exposure of the therapeutic transgene [87].

6.1. Acute thrombosis

In many pathologies, affected blood vessels have an increased propensity for thrombosis, in no small part due to suppression of the endothelium's natural anti-thrombotic mechanisms [274]. Delivery of ATA, such as TM or plasminogen activators (tissue type, tPA, or urokinase, uPA) to the luminal surface of the endothelium may help to subside some pro-thrombotic states. These results have been supplemented by nucleic acid-based delivery of these proteins [275].

In cell-based models, targeting anticoagulants to activated endothelium using anti-*E*-selectin mAbs was shown to inhibit thrombin expression, providing proof-of-principle of this approach [67]. As

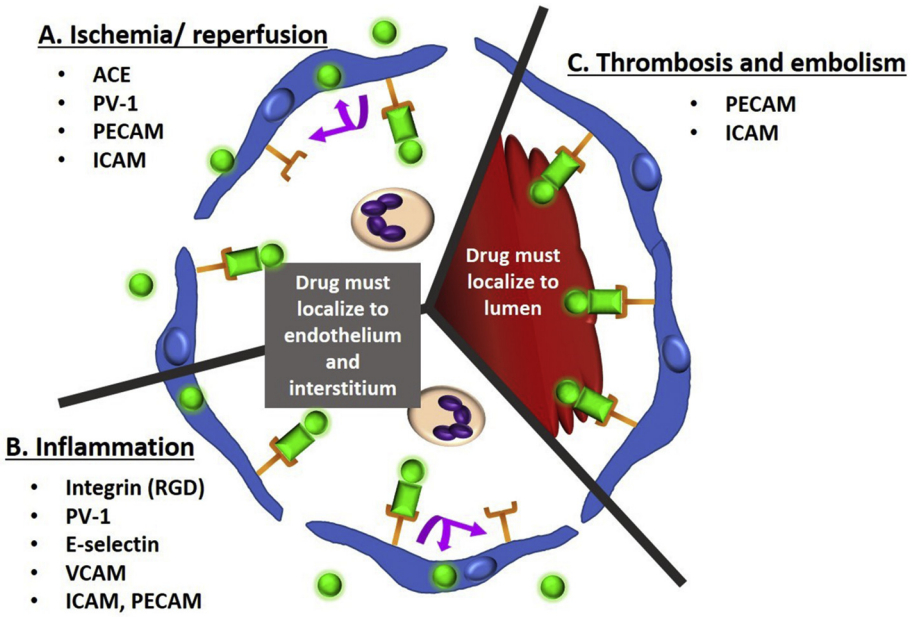

Fig. 6. Endothelial receptors for conjugate targeting in acute vascular pathology. Shown is a small vessel or capillary in cross-section, lined by endothelial cells (blue). Receptors (orange) are bound by drug conjugate (green circle) plus targeting moiety/ nanocarrier (green rectangle). Many receptors including PECAM and ICAM enable both intravascular and extravascular drug targeting. Factors that promote intracellular uptake (with retention of drug conjugate and recycling of receptor) include multivalent targeting moieties and low-flow state. Factors that modify uptake include ligand density, carrier geometry, and size. Ischemia/reperfusion injury (A) and acute vascular inflammation (B) both result in increased leukocyte recruitment and leaky tight junctions between endothelial cells. Treatment requires drug to be delivered intracellularly to the endothelium and to the interstitial space. Drug targeting to the specific receptors shown has resulted in treatment effect in animal models of these pathologies. (C) Shown is a blood clot within the lumen. Thrombosis and embolism both require the drug to remain in the vessel lumen to achieve direct therapeutic effect. Drug targeting to the receptors shown has resulted in treatment effect in animal models of thrombosis.

Table 3
Examples of endothelial-targeted drug delivery in non-oncology settings.

Pathology	Target Epitope(s)	Drug(s)	DDS	Ref
Pulmonary Embolism	PECAM-1	scuPA	Fusion Protein	[267,268]
	ICAM-1	tPA	Protein Conjugate	[269]
	N/A	Reteplase	RBC-H	[232]
Ischemic Stroke	PECAM-1	scuPA	Fusion Protein	[230]
Lung Transplant	ACE	Catalase	Protein Conjugate	[324]
	PECAM-1	Catalase	Protein Conjugate	[278,366]
Acute Lung Injury	PECAM-1	Catalase	Protein Conjugate	[321,332]
		Thrombomodulin	Fusion Protein	[270]
		MJ33	Liposomes	[333]
		EUK-134	Liposomes	[205]
		SOD/Catalase	Nanoparticles	[367]
	ICAM-1	Thrombomodulin	Fusion Protein	[271]
		Dexamethasone	Nanogel	[273]
	PLVAP	SOD	Protein Conjugate	[318]
Type B NPD	ICAM-1	Acid Sphingomyelinase	Nanocarrier	[166]
Neurovascular Inflammation	VCAM-1	Thrombomodulin mRNA	LNP	[87]
Fabry Disease	ICAM-1	α-Galactosidase A	Nanocarriers	[167]
Glomerulonephritis	E-Selectin	Dexamethasone	Liposome	[284,285]
		DnlκB Transgene	Adenovirus	[289]
Arthritis	Integrin	Dexamethasone	Liposome	[291]
Atherosclerosis	VCAM-1	Prostaglandins	Liposome	[293]
Pulmonary Hypertension	ACE	Tph1 shRNA	Adenovirus	[323]
Pulmonary Ischemia-Reperfusion	ACE	Catalase	Protein Conjugate	[325]
Endotoxemia (IP LPS)	VCAM-1	Dexamethasone	SAINT-O-Somes	[368]
Idiopathic Pulmonary Fibrosis	PLVAP	PGE_2	Protein Conjugate	[369]

Abbreviations used in table: ACE: angiotensin-converting enzyme, ICAM-1: intercellular adhesion molecule-1, LNP: lipid nanocarrier, NPD: Neimann-Pick disease, PECAM-1: platelet-endothelial cell adhesion molecule-1, PGE_2: prostaglandin E2, PLVAP: plasmalemma vesicle-associated protein, RBC–H: red blood cell hitchhiking, scuPA: single chain urokinase-like plasminogen activator, SOD: superoxide dismutase, tPA: tissue-type plasminogen activator.

investigations moved into rodent studies, both tPA and uPA were shown to be concentrated within the lungs by targeting to ACE; however, there were limited pharmacologic benefits due to rapid endocytosis [99,276]. However, delivery of ATA to non-internalizing endothelial epitopes (e.g., ICAM-1, PECAM) permitted not only efficient targeting of the lung, but also clot dissolution [269]. Fusion proteins between anti-PECAM scFv and ATA were able to provide thromboprophylaxis due to prolonged residence in the pulmonary lumen [267,277,278]. Local infusion of scFv/uPA via the carotid artery prevented cerebrovascular thrombosis [230]. Fusions containing uPA mutant activated by thrombin [279–281] had similar high degree of lung uptake and thromboprophylaxis, with additional improvements in reductions of fibrin deposition and improvement of arterial oxygen tension relative to a non-thrombin-dependent scFv/uPA [268].

In rat models, pharmacologic benefits have been observed using both recombinant soluble TM [34] and tissue factor-targeted TM [282], but this fusion rapidly disappeared from the vascular lumen. PECAM-targeted scFv/TM fusion accumulated in the pulmonary vasculature, and reduced both thrombosis and other tissue damage in mice, without causing bleeding [270]. By fusing TM with scFvs directed against PECAM and ICAM, the antithrombotic and anti-inflammatory effects of untargeted soluble TM are dwarfed, due to prolonged endothelial anchoring of TM [268]. Targeting scFv/TM fusions to ICAM afforded more potent protection than to PECAM, consistent with the notion of ICAM localization close to the TM cofactor EPCR in the plasmalemma [271]. As discussed above, by taking advantage of the CEPAL effect observed for PECAM-targeted ligands, TM and EPCR were able to be co-delivered, providing a substantially increased pharmacologic effect. This benefit was likely due to enhanced targeting (CEPAL effect) and optimal special arrangement of TM and EPCR, allowing enhanced interactions between the partner molecules [184]. Thrombin-activated, targeted ATA have the potential to provide a safe thrombophrophlyactic option in patients at high risk of acute thrombosis.

6.2. Inflammation

Thrombosis and inflammation are closely intertwined. Endothelium controls vascular permeability of leukocytes in response to tissue damage and infection, but also become a victim of injurious inflammatory mediators. Targeting anti-inflammatory drugs to protect the endothelium from friendly fire without impeding host defense is an attractive strategy. Inducible and constitutive endothelial determinants involved in inflammation appear to be good targets for this approach.

The use of liposomes targeted to *E*-selectin has shown utility in delivering dexamethasone (DEX) to a variety of tissues in animal models of chronic inflammation. For example, these particles showed accumulation in skin and kidneys in models of dermal [283] and renal [284] inflammation. In the latter model, this treatment conferred anti-inflammatory effects in mice [284] and in rats [285]. Within minutes of injection, similar formulations accumulated in the inflamed eye and reduced the level of inflammatory markers [286]. Similar DDS were used for nucleic acid delivery to the kidney and tumors [287,288]. Both E-selectin and ICAM-1 have been used as target molecules for delivery of siRNA in vitro [289,290]. Coupling of an E-selectin targeting ligand to adenovirus allowed targeting to the kidneys and displayed functional activity in a glomerulonephritis model [289].

Delivery of DEX liposomes to integrins was achieved in a rat arthritis model, using RGD peptides. These particles provided improved protection when compared to untargeted particles [291]. A similar strategy was also used to delivery anti-inflammatory siRNA in animals [292]. Delivery of the anti-inflammatory prostaglandin, PGE_2, was achieved by targeting to VCAM-1. Chronic administration of VCAM-1-targeted particles provided improved PGE_2 delivery and therapeutic benefits in a mouse model of 'atherosclerosis' [293]. Targeting dexamethasone-loaded liposomes and nanogels to ICAM, PECAM and PLVAP supersedes protective effects of untargeted counterparts in a mouse model of ARDS [273,294].

6.3. Inflammatory signaling and reactive oxygen species

A plethora of recent findings have revealed close links between endocytosis and signaling pathways that have changed our understanding of the role that endocytosis plays in a cell's make-up [295,296]. Traditionally, endocytosis and signaling were considered as distinct processes. However, it has now become clear that receptor-regulated endocytosis is critical for modulation of numerous signaling pathways, including attenuation of signal, its prolongation, activation, signal distinction from several possible paths, etc. [297]. This is also the case for many cytokine receptors, including TNFR1 and IL-1R [298]. TNFR1 activated by interaction with TNF co-localizes with caveolin in lipid raft and receptor complex internalization may modulate TNF signaling outcome [299–301]. Similarly, IL-1R translocates to caveolae upon activation and receptor internalization is required for complete NF-κB signaling processes [302,303].

Redox-active endosomes (i.e. containing reactive oxygen species (ROS)) may play an important role in pro-inflammatory signaling [304,305] indicating potential therapeutic applications of antioxidant drug delivery [306]. PECAM- and ICAM-targeted delivery of SOD is able to considerably block the inflammatory response caused by cytokines and by TLR activation [307–309]. In addition, caveolar targeting of nanocarriers may have significant potential, particularly for endothelial and trans-endothelial delivery, due to the abundance of caveolae in endothelial cells [310]. Effective caveolar targeting has been demonstrated using albumin-coated nanocarriers [130]. The profound involvement of caveolae in cellular signaling [311] should be of particular interest for the drug delivery field (Fig. 7).

The role of localization and receptor trafficking of TLRs (and particularly TLR4 as the best characterized TLR) was clarified from recent works [312,313]. However, the mechanisms of TLRs endocytosis and its exact role in cellular response to pro-inflammatory signals still require more studies for our understanding. Moreover, it may be cell-specific. Indeed, TLR4 endocytosis in macrophages is not required for their response. In contrast, astrocytes internalized activated TLR4 by both caveolae and clathrin endocytosis, and caveolar endocytosis is required for both MyD88- and TRIF-dependent signaling pathways [314], while epithelial cells are characterized by intracellular localization of TLR4 at Golgi apparatus and CD14-dependent trafficking of LPS via clathrin-dependent endocytosis [315,316]. Interestingly, the proinflammatory effects of LPS are significantly attenuated in caveolin-1 knockout mice. NF-κB activation by LPS is decreased; ICAM expression is lower as well as PMN accumulation in lungs, while NO level is higher in Cav-$1^{-/-}$ mice compared to wild type [317] suggesting an important role of caveolae in LPS-induced response in vivo. Indeed, caveolar targeting and delivery of SOD using PLVAP has a significant advantage compared to less specific delivery via PECAM and ICAM [133,318]. Caveolar targeting potentially can deliver SOD right to the signaling endosomes responsible for activation of NFκB signal transduction pathway.

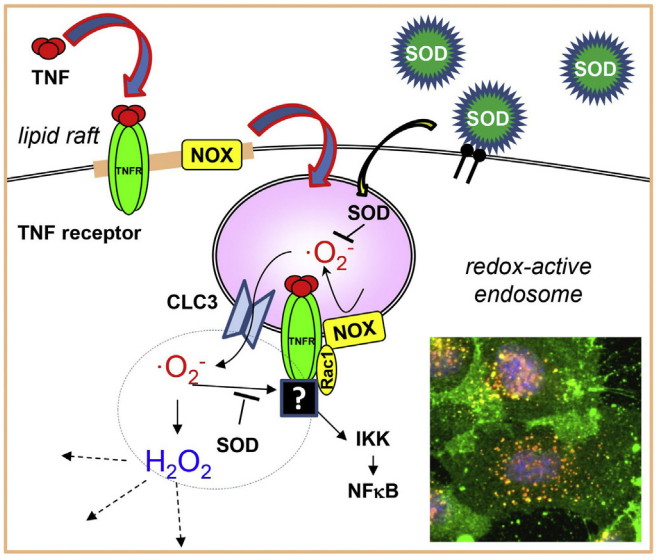

Fig. 7. Role of ROS in cytokine- and TLR-induced signaling and potential use of intracellular antioxidant delivery for anti-inflammatory protection. Cytokine or PAMP binds to counterpart receptor, causing NOX activation and the complex internalization forming redox-active endosome. Generated ROS transfers into cytosol via CLC3 channel and stimulates proinflammatory NFκB cascade. Delivery of antioxidant to the endosome or overexpression of cytosolic SOD can reduce the inflammatory signaling and attenuate cellular injury [307,308]. Inset shows SOD-loaded nanoparticles (green) enter endosomes (red), SOD-containing endosomes seen as yellow. Cell nuclei are shown blue.

6.4. Ischemia-reperfusion and acute oxidative stress

Endothelial involvement in oxidative mechanisms of inflammation and other conditions is not limited to mediation of pro-inflammatory signaling via endosomal pathways discussed above. Endothelial cells are common victims of ROS in ischemia-reperfusion injury, acute inflammation, and infections. ROS-quenching enzymes, catalase and SOD, conjugated with mAbs directed against several endothelial targets (PECAM, ICAM-1, ACE) improved protection in several models of acute oxidative stress compared to untargeted formulations [263,277,278,319–329].

PECAM- and ICAM-targeted catalase protected against H_2O_2-induced vascular leak [330], alleviated vascular [331] and oxidative stresses [332], and pulmonary ischemia-reperfusion injuries [278,321]. These results include lung transplantation in multiple animal models [324,325]. Targeting to PECAM and ICAM of SOD, liposomal SOD, and liposomal SOD/catalase mimetic, as well as inhibitors of ROS-producing enzymes, reduced ROS toxicities in endothelial cells [205,306,326], normalized vasoconstriction in mice [321], attenuated VEGF-induced endothelial leakage [330], and endotoxin-induced acute pulmonary inflammation in animals [333].

Pharmacological agents decelerating vesicular trafficking prolong endothelial antioxidant protection conferred by ICAM- and PECAM-targeted catalase and SOD via delaying their lysosomal degradation [106,152]. Targeting to PECAM of catalase loaded into nanocarriers selectively permeable for ROS provided prolonged protection [334–337].

Overall, delivery of both antioxidants and anti-inflammatories to the endothelium provides a variety of mechanistically precise, spatiotemporally controlled protective effects in the variety of animal models emulating pathological pathways typical of acute human diseases and conditions associated with or driven by vascular oxidative stress, ischemia-reperfusion and inflammation.

7. Current challenges and perspectives

7.1. Modeling of vascular targeting

Many reasons drive attempts to minimize in vivo investigations by pursuing in vitro models, despite the fact that no in vitro system fully recapitulates the situation in vivo and most, if not all, lack effects of an organism on behavior of a drug or DDS systemically (PK, clearance, effect of pathology, as well as humoral, nervous and defense factors) and locally (cellular microenvironment, histological, and pathological factors). In case of vascular targeting, these challenges are aggravated by rapid phenotypic bastardization of cultivated endothelial cells, which quickly lose specific markers like ACE [338].

The introduction of HUVEC (human umbilical vein endothelial cells) as an in vitro endothelial model by Eric Jaffe, Michael Gimbrone, Jordan Pober and colleagues nearly half a century ago was a truly major

breakthrough [339]. Further advances, such as adaptation of the Boyden's chamber to study endothelial permeability, upgrades of static cell cultures to flow-adapted and flow-exposed cell cultures, microfluidic systems and flow chambers with cells grown on semi-permeable supports, and recent advent of the "organ on the chip", including exquisite 3D multichannel models of vascular segments have yielded significant results [91,272,340–343].

In vitro models are useful to address specific questions, such as analysis of hydrodynamic parameters and effects of flow adaptation on binding, uptake, vesicular transport, and effects of DDS targeted to endothelium. Static 2D cultures are useful to determine specificity and other parameters of binding DDS to target vs. non-target cells (K_d, B_{max}, kinetics of binding and dissociation).

Sometimes, there is no choice but to retreat to in vitro models, since our methods do not work in vivo. Thus, parameters of resolution, dynamics, objectivity, and attribution to histological and cellular structures of current methods afford rather limited information on DDS sub-cellular addressing and intracellular transport. Here, endothelial cell culture seems to be a reasonable resolution. More technically challenging models of perfusion of isolated vessels and organs provide a valuable resource, permitting real-time direct measurements of the uptake, tissue localization, and activity of a DDS in conditions maximally close to in vivo [39,319].

Computational modeling and systems biology represent further interesting in silico approaches. Major challenges in this area include variable compatibility and quality of the feed data, oversimplification of conditions, parameters, and margins, and relatively arbitrary selection of the focus and readouts. Yet, mutually enriching collaboration of computational researchers with experimentalists and physicians may enable modeling with high predictive value that allows for a reduction of in vivo experimentation [344–347].

7.2. Characterization and analysis of PK/BD

A key consideration in development of a DDS is selection of appropriate methodologies for characterization of blood and tissue pharmacokinetics. An ideal bioassay would have the capacity to reliably quantify DDS in all relevant matrices (e.g. whole blood, plasma, tissues, etc.) in an unbiased and highly precise manner.

There are several methods that are often utilized to describe PK/biodistribution of DDS, each with their own unique strengths and weaknesses. Measurement of radioisotope-labeled DDS can be considered as the 'gold standard' for assessment of blood and tissue PK [348,349]. Radioisotope-based measurements do not require tissue processing, have no background signal, and can be directly related to the injected dose, allowing calculation of key PK parameters with no intrinsic bias. However, a key drawback of radioisotope labeling is the potential introduction of artifacts due to the labeling process, including, but not limited to, changes in PK due to introduction of the label and loss of the label from the DDS. By labeling and tracing multiple components of the DDS, these artifacts can be minimized.

A second approach that is often reported in the literature is optical tracing of fluorescent components of a DDS. While fluorescent labels are often useful for visualizing distribution on a sub-tissue level, the presence of high (and variable) background signals in biological matrices and non-linear signal vs. concentration relationships reduces their utility in measuring PK/BD.

Finally, mass spectrometry (MS) is being used frequently for PK measurements in both preclinical and clinical investigations [350–352]. Due to the complex nature of many DDS, it would be challenging to validate an assay for direct measurement of unlabeled DDS. However, MS should be considered as the gold standard for assessment of PK/BD of small molecule cargo drugs, as it is able to make highly precise measurements in both blood and tissues.

Collection of high quality PK/BD data permits development of mathematical models that can be used to guide further development and optimization of DDS. The types of models that can be developed range in terms of complexity and degree of mechanistic information, from empirical models that simply describe the concentration vs. time curve, to physiologically-based models that are able to predict drug behavior. At the preclinical academic level, more mechanistic models are often useful as tools to predict the impact of DDS properties and pathology on behavior, permitting optimization of a delivery strategy early in development.

Additionally, physiologically-based models have the potential for relatively straightforward scaling to higher species, permitting prediction of clinical PK/PD profiles [353–355]. Once a DDS moves into the clinical domain, modeling often shifts to simpler models, with the goal of identifying patient-specific factors, termed covariates that may impact drug behavior and patient outcomes. This application of population PK is then used to identify special patient populations (e.g. renal failure, pediatrics, obesity, etc.) that may require dose adjustments from the 'standard' dose [356,357].

7.3. Benefit/risk ratio appraisal: Omnes viae Romam ducunt

Ultimately, preclinical studies culminate in analysis of the beneficial vs. unintended and adverse effects of the devised DDS in animal models. Admittedly, no animal model fully recapitulates human diseases, but, as physicians know, no individual patient's condition fully recapitulates another patient's condition. Of course, the closer to human the species is and the closer the pathogenesis is to the relevant human condition, the greater the confidence in the translatability of results.

Parameters of the amplitude, timing of the onset, and duration of the desirable vs. unintended effects are the key readouts deciding the fate of a DDS. Statistics are necessary, but not always sufficient to prove the therapeutic significance. The rigor and scrutiny of characterization of DDS effects are painstaking. These studies, performed in healthy and sick animals in an obligatory double-blinded manner, must include direct comparison of experimental groups with controls including healthy and sick animals treated with: A) Untargeted agents - free and loaded in untargeted DDS; B) A mixture of targeted DDS and free drug; C) Drugless targeted and untargeted DDS. Not all, but many studies appraising benefit/risk ratio of vascular targeting in fact meet these draconian criteria, giving a certain level of comfort and confidence that this drug delivery approach will inevitably reach the clinical utility.

8. Conclusions

The wealth of knowledge garnered in three decades of studies of vascular targeting is enormous. These studies yielded diverse endothelial target molecules for drug delivery, multitudes of ligands, nanocarriers, and methodologies for their conjugation and assembly. These means provide specific and effective targeting of drugs to, into and across of the endothelia in several important organs and vascular areas of significant biomedical interest. It seems safe to state that the general progress in this area of the drug delivery field is impressive, encouraging and brings us to the brink of industrial development and clinical translation.

These studies have also advanced our understanding of physiological and pathological processes localized in or involving endothelial interface, especially in the lungs and brain. Targeted pharmacological interventions designed to yield functional responses in the specific components and compartments of vascular system and blood represent valuable investigational tools. Indeed, both positive and negative outcomes of such interventions may give mechanistic insight about the role of implicated molecules and cells, on the *sine qua none* condition that delivery, localization and activity of the targeted pharmacological agent are confirmed "beyond any reasonable doubt", and therefore, the lack of the effect is attributable to the intricacies of the pathophysiological mechanism, not failure of delivery.

Notwithstanding, the main biomedical purpose of drug delivery is, of course, advancement of the treatment of patients - diagnostic, prophylaxis and therapy. The pressure to come up with the winning formulations is palpable, but it would be a mistake to put on the back burner, suspend or abandon devising and research of new DDS, especially in academia, for the sake of concentrating efforts on the industrial development and clinical testing of DDS showing favorable profile in experimental settings. It is unpredictable which specific DDS iterations will emerge as new therapeutic options. Yet, focus on promising candidates seems timely and necessary. Selection of these lead candidates is a complex and multifaceted interdisciplinary affair that must involve basic scientists, clinical investigators, industrial, and regulatory counterparts.

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